

HONEY TRUFFLE SWEETENER (HTS) VARIANTS**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of U.S. provisional application no. 63/444,185, filed February 8, 2023, U.S. provisional application no. 63/524,794, filed July 3, 2023, U.S. provisional application no. 63/541,591, filed September 29, 2023, and U.S. provisional application no. 63/618,616, filed January 8, 2024, each of which are incorporated by reference herein in their entireties.

**INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED
ELECTRONICALLY**

[0002] Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing identified as follows: One 282 KB XML file named "0640-47_WO.xml" created on February 8, 2024.

FIELD OF THE INVENTION

[0003] This invention includes embodiments of sweet-tasting (Honey Truffle Sweetener (HTS)) proteins, genes, and cDNA encoding said proteins, and to methods of using such proteins, genes, and cDNA in the modulation of the taste of foods. More particularly, the invention includes embodiments of HTS variant sweet proteins, and to the genes and cDNA encoding such proteins.

BACKGROUND OF THE INVENTION

[0004] Excess intake of nutritive sweeteners has long been associated with diet-related health issues, such as obesity, heart disease, metabolic disorders and dental problems. Consequently, consumers are increasingly looking for ways to decrease the amount of nutritive sweeteners in their diets; and manufacturers are trying to respond to this demand by attempting to replace nutritive sweeteners with substitutes to mimic the desirable taste and functional properties of the nutritive sweeteners.

[0005] Zero or low-calorie sweeteners derived from, preferably, natural sources are desired to limit the negative effects of high sugar consumption (e.g., diabetes and obesity, among others). But, commonly-known zero or low-calorie sweetener substitutes such as aspartame, acesulfame potassium, Luo Han Guo (monk) fruit extract, neotame, saccharin, stevia and sucralose have undesirable taste defects such as bitterness.

[0006] Zero or low-calorie sweeteners derived from natural sources may be preferred to limit the negative effects of high sugar consumption. So far there are only seven known sweet and taste-modifying proteins, namely monellin, thaumatin, brazzein, curculin, mabinlin, miraculin and pentadin. The key residues on the protein surface responsible for biological activity have not yet been identified

with certainty for any of these proteins. Monellin was found to be 100,000 times sweeter than sucrose on a molar basis, followed by thaumatin and brazzein which are 3000 times and 500 times sweeter than sucrose, respectively, on a gram basis. Most of them share no sequence homology or structural similarity; and thaumatin shares extensive similarity at the protein sequence level with certain non-sweet proteins found in other plants.

[0007] International patent application PCT/US2020/012955, filed 01/09/2020, published as WO2020/146650 on 07/16/2020 relates to a sweetening composition comprising (i) mycelia of an ascomycete or an aqueous extract thereof or (ii) an aqueous extract of a fruiting body of an ascomycete and use of such composition to provide improved flavor to a product for oral administration. The application also relates to compositions comprising combinations of sweetening compositions and a product for oral administration.

[0008] International patent application PCT/US2021/039176, filed June 25, 2021, published as WO2021/263158 on December 30, 2021, relates to newly identified fungal sweet-taste modifying proteins and the cDNA encoding the proteins. Sweet-taste modifying proteins were identified in the truffle, *Mattirolomyces* terfeziodes. The application further relates to Myd proteins as sweeteners and in sweet taste activation/modulation and the cDNA encoding the same and methods for isolating such cDNA and for isolating and expressing such proteins. The application also relates to a sweetening composition which includes the proteins and methods to provide improved flavor to a product for oral administration.

[0009] International patent application PCT/US2022/82443, filed December 27, 2022, and published as WO2023/129938, on July 6, 2023, relate to sweet protein variants from truffle, e.g., *Mattirolomyces* terfeziodes, including proteins, genes, cDNA encoding said proteins, and compositions thereof, and are hereby incorporated by reference in their entirety.

[0010] A need still remains for new low or zero calorie sweeteners with improved taste from natural sources, particularly from ascomycetes fungal species. A need also remains for producing fungally-derived low or zero calorie sweeteners with improved taste and methods of using such ingredients in final food products without compromising on taste and with superior taste profiles.

SUMMARY OF THE DISCLOSURE

[0011] The invention meets these and other needs by providing fungally-derived sweet-tasting proteins identified herein as the Myd family of proteins, as well as the genes and cDNA encoding such proteins, and methods of using such proteins, genes, and cDNA in the modulation of the taste of foods. The invention provides variants of MYD1 (also referred to as mycodulcein, and Honey Truffle Sweetener protein (HTS protein)). Polypeptides of the invention exhibit flavor-modifying properties

and, in particular, modulate sweet taste and perception, either alone, or in combination with food, beverages, dietary supplements, or pharmaceuticals. In embodiments, Myd1 variants of the invention can provide sweet-taste to materials, such as food, beverages, dietary supplements, or pharmaceuticals that are ingested. In embodiments, Myd1 variants of the invention can reduce the sourness, bitterness or astringency of foods and drinks. In embodiments, Myd1 variants of the invention can exhibit enhancement of the taste of foods and drinks, namely a taste-modifying activity. In embodiments, Myd1 variants, can exhibit in addition to sweet-taste modulation activity, enhanced thermal stability compared to native forms of HTS protein. In embodiments, Myd1 variants can exhibit flavor-modifying properties. In embodiments, Myd1 variants, can exhibit in addition to flavor modifying properties, enhanced thermal stability compared to native forms of HTS protein. In an embodiment, the invention provides naturally-occurring Myd proteins. In an embodiment, the invention provides variant Myd proteins other than naturally-occurring proteins.

[0012] Naturally-occurring Myd protein exists as two isoforms; HTS-1 (SEQ ID NO:3) and HTS-2 (SEQ ID NO:141), as isolated from *Mattirolomyces terfezioides*. The relative amounts of HTS-1 and HTS-2 isolated from *Mattirolomyces terfezioides* range from about 40% to 60% by weight HTS-2 to 60% to 40% by weight HTS-1. In embodiments, the invention provides non-naturally occurring sweet proteins which include non-naturally occurring mixtures of the two isoforms HTS-1 and HTS-2. In embodiments, the invention provides sweet protein generated by recombinant expression of the coding sequence of SEQ ID NO:2 or a codon-optimized version of the coding sequence of SEQ ID NO:2 in a non-native host (e.g., a host other than *Mattirolomyces terfezioides* truffle), as well as methods of generating sweet protein by recombinant expression.

[0013] In an embodiment the encoded polypeptide is the polypeptide having the amino acid sequence of SEQ ID NO:3 (also designated as the HTS-1 isoform). In an embodiment the encoded polypeptide is the polypeptide having the amino acid sequence of SEQ ID NO:3, except that the methionine at position 1 in SEQ ID NO:3 is absent, designated hereinafter as the polypeptide having the amino acid sequence of SEQ ID NO:141(also designated as the HTS-2 isoform). In embodiments, the encoded polypeptide is the polypeptide having amino acid sequence SEQ ID NO:5 which is the HTS-1 isoform with a (His-tag)₆. In embodiments, the encoded polypeptide is the polypeptide having amino acid sequence SEQ ID NO:142 which is the HTS-2 isoform with a (His-tag)₆.

[0014] Accordingly, one aspect of the invention provides a polynucleotide (e.g., isolated polynucleotide) encoding a polypeptide, wherein the encoded polypeptide has a sweet-taste modulation activity and which is different in amino acid sequence from the polypeptide of SEQ ID NO:3 or optionally different from the polypeptide of SEQ ID NO:141. In embodiments, the encoded polypeptide is selected from the group consisting of: (a) a polypeptide sequence selected from the

group consisting of the amino acid sequences of a variant listed in Table 8; Table 9 or Table 10 (b) a polypeptide having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of the amino acid sequences of a variant listed in Table 8, Table 9 or Table 10; and (c) a polypeptide sequence modified from the polypeptide sequence selected from the group consisting of the amino acid sequences of a variant listed in Table 8, Table 9 or Table 10 by deletion, insertion, substitution, or addition of no more than 24 amino acids, and wherein the polypeptide sequence is different from that of SEQ ID NO:3 and optionally different from that of SEQ ID NO:141. In an embodiment, the polynucleotide encoding the polypeptide is other than the polynucleotide of SEQ ID NO:2. In an embodiment, the polynucleotide encoding the polypeptide is the polynucleotide of SEQ ID NO:2 which is codon optimized for expression in a bacterium, yeast or fungus. In an embodiment, the polynucleotide encoding the polypeptide is the polynucleotide of SEQ ID NO:6 with optional His-tag which is codon optimized for expression in *E. coli*. In an embodiment, the polynucleotide encoding the polypeptide is the polynucleotide of SEQ ID NO:7 with optional His-tag which is codon optimized for expression in *Saccharomyces cerevisiae*.

[0015] In an embodiment, the encoded polypeptide is a polypeptide having the amino acid sequence of SEQ ID NO:3, except that the methionine at position 1 in SEQ ID NO:3 is absent, presented herein as the polypeptide having the amino acid sequence of SEQ ID NO:141. In an embodiment, the encoded polypeptide is a polypeptide other than the polypeptide having the amino acid sequence of SEQ ID NO:141. In embodiments, the encoded polypeptide exhibits sweet taste. In embodiments, the encoded polypeptide exhibits sweet taste, when present in a composition at a concentration of 30 ppm or higher. In embodiments, the encoded polypeptide exhibits sweet taste, when present in a composition at a concentration of 40 ppm or higher.

[0016] In a related aspect, the invention provides a polynucleotide (e.g., isolated polynucleotide) encoding a polypeptide, wherein the encoded polypeptide has flavor modifying properties, and which is different in amino acid sequence from polypeptide SEQ ID NO:3 and optionally from SEQ ID NO:141. In embodiments, the encoded polypeptide is selected from the group consisting of: (a) a polypeptide sequence selected from the group consisting of the amino acid sequences of a variant listed in Table 8; (b) a polypeptide having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of the amino acid sequences of a variant listed in Table 8; and (c) a polypeptide sequence modified from the polypeptide sequence selected from the group consisting of the amino acid sequences of a variant listed in Table 8, by deletion, insertion, substitution, or addition of no more than 24 amino acids, and wherein the polypeptide sequence is different from that of SEQ ID NO:3 and optionally from SEQ ID NO:141. In embodiments, the encoded polypeptide is a polypeptide selected from the group consisting of the amino acid sequences of a variant listed in Table

8, that is other than a variant listed in Table 9 or Table 10. In an embodiment, the polynucleotide encoding the polypeptide is a polynucleotide other than the polynucleotide of SEQ ID NO:2. In an embodiment, the encoded polypeptide is a polypeptide having the amino acid sequence of SEQ ID NO:141. In an embodiment, the encoded polypeptide is a polypeptide other than the polypeptide having the amino acid sequence of SEQ ID NO:141. In embodiments, the encoded polypeptide does not exhibit sweet taste. In embodiments, the encoded polypeptide does not exhibit sweet taste when present in a composition at a concentration of 30 ppm or higher. In embodiments, the encoded polypeptide does not exhibit sweet taste when present in a composition at a concentration of 40 ppm or higher. In embodiments, the encoded polypeptide only exhibits sweet taste when present in a composition at a concentration of 30 ppm or higher.

[0017] In another aspect of the invention, amino acid mutations of HTS polypeptide and protein variants are summarized in Table 8. The mutations of Table 8 are identified relative to the amino acid sequence of SEQ ID NO:3. Thus, a polypeptide having an amino acid sequence of a variant listed in Table 8 will have the amino acid sequence of SEQ ID NO:3, but with one or more site mutations identified in Table 8. Mutants of Table 8 also include those in which the methionine at position 1 of the protein is absent. In a related aspect, the invention provides HTS polypeptide and protein variants, wherein the variants have the single site mutations of Table 8 relative to the amino acid sequence of SEQ ID NO:3 and in addition wherein the methionine at position 1 in SEQ ID NO:3 is absent.

[0018] In another aspect of the invention, amino acid mutations of HTS polypeptide and protein variants are summarized in Table 9. The mutations of Table 9 are identified relative to the amino acid sequence of SEQ ID NO:3. Thus, a polypeptide having an amino acid sequence of a variant listed in Table 9 will have the amino acid sequence of SEQ ID NO:3, but with one or more site mutations identified in Table 9. Mutants of Table 9 also include those in which the methionine at position 1 of the protein is absent. In a related aspect, the invention provides HTS polypeptide and protein variants, wherein the variants have the single site mutations of Table 9 relative to the amino acid sequence of SEQ ID NO:3 and in addition wherein the methionine at position 1 in SEQ ID NO:3 is absent.

[0019] In another aspect of the invention, amino acid mutations of HTS polypeptide and protein variants are summarized in Table 10. The mutations of Table 10 are identified relative to the amino acid sequence of SEQ ID NO:3. Thus, a polypeptide having an amino acid sequence of a variant listed in Table 10 will have the amino acid sequence of SEQ ID NO:3, but with one or more site mutations identified in Table 10. Mutants of Table 10 also include those in which the methionine at position 1 of the protein is absent. In a related aspect, the invention provides HTS polypeptide and protein variants, wherein the variants have the single site mutations of Table 8 relative to the amino acid sequence of SEQ ID NO:3 and in addition wherein the methionine at position 1 in SEQ ID NO:3 is absent.

[0020] In another aspect of the invention, the HTS polypeptide and protein variants are those having the single-site mutations listed in Table 7, which are identified relative to the amino acid sequence of SEQ ID NO:3. In a related aspect of the invention, the HTS polypeptide and protein variants are those having the single-site mutations listed in Table 7 relative to the amino acid sequence of SEQ ID NO:3, and in addition wherein the methionine at position 1 in SEQ ID NO:3 is absent.

[0021] In another aspect of the invention, the HTS polypeptide and protein variants are other than those having the single-site mutations listed in Table 7 which are identified relative to the amino acid sequence of SEQ ID NO:3. In a related aspect, the present invention excludes polynucleotides encoding the HTS polypeptide and protein variants having the single-site mutations listed in Table 7. In a related aspect of the invention, the HTS polypeptide and protein variants are other than those having the single-site mutations listed in Table 7 and wherein the methionine at position 1 in SEQ ID NO:3 is absent. In a related aspect, the present invention excludes polynucleotides encoding the HTS polypeptide and protein variants having the single-site mutations listed in Table 7 and wherein the methionine of SEQ ID NO:3 is absent.

[0022] In another aspect of the invention, the HTS polypeptide or protein variants do not include a mutation of Table 7. In a related aspect, the present invention excludes HTS polypeptide or protein variants comprising a mutation listed in Table 7. In a related aspect, the present invention excludes polynucleotides encoding a HTS polypeptide or protein variant comprising a mutation listed in Table 7.

[0023] In another aspect of the invention, the HTS polypeptide or protein variants do not include a mutation of Table 3 or Table 6. In a related aspect, the present invention excludes HTS polypeptide or protein variants comprising a mutation listed in Table 3 or Table 6. In a related aspect, the present invention excludes polynucleotides encoding a HTS polypeptide or protein variant comprising a mutation listed in Table 3 or Table 6.

[0024] In yet another aspect of the invention, the HTS polypeptide or protein variant does not include a mutation of Table 8, Table 9 or Table 10, but does include 1 or more conservative mutations of the amino acid sequence of SEQ ID NO:3 or optionally of SEQ ID NO:141, wherein, in the variant, the methionine at position 1 is present or absent. In a related aspect, the present invention includes polynucleotides encoding a HTS polypeptide or protein variant that does not include a mutation of Table 8, Table 9 or Table 10, but does include 1 or more conservative mutations of the amino acid sequence of SEQ ID NO:3, wherein, in the variant, the methionine at position 1 is present or absent.

[0025] In other aspects, the polynucleotide encoding the polypeptide having flavor-modulation or sweet-taste modulation activity is optionally operably linked to a heterologous regulatory element. Additionally or alternatively, the polynucleotide sequence further encodes a protein/peptide tag or

label. The protein/peptide tag is optionally an affinity tag. The protein tag is optionally a histidine tag (His tag). Optionally the protein tag is (His)₆. In an embodiment, the polynucleotide encoding the polypeptide having sweet-taste modulation activity is other than the nucleotide of SEQ ID NO:2 further encoding a protein/peptide tag or label.

[0026] An aspect of the invention provides a polynucleotide (e.g., isolated polynucleotide) encoding a polypeptide, where the encoded polypeptide is selected from the group consisting of: (a) a polypeptide sequence selected from the group consisting of the amino acid sequences of a variant listed in Table 8, Table 9 or Table 10, where the polypeptide further consists of a protein/peptide tag, optionally an affinity tag, or optionally a histidine tag; (b) a polypeptide having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of the amino acid sequences of a variant listed in Table 8, Table 9 or Table 10, where the polypeptide further consists of a protein/peptide tag, optionally an affinity tag, or optionally a histidine tag; and (c) a polypeptide sequence modified from the polypeptide sequence selected from the group consisting of the amino acid sequences of a variant listed in Table 8, Table 9 or Table 10 by deletion, insertion, substitution, or addition of no more than 24 amino acids and wherein the polypeptide further consists of a protein/peptide tag, optionally an affinity tag or optionally a histidine tag and where the polypeptide sequence is different from that of SEQ ID NO:3 containing the histidine tag. In a related embodiment, the encoded polypeptide is different from that of SEQ ID NO:142 containing a histidine tag.

[0027] In specific aspects, the polynucleotide encoding the polypeptide having sweet-taste modulation activity is other than SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, SEQ ID NO:120, SEQ ID NO:121, SEQ ID NO:122, SEQ ID NO:123, SEQ ID NO:125, SEQ ID NO:126, SEQ ID NO:128, SEQ ID NO:129, SEQ ID NO:130, SEQ ID NO:131, SEQ ID NO:133, SEQ ID NO:134, SEQ ID NO:135, SEQ ID NO:136, SEQ ID NO:138, SEQ ID NO:139 or SEQ ID NO:140 as identified in international patent application PCT/US2022/82443 and U.S. patent application 18/146,958.

[0028] Another aspect of the invention provides a polynucleotide other than the polynucleotide of SEQ ID NO:4, which corresponds to the coding sequence optimized for His tagged HTS in *E. coli* (wherein residues 364-381 correspond to an optional His tag sequence). Another aspect of the invention provides a polynucleotide other than the polynucleotide of SEQ ID NO:6, which corresponds to the

coding sequence of HTS optimized for expression in *S. cerevisiae* (to which a coding sequence for a His-tag, e.g., (His)₆ is added).

[0029] In a specific embodiment, the invention provides Myd variants which exhibit enhanced thermostability with respect to native isoforms of Myd proteins (HTS-1 and HTS-2). In specific embodiment, the invention provides a

[0030] In additional aspects, the invention provides an expression cassette comprising a polynucleotide herein and a vector comprising the polynucleotide, as well as a host cell transformed with the vector. Additionally, a method of producing a protein having sweet-taste modulation activity, comprising culturing the transformed host cell in a medium under conditions that result in producing the protein having sweet-taste modulation activity also is provided.

[0031] In additional aspects, the invention provides a host cell which expresses a polypeptide as described herein. In embodiments, the host cell is *E. coli*. In embodiments, the host cell is a plant cell.

[0032] Another aspect of the invention provides a polypeptide (e.g., isolated polypeptide) having sweet-taste modulation activity and comprising (i) a polypeptide sequence selected from those amino acid sequences of a variant listed in Table 8, Table 9 or Table 10; wherein the polypeptide sequence is different from SEQ ID NO:3 and optionally different from SEQ ID NO:141 or (ii) a polypeptide having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of those amino acid sequences of a variant listed in Table 8, Table 9 or Table 10, wherein the polypeptide sequence is different from SEQ ID NO:3 and optionally different from SEQ ID NO:141. In additional aspects, the polypeptide (a) contains at least one modification relative to a polypeptide sequence selected from the group consisting of an amino acid sequence of a variant listed in Table 8, Table 9 or Table 10 by deletion, insertion, substitution, or addition of no more than 24 amino acids, wherein the polypeptide is different from polypeptide SEQ ID NO:3 and optionally different from SEQ ID NO:141, and/or (b) further comprises a protein/peptide tag, optionally an affinity tag, and particularly a histidine tag, and wherein the polypeptide has sweet-taste modulation activity.

[0033] In another aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by at least one mutation selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by two mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by three mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a

polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by four mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by five mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by six mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In further embodiments of the preceding embodiments, the 1-6 modifications are other than removal of the methionine at position 1. In embodiments, in the polypeptides having 1-6 amino acid modifications, the methionine at position 1 is present or absent. The invention also provides polynucleotides encoding the foregoing mutant (variant) polypeptides of SEQ ID NO:3. The invention further provides the foregoing mutant polypeptides which further comprise a protein/peptide tag, optionally an affinity tag and more specifically a histidine tag. The invention also provides polynucleotides encoding the foregoing mutant polypeptides of SEQ ID NO:3 which mutants further comprise a protein/peptide tag, optionally an affinity tag and more specifically a histidine tag. In an embodiment, the polypeptide excludes polypeptides comprising a mutation listed in Table 7.

[0034] In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by seven mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by eight mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by nine mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by ten mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by eleven mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twelve mutations at

different positions selected from those listed in Table 8, Table 9 or Table 10. In further embodiments of the preceding embodiments, the 1-12 modifications are other than removal of the methionine at position 1. In embodiments, in the polypeptides having 1-12 amino acid modification, the methionine at position 1 is present or absent. The invention also provides polynucleotides encoding the foregoing mutant polypeptides of SEQ ID NO:3. The invention further provides the foregoing mutant polypeptides which further comprise a protein/peptide tag, optionally an affinity tag and more specifically a histidine tag. The invention also provides polynucleotides encoding the foregoing mutant polypeptides of SEQ ID NO:3 which further comprise a protein/peptide tag, optionally an affinity tag and more specifically a histidine tag. In an aspect, the polypeptide excludes polypeptides comprising a mutation listed in Table 7.

[0035] In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by thirteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by fourteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by fifteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by sixteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by seventeen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by eighteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In further embodiments of the preceding embodiments, the 1-18 modifications are other than removal of the methionine at position 1. In embodiments, in the polypeptides having 1-18 amino acid modification, the methionine at position 1 is present or absent. The invention also provides polynucleotides encoding the foregoing mutant polypeptides of SEQ ID NO:3. The invention further provides the foregoing mutant polypeptides which further comprise a protein/peptide tag, optionally an affinity tag and more specifically a histidine tag. The invention also provides polynucleotides encoding the foregoing mutant polypeptides of SEQ ID NO:3 which further comprise a protein/peptide

tag, optionally an affinity tag and more specifically a histidine tag. In an aspect, the polypeptide excludes polypeptides comprising a mutation listed in Table 7.

[0036] In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by nineteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twenty mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twenty-one mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twenty-two mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twenty-three mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twenty-four mutations at different positions selected from those listed in Table 8, Table 9 or Table 10. In further embodiments of the preceding embodiments, the 1-24 modifications are other than removal of the methionine at position 1. In embodiments, in the polypeptides having 1-24 amino acid modification, the methionine at position 1 is present or absent. The invention also provides polynucleotides encoding the foregoing mutant polypeptides of SEQ ID NO:3. The invention further provides the foregoing mutant polypeptides which further comprise a protein/peptide tag, optionally an affinity tag, and more specifically a histidine tag. The invention also provides polynucleotides encoding the foregoing mutant polypeptides of SEQ ID NO:3 which further comprise a protein/peptide tag, optionally an affinity tag and more specifically a histidine tag. In an aspect, the polypeptide excludes polypeptides comprising a mutation listed in Table 7.

[0037] Another aspect of the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by at least one mutation, where the at least one mutation is selected from the mutations listed in Table 8, Table 9 or Table 10 and is not a mutation listed in Table 7, and where the polypeptide is also modified by one or more additional mutations, where the one or more additional mutations are selected from the

mutations listed in Table 7. In a further aspect, the polypeptide is modified by two or more mutations selected from the mutations listed in Table 8, Table 9 or Table 10. In an aspect, the polypeptide is also modified by two or more additional mutations, where the two or more additional mutations are selected from the mutations listed in Table 7. In an aspect, the polypeptide has at least 80% sequence identity to a polypeptide sequence selected from the group consisting of those amino acid sequences of variants listed in Table 8, Table 9 or Table 10. The forgoing polypeptides optionally further comprise a protein/peptide tag, optionally an affinity tag or optionally a histidine tag.

[0038] In another aspect, the polypeptides of the invention are optionally isolated and/or optionally purified. In another aspect, the polynucleotides of the invention are optionally isolated and/or purified. In another aspect, the polypeptides of the invention optionally comprise a protein/peptide tag, optionally an affinity tag or optionally a histidine tag. In another aspect, the polypeptides of the invention optionally comprise an affinity tag. In another aspect, the polypeptides of the invention optionally comprise a histidine tag. In another aspect, the polypeptides of the invention optionally have a methionine at position 1. In another aspect, the polypeptides of the invention are optionally derivatized as described herein. In another aspect, the polypeptides of the invention are acylated, and optionally acetylated, at the N-terminus. In another aspect, the polypeptides of the invention are acylated at the N-terminus. In another aspect, the polypeptides of the invention have a methionine at position 1 and the methionine is derivatized and optionally the S of the methionine is oxidized. In another aspect, the polypeptides of the invention have a methionine at position 1 and the methionine is derivatized wherein the S of the methionine is oxidized. In another aspect, the polypeptides of the invention are pegylated at the N-terminus. In another aspect, the polypeptides of the invention are derivatized at the C-terminus as described herein. In another aspect, the polypeptides of the invention are esterified at the C-terminus.

[0039] In other aspects, certain polypeptides of the invention exhibit sweet-taste modulation activity that is enhanced compared to the polypeptide of SEQ ID NO:3. In other aspects certain polypeptides of the invention exhibit thermal stability that is enhanced compared to the polypeptide of SEQ ID NO:3.

[0040] Another aspect of the invention provides a composition which comprises one or more polypeptides of this invention which exhibit flavor modulation and/or sweet taste modulation activity and/or exhibit sweet taste. The composition includes a combination of (a) a product for oral administration other than *Mattirolomyces terfezioides* truffle, and (b) a sweetening composition comprising the polypeptide, wherein the combination has enhanced sweet taste and/or change or enhancement in flavor compared to the product for oral administration. The polypeptide includes one or more polypeptide sequences, either individually or in a combination of two or more thereof having

flavor modulation and/or sweet-taste modulation activity and comprising (i) a polypeptide sequence selected from an amino acid sequence of a variant of Table 8, Table 9 or Table 10, wherein the polypeptide sequence is different from SEQ ID NO:3 and optionally different from SEQ ID NO:141; or (ii) a polypeptide having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of an amino acid sequence of a variant of Table 8, Table 9 or Table 10, wherein the polypeptide sequence is different from SEQ ID NO:3 and optionally different from SEQ ID NO:141; or (iii) wherein the polypeptide of a variant of Table 8, Table 9 or Table 10 further comprises a protein/peptide tag, or affinity tag, wherein the protein/peptide tag is optionally a histidine tag.

[0041] Another aspect of the invention is a sweetening composition which comprises one or more polypeptides of the invention (e.g., HTS variants) exhibiting sweet taste. A related aspect of the invention is a flavor modulation composition which comprises one or more polypeptides of the invention (e.g., HTS variants) exhibiting flavor modulation activity. In embodiments, sweetening compositions contain 30 ppm or more of total HTS variant polypeptide. In embodiments, flavor modulation compositions contain 30 ppm or less of total HTS variant polypeptide.

[0042] Another more specific aspect of the invention provides a composition including a combination of (a) a product for oral administration other than *Mattirolomyces terfezioides* truffle, and (b) a sweetening composition comprising the polypeptide, wherein the combination has enhanced sweet taste compared to the product for oral administration. In embodiments, the polypeptide includes (a) one or more amino acid sequences selected from the group consisting of the amino acid sequences of a variant of Table 8, Table 9 or Table 10. In embodiments, the polypeptide is not a variant listed in Table 7, Table 3 or Table 6.

[0043] Another aspect of the invention provides a method for modulating the taste of a product for oral administration. The method comprises combining the product for oral administration with an effective amount of a flavor modulation composition or a sweetening composition comprising the polypeptide (e.g., a HTS variant), wherein the product for oral administration differs from *Mattirolomyces terfezioides* truffle, and wherein the combination has enhanced flavor or sweet taste, respectively, compared to the product for oral administration. In embodiments, the polypeptide includes one or more sequences as listed herein, particularly those having one or more mutations of Table 8, Table 9 or Table 10, either individually or in a combination of two or more thereof. In embodiments, the polypeptide is not a variant listed in Table 7, Table 3 or Table 6.

[0044] Another aspect of the invention provides a method of purifying a polypeptide having flavor modulation activity or sweet-taste modulation activity. The method includes: (a) conducting hydrophobic interaction chromatography (HIC) on a composition comprising a polypeptide, and/or (b) conducting size exclusion chromatography (SEC) on the composition comprising the polypeptide. In

embodiments, the method includes (a) conducting hydrophobic interaction chromatography (HIC) on a composition comprising a polypeptide, or (b) conducting size exclusion chromatography (SEC) on the composition comprising the polypeptide. In embodiments, the method includes (a) conducting hydrophobic interaction chromatography (HIC) on a composition comprising a polypeptide to generate a purified composition, and thereafter (b) conducting size exclusion chromatography (SEC) on the purified composition comprising the polypeptide. In embodiments, the polypeptide includes one or more sequences, either individually or in a combination of two or more thereof having sweet-taste modulation activity and comprising (i) a polypeptide sequence selected from that of a variant of Table 8, Table 9 or Table 10, wherein the polypeptide sequence is different from SEQ ID NO:3 and optionally different from SEQ ID NO:141; or (ii) a polypeptide having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of that of a variant of Table 8, Table 9 or Table 10, wherein the polypeptide sequence is different from SEQ ID NO:3 and optionally different from SEQ ID NO:141; (iii) wherein the polypeptide sequence of the variants of Table 8, Table 9 or Table 10 further comprises a protein/peptide tag, wherein the protein tag is optionally an affinity tag or more specifically a histidine tag; (iv) wherein the polypeptide contains 1-24 mutations selected from those listed in Table 8, Table 9 or Table 10 and optionally further comprises a protein/peptide tag, wherein the protein tag is optionally an affinity tag and more specifically a histidine tag. In a related aspect, the invention provided polypeptides that are purified by the method described herein.

[0045] Another aspect of the invention provides sweetener compositions. In some embodiments, the sweetener compositions include: (a) one or more polypeptide, wherein the one or more polypeptide comprises: (i) a polypeptide sequence selected from amino acid sequences of any variant listed in Table 8, Table 9 or Table 10; (ii) a polypeptide sequence having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of amino acid sequences of any variant listed in Table 8, Table 9 or Table 10; (iii) a polypeptide sequence containing at least one modification by deletion, insertion, substitution, or addition of no more than 24 amino acids, relative to a polypeptide sequence selected from the group consisting of an amino acid sequence of a variant listed in Table 8, Table 9 or Table 10; or (iv) a polypeptide of any of (i)-(iii), wherein the methionine at position 1 is absent; and/or the polypeptide further comprises a protein/peptide tag, optionally an affinity tag or optionally a histidine tag or optionally a (His)₆ tag; and (b) at least one additional sweetener other than a Myd sweet protein.

[0046] The additional sweetener is optionally selected from a steviol glycoside sweetener, a mogroside sweetener, sucrose, allulose, sucralose, a polyol, and high fructose corn syrup (HFCS). In other embodiments, the sweetener compositions include: (a) one or more polypeptide, wherein the one or more polypeptide comprises: (i) a polypeptide sequence selected from amino acid sequences of any

variant listed in Table 8, Table 9 or Table 10; (ii) a polypeptide sequence having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of amino acid sequences of any variant listed in Table 8; Table 9 or Table 10; (iii) a polypeptide sequence containing at least one modification by deletion, insertion, substitution, or addition of no more than 24 amino acids, relative to a polypeptide sequence selected from the group consisting of an amino acid sequence of a variant listed in Table 8, Table 9 or Table 10; or (iv) a polypeptide of any of (i)- (iii), wherein the methionine at position 1 is absent; and/or the polypeptide further comprises a protein/peptide tag, optionally an affinity tag or optionally a histidine tag or optionally a (His)₆ tag; and (b) at least one additional component selected from monosaccharides, disaccharides (e.g., sucrose), sugar alcohols (e.g., mannitol), amino acids (e.g., leucine), organic acids (e.g., citric acid), hypoxanthine, theophylline, vitamins and combinations thereof.

[0047] In embodiments, sweetener composition herein comprise at least one additional component selected from sucrose, mannitol, citric acid, hypoxanthine, theophylline, leucine, and combinations thereof.

[0048] In specific embodiments, the polypeptide of the sweetener compositions has the amino acid sequence of SEQ ID NO:3. In specific embodiments, the polypeptide of the sweetener composition has the amino acid sequence of SEQ ID NO:3 where the methionine at position 1 is missing. In embodiments, the polypeptide of the sweetener is a mixture of a polypeptide having amino acid sequence of SEQ ID NO:3 and a polypeptide having amino acid sequence of SEQ ID NO:3 where the methionine at position 1 is missing.

[0049] In embodiments, the polypeptide of the sweetener compositions is present in an amount between about 1 ppm and about 50 ppm. In some embodiments, the polypeptide is present in an amount between about 1 ppm and about 40 ppm. In further embodiments, the polypeptide is present in an amount selected from an amount between about 1 ppm and about 30 ppm, about 1 ppm and about 25 ppm, about 1 ppm and about 20 ppm or about 1 ppm and about 15 ppm. In some embodiments, the polypeptide is present in an amount between about 5 ppm and about 50 ppm. In some embodiments, the polypeptide is present in an amount between about 10 ppm and about 50 ppm. In some embodiments, the polypeptide is present in an amount between about 15 ppm and about 50 ppm. In some embodiments, the polypeptide is present in an amount between about 20 ppm and about 50 ppm. In some embodiments, the polypeptide is present in an amount between about 25 ppm and about 50 ppm. In some embodiments, the polypeptide is present in an amount between about 30 ppm and about 50 ppm. In some embodiments, the polypeptide is present in an amount between about 35 ppm and about 50 ppm. In some embodiments, the polypeptide is present in an amount between about 40 ppm and about 50 ppm. In some embodiments, the polypeptide is present in an amount between about 45 ppm and about 50 ppm. In some embodiments, the polypeptide is present in an amount between about

10 ppm and about 40 ppm. In some embodiments, the polypeptide is present in an amount between about 20 ppm and about 30 ppm. In some embodiments, the steviol glycoside sweetener is selected from rebaudioside M (“Reb M”), RebM80, rebaudioside D (“Reb D”), Reb A95 and rebaudioside A (“Reb A”). In embodiments, the mogroside sweetener is selected from siamenoside I and mogroside V.

[0050] In another aspect, the invention provides a combination of a product for oral administration, wherein the product is not *Mattirolomyces terfezioides* truffle, and a sweetening composition described herein. In some embodiments, the product for oral administration has at least one improved organoleptic property compared to a product for oral administration that does not contain the sweetener composition, wherein the organoleptic property is selected from the group consisting of aroma, flavor, basic taste (sweetness, sour, saltiness, bitterness or umami), aftertaste or linger, temporal profile, mouthfeel or a combination thereof. In embodiments, the product for oral administration is a solid food product or a beverage product. In embodiments, the product for oral administration is a yogurt product. In embodiments, the product for oral administration is a chewing gum product.

[0051] In another aspect, the invention provides a beverage or beverage product comprising the sweetener composition described herein. In some embodiments, the beverage or beverage product has at least one improved organoleptic property compared to a beverage or beverage product that does not contain the sweetener composition, wherein the organoleptic property is selected from the group consisting of aroma, flavor, basic taste (sweetness, sour, saltiness, bitterness or umami), aftertaste or linger, temporal profile, mouthfeel or a combination thereof. In some embodiments, the beverage or beverage product is selected from a low-calorie or no-calorie beverage or beverage product. In embodiments, the beverage is selected from cola, ginger-ale, soft drinks, root beer, fruit juice, fruit-flavored juice, vegetable juice, vegetable-flavored juice, sports drinks, energy drinks, plant protein beverage, near water drinks (e.g., water with natural or synthetic flavorants), tea type (e.g. black tea, green tea, red tea, oolong tea), coffee, cocoa drink, beverage containing milk components (e.g. milk beverages, coffee containing milk components, café au lait, milk tea, fruit milk beverages). In embodiments, the beverage or beverage product includes at least one organic acid additive salt that is a sodium, calcium, potassium, or magnesium salt of an organic acid. In embodiments, the organic acid is selected from citric acid, malic acid, tartaric acid, fumaric acid, lactic acid, alginic acid, ascorbic acid, benzoic acid and adipic acid.

[0052] In another aspect, the invention provides methods of improving at least one organoleptic property of the product for oral administration described herein. In embodiments the product for oral administration is a solid food product or a liquid beverage product. In an embodiment, the invention

provides methods of improving at least one organoleptic property of the product for oral administration described herein. The methods include adding a sweetener composition as described herein to a solid or liquid matrix, thereby providing the product having at least one improved organoleptic property. In embodiments, the improved organoleptic property is selected from the group consisting of aroma, flavor, basic taste (sweetness, sour, saltiness, bitterness or umami), aftertaste or linger, temporal profile, mouthfeel or a combination thereof. In embodiments, the amount of polypeptide present in the product improved by the methods is an amount between about 1 ppm and about 50 ppm, about 1 ppm and about 40 ppm, about 1 ppm and about 30 ppm, about 1 ppm and about 25 ppm, about 1 ppm and about 20 ppm or about 1 ppm and about 15 ppm. In embodiments, the product is a solid food product and the sweetener composition is added to, combined with or mixed into the solid food product.

[0053] In embodiments, the invention provides methods of improving at least one organoleptic property of the beverage or beverage product described herein. The methods include adding a sweetener composition as described herein to a liquid matrix, thereby providing a beverage or beverage product having at least one improved organoleptic property. In embodiments, the improved organoleptic property is selected from the group consisting of aroma, flavor, basic taste (sweetness, sour, saltiness, bitterness or umami), aftertaste or linger, temporal profile, mouthfeel or a combination thereof. In embodiments, the amount of polypeptide present in the beverage or beverage product improved by the methods is an amount between about 1 ppm and about 50 ppm, about 1 ppm and about 40 ppm, about 1 ppm and about 30 ppm, about 1 ppm and about 25 ppm, about 1 ppm and about 20 ppm or about 1 ppm and about 15 ppm.

[0054] In another aspect, the invention provides methods of changing/modifying at least one organoleptic property of a product for oral administration as described herein. The methods include adding a sweetener composition as described herein to the product, thereby providing a product having at least one changed/modified organoleptic property. In embodiments, the changed/modified organoleptic property is selected from the group consisting of aroma, flavor, basic taste (sweetness, sour, saltiness, bitterness or umami), aftertaste or linger, temporal profile, mouthfeel or a combination thereof. In embodiments, the amount of polypeptide present in the product changed/modified by the methods is an amount between about 1 ppm and about 50 ppm, about 1 ppm and about 40 ppm, about 1 ppm and about 30 ppm, about 1 ppm and about 25 ppm, about 1 ppm and about 20 ppm or about 1 ppm and about 15 ppm. In embodiments, the product for oral administration is a food product or a beverage product. In embodiments, the product is a solid food product and the sweetener composition is added to, combined with or mixed into the solid food product.

[0055] In another aspect, the invention provides methods of changing/modifying at least one organoleptic property of the beverage or beverage product described herein. The methods include

adding a sweetener composition as described herein to a liquid matrix, thereby providing a beverage or beverage product having at least one changed/modified organoleptic property. In embodiments, the changed organoleptic property is selected from the group consisting of aroma, flavor, basic taste (sweetness, sour, saltiness, bitterness or umami), aftertaste or linger, temporal profile, mouthfeel or a combination thereof. In embodiments, the amount of polypeptide present in the beverage or beverage product changed/modified by the methods is an amount between about 1 ppm and about 50 ppm, about 1 ppm and about 40 ppm, about 1 ppm and about 30 ppm, about 1 ppm and about 25 ppm, about 1 ppm and about 20 ppm or about 1 ppm and about 15 ppm.

[0056] Other aspects and embodiments of the invention will be apparent on review of the figures, detailed description and non-limiting examples herein.

BRIEF DESCRIPTION OF THE FIGURES

[0057] Figure 1 shows a Coomassie-stained SDS-PAGE gel of proteins obtained from fractions of partially purified *M. terfezioides* gleba.

[0058] Figure 2 shows a SDS-PAGE gel, Coomassie stained, of purification steps of His-tagged sweet polypeptide expressed in *E. coli* from the coding region of SEQ ID NO:4 lane 1: molecular weight standards; lane 3, crude lysate; lane 4, flow through fraction from HisPur™ Ni-NTA; lane 5, wash 1; lane 6, wash 2; lane 7, wash 3; lane 8, elution fraction. The Hist-tagged sweet protein expressed in *E. coli* is the His-tagged version of HTS-2 in which methionine is absent (SEQ ID NO:142)

[0059] Figure 3 shows the concentration-response functions for sweet taste for mycodulcein (Honey Truffle Sweetener (HTS)), aspartame, thaumatin, rebaudioside A. Data are plotted as the proportion (*p*) of responses occurring on the 200 mM sucrose-associated (“sweet”) target. Each data point in the curves for mycodulcein, aspartame, thaumatin, rebaudioside A was calculated as the average across 32 replicates and averaged across 16 replicates for the sucrose curve; error bars are SEM. Points for water and sucrose controls were similarly calculated as the average across 128 and 64 replicates, respectively. Curves were fit by non-linear regression.

[0060] Figure 4A shows SDS-PAGE analysis, Coomassie stain, of the eluted fraction from Capto MMC a multimodal weak cation exchange resin. M: protein marker; lane 1: eluted fraction, showing low purity after cation exchange. Arrow indicates mycodulcein (native HTS) band.

[0061] Figure 4B shows SDS-PAGE analysis, Coomassie stain, of two eluted fractions collected during the gradient elution from the HiScreen Capto Butyl (Cytiva Sweden AB, Uppsala, Sweden) column analyzed on SDS-PAGE. Lane 1 shows eluted fraction 1 not containing mycodulcein and Lane 2 shows eluted mycodulcein. The purity of the eluted fraction was determined by GelAnalyzer to be ~86%. Arrow indicates mycodulcein (native HTS) band.

[0062] Figure 4C shows SDS-PAGE analysis, Coomassie stain, the eluted protein from the HIC column after chromatographing on HiPrep® 26/60 Sephacryl® S-200 (Cytiva Sweden AB, Upsala, Sweden). Lane 1 shows purified his-tag mycodulcein and Lane 2 shows purified native mycodulcein. The purity of the eluted fraction was determined by GelAnalyzer to be ~98%. Arrow indicates mycodulcein (native HTS) band.

[0063] Figure 5 is a map of the pD44-CH vector which is isopropyl beta-D-1-thiogalactopyranoside (IPTG) inducible.

[0064] Figures 6A and 6B include graphs of exemplary melting temperature results obtained from GloMelt™ thermostability assays comparing native HTS (1) and HTS variant S33C_I49C (double mutant, 2). Fig. 6A compares melt curves for native HTS (1) and the double mutant (2). Fig. 6B compares melt peak curves for 1 and 2. Relative fluorescence (RFU) is measured as a function of temperature. An increase in average melting temperature of 7.5 °C is observed with the double mutant compared to native HTS protein.

[0065] Figure 7 shows melting temperature results obtained from GloMelt™ thermostability assays comparing native HTS protein with HTS variant double mutation S33C_I49C. In this assay, the sample was brought to a low pasteurization temperature (63 °C) and held for an hour.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0066] The invention provides embodiments of isolated nucleotides encoding Myd1 polypeptide variants, the encoded Myd1 polypeptide variants (also called Honey Truffle Sweetener (HTS) proteins and variants thereof) that are capable of flavor modulation and/or modulating sweet taste and perception, and methods of making and using such. The invention provides embodiments of MYD1 variants and Myd1 polypeptide variants either alone, or in combination with food, beverage, dietary supplement, or pharmaceuticals; and methods of modifying the taste of such foods, beverages, dietary supplements, or pharmaceutical compositions with the isolated polynucleotides and polypeptides of the invention. The invention provides naturally occurring Myd1 polypeptides and nucleic acids encoding these polypeptides. The invention provides non-naturally occurring Myd1 polypeptide variants and nucleic acids encoding these polypeptides.

[0067] An embodiment of the invention provides Myd polypeptide variants (also referred to as HTS variants). The term “Myd polypeptide variants” is used herein to identify non-naturally occurring HTS polypeptides and includes any of the polypeptides according to the present invention which e.g., have at least 80% sequence identity to a variant of Table 8, Table 9 or Table 10 and also have flavor modulation (or modifying) and/or sweet-taste modifying activity.

[0068] A sweet tasting partially purified extract of *terfezioides* gleba was subjected to *de novo* amino acid sequencing to identify a 20-mer N-terminal sequence. The Myd1 coding sequence (putatively derived from the *MYD1* gene) was identified after the whole transcriptome of the *Mattirolomyces. terfezioides* gleba was *de novo* assembled using RNAseq reads. Screening the *M. terfezioides* whole transcriptome using the 20-mer N-terminus sequences identified a transcript predicted to encode a protein with 100% identity at the N-terminus. The identified transcript is predicted to encode a 121 amino acid protein. This method identified the polynucleotide of SEQ ID NO:1. Start and stop codons were identified in the transcript to identify putative coding sequence SEQ ID NO:2. SEQ ID NO:3 is the putative encoded protein, a 121 amino acid protein. The naturally-occurring protein isolated from *M. terfezioides* gleba was subsequently found to be a mixture of two isoforms the protein of SEQ ID NO:3 and a mature protein of SEQ ID NO: 3 with the methionine (Met) residue at amino acid position 1 deleted (designated SEQ ID NO:141 hereinafter). Identity between the predicted protein SEQ ID NO:3, and other protein sequences in GENBANK were 31% or less.

[0069] Native HTS appears to be a mixture of two isoforms (HTS-1 and HTS-2). HTS-1 is the polypeptide of SEQ ID NO:3. HTS-2 is the polypeptide of SEQ ID NO:141. Native HTS isolated from *M. terfezioides*, as described herein, is a mixture of about 40-60% by weight HTS-2 and 60 to 40% by weight HTS-1. The relative amount of HTS-1 and HTS-2 isolated from *M. terfezioides* varies at least by truffle source. As isolated, about 80% of HTS-1 is acetylated at the N-terminal amine group. No significant difference in sweet taste has as yet been observed for the two sequence isoforms of HTS. Acetylation of the N-terminus of the HTS polypeptide HTS-1 also does not have a significant effect on sweet taste.

[0070] The polynucleotide encoding the polypeptide of SEQ ID NO:3 has been successfully expressed in multiple hosts. In some cases, the polynucleotide sequence encoding the polypeptide of SEQ ID NO:3 has been codon-optimized, as known in the art, for expression in a given host. For example, intracellular expression in *E. coli* using optimized codons to express the polynucleotide encoding SEQ ID NO:3 using nucleotide coding sequence of SEQ ID NO:4 results in a sweet tasting protein. For example, intracellular expression in *E. coli* using optimized codons to express the polynucleotide encoding SEQ ID NO:3 and a (His)₆ tag results in a sweet tasting protein. The sweet protein with His-tag expressed from SEQ ID NO:4 in *E. coli* has subsequently been determined by proteome analysis to be SEQ ID NO:141 (SEQ ID NO:3 without methionine in position 1) with a His-tag (SEQ ID NO: 148) expression of the HTS-1 isoform of the protein is not observed. The sweet protein of SEQ ID NO:141 (without His-tag) is the protein expressed on expression of the optimized *E. coli* coding region without the His-tag (SEQ ID NO:148).

[0071] For example, intracellular expression in *Saccharomyces cerevisiae* using optimized codons (SEQ ID NO:6) to express the polynucleotide encoding SEQ ID NO:3 with an additional His-tag coding sequence results in sweet tasting protein. The protein expressed is a mixture of HTS-1 and HTS-2 isoforms each with His-tags. The relative amounts of HTS-2 to HTS-1 is about 60% to 40% by weight. Extracellular expression in *Saccharomyces cerevisiae* using optimized codons and an appropriate signal peptide at the N-terminal to facilitate secretion into the fermentation media resulted in an expression product that was not sweet.

[0072] For example, intracellular expression in *Yarrowia lipolytica* using optimized codons to express the polynucleotide encoding SEQ ID NO:3 results in sweet tasting protein. The protein expressed is a mixture of HTS-1 and HTS-2 isoforms. The relative amounts of HTS-2 to HTS-1 is about 20% to 80% by weight.

[0073] For example, intracellular expression in *Pichia pastoris* using optimized codons to express the polynucleotide encoding SEQ ID NO:3 results in sweet tasting protein. The protein expressed is a mixture of HTS-1 and HTS-2 isoforms. The relative amounts of HTS-2 to HTS-1 is about 90% to 10% by weight.

[0074] The coding sequences for native mycodulcein (HTS), which have been codon-optimized for expression in *E. coli* and *Saccharomyces cerevisiae* correspond to the nucleic acid sequences of SEQ ID NO:4 and SEQ ID NO:6, respectively (which both encode the amino acid sequence of SEQ ID NO:3 with an optional 6 residue histidine tag, i.e., the amino acid sequence of SEQ ID NO:5).

[0075] In embodiments, the invention provides non-naturally occurring sweet proteins which include non-naturally occurring mixtures of the two isoforms HTS-1 and HTS-2. In embodiments, the invention provides HTS-2 (SEQ ID NO:141) substantially free of the other isoform HTS-1, where substantially free means less than 10% by weight of HTS-1 (SEQ ID NO:3). In embodiments, the invention provides HTS-2 (SEQ ID NO:141) free of the other isoform HTS-1, where free means less than 1% by weight of HTS-1 (SEQ ID NO:3). In embodiments, the invention provides HTS-1 (SEQ ID NO:3) substantially free of the other isoform HTS-2, where substantially free means less than 10% by weight of HTS-1 (SEQ ID NO:141). In embodiments, the invention provides HTS-1 (SEQ ID NO:3) free of the other isoform HTS-2, where free means less than 1% by weight of HTS-2 (SEQ ID NO:141). In embodiments, the invention provides non-naturally occurring mixtures of HTS-1 and HTS-2, specifically those enriched in HTS-2 where the amount of HTS-2 is greater than 60% by weight and for example is 65% or more by weight, 70% or more by weight, 75% or more by weight, 80% or more by weight, 85% or more by weight, 90% or more by weight, or 95% or more by weight. In embodiments, the invention provides non-naturally occurring mixtures of HTS-1 and HTS-2, specifically those enriched in HTS-1 where the amount of HTS-1 is greater than 60% by weight and

for example is 65% or more by weight, 70% or more by weight, 75% or more by weight, 80% or more by weight, 85% or more by weight, 90% or more by weight, or 95% or more by weight.

[0076] In embodiments, the invention provides sweet protein generated by recombinant expression of the coding sequence of SEQ ID NO:2 or a codon-optimized version of the coding sequence of SEQ ID NO:2 in a non-native host (e.g., a host other than *Mattirolomyces terfezioides* truffle. In specific embodiment, the sweet protein is generated by recombinant expression in a bacterium, yeast or fungus. In specific embodiments, the non-native host is *Escherichia coli*. In specific embodiments, the non-native host is *Saccharomyces cerevisiae*. In specific embodiments, the non-native host is *Yarrowia lipolytica*. In specific embodiments, the non-native host is *Pichia pastoris*. In embodiments, recombinant expression in a non-native host results in non-naturally occurring mixtures of the HTS-1 and HTS-2 naturally-occurring isoforms of mycodulcein (Myd).

[0077] POLYNUCLEOTIDES

[0078] An embodiment of the invention comprises a polynucleotide (e.g., isolated polynucleotide) encoding a polypeptide variant having sweet-taste modulation activity. Examples of polynucleotides encoding a polypeptide having sweet-taste modulation activity include polynucleotides able to encode polypeptides such as, but not limited to, a variant of Table 8, Table 9 or Table 10, or a polypeptide thereof further having a histidine tag, or an nucleic acid sequence with at least 80% (e.g., 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) sequence identity to a polynucleotides encoding a variant of Table 8, Table 9, or Table 10 or a polypeptide thereof further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag. It will be understood that the polynucleotides, including or excluding the sequence encoding the protein/peptide tag, affinity tag or histidine tag, are both provided and useful in this invention. It will be further understood that for any specific polynucleotide indicated to encode a given histidine tag (e.g., (His)₆), the sequence encoding the histidine tag can be substituted with a sequence encoding a different His tag or a sequence encoding a different protein/peptide tag or affinity tag. In embodiments, the protein/peptide, affinity or histidine tag is one that is encoded by 3-30 or 3-18 nucleotides. In embodiments, the variants recited herein above include a methionine at position 1 or the methionine at position 1 is absent. In embodiments, variant HTS polypeptides are other than the polypeptides of SEQ ID NO:3 or SEQ ID NO:141.

[0079] In an embodiment, the polynucleotide comprises, consists essentially of, or consists of a polynucleotide selected from the group consisting of polynucleotide encoding a variant of Table 8, Table 9 or Table 10, or a nucleic acid sequence with at least 80% (e.g., 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) sequence identity

to a polynucleotide able to encode a variant of Table 8, Table 9 or Table 10. In embodiments, the encoded variant polypeptide is other than the polypeptides of SEQ ID NO:3 or SEQ ID NO:141.

[0080] Unless expressly stated otherwise, when a polynucleotide sequence has a plurality of nucleotide modifications, each modification may independently be modified with a modification of choice such as deletion, insertion, substitution, or addition, regardless of what the other modifications are. Additionally, the plurality of modifications in the polynucleotide sequence may be the same or differ from each other; and the modification options for each modification may independently vary such deletion, insertion, substitution, or addition, etc. unless expressly stated otherwise. When there are a plurality of modifications, each modification may independently be a substitution, addition, insertion, or deletion regardless of what the other modification is or are. In an embodiment, the polynucleotide sequence has at least one substitute modification. In a particular embodiment, the polynucleotide sequence has a plurality of substitution modifications; and each substitution may independently be substituted with a substitution of choice regardless of what the other substitutions are. Additionally, the plurality of substitutions in the polynucleotide sequence may be the same or differ from each other; and the options for nucleotide substitutions for each substitution may independently vary unless expressly stated otherwise. When the polynucleotide sequence has a plurality of substitutions, each substitution may independently be chosen regardless of what the other substitutions is or are. In embodiments, polynucleotides herein are optionally isolated and/or optionally purified.

[0081] In embodiments, at least 80% sequence identity with respect to a polynucleotide includes, without limitation, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% and at least 99% sequence identity. In embodiments, at least 80% sequence identity with respect to a polynucleotide also includes, without limitation, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99% sequence identity.

[0082] In an embodiment, the polynucleotide encodes a polypeptide sequence comprising, consisting essentially of, or consisting of a polypeptide selected from the group consisting of: (a) the amino acid sequence of a variant of Table 8, Table 9 or Table 10, or a polypeptide thereof further having a protein/peptide tag, an affinity tag or a histidine tag; (b) an amino acid sequence having at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to the amino acid sequence of a variant of Table 8, Table 9 or Table 10, or a polypeptide thereof further having a protein/peptide tag, an affinity tag or a histidine tag; and (c) an amino acid sequence modified from an

amino acid sequence of a variant of Table 8, Table 9 or Table 10 by deletion, insertion, substitution, or addition of no more than 24 amino acids (e.g., modification of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 amino acids), or a polypeptide thereof further having a histidine tag. In the above listed embodiments, the polypeptide encoded by the polynucleotide is optionally a polypeptide other than that of SEQ ID NO:3 and optionally is other than that of SEQ ID NO:141. When the amino acid sequence has a plurality of modifications, the number of modifications the amino acid has may range from at least one and up to 24 modifications, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 modifications, as desired. Furthermore, unless expressly stated otherwise, when an amino acid sequence has a plurality of modifications, each modification may independently be modified with a modification of choice such as deletion, insertion, substitution, or addition, regardless of what the other modifications are. Additionally, the plurality of modifications in the amino acid sequence may be the same or differ from each other; and the modification options for each modification may independently vary such deletion, insertion, substitution, or addition, etc. unless expressly stated otherwise. When the amino acid sequence has a plurality of modifications, each modification may independently be a substitution, addition, insertion, or deletion regardless of what the other modification is or are. In an embodiment, the amino acid sequence has at least one substitute modification. In a particular embodiment, the amino acid sequence has a plurality of substitution modifications; and each substitution modification may independently be substituted with a substitution of choice regardless of what the other substitutions are. In an embodiment, the amino acid sequence has one substitution modification (a single mutant) or two substitution modifications (a double mutant) compared to SEQ ID NO:3 or SEQ ID NO:141. Additionally, the plurality of substitutions in the amino acid sequence may be the same or differ from each other; and the options for substitutions for each amino acid may independently vary unless expressly stated otherwise. When the amino acid sequence has a plurality of substitutions, each substitution may independently be chosen regardless of what the other substitutions is or are. In embodiments, the polypeptide encoded may include 1-6, 1-12, 1-18 or 1-24 substitutions compared to the polypeptide of SEQ ID NO:3, wherein the substitutions are those listed in Table 8. In embodiments, the polypeptide encoded may include 1-6, 1-12, 1-18 or 1-24 substitutions compared to the polypeptide of SEQ ID NO:3, wherein the substitutions are those listed in Table 9. In embodiments, the polypeptide encoded may include 1-6, 1-12, 1-18 or 1-24 substitutions compared to the polypeptide of SEQ ID NO:3, wherein the substitutions are those listed in Table 10. In embodiments, the polypeptide encoded may include 1-6, 1-12, 1-18 or 1-24 substitutions compared to the polypeptide of SEQ ID NO:3, wherein the substitutions are any one or more of those listed in Table 3, Table 6, Table 7, Table 8, Table 9 or Table 10. In embodiments, the polypeptide encoded may include 1-6, 1-12, 1-18 or 1-24 substitutions compared to the polypeptide of SEQ ID NO:3, wherein

the substitutions are other than those listed in Table 7. In embodiments, the polypeptide encoded may include 1-6, 1-12, 1-18 or 1-24 substitutions compared to the polypeptide of SEQ ID NO:3, wherein the substitutions are other than those listed in Table 3 or Table 6. In embodiments, the polypeptide encoded may include 1-6, 1-12, 1-18 or 1-24 substitutions compared to the polypeptide of SEQ ID NO:3, wherein the substitutions are other than those listed in Table 3 or Table 6. In embodiments, the polypeptide encoded may include 1-6, 1-12, 1-18 or 1-24 substitutions compared to the polypeptide of SEQ ID NO:3, wherein the substitutions are those listed in Tables 9 and 10 and are other than those listed in Table 3 or Table 6 or Table 7.

[0083] In certain embodiments of polynucleotides herein, the polynucleotide encodes a polypeptide herein and also encodes a protein/peptide tag, an affinity tag or a histidine tag. In all such cases in which a specific polynucleotide encodes such a tag, the invention also provides the polynucleotide excluding the sequence encoding the tag. In additional embodiments, in those polynucleotides herein encoding a histidine tag, the sequence encoding the histidine tag can be deleted or excluded, or can be replaced with a coding sequence for a different protein/peptide tag or a different affinity tag, including a different histidine tag.

[0084] The invention provides isolated and/or purified embodiments of the respective polynucleotides, when applicable. The invention provides embodiments of each of the polynucleotides able to encode the variants of Table 8, Table 9 or Table 10 herein with or without being isolated and with or without being purified, when applicable. The invention provides embodiments of each of the polynucleotides able to encode the variants of Table 8, Table 9 or Table 10 herein with one or more mutations, when applicable.

[0085] The invention also provides an expression cassette comprising one or more polynucleotides encoding a Myd polypeptide variant (HTS variant) and a host cell transformed with the vector.

[0086] The polynucleotide encoding the HTS variant of the present invention can be in the form of a single-stranded or double-stranded DNA, RNA or an artificial nucleic acid, or can be a cDNA or a chemically synthesized DNA which does not comprise any intron. The term “*MYD* family” can refer to polymorphic variants, including natural alleles, mutants, alleles, and interspecies homologs that encode polypeptides that: (1) have at least about 35 to 50% amino acid sequence identity, optionally about 60, 75, 80, 85, 90, 95, 96, 97, 98, or 99% amino acid sequence identity to SEQ ID NO:3 over a window of about 25 amino acids, optionally 50-100 amino acids. In one aspect, the term “isolated” encompasses products that have been removed from a biological environment (e.g., a cell, tissue, culture medium, body fluid, etc.), or otherwise increased in purity to any degree (e.g., isolated from a synthesis medium). Isolated products, thus, can be synthetic or naturally produced. In an embodiment, the term “isolated” encompasses products that have been isolated from other proteins that are present

in the natural environment. In embodiments, the term "isolated" encompasses products that have been isolated from carbohydrates that are present in the natural environment.

[0087] The term "nucleic acid" or "nucleic acid sequence" refers to a deoxy-ribonucleotide or ribonucleotide oligonucleotide in either single- or double-stranded form. The term encompasses nucleic acids, i.e., oligonucleotides, containing known analogs of natural nucleotides. The term also encompasses nucleic-acid-like structures with synthetic backbones (see e.g., *Oligonucleotides and Analogues, a Practical Approach*, ed. F. Eckstein, Oxford Univ. Press (1991); *Antisense Strategies*, *Annals of the N.Y. Academy of Sciences*, Vol. 600, Eds. Baserga et al. (NYAS 1992); Milligan J. *Med. Chem.* 36:1923-1937 (1993); *Antisense Research and Applications* (1993, CRC Press), WO 97/03211; WO 96/39154; Mata, *Toxicol. Appl. Pharmacol.* 144:189-197 (1997); Strauss-Soukup, *Biochemistry* 36:8692-8698 (1997); Samstag, *Antisense Nucleic Acid Drug Dev.* 6:153-156 (1996)).

[0088] As used herein a "nucleic acid probe or oligonucleotide" is defined as a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (i.e., A, G, C, or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in a probe may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, for example, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are optionally directly labeled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labeled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

[0089] The polynucleotide or polypeptide can be naturally occurring or non-naturally occurring (e.g., synthetic, recombinant, modified, and/or variant products). In one aspect, the naturally occurring or non-naturally occurring products are isolated or purified. In another aspect, the naturally occurring or non-naturally occurring products are not isolated or are not purified. In embodiments herein, the polynucleotides and polypeptides are non-naturally occurring. In embodiments herein, the polynucleotides and polypeptides are non-naturally occurring variants of the naturally-occurring mycoduclein (HTS) polypeptide and the naturally-occurring polynucleotide sequence that encodes the naturally-occurring mycoduclein (HTS).

[0090] As used herein, "recombinant" refers to a polynucleotide synthesized or otherwise manipulated *in vitro* (e.g., "recombinant polynucleotide"), to methods of using recombinant polynucleotides to

produce gene products in cells or other biological systems, or to a polypeptide ("recombinant protein") encoded by a recombinant polynucleotide. "Recombinant means" also encompass the ligation of nucleic acids having various coding regions or domains or promoter sequences from different sources into an expression cassette or vector for expression of, e.g., inducible or constitutive expression of a fusion protein comprising a translocation domain of the invention and a nucleic acid sequence amplified using a primer of the invention.

[0091] As used herein, the terms "amplifying" and "amplification" refer to the use of any suitable amplification methodology for generating or detecting recombinant or naturally expressed nucleic acid, as described in detail, below. For example, the invention provides methods and reagents (e.g., specific degenerate oligonucleotide primer pairs) for amplifying (e.g., by polymerase chain reaction, PCR) naturally expressed (e.g., genomic or mRNA) or recombinant (e.g., cDNA) nucleic acids of the invention (e.g., taste stimulus-binding sequences of the invention) in vivo or in vitro.

[0092] As used herein, the term "isolated," when referring to a nucleic acid or polypeptide refers to a state of purification or concentration different than that which occurs naturally. Any degree of purification or concentration greater than that which occurs naturally, including (1) the purification from other naturally occurring associated structures or compounds (e.g., other proteins, carbohydrates), or (2) the association with structures or compounds to which it is not normally associated in the body are within the meaning of "isolated" as used herein. The nucleic acids or polypeptides described herein may be isolated or otherwise associated with structures or compounds to which they are not normally associated in nature, according to a variety of methods and processes known to those of skill in the art. In one embodiment, the polypeptides described herein contain at most 5% (e.g., at most 4%, at most 3%, at most 2% at most 1%) by weight of other fungal proteins, e.g., fungal proteins other than Myd proteins.

[0093] "Modified" or "variant" products refer to products (e.g., polynucleotides or polypeptides) that have been altered from the original (e.g., naturally occurring) structure. As described herein, variants encompass polynucleotides or polypeptides with one or more changes to the nucleic acid or amino acid sequences, respectively. Changes include modifications to the nucleic acid or amino acid sequence, including additions, deletions, insertions, and substitutions. Modified or variant products also can encompass those modified to include disulfide bond formation, as well as those that are derivatized by glycosylation using post-translation modification methods, lipidation, acylation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component, relative to the original structure. As is known in the art, derivatization can be accomplished by chemical or enzymatic methods after translation of a polypeptide. Derivatization can

also be accomplished with post-translation modification during expression of the polypeptide in a selected host.

[0094] Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating, e.g., sequences in which the third position of one or more selected codons is substituted with mixed-base and/or deoxyinosine residues (Batzner et al., *Nucleic Acid Res.*, 19:5081 (1991); Ohtsuka et al., *J. Biol. Chem.*, 260:2605-2608 (1985); Rossolini et al., *Mol. Cell. Probes*, 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

[0095] Herein where a specific polynucleotide is indicated to encode a histidine tag, it will be understood that the polynucleotide excluding the sequence encoding the histidine tag is also provided. It will be further understood that for any specific polynucleotide indicated to encode a given histidine tag (e.g., (His)₆), the sequence encoding the histidine tag can be substituted with a sequence encoding a different His tag or a sequence encoding a different protein/peptide tag or affinity tag.

[0096] POLYPEPTIDES

[0097] It should be appreciated that embodiments of the invention also encompass Myd polypeptide variants (HTS variants) encoded by one or more polynucleotides. The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms also apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers.

[0098] An embodiment of the invention includes a polypeptide comprising, consisting essentially of, or consisting of a polypeptide sequence having at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a sequence selected from the group consisting of a variant of Table 8 or a variant of Table 8 further having a histidine tag. In embodiments, the polypeptide is not the polypeptide of SEQ ID NO:3. In embodiments, the polypeptide is not the polypeptide of SEQ ID NO:141. In embodiments, the polypeptide is the polypeptide of SEQ ID NO:3 which is substantially free (less than 95% or more specifically less than 99% by weight being present) of the polypeptide of SEQ ID NO:141. In embodiments, the polypeptide is the polypeptide of SEQ ID NO:141 which is substantially free (less than 95% or more specifically less than 99% by weight being present) of the polypeptide of SEQ ID NO:3. Optionally, the amino acid sequence has at least one and up to 24

modifications. When the amino acid sequence has a plurality of modifications, the number of modifications the amino acid has may range from at least one and up to 24 modifications such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 modifications, as desired. Furthermore, unless expressly stated otherwise, when an amino acid sequence has a plurality of modifications, each modification may independently be modified with a modification of choice such as deletion, insertion, substitution, or addition, regardless of what the other modifications are. Additionally, the plurality of modifications in the amino acid sequence may be the same or differ from each other; and the modification options for each modification may independently vary such deletion, insertion, substitution, or addition, etc. unless expressly stated otherwise. When the amino acid sequence has a plurality of modifications, each modification may independently be a substitution, addition, insertion, or deletion regardless of what the other modification is or are. In an embodiment, the amino acid sequence has at least one substitute modification. In a particular embodiment, the amino acid sequence has a plurality of substitution modifications; and each substitution modification may independently be substituted with a substitution of choice regardless of what the other substitutions are. Additionally, the plurality of substitutions in the amino acid sequence may be the same or differ from each other; and the options for substitutions for each amino acid may independently vary unless expressly stated otherwise. When the amino acid sequence has a plurality of substitutions, each substitution may independently be chosen regardless of what the other substitutions is or are. The term “consisting essentially of” allows for the inclusion of components that are not essential to the function or activity of the product and do not materially affect the function or activity, such as an anti-caking agent, filler, stabilizer (e.g., thermal stabilizer), and bulking agent (e.g., maltodextrose, gum acacia and the like).

[0099] Another embodiment of the invention includes a recombinant polypeptide having sweet modulation activity comprising, consisting essentially of, or consisting of a sequence having at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a variant of Table 8, Table 9 or Table 10, or a variant of these Tables further having a histidine tag, or being fused to a heterologous signal peptide or transit peptide. The term “consisting essentially of” allows for the inclusion of components that are not essential to the function or activity of the product and do not materially affect the function or activity, such as an anti-caking agent, filler, stabilizer (e.g., thermal stabilizer), and bulking agent (e.g., maltodextrose, gum acacia and the like).

[0100] In an embodiment, the polypeptide having sweet-taste modulation activity comprises, consists essentially of or consists of a modified SEQ ID NO:3 wherein the peptide has at least 1 and up to 24

amino acid modifications as shown in Table 8, Table 9 or Table 10. In another embodiment, the polypeptide has at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a modified polypeptide of SEQ ID NO: 3 with at least 1 and up to 24 amino acid modifications as shown in Table 8, Table 9 or Table 10; and the polypeptide differs from the polypeptide of amino acid sequence SEQ ID NO:3 and optionally differs from the polypeptide of amino acid sequence SEQ ID NO:141.

[0101] In an embodiment, the polypeptide having sweet-taste modulation activity comprises, consists essentially of or consists of a modified SEQ ID NO:3 wherein the peptide has at least 1 and up to 24 amino acid modifications as shown in Table 8, and further has 1-24 amino acid modifications as shown in Table 7, wherein the total number of modifications is 1-24. In another embodiment, the polypeptide has at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a modified polypeptide of SEQ ID NO: 3 with at least 1 and up to 24 amino acid modifications as shown in Table 8 and 1-24 amino acid modifications as shown in Table 7.

[0102] In an embodiment, the polypeptide having sweet-taste modulation activity comprises, consists essentially of or consists of a modified SEQ ID NO:3 wherein the peptide has at least 1 and up to 12 amino acid modifications as shown in Table 8, and further has 1-12 amino acid modifications as shown in Table 7. In another embodiment, the polypeptide has at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a modified polypeptide of SEQ ID NO: 3 with at least 1 and up to 12 amino acid modifications as shown in Table 8 and 1-12 amino acid modifications as shown in Table 7.

[0103] In an embodiment, the polypeptide having sweet-taste modulation activity comprises, consists essentially of or consists of a modified SEQ ID NO:3 wherein the peptide has at least 1 and up to 6 amino acid modifications as shown in Table 8, and further has 1-6 amino acid modifications as shown in Table 7. In another embodiment, the polypeptide has at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a modified polypeptide of SEQ ID NO: 3 with at least

1 and up to 6 amino acid modifications as shown in Table 8 and 1-6 amino acid modifications as shown in Table 7.

[0104] In an embodiment, the polypeptide having sweet-taste modulation activity comprises, consists essentially of or consists of a modified SEQ ID NO:3 wherein the peptide has at least 1 and up to 24 amino acid modifications as shown in Table 8, Table 9, Table 10, Table 7, Table 6 or Table 3. In another embodiment, the polypeptide has at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a modified polypeptide of SEQ ID NO: 3 with at least 1 and up to 24 amino acid modifications as shown in Table 8, Table 9, Table 10, Table 7, Table 6 or Table 3.

[0105] In an embodiment, the polypeptide having sweet-taste modulation activity comprises, consists essentially of or consists of a modified SEQ ID NO:3 wherein the peptide has at least 1 and up to 24 amino acid modifications as shown in Table 8, Table 9 or Table 10 and no modifications as shown in Table 7, Table 3 or Table 6. In another embodiment, the polypeptide has at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a modified polypeptide of SEQ ID NO: 3 with at least 1 and up to 24 amino acid modifications as shown in Table 8, Table 9, Table 10 and no modifications as shown in Table 7, Table 3 or Table 6.

[0106] In an embodiment, the polypeptide having sweet-taste modulation activity comprises, consists essentially of or consists of a modified SEQ ID NO:3 wherein the peptide has at least 1 and up to 6 amino acid modifications as shown in Table 8, Table 9 or Table 10. In another embodiment, the polypeptide has at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a modified polypeptide of SEQ ID NO:3 with at least 1 and up to 6 amino acid modifications as shown in Table 8, Table 9 or Table 10.

[0107] In certain embodiments herein, a specific polypeptide is described as having a histidine tag or as optionally having a histidine tag (noted as XXXXXX, where X is His, in the sequence listing herein). The invention provides all specific polypeptides including or optionally including a histidine tag as well as the corresponding polypeptides excluding the histidine tag. The invention also provides all specific polypeptides having a histidine tag or an optional histidine tag as well as the polypeptides excluding the histidine tag or wherein the histidine tag is replaced with a different protein tag including a different histidine tag.

[0108] In an embodiment, the Myd peptide (HTS) variants as described herein are capable of sweet-taste modulation activity. HTS polypeptide variants of the invention may have e.g., functional, physical and chemical effects at taste receptors, such as sweet taste receptors. “Sweet-taste modulation activity” may refer to inhibitory, activating, e.g., agonist or antagonist properties of a polypeptide of the invention, identified using *in vitro* and *in vivo* assays for taste transduction. Proteins with inhibitory activity may bind to, partially or totally block stimulation, decrease, prevent, delay activation, inactivate, desensitize, or down regulate taste transduction, e.g., antagonists. Activating polypeptides may bind to, stimulate, increase, open, activate, facilitate, enhance activation, sensitize, or up regulate taste transduction, e.g., agonists. Activating polypeptides are preferred.

[0109] Sweet taste modulation also refers to enhancing the taste, such as a sweet taste, of a particular product for oral administration when administered as a combination. In addition to sweet taste, HTS variants, may also exhibit differences in intensity of sweetness and duration of sweetness. These attributes of sweetness can be assessed in taste tests as described herein.

[0110] Sweet taste modulation also refers to polypeptide variants herein which exhibit sweet taste or convey sweet taste to compositions or formulations that do not themselves exhibit sweet taste when the polypeptide variant is added to the compositions or formulations.

[0111] In some embodiments, the Myd (HTS) polypeptide variants of the invention include polypeptides that are at least as sweet as sucrose (on a w/w basis) (e.g., 1X), or alternatively, are 2X, 5X, 10X, 50X, 100X, 200X, 400X, 600X, 800X, 1000X, 1500X, 2000X, 3000X, 5000X, 10,000X, 20,000X or sweeter than sucrose, as measured by any of the methods described above or known in the art. In other embodiments, the Myd polypeptide variants are at least 1% (at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%) as sweet as sucrose.

[0112] In embodiments, the polypeptides having sweet-taste modulation activity include modified SEQ ID NO:3 with 1 to up to 24 different amino acid modifications as shown in Table 8, Table 9 or Table 10. In embodiments, the polypeptides having sweet-taste modulation activity include modified SEQ ID NO:141, with 1 to up to 24 different amino acid modifications as shown in Table 8, Table 9 or Table 10.

[0113] In an embodiment, the Myd (HTS) peptide variants as described herein have flavor modifying properties (FMP) or activity. A compound having FMP is a compound, including a protein or polypeptide that causes a change in any attributes of perceived flavor of a formulation relative to a formulation without the compound, but does not directly provide a flavor attribute. Compounds may have flavor modifying properties below a perceptible sweetness threshold concentration and provide a

direct flavor sensation above that threshold. HTS proteins/polypeptide or variants can exhibit a FMP threshold for the wild-type protein in a formulation, where below the threshold, FMP are observed, and above the threshold the HTS proteins or variants directly causes sweetness perception. The exact threshold for this perception change is HTS protein and application specific.

[0114] In an embodiment, a composition or formulation containing HTS proteins or variants at about 1 to about 50 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 1 to about 40 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 1 to about 30 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 1 to about 25 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 1 to about 20 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 1 to about 15 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 5 to about 50 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 10 to about 50 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 15 to about 50 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 20 to about 50 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 25 to about 50 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 30 to about 50 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 35 to about 50 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 40 to about 50 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an

embodiment, a composition or formulation containing HTS proteins or variants at about 45 to about 50 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 10 to about 40 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation. In an embodiment, a composition or formulation containing HTS proteins or variants at about 20 to about 30 ppm exhibits flavor modification, but does not provide additive sweetness to the composition or formulation.

[0115] In embodiments, a composition or formulation containing HTS proteins or variants at concentrations above 30 ppm, and particularly at concentrations above 40 ppm, provides sweetness to the composition or formulation. In embodiments, compositions or formulations can contain from 1-100 ppm of HTS proteins or variants. In embodiments, compositions or formulations can contain from 1-5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 ppm of total HTS protein or variant. In embodiments, compositions or formulations can contain from 1-5, 5-10, 1-10, 1-30, or 1-30 ppm of total HTS protein or variant. In embodiments, compositions or formulations can contain from 30-100, 30-40, 40-50, 50-60, 70-80, 80-90, 90-100, 40-100 or 50-100 ppm of total HTS protein or variant.

[0116] In embodiments, at least 80% sequence identity with respect to a polypeptide includes, without limitation, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% and at least 99% sequence identity. In further embodiments, at least 80% sequence identity with respect to a polypeptide also includes, without limitation, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99% sequence identity.

[0117] SWEETNESS AND THERMAL STABILITY COMPARISON OF MODIFIED PEPTIDE TO UNMODIFIED POLYPEPTIDE

[0118] The Myd (HTS) protein variants described herein also include "analogs," or "conservative variants" and "mimetics" ("peptidomimetics") with structures and activity that substantially correspond to the exemplary sequences. Thus, the terms "conservative variant" or "analog" or "mimetic" refer to a polypeptide which has a modified amino acid sequence, such that the change(s) do not substantially alter the polypeptide's (the conservative variant's) structure and/or activity, as defined herein. These include conservatively modified variations of an amino acid sequence, i.e., amino acid substitutions, additions or deletions of those residues that are not critical for protein activity, or substitution of amino acids with residues having similar properties (e.g., acidic, basic,

positively or negatively charged, polar or non-polar, etc.) such that the substitutions of even critical amino acids does not substantially alter structure and/or activity.

[0119] More particularly, "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein.

[0120] For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide.

[0121] Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

[0122] When it is desired to express a coding sequence in a heterologous host (i.e., a non-naturally occurring host), codon optimization can be employed as is known in the art to enhance expression levels in a selected heterologous host. Codon optimization involves replacing codons in a given naturally-occurring coding sequence with codons that have a higher usage level in the selected heterologous host. In general, codon optimization is performed by comparing the codon frequency in the naturally-occurring coding sequence with the codon frequency in the selected heterologous host and where a given codon is not one typically employed in the selected heterologous host replacing that codon with one that is used by the selected heterologous host more frequently. One to all codons in a given naturally-occurring coding sequence can be optimized. Dependent upon the codon frequencies in the naturally-occurring coding sequence and the heterologous host and the specific coding sequence, at least 50%, at least 75%, at least 85%, or at least 95% of the codons can be replaced. A number of codon optimization tools are known and readily available in the art from various sources. For example, OPTIMIZER is an on-line application for codon optimization (P. Puigbo et al. (2007) "OPTIMIZER; a web server for optimizing the codon usage of DNA sequences" *Nucleic Acids Res.* 35: W126-W131). A recent review of codon optimization methods is provided in H. Fu et al. "Codon optimization with deep learning to enhance protein expression" *Nature Research Scientific Reports* (2020) 10:17617.

Additional references regarding methods of codon optimization include, among others: N. M. Marlatt et al. (2010) "Codon optimization for enhanced *Escherichia coli* expression of human S100A11 and S100A1 proteins" *Protein Expr. Purif.* 73(1):58-64; A. Mellitzer et al. (2012) "Expression of lignocellulolytic enzymes in *Pichia pastoris*" *Microb. Cell Fact.* 11(1) 61; and E. Angov et al. (2008) "Heterologous protein expression is enhanced by harmonizing the codon usage frequencies of the target gene with those of the expression host" *PLoS ONE* 3(5): e21899).

[0123] Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, one exemplary guideline to select conservative substitutions includes (original residue followed by exemplary substitution): ala/gly or ser; arg/lys; asn/gln or his; asp/glu; cys/ser; gln/asn; gly/asp; gly/ala or pro; his/asn or gln; ile/leu or val; leu/ile or val; lys/arg or gln or glu; met/leu or tyr or ile; phe/met or leu or tyr; ser/thr; thr/ser; trp/tyr; tyr/trp or phe; val/ile or leu. An alternative exemplary guideline uses the following six groups, each containing amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (I); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); (see also, e.g., Creighton, *Proteins*, W.H. Freeman and Company (1984); Schultz and Schimer, *Principles of Protein Structure*, Springer-Verlag (1979)). Another alternative exemplary guidelines uses the following six groups, where proline is unique: 1) Gly (G), Ala (A), Val (V), Leu (L), Ile (I); 2) Ser (S), Cys (C), Thr (T), Met (M); 3) Pro (P); 4) Phe (F), Tyr (Y), Try (W); 5) His (H), Lys (K), Arg (R); and 6) Asp (D), Glu (E), Gln (N). One of skill in the art will appreciate that the above-identified substitutions are not the only possible conservative substitutions. For example, for some purposes, one may regard all charged amino acids as conservative substitutions for each other whether they are positive or negative. In addition, individual substitutions, deletions or additions that alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence can also be considered "conservatively modified variations." One of skill in the art would be familiar with codon selection in a given host that is expressing the protein of interest.

[0124] Nucleotide and amino acid sequence information for *MYD* family members may also be used to construct models of sweet modulating polypeptides in a computer system and how they interact with sweet receptors and computer system models of same. Sweet taste receptors are composed of a heterodimer of taste 1 receptor member 2 (T1R2) and taste 1 receptor member 3 (T1R3). These models can be subsequently used to identify variants and mutations of Myd that can increase activation of sweet receptors and identify more active versions of Myd.

[0125] Various conservative mutations as well as the various less conservative mutations as listed in Table 8, Table 9, Table 10, Table 7, Table 6 and Table 3, and substitutions are envisioned to be within

the scope of the invention. Mutations of Table 9, Table 10, Table 7, Table 3 and Table 6, which exhibit sweet taste, are currently preferred mutants. For instance, it is within the level of skill in the art to perform amino acid substitutions using known protocols of recombinant gene technology including PCR, gene cloning, site-directed mutagenesis of cDNA, transfection of host cells, and *in-vitro* transcription. The variants are then be screened for sweet-taste modulation activity and particularly for sweet taste as well as for organoleptic properities or changes in such activities or properties. For example, variants generated are screened employing sensory tested as described herein and as understood in the art. For example, variants generated are screened for taste receptor agonist functional activity as is known in the art.

[0126] In embodiments, HTS variant polypeptides herein exhibit enhanced sweetness compared to the unmodified polypeptide SEQ ID NO:3. In some embodiments, the sweetness of respective HTS variants is enhanced at least 10% (or an enhancement ranging from 10% to 100%, or an enhancement ranging from 10 % to 200%, or at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or 100% or more) compared to the unmodified polypeptide of SEQ ID NO:3. In other embodiments, HTS variants exhibit comparable sweetness compared to the unmodified polypeptide of SEQ ID NO:3. Comparable sweetness of a modified polypeptide, as described above, compared to the unmodified polypeptide SEQ ID NO:3 is at least 10% (or at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or 100%) as sweet as the unmodified polypeptide of SEQ ID NO:3. Sweetness is measured by any method known in the art for comparison of sweetness, and more particularly by a method for assessing sweetness as described herein.

[0127] Flavor modification activity and sweet-taste modulation activity may be detected by methods known in the art, e.g., *in vitro* methods, or *in vivo* by animal or human sensory testing. While not wishing to be bound to any particular theory, Myd (HTS) is involved in sweet taste activation e.g., is an agonist of taste 1 receptor member 2 (Tas1R2) and/or taste 1 receptor member 3 (Tas1R3). However, Myd (HTS) agonizes other taste receptors, such as bitter, umami, sour and salty. Such functional effects can be measured by any means known to those skilled in the art, e.g., measurement of binding to taste receptors Tas1Rs via changes in spectroscopic characteristics (e.g., fluorescence, absorbance, refractive index), hydrodynamic (e.g., shape), chromatographic, or solubility properties, patch clamping, voltage-sensitive dyes, whole cell currents, radioisotope efflux, inducible markers, transcriptional activation of Tas1R genes; ligand-binding assays; voltage, membrane potential and conductance changes; ion flux assays; changes in intracellular second messengers such as cAMP, cGMP, and inositol triphosphate (IP3); changes in intracellular calcium levels; neurotransmitter release, and the like.

[0128] Sensory testing (human or animal) may also be employed to determine whether a Myd (HTS) candidate polypeptide has sweet-taste modulation activity. Sensory evaluation is a scientific discipline that analyses and measures human responses to the composition of food and drink, e.g., appearance, touch, odor, texture, temperature and taste. Measurements using people as the instruments are sometimes necessary. Selection of an appropriate method to determine sweetening can be determined by one of skill in the art, and includes, e.g., discrimination tests or difference tests, designed to measure the likelihood that two products are perceptibly different. Responses from the evaluators are tallied for correctness, and statistically analyzed to see if there are more correct than would be expected due to chance alone. The food industry had the first need to develop this measurement tool as the sensory characteristics of flavor and texture were obvious attributes that cannot be measured easily by instruments. For sweetness perception, for example, samples of, for example, one or more of 5% sucrose, 6% sucrose, 7% sucrose, 8% sucrose, 9% sucrose, 10% sucrose, and a test sample can be ranked by trained panelists in order of sweet taste intensity from low sweet to high sweet. In the instant invention, it should be understood that there are any number of ways one of skill in the art could measure the organoleptic properties (e.g., sensory differences). For example, organoleptic response can be measured using trained panelist who rate their responses on an accepted scale or rating system, such as a hedonic rating system. Alternatively or in addition, instrumental methods are available or can be readily adapted when a subject's (panelist's) response to an organoleptic property can be linked to a property measured by the instrumental method. See, for example, Ray, S. (2221) "Sensory Properties of Foods and Their Measurement Methods." In: Khan, M.S., Shafiur Rahman, M. (eds) Techniques to Measure Food Safety and Quality. Springer, Cham. https://doi.org/10.1007/978-3-030-68636-9_15.

[0129] Brix measurement (or Brix scale) is a well-known application in the food and beverage industry that determines pure sucrose content in water: $1 \text{ degree Brix } (^{\circ}\text{Bx}) = 1\text{g of sucrose} / 100\text{g of solution}$ and represents the strength of the solution as percentage by mass. 8°Bx is equivalent to approximately an 8% sucrose solution. As described in the Examples, purified polypeptide corresponding to SEQ ID NO:5 was tasted at 0.03 mg/ml by a trained sensory scientist (0.2 mL aliquot) and found to have a sweetness equivalent to 8°Bx (approximately 8% sucrose solution) (see Examples 4, 5, 9, and 10).

[0130] THERMAL STABILITY

[0131] Thermal stability of sweet-taste modifying polypeptides can impact the potential applications of the polypeptide, for example, for food applications at elevated temperature. In embodiments, HTS variants herein exhibit comparable thermal stability compared to the unmodified polypeptide of SEQ ID NO:3. Comparable thermal stability of a modified polypeptide, as described above, compared to the unmodified polypeptide of SEQ ID NO:3 is substantially the same which herein means less than or

equal to a change in thermal stability of 4.5 % (including less than or equal to 1%, less than or equal to 2%, less than or equal to 3%, less than or equal to 4%) compared to the thermal stability of the unmodified polypeptide of SEQ ID NO:3.

[0132] In some embodiments, HTS variants herein exhibit enhanced thermal stability compared to the unmodified SEQ ID NO:3. In other embodiments, enhanced thermal stability of a modified polypeptide, as described above, compared to the unmodified polypeptide of SEQ ID NO:3 is enhanced greater than 4.5% (including, 4.5 to 15% enhanced, greater than 5% enhanced, greater than 6% enhanced, greater than 7% enhanced, greater than 8% enhanced, greater than 9% enhanced, greater than 10% enhanced, greater than 11% enhanced greater than 12% enhanced, greater than 13% enhanced, greater than 14% enhanced, greater than 5% up to 15% enhanced, greater than 6% up to 15% enhanced, greater than 7% up to 15% enhanced, greater than 8% up to 15% enhanced, greater than 9% up to 15% enhanced, greater than 10% up to 15% enhanced, greater than 11% up to 15% enhanced, greater than 12% up to 15% enhanced, greater than 13% up to 15% enhanced, greater than 14% up to 15% enhanced, or up to 15% enhanced) compared to the thermal stability of the unmodified polypeptide of SEQ ID NO:3. Thermal stability is measured by any method known in the art for assessing thermal stability, and more particularly by a method for assessing thermal stability as described herein. In embodiments, sweet-taste modifying polypeptides herein exhibit comparable sweetness and comparable thermal stability compared to that of the unmodified polypeptide of SEQ ID NO:3. In embodiments, sweet-taste modifying polypeptides herein exhibit comparable sweetness and enhanced thermal stability compared to that of the unmodified polypeptide of SEQ ID NO:3. In embodiments, sweet-taste modifying polypeptides herein exhibit enhanced sweetness and comparable thermal stability compared to that of the unmodified polypeptide of SEQ ID NO:3. In embodiments, sweet-taste modifying polypeptides herein exhibit enhanced sweetness and enhanced thermal stability compared to that of the unmodified polypeptide of SEQ ID NO:3.

[0133] In some embodiments, the HTS variant herein exhibits comparable sweetness and comparable thermal stability compared to that of the unmodified polypeptide of SEQ ID NO:3. In some embodiments, the HTS variant herein exhibits enhanced sweetness and comparable thermal stability compared to that of the unmodified polypeptide of SEQ ID NO:3. In some embodiments, the HTS variant herein exhibits comparable sweetness and enhanced thermal stability compared to that of the unmodified polypeptide of SEQ ID NO:3. In some embodiments, the HTS variant herein exhibits enhanced sweetness and enhanced thermal stability compared to that of the unmodified polypeptide of SEQ ID NO:3.

[0134] Comparable thermal stability of a modified polypeptide, as described above, compared to the unmodified polypeptide of SEQ ID NO:3 is substantially the same, which herein means less than or

equal to a change in thermal stability of 4.5 % (including less than or equal to 1%, less than or equal to 2%, less than or equal to 3%, less than or equal to 4%) compared to the thermal stability of the unmodified polypeptide of SEQ ID NO:3.

[0135] Enhanced thermal stability of a modified polypeptide, as described above, compared to the unmodified polypeptide of SEQ ID NO:3 is enhanced greater than 4.5% (including, 4.5 to 15% enhanced, greater than 5% enhanced, greater than 6% enhanced, greater than 7% enhanced, greater than 8% enhanced, greater than 9% enhanced, greater than 10% enhanced, greater than 11% enhanced, greater than 12% enhanced, greater than 13% enhanced, greater than 15% enhanced, greater than 5% up to 15% enhance, greater than 6% up to 15% enhanced, greater than 7% up to 15 % enhanced, greater than 8% up to 15% enhanced, greater than 9% up to 15% enhanced, greater than 10% up to 15% enhanced, greater than 11% up to 15% enhanced, greater than 12% up to 15% enhanced, greater than 13% up to 15% enhanced, greater than 14% up to 15% enhanced, or up to 15% enhanced) compared to the thermal stability of the unmodified polypeptide of SEQ ID NO:3.

[0136] In embodiments, the polypeptide variant herein exhibits sweet-taste modulation activity. Non-limiting examples of sweet-taste modulation activity include providing a sweet taste.

[0137] In another aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by at least one mutation selected from those listed in Tables 8 and optionally one deletion such as the deletion of Met at position 1. In another aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by at least one mutation up to 24 mutations at different amino acid positions selected from those listed in Tables 8.

[0138] In another aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by at least one mutation selected from those listed in Table 8 and at least one mutation selected from Table 7 and optionally one deletion such as the deletion of Met at position 1. In another aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by at least one mutation up to 24 mutations at different amino acid positions selected from those listed in Table 8 and from one to 24 mutations selected from Table 7.

[0139] In another aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by at least one mutation up to 24 selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In another aspect, the invention provides a

polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by at least one mutation up to 24 mutations at different amino acid positions selected from those listed in Table 8, Table 9 or Table 10.

[0140] In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by two mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by three mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by four mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by five mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by six mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1.

[0141] In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by seven mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by eight mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by nine mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated

polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by ten mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by eleven mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twelve mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1.

[0142] In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by thirteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by fourteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by fifteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by sixteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by seventeen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by eighteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1.

[0143] In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by

nineteen mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twenty mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twenty-one mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twenty-two mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twenty-three mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In a related aspect, the invention provides a polypeptide having sweet-taste modulation activity (e.g., isolated polypeptide) comprising the amino acid sequence of SEQ ID NO:3 which is modified by twenty-four mutations at different positions selected from those listed in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. The invention also provides polynucleotides encoding the foregoing mutant polypeptides of SEQ ID NO:3 or SEQ ID NO:141. The invention further provides the foregoing mutant polypeptides which further comprise a protein tag and more specifically a histidine tag. The invention also provides polynucleotides encoding the foregoing mutant polypeptides of SEQ ID NO:3 or SEQ ID NO:141 which further comprise a protein tag and more specifically a histidine tag. In embodiments, HTS variants do not include any one or more of the mutations listed in Table 3, Table 6 or Table 7.

[0144] In some embodiments, the polypeptide having sweet-taste modulation activity also has enhanced thermal stability compared to the polypeptide of SEQ ID NO:3. In an embodiment, the polypeptide having sweet-taste modulation activity and enhanced thermal stability comprises a modified SEQ ID NO:3, wherein the polypeptide has at least 1 and up to 6 amino acid modifications as shown in Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In an embodiment, the polypeptide having sweet-taste modulation activity and enhanced thermal stability comprises a modified SEQ ID NO:3, wherein the polypeptide has at least 1 and up to 6 amino acid modifications as shown in Table 7 and optionally one deletion such as the deletion of Met at position 1. In an embodiment, the polypeptide having sweet-taste modulation activity and

enhanced thermal stability comprises a modified SEQ ID NO:3, wherein the polypeptide has at least 1 and up to 6 amino acid modifications as shown in Table 8, Table 9 or Table 10 and further has 1-6 amino acid modifications as shown in Table 7 and optionally one deletion such as the deletion of Met at position 1. In further embodiments of the preceding embodiments, the polypeptide having sweet-taste modulation activity (including sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:141.

[0145] In another embodiment, the polypeptide having sweet-taste modulation activity (and particularly sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:3 has at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a modified polypeptide of SEQ ID NO:3 with at least 1 and up to 6 amino acid modifications as shown in Table 8, Table 9 or Table 10 and 1-6 amino acid modifications as shown in Table 7 and optionally one deletion such as the deletion of Met at position 1. In further embodiments of the preceding embodiments, the polypeptide has sweet-taste modulation activity (including sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:141.

[0146] In another embodiment, the polypeptide having sweet-taste modulation activity (and particularly sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:3 has both mutations I49C and S33C (compared to SEQ ID NO:3) and optionally 1, 2, 3, 4, 5 or 6 other mutations as shown in Table 8, Table 9, Table 10, Table 3, Table 6 or Table 7 and in particular optionally one deletion such as the deletion of Met at position 1. In a related embodiment, the polypeptide having sweet-taste modulation activity (and particularly sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:3 has both mutations I49C and S33C (compared to SEQ ID NO:3) and has at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a modified polypeptide of SEQ ID NO:3. In further embodiments of the preceding embodiments, the polypeptide has sweet-taste modulation activity (including sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:141.

[0147] In another embodiment, the polypeptide having sweet-taste modulation activity (and particularly sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:3 has both mutations Y23C and Y61C (compared to SEQ ID NO:3) and optionally 1, 2, 3, 4, 5 or 6 other mutations as shown in Table 8, Table 9, Table 10, Table 3, Table 6 or Table 7 and optionally one deletion such as the deletion of Met at position 1. In a related embodiment, the polypeptide having

sweet-taste modulation activity (and particularly sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:3 has both mutations I49C and S33C (compared to SEQ ID NO:3) and has at least 80% (e.g., at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%) sequence identity to a modified polypeptide of SEQ ID NO:3. In further embodiments of the preceding embodiments, the polypeptide has sweet-taste modulation activity (including sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:141.

[0148] In an embodiment, the polypeptide having sweet-taste modulation activity and enhanced thermal stability comprises a modified SEQ ID NO:3 wherein the polypeptide has two modifications as shown in Table 7 and/or Table 8, Table 9 or Table 10 and optionally one deletion such as the deletion of Met at position 1. In an embodiment, the polypeptide having sweet-taste modulation activity and enhanced thermal stability comprises a modified SEQ ID NO:3 wherein the polypeptide has two modifications as shown in Table 7 and/or Table 8, Table 9 or Table 10 that result in disulfide bond formation between the two modified amino acids and optionally one deletion such as the deletion of Met at position 1. In one embodiment, the polypeptide having sweet-taste modulation activity and enhanced thermal stability comprises a modified SEQ ID NO:3, wherein the polypeptide has at least two modifications as shown in Table 7 and/or Table 8, Table 9 or Table 10 that result in disulfide bond formation between the at least two modified amino acids and optionally one deletion such as the deletion of Met at position 1. In further embodiments of the preceding embodiments, the polypeptide has sweet-taste modulation activity (including sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:141.

[0149] In an embodiment, the polypeptide having sweet-taste modulation activity (and particularly sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:3 comprises the polypeptide of SEQ ID NO:143 (double mutant S33C_I49C) or SEQ ID NO:145 (double mutant Y24C_Y62C). In a related embodiment, the polypeptide having sweet-taste modulation activity (and particularly sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:3 comprises the polypeptide of SEQ ID NO:142 or SEQ ID NO:145 and in addition the methionine at position 1 is absent SEQ ID NO:146 and SEQ ID NO:147. In an embodiment, the polypeptide having sweet-taste modulation activity (and particularly sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:3 is the polypeptide of SEQ ID NO: 143. In an embodiment, the polypeptide having sweet-taste modulation activity (and particularly sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO:3 is the polypeptide of SEQ ID NO:145. In an embodiment, the polypeptide having sweet-taste modulation activity (and particularly sweet

taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO: 3 is the polypeptide of SEQ ID NO:146. In an embodiment, the polypeptide having sweet-taste modulation activity (and particularly sweet taste) and enhanced thermal stability compared to the polypeptide of SEQ ID NO: 3 is the polypeptide of SEQ ID NO: 147.

[0150] In some embodiments, polypeptides of the invention exhibit stability to low pH (<pH 7) that is enhanced compared to the polypeptide of SEQ ID NO:3. In some embodiments, polypeptides of the invention exhibit stability to high pH (> pH 7) that is enhanced compared to the polypeptide of SEQ ID NO:3. In one embodiment, polypeptides of the invention exhibit greater stability at pH of about 2 as compared to the polypeptide of SEQ ID NO:3. In another embodiment, polypeptides of the invention exhibit greater stability at pH of about 10 as compared to the polypeptide of SEQ ID NO:3. In one embodiment, polypeptides of the invention having methionine present at position 1 exhibit greater stability at pH of about 2 as compared to the polypeptide of SEQ ID NO:3. In another embodiment, polypeptides of the invention having methionine present at position 1 exhibit greater stability at pH of about 10 as compared to the polypeptide of SEQ ID NO:3.

[0151] The term "expression vector" or "expression cassette" refers to any recombinant expression system for the purpose of expressing a nucleic acid sequence of the invention *in vitro* or *in vivo*, constitutively or inducibly, in any cell, including prokaryotic, yeast, fungal, plant, insect or mammalian cell. The term includes linear or circular expression systems. The term includes expression systems that remain episomal or integrate into the host cell genome. The expression systems can have the ability to self-replicate or not, i.e., drive only transient expression in a cell. The term includes recombinant expression cassettes which contain only the minimum elements needed for transcription of the recombinant nucleic acid.

[0152] A recent review of methods for expression of recombinant proteins is found in Tripathi & Shrivastava (2019) "Recent Developments in Bioprocessing of Recombinant Proteins; Expression Hosts and Process Development," *Frontiers in Bioeng. Biotech.* 7:420, doi:10.3389/ fbio.2019.00420. This reference is incorporated by reference herein in its entirety for details of host expression systems and methods for expression of recombinant proteins.

[0153] By "host cell" is meant a cell that contains an expression vector and supports the replication or expression of the expression vector. In one embodiment, the host cell is a prokaryotic cell. In one embodiment, the host cell is a eukaryotic cell. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells such as CHO, HeLa, HEK-293, and the like, e.g., cultured cells, explants, and cells *in vivo*.

[0154] In an embodiment, the host cell is selected from the group consisting of *Escherichia coli*, *Klebsiella oxytoca*, *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia*

succiniciproducens, *Agrobacterium tumefaciens*, *Rhizobium etli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Gluconobacter oxydans*, *Zymomonas mobilis*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Streptomyces coelicolor*, *Clostridium acetobutylicum*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Khuyveromyces lactis*, *Khuyveromyces marxianus*, *Aspergillus terreus*, *Aspergillus niger*, *Pichia pastoris*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Yarrowia lipolytica*, *Candida albicans*, *Issatchenkia orientalis*, *Scheffersomyces stipitis*, *Yarrowia lipolytica*, *Ogataea polymorpha*, *Phaffia rhodozyma*, *Candida utilis*, *Arxula adenivorans*, *Debaryomyces hansenii*, *Debaryomyces polymorphus*, and *Schwanniomyces occidentalis*.

[0155] In an embodiment, the host cell is selected from the group consisting of Qualified Presumption of Safety (QPS) recommended biological agents. A list of such hosts is available on the website: efsa.europa.eu/efsajournal EFSA Journal 2021;19(7):6689. In an embodiment, the host organism is selected from the group consisting of *Bacillus megaterium*, *Trichoderma reesei*, *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium Longum*, *Carnobacterium divergens*, *Lactobacillus acidophilus*, *Lactobacillus amylolyticus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus alimentarius*, *Lactobacillus aviaries*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus collinoides*, *Lactobacillus coryniformis*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus dextrinicus*, *Lactobacillus diolivorans*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Lactobacillus hilgardii*, *Lactobacillus johnsonii*, *Lactobacillus kefiranoferiens*, *Lactobacillus kefir*, *Lactobacillus mucosae*, *Lactobacillus panis*, *Lactobacillus paracasei*, *Lactobacillus parafarraginis*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactobacillus salivarius*, *Lactobacillus Sanfranciscensis*, *Leuconostoc citreum*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Leuconostoc Pseudomesenteroides*, *Microbacterium imperial*, *Oenococcus oeni*, *Pasteuria nishizawae*, *Pediococcus acidilactici*, *Pediococcus parvulus*, *Pediococcus pentosaceus*, *Propionibacterium acidipropionic*, *Propionibacterium freudenreichii*, *Streptococcus thermophilus*, *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus flexus*, *Bacillus fusiformis*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus mojavensis*, *Bacillus paralicheniformis*, *Bacillus pumilus*, *Bacillus smithii*, *Bacillus subtilis*, *Bacillus vallismortis*, *Bacillus velezensis*, *Geobacillus stearothermophilus*, *Paenibacillus illinoisensis*, *Parageobacillus thermoglucosidasius*, *Gluconobacter oxydans*, *Komagataeibacter sucrofermentans*, *Xanthomonas campestris*, *Candida cylindracea*, *Cyberlindnera jadinii*, *Debaryomyces hansenii*,

Hanseniaspora uvarum, *Khuyveromyces lactis*, *Khuyveromyces marxianus*, *Komagataella pastoris*, *Komagataella phaffi*, *Lindnera jadinii*, *Ogataea angusta*, *Saccharomyces bayanus*, *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Xanthophyllomyces dendrorhous*, or *Zygosaccharomyces rouxii*.

[0156] In another aspect, the host cell is selected from the group consisting of gram-positive non-spore forming bacteria, gram-positive spore forming bacteria, gram negative bacteria, yeast, and protists/algae. In other aspects, the host cell is selected from plant cells. In other aspects, the host cell is selected from insect cells. With respect to insect cells, Baculo virus insect expression systems are useful. Insect cells useful as hosts for production of recombinant protein include, among others, *Spodoptera frugiperda* cells (e.g., Sf9, Sf21), *Drosophila* cells (e.g., S2), *Trichoplusia ni* cells (e.g., Tn-368, High-Five™ (Thermo Fisher Scientific, Waltham, MA)). Various host cells are known in the art and available from commercial sources, among others.

[0157] Non-limiting examples of gram-positive non-spore forming bacteria include *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Carnobacterium divergens*, *Corynebacterium ammoniagenes*, *Corynebacterium glutamicum*, *Lactobacillus acidophilus*, *Lactobacillus amylolyticus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus alimentarius*, *Lactobacillus aviaries*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus collinoides*, *Lactobacillus coryniformis*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus dextrinicus*, *Lactobacillus diolivorans*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Lactobacillus hilgardii*, *Lactobacillus johnsonii*, *Lactobacillus kefiranoferiens*, *Lactobacillus kefir*, *Lactobacillus mucosae*, *Lactobacillus panis*, *Lactobacillus paracasei*, *Lactobacillus parafarraginis*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactobacillus salivarius*, *Lactobacillus sanfranciscensis*, *Lactococcus lactis*, *Leuconostoc citreum*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides*, *Microbacterium imperial*, *Oenococcus oeni*, *Pasteuria nishizawae*, *Pediococcus acidilactic*, *Pediococcus parvulus*, *Pediococcus pentosaceus*, *Propionibacterium acidipropioni*, *Propionibacterium freudenreichii*, and *Streptococcus thermophiles*.

[0158] Non-limiting examples of gram-positive spore forming bacteria include *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus flexus*, *Bacillus fusiformis*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus mojavensis*, *Bacillus pumilus*, *Bacillus smithii*, *Bacillus subtilis*, *Bacillus vallismortis*,

Bacillus velezensis, *Geobacillus stearothermophilus*, *Paenibacillus illinoisensis*, and *Parageobacillus thermoglucosidasius*. Non-limiting examples of gram-negative bacteria include *Cupriavidus necator*, *Gluconobacter oxydans*, *Komagataeibacter sucrofermentans*, and *Xanthomonas campestris*.

[0159] Non-limiting examples of yeast include *Candida cylindracea*, *Debaryomyces hansenii*, *Hanseniaspora uvarum*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Komagataella pastoris*, *Komagataella phaffii*, *Lindnera jadinii*, *Ogataea angusta*, *Saccharomyces bayanus*, *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Xanthophyllomyces dendrorhous*, *Yarrowia lipolytica*, and *Zygosaccharomyces rouxii*.

[0160] Non-limiting examples of protists/algae include *Aurantiochytrium limacinum*, *Euglena gracilis*, and *Tetraselmis chuii*.

[0161] In certain embodiments, recombinant HTS is produced by a transgenic mammal, i.e., in milk.

[0162] The expression of HTS (or a variant thereof) may be stable or transient. In a stable expression system, the exogenous DNA is integrated into the chromosomes, or as an episome (a separate piece of nuclear DNA) and is passed on to future generations of the host cell.

[0163] The terms "mimetic" and "peptidomimetic" refer to a synthetic chemical compound that has substantially the same structural and/or functional characteristics of the polypeptides, e.g., translocation domains, ligand-binding domains, or chimeric receptors of the invention. The mimetic can be either entirely composed of synthetic, non-natural analogs of amino acids, or may be a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic's structure and/or activity.

[0164] As with polypeptides of the invention which are conservative variants, routine experimentation will determine whether a mimetic is within the scope of the invention, i.e., that its structure and/or function is not substantially altered. Polypeptide mimetic compositions can contain any combination of non-natural structural components, which are typically from three structural groups: a) residue linkage groups other than the natural amide bond ("peptide bond") linkages; b) non-natural residues in place of naturally occurring amino acid residues; or c) residues which induce secondary structural mimicry, i.e., to induce or stabilize a secondary structure, e.g., a beta turn, gamma turn, beta sheet, alpha helix conformation, and the like. A polypeptide can be characterized as a mimetic when all or some of its residues are joined by chemical means other than natural peptide bonds. Individual peptidomimetic residues can be joined by peptide bonds, other chemical bonds or coupling means, such as, e.g., glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, N,N'-dicyclohexylcarbodiimide (DCC) or N,N'-diisopropylcarbodiimide (DIC). Linking groups that can be

an alternative to the traditional amide bond ("peptide bond") linkages include, e.g., ketomethylene (e.g., --C(O)--CH₂-- for --C(O)--NH--), aminomethylene (CH₂--NH), ethylene, olefin (CH=CH), ether (CH₂--O), thioether (CH--S), tetrazole (CN₄), thiazole, retroamide, thioamide, or ester (see, e.g., Spatola, Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp 267-357, "Peptide Backbone Modifications," Marcell Dekker, NY (1983)). A polypeptide can also be characterized as a mimetic by containing all or some non-natural residues in place of naturally occurring amino acid residues; non-natural residues are well described in the scientific and patent literature. Phyre2 is a suite of tools available on the web to predict and analyze protein structure, function and mutations.

[0165] Examples of conservatively modified variations of Myd1 protein structure may be derived using homology-modelling algorithms: SWISS-MODEL, PHYRE2.0, and Jpred to identify a sequence-based consensus loop regions, as known in the art, see, e.g., Pechmann, S. & Frydman, J. Interplay between Chaperones and Protein Disorder Promotes the Evolution of Protein Networks. PLoS Computational Biology 10, e1003674 (2014).

[0166] Specific regions of the *MYD*/Myd nucleotide and amino acid sequences may be used to identify polymorphic variants, interspecies homologs, and alleles of Myd family members. This identification can be made *in vitro*, e.g., under stringent hybridization conditions or PCR (e.g., using primers encoding the Myd sequences identified herein), or by using the sequence information in a computer system for comparison with other nucleotide sequences. Different alleles of *MYD* genes within a single species population will also be useful in determining whether differences in allelic sequences correlate to differences in taste perception between members of the population. Classical PCR-type amplification and cloning techniques are useful for isolating orthologs, for example, where degenerate primers are sufficient for detecting related genes across species.

[0167] For instance, primers designed using the sequences disclosed herein can be used to amplify and clone *MYD* -related genes from different fungal genomes. In contrast, genes within a single species that are related to *MYD* are best identified using sequence pattern recognition software to look for related sequences. Typically, identification of polymorphic variants and alleles of *MYD* family members can be made by comparing an amino acid sequence of about 25 amino acids or more, e.g., 50-100 amino acids. Amino acid identity of approximately at least 35 to 50%, and optionally 60%, 70%, 75%, 80%, 85%, 90%, 95-99%, or above typically demonstrates that a protein is a polymorphic variant, interspecies homolog, or allele of a *MYD* family member. Sequence comparison can be performed using any of the sequence comparison algorithms discussed below. Antibodies that bind specifically to Myd polypeptides or a conserved region thereof can also be used to identify alleles, interspecies homologs, and polymorphic variants.

[0168] In an embodiment, hybrid protein-coding sequences comprising nucleic acids encoding Myd variant fusion proteins may be constructed. These nucleic acid sequences can be operably linked to transcriptional or translational control elements, e.g., transcription and translation initiation sequences, promoters and enhancers, transcription and translation terminators, polyadenylation sequences, and other sequences useful for transcribing DNA into RNA. Fusion proteins may include C-terminal or N-terminal translocation sequences. Further, fusion proteins can comprise additional elements, e.g., for protein detection, purification, or other applications. Detection and purification facilitating domains include, e.g., metal chelating peptides such as polyhistidine tracts, histidine-tryptophan modules, or other domains that allow purification on immobilized metals; maltose binding protein; protein A domains that allow purification on immobilized immunoglobulin; or the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle Wash.).

[0169] In an embodiment, the fusion protein comprises a peptide or protein tag (e.g., for protein purification or detection). Protein/peptide tags are peptide sequences genetically grafted into a recombinant (e.g., fusion) protein. Peptide/protein tags are known in the art, such as those described in Johnson, "Protein/Peptide Tags," DOI //dx.doi.org/10.13070/mm.en.2.116 including but not limited to green fluorescent protein (GFP), FLAG, Myc epitope, polyhistidine, glutathione-S-transferase (GST), HA, V5, ABDz1-tag, Adenylate kinase (AK-tag), BC2-tag, Calmodulin-binding peptide, CusF, Fc, Fh8, Halo tag, Heparin binding peptide (HB-tag), Ketosteroid isomerase (KSI), maltose-binding protein (MBP), thioredoxin, PA(NZ-1), Poly-Arg, Poly-Lys, S-tag, SBP / Streptavidin-Binding Peptide, SNAP, Strep-II (Twin-Strep), and SUMO / SUMO2.

[0170] Affinity tags are a type of protein tag that is appended to proteins so that they can be purified from their crude biological source using an affinity technique. Affinity tags are known in the art, such as those described in Kimple et al. *Curr Protoc Protein Sci.*; 73: Unit-9.9, doi:10.1002/0471140864.ps0909s73. These include, among others, polyhistidine, GST, MBP, Calmodulin-binding peptide, intein-chitin binding domain, Streptavidin/Biotin-based tags, and His-Patch ThioFusion (thioredoxin). Affinity tags include small (e.g., 20 or less amino acid residues) or large affinity tags. Examples of small affinity tags include His, FLAG, Strep II, and S-peptide, and examples of large affinity tags include MBP, GST, cellulose binding domains, calmodulin binding peptide, and His-patch thioredoxin.

[0171] Protein/peptide tags include epitope tags and reporter tags. Reporter tags serve as reporters of protein expression and protein-protein interaction. Reporter tags include, but are not limited to, enzymes such as β -galactosidase (β -gal), alkaline phosphatase (AP), chloramphenicol acetyl transferase (CAT), and horseradish peroxidase (HRP).

[0172] Epitope tags include FLAG, hemagglutinin (HA), c-myc, T7, and Glu-Glu, which are used for the detection of fusion proteins in vitro and in cell culture. Their short, linear recognition motifs rarely affect the properties of the protein of interest and are usually very specific for their respective primary antibodies. If the anti-myc antibody is used, specificity can be increased by using an enzyme-linked secondary antibody to detect a conjugated anti-myc primary antibody instead of using an HRP- or AP-anti-myc conjugate alone.

[0173] Protein/peptide tags also include solubilization tags which are used to assist in the proper folding of proteins and keep them from aggregating in inclusion bodies. In embodiments, solubilization tags are employed for proteins expressed in *E. coli*. Solubilization tags include thioredoxin and poly(NANP), among others. Some affinity tags can also assist in solubilization, such as MBP and GST.

[0174] Protein/peptide tags can be at either end of the target protein. Some tags, such as FLAG, are often used in tandem to increase their desired features, or in combination with another tag, such as in the construct of His-Myc and His-V5.

[0175] Tandem affinity purification (TAP) is a dual-affinity purification method based on the fusion of two affinity tags to a protein of interest, which allows purification of a tagged protein and isolation of protein complexes interacting with the protein of interest. The use of TAP is encompassed within the present invention.

[0176] In one embodiment, the fusion protein comprises a histidine tag that comprises 2-10 (e.g., 2, 3, 4, 5, 6, 7, 8, 9, or 10) histidine residues. For example, the histidine tag can comprise 6 histidine residues.

[0177] The inclusion of a cleavable linker sequences such as Factor Xa (see, e.g., Ottavi, *Biochimie* 80:289-293 (1998)), subtilisin protease recognition motif (see, e.g., Polyak, *Protein Eng.* 10:615-619 (1997)); enterokinase (Invitrogen, San Diego, Calif.), and the like, between the translocation domain (for efficient plasma membrane expression) and the rest of the newly translated polypeptide may be useful to facilitate purification. For example, one construct can include a polypeptide encoding a nucleic acid sequence linked to six histidine residues followed by a thioredoxin, an enterokinase cleavage site (see, e.g., Williams, *Biochemistry* 34:1787-1797 (1995)), and a C-terminal translocation domain. The histidine residues facilitate detection and purification while the enterokinase cleavage site provides a means for purifying the desired protein(s) from the remainder of the fusion protein. Technology pertaining to vectors encoding fusion proteins and application of fusion proteins are well described in the scientific and patent literature, see, e.g., Kroll, *DNA Cell. Biol.* 12:441-53 (1993).

[0178] The fusion protein can contain one or more linkers (e.g., flexible linkers, rigid linkers, and *in vivo* cleavable linkers). Besides the basic role in linking the functional domains together (as in flexible and rigid linkers) or releasing free functional domain *in vivo* (as in *in vivo* cleavable linkers), linkers offer many other advantages for the production of fusion proteins, such as improving biological activity, increasing expression yield, and achieving desirable pharmacokinetic profiles. Linkers are known in the art (see, e.g., Chen et al., Adv Drug Deliv Rev. 65(10): 1357–1369 (2013)).

[0179] Flexible linkers are used when the joined domains require a certain degree of movement or interaction. They are generally composed of small, non-polar (e.g. Gly) or polar (e.g. Ser or Thr) amino acids. The small size of these amino acids provides flexibility and allows for mobility of the connecting functional domains. The incorporation of Ser or Thr can maintain the stability of the linker in aqueous solutions by forming hydrogen bonds with the water molecules, and therefore reduces the unfavorable interaction between the linker and the protein moieties.

[0180] The most commonly used flexible linkers have sequences consisting primarily of stretches of Gly and Ser residues (“GS” linker). An example of the most widely used flexible linker has the sequence of (Gly-Gly-Gly-Gly-Ser)_n (SEQ ID NO:7). By adjusting the copy number “n”, the length of this GS linker can be optimized to achieve appropriate separation of the functional domains, or to maintain necessary inter-domain interactions. Besides the GS linkers, many other flexible linkers have been designed for recombinant fusion proteins. These flexible linkers are also rich in small or polar amino acids such as Gly and Ser but can contain additional amino acids such as Thr and Ala to maintain flexibility, as well as polar amino acids such as Lys and Glu to improve solubility.

[0181] Rigid linkers keep a fixed distance between the domains and to maintain their independent functions. Examples of rigid linkers include alpha helix-forming linkers with the sequence of (EAAAK)_n (SEQ ID NO:8) and linkers with a Pro-rich sequence, (XP)_n, with X designating any amino acid, preferably Ala, Lys, or Glu.

[0182] The polypeptide of the present invention also can contain a signal peptide (i.e., a signal sequence, targeting signal, localization signal, localization sequence, transit peptide, leader sequence, or leader peptide), which is a short peptide present at the N-terminus or occasionally C-terminus of most newly synthesized proteins that are destined toward the secretory pathway. These proteins include those that reside either inside certain organelles (the endoplasmic reticulum, Golgi or endosomes), secreted from the cell, or inserted into most cellular membranes. Exemplary signal peptides are known in the art and a person of ordinary skill in the art would recognize how to select a particular signal peptide for use in the invention.

[0183] PROTEIN DERIVATIZATION

[0184] HTS proteins/polypeptide and sequence variants thereof can be further derivatized or modified other than by substitution of one or more amino acids. HTS wild-type (native) protein/polypeptide and HTS protein/polypeptide variants of this disclosure can be derivatized at the N-terminus, the C-terminus or at an amino acid side chain without loss of taste modulation activity. Derivatization, in general includes, acylation, esterification, glycosylation, oxidation, methylation, reductive alkylation, phosphorylation (as phospho-amino acids), sulfurylation, sulfonylation, or oxidation or reduction of side chain heteroatoms. Derivatization can be accomplished by chemical methods using reagents and methods that are well-known in the art. Alternatively, derivatization can be accomplished by biological methods, such as treatment with one or more enzymes, or by post-translational methods (post-translational modification). One of ordinary skill in the art can select one or more enzymes to effect desired derivatization of HTS proteins/polypeptides or variants thereof. The HTS protein or polypeptide, preferably isolated, is treated with the selected enzyme to achieve the desired derivatization. Post-translational modifications (PTMs) are covalent modification to a protein/polypeptide by proteolytic cleavage (e.g., removal of N-terminal methionine) and/or addition of a modifying group, such as acetyl (more broadly acyl), a phosphoryl, a glycosyl and/or a methyl, to one or more amino acids. Other chemical modification of an amino acid of a protein/polypeptide (e.g., oxidation of the sulfur of a methionine group) can be achieved by both chemical and biological means to one skilled in the art. Post-translational modification can occur during native expression of a protein/polypeptide or post-translational modification can be controlled during protein expression in a non-native host using recombinant methods. Such recombinant methods rely on the use of specially constructed expression vectors including, for example, coding sequences for expression of one or more enzyme to actuate a selected post-translational modification. One of ordinary skill in the art, is aware of chemical or recombinant techniques to derivatize proteins at one or more position on a given protein. In an embodiment, preferred protein/polypeptide derivatizations are those that do not significantly affect the sweet taste-modulation activity of the HTS protein/polypeptide variants of this disclosure. In an embodiment, preferred protein/polypeptide derivatizations are those that do not significantly detrimentally affect the sweet taste of the HTS protein/polypeptide variants of this disclosure (e.g., do not significantly decrease the sweet taste of the HTS variants herein). In embodiments, derivatization of the HTS variant can enhance flavor modification, sweet taste modification or sweet taste activity of HTS variants.

[0185] Derivatization of an HTS protein/polypeptide or variant, by any known method, can occur at the N-terminus, the C-terminus, or at one or more amino acid side group (e.g., a side-chain sulfur, a side chain amine or a side-chain carboxylic acid). In embodiments, a HTS protein/polypeptide or HTS variant of this disclosure can have 1, 2, 3, 4, 5 or 6 different derivations. Preferably, the HTS protein or HTS variant has a single derivatization. For example, the N-terminus of the HTS

protein/polypeptide or variant can be derivatized by acylation and more specifically by acetylation. In more specific embodiments, the N-terminus of the protein/polypeptide or variant is a methionine that is N-acetylated. In embodiments, the derivatization is of the N-terminal amino acid or of an amine side chain of an amino acid of the HTS protein/polypeptide. The N-terminal amine acid or an amine side chain (e.g., a lysine side-chain) can be derivatized by acylation; glycosylation; methylation; reductive amination to form $\text{-NH-CH}_2\text{-R}$, where R is an alkyl group (e.g., an alkyl group having 1-19 carbon atoms); phosphorylation of amino acid side chains such as serine or threonine (-OH) with kinases to form a phospho-polypeptides; reaction of sulfur in amino acid side chains with monooxygenases to produce sulfoxide derivatives. The N-terminal amine or an amine side-chain can be derivatized by chemical methods or by biological processes after the translation of the polypeptide, resulting in a post-translational modification of the nitrogen. Reactive side chains of other amino acids may be derivatized by chemical methods or biological processes after the translation of the polypeptide, resulting in post-translational modification of those side chains.

[0186] In specific embodiments, the derivatization is an addition, subtraction, or substitution of one or more of the following chemical moieties to an amine nitrogen: acyl (R-CO- , where R is a straight-chain or branched alkyl group having 1-20 carbon atoms); acetyl (CH_3CO), formyl (HCO), glycosyl (e.g., $\text{C}_6\text{H}_{11}\text{O}_6\text{-}$), hydroxyl (HO-), methyl ($\text{CH}_3\text{-}$) or other alkyl group, a phosphatidyl (PO_4), a phosphonyl (PO_2), a sulfhydryl (SH-), or a sulfonyl ($\text{HSO}_2\text{-}$).

[0187] In specific embodiments, wherein the first amino acid of the HTS protein/polypeptide is a methionine, the delta-position sulfur of the first amino acid is modified, by chemical methods, by enzymatic methods (*in vitro* or *in vivo*) or by biological processes after the translation of the polypeptide, resulting in a post-translational modification of the sulfur. In embodiments, the modification of the delta-position sulfur of the first amino acid is an addition, subtraction, or substitution of any of the following chemical moieties to the delta sulfur: acyl (R-CO- , where R is a straight-chain or branched alkyl group having 1-20 carbon atoms); acetyl (CH_3CO), formyl (HCO), glycosyl (e.g., $\text{C}_6\text{H}_{11}\text{O}_6\text{-}$), hydroxyl (HO-), methyl ($\text{CH}_3\text{-}$) or other alkyl group, a phosphatidyl (PO_4), a phosphonyl (PO_2), a sulfhydryl (SH-), or a sulfonyl ($\text{HSO}_2\text{-}$). In embodiments, the modification of the delta-position sulfur of the first amino acid is the oxidation of the sulfur, resulting in a sulfoxide derivative.

[0188] As used herein, "at least 80% identity" with reference to an amino acid sequence or a nucleotide sequence refers to 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more identity.

[0189] As used herein, examples of "an amino acid sequence modified by deletion, insertion, substitution, or addition of one or more amino acids" include an amino acid sequence modified by

deletion, insertion, substitution, or addition of 1 or more to 30 or less, preferably 20 or less, more preferably 10 or less, and further preferably 5 or less amino acids (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or any ranges thereof). As used herein, examples of "a nucleotide sequence modified by deletion, insertion, substitution, or addition of one or more nucleotides" include a nucleotide sequence modified by deletion, insertion, substitution, or addition of 1 or more to 90 or less, preferably 60 or less, more preferably 30 or less, further preferably 15 or less, and further more preferably 10 or less nucleotides (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, or any ranges thereof).

[0190] For example, in sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, as described below for the BLASTN and BLASTP programs, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0191] A "comparison window," as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology* (Ausubel et al., eds. 1995 supplement)).

[0192] A preferred example of an algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., *Nuc. Acids Res.* 25:3389-3402 (1997) and Altschul et al., *J Mol. Biol.* 215:403-410 (1990), respectively. Software for performing BLAST analyses is publicly available through the National

Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., Altschul et al., *Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul et al., *J Mol. Biol.* 215:403-410 (1990)). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W , T , and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, $M=5$, $N=-4$ and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, $M=5$, $N=-4$, and a comparison of both strands.

[0193] Another example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a so-called "tree" or "dendrogram" showing the clustering relationships used to create the alignment (see, e.g., FIG. 1). PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153 (1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap

weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, e.g., version 7.0 (Devereaux et al., Nuc. Acids Res. 12:387-395 (1984) encoded by the genes were derived by conceptual translation of the corresponding open reading frames.

[0194] The polynucleotides encoding the polypeptides of the present invention can be synthesized chemically or by genetic engineering based on the amino acid sequence of a Myd. For example, the polynucleotide can be synthesized chemically based on the amino acid sequence of the polypeptides of the present invention or preprotein thereof. A contract synthesis service of nucleic acid (provided from, for example, Medical & Biological Laboratories Co., Ltd., Genscript etc.) can be used for the chemical synthesis of the polynucleotide. Further, the synthesized polynucleotide can be amplified by PCR and cloning etc.

[0195] The polypeptides of the present invention can be produced, for example, by expressing a gene encoding a Myd polypeptide variant of the present invention. Preferably, a Myd polypeptide variant of the present invention can be produced from a transformant in which the polynucleotide encoding a Myd polypeptide variant of the present invention is introduced. For example, a Myd polypeptide variant of the present invention is produced from a polynucleotide encoding a Myd polypeptide variant of the present invention introduced in a transformant after a polynucleotide encoding a Myd polypeptide variant of the present invention or a vector comprising it is introduced into a host to obtain a transformant and the transformant is cultured in an appropriate medium. The proteins of the present invention can be obtained by isolating or purifying the produced Myd polypeptide variant from the culture.

[0196] Therefore, the present invention further provides a polynucleotide encoding a Myd polypeptide variant of the present invention and a vector comprising it. The present invention further provides a method of manufacturing a transformant, comprising introducing a polynucleotide encoding a Myd polypeptide variant of the present invention or a vector comprising it into a host. The present invention further provides a transformant comprising a polynucleotide encoding a Myd polypeptide variant of the present invention or a vector comprising it introduced from the outside of a cell. The present invention further provides a method of manufacturing a Myd polypeptide variant of the present invention, comprising culturing the transformant.

[0197] The present invention also includes the polynucleotides of the invention, operably linked to a heterologous regulatory element. The invention may include an expression cassette or vector comprising the polynucleotides of the present invention, and a host cell transformed with a vector of the invention.

[0198] Alternatively, the polynucleotide encoding a Myd polypeptide variant of the present invention can be produced by introducing one or more mutation into the polynucleotide synthesized according to the procedure with known mutagenesis methods such as the ultraviolet irradiation and site-directed mutagenesis. For example, the polynucleotide encoding the polypeptides of the present invention can be obtained by introducing one or more mutation into the polynucleotide of SEQ ID NO:1 or SEQ ID NO:2 with a known method, expressing the obtained polynucleotide, investigating the expressed protein's sweet-modification activity, and selecting a polynucleotide encoding the protein having desired sweet modification activity.

[0199] Site-directed mutagenesis of a polynucleotide can be performed with any methods such as, for example, inverse PCR and annealing (Muramatsu et al. edit., "Revised 4th edition New genetic engineering handbook", YODOSHA, p. 82-88). A variety of commercially available kits for site-directed mutagenesis such as QuickChange II Site-Directed Mutagenesis Kit from Stratagene and QuickChange Multi Site-Directed Mutagenesis Kit can be used as needed.

[0200] Examples of the type of a vector comprising the polynucleotide encoding the polypeptides of the present invention include, without limitation, a vector usually used for gene cloning, for example, a plasmid, a cosmid, a phage, a virus, a YAC and a BAC. Examples of vectors include plasmids (e.g., DNA plasmids), yeast (e.g., *Saccharomyces*), and viral vectors, such as poxvirus, retrovirus, adenovirus, adeno-associated virus, herpes virus, polio virus, alphavirus, baculovirus, Sindbis virus, plant viruses (e.g., *Alphaflexiviridae* or *Potyviridae*), and insect viruses (e.g., *Baculoviridae*).

[0201] Among these, a plasmid vector is preferred and for example a commercially available plasmid vector for protein expression, for example, pUC19, pUC118, pUC119, pBR322 etc. (all of which are from TAKARA BIO INC.) can be used.

[0202] The vector can comprise a DNA region comprising a replication initiation region or a replication origin of DNA. Alternatively, a regulatory sequence such as a promoter region for initiating transcription of the gene, a terminator region or a secretory signal region for secreting an expressed protein to the outside of a cell can be operably linked to the upstream of the polynucleotide encoding the proteins of the present invention (i.e. the *MYD* gene of the present invention) in the vector. As used herein, a gene and a regulatory sequence being "operably linked" refers to a condition in which the gene and the regulatory region are positioned so that the gene can be expressed under the regulation by the regulatory region.

[0203] The type of the regulatory sequence of a promoter region, a terminator, and a secretory signal region etc. is not specifically limited, and a promoter and a secretory signal sequence usually used can be selected to use as appropriate depending on the host into which the sequence is introduced. For example, preferred examples of the regulatory sequence which can be incorporated to the vector of the

present invention include the *cbh1* promoter sequence derived from *Trichoderma reesei* (Curr, Genet, 1995, 28 (1): 71-79).

[0204] Alternatively, a marker gene to select a host into which the vector is appropriately introduced (for example, a resistance gene to an agent such as ampicillin, neomycin, kanamycin and chloramphenicol) can be further incorporated into the vector of the present invention. Alternatively, a gene encoding a synthase of a required nutrient can be incorporated into the vector as a marker gene, when an auxotrophic strain is used as a host. Alternatively, a related gene of the metabolism can be incorporated into the vector as a marker gene, when a selective medium requiring specific metabolism for growth is used. Examples of such a metabolism related gene include an acetamidase gene for using acetamide as a nitrogen source.

[0205] Ligation between the polynucleotide encoding a Myd polypeptide variant of the present invention and a regulatory sequence and a marker gene can be performed by a known method in the art such as SOE (splicing by overlap extension) -PCR (Gene, 1989, 77: 61-68). The procedure for introducing a ligated fragment into a vector is known in the art.

[0206] Examples of a host of a transformant into which the vector is introduced include a microorganism such as a bacterium or filamentous fungus. Examples of the bacterium include *Escherichia coli* and a bacterium belonging to *Staphylococcus*, *Enterococcus*, *Listeria* and *Bacillus*, of which *Escherichia coli* and *Bacillus bacteria* (for example, *Bacillus subtilis* or a mutant thereof) are preferred. Examples of the *Bacillus subtilis* mutant can include protease 9 double deficient strain KA8AX described in J. Biosci. Bioeng., 2007, 104 (2): 135-143 and a DBPA strain, a mutant from protease 8 double deficient strain described in Biotechnol. Lett., 2011, 33 (9): 1847-1852, of which protein folding efficiency is improved. Examples of the filamentous fungus include *Trichoderma*, *Aspergillus* and *Rhizopus*. Also, for example, *Pichia pastoris*, *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Yarrowia lipolytica*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis* are appropriate expression hosts. In embodiments, the host cell is a cell of a fungus other than *Mattiolomyces terfezioides*. In embodiments, the HTS protein variant can be expressed in mycelium.

[0207] In embodiments, the HTS protein variant can be expressed in a plant cell, a plant organ, a leaf, a root or in a whole plant.

[0208] In yet another aspect, the invention includes a host cell comprising one or more of the expression cassettes described herein operably linked to control elements compatible with expression in the cell. The cell can be, for example, a mammalian cell (e.g., BHK, VERO, HT1080, 293, RD, COS-7, or CHO cells), an insect cell (e.g., *Trichoplusia ni* (Tn5) or Sf9), a bacterial cell, a plant cell, or a yeast cell.

[0209] In a particular embodiment, the HTS (or a variant thereof) is produced in a yeast expression system (i.e., yeast-derived HTS or variant thereof), for example, in the genera *Kluyveromyces* (e.g., *K. lactis*), *Lactococcus* (e.g., *L. lactis*), *Lactobacillus*, *Saccharomyces* (e.g., *S. cerevisiae*) *Pichia* (e.g., *P. pastoris*), *Hansenula* (e.g., *H. polymorpha*) or *Yarrowia* (e.g., *Y. lipolytica*).

[0210] In another particular embodiment, the HTS (or a variant thereof) is produced in a bacterial expression system (i.e., bacteria-derived HTS or variant thereof), for example, in *Escherichia coli* or *Bacillus subtilis*. In one embodiment, the HTS (or a variant thereof) is not produced in *E. coli*.

[0211] In a further particular embodiment, the HTS (or a variant thereof) is produced in an insect expression system (i.e., insect-derived HTS or variant thereof), for example, in baculovirus infected or non-lytic insect cells (e.g., sf9, Sf21).

[0212] In another embodiment, the HTS (or a variant thereof) is produced in a fungal expression system (i.e., fungi-derived HTS or variant thereof), for example in the genera *Chrysosporium*, *Thielavia*, *Talaromyces*, *Trichoderma*, *Thermomyces* or *Thermoascus*.

[0213] In yet another embodiment, HTS (or a variant thereof) is produced in a mammalian expression system (i.e., mammalian-derived HTS or variant thereof), for example in Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK), COS and baby hamster kidney (BHK) cells. Alternatively, the HTS (or a variant thereof) may be produced *in vitro* using a cell-free expression system, such as an *E. coli* S30 extract.

[0214] PURIFICATION

[0215] The recombinantly expressed polypeptides from Myd-encoding expression cassettes are typically isolated from lysed cells or culture media. Purification can be carried out by methods known in the art including salt fractionation, ion exchange chromatography, gel filtration, size-exclusion chromatography, size-fractionation, affinity chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential precipitation or solubilization. Immunoaffinity chromatography can be employed using antibodies generated based on, for example, Gag antigens.

[0216] The invention provides a method of purifying a polypeptide having sweet-taste modulation activity comprising (a) obtaining a composition comprising the polypeptide, and (b) purifying the composition via hydrophobic interaction chromatography (HIC) followed by size exclusion chromatography (SEC).

[0217] One of skill in the art is familiar with the purification techniques of hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC), including the selection of

appropriate columns, buffers, and eluting solutions. Exemplary HIC and SEC purification techniques are described herein in Example 11. In an exemplary aspect, the purity of the polypeptide following purification by HIC and SEC is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or any ranges of values thereof.

[0218] In a cell-based system, the first step in the protein purification process extracts the protein from the cells by lysing or breaking them open. Any suitable cell lysis method may be used, for example, mechanical disruption, chemical breakdown, freeze-thaw cycles or enzymatic digestion. The protein can then be purified by any suitable protein purification method for example, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential precipitation or solubilization.

[0219] The yield of *in vivo* production of HTS (or a variant thereof) may vary. In a particular embodiment, HTS represents at least about 1% of total cellular proteins. In a particular embodiment, HTS represents between about 1% and about 5% of total cellular proteins. In another embodiment, HTS represents between about 5% and about 10% of total cellular proteins. In a further embodiment, HTS represents between 10% and about 20% of total cellular proteins. In certain embodiments, HTS represents more than 20% of total cellular proteins.

[0220] In another particular embodiment, HTS is purified to provide a yield between about 1 mg/mL and about 200 mg/mL, more particularly, between about 5 mg/mL and about 195 mg/mL, between about 10 mg/mL and about 190 mg/mL, between about 15 mg/mL and about 185 mg/mL, between about 20 mg/mL and about 180 mg/mL, between about 25 mg/mL and about 175 mg/mL, between about 30 mg/mL and about 170 mg/mL or about 35 mg/mL and about 165 mg/mL. In one embodiment, the HTS is purified to provide a yield of about 5 mg/mL, about 10 mg/mL, about 15 mg/mL, about 20 mg/mL, about 25 mg/mL, about 30 mg/mL, about 35 mg/mL, about 40 mg/mL, about 45 mg/mL, about 50 mg/mL, about 75 mg/mL, about 100 mg/mL, about 125 mg/mL, about 150 mg/mL, about 175 mg/mL, or about 200 mg/mL or more. Optionally, the HTS is produced as a fusion protein further comprising a tag and the yields described above reflect both purification and removal of the tag.

[0221] In a particular embodiment, the HTS (or a variant thereof) is substantially pure. In one embodiment, HTS (or a variant thereof) is at least about 80% pure, at least about 85% pure, at least about 90% pure, at least about 95% pure or at least about 99% pure. In another embodiment, HTS (or a variant thereof) is about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98% or about 99% pure.

[0222] PLANTS

[0223] The invention also contemplates a transgenic plant comprising a heterologous polynucleotide and/or heterologous polypeptide of the invention as described herein. The plant has an altered phenotype due to the expression of the heterologous nucleic acid sequence. The altered phenotype may include a phenotype with increased sweetness in any plant part, including fruits. The transgenic plant may contain an expression cassette as defined herein as a part of the plant, the cassette having been introduced by transformation of a plant with a vector of this invention. Such expression cassettes include regulatory sequences for expression of heterologous coding sequences in plants, including plant-expressible promoters and terminators. A transgenic plant can be any type of plant which can express the heterologous nucleic acid sequences described herein. The term “plant” includes whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. The class of plants which can be used in the method of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous (monocots) and dicotyledonous (dicots) plants. It includes plants of a variety of ploidy levels, including polyploid, diploid and haploid. For example, the transgenic plant can be an apple or strawberry. An HTS protein/polypeptide or variant of this disclosure can be produced in a plant or a plant culture.

[0224] Techniques for transforming a wide variety of plant species are well known in the art and described in the technical and scientific literature. See, for example, Weising et al. (1988) *Ann. Rev. Genet.*, 22:421-477 and Joung et al. (2015) “Plant Transformation Methods and Applications,” in *Current Technology in Plant Molecular Breeding*, (Koh et al., eds) Springer Dordrecht Heidelberg New York London, Chapter 9, pages 297-344. Any method known in the art for transformation of plant cells, including plant protoplasts, or plant tissue can be employed for plant transformation. Specific methods for plant transformation include among others, bolistic methods (gene guns), electroporation, microinjection, protoplast fusion and *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformation can, for example, employ binary vectors that replicate in *Escherichia coli* and *Agrobacterium tumefaciens* or other *Agrobacterium* strains. A variety of such binary vectors are known in the art and can be employed to introduce heterologous polynucleotides into plant cells and plant tissue. Plant expression vectors which include regulatory sequences for expression of heterologous coding sequences, including plant-expressible promoter sequences and other plant regulatory sequences, in plant cells and plant tissue are known in the art and can be employed to transform plants to express polypeptides as described herein.

[0225] A variety of plant-expressible promoters are known in the art and are available for use in heterologous constructs, vectors and transformed plant materials herein which contain polynucleotides encoding protein having sweet-taste modulation activity. Plant-expressible promoters can derive from natural plant sources, plant virus sources and from bacteria, such as *Agrobacterium* strains, having

promoters that are plant-expressible. Plant-expressible promoters include, among others, Cauliflower Mosaic Virus promoter (CaMV 35S), octopine and nopaline synthase promoters (e.g., *nos* promoter), plant ubiquitin promoter (Ubi), rice actin promoter (*Act-1*), and maize alcohol dehydrogenase (*Adh-1*). Plant-expressible promoters include constitutive promoters, inducible promoters, tissue-specific promoters, developmental stage-specific promoters and examples of each type of promoter are known in the art. Tissue-specific promoters include, among others, those that direct expression in plant roots, plant leaves, fruit, flowers, pollen or cells engaged in active photosynthesis (e.g., phosphoenolpyruvate promoters (PEP)). Development stage-specific promoters include those that direct expression during fruit ripening, flowering or seed set. Synthetic plant promoters are also known in the art and are useful in heterologous constructs, vectors and transformed plant materials (see, e.g., Ali S. & Kim W-C (2019) *Frontiers in Plant Science*, 10, article 1433).

[0226] Techniques for regeneration of plants from transformed protoplasts, plant cells, callus, or other plant tissue are well known in the art and can be employed to regenerate whole plants and plant parts from such transformed plant material. Regeneration methods include organogenesis and embryogenesis. See: *Handbook of plant cell culture. Volume 1: Techniques for propagation and breeding* (1983) Edited by D. A. Evans et al., Macmillan (New York); R.H. Smith, *Plant Tissue Culture: Techniques and Experiments*, 3rd Edition (2012) Academic Press (New York); M.R. Davey & P. Anthony, *Plant Cell Culture: Essential Methods* (2010) John Wiley & Sons (New York), particularly Chapters 3 and 9.

[0227] In embodiments, HTS (or a variant thereof) is produced in an algal expression system (i.e., algae-derived HTS or variant thereof).

[0228] In embodiments, HTS (or a variant thereof) is produced in a plant expression system (i.e., plant-derived HTS or variant thereof), for example, in maize, corn, tobacco, melon (e.g., watermelon), potatoes, strawberries, duckweed or sugarcane. In one embodiment, the plant expression system is a plant cell culture expression system.

[0229] In a particular embodiment, HTS (or a variant thereof) is produced in maize, and more particularly, the seeds of maize. In another particular embodiment, HTS (or a variant thereof) is produced in corn, and more particularly, the seeds of corn. According to these embodiments, HTS (or a variant thereof) may be utilized as HTS-containing germ flour.

[0230] METHODS OF PRODUCING A PROTEIN HAVING SWEET-TASTE MODULATION ACTIVITY (IN A HOST OR CELL-FREE EXPRESSION SYSTEM)

[0231] A method usually used in the field such as protoplast method and electroporation can be used as a method of introducing a vector into a host. A transformant of interest can be obtained by selecting a

strain in which a vector is appropriately introduced using an index such as the expression of a marker gene and/or auxotrophy.

[0232] Alternatively, a fragment in which the polynucleotide encoding a Myd polypeptide variant of the present invention, a regulatory sequence and a marker gene are ligated can be directly introduced into the genome of a host. For example, the polynucleotide encoding a Myd polypeptide variant of the present invention is introduced into the genome of a host by constructing a DNA fragment added with a sequence complementary to the genome of the host at both ends of the ligated fragment, introducing the fragment into the host and inducing homologous recombination between the host genome and the DNA fragment by SOE-PCR.

[0233] Culturing the thus obtained transformant, in which the polynucleotide encoding a Myd polypeptide variant of the present invention or a vector comprising is introduced, in an appropriate medium, results in the expression of the *MYD* cDNA on the vector, and then the production of a Myd polypeptide variant of the present invention. The medium used for the culture of such transformant can be selected depending on the type of microorganism of such transformant by those skilled in the art as appropriate.

[0234] Alternatively, a Myd polypeptide variant of the present invention can be expressed from the polynucleotide encoding a Myd polypeptide variant of the present invention or a transcription product thereof using a cell-free translation system. "Cell-free translation system" refers to an *in vitro* transcription-translation system or an *in vitro* translation system constructed by adding reagents such as amino acids required for the translation of a protein into a suspension obtained by mechanically destructing cells to be a host.

[0235] Cell-free systems that can be used to produce HTS for use in the compositions described herein include, but are not limited to, protein expression components from eukaryotic, prokaryotic, and/or viral sources. For example, cell-free systems as used herein can include mammalian and/or bacterial protein expression systems derived from mammalian and/or bacterial lysates.

[0236] A Myd polypeptide variant of the present invention produced in the culture or cell-free translation system can be isolated or purified, if desired, by using a general method used for the purification of a protein, for example, centrifugation, ammonium sulfate precipitation, gel chromatography, ion-exchange chromatography and affinity chromatography etc. alone or in combination as appropriate. Here, when the gene encoding a Myd polypeptide variant of the present invention and the secretory signal sequence are operably linked on the vector within the transformant, the produced Myd polypeptide variant can be collected more easily from the culture because the Myd polypeptide variant is secreted to the outside of a cell. The Myd polypeptide variant collected from the culture can be further purified by any known means.

[0237] In embodiments, the Myd protein is solubilized in a liquid solution, such as e.g., a buffered solution or any solution that readily dissolves the Myd protein into solution. In embodiments, the Myd protein contained in a liquid solution is lyophilized to form a powder. In embodiments, the Myd protein contained in a liquid solution is dried to form a powder, such as e.g., by using a spray dryer. In embodiments, spray drying includes the use of a carrier as is known in the art. In embodiments, the carrier used for spray drying is maltodextrin, gum Arabic or whey protein concentrate,

[0238] The present invention also includes a method for producing a protein having sweet-taste modulation activity, comprising culturing the host cells of the invention in a medium under conditions that result in producing the protein having sweet-taste modulation activity, similar to a known sweet flavoring agent or compound.

[0239] SWEET COMPOSITIONS-FOODS, BEVERAGES, SUPPLEMENTS, MEDICINAL PRODUCTS

[0240] Disclosed herein are sweetener compositions and flavor modifying compositions, in each case, containing Myd (HTS) or variants thereof. In certain embodiments, the sweetener compositions and flavor modifying compositions change (e.g., improve) one or more sensory experiences of a subject who consumes the same. In a particular embodiment, the sweetener composition and flavor modifying compositions disclosed herein comprise a HTS variant. In one embodiment, the HTS variant differs from wild-type HTS at least one amino acid position and more particularly, at one, two, three or more amino acid positions.

[0241] As used herein, a “sweet flavoring agent,” “sweet compound” or “sweet receptor activating compound” refers to a composition that elicits a detectable sweet flavor in a subject, e.g., sucrose, fructose, glucose, and other known natural saccharide-based sweeteners, or known artificial sweeteners such as saccharine, cyclamate, aspartame, and the like as is further discussed herein, or a material that activates a T1R2/T1R3 receptor *in vitro*. The subject may be a human or an animal.

[0242] A sweet flavoring agent or sweetening composition may be used in an effective amount, which refers to an amount of a sweetening composition of the invention that is sufficient to induce sweet taste in a subject when present in a product for oral administration.

[0243] An embodiment of the present invention includes a composition. In an embodiment, the composition comprises, consists essentially of, or consists of a combination of a product for oral administration and one or more sweetening composition comprising an isolated Myd polypeptide variant according to the invention, as described herein. In an embodiment, the combination has enhanced sweet taste compared to a product for oral administration lacking the Myd polypeptide variant (control). In one embodiment, the product for oral administration is not *Mattirolomyces*

terfezioides truffle. The term “consisting essentially of” allows for the inclusion of components that are not essential to the function or activity of the product and do not materially affect the function or activity, such as an anti-caking agent, filler, stabilizer (e.g., thermal stabilizer), and bulking agent (e.g., maltodextrose, gum acacia and the like). In an embodiment, the composition includes a plurality of isolated Myd polypeptides. In a particular embodiment, the composition includes a plurality of isolated Myd polypeptides which differ from each other to enhance taste. It should be appreciated that compositions comprising one or more Myd polypeptides of the invention are not limited by the form, shape, and means of administration, and encompass solids, liquids, powder, and other forms, either individually or in combinations of two or more thereof. Furthermore, the compositions may be administered or consumed orally, by injection, etc.

[0244] In another embodiment, compositions comprising an isolated Myd protein of the invention include formulations that provide enhanced functionality to the isolated Myd protein. For example, a composition may include a formulation that stabilizes the Myd protein against thermal, osmotic, pH, or other types of degradation. In one embodiment, the formulation stabilizes the Myd protein against thermal degradation. Exemplary compounds for stabilizing the Myd protein includes, for example, L-arginine glycine, L-proline, L-histidine, β -alanine, L-serine, L-arginine ethyl ester dihydrochloride, L-argininamide dihydrochloride, 6-aminohexanoic acid, gly-gly, gly-gly-gly, tryptone, betaine monohydrate, D-(+)-trehalose dihydrate, xylitol, D-sorbitol, sucrose, hydroxyectoine, trimethylamine n-oxide dihydrate, methyl- α -d-glucopyranoside, triethylene glycol, spermine tetrahydrochloride, spermidine, 5-aminovaleric acid, glutaric acid, adipic acid, ethylenediamine dihydrochloride, guanidine hydrochloride, urea, N-methylurea, N-ethylurea, N-methylformamide, hypotaurine, TCEP hydrochloride, GSH (l-glutathione reduced), benzamidine hydrochloride, ethylenediaminetetraacetic acid disodium salt dihydrate, magnesium chloride hexahydrate, cadmium chloride hydrate, non-detergent sulfobetaine 195 (nds-195), non-detergent sulfobetaine 201 (NDSB-201), non-detergent sulfobetaine 211 (NDSB-211), non-detergent sulfobetaine 221 (NDSB-221), non-detergent sulfobetaine 256 (NDSB-256), taurine, acetamide, oxalic acid dihydrate, sodium malonate pH 7.0, succinic acid pH 7.0, tacsimate pH 7.0, tetraethylammonium bromide, choline acetate, 1-ethyl-3-methylimidazolium acetate, 1-butyl-3-methylimidazolium chloride, ethylammonium nitrate, ammonium sulfate, ammonium chloride, magnesium sulfate hydrate, potassium thiocyanate, gadolinium(III) chloride hexahydrate, cesium chloride, 4-aminobutyric acid (GABA), lithium nitrate, DL-malic acid pH 7.0, lithium citrate tribasic tetrahydrate, ammonium acetate, sodium benzenesulfonate, sodium p-toluenesulfonate, sodium chloride, potassium chloride, sodium phosphate monobasic monohydrate, sodium sulfate decahydrate, lithium chloride, sodium bromide, glycerol, ethylene glycol, polyethylene glycol 200, polyethylene glycol monomethyl ether 550, polyethylene glycol monomethyl ether 750, formamide, polyethylene glycol 400, pentaerythritol ethoxylate (15/4

EO/OH), 1,2-propanediol, polyethylene glycol monomethyl ether 1,900, polyethylene glycol 3,350, polyethylene glycol 8,000, polyvinylpyrrolidone k15, polyethylene glycol 20,000, (2-hydroxypropyl)- β -cyclodextrin, α -cyclodextrin, β -cyclodextrin, methyl- β -cyclodextrin.

[0245] In embodiments, the sweetening composition includes one or more Myd polypeptides described above. In an embodiment, the sweetening composition includes a plurality of Myd polypeptides described above. In a particular embodiment, the plurality of Myd polypeptides differ from each other.

[0246] The present invention also includes a method for modulating the taste of a product for oral administration, comprising combining the product for oral administration with an effective amount of an isolated Myd polypeptide variant, as described herein. In one aspect, the combination has enhanced sweet taste compared to a product for oral administration lacking the Myd polypeptide variant (control). In one embodiment, the product for oral administration is not *Mattirolomyces terfezioides* truffle.

[0247] The product for oral administration may be a food, a beverage, a dietary supplement composition, or a pharmaceutical composition.

[0248] The term “product for oral administration” may refer to a comestible product (consumable) such as a food product, a beverage product, a medicinal (pharmaceutical) product, or a dietary supplement product such as an herbal supplement. As used herein, the term “consumable” may be used interchangeably with the term “product(s) for oral administration”. As used herein, the term “medicinal product” includes both solids and liquid compositions which are ingestible non-toxic materials which have medicinal value or comprise medicinally active agents such as cough syrups, cough drops, aspirin and chewable medicinal tablets. An oral hygiene product is also a product for oral administration and includes solids and liquids such as toothpaste or mouthwash. In general terms, the present invention contemplates that food or beverage products may include an isolated sweet protein of the invention in an effective amount, e.g., in an amount of up to about 99% by weight relative to the total weight of the food or beverage product, for example in an amount from about 0.01% by weight to about 99% by weight. All intermediate weights (i.e., 0.01%, 0.05%, 0.1%, 0.5%, 1%, 2%, 3%, 4%, . . . 90%, 95%, 99%) by weight relative to the total weight of the food or beverage products are contemplated, as are all intermediate ranges based on these amounts. The compositions of the invention may include a “comestibly, biologically or medicinally acceptable carrier or excipient” which can include a solid or liquid medium and/or composition that is used to prepare a desired dosage form of a Myd polypeptide variant, in order to administer a Myd polypeptide variant in a dispersed/diluted form, so that the biological effectiveness of a Myd polypeptide variant is maximized. A comestibly, biologically or medicinally acceptable carrier includes many common food ingredients, such as water at neutral, acidic, or basic pH, fruit or vegetable juices, vinegar, marinades, beer, wine,

natural water/fat emulsions such as milk or condensed milk, edible oils and shortenings, fatty acids, low molecular weight oligomers of propylene glycol, glyceryl esters of fatty acids, and dispersions or emulsions of such hydrophobic substances in aqueous media, salts such as sodium chloride, wheat flours, solvents such as ethanol, solid edible diluents such as vegetable powders or flours, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents; thickening or emulsifying agents, preservatives, solid binders, lubricants and the like.

[0249] A medicinally acceptable carrier or excipient can include excipients which allow for microencapsulation of the Myd polypeptide variant to enhance functionality, such as protecting and extending the sweetness sensation. In fact, microencapsulation is known in the art to be a technology that can facilitate regular use in addition to creating many possible new uses for sweeteners. See, e.g., Favaro-Trindade, Carmen & Rocha-Selmi, Glaucia & dos Santos, Milla. (2015). Microencapsulation of Sweeteners. 10.1016/B978-0-12-800350-3.00022-4. In one embodiment, microencapsulation methods known in the art for stabilizing and/or modifying (e.g., extending) the sweetness release of Myd. For example, sugar-free chewing gums and chewable confections usually have encapsulated sweeteners in their formulations to prolong their sweet taste during chewing. It should be appreciated that food or beverage products and compositions comprising one or more Myd polypeptides as described are not limited by the form and shape and encompass solids, liquids, powder, and other forms, either individually or in combinations of two or more thereof. Examples of food or beverage products of the present invention include such as, but not limited to, baked goods; sweet bakery products, (including, but not limited to, rolls, cakes, pies, pastries, and cookies); pre-made sweet bakery mixes for preparing sweet bakery products; pie fillings and other sweet fillings (including, but not limited to, fruit pie fillings and nut pie fillings such as pecan pie filling, as well as fillings for cookies, cakes, pastries, confectionary products and the like, such as fat-based cream fillings); desserts, gelatins and puddings; frozen desserts (including, but not limited to, frozen dairy desserts such as ice cream—including regular ice cream, soft serve ice cream and all other types of ice cream—and frozen non-dairy desserts such as non-dairy ice cream, sorbet and the like); carbonated beverages (including, but not limited to, soft carbonated beverages); non-carbonated beverages (including, but not limited to, soft non-carbonated beverages such as flavored waters and sweet tea or coffee based beverages); beverage concentrates (including, but not limited to, liquid concentrates and syrups as well as non-liquid concentrates, such as freeze-dried and/or powder preparations); yogurts (including, but not limited to, full fat, reduced fat and fat-free dairy yogurts, as well non-dairy and lactose-free yogurts and frozen equivalents of all of these); snack bars (including, but not limited to, cereal, nut, seed and/or fruit bars); bread products (including, but not limited to, leavened and unleavened breads, yeasted and un-yeasted breads such as soda breads, breads comprising any type of wheat flour, breads comprising any type of non-wheat flour (such as potato, rice and rye flours),

gluten-free breads); pre-made bread mixes for preparing bread products; sauces, syrups and dressings; sweet spreads (including, but not limited to, jellies, jams, butters, nut spreads and other spreadable preserves, conserves and the like); confectionary products (including, but not limited to, jelly candies, soft candies, hard candies, chocolates and gums); sweetened breakfast cereals (including, but not limited to, extruded (KIX type) breakfast cereals, flaked breakfast cereals and puffed breakfast cereals); and cereal coating compositions for use in preparing sweetened breakfast cereals. Other types of food and beverage products not mentioned here but which conventionally include one or more nutritive sweetener may also be contemplated in the context of the present invention.

[0250] In embodiments, the product for oral administration is a food product which is warmed or heated prior to eating or which is served for consumption warm or hot. In embodiments, polypeptide variants herein which exhibits enhanced thermal stability compared to the polypeptide of SEQ ID NO:3 are preferred for application in compositions for oral administration (e.g., food products) which are to be cooked, warmed or heated prior to administration or consumption or which are to be administered or consumed warm or hot.

[0251] As a consequence of the complete or partial replacement of nutritive sweeteners in the food or beverage products of the present invention, the food or beverage products of the present invention may be useful as low calorie or dietetic products, medical foods/products (including pills and tablets), and sports nutrition products, and may be particularly suitable for food or beverage products requiring a lower sweetness at a given soluble solids level.

[0252] In some embodiments, the sweetening composition of the invention can be supplemented with other nutritional or non-nutritional sweeteners to form a sweetener system. The sweetener system may comprise the sweetening composition of the invention, a bulking agent such as maltodextrose, gum acacia and the like, and at least one high intensity sweetener. The composition may be provided as liquid composition or a dried blend.

[0253] As used herein the term “high-intensity sweetener,” refers to any synthetic or semi-synthetic sweetener or sweetener found in nature. High-intensity sweeteners are compounds or mixtures of compounds which are sweeter than sucrose. High-intensity sweeteners are typically many times (e.g., 20 times and more, 30 times and more, 50 times and more or 100 times or more) sweeter than sucrose).

[0254] In an embodiment, the present invention includes a process for enhancing the sweet taste of a product for oral administration, comprising the addition of a Myd polypeptide variant of the invention.

[0255] In another embodiment, the methods of the invention include a method for improving the sweet flavor of a product for oral administration, comprising adding to the product for oral administration a

sweetening composition made by the methods of the invention. Amounts to add can be determined by methods known in the art, e.g., using sensory testing as a guide.

[0256] In another embodiment, the methods of the invention include methods for modifying the flavor of a product for oral administration, comprising adding to the product for oral administration a flavor modifying composition made by the methods of the invention. Amounts to add can be determined by methods known in the art, e.g., using sensory testing as a guide. A flavor modifying composition may modify (e.g., enhance, inhibit or change) a taste, aroma and/or texture of a given composition, e.g., a consumable. In a particular embodiment, the flavor modifying composition modifies (e.g., enhances, inhibits or changes) a particular taste(s). In another embodiment, the flavor modifying composition modifies (e.g., enhances, inhibits or changes) a given texture. In certain embodiments, the flavor modifying composition modifies (e.g., enhances, inhibits or changes) both a given taste(s) and texture.

[0257] A flavor modifying composition may be sweetened or unsweetened. Therefore, in some embodiments, the addition of a flavor modifying composition may serve both to add flavor modifiers and may further provide sweetness to a composition selected for taste adjustment. The addition of a sweetened flavor modifying composition may be used in addition to or alternatively to addition of another sweetening composition.

[0258] The sweetener compositions and flavor modifying compositions disclosed herein contain Myd (HTS) and variants thereof. In certain embodiments, HTS (or variant thereof) is the only sweet tasting component in the sweetener composition or flavor modifying composition. In certain embodiments, the sweetener composition or flavor modifying composition further comprises one or more additional sweet tasting components (i.e., additional sweetener or high-intensity sweetener). In embodiments, the additional sweetener is a polypeptide or protein sweetener other than HTS or a variant thereof. In embodiments, the sweetener is a carbohydrate sweetener. In embodiments, the additional sweetener is a synthetic sweetener. In a particular embodiment, the one or more sweet tasting components include steviol glycosides (e.g., Reb M, Reb A) and high fructose corn syrup (HFCS).

[0259] High-fructose corn syrup (HFCS), also known as glucose-fructose, isoglucose and glucose-fructose syrup, is a sweetener made from corn starch. As in the production of conventional corn syrup, the starch is broken down into glucose by enzymes.

[0260] Steviol glycosides are compounds responsible for the sweet taste of leaves of the plant *Stevia rebaudiana* and several related plants. Certain steviol glycosides are the ingredients in or are precursors to ingredients in stevia sweeteners. Steviol glycoside may be a single compound or a mixture of compounds. Steviol glycosides include, among others, stevioside, dulcoside A, rebaudioside A (Reb A), rebaudioside M (Reb M), rebaudioside B (Reb B), rebaudioside C (Reb C),

rebaudioside D (Reb D), rebaudioside E, rebaudioside F, rubusoside, steviolbioside and combinations thereof.

[0261] Mogrosides are glycosides of cucurbitane derivatives, including mogrol, associated with the sweet taste of extracts of *Siraitia grosvenorii* (monkfruit or luo han guo). Certain mogrosides are the ingredients in monkfruit sweeteners. Mogrosides include, among others, mogroside II A₁, mogroside II B, 7-oxomogroside II E, 11-oxomogroside A₁, mogroside III A₂, 11-deoxymogroside III, 11-oxomogroside IV A, mogroside V, 7-oxomogroside V, 11-oxo-mogroside V, mogroside VI, siamenoside I and combinations thereof. Preferred mogrosides are mogroside V, mogroside VI and siamenoside.

[0262] In one embodiment, the one or more additional sweetener may be a carbohydrate sweetener. Non-limiting examples of suitable carbohydrate sweeteners include sucrose, fructose, glucose, erythritol, maltitol, lactitol, sorbitol, mannitol, xylitol, D-tagatose, trehalose, galactose, rhamnose, cyclodextrin (e.g., α -cyclodextrin, β -cyclodextrin, and γ -cyclodextrin), ribulose, threose, arabinose, xylose, lyxose, allose, altrose, mannose, idose, lactose, maltose, invert sugar, isotrehalose, neotrehalose, palatinose or isomaltulose, erythrose, deoxyribose, gulose, idose, talose, erythrulose, xylulose, psicose, turanose, cellobiose, glucosamine, mannosamine, fucose, fuculose, glucuronic acid, gluconic acid, glucono-lactone, abequose, galactosamine, xylo-oligosaccharides (xylotriose, xylobiose and the like), gentio-oligosaccharides (gentiobiose, gentiotriose, gentiotetraose and the like), galacto-oligosaccharides, sorbose, ketotriose (dehydroxyacetone), aldotriose (glyceraldehyde), nigero-oligosaccharides, fructooligosaccharides (kestose, nystose and the like), maltotetraose, maltotriol, tetrasaccharides, mannan-oligosaccharides, malto-oligosaccharides (maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and the like), dextrans, lactulose, melibiose, raffinose, rhamnose, ribose, isomerized liquid sugars such as high fructose corn/starch syrup (HFCS/HFSS) (e.g., HFCS55, HFCS42, or HFCS90), coupling sugars, soybean oligosaccharides, glucose syrup and combinations thereof.

[0263] In other embodiments, the at least one additional sweetener is a synthetic sweetener. As used herein, the phrase “synthetic sweetener” refers to any composition which is not found naturally in nature and characteristically has a sweetness potency greater than sucrose, fructose, or glucose, yet has less calories. Non-limiting examples of synthetic high-potency sweeteners suitable for embodiments of this disclosure include sucralose, potassium acesulfame, aspartame, alitame, saccharin, neohesperidin dihydrochalcone, cyclamate, neotame, advantame, glucosylated steviol glycosides (GSGs) and combinations thereof.

[0264] In other embodiments, the at least one additional sweetener is a protein sweetener (i.e., a polypeptide or protein that tastes sweet). Typically, such protein sweeteners are extracted from plants.

Non-limiting examples of protein sweeteners are monellin, thaumatin, brazzein, curculin, pentadin, and mabinlin.

[0265] In a particular embodiment, the sweetener composition and flavor modifying compositions disclosed herein comprise a Myd (HTS) variant. In one embodiment, the HTS variant differs from wild-type HTS (with or without methionine at position 1) at least one amino acid position and more particularly, at one, two, three or more amino acid positions.

[0266] In one embodiment, the sweetener composition and flavor modifying composition disclosed herein comprises a HTS variant having a sweetness equal to or greater than wild-type HTS having an amino acid sequence of SEQ ID NO:3.

[0267] In one embodiment, the sweetener composition and flavor modifying composition disclosed herein comprise a HTS variant having a stability equal to or greater than wild-type HTS having an amino acid sequence of SEQ ID NO:3.

[0268] In one embodiment, the sweetener composition and flavor modifying composition disclosed herein comprises a HTS variant having a sweetness equal to or greater than wild-type HTS having an amino acid sequence of SEQ ID NO:3 without methionine at position 1.

[0269] In one embodiment, the sweetener composition and flavor modifying composition disclosed herein comprise a HTS variant having a stability equal to or greater than wild-type HTS having an amino acid sequence of SEQ ID NO:3 without methionine at position 1.

[0270] HTS (or variants thereof) suitable for use in compositions disclosed herein (e.g., sweetener compositions, flavor and/or taste modifying compositions, consumables) may be produced in any suitable manner as previously described herein. Representative methods of production include extraction, chemical synthesis (i.e., solid state synthesis) or recombinant production (i.e., *in vivo* production or *in vitro* production).

[0271] In one embodiment, HTS used in the compositions disclosed herein is isolated from the edible (i) mycelia truffle of family Terfeziaceae or an aqueous extract thereof or (ii) an aqueous extract of a fruiting body of truffle of family Terfeziaceae. In one embodiment, the HTS is isolated from the mycelia or fruiting body of a *Mattirolomyces terfezioides* truffle.

[0272] In another embodiment, HTS (or variant thereof) used in compositions disclosed herein is produced *in vivo*. In one embodiment, the nucleic acid coding sequence for HTS isolated from *Mattirolomyces terfezioides* truffle, optionally optimized, is introduced into a suitable vector, which is then cloned into a host cell in an appropriate growth system/environment—resulting in expression of the protein in recombinant fashion. Suitable host cells and expression systems are previously described herein.

[0273] The amount of HTS (or a variant thereof) in the sweetener composition and flavor modifying compositions disclosed herein may vary. In one embodiment, the HTS (or a variant thereof) is present in the sweetener composition above its sweetness threshold concentration.

[0274] In one embodiment, HTS (or a variant thereof) is present in the sweetener composition or flavor modifying composition in any amount to impart the desired sweetness when the sweetener composition or flavor modifying composition is added to a consumable (e.g., beverage), either alone or in combination with one or more additional sweet tasting components (e.g., steviol glycosides, HFCS) present in the sweetener composition or flavor modifying composition, i.e., before such compositions are added to the consumable.

[0275] In a particular embodiment, the desired sweetness of the consumable is isosweet to a sucrose-sweetened consumable having a sweetness from at least about 8 degrees Brix, such as, for example, about 9 degrees Brix, about 10 degrees Brix, about 11 degrees Brix, about 12 degrees Brix, about 13 degrees Brix, about 14 degrees Brix or about 15 degrees Brix.

[0276] In another embodiment, the desired sweetness of the consumable is isosweet to a sucrose-sweetened consumable having a sweetness from about 10 degrees Brix to about 15 degrees Brix, such as, for example, from about 10 degrees Brix to about 14 degrees Brix, from about 10 degrees Brix to about 13 degrees Brix, from about 10 degrees Brix to about 12 degrees Brix, from about 10 degrees Brix to about 11 degrees Brix, from about 11 degrees Brix to about 15 degrees Brix, from about 11 degrees Brix to about 14 degrees Brix, from about 11 degrees Brix to about 13 degrees Brix, from about 11 degrees Brix to about 12 degrees Brix, from about 12 degrees Brix to about 15 degrees Brix, from about 12 degrees Brix to about 14 degrees Brix, from about 12 degrees Brix to about 13 degrees Brix, from about 13 degrees Brix to about 15 degrees Brix, from about 13 degrees Brix to about 14 degrees Brix and from about 14 degrees Brix to about 15 degrees Brix.

[0277] In one embodiment, the Myd polypeptide (or variant thereof) is present in the sweetener composition or flavor modifying composition in an amount that enhances the sweetness of the consumable to which it is added by about 1.0% (w/v) sucrose equivalence (SE) or greater, either alone or in combination with one or more additional sweet tasting component (e.g. steviol glycosides, HFCS) present in the sweetener composition or flavor modifying composition, i.e., before such compositions are added to the consumable.

[0278] In a particular embodiment, the Myd polypeptide (or variant thereof) is present in the sweetener composition in an amount that enhances the sweetness of the consumable to which it is added from about 1.0% to about 3.0% (w/v) sucrose equivalence (SE), such as, for example, about 1.1%, about 1.2%, about 1.3%, about 1.4%, about 1.5%, about 1.6%, about 1.7%, about 1.8%, about 1.9%, about 2.0%, about 2.1%, about 2.2%, about 2.3%, about 2.4%, about 2.5%, about 2.6%, about 2.7%, about

2.8%, about 2.9% or about 3.0% sucrose equivalence, either alone or in combination with one or more additional sweet tasting components (e.g., steviol glycosides, HFCS) present in the sweetener composition or flavor modifying composition, i.e., before such compositions are added to the consumable.

[0279] In another particular embodiment, the Myd polypeptide (or variant thereof) is present in the sweetener composition or flavor modifying composition in an amount that enhances the sweetness of the consumable to which it is added by about 3.0% to about 5% (w/v) sucrose equivalence (SE), for example, about 3.1%, about 3.2%, about 3.3%, about 3.4%, about 3.5%, about 3.6%, about 3.7%, about 3.8%, about 3.9%, about 4.0%, about 4.1%, about 4.2%, about 4.3%, about 4.4%, about 4.5%, about 4.6%, about 4.7%, about 4.8%, about 4.9% or about 5.0%, either alone or in combination with one or more additional sweet tasting components (e.g., steviol glycosides, HFCS) present in the sweetener composition or flavor modifying composition, i.e., before such compositions are added to the consumable.

[0280] The sweetness of a given composition is typically measured with reference to a solution of sucrose. See generally "A Systematic Study of Concentration-Response Relationships of Sweeteners," G. E. DuBois, D. E. Walters, S. S. Schiffman, Z. S. Warwick, B. J. Booth, S. D. Pecore, K. Gibes, B. T. Carr, and L. M. Brands, in *Sweeteners: Discovery, Molecular Design and Chemoreception*, D. E. Walters, F. T. Orthoefer, and G. E. DuBois, Eds., American Chemical Society, Washington, D.C. (1991), pp 261-276.

[0281] The amount of sucrose in a reference solution may be described in degrees Brix (° Bx). One degrees Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by weight (% w/w) (strictly speaking, by mass).

[0282] In one embodiment, a sweetener composition is provided that contains Myd polypeptide (or variant thereof) in an amount effective to provide sweetness equivalent from about 1 to about 12 degrees Brix of sugar when added to a consumable, such as, for example, from about 2 to about 9 degrees Brix, from about 3 to about 8 degrees Brix, from about 4 to about 7 degrees Brix, or about 5 degrees Brix, either alone or together with one or more sweet tasting components (e.g., steviol glycosides, HFCS) present in the sweetener composition or flavor modifying composition or consumable.

[0283] In another embodiment, Myd polypeptide (or variant thereof) is present in an amount effective to provide sweetness equivalent to about 10 degrees Brix when added to a sweetenable composition, either alone or in combination with one or more sweet tasting component (e.g., steviol glycosides, HFCS) present in the sweetener composition, flavor modifying composition or the consumable to which it is added.

[0284] The sweetness of a non-sucrose sweetener can also be measured against a sucrose reference by determining the non-sucrose sweetener's sucrose equivalence. Typically, taste panelists are trained to detect sweetness of reference sucrose solutions containing between 1-15% sucrose (w/v). Other non-sucrose sweeteners are then tasted at a series of dilutions to determine the concentration of the non-sucrose sweetener that is as sweet as a given percent sucrose reference. For example, if a 1% solution of a sweetener is as sweet as a 10% sucrose solution, then the sweetener is said to be 10 times as potent as sucrose.

[0285] In one embodiment, the amount of Myd polypeptide (or variant thereof) present in the sweetener composition or flavor modifying composition disclosed herein is any amount that contributes to one or more improved organoleptic properties of the consumable (e.g., beverage) to which the sweetener composition or taste modifying composition is added. In a particular embodiment, the improved organoleptic property is associated with basic taste. In one embodiment, improving one or more organoleptic property results in the improvement of the taste profile. The overall taste profile of a composition is an interplay of several different tastes, such as sweetness, sourness, saltiness, bitterness, umami and the like.

[0286] As used herein, "organoleptic properties" are the aspects of food, water or other substances that create an individual experience via the senses—including taste, sight, smell, and touch. Organoleptic properties include, e.g., appearance, texture, color, odor, size, shape and flavor. It is the qualitative evaluation based on the study of morphological and sensory profiles of the food, water, or other substance (such as, e.g., sweetener compositions).

[0287] Examples of improved organoleptic properties can include, for example, a reduction in bitterness, a reduction in astringent and liquorice notes, slower onset of sweetness, a reduction in lingering sweetness, a reduction in lingering bitterness, a reduction in bitter aftertaste, a reduction in metallic aftertaste, a reduction in chemical and synthetic aftertaste, and a combination thereof. In a particular embodiment, the term "improved organoleptic properties" means that the sweetened or taste modified composition (e.g., a beverage) will have one or more improved organoleptic properties for the majority of users. The improvement may be expressed qualitatively or quantitatively, e.g. as a percentage improvement.

[0288] The improved organoleptic property may be measured by or using technical means such as a taste sensing system (TSS), a term referring to analytical sensory array units (e.g., electrochemical, gravimetric, optic or biosensors) which can detect specific substances. Sliwińska, M et al. *J. Agric. Food Chem.* (2014), 62, 1423-1448.

[0289] In a particular embodiment, the Myd polypeptide (or variant thereof) is present in the sweetener composition or flavor modifying composition in any amount that reduces, suppresses or masks the

bitterness of a consumable (e.g., a beverage) to which the sweetener or flavor modifying composition is added, either alone or together with one or more sweet tasting components (e.g., steviol glycosides, HFCS) in the sweetener composition or flavor modifying composition, i.e., before it is added to the consumable. The comparison is made to a consumable to which the sweetener or flavor modifying composition has not been added.

[0290] In a particular embodiment, the Myd polypeptide (or variant thereof) is present in the sweetener composition or flavor modifying composition in an amount that reduces the bitterness of consumable (e.g., beverage) to which it is added by at least about 5%, at least about 10%, at least about 15%, at least about 20% or at least about 25% or more, either alone or in combination with one or more sweet taste components (e.g., steviol glycosides, HFCS) in the sweetener composition or flavor modifying composition, i.e., before it is added to the consumable. In one embodiment, the reduction in bitterness is experienced by a majority of subjects. The comparison is made to a consumable to which the sweetener or flavor modifying composition has not been added.

[0291] In a particular embodiment, the Myd polypeptide (or variant thereof) is present in the sweetener composition or flavor modifying composition in any amount that reduces the bitter aftertaste of a consumable (e.g., a beverage) to which the sweetener or flavor modifying composition is added, either alone or in combination with one or more sweet taste components (e.g., steviol glycosides, HFCS) in the sweetener composition or flavor modifying composition, i.e., before it is added to the consumable. In a particular embodiment, the Myd polypeptide (or variant thereof) is present in the sweetener composition or flavor modifying composition in an amount that reduces bitter aftertaste of consumable (e.g., beverage) to which it is added by at least about 5%, at least about 10%, at least about 15%, at least about 20% or at least about 25% or more. In one embodiment, the reduction in bitter aftertaste is experienced by a majority of subjects. The comparison is made to a consumable to which the sweetener or flavor modifying composition has not been added.

[0292] In another embodiment, the Myd polypeptide (or variant thereof) is present in the sweetener composition or taste modifying composition in any amount reduces the sweetness linger of a consumable (e.g., a beverage) to which the sweetener or taste modifying composition is added. Sucrose exhibits a sweet taste in which the maximal response is perceived quickly and where perceived sweetness disappears relatively quickly on swallowing a food or beverage. In contrast, the sweet tastes of essentially all high-potency sweeteners reach their maximal responses somewhat more slowly and they then decline in intensity more slowly than is the case for sucrose. This decline in sweetness is often referred to as “sweetness linger” and is a major limitation for high-potency sweeteners including NHPs. Slow onset of sweetness also can be a problem. In general, however,

sweetness linger is a more significant problem. And so, preferred embodiments of this invention exhibit significant reductions in sweetness linger.

[0293] In a particular embodiment, the Myd polypeptide (or variant thereof) is present in the sweetener composition or flavor modifying compositions in an amount that reduces the sweetness linger of the consumable (e.g., beverage) to which it is added by at least about 5%, at least about 10%, at least about 15%, at least about 20% or at least about 25% or more, either alone or in combination with one or more sweet tasting components (e.g., steviol glycosides, HFCS) in the sweetener composition or flavor modifying composition before it is added to the consumable. In one embodiment, the majority of subjects perceive the reduction in sweetness linger. In a particular embodiment, the comparison is made to a consumable to which the sweetener composition or flavor composition has not been added.

[0294] In a particular embodiment, the Myd polypeptide (or variant thereof) is present in the sweetener composition or flavor modifying compositions in an amount that results in at least one change/modification in organoleptic property in a consumable (e.g., beverage) compared to a consumable that does not contain the sweetener composition, wherein the organoleptic property is selected from the group consisting of aroma, flavor, basic taste (sweetness, sour, saltiness, bitterness or umami), aftertaste or linger, temporal profile, mouthfeel or a combination thereof. In this embodiment, a change or modification can be any perceived difference in the organoleptic property or properties that may or may not be considered an improvement. For example, if the Myd polypeptide being present in a consumable results in a change of a flavor from chocolate to caramel, this would be considered a “change” but not necessarily an “improvement”.

[0295] In certain embodiments, the sweetener composition or flavor and/or taste modifying compositions contain one or more additional sweeteners. In one embodiment, the additional sweetener is present above its sweetness threshold concentration. In certain embodiments, the sweetener composition containing Myd and the one or more additional sweeteners synergistically enhance the sweetness of the consumable to which the sweetener composition is added. In one embodiment, the sweetness of the consumable is enhanced in a manner that would be unexpected to one of skill in the art.

[0296] The additional sweetener can be any type of sweetener, for example, a natural, non-natural, or synthetic sweetener.

[0297] In at least one embodiment, the at least one additional sweetener is chosen from natural sweeteners other than *Stevia* sweeteners. In another embodiment, the at least one additional sweetener is chosen from synthetic high potency sweeteners (SHPS).

[0298] In a particular embodiment, the one or more additional sweetener may be a natural high potency sweetener (NHPS). Suitable natural high potency sweeteners include, but are not limited to, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, dulcoside A, dulcoside B, rubusoside, *Stevia*, stevioside, mogroside IV, mogroside V, Luo Han Guo sweetener, siamenoside, monatin and its salts (monatin SS, RR, RS, SR), curculin, glycyrrhizic acid and its salts, thaumatin, monellin, mabinlin, brazzein, hernandulcin, phyllodulcin, glycyphyllin, phloridzin, trilobatin, baiyunoside, osladin, polypodoside A, pterocaryoside A, pterocaryoside B, mukurozioside, phlomisioside I, periandrin I, abrusoside A, steviolbioside and cyclocarioside I. The natural high potency sweetener can be provided as a pure compound or, alternatively, as part of an extract. For example, rebaudioside A can be provided as a sole compound or as part of a *Stevia* extract.

[0299] In one embodiment, the one or more additional sweeteners is selected from the group consisting of rebaudioside M, rebaudioside A, siamenoside I and mogroside V.

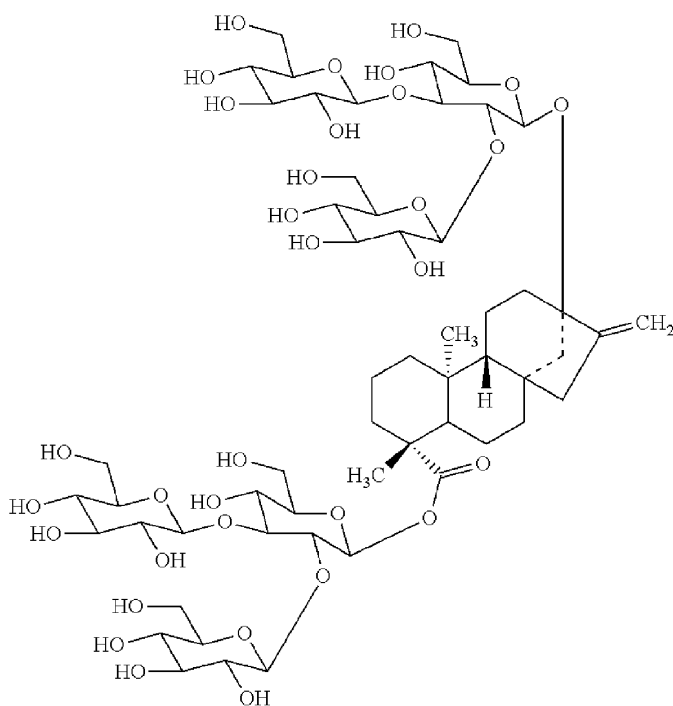
[0300] In a particular embodiment, the sweetener composition and/or flavor modifying composition of the present invention comprises Myd polypeptide (or variant thereof) and siamenoside I.

[0301] In another particular embodiment, the sweetener composition and/or flavor modifying composition of the present invention comprises Myd polypeptide (or variant thereof) and mogroside V.

[0302] In another embodiment, the one or more additional sweeteners is selected from the group consisting of rebaudioside D, rebaudioside N, rebaudioside O, rebaudioside E, steviolmonoside, steviolbioside, rubusoside, dulcoside B, dulcoside A, rebaudioside B, rebaudioside G, stevioside, rebaudioside C, rebaudioside F, rebaudioside I, rebaudioside H, rebaudioside L, rebaudioside K, rebaudioside J, rebaudioside M2, rebaudioside D2, rebaudioside S, rebaudioside T, rebaudioside U, rebaudioside V, rebaudioside W, rebaudioside Z1, rebaudioside Z2, rebaudioside IX, enzymatically glucosylated steviol glycosides and combinations thereof.

[0303] In a further embodiment, the one or more additional sweeteners is selected from the group consisting of mogroside IA, mogroside IE, 11-oxomogroside IA, mogroside II, mogroside II A, mogroside II B, mogroside II E, 7-oxomogroside II E, mogroside III, Mogroside IIIe, 11-deoxymogroside III, mogroside IV, 11-oxomogroside IV, 11-oxomogroside IV A, 11-deoxymogroside V, 7-oxomogroside V, 11-oxomogroside V, isomogroside V, mogroside VI, mogrol, 11-oxomogrol, the 1,6- α isomer of siamenoside I, monk fruit extract, and combinations thereof.

[0304] In a particular embodiment, the one or more additional sweeteners is rebaudioside M (13-[2-O- β -D-glucopyranosyl-3-O- β -D-glucopyranosyl- β -D-glucopyranosyl]oxy] Ent Kaur-16-end-19-oil acid-[2-O- β -D-glucopyranosyl-3-O- β -D-glycopyranosyl) ester having the formula of Formula (I).



Formula (I).

[0305] Reb M may be provided in a purified or unpurified form, i.e., as part of a naturally occurring mixture that contains Reb M. In one embodiment, Reb M can be obtained from a *Stevia* extract by any suitable purification method. Suitable purification methods are known in the art, including, but not limited to, column chromatography, recrystallization, phase separation, extraction, high performance liquid chromatography and combinations thereof.

[0306] In another embodiment, the additional sweetener is a steviol glycoside composition. An exemplary steviol glycoside composition is A95, which contains primarily reb D and reb M with minor amounts of one or more of the following: Reb E, Reb O, Reb N, Reb A, Stevioside, Reb C and Reb B. Methods of obtaining A95 are provided in WO 2017/059414, incorporated herein by reference.

[0307] The amount of Reb M in the sweetener composition or taste modifying composition may vary. In one embodiment, Reb M is present in a sweetener composition in any amount to impart the desired sweetness when the sweetener composition is added to a consumable (e.g., a beverage). In a particular embodiment, the desired sweetness of the consumable is greater than about 10 degrees Brix.

[0308] In one embodiment, the sweetener composition contains Reb M in an amount effective to provide sweetness equivalent from about 1 to 12 degrees Brix when added to a consumable (e.g., a beverage), such as, for example, from about 2 to about 9 degrees Brix, from about 3 to about 8 degrees Brix, from about 4 to about 7 degrees Brix, or about 5 degrees Brix.

[0309] In a particular embodiment, Reb M is present in an effective amount to provide a sucrose equivalence (SE) of about 8 or less, such as for example, about 7, about 6.5, about 6, about 5.5, or about 5 SE.

[0310] In another particular embodiment, Reb M is present in an effective amount to provide a sucrose equivalence of about 8 or greater, such as, for example, about 9, about 9.5, about 10, about 10.5, about 11, about 11.5, about 12, about 12.5, about 13, about 13.5, about 14, about 14.5 or about 15.

[0311] In one embodiment, Reb M is present in the flavor modifying composition in any amount to impart the desired flavor when the flavor modifying composition is added to a flavor modifiable composition (e.g., a beverage). In a particular embodiment, the desired flavor is a more sugar-like temporal or taste profile.

[0312] In one embodiment, the Reb M and the Myd polypeptide (or variant thereof) produce a synergistic effect, e.g., synergistic sweetness, i.e., the sweetness of combination is greater than the sum of the individual sweeteners. In a particular embodiment, the Reb M and the Myd polypeptide (or variant thereof) produce an effect that would be unexpected by one of skill in the art.

[0313] Reb M may be provided in a purified form or as a component of a mixture containing Reb M and one or more additional components. In one embodiment, Reb X is provided as a component of a mixture. In a particular embodiment, the mixture is a *Stevia* extract. The *Stevia* extract may contain Reb M in an amount that ranges from about 5% to about 100% by weight on a dry basis, such as, for example, from about 10% to about 100%, from about 20% to about 100%, from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100% and from about 90% to about 100%. In still further embodiments, the *Stevia* extract contains Reb M in an amount greater than about 90% by weight on a dry basis, for example, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98% and greater than about 99%.

[0314] In one embodiment, Reb M is provided as a component of a steviol glycoside mixture, i.e., a mixture of steviol glycosides wherein the remainder of the non-Reb M portion of the mixture is comprised entirely of steviol glycosides. The identities of steviol glycosides are known in the art and include, but are not limited to, steviol, steviol monoside, rubososide, steviolbiocide, stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F and dulcoside A. The steviol glycoside mixture may contain from about 5% to about 100% Reb M by weight on a dry basis. For example, a steviol glycoside mixture may contain from about 10% to about 100%, from about 20% to about 100%, from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about

100%, from about 80% to about 100% and from about 90% to about 100% Reb M by weight on a dry basis. In still further embodiments, the steviol glycoside mixture may contain greater than about 90%, for example, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98% and greater than about 99% Reb M by weight on a dry basis.

[0315] RebM80 refers to a *Stevia* extract or steviol glycoside composition having about 80% Reb M by weight.

[0316] In a particular embodiment, the one or more additional sweeteners is rebaudioside A.

[0317] Reb A may be provided in a purified or unpurified form, i.e., as part of a naturally occurring mixture that contains Reb a. In one embodiment, Reb A can be obtained from a *Stevia* extract by any suitable purification method. Suitable purification methods are known in the art, including, but not limited to, column chromatography, recrystallization, phase separation, extraction, high performance liquid chromatography and combinations thereof.

[0318] Reb A may be provided in a purified form or as a component of a mixture containing Reb A and one or more additional components. In one embodiment, Reb A is provided as a component of a mixture. In a particular embodiment, the mixture is a *Stevia* extract. The *Stevia* extract may contain Reb A in an amount that ranges from about 5% to about 100% by weight on a dry basis, such as, for example, from about 10% to about 100%, from about 20% to about 100%, from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100% and from about 90% to about 100%. In still further embodiments, the *Stevia* extract contains Reb A in an amount greater than about 90% by weight on a dry basis, for example, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98% and greater than about 99%.

[0319] In one embodiment, Reb A is provided as a component of a steviol glycoside mixture, i.e., a mixture of steviol glycosides wherein the remainder of the non-Reb A portion of the mixture is comprised entirely of steviol glycosides. The steviol glycoside mixture may contain from about 5% to about 100% Reb A weight on a dry basis. For example, a steviol glycoside mixture may contain from about 10% to about 100%, from about 20% to about 100%, from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100% and from about 90% to about 100% Reb A by weight on a dry basis. In still further embodiments, the steviol glycoside mixture may contain greater than about 90%, for example, greater than about 91%, greater than about 92%, greater than

about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98% and greater than about 99% Reb A by weight on a dry basis.

[0320] The amount of Reb A in the sweetener composition or taste modifying composition may vary. In one embodiment, Reb A is present in the sweetener composition in any amount to impart the desired sweetness when the sweetener composition is added to a sweetenable composition. In a particular embodiment, the desired sweetness of the sweetened composition is greater than about 10 degrees Brix.

[0321] In one embodiment, the Reb A and the Myd polypeptide (or variant thereof) produce a synergistic effect, e.g., synergistic sweetness, i.e., the sweetness of combination is greater than the sum of the individual sweeteners. In certain embodiments, the Reb A and the Myd polypeptide (or variant thereof) produce an effect that one of skill in the art would not have expected.

[0322] In a particular embodiment, Reb A is present in an effective amount to provide a sucrose equivalence (SE) of about 8 or less, such as for example, about 7, about 6.5, about 6, about 5.5, or about 5 SE.

[0323] In another particular embodiment, Reb A is present in an effective amount to provide a sucrose equivalence (SE) of about 8 or greater, such as, for example, about 9, about 9.5, about 10, about 10.5, about 11, about 11.5, about 12, about 12.5, about 13, about 13.5, about 14, about 14.5 or about 15.

[0324] In another particular embodiment, Reb A is present in an effective amount to provide a sucrose equivalence of greater than about 8 SE, e.g., about 9 SE, about 9.5 SE, about 10 SE.

[0325] In one embodiment, Reb A is present in the flavor modifying composition in any amount to impart the desired taste when the taste modifying composition is added to a taste modifiable composition (e.g., a beverage). In a particular embodiment, the desired taste is a sugar-like taste.

[0326] In a particular embodiment, the Myd polypeptide (or variant thereof) and Reb A produce a synergistic effect. In one embodiment, the Myd polypeptide (or variant thereof) and Reb A produce an effect that would be unexpected to one of skill in the art.

[0327] The sweetener compositions can be customized to obtain a desired calorie content. For example, sweetener compositions can be “high-calorie”, such that they impart the desired sweetness when added to a sweetenable composition (such as, for example, as beverage) and have about 120 calories per 8 oz serving.

[0328] The sweetener compositions can be customized to obtain a desired calorie content. For example, sweetener compositions can be “mid-calorie”, such that they impart the desired sweetness when added

to a sweetenable composition (such as, for example, as beverage) and have about 80 calories per 8 oz serving.

[0329] For example, sweetener compositions can be “low-calorie”, such that they impart the desired sweetness when added to a sweetenable composition (such as, for example, as beverage) and have less than 40 calories per 8 oz serving.

[0330] In other embodiments, the sweetener compositions can be “zero-calorie”, such that they impart the desired sweetness when added to a sweetenable composition (such as, for example, a beverage) and have less than 5 calories per 8 oz. serving.

[0331] ADDITIVES

[0332] In addition to Myd (HTS) polypeptides (or variants thereof) and, optionally, one or more additional sweeteners (e.g., one or more steviol glycosides), the sweetener compositions or flavor modifying compositions disclosed herein can optionally include additional additives, detailed herein below. In some embodiments, the sweetener composition contains additives including, but not limited to, carbohydrates, polyols, amino acids and their corresponding salts, poly-amino acids and their corresponding salts, sugar acids and their corresponding salts, nucleotides, organic acids, inorganic acids, organic salts including organic acid salts and organic base salts, inorganic salts, bitter compounds, flavorants and flavoring ingredients, astringent compounds, proteins or protein hydrolysates, surfactants, emulsifiers, flavonoids, alcohols, polymers and combinations thereof. In some embodiments, the additives act to improve the temporal and flavor profile of the sweetener to provide a sweetener composition with a taste similar to sucrose.

[0333] In one embodiment, the sweetener compositions or flavor modifying compositions contain one or more polyols. The term “polyol”, as used herein, refers to a molecule that contains more than one hydroxyl group. A polyol may be a diol, triol, or a tetraol which contains 2, 3, and 4 hydroxyl groups respectively. A polyol also may contain more than 4 hydroxyl groups, such as a pentaol, hexaol, heptaol, or the like, which contain 5, 6, or 7 hydroxyl groups, respectively. Additionally, a polyol also may be a sugar alcohol, polyhydric alcohol, or polyalcohol which is a reduced form of carbohydrate, wherein the carbonyl group (aldehyde or ketone, reducing sugar) has been reduced to a primary or secondary hydroxyl group.

[0334] Non-limiting examples of polyols in some embodiments include erythritol, maltitol, mannitol, sorbitol, lactitol, xylitol, isomalt, propylene glycol, glycerol (glycerin), threitol, galactitol, palatinose, reduced isomalto-oligosaccharides, reduced xylo-oligosaccharides, reduced gentio-oligosaccharides, reduced maltose syrup, reduced glucose syrup, and sugar alcohols or any other carbohydrates capable of being reduced which do not adversely affect taste.

[0335] Suitable sweet taste improving amino acid additives include, but are not limited to, aspartic acid, arginine, glycine, glutamic acid, proline, threonine, theanine, cysteine, cystine, alanine, valine, tyrosine, leucine, arabinose, trans-4-hydroxyproline, isoleucine, asparagine, serine, lysine, histidine, ornithine, methionine, carnitine, aminobutyric acid (α -, β -, and/or δ -isomers), glutamine, hydroxyproline, taurine, norvaline, sarcosine, and their salt forms such as sodium or potassium salts or acid salts. The sweet taste improving amino acid additives also may be in the D- or L-configuration and in the mono-, di-, or tri-form of the same or different amino acids. Additionally, the amino acids may be α -, β -, γ - and/or δ -isomers if appropriate. Combinations of the foregoing amino acids and their corresponding salts (e.g., sodium, potassium, calcium, magnesium salts or other alkali or alkaline earth metal salts thereof, or acid salts) also are suitable sweet taste improving additives in some embodiments. The amino acids may be natural or synthetic. The amino acids also may be modified. Modified amino acids refers to any amino acid wherein at least one atom has been added, removed, substituted, or combinations thereof (e.g., N-alkyl amino acid, N-acyl amino acid, or N-methyl amino acid). Non-limiting examples of modified amino acids include amino acid derivatives such as trimethyl glycine, N-methyl-glycine, and N-methyl-alanine. As used herein, modified amino acids encompass both modified and unmodified amino acids. As used herein, amino acids also encompass both peptides and polypeptides (e.g., dipeptides, tripeptides, tetrapeptides, and pentapeptides) such as glutathione and L-alanyl-L-glutamine. Suitable sweet taste improving polyamino acid additives include poly-L-aspartic acid, poly-L-lysine (e.g., poly-L- α -lysine or poly-L- ϵ -lysine), poly-L-ornithine (e.g., poly-L- α -ornithine or poly-L- ϵ -ornithine), poly-L-arginine, other polymeric forms of amino acids, and salt forms thereof (e.g., calcium, potassium, sodium, or magnesium salts such as L-glutamic acid mono sodium salt). The sweet taste improving poly-amino acid additives also may be in the D- or L-configuration. Additionally, the poly-amino acids may be α -, β -, γ -, δ -, and ϵ -isomers if appropriate. Combinations of the foregoing poly-amino acids and their corresponding salts (e.g., sodium, potassium, calcium, magnesium salts or other alkali or alkaline earth metal salts thereof or acid salts) also are suitable sweet taste improving additives in some embodiments. The poly-amino acids described herein also may comprise co-polymers of different amino acids. The poly-amino acids may be natural or synthetic. The poly-amino acids also may be modified, such that at least one atom has been added, removed, substituted, or combinations thereof (e.g., N-alkyl poly-amino acid or N-acyl poly-amino acid). As used herein, poly-amino acids encompass both modified and unmodified poly-amino acids. For example, modified poly-amino acids include, but are not limited to, poly-amino acids of various molecular weights (MW), such as poly-L- α -lysine with a MW of 1,500, MW of 6,000, MW of 25,200, MW of 63,000, MW of 83,000, or MW of 300,000.

[0336] Suitable sugar acid additives include, but are not limited to, aldonic, uronic, aldaric, alginic, gluconic, glucuronic, glucaric, galactaric, galacturonic, and salts thereof (e.g., sodium, potassium, calcium, magnesium salts or other physiologically acceptable salts), and combinations thereof.

[0337] Suitable nucleotide additives include, but are not limited to, inosine monophosphate ("IMP"), guanosine monophosphate ("GMP"), adenosine monophosphate ("AMP"), cytosine monophosphate (CMP), uracil monophosphate (UMP), inosine diphosphate, guanosine diphosphate, adenosine diphosphate, cytosine diphosphate, uracil diphosphate, inosine triphosphate, guanosine triphosphate, adenosine triphosphate, cytosine triphosphate, uracil triphosphate, alkali or alkaline earth metal salts thereof, and combinations thereof. The nucleotides described herein also may comprise nucleotide-related additives, such as nucleosides or nucleic acid bases (e.g., guanine, cytosine, adenine, thymine, uracil). In particular embodiments, the nucleotide is present in the sweetener composition in an amount from about 5 ppm to about 1,000 ppm.

[0338] Suitable organic acid additives include any compound which comprises a ---COOH moiety, such as, for example, C2-C30 carboxylic acids, substituted hydroxyl C2-C30 carboxylic acids, benzoic acid, substituted benzoic acids (e.g., 2,4-dihydroxybenzoic acid), substituted cinnamic acids, hydroxyacids, substituted hydroxybenzoic acids, substituted cyclohexyl carboxylic acids, tannic acid, lactic acid, tartaric acid, citric acid, gluconic acid, glucoheptonic acids, adipic acid, hydroxycitric acid, malic acid, frutaric acid (a blend of malic, fumaric, and tartaric acids), fumaric acid, maleic acid, succinic acid, chlorogenic acid, salicylic acid, creatine, caffeic acid, bile acids, acetic acid, ascorbic acid, alginic acid, erythorbic acid, polyglutamic acid, glucono delta lactone, and their alkali or alkaline earth metal salt derivatives thereof. In addition, the organic acid additives also may be in either the D- or L-configuration.

[0339] Suitable organic acid additive salts include, but are not limited to, sodium, calcium, potassium, and magnesium salts of all organic acids, such as salts of citric acid, malic acid, tartaric acid, fumaric acid, lactic acid (e.g., sodium lactate), alginic acid (e.g., sodium alginate), ascorbic acid (e.g., sodium ascorbate), benzoic acid (e.g., sodium benzoate or potassium benzoate), and adipic acid. The examples of the sweet taste improving organic acid additives described optionally may be substituted with at least one group chosen from hydrogen, alkyl, alkenyl, alkynyl, halo, haloalkyl, carboxyl, acyl, acyloxy, amino, amido, carboxyl derivatives, alkylamino, dialkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfo, thiol, imine, sulfonyl, sulfenyl, sulfinyl, sulfamyl, carboxalkoxy, carboxamido, phosphonyl, phosphinyl, phosphoryl, phosphino, thioester, thioether, anhydride, oximino, hydrazino, carbamyl, phosphor or phosphonato. In particular embodiments, the organic acid additive is present in the sweetener composition in an amount from about 10 ppm to about 5,000 ppm.

[0340] Suitable inorganic acid additives include, but are not limited to, phosphoric acid, phosphorous acid, polyphosphoric acid, hydrochloric acid, sulfuric acid, carbonic acid, sodium dihydrogen phosphate, and alkali or alkaline earth metal salts thereof (e.g., inositol hexaphosphate Mg/Ca).

[0341] Suitable bitter compound additives include, but are not limited to, caffeine, quinine, urea, bitter orange oil, naringin, quassia, and salts thereof.

[0342] Suitable flavorant and flavoring ingredient additives for include, but are not limited to, vanillin, vanilla extract, mango extract, cinnamon, *Citrus*, coconut, ginger, viridiflorol, almond, menthol (including menthol without mint), grape skin extract, and grape seed extract. "Flavorant" and "flavoring ingredient" are synonymous and can include natural or synthetic substances or combinations thereof. Flavorants also include any other substance which imparts flavor and may include natural or non-natural (synthetic) substances which are safe for human or animals when used in a generally accepted range. Non-limiting examples of proprietary flavorants include Döhler™ Natural Flavoring Sweetness Enhancer K14323 (Döhler™, Darmstadt, Germany), Symrise™ Natural Flavor Mask for Sweeteners 161453 and 164126 (Symrise™, Holzminden, Germany), Natural Advantage™ Bitterness Blockers 1, 2, 9 and 10 (Natural Advantage™, Freehold, N.J., U.S.A.), and Sucramask™ (Creative Research Management, Stockton, Calif., U.S.A.).

[0343] Suitable polymer additives include, but are not limited to, chitosan, pectin, pectic, pectinic, polyuronic, polygalacturonic acid, starch, food hydrocolloid or crude extracts thereof (e.g., gum acacia senegal (Fibergum™), gum acacia seyal, carageenan), poly-L-lysine (e.g., poly-L- α -lysine or poly-L- ϵ -lysine), poly-L-ornithine (e.g., poly-L- α -ornithine or poly-L- ϵ -ornithine), polypropylene glycol, polyethylene glycol, poly(ethylene glycol methyl ether), polyarginine, polyaspartic acid, polyglutamic acid, polyethylene imine, alginic acid, sodium alginate, propylene glycol alginate, and sodium polyethyleneglycolalginate, sodium hexametaphosphate and its salts, and other cationic polymers and anionic polymers.

[0344] Other suitable polymer additives that also provide gelling and thickening properties include conventional low methoxyl (LMC) pectins. LMC pectin also acts as a food stabilizer. LMC apple pectin is a conventional low methoxyl pectin, extracted from apple pomace and standardized with sucrose. It is utilized for low-calorie jams and jellies since it relies on calcium instead of sugar to solidify. LMC pectin gets increasingly firmer as calcium is added until it hits a saturation point. At that time, the process reverses and it becomes less firm. In some embodiments, the products for oral administration described herein may comprise one or more food product selected from the group consisting of pie fillings and other sweet fillings, gelatins and puddings; yogurts; sauces, syrups and dressings; and sweet spreads. In one embodiment, the product for oral administration further comprises a low methoxyl pectin.

[0345] Suitable protein or protein hydrolysate additives include, but are not limited to, bovine serum albumin (BSA), whey protein (including fractions or concentrates thereof such as 90% instant whey protein isolate, 34% whey protein, 50% hydrolyzed whey protein, and 80% whey protein concentrate), soluble rice protein, soy protein, protein isolates, protein hydrolysates, reaction products of protein hydrolysates, glycoproteins, and/or proteoglycans containing amino acids (e.g., glycine, alanine, serine, threonine, asparagine, glutamine, arginine, valine, isoleucine, leucine, norvaline, methionine, proline, tyrosine, hydroxyproline, and the like), collagen (e.g., gelatin), partially hydrolyzed collagen (e.g., hydrolyzed fish collagen), and collagen hydrolysates (e.g., porcine collagen hydrolysate).

[0346] Suitable surfactant additives include, but are not limited to, polysorbates (e.g., polyoxyethylene sorbitan monooleate (polysorbate 80), polysorbate 20, polysorbate 60), sodium dodecylbenzenesulfonate, dioctyl sulfosuccinate or dioctyl sulfosuccinate sodium, sodium dodecyl sulfate, cetylpyridinium chloride (hexadecylpyridinium chloride), hexadecyltrimethylammonium bromide, sodium cholate, carbamoyl, choline chloride, sodium glycocholate, sodium taurodeoxycholate, lauric arginate, sodium stearyl lactylate, sodium taurocholate, lecithins, sucrose oleate esters, sucrose stearate esters, sucrose palmitate esters, sucrose laurate esters, and other emulsifiers, and the like.

[0347] Suitable flavonoid additives are classified as flavonols, flavones, flavanones, flavan-3-ols, isoflavones, or anthocyanidins. Non-limiting examples of flavonoid additives include, but are not limited to, catechins (e.g., green tea extracts such as Polyphenon™ 60, Polyphenon™ 30, and Polyphenon™ 25 (Mitsui Norin Co., Ltd., Japan), polyphenols, rutins (e.g., enzyme modified rutin Sanmelin™ AO (San-fi Gen F.F.I., Inc., Osaka, Japan)), neohesperidin, naringin, neohesperidin dihydrochalcone, and the like.

[0348] Suitable alcohol additives include, but are not limited to, ethanol.

[0349] Suitable astringent compound additives include, but are not limited to, tannic acid, europium chloride (EuCl₃), gadolinium chloride (GdCl₃), terbium chloride (TbCl₃), alum, tannic acid, and polyphenols (e.g., tea polyphenols). In particular embodiments, the astringent additive is present in an amount from about 10 ppm to about 5,000 ppm.

[0350] In particular embodiments, sweetener compositions or flavor modifying compositions comprise the Myd polypeptide (or variant thereof), optionally in combination with one or more steviol glycosides (e.g., Reb M, Reb A), a polyol selected from erythritol, maltitol, mannitol, xylitol, sorbitol, and combinations thereof; and optionally at least one additional sweetener and/or functional ingredient. In a particular embodiment, the polyol is erythritol. The steviol glycoside (e.g., Reb M, Reb A) can be provided as a pure compound or as part of a *Stevia* extract or steviol glycoside mixture, as described above. The steviol glycoside (e.g., Reb M, Reb A) can be present in an amount from

about 5% to about 100% by weight on a dry basis in either a steviol glycoside mixture or a *Stevia* extract.

[0351] In particular embodiments, the sweetener compositions or flavor modifying compositions comprise the Myd polypeptide (or variant thereof), optionally in combination with one or more steviol glycosides (e.g., Reb M, Reb A); a carbohydrate sweetener selected from sucrose, fructose, glucose, maltose and combinations thereof; and optionally at least one additional sweetener and/or functional ingredient. The steviol glycoside (e.g., Reb M, Reb A) can be provided as a pure compound or as part of a *Stevia* extract or steviol glycoside mixture, as described above. The steviol glycoside (e.g., Reb M, Reb A) can be present in an amount from about 5% to about 100% by weight on a dry basis in either a steviol glycoside mixture or a *Stevia* extract.

[0352] In particular embodiments, the sweetener compositions or flavor modifying compositions comprise the Myd polypeptide (or variant thereof), optionally in combination with one or more steviol glycosides (e.g., Reb M, Reb A); an amino acid selected from glycine, alanine, proline and combinations thereof; and optionally at least one additional sweetener and/or functional ingredient. The steviol glycoside can be provided as a pure compound or as part of a *Stevia* extract or steviol glycoside mixture, as described above. The steviol glycoside (e.g., Reb M, Reb A) can be present in an amount from about 5% to about 100% by weight on a dry basis in either a steviol glycoside mixture or a *Stevia* extract.

[0353] In particular embodiments, sweetener compositions or flavor modifying compositions comprise the Myd polypeptide (or variant thereof), optionally in combination with one or more steviol glycosides (e.g., Reb M, Reb A), a salt selected from sodium chloride, magnesium chloride, potassium chloride, calcium chloride and combinations thereof; and optionally at least one additional sweetener and/or functional ingredient. The steviol glycoside (e.g., Reb M, Reb A) can be provided as a pure compound or as part of a *Stevia* extract or steviol glycoside mixture, as described above.

[0354] FUNCTIONAL INGREDIENTS

[0355] The sweetener compositions or flavor modifying compositions disclosed herein can also contain one or more functional ingredients, which provide a real or perceived health benefit to the composition. Functional ingredients include, but are not limited to, saponins, antioxidants, dietary fiber sources, fatty acids, vitamins, glucosamine, minerals, preservatives, hydration agents, probiotics, prebiotics, weight management agents, osteoporosis management agents, phytoestrogens, long chain primary aliphatic saturated alcohols, phytosterols and combinations thereof.

[0356] Examples of suitable antioxidants for embodiments of this invention include, but are not limited to, vitamins, vitamin cofactors, minerals, hormones, carotenoids, carotenoid terpenoids, non-carotenoid terpenoids, flavonoids, flavonoid polyphenolics (e.g., bioflavonoids), flavonols, flavones,

phenols, polyphenols, esters of phenols, esters of polyphenols, nonflavonoid phenolics, isothiocyanates, and combinations thereof. In some embodiments, the antioxidant is vitamin A, vitamin C, vitamin E, ubiquinone, mineral selenium, manganese, melatonin, α -carotene, β -carotene, lycopene, lutein, zeaxanthin, cryptoxanthin, resveratrol, eugenol, quercetin, catechin, gossypol, hesperetin, curcumin, ferulic acid, thymol, hydroxytyrosol, tumeric, thyme, olive oil, lipoic acid, glutathione, glutamine, oxalic acid, tocopherol-derived compounds, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA), tert-butylhydroquinone, acetic acid, pectin, tocotrienol, tocopherol, coenzyme Q10, zeaxanthin, astaxanthin, canthaxanthin, saponins, limonoids, kaempferol, myricetin, isorhamnetin, proanthocyanidins, quercetin, rutin, luteolin, apigenin, tangeretin, hesperetin, naringenin, erodictyol, flavan-3-ols (e.g., anthocyanidins), gallic acid, epigallocatechin and its gallate forms, epigallocatechin and its gallate forms (EGCG), theaflavin and its gallate forms, thearubigins, isoflavone phytoestrogens, genistein, daidzein, glycitein, anthocyanins, cyaniding, delphinidin, malvidin, pelargonidin, peonidin, petunidin, ellagic acid, gallic acid, salicylic acid, rosmarinic acid, cinnamic acid and its derivatives (e.g., ferulic acid), chlorogenic acid, chicoric acid, gallotannins, ellagitannins, anthoxanthins, betacyanins and other plant pigments, silymarin, citric acid, lignan, antioxidants, bilirubin, uric acid, R- α -lipoic acid, N-acetylcysteine, emblicanin, and phytic acid, or combinations thereof. In alternate embodiments, the antioxidant is a synthetic antioxidant such as butylated hydroxytoluene or butylated hydroxyanisole, for example. Other sources of suitable antioxidants for embodiments of this invention include, but are not limited to, fruits, vegetables, tea, cocoa, chocolate, spices, herbs, rice, organ meats from livestock, yeast, whole grains, or cereal grains.

[0357] Particular antioxidants belong to the class of phytonutrients called polyphenols (also known as “polyphenolics”), which are a group of chemical substances found in plants, characterized by the presence of more than one phenol group per molecule. Suitable polyphenols for embodiments of this invention, include catechins, proanthocyanidins, procyanidins, anthocyanins, quercetin, rutin, resveratrol, isoflavones, curcumin, punicalagin, ellagitannin, hesperidin, naringin, *Citrus* flavonoids, chlorogenic acid, other similar materials, and combinations thereof.

[0358] In particular embodiments, the antioxidant is a catechin such as, for example, epigallocatechin gallate (EGCG). Suitable sources of catechins for embodiments of this invention include, but are not limited to, green tea, white tea, black tea, oolong tea, chocolate, cocoa, red wine, grape seed, red grape skin, purple grape skin, red grape juice, purple grape juice, berries, pycnogenol, and red apple peel.

[0359] In some embodiments, the antioxidant is chosen from proanthocyanidins, procyanidins or combinations thereof. Suitable sources of proanthocyanidins and procyanidins for embodiments of this invention include, but are not limited to, red grapes, purple grapes, cocoa, chocolate, grape seeds, red

wine, cacao beans, cranberry, apple peel, plum, blueberry, black currants, choke berry, green tea, sorghum, cinnamon, barley, red kidney bean, pinto bean, hops, almonds, hazelnuts, pecans, pistachio, pycnogenol, and colorful berries.

[0360] In particular embodiments, the antioxidant is an anthocyanin. Suitable sources of anthocyanins for embodiments of this invention include, but are not limited to, red berries, blueberries, bilberry, cranberry, raspberry, cherry, pomegranate, strawberry, elderberry, choke berry, red grape skin, purple grape skin, grape seed, red wine, black currant, red currant, cocoa, plum, apple peel, peach, red pear, red cabbage, red onion, red orange, and blackberries.

[0361] In some embodiments, the antioxidant is chosen from quercetin, rutin or combinations thereof. Suitable sources of quercetin and rutin for embodiments of this invention include, but are not limited to, red apples, onions, kale, bog whortleberry, lingonberries, chokeberry, cranberry, blackberry, blueberry, strawberry, raspberry, black currant, green tea, black tea, plum, apricot, parsley, leek, broccoli, chili pepper, berry wine, and ginkgo.

[0362] In some embodiments, the antioxidant is resveratrol. Suitable sources of resveratrol for embodiments of this invention include, but are not limited to, red grapes, peanuts, cranberry, blueberry, bilberry, mulberry, Japanese Itadori tea, and red wine.

[0363] In particular embodiments, the antioxidant is an isoflavone. Suitable sources of isoflavones for embodiments of this invention include, but are not limited to, soy beans, soy products, legumes, alfalfa sprouts, chickpeas, peanuts, and red clover.

[0364] In some embodiments, the antioxidant is curcumin. Suitable sources of curcumin for embodiments of this invention include, but are not limited to, turmeric and mustard.

[0365] In particular embodiments, the antioxidant is chosen from punicalagin, ellagitannin or combinations thereof. Suitable sources of punicalagin and ellagitannin for embodiments of this invention include, but are not limited to, pomegranate, raspberry, strawberry, walnut, and oak-aged red wine.

[0366] In some embodiments, the antioxidant is a *Citrus* flavonoid, such as hesperidin or naringin. Suitable sources of *Citrus* flavonoids, such as hesperidin or naringin, for embodiments of this invention include, but are not limited to, oranges, grapefruits, and *Citrus* juices.

[0367] In particular embodiments, the antioxidant is chlorogenic acid. Suitable sources of chlorogenic acid for embodiments of this invention include, but are not limited to, green coffee, yerba mate, red wine, grape seed, red grape skin, purple grape skin, red grape juice, purple grape juice, apple juice, cranberry, pomegranate, blueberry, strawberry, sunflower, *Echinacea*, pycnogenol, and apple peel.

[0368] Suitable dietary fibers include, but are not limited to, non-starch polysaccharides, lignin, cellulose, methylcellulose, the hemicelluloses, β -glucans, pectins, gums, mucilage, waxes, inulins, oligosaccharides, fructooligosaccharides, cyclodextrins, chitins, and combinations thereof.

[0369] Food sources of dietary fiber include, but are not limited to, grains, legumes, fruits, and vegetables. Grains providing dietary fiber include, but are not limited to, oats, rye, barley, and wheat. Legumes providing fiber include, but are not limited to, peas and beans such as soybeans. Fruits and vegetables providing a source of fiber include, but are not limited to, apples, oranges, pears, bananas, berries, tomatoes, green beans, broccoli, cauliflower, carrots, potatoes, celery. Plant foods such as bran, nuts, and seeds (such as flax seeds) are also sources of dietary fiber. Parts of plants providing dietary fiber include, but are not limited to, the stems, roots, leaves, seeds, pulp, and skin.

[0370] Fatty acids include any straight chain or branched monocarboxylic acid and include saturated fatty acids, unsaturated fatty acids, long chain fatty acids, medium chain fatty acids, short chain fatty acids, fatty acid precursors (including omega-9 fatty acid precursors), and esterified fatty acids. In an embodiment, the fatty acid is a straight chain monocarboxylic acid. As used herein, "long chain polyunsaturated fatty acid" refers to any polyunsaturated carboxylic acid or organic acid with a long aliphatic tail. Suitable omega-3 fatty acids include, but are not limited to, linolenic acid, alpha-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid, stearidonic acid, eicosatetraenoic acid and combinations thereof.

[0371] Suitable omega-6 fatty acids include, but are not limited to, linoleic acid, gamma-linolenic acid, dihomo-gamma-linolenic acid, arachidonic acid, eicosadienoic acid, docosadienoic acid, adrenic acid, docosapentaenoic acid and combinations thereof. Suitable esterified fatty acids for embodiments of the present invention include, but are not limited to, monoacylglycerols containing omega-3 and/or omega-6 fatty acids, diacylglycerols containing omega-3 and/or omega-6 fatty acids, or triacylglycerols containing omega-3 and/or omega-6 fatty acids and combinations thereof.

[0372] Suitable vitamins include, vitamin A, vitamin D, vitamin E, vitamin K, vitamin B1, vitamin B2, vitamin B3, vitamin B5, vitamin B6, vitamin B7, vitamin B9, vitamin B12, and vitamin C. Various other compounds have been classified as vitamins by some authorities. These compounds may be termed pseudo-vitamins and include, but are not limited to, compounds such as ubiquinone (coenzyme Q10), pangamic acid, dimethylglycine, taestrile, amygdaline, flavanoids, para-aminobenzoic acid, adenine, adenylic acid, and s-methylmethionine. As used herein, the term vitamin includes pseudo-vitamins.

[0373] Minerals are selected from bulk minerals, trace minerals or combinations thereof. Non-limiting examples of bulk minerals include calcium, chlorine, magnesium, phosphorous, potassium, sodium, and sulfur. Non-limiting examples of trace minerals include chromium, cobalt, copper, fluorine, iron,

manganese, molybdenum, selenium, zinc, and iodine. Although iodine generally is classified as a trace mineral, it is required in larger quantities than other trace minerals and often is categorized as a bulk mineral.

[0374] In other particular embodiments of this invention, the mineral is a trace mineral, believed to be necessary for human nutrition, non-limiting examples of which include bismuth, boron, lithium, nickel, rubidium, silicon, strontium, tellurium, tin, titanium, tungsten, and vanadium.

[0375] Preservatives are selected from antimicrobials, antioxidants, antienzymatics or combinations thereof. Non-limiting examples of antimicrobials include sulfites, propionates, benzoates, sorbates, nitrates, nitrites, bacteriocins, salts, sugars, acetic acid, dimethyl dicarbonate (DMDC), ethanol, and ozone. Sulfites include, but are not limited to, sulfur dioxide, sodium bisulfite, and potassium hydrogen sulfite. Propionates include, but are not limited to, propionic acid, calcium propionate, and sodium propionate. Benzoates include, but are not limited to, sodium benzoate and benzoic acid. Sorbates include, but are not limited to, potassium sorbate, sodium sorbate, calcium sorbate, and sorbic acid. Nitrates and nitrites include, but are not limited to, sodium nitrate and sodium nitrite. In yet another particular embodiment, the at least one preservative is a bacteriocin, such as, for example, nisin. In another particular embodiment, the preservative is ethanol. In still another particular embodiment, the preservative is ozone. Antienzymatics suitable for use as preservatives in particular embodiments of the invention include ascorbic acid, citric acid, and metal chelating agents such as ethylenediaminetetraacetic acid (EDTA).

[0376] Hydration products can be electrolytes, non-limiting examples of which include sodium, potassium, calcium, magnesium, chloride, phosphate, bicarbonate, and combinations thereof. Suitable electrolytes for use in particular embodiments of this invention are also described in U.S. Pat. No. 5,681,569, the disclosure of which is expressly incorporated herein by reference. Non-limiting examples of salts for use in particular embodiments include chlorides, carbonates, sulfates, acetates, bicarbonates, citrates, phosphates, hydrogen phosphates, tartrates, sorbates, citrates, benzoates, or combinations thereof.

[0377] In particular embodiments of this invention, the hydration product is a carbohydrate to supplement energy stores burned by muscles. Suitable carbohydrates for use in particular embodiments of this invention are described in U.S. Pat. Nos. 4,312,856, 4,853,237, 5,681,569, and 6,989,171, the disclosures of which are expressly incorporated herein by reference. Non-limiting examples of suitable carbohydrates include monosaccharides, disaccharides, oligosaccharides, complex polysaccharides or combinations thereof. Non-limiting examples of suitable types of monosaccharides for use in particular embodiments include trioses, tetroses, pentoses, hexoses, heptoses, octoses, and nonoses. Non-limiting examples of specific types of suitable monosaccharides

include glyceraldehyde, dihydroxyacetone, erythrose, threose, erythrulose, arabinose, lyxose, ribose, xylose, ribulose, xylulose, allose, altrose, galactose, glucose, gulose, idose, mannose, talose, fructose, psicose, sorbose, tagatose, mannoheptulose, sedoheltulose, octulose, and sialose. Non-limiting examples of suitable disaccharides include sucrose, lactose, and maltose. Non-limiting examples of suitable oligosaccharides include saccharose, maltotriose, and maltodextrin. In other particular embodiments, the carbohydrates are provided by a corn syrup, a beet sugar, a cane sugar, a juice, or a tea. In another particular embodiment, the hydration is a flavanol that provides cellular rehydration. Non-limiting examples of suitable flavanols for use in particular embodiments of this invention include catechin, epicatechin, galocatechin, epigallocatechin, epicatechin gallate, epigallocatechin 3-gallate, theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate, theaflavin 3,3' gallate, thearubigin or combinations thereof. In a particular embodiment, the hydration product is a glycerol solution to enhance exercise endurance.

[0378] Probiotics comprise microorganisms that benefit health when consumed in an effective amount. Probiotics may include, without limitation, bacteria, yeasts, and fungi. Examples of probiotics include, but are not limited to, bacteria of the genus *Lactobacilli*, *Bifidobacteria*, *Streptococci*, or combinations thereof. In particular embodiments of the invention, the at least one probiotic is chosen from the genus *Lactobacilli*. *Lactobacilli* (i.e., bacteria of the genus *Lactobacillus*, hereinafter "L."). Non-limiting examples of species of *Lactobacilli* found in the human intestinal tract include *L. acidophilus*, *L. casei*, *L. fermentum*, *L. salivarius*, *L. brevis*, *L. leichmannii*, *L. plantarum*, *L. cellobiosus*, *L. reuteri*, *L. rhamnosus*, *L. GG*, *L. bulgaricus*, and *L. thermophilus*. According to other particular embodiments of this invention, the probiotic is chosen from the genus *Bifidobacteria*. Non-limiting species of *Bifidobacteria* found in the human gastrointestinal tract include *B. angulatum*, *B. animalis*, *B. asteroides*, *B. bifidum*, *B. boum*, *B. breve*, *B. catenulatum*, *B. choerinum*, *B. coryneforme*, *B. cuniculi*, *B. dentium*, *B. gallicum*, *B. gallinarum*, *B. indicum*, *B. longum*, *B. magnum*, *B. merycicum*, *B. minimum*, *B. pseudocatenulatum*, *B. pseudolongum*, *B. psychraerophilum*, *B. pullorum*, *B. ruminantium*, *B. saeculare*, *B. scardovii*, *B. simiae*, *B. subtile*, *B. thermacidophilum*, *B. thermophilum*, *B. urinalis*, and *B. sp.* According to other particular embodiments of this invention, the probiotic is chosen from the genus *Streptococcus*. *Streptococcus thermophilus* is a gram-positive facultative anaerobe. Other non-limiting probiotic species of this bacteria include *Streptococcus salivarius* and *Streptococcus cremoris*.

[0379] Probiotics are compositions that promote the growth of beneficial bacteria in the intestines. Probiotics include, without limitation, mucopolysaccharides, oligosaccharides, polysaccharides, amino acids, vitamins, nutrient precursors, proteins and combinations thereof. According to a particular embodiment, the probiotic is chosen from dietary fibers, including, without limitation, polysaccharides

and oligosaccharides. Non-limiting examples of oligosaccharides that are categorized as prebiotics in accordance with particular embodiments of this invention include fructooligosaccharides, inulins, isomalto-oligosaccharides, lactitol, lactosucrose, lactulose, pyrodextrins, soy oligosaccharides, transgalacto-oligosaccharides, and xylo-oligosaccharides. According to other particular embodiments, the prebiotic is an amino acid.

[0380] As used herein, “a weight management agent” includes an appetite suppressant and/or a thermogenesis agent. As used herein, the phrases “appetite suppressant”, “appetite satiation compositions”, “satiety agents”, and “satiety ingredients” are synonymous. The phrase “appetite suppressant” describes macronutrients, herbal extracts, exogenous hormones, anorectics, anorexigenics, pharmaceutical drugs, and combinations thereof, that when delivered in an effective amount, suppress, inhibit, reduce, or otherwise curtail a person's appetite. The phrase “thermogenesis agent” describes macronutrients, herbal extracts, exogenous hormones, anorectics, anorexigenics, pharmaceutical drugs, and combinations thereof, that when delivered in an effective amount, activate or otherwise enhance a person's thermogenesis or metabolism.

[0381] Suitable weight management agents include macronutrients selected from the group consisting of proteins, carbohydrates, dietary fats, and combinations thereof. Carbohydrates generally comprise sugars, starches, cellulose and gums that the body converts into glucose for energy. Non-limiting examples of carbohydrates include polydextrose; inulin; monosaccharide-derived polyols such as erythritol, mannitol, xylitol, and sorbitol; disaccharide-derived alcohols such as isomalt, lactitol, and maltitol; and hydrogenated starch hydrolysates. Carbohydrates are described in more detail herein below. Dietary fats are lipids comprising combinations of saturated and unsaturated fatty acids. Polyunsaturated fatty acids have been shown to have a greater satiating power than mono-unsaturated fatty acids. Accordingly, the dietary fats embodied herein desirably comprise poly-unsaturated fatty acids, non-limiting examples of which include triacylglycerols.

[0382] In a particular embodiment, the weight management agent is a herbal extract. Non-limiting examples of plants whose extracts have appetite suppressant properties include plants of the genus *Hoodia*, *Trichocaulon*, *Caralluma*, *Stapelia*, *Orbea*, *Asclepias*, and *Camelia*. Other embodiments include extracts derived from *Gymnema sylvestre*, Kola Nut, *Citrus Aurantium*, Yerba Mate, *Griffonia simplicifolia*, Guarana, myrrh, guggul Lipid, and black current seed oil. In a particular embodiment, the herbal extract is derived from a plant of the genus *Hoodia*, species of which include *H. alstonii*, *H. currorii*, *H. dregei*, *H. flava*, *H. gordonii*, *H. jutatae*, *H. mossamedensis*, *H. officinalis*, *H. parviflorai*, *H. pedicellata*, *H. pifera*, *H. ruschii*, and *H. triebneri*. *Hoodia* plants are stem succulents native to southern Africa.

[0383] In another particular embodiment, the herbal extract is derived from a plant of the genus *Caralluma*, species of which include *C. indica*, *C. fimbriata*, *C. attenuata*, *C. tuberculata*, *C. edulis*, *C. adscendens*, *C. stalagmifera*, *C. umbellata*, *C. penicillata*, *C. russeliana*, *C. retrospiciens*, *C. arabica*, and *C. lasiantha*. *Caralluma* plants belong to the same Subfamily as *Hoodia*, Asclepiadaceae.

[0384] In another particular embodiment, the at least one herbal extract is derived from a plant of the genus *Trichocaulon*. *Trichocaulon* plants are succulents that generally are native to southern Africa, similar to *Hoodia*, and include the species *T. piliferum* and *T. officinale*. In another particular embodiment, the herbal extract is derived from a plant of the genus *Stapelia* or *Orbea*, species of which include *S. gigantea* and *O. variegata*, respectively. Both *Stapelia* and *Orbea* plants belong to the same Subfamily as *Hoodia*, Asclepiadaceae.

[0385] In another particular embodiment, the herbal extract is derived from a plant of the genus *Asclepias*. *Asclepias* plants also belong to the Asclepiadaceae family of plants. Non-limiting examples of *Asclepias* plants include *A. incarnate*, *A. curassayica*, *A. syriaca*, and *A. tuberosa*. Not wishing to be bound by any theory, it is believed that the extracts comprise steroidal compounds, such as pregnane glycosides and pregnane aglycone, having appetite suppressant effects. In a particular embodiment, the weight management agent is an exogenous hormone having a weight management effect. Non-limiting examples of such hormones include CCK, peptide YY, ghrelin, bombesin and gastrin-releasing peptide (GRP), enterostatin, apolipoprotein A-IV, GLP-1, amylin, somastatin, and leptin.

[0386] In certain embodiments, the osteoporosis management agent is at least one calcium source, i.e. any compound containing calcium, including salt complexes, solubilized species, and other forms of calcium. Non-limiting examples of calcium sources include amino acid chelated calcium, calcium carbonate, calcium oxide, calcium hydroxide, calcium sulfate, calcium chloride, calcium phosphate, calcium hydrogen phosphate, calcium dihydrogen phosphate, calcium citrate, calcium malate, calcium citrate malate, calcium gluconate, calcium tartrate, calcium lactate, solubilized species thereof, and combinations thereof. According to a particular embodiment, the osteoporosis management agent is a magnesium source, i.e. any compound containing magnesium, including salt complexes, solubilized species, and other forms of magnesium. Non-limiting examples of magnesium sources include magnesium chloride, magnesium citrate, magnesium gluceptate, magnesium gluconate, magnesium lactate, magnesium hydroxide, magnesium picolate, magnesium sulfate, solubilized species thereof, and mixtures thereof. In another particular embodiment, the magnesium source comprises an amino acid chelated or creatine chelated magnesium. In other embodiments, the osteoporosis agent is chosen from vitamins D, C, K, their precursors and/or beta-carotene and combinations thereof. Numerous

plants and plant extracts also have been identified as being effective in the prevention and treatment of osteoporosis. Not wishing to be bound by any theory, it is believed that the plants and plant extracts stimulates bone morphogenic proteins and/or inhibits bone resorption, thereby stimulating bone regeneration and strength. Non-limiting examples of suitable plants and plant extracts as osteoporosis management agents include species of the genus *Taraxacum* and *Amelanchier*, as disclosed in U.S. Patent Publication No. 2005/0106215, and species of the genus *Lindera*, *Artemisia*, *Acorus*, *Carthamus*, *Carum*, *Cnidium*, *Curcuma*, *Cyperus*, *Juniperus*, *Prunus*, *Iris*, *Cichorium*, *Dodonaea*, *Epimedium*, *Erigonoum*, *Soya*, *Mentha*, *Ocimum*, *thymus*, *Tanacetum*, *Plantago*, *Spearmint*, *Bixa*, *Vitis*, *Rosemarinus*, *Rhus*, and *Anethum*, as disclosed in U.S. Patent Publication No. 2005/0079232.

[0387] Examples of suitable phytoestrogens for embodiments of this invention include, but are not limited to, isoflavones, stilbenes, lignans, resorcylic acid lactones, coumestans, coumestrol, equol, and combinations thereof. Isoflavones belong to the group of phytonutrients called polyphenols. In general, polyphenols (also known as “polyphenolics”), are a group of chemical substances found in plants, characterized by the presence of more than one phenol group per molecule. Suitable phytoestrogen isoflavones in accordance with embodiments of this invention include genistein, daidzein, glycitein, biochanin A, formononetin, their respective naturally occurring glycosides and glycoside conjugates, matairesinol, secoisolariciresinol, enterolactone, enterodiol, textured vegetable protein, and combinations thereof.

[0388] Long-chain primary aliphatic saturated alcohols are a diverse group of organic compounds. The term long-chain refers to the fact that the number of carbon atoms in these compounds is at least 8 carbons. Non-limiting examples of particular long-chain primary aliphatic saturated alcohols for use in particular embodiments of the invention include the 8 carbon atom 1-octanol, the 9 carbon 1-nonanol, the 10 carbon atom 1-decanol, the 12 carbon atom 1-dodecanol, the 14 carbon atom 1-tetradecanol, the 16 carbon atom 1-hexadecanol, the 18 carbon atom 1-octadecanol, the 20 carbon atom 1-eicosanol, the 22 carbon 1-docosanol, the 24 carbon 1-tetracosanol, the 26 carbon 1-hexacosanol, the 27 carbon 1-heptacosanol, the 28 carbon 1-octanosol, the 29 carbon 1-nonacosanol, the 30 carbon 1-triacontanol, the 32 carbon 1-dotriacontanol, and the 34 carbon 1-tetracontanol. In a particularly desirable embodiment of the invention, the long-chain primary aliphatic saturated alcohols are policosanol. Policosanol is the term for a mixture of long-chain primary aliphatic saturated alcohols composed primarily of 28 carbon 1-octanosol and 30 carbon 1-triacontanol, as well as other alcohols in lower concentrations such as 22 carbon 1-docosanol, 24 carbon 1-tetracosanol, 26 carbon 1-hexacosanol, 27 carbon 1-heptacosanol, 29 carbon 1-nonacosanol, 32 carbon 1-dotriacontanol, and 34 carbon 1-tetracontanol.

[0389] At least 44 naturally-occurring phytosterols have been discovered, and generally are derived from plants, such as corn, soy, wheat, and wood oils; however, they also may be produced synthetically to form compositions identical to those in nature or having properties similar to those of naturally-occurring phytosterols. According to particular embodiments of this invention, non-limiting examples of phytosterols well known to those of ordinary skill in the art include 4-desmethylsterols (e.g., β -sitosterol, campesterol, stigmasterol, brassicasterol, 22-dehydrobrassicasterol, and Δ^5 -avenasterol), 4-monomethyl sterols, and 4,4-dimethyl sterols (triterpene alcohols) (e.g., cycloartenol, 24-methylenecycloartanol, and cyclobranol).

[0390] According to particular embodiments of this invention, non-limiting examples of phytostanols include β -sitostanol, campestanol, cycloartanol, and saturated forms of other triterpene alcohols.

[0391] Both phytosterols and phytostanols, as used herein, include the various isomers such as the α and β isomers (e.g., α -sitosterol and β -sitostanol, which comprise one of the most effective phytosterols and phytostanols, respectively, for lowering serum cholesterol in mammals). The phytosterols and phytostanols of the present invention also may be in their ester form. Non-limiting examples of suitable phytosterol and phytostanol esters include sitosterol acetate, sitosterol oleate, stigmasterol oleate, and their corresponding phytostanol esters. The phytosterols and phytostanols of the present invention also may include their derivatives.

[0392] Generally, the amount of functional ingredient in the composition varies widely depending on the particular composition and the desired functional ingredient. Those of ordinary skill in the art will readily ascertain the appropriate amount of functional ingredient for each composition.

[0393] CONSUMABLES/PRODUCTS FOR ORAL ADMINISTRATION

[0394] Disclosed herein are consumables (e.g., food and/or beverages) comprising the sweetener compositions or flavor modifying compositions disclosed herein.

[0395] Myd (HTS) polypeptides (or variants thereof) or the Myd-containing sweetener compositions or flavor modifying compositions disclosed herein can be incorporated in any known edible material (referred to herein as a “consumable,” “consumable product”, a “sweetenable composition” or “flavor and/or taste modified composition”), such as, edible gel mixes and compositions, dental compositions, foodstuffs (confections, condiments, chewing gum, cereal compositions, baked goods, dairy products, and tabletop sweetener compositions) beverages and beverage products. “A consumable product” herein includes concentrates, mixes, powders and other solid materials which can be combined with other edible ingredients to produce a final consumable product (e.g., cake, cookie, bread or related mixes; or seasoning, salad dressing or gravy mixes or packets).

[0396] In one embodiment, the consumable is a beverage or beverage product. Put another way, disclosed herein is a beverage or beverage product comprising Myd polypeptides (or variants thereof) or the Myd-containing sweetener compositions or flavor modifying compositions disclosed herein.

[0397] As used herein a “beverage product” is a ready-to-drink beverage, a beverage concentrate, a beverage syrup, or a powdered beverage. Suitable ready-to-drink beverages include carbonated and non-carbonated beverages. Carbonated beverages include, but are not limited to, cola, ginger-ale, soft drinks and root beer. Non-carbonated beverages include, but are not limited to fruit juice, fruit-flavored juice, vegetable juice, vegetable-flavored juice, sports drinks, energy drinks, plant protein beverage (including plant-based milks), near water drinks (e.g., water with natural or synthetic flavorants), tea type (e.g. black tea, green tea, red tea, oolong tea), coffee, cocoa drink, beverage containing milk components (e.g. milk beverages, coffee containing milk components, café au lait, milk tea, fruit milk beverages).

[0398] In particular embodiments, the beverage is a flavored black tea beverage, a zero calorie enhanced water beverage or an orange-flavored sparkling beverage.

[0399] Beverage concentrates and beverage syrups are prepared with an initial volume of liquid matrix (e.g. water) and the desired beverage ingredients. Full strength beverages are then prepared by adding further volumes of water. Powdered beverages are prepared by dry-mixing all of the beverage ingredients in the absence of a liquid matrix. Full strength beverages are then prepared by adding the full volume of water.

[0400] Beverages contain water as the liquid matrix. As used herein, a “liquid matrix” refers to the basic ingredient in which the ingredients—including the sweetener or sweetener compositions—are dissolved. Water of beverage quality, such as, for example deionized water, distilled water, reverse osmosis water, carbon-treated water, purified water, demineralized water and combinations thereof, can be used. Additional suitable liquid matrices include, but are not limited to phosphoric acid, phosphate buffer, citric acid, citrate buffer and carbon-treated water.

[0401] In one embodiment, a beverage or beverage product is disclosed that contains Myd polypeptides or a variant thereof. In a particular embodiment, the beverage contains a Myd variant that has the same or increased sweetness relative to a beverage containing the same amount of wild-type Myd.

[0402] In one embodiment, the concentration of Myd (or a variant thereof) in the beverage is at or above its sweetness recognition threshold concentration or flavor recognition threshold concentration. In a particular embodiment, the concentration of Myd is at least about 1%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, about least

about 35%, at least about 40%, about least about 45%, at least about 50% or more above its sweetness or flavor recognition threshold concentration.

[0403] In one embodiment, the beverage or beverage product contains Myd (or variant thereof) in an amount from about 1 ppm to about 50 ppm, such as, for example, from about 5 ppm to about 50 ppm, from about 5 ppm to about 40 ppm, from about 5 ppm to about 30 ppm, from about 5 ppm to about 20 ppm, from about 5 ppm to about 10 ppm, from about 10 ppm to about 50 ppm, from about 10 ppm to about 40 ppm, from about 10 ppm to about 30 ppm, from about 10 ppm to about 20 ppm, from about 20 ppm to about 50 ppm, from about 20 ppm to about 40 ppm, from about 20 ppm to about 30 ppm, from about 30 ppm to about 50 ppm, from about 30 ppm to about 40 ppm or from about 40 ppm to about 50 ppm.

[0404] In a more particular embodiment, the beverage or beverage product contains Myd (or variant thereof) in an amount from about 10 ppm to about 25 ppm.

[0405] In another embodiment, the beverage contains Myd (or variant thereof) in an amount from about 1 ppm to less than about 15 ppm. In one embodiment, the beverage contains Myd (or variant thereof) in an amount from about 1 ppm to about 5 ppm, about 5 ppm to about 10 ppm or about 10 ppm to about 15 ppm.

[0406] In another embodiment, the beverage contains Myd (or variant thereof) in an amount greater than about 15 ppm. In one embodiment, the beverage contains Myd (or variant thereof) in an amount between about 15 ppm and about 20 ppm, about 20 ppm and about 25 ppm, about 25 ppm and about 30 ppm, about 30 ppm and about 35 ppm, about 35 ppm and about 40 ppm, about 40 ppm and about 50 ppm, or about 50 ppm or greater.

[0407] In one embodiment, the beverage contains Myd (or variant thereof) in an amount of about 1 ppm, about 3 ppm, about 5 ppm, about 10 ppm, about 12 ppm, about 15 ppm, about 18 ppm, about 20 ppm, about 22 ppm, about 25 ppm, about 28 ppm, about 30 ppm, about 35 ppm, about 40 ppm, about 45 ppm, about 50 ppm, about 55 ppm, about 60 ppm, about 65 ppm, about 70 ppm or about 75 ppm or greater.

[0408] The beverage can further include at least one additional sweetener. Any of the sweeteners detailed herein can be used, including natural, non-natural, or synthetic sweeteners.

[0409] In a particular embodiment, the at least one addition sweetener is one or more steviol glycosides. In a particular embodiment, the steviol glycoside is Reb M. In another particular embodiment, the steviol glycoside is Reb A. In one embodiment, the beverage contains, in addition to Myd (or variant thereof) a steviol glycoside blend, e.g., Reb M and Reb A.

[0410] The amount of the steviol glycoside (e.g., Reb M, Reb A) may vary. In one embodiment, the amount of the steviol glycoside provides less than about 8 sucrose equivalence (SE), e.g., about 7.5 SE, about 7.0 SE, about 6.5 SE or about 6.0 SE or less. In another embodiment, the amount of steviol glycoside provides greater than about 8 sucrose equivalence (SE), e.g., about 8.5 SE, about 9.0 SE, about 9.5 SE, about 10 SE, about 10.5 SE, about 11 SE or about 12 SE or more.

[0411] In another embodiment, the amount of steviol glycoside (e.g., Reb M, Reb A) is less than about 450 ppm. In a particular embodiment, the amount of steviol glycoside is less than about 400 ppm, less than about 350 ppm, less than about 300 ppm, less than about 300 ppm, less than about 250 ppm, less than about 200 ppm, less than about 150 ppm or about 100 ppm or less.

[0412] In another embodiment, the amount of steviol glycoside (e.g., Reb M, Reb A) is between about 100 ppm and about 450 ppm, more particularly, between about 100 ppm and about 400 ppm, between about 100 ppm and about 350 ppm, between about 100 ppm and about 300 ppm, between about 100 ppm and about 250 ppm, between about 100 ppm and about 200 ppm, or between about 100 ppm and 250 ppm.

[0413] In a further embodiment, the amount of the steviol glycoside (e.g., Reb M, Reb A) is between about 100 ppm and about 200 ppm, more particularly, about 110 ppm, about 120 ppm, about 130 ppm, about 140 ppm, about 150 ppm, about 160 ppm, about 170 ppm, about 180 ppm, about 190 ppm or about 200 ppm.

[0414] In a further embodiment, the amount of the steviol glycoside (e.g., Reb M, Reb A) is between about 200 ppm and about 300 ppm, and more particularly, about 210 ppm, about 220 ppm, about 230 ppm, about 240 ppm, about 250 ppm, about 260 ppm, about 270 ppm, about 280 ppm, about 290 ppm, or about 300 ppm.

[0415] In another embodiment, the amount of the steviol glycoside (e.g., Reb M, Reb A) is between about 300 ppm and about 400 ppm, and more particularly, about 310 ppm, about 320 ppm, about 330 ppm, about 340 ppm, about 350 ppm, about 360 ppm, about 370 ppm, about 380 ppm, about 390 ppm or about 400 ppm.

[0416] In another embodiment, the amount of the steviol glycoside (e.g., Reb M, Reb A) is between about 400 ppm and about 500 ppm, and more particularly, about 410 ppm, about 420 ppm, about 430 ppm, about 440 ppm, about 450 ppm, about 460 ppm, about 470 ppm or about 480 ppm.

[0417] In a particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 1 ppm and about 100 ppm and at least one steviol glycoside (e.g., Reb M, Reb A), wherein the amount of the at least one steviol glycoside is sufficient to provide less than about 8 SE,

e.g., about 7.5 SE, about 7.0 SE, about 6.5 SE, about 6 SE, about 5.5 SE, about 5.0 SE, about 4.5 SE, about 4.0 SE, about 3.5 SE, about 3.0 SE, about 2.5 SE, about 2.0 SE, about 1.5 SE or about 1.0 SE.

[0418] In another particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 1 ppm and about 100 ppm and at least one steviol glycoside (e.g., Reb M, Reb A), wherein the amount of the at least one steviol glycoside is about 450 ppm or less, e.g., about 425 ppm, about 400 ppm, about 375 ppm, about 350 ppm, about 325 ppm, about 300 ppm, about 275 ppm, about 250 ppm, about 225 ppm, about 200 ppm, about 175 ppm, about 150 ppm, about 125 ppm or about 100 ppm or less.

[0419] In a particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 1 ppm and about 30 ppm, more particularly, about 1 ppm and about 15 ppm or about 15 ppm and about 30 ppm, and Reb M in an amount sufficient to provide less than about 8 SE, for example, e.g., about 7.5 SE, about 7.0 SE, about 6.5 SE, about 6 SE, about 5.5 SE, about 5.0 SE, about 4.5 SE, about 4.0 SE, about 3.5 SE, about 3.0 SE, about 2.5 SE, about 2.0 SE, about 1.5 SE or about 1.0 SE.

[0420] In another particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 1 ppm and about 30 ppm, more particularly, between about 1 ppm and about 15 ppm or between about 15 ppm and about 30 ppm, and Reb M in an amount about 450 ppm or less, e.g., about 425 ppm, about 400 ppm, about 375 ppm, about 350 ppm, about 325 ppm, about 300 ppm, about 275 ppm, about 250 ppm, about 225 ppm, about 200 ppm, about 175 ppm, about 150 ppm, about 125 ppm or about 100 ppm or less.

[0421] In another particular embodiment, a beverage comprises Myd (or variant thereof) in an amount between about 1 ppm and about 30 ppm, more particularly, between about 1 ppm and about 15 ppm or between about 15 ppm and about 30 ppm, and Reb M in an amount about 450 ppm or less, e.g., about 425 ppm, about 400 ppm, about 375 ppm, about 350 ppm, about 325 ppm, about 300 ppm, about 275 ppm, about 250 ppm, about 225 ppm, about 200 ppm, about 175 ppm, about 150 ppm, about 125 ppm or about 100 ppm or less.

[0422] For example, a beverage comprises Myd (or variant thereof) in an amount from about 1 ppm to about 30 ppm and RebM80 in an amount from about 200 ppm to about 400 ppm. In a more particular embodiment, a beverage comprises Myd (or variant thereof) in an amount from about 20 ppm to about 30 ppm and RebM80 in an amount from about 300 to about 400 ppm. In a still more particular embodiment, a beverage comprises Myd (or variant thereof) in an amount of about 25 ppm and RebM80 in an amount of about 315 ppm.

[0423] A beverage of the present invention comprises Myd (or variant thereof) in an amount from about 1 ppm to about 50 ppm and RebM80 in an amount from about 200 ppm to about 400 ppm. In preferred embodiments, the beverage of the present invention tastes similar to a beverage sweetened with RebM or RebM80 only, wherein the beverage of the present invention and the RebM or RebM80-sweetened beverages have similar sucrose equivalence. In a more particular embodiment, a beverage having a citric acid matrix comprises Myd (or variant thereof) in an amount from about 20 ppm to about 30 ppm and RebM80 in an amount from about 300 to about 400 ppm. In a still more particular embodiment, a beverage having a citric acid matrix comprises Myd (or variant thereof) in an amount of about 25 ppm and RebM80 in an amount of about 315 ppm. In another more particular embodiment, a lemon-lime diet carbonated beverage comprises Myd (or variant thereof) in an amount from about 30 ppm to about 50 ppm and RebM80 in an amount from about 150 ppm to about 350 ppm. In a still more particular embodiment, a lemon-lime diet carbonated beverage comprises Myd (or variant thereof) in an amount of about 40 ppm and RebM80 in an amount of about 210 ppm.

[0424] In a particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 10 ppm and about 25 ppm and Reb M in an amount sufficient to provide less than about 8 SE, e.g., about 7.5 SE, about 7.0 SE, about 6.5 SE, about 6 SE, about 5.5 SE, about 5.0 SE, about 4.5 SE, about 4.0 SE, about 3.5 SE, about 3.0 SE, about 2.5 SE, about 2.0 SE, about 1.5 SE or about 1.0 SE.

[0425] In another particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 10 ppm and about 25 ppm, and Reb M in an amount about 450 ppm or less, e.g., about 425 ppm, about 400 ppm, about 375 ppm, about 350 ppm, about 325 ppm, about 300 ppm, about 275 ppm, about 250 ppm, about 225 ppm, about 200 ppm, about 175 ppm, about 150 ppm, about 125 ppm or about 100 ppm or less.

[0426] In another particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 1 ppm and about 50 ppm and at least one additional sweetener (e.g., Reb M, Reb A, siamenoside I, mogroside IV), wherein the amount of the at least one additional sweetener is less than about 450 ppm, e.g., about 425 ppm, about 400 ppm, about 375 ppm, about 350 ppm, about 325 ppm, about 300 ppm, about 275 ppm, about 250 ppm, about 225 ppm, about 200 ppm, about 175 ppm, about 150 ppm, about 125 ppm or about 100 ppm or less.

[0427] In a particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 1 ppm and about 100 ppm and Reb A in an amount sufficient to provide less than about 8 SE, e.g., about 7.5 SE, about 7.0 SE, about 6.5 SE, about 6 SE, about 5.5 SE, about 5.0 SE, about 4.5 SE, about 4.0 SE, about 3.5 SE, about 3.0 SE, about 2.5 SE, about 2.0 SE, about 1.5 SE or about 1.0 SE.

[0428] In a particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 1 ppm and about 30 ppm, more particularly between about 1 ppm and about 15 ppm or between about 15 ppm and about 30 ppm, Reb A in an amount less than about 450 ppm, e.g., about 425 ppm, about 400 ppm, about 375 ppm, about 350 ppm, about 325 ppm, about 300 ppm, about 275 ppm, about 250 ppm, about 225 ppm, about 200 ppm, about 175 ppm, about 150 ppm, about 125 ppm or about 100 ppm or less.

[0429] In a particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 10 ppm and about 25 ppm and Reb A in an amount sufficient to provide less than about 8 SE, e.g., about 7.5 SE, about 7.0 SE, about 6.5 SE, about 6 SE, about 5.5 SE, about 5.0 SE, about 4.5 SE, about 4.0 SE, about 3.5 SE, about 3.0 SE, about 2.5 SE, about 2.0 SE, about 1.5 SE or about 1.0 SE.

[0430] In a particular embodiment, the beverage comprises Myd (or variant thereof) in an amount between about 10 ppm and about 25 ppm, Reb A in an amount less than about 450 ppm, e.g., about 425 ppm, about 400 ppm, about 375 ppm, about 350 ppm, about 325 ppm, about 300 ppm, about 275 ppm, about 250 ppm, about 225 ppm, about 200 ppm, about 175 ppm, about 150 ppm, about 125 ppm or about 100 ppm or less.

[0431] The ppm ratio of Myd (or variant thereof) and the one or more steviol glycosides in the beverage may vary. In one embodiment, the ppm ratio of Myd (or variant thereof) and the one or more steviol glycosides is between about 1:2 and about 1:40, more particularly, between about 1:5 and about 1:35, even more particularly, between about 1:10 and about 1:25.

[0432] In a particular embodiment, the ppm ratio of Myd (or variant thereof) and the one or more steviol glycosides is about 1:30 or less, more particularly, about 1:25.

[0433] In one embodiment, the steviol glycoside is Reb M and the ppm ratio of Myd (or variant thereof) and Reb M is between about 1:10 and about 1:20, more particularly, about 1:10, about 1:12, about 1:14, about 1:16, about 1:18 or about 1:20.

[0434] In another embodiment, the steviol glycoside is Reb A and the ppm ratio of Myd (or variant thereof) and Reb A is about 1:40 or less, more particularly, between about 1:20 and about 1:30, even more particularly, about 1:20, about 1:22, about 1:24, about 1:26, about 1:28 or about 1:30.

[0435] In another embodiment, the steviol glycoside is Reb A and the ppm ratio of Myd (or variant thereof) and Reb A is about 1:70 or less, more particularly, less than about 1:60, less than about 1:50 or less than about 1:40. In a particular embodiment, the ppm ratio of Myd (or variant thereof) and Reb A is about 1:35.

[0436] In another embodiment, the steviol glycoside is Reb A and the ppm ratio of Myd (or variant thereof) and Reb A is about 1:15 or greater, more particularly, greater than about 1:17, about 1:18, about 1:19 or about 1:20.

[0437] In another embodiment, the steviol glycoside is Reb A and the ppm ratio of Myd (or variant thereof) and Reb A is about 1:10 or greater, more particularly, greater than about 1:12, about 1:14 or about 1:16.

[0438] In one embodiment, the beverage comprises Myd (or variant thereof) and Reb M, wherein Myd is present in an amount from about 15 ppm to about 30 ppm, more particularly, about 20 ppm to about 25 ppm, and Reb M (e.g., Reb M80) is present in an amount from about 300 ppm to about 350 ppm, more particularly, about 315 ppm to about 325 ppm (i.e. the Reb M provides about 8 SE). According to this embodiment, the ratio of Myd (or variant thereof) to Reb M is between about 1:10 and about 1:20, more particularly about 1:10 1:12, about 1:14, about 1:16, about 1:18 or about 1:20. In one embodiment, the amount of Reb M provides about 8 sucrose equivalence (SE).

[0439] In one embodiment, the beverage comprises Myd (or variant thereof) and Reb M, wherein Myd is present in an amount from about 1 ppm to about 20 ppm, more particularly about 1 ppm to about 15 ppm, and Reb M is present in an amount of about 110 ppm to about 140 ppm, more particularly about 115 ppm, and even more particularly, about 120 ppm. In a particular embodiment, the ppm ratio of Myd (or variant thereof) to Reb M is about 1:5 to about 1:10, more particularly about 1:7.

[0440] In one embodiment, the beverage comprises Myd (or variant thereof) and Reb M, wherein Myd is present in an amount from about 1 ppm to about 15 ppm, more particularly, about 1 ppm to about 5 ppm, even more particularly, about 3 ppm, and Reb M is present in an amount between about 450 ppm and about 500 ppm, more particularly, about 470 ppm to about 475 ppm, even more particularly, about 472 ppm. In a particular embodiment, Reb M is present in an amount that provides greater than about 9 sucrose equivalence (SE).

[0441] In one embodiment, the beverage comprises Myd (or variant thereof) and Reb A, wherein Myd is present in an amount from about 10 ppm to about 20 ppm, more particularly, about 15 ppm to about 20 ppm, and Reb A is present in an amount of about 375 ppm to about 425 ppm, more particularly, about 400 ppm. In one embodiment, the amount of Reb A provides about 7 SE. According to this embodiment, a ppm ratio of Myd (or variant thereof) to Reb A between about 1:20 ppm and about 1:30 ppm ratio of Myd (or variant thereof) to Reb A has at least one improved organoleptic property (e.g., taste) compared to a ppm ratio of Myd (or variant thereof) to Reb A, more particularly about 1:20 ppm, about 1:22 ppm, about 1:24 ppm, about 1:26 ppm, about 1:28 ppm or about 1:30 ppm.

[0442] In one embodiment, the beverage comprises Myd (or variant thereof) and Reb A, wherein Myd is present in an amount between about 5 ppm and about 15 ppm, more particular, about 10 ppm, and Reb A is present in an amount of between about 325 ppm and about 375 ppm, more particularly, about 350 ppm. In one embodiment, the amount of Reb A provides between about 6 and about 7 sucrose equivalence (SE). According to this embodiment, a ppm ratio of Myd (or variant thereof) to Reb A of about 1:30 has at least one improved organoleptic property compared to a ppm ratio of Myd (or variant thereof) to Reb A of about 1:70 or about 1:17.

[0443] In one embodiment, a beverage is provided that comprises Myd (or variant thereof) and Reb A, wherein the amount of Myd (or variant thereof) is between about 25 ppm and about 35 ppm, more particularly, about 30 ppm, and the amount of Reb A is between about 220 ppm and about 240 ppm, more particularly, about 230 ppm. According to this embodiment, a ppm ratio of Myd (or variant thereof) to Reb A of greater than about 1:10 has at least one improved organoleptic property than a ppm ratio of Myd (or variant thereof) to Reb A of less than about 1:10 ppm. In a particular embodiment, a ppm ratio of Myd (or variant thereof) to Reb A of about 1:12, about 1:14, about 1:16 or about 1:18 has at least one improved organoleptic property than a ppm ratio of Myd (or variant thereof) of about 1:8, about 1:6, about 1:4, about 1:2 or about 1:1 ppm.

[0444] In one embodiment, a beverage is provided that comprises Myd (or variant thereof) and Reb A, wherein the amount of Myd (or variant thereof) is between about 50 ppm and about 70 ppm, more particularly, about 60 ppm, and the amount of Reb A is between about 100 ppm and about 130 ppm, more particularly, about 120 ppm. According to this embodiment, the ppm ratio of Myd (or variant thereof) to Reb A is about 1:2.

[0445] In one embodiment, a beverage comprises from about 300 ppm to about 350 ppm Reb A and from about 10 ppm to about 50 ppm Myd (or variant thereof). In preferred embodiments, the beverage comprising Reb A and Myd (or variant thereof) tastes better and/or has positive synergy compared to a Reb A-sweetened beverage, wherein the beverage comprising Reb A and Myd (or variant thereof) and beverage sweetened with Reb A have the same sucrose equivalence.

[0446] In one embodiment, a beverage comprises from about 30 ppm to about 50 ppm Myd (or variant thereof), from about 300 ppm to about 450 ppm siamenoside I, and optionally from about 50 ppm to about 100 ppm RebM80. In preferred embodiments, the beverage tastes similar or better than either a siamenoside I-sweetened control or a RebM80-sweetened control, wherein the beverage of the present invention and the siamenoside I- and/or RebM80-sweetened control beverages have the same sucrose equivalence. In a particular embodiment, a beverage comprises from about 40 ppm to about 50 ppm Myd (or variant thereof) and from about 350 ppm to about 450 ppm siamenoside I, with no RebM80. In a more particular embodiment, the beverage comprises from about 40 ppm to about 45

ppm Myd (or variant thereof) and from about 350 ppm to about 450 ppm siamenoside I, with no RebM80. In a still more particular embodiment, the beverage comprises about 45 ppm Myd (or variant thereof) and about 410 ppm siamenoside I, with no RebM80. In another embodiment, a beverage comprises from about 25 to about 30 ppm Myd (or variant thereof), from about 300 ppm to about 400 ppm siamenoside I and from about 50 ppm to about 100 ppm RebM80. In a more particular embodiment, a beverage comprises about 30 ppm Myd (or variant thereof), about 350 ppm siamenoside I and about 70 ppm RebM80.

[0447] In one embodiment, a beverage comprises from about 30 to about 50 ppm Myd (or variant thereof) and from about 7 Brix to about 10 Brix high fructose corn syrup (HFCS). In preferred embodiments, the beverage of the present invention tastes similar or better than a HFCS-sweetened control (10 Brix). In a more particular embodiment, the beverage comprises from about 35 ppm to about 45 ppm Myd (or variant thereof) and about 7 Brix to about 9 Brix HFCS. In a still more particular embodiment, the beverage comprises about 40 ppm Myd (or variant thereof) and about 8 Brix HFCS.

[0448] In one embodiment, a reduced calorie beverage comprises from about 5 ppm to about 20 ppm Myd (or variant thereof) and from about 100 ppm to about 200 ppm RebM. In preferred embodiments, the beverage of the present invention has a taste profile similar to a sucrose-sweetened control (10 Brix). In a particular embodiment, a reduced calorie still (non-carbonated) beverage comprises from about 10 ppm to about 15 ppm Myd (or variant thereof) and from about 100 ppm to about 150 ppm RebM. In a still more particular embodiment, a reduced calorie still beverage comprises from about 10 ppm to about 15 ppm Myd (or variant thereof) and about 115 ppm RebM. In another particular embodiment, a reduced calorie carbonated beverage comprises about 20 ppm Myd (or variant thereof) and from about 100 to about 150 ppm RebM.

[0449] In one embodiment, a beverage comprises from about 5 ppm to about 20 ppm Myd (or variant thereof) and from about 300 ppm to about 500 ppm A95. In a particular embodiment, the beverage comprises from about 15 ppm to about 20 ppm Myd (or variant thereof) and about 300 ppm to about 500 ppm A95. In a further embodiment, the beverage comprises about 15 ppm or about 20 ppm Myd (or variant thereof) and about 400 ppm A95.

[0450] In one embodiment, the temporal profile, flavor profile and/or taste profile of the beverage is improved relative to a beverage that either does not contain Myd (or variant thereof) or contains Myd (or variant thereof) in an amount less than from about 20 ppm, even more particularly, less than about 15 ppm. In a particular embodiment, the temporal profile, flavor profile and/or taste profile of the beverage is more sugar-like. In one embodiment, the beverage has reduced sweetness

linger, reduced bitterness, reduced bitter aftertaste, reduced astringency, improved mouthfeel (e.g., greater fullness or body) or the like.

[0451] The beverages can further include additives including, but are not limited to, carbohydrates, polyols, amino acids and their corresponding salts, poly-amino acids and their corresponding salts, sugar acids and their corresponding salts, nucleotides, organic acids, inorganic acids, organic salts including organic acid salts and organic base salts, inorganic salts, bitter compounds, flavorants and flavoring ingredients, astringent compounds, proteins or protein hydrolysates, surfactants, emulsifiers, flavonoids, alcohols, polymers and combinations thereof. Any suitable additive described herein can be used.

[0452] The beverage can further contain one or more functional ingredients, detailed above. Functional ingredients include, but are not limited to, vitamins, minerals, antioxidants, preservatives, glucosamine, polyphenols and combinations thereof. Any suitable functional ingredient described herein can be used.

[0453] It is contemplated that the pH of the sweetened composition, such as, for example, a beverage, does not materially or adversely affect the taste of the sweetener. A non-limiting example of the pH range of the sweetenable composition may be from about 1.8 to about 8. A further example includes a pH range from about 2 to about 5. In a particular embodiment, the pH of a beverage is from about 3 to about 3.25. In a particular embodiment, the pH of a beverage is from about 2.5 to about 3.0.

[0454] The temperature of a beverage comprising Myd (or variant thereof) may, for example, range from about 4° C. to about 100° C., such as, for example, from about 4° C. to about 25° C.

[0455] The beverage can be a high-calorie beverage that has about 120 calories per 8 oz serving.

[0456] The beverage can be a mid-calorie beverage that has about 80 calories per 8 oz serving.

[0457] The beverage can be a low-calorie beverage that has less than 40 calories per 8 oz serving.

[0458] The beverage can be a zero-calorie that has less than 5 calories per 8 oz. serving.

[0459] In one embodiment, the beverage or beverage product has a low glycemic index. The glycemic index is a value assigned to foods based on how slowly or how quickly those foods cause increases in blood glucose level. Low glycemic index diets (including but not limited to beverages) are intended to achieve a more beneficial effect on blood glucose control in people with diabetes mellitus and may also provide metabolic benefits for the general population.

[0460] In a particular embodiment, the beverage or beverage product has a glycemic index that is at least 10% lower than the glycemic index of a substantially similar product made using conventional sweeteners (e.g., sucrose-based sweeteners). Methods for testing the glycemic index of a beverage

have been described, e.g., as provided in Wolever, et al. *Nutrition Research* 23:621-629, 2003. In a particular embodiment, the beverage or beverage product has a glycemic index (GI) of about 55 or less. In a particular embodiment, the beverage or beverage product is a nutritional beverage with a low glycemic index.

[0461] In one embodiment, a beverage is disclosed that comprises between about 1 and about 30 ppm and less than about 500 ppm of one or more steviol glycosides, wherein the liquid matrix of the beverage is selected from the group consisting of water, phosphoric acid, phosphate buffer, citric acid, citrate buffer, carbon-treated water and combinations thereof. The pH of the beverage can be from about 3 to about 3.5 or from about 2.5 to about 3. The beverage can further include additives, such as, for example, erythritol. The beverage can further include functional ingredients, such as, for example vitamins.

[0462] METHODS FOR IMPROVING TEMPORAL OR FLAVOR PROFILE

[0463] A method is disclosed for imparting a more sugar-like temporal profile, flavor profile and/or taste profile to a consumable (e.g. a beverage), comprising adding Myd (HTS) (or variant thereof) or the Myd-containing sweetener compositions or flavor modifying compositions disclosed herein to the consumable. The consumable may be referred to as a sweetenable composition or a flavor modifiable composition.

[0464] Without being bound by any particular theory, it is believed that the HTS may bind to mucins in the oral cavity. Mucins are the principal organic constituents of mucus, the slimy visco-elastic material that coats all mucosal surfaces. Structurally, mucins are high-molecular weight epithelial glycoproteins with a high content of clustered oligosaccharides O-glycosidically linked to tandem repeat peptides rich in threonine, serine, and proline.

[0465] Within the mouth, mucins coat the hard and soft tissues. Several salivary mucins are known, including MUC5B, MUC7 (previously known as MG2), MUC19, MUC1, and MUC4. Based on macromolecular characteristics, they can be classified into high (>1000 kD) and low (200-300 kD) molecular weight forms. Salivary mucins are synthesized by the mucus acinar cells of the paired submandibular (SMG) and sublingual (SLG) glands, as well as minor salivary glands distributed throughout the palatal and buccal mucosa.

[0466] The method can further include the addition of other sweeteners, additives, functional ingredients and combinations thereof. Any sweetener, additive or functional ingredient detailed herein can be used.

[0467] In one embodiment, the method of imparting a more sugar-like temporal profile, flavor profile to a consumable (e.g., a beverage) comprises adding Myd (or variant thereof) or the Myd-containing

sweetener composition or flavor modifying composition to the consumable, thereby imparting a more sugar-like temporal profile or flavor profile.

[0468] As used herein, the “sugar-like” characteristics include any characteristic similar to that of sucrose.

[0469] A sweetener is generally a compound or mixture of compounds which taste sweet, particularly to a human. It will be appreciated that the sweetness or other characteristic of a sweetener perceived from a given sweetener may vary by the individual tasting the sweetener. Natural sweeteners generally include sugars, particularly sucrose, fructose, galactose, glucose, lactose, maltose and mixtures thereof. Non-sugar sweeteners include polyols such as erythritol, isomalt, lactitol, maltitol, sorbitol and xylitol. Some sweeteners are lower in calories than sucrose and others are described as non-caloric. Artificial sweeteners include, among others, acesulfame potassium (Ace-K), Advantame, Aspartame, Neotame, Saccharin and Sucralose. There is also a group of plant-derived non caloric or low caloric sweeteners including allulose, Monk fruit, Stevia and Tagatose. In general, each sweetener or mixture of sweeteners will exhibit different levels of sweetness and different taste and flavor characteristics.

[0470] Characteristics inherent to sweeteners include maximal response, flavor profile (including taste), temporal profile, mouthfeel, dose response, taste/flavor interactions, and temperature effects. As used herein, “maximal response” refers to peak sweetness intensity. As used herein, “flavor profile” refers to the combination of aromatic and basic taste elicited by a substance. As used herein, “temporal profile” refers to how the flavor profile changes throughout the perception of a substance over time. As used herein, “mouthfeel” refers to sensations perceived in the mouth that are elicited by the chemical or physical properties of a substance. As used herein, “dose response” refers to the function of concentration of the substance versus the intensity of the measured flavor or taste, e.g., sweetness.

[0471] In some instances, the perception of a sweetener may be affected by adaptation. “Adaptation” refers to the decrease in perceived intensity of a substance over a prolonged period of exposure.

[0472] These characteristics are dimensions in which the taste of sucrose is different from the tastes of Myd (or variant thereof). Of these, however, the flavor profile and temporal profile are particularly important. In a single tasting of a sweet food or beverage, differences (1) in the attributes that constitute a sweetener’s flavor profile and (2) in the rates of sweetness onset and dissipation, which constitute a sweetener’s temporal profile, between those observed for sucrose and for Myd (or variant thereof) can be noted.

[0473] In one embodiment, the method disclosed herein improves one or more organoleptic property of the consumable compared to a consumable that does not contain the sweetener composition or flavor modifying composition disclosed herein. In certain embodiments, the improvement is measured by a

sensory test. The sensory test may be a taste test, a blind test, or a combination thereof. A sensory test can use one or more various protocols. For example, a sensory test can be the “triangle method”, follow ISO requirements, or a combination thereof. The taste test can be the average of multiple trials.

[0474] A taste test may be a screening test, a professional taste test, or a market research test. A screening test may be performed by at least 1, 2, 3, 4, 5, 6, 7, 8, or 9 taste testers. A professional taste test may be performed by at least 10, 15, 20, 25, or 30 taste testers. A market research test may be performed by at least 31, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, 400, or 500 taste testers. In some cases, a taste tester can be a person with average taste perception, a professional taste tester, a person who has passed a tasting exam by correctly identifying foods or food components, or a person who can identify the relative amounts of a taste or flavor (e.g., correctly sequence varying amounts of sugar in water).

[0475] In one embodiment, whether or not a characteristic is more sugar-like is determined by an expert sensory panel who taste compositions comprising sugar and compositions comprising HTS (or variant thereof), optionally in combination with at least one steviol (e.g., Reb M, Reb A) or HFCS, both with and without additives and provide their impression as to the similarities of the characteristics of the sweetener compositions, both with and without additives, with those comprising sugar. A suitable procedure for determining whether a composition has a more sugar-like taste is described in embodiments described herein below.

[0476] In a particular embodiment, a panel of assessors is used to measure the reduction of sweetness linger. Briefly described, a panel of assessors (generally 8 to 12 individuals) is trained to evaluate sweetness perception and measure sweetness at several time points from when the sample is initially taken into the mouth until 3 minutes after it has been expectorated. Using statistical analysis, the results are compared between samples containing additives and samples that do not contain additives. A decrease in score for a time point measured after the sample has cleared the mouth indicates there has been a reduction in sweetness perception.

[0477] The panel of assessors may be trained using procedures well known to those of ordinary skill in the art. In a particular embodiment, the panel of assessors may be trained using the Spectrum™ Descriptive Analysis Method (Melgaard et al, *Sensory Evaluation Techniques*, 3rd edition, Chapter 11). Desirably, the focus of training should be the recognition of and the measure of the basic tastes; specifically, sweet. In order to ensure accuracy and reproducibility of results, each assessor should repeat the measure of the reduction of sweetness linger about three to about five times per sample, taking at least a five-minute break between each repetition and/or sample and rinsing well with water to clear the mouth.

[0478] Generally, the method of measuring sweetness comprises taking a 10 mL sample into the mouth, holding the sample in the mouth for 5 seconds and gently swirling the sample in the mouth, rating the sweetness intensity perceived at 5 seconds, expectorating the sample (without swallowing following expectorating the sample), rinsing with one mouthful of water (e.g., vigorously moving water in mouth as if with mouth wash) and expectorating the rinse water, rating the sweetness intensity perceived immediately upon expectorating the rinse water, waiting 45 seconds and, while waiting those 45 seconds, identifying the time of maximum perceived sweetness intensity and rating the sweetness intensity at that time (moving the mouth normally and swallowing as needed), rating the sweetness intensity after another 10 seconds, rating the sweetness intensity after another 60 seconds (cumulative 120 seconds after rinse), and rating the sweetness intensity after still another 60 seconds (cumulative 180 seconds after rinse). Between samples take a 5-minute break, rinsing well with water to clear the mouth.

[0479] In some embodiments, the temporal profile, flavor profile and/or taste profile is evaluated *in vitro*, e.g., in a suitable assay such an *in vitro* system based on a cellular model overexpressing sweet and/or bitter receptor. In a particular embodiment, the *in vitro* system comprises TAS1R2/TAS1R3 receptors.

[0480] In one embodiment, the sweetener composition or flavor and/or taste modifying composition disclosed herein is capable of modifying one or more properties of the consumable including but not limited to mouthfeel, bitterness, bitter aftertaste or sweetness linger.

[0481] Mouthfeel refers to the textural aspects of a food or beverage responsible for producing characteristic tactile sensations perceived at the lining of the mouth, including the tongue, gums and teeth. These may include, but are not limited to, astringency, viscosity, slipperiness and mouth-coating. Mouthfeel is a fundamental sensory attribute which, along with taste and smell, determines the overall flavor of a consumable.

[0482] In one embodiment, Myd (or variant thereof) or the Myd-containing sweetener composition and/or flavor modifying composition disclosed herein imparts an improved mouthfeel to a consumable (e.g., beverage) to which it is added relative to a conventional sweetener composition or flavor and/or taste modifying composition. In one embodiment, the “improved mouthfeel” can be determined by a taste panel consuming said beverage in comparison to the same beverage without the taste modifying composition or an active component thereof. In a particular embodiment, the mouthfeel is improved by about 5%, about 10%, about 15%, about 20% or about 25% or more. In a particular embodiment the improvement in mouthfeel is characterized as increased fullness, body or richness. In a particular embodiment, the improved mouthfeel is characterized as a more syrup-y feel.

[0483] In another embodiment, the sweetener composition or flavor modifying composition disclosed herein improves (reduces) bitterness in a consumable (e.g., a beverage) to which it is added, relative to a conventional sweetener composition or flavor and/or taste modifying composition. This “improved bitterness” or “reduced bitterness” can be determined by a taste panel consuming said consumable in comparison to the same consumable without the taste modifying composition or an active component thereof. In a particular embodiment, the bitterness is reduced by about 5%, about 10%, about 15%, about 20% or about 25% or more.

[0484] In another embodiment, the sweetener composition or flavor modifying composition disclosed herein reduces the bitter aftertaste in a consumable (e.g., a beverage) to which it is added, relative to a conventional sweetener composition or flavor and/or taste modifying composition. This “reduced bitter aftertaste” can be determined by a taste panel consuming said consumable in comparison to the same consumable without the taste modifying composition or an active component thereof. In a particular embodiment, the bitter aftertaste is reduced by about 5%, about 10%, about 15%, about 20% or about 25% or more.

[0485] In a particular embodiment, the sweetener composition or flavor modifying composition disclosed herein reduces the bitter taste of a consumable (e.g., a beverage) to which it is added where the reduction is measured by the Metachronic VAS Score Profile for Bitterness and more particularly, the reduction is at least about 0.5 points, about 1 point, about 1.5 points, about 2.0 points, about 2.5 points, about 3.0 points, about 3.5 points or about 4.0 points or more. In certain embodiments, the reduction is between about 0.5 and about 4.0 points, about 1.0 and about 3.5 points or about 1.5 and about 3.0 points.

[0486] In a further embodiment, the sweetener composition and/or flavor modifying composition disclosed herein improves (reduces) sweetness linger of a consumable (e.g., a beverage) to which it is added. In one embodiment, this “improved sweetness linger” can be examined by a taste panel consuming said beverage in comparison to the same beverage without the taste modifying composition or an active component thereof. In a particular embodiment, the sweetness linger is reduced by about 5%, about 10% about 15%, about 20% or about 25% or more.

[0487] In a further embodiment, a flavor modifying composition disclosed herein provides a reduction in sourness of a consumable (e.g., a beverage) to which it is added. In one embodiment, this “reduced sourness” can be examined by a taste panel consuming said consumable in comparison to the same consumable without the taste modifying composition or an active component thereof. In a particular embodiment, the sourness is reduced by about 5%, about 10% about 15%, about 20% or about 25% or more.

[0488] Additional details relating to HTS and HTS variants are found in the Table 31 which includes a list of amino acid and nucleic acid sequences.

[0489] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLES

[0490] EXAMPLE 1

[0491] Fresh *Mattirolomyces terfezioides* truffles were obtained *in situ* using appropriate procedures and permissions in their natural range. Fresh samples (29 in total) were shipped to MycoTechnology, Inc. facilities and were gently washed in RO water, then frozen in liquid nitrogen and stored at -80° C. The average moisture content of the truffles was 83.6% plus or minus 4.6 %.

[0492] Aqueous extraction of the truffles was performed as follows. Eight different samples of truffle were pestled in liquid nitrogen to grind into a powder, then 5:1 v/w truffle of 4° C water was added and allowed to incubate at 30 minutes at 4°C. The extracted material was then subjected to low-speed brief centrifugation and the filtrate was tasted “neat.” The sweetness intensity was rated between 0 for no sweetness and 10 for extremely sweet. Of the samples, the sweetness was rated as follows:

[0493] Table 1. Sweetness of various truffle samples.

sample	Sweetness intensity	Notes
1	5	Sweet taste at end
2	8	Sweet taste is upfront and intensifies at mid-end and lingers
3	7	Sweetness is upfront and intensifies at mid-end and lingers.
4	5	Sweet upfront, low sweet linger
5	4	Sweetness less strong
6	3	Low sweetness
7	6	A mild and clean sweet taste

[0494] Aqueous extract was stored at 4°C at pH 7 and pH 2 in sodium phosphate buffer, and little to no change in sweetness was observed over an 8-day period.

[0495] EXAMPLE 2

[0496] Purification of sweet protein Myd from *M. terfezoides*. Fresh *Mattirolomyces terfezioides* truffles were obtained *in situ* using appropriate procedures and permissions in their natural range and stored at -80°C. A 16.3 g sample was removed from the freezer and was ground with a mortar and pestle (white ceramic) in liquid nitrogen. Grinding proceeded for 15 minutes to fully pulverize tissue and obtain a fine frozen powder. Powder was added to 50 mL Falcon tubes and 20 mL RO-H₂O was

added, and vortexed to mix tissue, until no ice crystals were observed. A Rotor-Stator at a setting of 20 for 2 X 1 minutes at 4°C was used to breakup fragment and make a homogenous solution (H1). The volume of slurry was adjusted to 53 mL with RO-H₂O and centrifuged at 7500 x G for 30 minutes at 4°C. The supernatant (S1) from this step was collected into 2 mL Eppendorf tubes and centrifuged in a 5417 R and Eppendorf centrifuge at 20,000 x G for 15 minutes at 4°C. The pellet (P1) was discarded. The sweet taste (via human sensory) was perceived in the supernatant. Supernatant from this step was collected and pooled. The supernatant was then filtered through a 0.45 micron syringe filter (Cellulose, VWR International), 25 mm, and called S1 + 0.22 um Filtration. The filtrate was then washed with hexane (38 mL to 50 mL hexane 2 times, and the water phase was collected. S1FH is S1F + hexane wash. The hexane phase was saved and dried. The water phase was then precipitated with acetone (50 mL at -20°C was added to 33 ml of fraction S1FH and set for 30 minutes at -20°C). The sample was centrifuged at 3,000 x g and the precipitate was collected. The precipitate was resuspended in 10 mM sodium phosphate pH 6, with the supernatant from this step called S2, and the precipitate called P2. The supernatant S2 was first applied to an AMICON centrifugal filter units with a molecular weight cutoff of 100 kD, obtaining a filtrate (flow-through) portion (called 100F) and a retentate (called 100R); followed by applying the 100F to a unit with a molecular weight cutoff of 30 kD, resulting in a filtrate (30F) and a retentate (30R). The sweet taste fraction flowed through the 100 kD column and appeared in 100F, and was retained on the 30 kD column (30R). A band was observed at approximately 13 kD on the SDS-PAGE of Figure 1, shown by the arrow, and this band was cut out and subjected to N-terminal sequence analysis by Edman degradation using standard methods of detection e.g. liquid chromatography and mass spectrometry to identify the residues for each cycle.

[0497] EXAMPLE 3 (Identification of RNA)

[0498] Sample Collection

[0499] Fresh *Mattirolomyces terfezioides* truffles were obtained *in situ* using appropriate procedures and permissions in their natural range. A wild isolate (BDP2_18) of *Mattirolomyces terfezioides* truffle (gleba) was sourced from a natural environment. BDP2_18 was the largest wild truffle scavenged and the truffle had a sweetness characteristic of “sweet upfront, more fungal and earthy, low sweet linger.”

[0500] Sample Identification

[0501] The wild isolates of gleba were shipped frozen for GeneWiz® sequencing (Azenta US Inc.) Sequencing for Internal Transcribed Spacer (ITS) Sequencing. The genome loci sequenced are the ITS 1 & 2 region. The resulting Sanger sequencing reads were then aligned and trimmed of low-quality bases. Each sequence was then subjected to an individual Basic Local Alignment Search Tool (BLAST) (Altschul, Gish, Miller, Myers, & Lipman, 1990) search to verify identity. BLASTn search was employed using nucleotide collection (nr/nt) with unpublished samples sequences excluded. The entry with the highest percent identity to the wild isolate is *Mattirolomyces terfezioides* strain rib02.

[0502] Sample Preparation

[0503] The wild isolate, BPD2_18, was superficially washed with sterile water and cut into cubes weighing ~ 100 mg and flash frozen in liquid N₂ to be stored at -80°C. Shipment was carried out on dry ice for GeneWiz® (Azenta US Inc) sequencing.

[0504] RNA Sequencing

[0505] The following samples were submitted through GeneWiz® sequencing for Standard RNASeq going through Illumina HiSeq, 2x150 bp, single index, per lane with ~350M raw paired-end reads per lane. This RNASeq study involved polyA⁺ selection for transcriptome profiling.

[0506] The GeneWiz® sequencing procedure extracted RNA using the RNeasy® Plus (Qiagen GmbH) Universal mini kit following manufacturer's instructions. RNA Library prep was done using the NEBNext® Ultra (New England Biolabs, Inc.) RNA Library Prep Kit for Illumina. The Illumina adapter sequences are outlined below.

[0507] 5'-AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACG

CTCTTCCGATCT-3' (SEQ ID NO:9)

[0508] 5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC[i7

barcode]ATCTCGTATGCCGTCTTCTGCTTG-3' (SEQ ID NO:10)

[0509] Fastq sequence files from the RNA-seq study on two strains of *Mattirolomyces terfezioides* (three replicates each) were trimmed and cleaned with HTStream to remove the following contaminants: PhiX (common contaminant from sequencing), rRNA reads, sequencing adapters, low quality and "N" bases, poly-a tracts, primers and reads < 50bp. Transcriptome assembler rnaSPAdes (Bushmanova E, Antipov D, Lapidus A, Prjibelski AD. rnaSPAdes: a de novo transcriptome assembler and its application to RNA-Seq data. Gigascience.(2019)8(9):giz100. doi:10.1093/gigascience/giz100) was then used for the de novo assembly of the transcriptome for each strain of *M. terfezioides* from the cleaned files. Bandage (a Bioinformatics Application for Navigating De novo Assembly Graphs Easily) (Ryan R. Wick, Mark B. Schultz, Justin Zobel, Kathryn E. Holt, Bandage: interactive

visualization of de novo genome assemblies, Bioinformatics, Volume 31, Issue 20, 15 October 2015, Pages 3350–3352) was used to visualize and search the assembly for the target peptide using a tBLASTn search (Gertz EM, Yu YK, Agarwala R, Schäffer AA, Altschul SF. Composition-based statistics and translated nucleotide searches: improving the TBLASTN module of BLAST. BMC Biol. 2006;4:41. Published 2006 Dec 7. doi:10.1186/1741-7007-4-41) that compares the protein query sequence against the assembled transcriptome sequence database dynamically translated in all six reading frames. Contigs that contained perfect matches to the query protein sequence were analyzed for open reading frames (ORFs) and eventually used to construct the full-length mRNA connected to the target peptide.

[0510] An RNA transcript was identified, and the DNA copy thereof has the sequence shown as SEQ ID NO:1, of which SEQ ID NO:2 is the predicted coding sequence based on the start and stop codons. A predicted protein, SEQ ID NO:3, is also provided, having 121 amino acids with an estimated size of 13.3 kD. Length: 122 aa, molecular weight: 13.381 kD, predicted isoelectric point: 8.64, and predicted charge at pH 7: 1.01.

[0511] Blast analyses. Identity between the predicted protein SEQ ID NO:3, and other protein sequences in GENBANK was 31% or less. A hypothetical protein from *Pisolithus tinctorius* was found (GenBank: KIN98154.1) with approximately 31% homology to SEQ ID NO:3; called hypothetical protein M404DRAFT_1005519 [*Pisolithus tinctorius* Marx 270]. It is hypothesized that this protein may also have sweet modulating activity. The complete cDNA copy of the *Pisolithus tinctorius* RNA transcript is identified in international patent application PCT/US2022/82443 (see also PCT/US2020/012955 and PCT/US2021/039176).

[0512] EXAMPLE 4 (Cloning and heterologous expression of mycodulcein HTS in *E. coli*; confirmation of sweet taste.)

[0513] Based on the nucleotide sequence identified as encoding SEQ ID NO:3, three expression vectors to express the nucleotide coding sequence SEQ ID NO:4 also containing sequence encoding a histidine tag were synthesized and cloned by Atum, Inc. (Newark, CA) into three different Atum vector backbones: pD454-SR (plasmid pMy_3000), pD454-MR (plasmid pMy_3001), and pD454-WR (plasmid pMy_3002), all of which are *E. coli* isopropyl beta-D-1-thiogalactopyranoside (IPTG)-inducible T7 promoter expression vectors with ampicillin-r, lacI, Lac01, Ori_pUC, and medium (M), strong (S) and weak (W) ribosome binding sites on the plasmid. *E. coli* BL21 DE3 (Studier et al. (1986) J. Mol. Biol. 189:113-130) (New England Biolabs) was transformed with pMy_3000, pMy_3001, pMy_3002 using manufacturer protocols. In short, previously frozen competent cells were thawed and mixed with 1 pg -100 ng of plasmid DNA and held on ice for 30 minutes. A heat shock of 42°C for 45 seconds was applied to this mixture. Immediately thereafter, the mixture was placed into

an ice bath for 10 minutes. 950 μ L of pre-warmed LB media was added and then subjected to 1 hour of 225 rpm shaking at 37°C. Dilution of cells at 1:10 and 1:100 and plating 100 μ L on the antibiotic plate was performed for each transformation reaction. An overnight growth at 37°C allowed colonies to fully recover and become visible. To confirm successful transformation, csPCR (colony screen PCR) was performed to interrogate the cDNA region of the plasmid and expression was confirmed through lysate SDS-PAGE. Shake flask-scale induced expression was used to confirm heterologous expression in the *E. coli* host. This process yielded strains Z14CE, Z15CE, Z16CE containing the plasmids pMy_3000, pMy_3001, and pMy_3002 respectively. The three strains (Z14CE, Z15CE, Z16CE) were maintained on LB + ampicillin 100 μ g/mL agar plates while the negative control (Eco_0001) was maintained on a LB agar plate. An overnight culture was grown for each strain in 50 mL of LB liquid media in un-baffled 250 mL culture shake flasks at 37°C, shaking at 150 rpm overnight with appropriate antibiotics. Each overnight culture was the following day seeded into 200 mL of TB liquid media in baffled 1000 mL culture shake flasks at 37°C, shaking at 200 rpm, and adjusted to 0.1 OD₆₀₀ with the appropriate antibiotic. Once the OD₆₀₀ reached 0.8, IPTG was added into the media to a final concentration of 0.66 mM. Shaking was continued at 37°C for an additional 5 hrs. After expression, the cells were centrifuged at 4000 g for 20 min. The supernatant was discarded, and the cell pellet was suspended in ~20 mL of wash buffer (10 mM Sodium Phosphate Buffer pH 7.0). Suspended cells were disrupted in a high-pressure homogenizer (C3 Emulsiflex, Avestin, Inc., Ottawa, ON, Canada) operated at a max of 2,000 bar. Disrupted cells were centrifuged at 13,000 g (30 min), the supernatant was collected, and the pellet was discarded. The supernatant containing solubilized protein was filtered through a 0.22 μ m PES membrane unit (Millipore, Burlington, MA, USA). SDS PAGE was run and a 13.1 kD band (Coomassie stain) was observed to confirm expression.

[0514] Thermo Scientific™ HisPur™ Ni-NTA resin was used to purify the his-tagged protein expressed from SEQ ID NO:4 using effective immobilized metal affinity chromatography (IMAC). The protein encoded by SEQ ID NO:4 was purified using nickel-charged nitrilotriacetic acid (NTA) chelate immobilized onto 6% crosslinked agarose resin. Lysate was loaded onto a prepared IMAC column, equilibrating with Binding Buffer: 20mM sodium phosphate monobasic, 0.5M sodium chloride, 0.1M imidazole, pH 7.4, and eluted using Elution Buffer: 20mM sodium phosphate monobasic, 0.5M sodium chloride, 0.5M imidazole. The column was washed three times with Binding Buffer followed by eluting his-tagged mycodulcein four times with elution buffer, followed by ultrafiltration of eluted fractions using a 30 kD MWCO filter followed by filtering filtrate through 10 kD filters at 4000x G for 15 minutes. The retentate was diluted and spun again for a wash step, which was repeated three times. Purification steps analysis by SDS-PAGE is shown in Figure 2.

[0515] The purified fraction (shown in Lane 8 from Figure 2, containing highly purified SEQ ID NO:5) was tasted at 0.03 mg/ml by a trained sensory scientist (0.2 mL aliquot) and found to have a sweetness equivalent to 8° brix (approximately 8% sucrose solution), confirming that mycodulcein (HTS) isolated from *M. terfezioides* was responsible for the sweet taste activity observed in Examples 1 and 2. The sweet taste was very noticeably sweet, with a “clean” sweetness (sugar-like taste) with no other flavors, with a slightly delayed onset and a sweet aftertaste.

[0516] EXAMPLE 5 (Pilot-Scale Production of Mycodulcein (His-Tag))

[0517] *E. coli* strain Z14CE prepared in Example 4 containing nucleotide coding sequence SEQ ID NO:4 was tested for its performance during fermentation in a lab-scale bioreactor. Bioreactor cultures were performed in 10.0 L Bioflo 320 round bottomed stirred fermentor (BioFlo / CelliGen 310, New Brunswick Scientific, Edison, NJ, USA). The fermenter was fitted with pH and dissolved oxygen sensors (Mettler Toledo, OH, USA). Temperature was controlled via a water-filled stainless-steel base. Agitation was provided by two mounted six-bladed Rushton turbines spaced 47 mm apart with the lowermost impeller positioned just above the bottom of the shaft. Aeration occurred through a perforated pipe sparger ring. Dissolved oxygen (DO) was controlled at 20% of air saturation by using a sequential cascade of agitation between 500 and 800 rpm and aeration between 5 and 8 L/min with air sparged at high-cell densities. The pH was controlled at 7.0 using 5.0 M ammonium hydroxide. Antifoam 204 (Sigma, St Louis, MO, USA) was added automatically to control the foaming. The latter was sensed using a conductivity probe mounted 10 cm above the culture level. The main fermentation medium contained (per liter) 24 g yeast extract, 12 g tryptone, 5.42 mL glycerol, 100 mL of phosphate buffer stock (0.17 M KH_2PO_4 , 0.72 M K_2HPO_4) The medium was adjusted to pH 7.0 using 2 M HCl. When the original glucose supply was depleted (signaled by a rise in pH), a feed medium (per liter) consisting of 200 g glucose, 21.1 g $(\text{NH}_4)_2\text{SO}_4$ and 19.7 g MgSO_4 was pumped into the fermenter at an initial flow rate of 1.00 mL/min. Unless otherwise stated, the initial volume of the medium in the vessel was 4.0 L. Inoculum (200 mL) consisted of a culture that had been grown for 16 h in a 1 L baffled shake flask (37°C, 200 rpm) in LB started culture media. The fermenter temperature was 37°C. Fermentations were induced with 0.66 mM IPTG after optical density reached 10-20 and continued thereafter for 24 hours. The broth was subsequently centrifuged at 4000 g for 20 min after the 24-hour induction period. The supernatant was discarded, and pellet was suspended in 1 L of wash buffer (10 mM sodium phosphate buffer pH 7.0). Suspended cells were disrupted in a high-pressure homogenizer (C3 Emulsiflex, Avestin, Inc., Ottawa, ON, Canada) operated up to a max of 1,500 bar. Disrupted cells were centrifuged at 13,000 g (30 min), the supernatant was collected, and the pellet was discarded. The supernatant containing solubilized protein was filtered through a 0.22 μm PES membrane unit (Millipore, Burlington, MA, USA).

[0518] The purified supernatant prepared in this Example was tasted at 0.03 mg/mL by a trained sensory scientist (0.2 mL aliquot) and found to have a sweetness equivalent to 8 brix (approximately 8% sucrose solution). The sweet taste was very noticeably sweet, with a “clean” sweetness (sugar-like taste) with no other flavors, with a slightly delayed onset and a sweet aftertaste.

[0519] Supernatant was stored in aliquots at -20 °C and used for further experiments.

[0520] EXAMPLE 6 (Mycodulcein (HTS) from *E. coli* ELISA Quantification)

[0521] A direct ELISA was developed to quantify his-tagged mycodulcein (HTS) (SEQ ID NO:5) with a horseradish peroxidase (HRP) conjugated antibody to the 6XHis-Tag sequence on the carboxy terminus of SEQ ID NO:5. The ELISA enables the measurement of mycodulcein (HTS) concentration in complex lysates and purified protein. Recombinant 6XHis-tagged *E. coli* mycodulcein has a molecular weight of 14.2 kD and a molar extinction coefficient of $27,960 \text{ M}^{-1} \text{ cm}^{-1}$, calculated from the amino acid sequence by methods known in the art. Purity was assessed by SDS-PAGE as $\geq 98\%$. Mycodulcein (HTS) protein concentration was determined by absorbance at 280nm using Beer-Lambert's Law, then a standard curve was generated using known concentrations of mycodulcein ($\mu\text{g/ml}$) for the ELISA.

[0522] ELISA procedure: Protein was bound to the walls of a high-protein binding 96-well plate in 50 mM carbonate buffer pH 9.4 coating buffer for 30 minutes at room temperature. The plates were washed 3 times with phosphate buffered saline (PBS) pH 7.4 with 0.02% Tween-20. Nonspecific binding sites on the microplate were blocked with 5% BSA in PBS pH 7.4 for 15 minutes at room temperature and washed three times with PBS 0.02% Tween-20. The primary antibody was diluted (1:1000) in blocking buffer and the microplates were incubated for 1 hour and washed 3 times in PBS 0.02% Tween-20. The colorimetric substrate, 3,3',5,5'-tetramethylbenzidine (TMB), was used to develop the HRP reaction for 8 minutes and stopped with 2N sulfuric Acid.

[0523] EXAMPLE 7 (Quantitative Characterization of The Concentration-Dependence Of Mycodulcein HTS)

[0524] Opertech Bio (Philadelphia, PA) performed a quantitative characterization of the concentration-dependence of the taste properties of the purified his-tagged mycodulcein HTS (SEQ ID NO:141 with His-tag, SEQ ID NO:142) obtained from *E. coli* as described in Example 5 and purified as described in Example 4. The sweet-taste potency and relative efficacy of mycodulcein (HTS) was compared with sucrose and with other sweeteners thaumatin, rebaudioside A, and aspartame. Control standards were solutions of 200 mM sucrose, 100 mM NaCl, 0.5 mM quinine, and 10 mM citric acid. Figure 3 shows the concentration-response functions for sweet taste for mycodulcein (HTS), aspartame, thaumatin,

rebaudioside A. Data are plotted as the proportion (p) of responses occurring on the 200 mM sucrose-associated (“sweet”) target. Each data point in the curves for mycodulcein, aspartame, thaumatin, and rebaudioside A was calculated as the average across 32 replicates and averaged across 16 replicates for the sucrose curve; error bars are SEM. Points for water and sucrose controls were similarly calculated as the average across 128 and 64 replicates, respectively. Curves were fit by non-linear regression.

[0525] The concentrations that elicit half-maximal sweet taste response (EC50s, or potencies) were derived from the nonlinear regression. The EC50s (and 95% confidence intervals) for mycodulcein (MYC) also called HTS, sucrose (SUC), aspartame (ASP), rebaudioside A (REB), and thaumatin (THN) are given in Table 2. Also provided are the equivalencies to sucrose on a molar basis and on a weight basis.

[0526] Table 2.

	<u>EC50 (molar units)</u>	<u>CI 95%</u>	<u>EC50 (mg/ml)</u>	<u>Relative to Sucrose (mM)</u>	<u>Relative to Sucrose (mg/ml)</u>
MYC	2 μ M	2 - 3 μ M	0.0286	18000	431
THN	0.3 μ M	0.2 - 0.3 μ M	0.0066	120000	1865
REB	42 μ M	33 - 57 μ M	0.0411	857	300
ASP	182 μ M	142 - 254 μ M	0.0532	198	231
SUC	36 mM	27 - 45 mM	12.3120	1	1

[0527] Evaluation of thaumatin, sucrose, and his-tagged mycodulcein (HTS) was carried out using a CATA (click all that apply) time intensity of the sweet sensation of named analytes in water at 0.045 mg/mL for proteins and 10% sucrose. Methodology: Time Intensity Technique; Data collection software: EyeQuestion, responses recorded every 2.57 seconds; Scaling Method: a 15-point sweet scale, e.g., score 5 = 5% sucrose, score 10 = 10% sucrose; Evaluation Protocol: small volume sip, tilt and spit test. There were 3-6 panelists and 2 replicates. All samples were blinded and presented with randomized 3-digit codes.

[0528] Training Strategies: An intense training on sweet scale over 6 weeks on 15-point sweet scale to confidently assign sweet values. Due to unique sweet behavioral patterns, it is necessary to train time intensity principles over 3 weeks. Samples were tasted with a stopwatch to note times and help reach a consensus. # of Panelists: 3-6, # of replications: 2. All samples were blinded and presented with randomized 3-digit codes. Statistical analysis: due to the small # of panelists, a statistical analysis cannot be performed.

[0529] The maximum intensity (Imax) of mycodulcein and thaumatin shows approximately 1 point higher on the 15 point scale at the amounts tested than sucrose. Thaumatin and mycodulcein have a flatter slope, indicating a longer peak time and more gradual/longer decline. Sucrose approaches threshold sweetness (Intensity <1) at 162 seconds, sooner than thaumatin and mycodulcein. When sucrose reaches threshold level, thaumatin and mycodulcein are recognizable at a low-moderate intensity. Mycodulcein and thaumatin appeared to be of similar potency in this experiment, which is on the order of 3000x sweeter than sucrose on a weight basis, or approximately 120,000x sweeter than sucrose on a molar basis. From these two experiments, mycodulcein is shown to be a high intensity sweet protein with a potency of between 400x sweeter than sucrose and 3000x sweeter than sucrose on a weight basis.

[0530] EXAMPLE 8 (Production and Testing of Variants of Mycodulcein (HTS) for Sweetness and Thermal Stability)

[0531] Method used to identify potential key residues in mycodulcein. Although sweet proteins have no primary sequence identity, the overall tertiary structures have a sweet finger motif (Tancredi, T., Pastore, A., Salvadori, S., Esposito, V. & Temussi, P. A. Interaction of sweet proteins with their receptor: A conformational study of peptides corresponding to loops of brazzein, monellin and thaumatin. *European Journal of Biochemistry* 271, (2004): 2231–2240.). Sweet proteins have antiparallel beta-sheets with an alpha-helix perpendicular to the beta-sheets. The sweet proteins thaumatin, monellin, brazzein, and lysozyme tertiary structures were analyzed using PyMOL 2.0 (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) and compared to a model of mycodulcein generated with Phyre2 (Kelley LA et al. The Phyre2 web portal for protein modeling, prediction, and analysis *Nature Protocols* 10, (2015): 845-858).

[0532] A number of single-site mutants were generated (International patent application PCT/US2022/82443) and are presented in Table 3.

[0533] Cloning: Eco_0001 aka *E. coli* BL21 DE3 (*E. coli* str. B F⁻ *ompT gal dcm lon hsdS_B(r_B m_B⁻)* λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB⁺*]_{K-12}(λ^S)) (obtained from New England Biolabs# C2527I), was transformed with twenty-three plasmids (pMy_3018 to pMy_3040) using manufacturer protocols. In short, previously frozen competent cells were thawed and mixed with 1 pg -100 ng of plasmid DNA and held on ice for 30 minutes. A heat shock of 42°C for 45 seconds was applied to this mixture. Immediately thereafter, the mixture was placed into an ice bath lasting for 10 minutes. 950 μL of pre-warmed LB media was added and then subjected to 1 hr of 225 rpm shaking at 37°C. Dilution of cells at 1:10 and 1:100 and plating 100 μL on the antibiotic plate was performed for each transformation reaction. An overnight growth at 37°C allowed colonies to fully recover and become visible. This process yielded strains Z18CE to Z40CE containing the plasmids pMy_3018 to

pMy_3040 sequentially. Shake flask-scale induced expression was used to confirm heterologous expression in the *E. coli* host. Post-transformational mutants were plated and maintained on LB + Ampicillin 100 µg/mL agar plates.

[0534] Plates were incubated for 16 hours at 37°C. Colony screen PCR was performed to confirm genotypes using colony screening primers designed to interrogate the flanking region along with the cDNA region of the plasmid. A successful transformation resulted in a DNA fragment of a certain size while a negative control and no template control result in no PCR band. Successful transformation was observed for all mutants.

[0535] Shake flask-scale induced expression was used to confirm heterologous expression in the *E. coli* host.

[0536] Strains were maintained on LB + ampicillin 100 µg/mL agar plates while the negative control (Eco_0001) was maintained on a LB agar plate. An overnight culture was grown for each strain in 50 mL of LB liquid media in un-baffled 250 mL culture shake flasks at 37°C shaking at 150 rpm overnight with appropriate antibiotics. Each overnight culture was subsequently seeded into 200 mL of TB liquid media the following day into baffled 1000 mL culture shake flask at 37°C shaking at 200 rpm and adjusted to 0.1 OD₆₀₀ with the appropriate antibiotic. Once the OD₆₀₀ reached 0.8, supplement of IPTG was added into the media to a final concentration of 0.66 mM. Continued the shaking at 37°C for an additional 5 hrs. Afterwards, cells were collected by centrifugation at 5000 g at 4°C for 5 min. *E. coli* cells were washed once with cold dH₂O and centrifuged again at 5000 g at 4°C for 10 minutes. For confirmation of successful expression, cell lysate was created using liquid nitrogen and a mortar and pestle. The cell pellet was resuspended in 10 mL of cold dH₂O and the crude lysate was spun at 20,000g at 4°C for 5 minutes. Finally, the supernatant was aspirated and filtered through 0.2 µm PES filter and run on SDS-PAGE protein electrophoresis. Crude lysates were tasted in order to identify sweet-tasting mutants. Table 3 shows exemplary results of the testing.

[0537] Table 3. Results of sweetness testing on single point mutations of mycodulcein (see international patent application nos. PCT/US2021/039176 and PCT/US2022/82443)

Content	Sweetness?	AA Substitution
Control-no mutation (SEQ ID NO:5)	Yes	N/A
Z38CE	Yes	D3E
Z39CE	Yes	K11R

Content	Sweetness?	AA Substitution
Z40CE	No	R20K
Z41CE	Yes	K26R
Z42CE	No	E35D
Z43CE	No	K44R
Z44CE	No	D46E
Z45CE	Yes	K51R
Z46CE	No	D52E
Z47CE	Yes	R57K
Z48CE	Yes	R66K
Z49CE	Yes	D69E
Z50CE	No	R75K
Z51CE	Yes	D85E
Z52CE	Yes	E86D
Z53CE	Yes	E89D
Z54CE	No	D94E
Z55CE	Yes	D97E
Z56CE	Yes	K103R
Z57CE	Yes	R106K
Z58CE	Yes	R110K
Z59CE	Yes	E117D
Z60CE	Yes	K120R

[0538] 16 of the variants (Z38CE, Z39CE, Z41CE, Z45CE, Z47CE, Z48CE, Z49CE, Z51CE, Z52CE, Z53CE, Z55CE, Z56CE, Z57CE, Z58CE, Z59CE, Z60CE) all of which had sweet taste were

additionally re-expressed using 200 mL of media. After expression, the cells were centrifuged at 4000 g for 20 min after the 24-hour induction period. The supernatant was discarded, and pellet was suspended in ~20 mL of wash buffer (10 mM sodium phosphate buffer pH 7.0). Suspended cells were disrupted in a high-pressure homogenizer (C3 Emulsiflex, Avestin, Inc., Ottawa, ON, Canada) operated at a max of 2,000 bar. Disrupted cells were centrifuged at 13,000 g (30 min), the supernatant was collected, and the pellet was discarded. The supernatant containing solubilized protein was filtered through a 0.22 µm PES membrane unit (Millipore, Burlington, MA, USA). The material was subsequently isolated through IMAC Purification as described in Example 4.

[0539] The purified samples were tasted by a trained sensory scientist. Mycodulcein stocks were diluted to equal protein concentration as measured by ELISA. Subjects followed a sip and spit protocol approved by an Institutional Review Board. 0.2 ml of each purified mutant was placed on the tongue and intensity of sweetness perception, time of onset of sweet perception, and duration of sweetness perception were noted. Single site mutations (see Table 8) are assessed for sweetness as described above.

[0540] References:

[0541] Korz, D. J., Rinas, U., Hellmuth, K., Sanders, E. A. & Deckwer, W.-D. Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *Journal of Biotechnology* 39, 59–65 (1995).

[0542] Norsyahida, A., Rahmah, N. & Ahmad, R. M. Y. Effects of feeding and induction strategy on the production of BmR1 antigen in recombinant *E. coli*. *Letters in Applied Microbiology* 49, 544–550 (2009).

[0543] EXAMPLE 9

[0544] Thermal Stability measurements are performed on protein samples from the IMAC Purification of HTS variants as described above in Example 8, normalized to 0.04 mg/mL, using the GloMelt™ Thermal Shift Protein Stability Kit (Biotium, Inc. Fremont, CA), using instructions provided by the manufacturer. In short, the individual reaction to measure thermal shift relies on mixing the following: mycodulcein or variant, 36 µg/ml in 25 mM sodium phosphate buffer pH 7.4 with reagents provided in the kit per manufacturer's instructions. For the thermal shift measurement, Bio-Rad CFX96 Touch system using BR Clear plates, scan-mode SYBER/FAM only, 25°C for 30 seconds, melt curve 25°C to 95°C, increment 0.5°C for 10 sec plus plate read was used. The *T_m* is determined based on the midpoint determined for a curve fitted to the experimental data with a five-parameter equation using the techniques as described in Schulz, M. N., Landström, J. & Hubbard, R. E. MTSA—A Matlab

program to fit thermal shift data. *Analytical Biochemistry* 433, 43–47 (2013). Results (Table 4) show the effect of amino acid changes on thermal stability.

[0545] Table 4. Exemplary results of thermal stability testing for mutants

Amino Acid Subs	Mutant STRAIN ID NUMBER	T _m
D3E	Z38CE	58.02135
K11R	Z39CE	58.9354
K26R	Z41CE	58.81657
K51R	Z45CE	60.66312
R57K	Z47CE	58.48098
R66K	Z48CE	58.99634
D6E	Z49CE	59.79641
D85E	Z51CE	59.99543
E86D	Z52CE	58.81274
E89D	Z53CE	57.96772
D97E	Z55CE	60.14034
K103R	Z56CE	59.29316
R106K	Z57CE	61.10271
R110K	Z58CE	57.92335
K120	Z60CE	60.168

[0546] EXAMPLE 10 (Cloning and Heterologous Expression of Mycodulcein or Variants in *Saccharomyces Cerevisiae*; Confirmation of Sweet Taste)

[0547] Based on the nucleotide sequence identified as SEQ ID NO:2, two expression vectors to express his-tagged mycodulcein and (native) mycodulcein were synthesized and cloned by Atum, Inc. (Newark, CA) into Atum vector non-secretory backbone pD1234 containing URA3 marker, and strong

constitutive promoter GPD (also described in International patent application nos. PCT/US2021/039176 and PCT/US2022/82443). The transformation procedure involves making electrocompetent cells and then introducing expression vectors through electroporation. In short, the electrocompetent cells are created by first growing the cells to between the early and mid-log phase with multiple washes to remove the salt from the growth medium. After mixing 1-5 μ g of the expression vector, the sample is subjected to the following settings on a Gene Pulser® II Electroporator (BioRad) Charging Voltage: 1.5 kV, Capacitance: 25 μ F, Resistance: 200 Ω) 1 mL of prewarmed 30°C YPD is added immediately, and the suspension is incubated for 1-2 h at 30°C shaking at 200-250 rpm. Post-transformational mutants were plated and maintained on SC-ura agar plates. This process yielded strains Z19ES, Z20ES containing the plasmids pMy_4002 and pMy_4003 respectively. csPCR (colony screen PCR) was performed to interrogate the cDNA region of the plasmid. Thus, a successful transformation would result in the DNA fragment of 303 bp while a negative control and no template control would result in no PCR band and expression was confirmed. Analogous methods are used to express various mycodulcein variants and his-tagged variants.

[0548] The two strains (Z19ES, Z20ES) were maintained on SC-ura agar plates while the negative control was maintained on SC agar plates. An overnight culture was grown for each strain in 50 mL of SC-ura/SC liquid media in un-baffled 250 mL culture shake flask at 37°C with shaking at 150 rpm overnight. Each overnight culture was inoculated into 200 mL of SC-ura/SC (O-RDL-R10_TB Media) liquid media in baffled 1000 mL culture shake flask at 30°C shaking at 200 rpm and adjusted to 0.02 OD₆₀₀. The shaking was continued at 30°C for an additional 48 hrs. Afterwards, cells were collected by centrifugation at 5000 g at 4°C for 5 min. Afterwards, *S. cerevisiae* cells were washed with cold dH₂O and centrifuged again at 5000 g at 4°C for 5 minutes. For confirmation of successful expression, cells were lysed using liquid nitrogen and a mortar and pestle. Cell pellets were resuspended in 10 mL of cold dH₂O and lysate was spun at 20,000g at 4°C for 5 minutes. Supernatant was filtered with a 0.2 μ m PES filter. The filtrate (both strains) was confirmed to taste sweet by methods described in Example 3.

[0549] Thermo Scientific™ HisPur™ Ni-NTA resin was used to purify the his-tagged mycodulcein (or variant) protein from *S. cerevisiae* using effective immobilized metal affinity chromatography (IMAC). His-tagged protein was purified using nickel-charged nitrilotriacetic acid (NTA) chelate immobilized onto 6% crosslinked agarose resin. Lysate was loaded onto a prepared IMAC column, the column was washed three times with 0.02 M imidazole in PBS followed by eluting his-tagged mycodulcein four times with 0.3 M imidazole in PBS. Eluted fractions were subjected to ultrafiltration using a 50 kD MWCO filter followed by concentration and desalting with a 3 kD MWCO filter.

[0550] EXAMPLE 11 (Purification of Mycodulcein expressed from Strain Z19ES (*S. Cerevisiae*))

[0551] Three chromatographical techniques were assessed for effectiveness for purification of mycodulcein expressed in *S. cerevisiae*: cation exchange (CIEX), hydrophobic interaction (HIC), and size exclusion chromatography (SEC).

[0552] Cation exchange assessment

[0553] The isoelectric point for the native mycodulcein (HTS) was determined to be ~9.5 by isoelectric focusing, suggesting a cation exchange column might be successful in purifying mycodulcein. The clarified cell lysate prepared as described in Example 10 was mixed with 2x starting buffer to obtain a cell lysate in 50mM sodium phosphate, 1M ammonium sulfate at pH 7.0, and it was stored at 4°C for future use. The purification procedure was performed on ÄKTA Explorer 100 system (GE Healthcare, Sweden), and the eluted proteins were monitored at 280 nm and 215 nm on a UV detector UV-900 (GE Healthcare, Sweden). A PreDicator plate (GE Healthcare, Sweden) prefilled with CIEX resins was used to screen binding conditions of native mycodulcein. The prefilled plate contains three main resins: Capto S (strong CIEX), Capto MMC (Weak CIEX), and SP Sepharose Fast Flow (strong CIEX). Lysate was dialyzed into 20 mM sodium phosphate dibasic, and different pH values ranging from 4 to 9 were screened and the optimal conditions were then scaled up using a HiScreen column. Equilibration was conducted using 25mM sodium phosphate dibasic at pH 5 at flow rate of 3 mL/min. Binding proteins were eluted by an increasing sodium chloride gradient from 0 to 1 M using 25mM sodium phosphate dibasic, 1M sodium chloride at pH 7. Different binding and eluting conditions were screened in which the weak cation exchanger Capto MMC showed the best binding capabilities at pH 5. However, due to low purity (25%) after this step, an alternative purification step was sought. Figure 4A shows PAGE analysis of the eluted fraction from Capto MMC. M: protein marker; lane 1: eluted fraction, showing low purity after cation exchange. Arrow indicates mycodulcein band.

[0554] HIC assessment.

[0555] Cell lysate was also subjected to hydrophobic interaction chromatography (HIC) using a HiScreen CaptoButyl column (Cytiva Sweden AB, Upsala, Sweden); equilibration was carried out using 5 column volumes of 50mM sodium phosphate, 1M ammonium sulfate at pH 7.0. Cell lysate was then loaded into the column at flow rate of 3 mL/min. Elution of bound proteins was performed by a decreasing ammonium sulfate gradient from 1 to 0 M using 50mM sodium phosphate at pH 7.0. All obtained fractions were analyzed by SDS-PAGE.

[0556] Figure 4B shows two eluted fractions collected during the gradient elution from the HiScreen Capto Butyl column analyzed on SDS-PAGE. Lane 1 shows eluted fraction 1 not containing

mycodulcein and Lane 2 shows eluted mycodulcein. The purity of the eluted fraction was determined by GelAnalyzer to be ~86%.

[0557] SEC assessment.

[0558] The eluted fraction containing native mycodulcein was then further purified using size exclusion chromatography (SEC) HiPrep 26/60 Sephacryl S-200 HR column (Cytiva Sweden AB, Upsala, Sweden), and eluted with buffer containing 50 mM sodium phosphate and 150 mM NaCl at pH 7.0. Fractions were collected and were then concentrated and desalted using 3kD molecular weight cut-offs (MWCO) centrifugal filters (Millipore-Sigma, Germany) and then analyzed by SDS-PAGE.

[0559] Summary: although mycodulcein binds successfully to the weak cation exchanger Capto MMC, the relatively low purity of the eluted fraction made CIEX a less favorable capture / intermediate purification step. On the other hand, a higher purity fraction was obtained from the HIC, Capto Butyl column. As per the SDS-PAGE analysis, impurities seemed to have a relatively high molecular weight which made SEC a great candidate to obtain a high pure mycodulcein.

[0560] Purity after HIC/SEC is approximately 98% by GelAnalyzer of SDS-PAGE. Figure 4C shows the eluted protein from the HIC column after chromatographing on HiPrep 26/60 Sephacryl S-200. Lane 1 shows purified his-tag mycodulcein and Lane 2 shows purified mycodulcein.

[0561] The purified native protein purified from *S. cerevisiae* was tasted at 0.03 mg/ml by a trained sensory scientist (0.2 mL aliquot) and found to have a sweetness equivalent to 8° brix (approximately 8% sucrose solution). The sweet taste was very noticeably sweet, with a “clean” sweetness (sugar-like taste) with no other flavors, with a slightly delayed onset and a sweet aftertaste. It has subsequently been determined that the mycodulcein expressed in *S. cerevisiae* is a mixture of two isoforms HTS-1 (SEQ ID NO:3) and HTS-2 (SEQ ID NO:141). The relative amounts of the HTS-1 to HTS-2 isoforms expressed are about 40% to 60% by weight. The two isoforms can be expressed in *S. cerevisiae* with or without His-tags or related protein tags.

[0562] **EXAMPLE 12** (Exemplary Food Compositions)

[0563] His-tagged mycodulcein prepared as in Example 5 and purified as described herein was tested in a yogurt base. The yogurt base had the following recipe in Table 5.

[0564] **Table 5:** Yogurt Base Recipe

Ingredient	%
Water	81.1200%

Pea Protein	10.6000%
Sunflower lecithin	0.0600%
Coconut Oil (AAK)	5.1000%
Ticaloid YG LP (TIC GUMS)	0.2000%
Citri-Fi Citrus Fiber	0.6000%
Canola Oil	1.0000%
Cane Sugar	1.3000%
YoFLEX YF-LO2 Cultures (CHR-Hansen)	0.0200%
	0.0000%
Total	100.0000%

[0565] The cane sugar is added as a carbon source for the yogurt cultures and is at least partially consumed by the cultures. Mycodulcein (HTS) was added to approximate the sweetness from 8° to 10° Brix of sucrose and the final concentration in the yogurt base is 0.05 mg/ml. Taste testing was performed by a trained sensory scientist and the yogurt was found to have a sweetness equivalent to 8° brix (approximately 8% sucrose solution). The sweet taste was very noticeably sweet, with a “clean” sweetness (sugar-like taste) with no other flavors, with a slightly delayed onset and a sweet aftertaste.

[0566] His-tagged mycodulcein (HTS-His-tagged) prepared as in Example 5 and purified as described herein was tested in whole milk; non-dairy pea-based milk (water, 93.75%, pea protein, 4.2%, canola oil, 1.7%, TIC Gum Blend Pro 181 AG (Acacia + Gellan) 0.3%, sunflower lecithin 0.05%); cold coffee; and water (control) at a final concentration of 0.04 mg/ml, predicted to provide a sweet level of between 8° to 10° Brix of sucrose. It was confirmed by taste testing that the sweet protein delivers a sweet level of between 8° to 10° Brix in all samples, and all samples had sweetness intensity, onset, and duration that was similar to the water control.

[0567] EXAMPLE 13 (Production and Testing of Various Mycodulcein (HTS) Variants for Sweetness and Thermal Stability)

[0568] Methods to identify potential key new mutants described herein able to be prepared in food compositions as described above. Specifically, single mutants as identified in Table 8 were generated.

These mutants were generated on a background of mycodulcein, e.g., SEQ ID NO:3. Thus, all Table 8 sequences are, for example, mutants with a single amino acid change relative to SEQ ID NO:3 as indicated in Table 8. All mycodulcein variants in Table 8 can be prepared with a His-tag. All mycodulcein variants in Table 8 can be prepared with the methionine at position 1 absent.

[0569] Cloning: Eco_0001 aka *E. coli* BL21 DE3 (obtained from New England Biolabs#C2527I), was transformed with twenty plasmids (pMy_3083 to pMy_3103) using manufacturing protocols. The competent cells were thawed and mixed with 1 ng – 100 ng of plasmid DNA and held on ice for 30 minutes. A heat shock of 42°C for 45 seconds was applied to this mixture. Immediately after, the suspension was placed into an ice bath for 10 minutes. Roughly 950 µL of pre-warmed LB media was added and then placed in a 37°C incubator for an hour, shaking at 225 rpm. For each transformation reaction, dilutions of 1:10 and 1:100 were made and 100 µL was plated on an antibiotic plate. These plates were allowed to grow overnight at 37°C so colonies can recover and become visible. This transformation process yielded strains Z83CE – Z103CE, containing the plasmids pMy_3083 – pMy_3103 sequentially. Shake flask-scale induced expression was used to confirm heterologous expression in the *E. coli* host. Post-transformational mutants were plated and maintained on LB + Ampicillin 100 µg/mL agar plates.

[0570] Plates were incubated for 16 hours at 37°C. Colony screen PCR was performed to confirm genotypes using colony screening primers designed to interrogate the flanking region along with the cDNA region of the plasmid. A successful transformation resulted in a DNA fragment of a certain size while a negative control and no template control result in no PCR band. Successful transformation was observed for all mutants.

[0571] Shake flask-scale induced expression was used to confirm heterologous expression in the *E. coli* host.

[0572] Strains were maintained on LB + ampicillin 100 µg/mL agar plates while the negative control was maintained on a LB plate. An overnight culture was grown for each strain in 50 mL of LB liquid media in a baffled 250 mL culture shake flask. These flasks were shaken at 37°C, at 150 rpm overnight with the appropriate antibiotics. Each overnight culture was subsequently seeded into 200 mL of TB liquid media after 16 hours of growth. These larger culture flasks were shaken at 37°C at 200 rpm and adjusted to 0.1 OD₆₀₀ with the appropriate antibiotic. Once the OD₆₀₀ reached 0.8, a supplement of IPTG was added into the media to a final concentration of 0.66 mM. The flasks then were shaken for 5 more hours. After this time had elapsed, cells were collected by centrifugation at 4000 rpm for 5 min. *E. coli* cells were washed once with cold dH₂O and centrifuged again at 4000 rpm for 10 minutes. For confirmation of successful expression, cell lysate was created via sonication of the cell membrane. The cell lysate and cell debris were separated via centrifugation at 10,000 rpm for 5

minutes. Finally, the supernatant was aspirated and filtered through 0.2 µm PES filter and run on SDS-PAGE protein electrophoresis. Crude lysates were tasted to identify sweet-tasting mutants.

[0573] Table 6 shows results of exemplary testing. Thermal stability was tested in accordance with the methods of Example 9. Sensory testing was performed in accordance with Example 8.

[0574] Table 6. Exemplary results of sweetness testing on single point mutations of mycodulcein

Mutant	Sweetness?	AA Substitution*	T _m (°C)
Control-No mutation	Yes	N/A	59.4
Z83CE	ND	S5Y	ND
Z84CE	ND	S6Y	ND
Z85CE	ND	F18Y	ND
Z86CE	ND	S25Y	ND
Z87CE	ND	V30A	ND
Z88CE	Yes	Q37K	60.9
Z89CE	Yes	Q37E	57.7
Z90CE	Yes	Q37N	58.9
Z91CE	ND	D46A	ND
Z92CE	ND	F48Y	ND
Z94CE	ND	V63A	ND
Z95CE	Yes	V74A	59.5
Z96CE	Yes	V76A	62.8
Z97CE	ND	F78Y	ND
Z98CE	Yes	V87A	54.4
Z99CE	Yes	V100A	56.5
Z100CE	Yes	Q102K	61.23

*Substitution numbered with respect to SEQ ID NO:3

[0575] Purified samples were tasted by a trained sensory scientist. Mycodulcein stocks were diluted to equal protein concentration as measured by ELISA. Subjects followed a sip and spit protocol approved by an Institutional Review Board. 0.2 ml of each purified mutant was placed on the tongue and intensity of sweetness perception, time of onset of sweet perception, and duration of sweetness perception were noted.

[0576] The exemplary results (Table 6) show that thermal stability is minimally affected by the amino acid changes in the mutants tested therein.

[0577] Additional single-site mutants for testing were generated by methods as described in this Example. Table 7 shows the mutants created. The mutations are identified relative to SEQ ID NO:3. The first column in Table 7 shows the amino acid position relative to SEQ ID NO:3, the second column shows the amino acid at that position in SEQ ID NO:3 and the third column shows the mutations (either none, or in the format of original amino acid, position, and new amino acid, using single letter code). All single site mutations of Table 7 can be introduced into the HTS-2 isoform of SEQ ID NO:141. All single site mutations of Table 7 can also have methionine missing at position 1. All single site mutations of Table 7 can be generated with or without a His-tag or other protein tag.

[0578] Table 7: Summary of Single Site Mutations

1	M	None
2	P	None
3	D	D3A, D3F, D3H, D3I, D3K, D3L, D3N, D3Q, D3R, D3V, D3W, D3Y
4	L	L4D, L4E, L4F, L4H, L4K, L4N, L4Q, L4R, L4W, L4Y
5	S	None
6	S	None
7	F	F7A, F7D, F7E, F7H, F7I, F7K, F7L, F7N, F7Q, F7R, F7V, F7W, F7Y
8	I	I8D, I8E, I8F, I8H, I8I, I8K, I8N, I8Q, I8R, I8V, I8W, I8Y
9	T	None
10	I	I10D, I10E, I10F, I10H, I10K, I10L, I10N, I10Q, I10R, I10V, I10W, I10Y
11	K	K11A, K11D, K11E, K11F, K11H, K11I, K11L, K11N, K11Q, K11V, K11W, K11Y
12	N	N12A, N12D, N12E, N12F, N12H, N12I, N12K, N12L, N12Q, N12R, N12V, N12W, N12Y

13	N	N13A, N13D, N13E, N13F, N13H, N13I, N13K, N13L, N13Q, N13R, N13V, N13W, N13Y
14	S	None
15	N	N15A, N15D, N15E, N15F, N15H, N15I, N15K, N15L, N15Q, N15R, N15V, N15W, N15Y
16	H	H16A, H16D, H16E, H16F, H16I, H16K, H16L, H16N, H16Q, H16R, H16V, H16W, H16Y
17	V	V17D, V17E, V17F, V17H, V17K, V17N, V17Q, V17R, V17W, V17Y
18	F	F18A, F18D, F18E, F18H, F18I, F18K, F18L, F18N, F18Q, F18R, F18V, F18W
19	T	None
20	R	R20A, R20D, R20E, R20F, R20H, R20I, R20L, R20N, R20Q, R20V, R20W, R20Y
21	T	None
22	A	A22D, A22E, A22F, A22H, A22K, A22N, A22Q, A22R, A22W, A22Y
23	I	I23D, I23E, I23F, I23H, I23K, I23L, I23N, I23Q, I23R, I23V, I23W, I23Y
24	Y	Y24A, Y24C, Y24D, Y24E, Y24F, Y24H, Y24I, Y24K, Y24L, Y24N, Y24Q, Y24R, Y24V, Y24W
25	S	None
26	K	K26A, K26D, K26E, K26F, K26H, K26I, K26L, K26N, K26Q, K26R, K26V, K26W, K26Y
27	Y	Y27A, Y27D, Y27E, Y27F, Y27H, Y27I, Y27K, Y27L, Y27N, Y27Q, Y27R, Y27V, Y27W
28	A	A28D, A28E, A28F, A28H, A28K, A28N, A28Q, A28R, A28W, A28Y
29	A	A29D, A29E, A29F, A29H, A29K, A29N, A29Q, A29R, A29W, A29Y
30	V	V30D, V30E, V30F, V30H, V30I, V30K, V30L, V30N, V30Q, V30R, V30W, V30Y
31	Q	Q31A, Q31D, Q31E, Q31F, Q31H, Q31I, Q31K, Q31L, Q31N, Q31R, Q31V, Q31W, Q31Y
32	W	W32A, W32D, W32E, W32F, W32H, W32I, W32K, W32L, W32N, W32Q, W32R, W32V, W32Y

33	S	S33T, S33M, S33C
34	P	P34A, P34D, P34E, P34F, P34H, P34I, P34K, P34L, P34N, P34Q, P34R, P34V, P34W, P34Y
35	E	E35A, E35F, E35H, E35I, E35K, E35L, E35N, E35Q, E35R, E35V, E35W, E35Y
36	P	P36A, P36D, P36E, P36F, P36H, P36I, P36K, P36L, P36N, P36Q, P36R, P36V, P36W, P36Y
37	Q	Q37A, Q37D, Q37F, Q37H, Q37I, Q37L, Q37N, Q37R, Q37V, Q37W, Q37Y
38	L	L38D, L38E, L38F, L38H, L38K, L38N, L38Q, L38R, L38W, L38Y
39	S	S39T, S39M
40	I	I40D, I40E, I40F, I40H, I40K, I40L, I40N, I40Q, I40R, I40V, I40W, I40Y
41	S	None
42	P	P42A, P42D, P42E, P42F, P42H, P42I, P42K, P42L, P42N, P42Q, P42R, P42V, P42W, P42Y
43	G	G43A, G43D, G43E, G43F, G43H, G43I, G43K, G43L, G43N, G43Q, G43R, G43V, G43W, G43Y
44	K	K44A, K44D, K44E, K44F, K44H, K44I, K44L, K44N, K44Q, K44R, K44V, K44W, K44Y
45	W	W45A, W45D, W45E, W45F, W45H, W45I, W45K, W45L, W45N, W45Q, W45R, W45V, W45Y
46	D	D46F, D46H, D46I, D46K, D46L, D46N, D46Q, D46R, D46V, D46W, D46Y
47	L	L47D, L47E, L47F, L47H, L47K, L47N, L47Q, L47R, L47W, L47Y
48	F	F48A, F48D, F48E, F48H, F48I, F48K, F48L, F48N, F48Q, F48R, F48V, F48W
49	I	I49D, I49E, I49F, I49H, I49K, I49N, I49Q, I49R, I49W, I49Y, I49C
50	L	L50D, L50E, L50F, L50H, L50K, L50N, L50Q, L50R, L50V, L50W, L50Y
51	K	K51A, K51D, K51E, K51F, K51H, K51I, K51L, K51N, K51Q, K51R, K51V, K51W, K51Y
52	D	D52F, D52H, D52I, D52K, D52L, D52N, D52Q, D52R, D52V, D52W, D52Y
53	I	I53D, I53E, I53F, I53H, I53K, I53N, I53Q, I53R, I53W, I53Y

54	L	L54D, L54E, L54F, L54H, L54K, L54N, L54Q, L54R, L54W, L54Y
55	S	S55T, S55M
56	I	I56D, I56E, I56F, I56H, I56K, I56N, I56Q, I56R, I56W, I56Y
57	R	R57A, R57D, R57E, R57F, R57H, R57I, R57K, R57L, R57N, R57Q, R57V, R57W, R57Y
58	G	G58A, G58D, G58E, G58F, G58H, G58I, G58K, G58L, G58N, G58Q, G58R, G58V, G58W, G58Y
59	T	None
60	S	None
61	G	G61A, G61D, G61E, G61F, G61H, G61I, G61K, G61L, G61N, G61Q, G61R, G61V, G61W, G61Y
62	Y	Y62A, Y62C, Y62D, Y62E, Y62F, Y62H, Y62I, Y62K, Y62L, Y62N, Y62Q, Y62R, Y62V, Y62W
63	V	V63D, V63E, V63F, V63H, V63L, V63K, V63N, V63Q, V63R, V63V, V63W, V63Y
64	Q	Q64A, Q64D, Q64E, Q64F, Q64H, Q64I, Q64K, Q64L, Q64N, Q64R, Q64V, Q64W, Q64Y
65	Y	Y65A, Y65D, Y65E, Y65F, Y65H, Y65I, Y65K, Y65L, Y65N, Y65Q, Y65R, Y65V, Y65W
66	R	R66A, R66D, R66E, R66F, R66H, R66I, R66K, R66L, R66N, R66Q, R66V, R66W, R66Y
67	V	V67D, V67E, V67F, V67H, V67K, V67N, V67Q, V67R, V67W, V67Y
68	G	G68A, G68D, G68E, G68F, G68H, G68I, G68K, G68L, G68N, G68Q, G68R, G68V, G68W, G68Y
69	D	D69A, D69E, D69F, D69H, D69I, D69K, D69L, D69N, D69Q, D69R, D69V, D69W, D69Y
70	G	G70A, G70D, G70E, G70F, G70H, G70I, G70K, G70L, G70N, G70Q, G70R, G70V, G70W, G70Y

71	P	P71A, P71D, P71E, P71F, P71H, P71I, P71K, P71L, P71N, P71Q, P71R, P71V, P71W, P71Y
72	G	G72A, G72D, G72E, G72F, G72H, G72I, G72K, G72L, G72N, G72Q, G72R, G72V, G72W, G72Y
73	W	W73A, W73D, W73E, W73F, W73H, W73I, W73K, W73L, W73N, W73Q, W73R, W73V, W73Y
74	V	V74D, V74E, V74F, V74H, V74I, V74K, V74L, V74N, V74Q, V74R, V74W, V74Y
75	R	R75A, R75D, R75E, R75F, R75H, R75I, R75K, R75L, R75N, R75Q, R75V, R75W, R75Y
76	V	V76D, V76E, V76F, V76H, V76K, V76I, V76L, V76N, V76Q, V76R, V76W, V76Y
77	T	None
78	F	F78A, F78D, F78E, F78H, F78I, F78K, F78L, F78N, F78Q, F78R, F78V, F78W
79	S	None
80	S	None
81	L	L81D, L81E, L81F, L81H, L81I, L81K, L81N, L81Q, L81R, L81V, L81W, L81Y
82	V	V82D, V82E, V82F, V82H, V82K, V82N, V82Q, V82R, V82W, V82Y
83	G	G83A, G83E, G83F, G83H, G83I, G83K, G83L, G83N, G83Q, G83R, G83V, G83W, G83Y
84	A	A84D, A84F, A84H, A84K, A84N, A84Q, A84R, A84W, A84Y
85	D	D85A, D85I, D85K, D85L, D85N, D85Q, D85R, D85V, D85W, D85Y
86	E	E86A, E86D, E86F, E86H, E86I, E86K, E86L, E86N, E86Q, E86R, E86V, E86W, E86Y
87	V	V87D, V87E, V87F, V87H, V87I, V87K, V87L, V87N, V87Q, V87R, V87W, V87Y
88	A	A88D, A88E, A88F, A88H, A88I, A88K, A88L, A88N, A88Q, A88R, A88V, A88W, A88Y
89	E	E89A, E89D, E89F, E89H, E89I, E89K, E89L, E89N, E89Q, E89R, E89V, E89W, E89Y

90	W	W90A, W90D, W90E, W90F, W90H, W90I, W90K, W90L, W90N, W90Q, W90R, W90V, W90Y
91	S	None
92	S	None
93	G	G93A, G93D, G93E, G93F, G93H, G93I, G93K, G93L, G93N, G93Q, G93R, G93V, G93W, G93Y
94	D	D94A, D94E, D94F, D94H, D94I, D94K, D94L, D94N, D94Q, D94R, D94V, D94W, D94Y
95	L	L95A, L95D, L95E, L95F, L95H, L95I, L95K, L95N, L95Q, L95R, L95V, L95W, L95Y
96	P	P96A, P96D, P96E, P96F, P96H, P96I, P96K, P96L, P96N, P96Q, P96R, P96V, P96W, P96Y
97	D	D97A, D97E, D97F, D97H, D97I, D97K, D97L, D97N, D97Q, D97R, D97V, D97W, D97Y
98	G	G98D, G98E, G98F, G98H, G98I, G98K, G98L, G98N, G98Q, G98R, G98V, G98W, G98Y
99	F	F99D, F99E, F99H, F99I, F99K, F99L, F99N, F99Q, F99R, F99V, F99W, F99Y
100	V	V100D, V100E, V100F, V100H, V100I, V100K, V100L, V100N, V100Q, V100R, V100W, V100Y
101	L	L101A, L101D, L101E, L101F, L101H, L101I, L101K, L101N, L101Q, L101R, L101V, L101W, L101Y
102	Q	Q102A, Q102D, Q102F, Q102H, Q102I, Q102L, Q102R, Q102V, Q102W, Q102Y
103	K	K103D, K103E, K103F, K103H, K103I, K103L, K103N, K103Q, K103R, K103V, K103W, K103Y
104	P	P104A, P104D, P104E, P104F, P104H, P104I, P104K, P104L, P104N, P104Q, P104R, P104V, P104W, P104Y
105	V	V105D, V105E, V105F, V105H, V105K, V105N, V105Q, V105R, V105W, V105Y
106	R	R106A, R106D, R106E, R106F, R106H, R106I, R106K, R106L, R106N, R106Q, R106V, R106W, R106Y

107	T	None
108	G	G108A, G108D, G108E, G108F, G108H, G108I, G108K, G108L, G108N, G108Q, G108R, G108V, G108W, G108Y
109	S	None
110	R	R110D, R110E, R110F, R110H, R110I, R110K, R110L, R110N, R110Q, R110V, R110W, R110Y
111	P	P111A, P111D, P111E, P111F, P111H, P111I, P111K, P111L, P111N, P111Q, P111R, P111V, P111W, P111Y
112	L	L112D, L112E, L112F, L112H, L112K, L112N, L112Q, L112R, L112W, L112Y
113	Q	Q113A, Q113D, Q113E, Q113F, Q113H, Q113I, Q113K, Q113L, Q113N, Q113R, Q113V, Q113W, Q113Y
114	A	A114D, A114E, A114F, A114H, A114I, A114K, A114L, A114N, A114Q, A114R, A114V, A114W, A114Y
115	T	None
116	F	F116D, F116E, F116H, F116I, F116K, F116L, F116N, F116Q, F116R, F116V, F116W, F116Y
117	E	E117A, E117F, E117H, E117I, E117K, E117L, E117N, E117Q, E117R, E117V, E117W, E117Y
118	A	A118F, A118H, A118K, A118N, A118Q, A118R, A118W, A118Y
119	T	None
120	K	K120F, K120H, K120I, K120L, K120N, K120Q, K120R, K120V, K120W, K120Y
121	Q	Q121F, Q121H, Q121I, Q121K, Q121L, Q121N, Q121R, Q121V, Q121W, Q121Y.

[0579] In variants of Table 7, variants in which the proline at any of positions 34, 36, 42, 71 or 111 (based on SEQ ID NO:3) is modified to any other amino acid are less preferred.

[0580] EXAMPLE 14: Generation and Characterization of HTS Variants With Increased Thermostability

[0581] This Example describes the design, expression, testing, and validation of Myd1 (wild-type HTS protein) variants that are more thermostable than the native (wild-type) protein for use, among others,

in the food industry. Current standard pasteurization and sterilization techniques employed in food manufacturing can degrade native Myd1 structure due to its relative low melting temperature. The thermostable variants as described in this example provide sweet taste HTS protein that is useful in food and other comestibles that are subject to heating, such as in pasteurization.

[0582] This example relates to the design of focused amino acid substitutions for disulfide bridge formation. Disulfide bridges can increase stability of a protein.

[0583] A specific region of the HTS protein (amino acids ~20~70) was chosen for these experiments. It was hypothesized that upon heating, this region would be the first to denature. Selected amino acids in this region were substituted with two cysteines, to determine if forming a disulfide bridge between the two cysteines would retain protein sweetness, as well as increase the melting temperature of the variant protein.

[0584] For one candidate double mutant (HTS S33C_I49C (SEQ ID NO: 143)), the *E. coli* optimized nucleotide sequence encoding the double mutant variant, provided as (SEQ ID NO: 144, with Met at position 1 with no stop codon), was inserted into the isopropyl beta-D-1-thiogalactopyranoside (IPTG)-inducible vector (pD444-CH, AUTM, Newark, CA) (FIG. 5). The HTS variant translated from the pD44-CH vector contains two cysteines indicated and a C-terminal 6X His tag (encoded in the vector).

[0585] Pure circular plasmids (pD444-CH, AUTM, Newark, CA) were transformed into chemically competent *E. coli* BL21-DE3 using heat shock transformation with transformant recovery on LB plates containing Ampicillin 100 ug/mL. Three colonies of successful transformants were grown overnight in LB_amp at 37 °C, with shaking at 200 rpm. Glycerol stocks were made from overnight cultures of each of the three transformants.

[0586] A glycerol stock for each was then used to seed 50 mL of LB_amp and the cultures were grown overnight at 37 °C, shaking at 200 rpm. The next morning, LB cultures were used to seed 200 mL of TB_amp in 2 L baffled flasks to bring the starting OD₆₀₀ values to 0.1. Cultures were then grown at 37 °C, with shaking at 200 rpm. OD₆₀₀ values were monitored for about 1.5 hours when values reached OD₆₀₀ of 0.6-0.8. 100 mM IPTG was added to the flasks to bring the final concentration to 0.66 mM to induce protein expression. Flasks were grown for an additional 5 hours and harvested by spinning down at 4,000 g for 20 minutes. Pellets were washed with water and re-spun at 4,000 g for 20 minutes. Cell pellets were placed in the freezer for future lysis and protein purification.

[0587] Cell pellets for BL21-DE3 mutants expressing double mutant HTS_S33C_I49C were thawed on ice and resuspended in ~25 mL HisTrap™ (Cytiva Sweden AB, Uppsala, Sweden) working buffer (20 mM sodium phosphate, 0.5 M sodium chloride and 25 mM imidazole; (the imidazole concentration

typically ranges from 20-40 mM and is adjusted for a given protein as is known in the art).

Resuspended mutants were then passed through an Emulsiflex C3 high pressure homogenizer (Avestin, Ottawa, Ontario, Canada) and lysed at a pressure $\geq 18,000$ psi. Cell lysate was collected on ice and then spun down at 4,000 g for 1 hour at 4 OC. Supernatant was collected and passed through a 0.22 μ m filter before loading on the FPLC (Fast Protein Liquid Chromatography) system.

[0588] Clarified lysate (20 mL) was loaded onto a 5 mL HisTrapTM FF column using an FPLC (ÄKTA FPLCTM, Cytiva Sweden AB, Uppsala, Sweden). The column was washed with 5 column volumes of working buffer and then protein was eluted using a gradient elution and protein eluted at ~45% elution buffer (250 mM imidazole). Resulting protein samples were desalted on 3 kD Molecular weight Cut Off (MWCO) filters and buffer exchanged with 5 mM sodium phosphate. Resulting protein samples were run on an 16.5% Tris Tricine gel, using a native HTS sample expressed from *S. cerevisiae* (~1 mg/mL) as a control. The purified double mutant variant also tasted sweet. Upon this observation, it was decided to move forward with testing to see if melting temperature was increased.

[0589] A GLoMELTTM thermostability assay (Biotrend Chemikalien GmbH, Köln, DE) was carried out to determine T_m of the purified variant. This assay uses a quantitative (q)PCR thermocycler to read fluorescence as temperature increases. This assay determines unfolding and denaturation of a protein as a function of temperature as measured by change in fluorescence of a fluorescent dye. The dye does not bond to the native protein, but bonds to the protein as it unfolds and fully denatures.

[0590] Using a CFX96TM (Bio-Rad, Hercules, CA) PCR thermocycler system, the parameters for the GlomeltTM assay (FIGs. 6A and 6B) are as follows:

[0591] 25° C: 30 seconds;

25° C: 10 seconds;

Increase 0.5° C every 10 seconds: Read RFU in SYBR (channel every 10 seconds).

[0592] Protein was observed to be pure enough for the assay requirements based on SDS-PAGE (at least 80% pure). Initial assays were run using unknown concentrations of the variant protein as a first pass. It was assumed that protein concentrations met the assay requirements of 0.5-5.0 μ g/ μ L. CFX MaestroTM (Bio-Rad, Hercules, CA) software was used to analyze the raw data and generate melt curves.

[0593] The results of the thermostability assay are shown in FIGs. 6A and 6B,

[0594] The increase in T_m seen with the HTS_S33C_I49C variant compared to native HTS shows that it is a protein that can survive the harsh conditions employed in food manufacturing to ensure food safety.

[0595] Since a shift in T_m was seen with the double mutant a more functional assay was designed to better mimic the conditions of a low temperature, long time pasteurization technique commonly used in the dairy industry (vat pasteurization). This assay employed the same GloMelt™ assay kit, but instead of ramping the temperature up over time, the sample is brought to a low pasteurization temperature (63 °C) and is held for an hour. If max fluorescence is seen before 30 minutes, then it is known the protein will not survive this type of manufacturing process (vat pasteurization). However, if there is a shift in max fluorescence over time vs. the native protein, then the variant is indeed more thermostable under vat pasteurization conditions and is a protein that can be added in food using this practice. The results of this assay are shown in FIG. 7 for Native HTS protein (1) and the double mutant (2).

[0596] Thermocycler parameters for FIG. 7:

Bring sample temp to 63° C ;

63° for 60 min °C: read RFU every 10 seconds.

[0597] As illustrated in FIG. 7, the shift in peak RFU of 6.22 minutes showed that the double mutant protein survives that much longer under these pasteurization conditions than the native HTS protein.

[0598] A similar thermostable HTS variant is prepared by introducing two cysteines into the polypeptide of SEQ ID NO:3 at amino acid positions 24 and 62 (double mutant Y24C_Y62C SEQ ID NO:145). Analogous thermostable variants of SEQ ID NO:141 are the variants of SEQ ID NO:146 and SEQ ID NO:147 which are optionally generated with a His-tag or other protein tag.

[0599] EXAMPLE 15 (Honey Truffle Sweetener (HTS) Protein Variants Generated by Site Mutations)

[0600] Amino acid mutations able to generate HTS protein variants are summarized in Table 8. The mutations of Table 8 are identified relative to the amino acid sequence of SEQ ID NO:3, with one column indicating the amino acid substitution of the mature protein (i.e., with no methionine at amino acid position 1) and a second column indicating the amino acid substitution of the protein where methionine is still present at amino acid position 1. The presence or absence of the methionine at position 1 of the HTS protein does not affect sweet taste of the variant protein. A polypeptide having an amino acid sequence of a variant listed in Table 8 will have the amino acid sequence of SEQ ID NO:3, but with one or more site mutations identified in Table 8.

[0601] Table 8 – Summary of single site mutations producing protein variants

No.	Amino Acid Substitution (no Met at position 1)	Amino Acid Substitution (Met present at position 1)	No.	Amino Acid Substitution (no Met at position 1)	Amino Acid Substitution (Met present at position 1)
1	AA113A to F	A114F	553	AA55I to H	I56H
2	AA113A to L	A114L	554	AA101Q to R	Q102R
3	AA113A to W	A114W	555	AA14N to A	N15A
4	AA113A to Y	A114Y	556	AA120Q to R	Q121R
5	AA117A to F	A118F	557	AA21A to Q	A22Q
6	AA87A to Q	A88Q	558	AA68D to N	D69N
7	AA2D to L	D3L	559	AA81V to N	V82N
8	AA45D to F	D46F	560	AA55I to K	I56K
9	AA45D to I	D46I	561	AA97G to N	G98N
10	AA45D to K	D46K	562	AA120Q to K	Q121K
11	AA45D to L	D46L	563	AA56R to N	R57N
12	AA45D to Q	D46Q	564	AA56R to Q	R57Q
13	AA45D to R	D46R	565	AA61Y to F	Y62F
14	AA45D to V	D46V	566	AA101Q to V	Q102V
15	AA45D to W	D46W	567	AA120Q to W	Q121W
16	AA45D to Y	D46Y	568	AA83A to K	A84K
17	AA51D to F	D52F	569	AA44W to A	W45A
18	AA51D to H	D52H	570	AA36Q to R	Q37R
19	AA51D to I	D52I	571	AA9I to E	I10E
20	AA51D to K	D52K	572	AA53L to Q	L54Q
21	AA51D to L	D52L	573	AA99V to F	V100F
22	AA51D to R	D52R	574	AA50K to I	K51I
23	AA51D to V	D52V	575	AA82G to E	G83E
24	AA51D to W	D52W	576	AA6F to L	F7L
25	AA51D to Y	D52Y	577	AA73V to F	V74F
26	AA34E to H	E35H	578	AA71G to Y	G72Y
27	AA34E to N	E35N	579	AA44W to H	W45H
28	AA34E to W	E35W	580	AA14N to E	N15E
29	AA85E to H	E86H	581	AA55I to W	I56W
30	AA85E to I	E86I	582	AA44W to K	W45K
31	AA115F to L	F116L	583	AA73V to I	V74I
32	AA115F to W	F116W	584	AA82G to Y	G83Y
33	AA115F to Y	F116Y	585	AA22I to Y	I23Y
34	AA17F to A	F18A	586	AA6F to E	F7E
35	AA17F to D	F18D	587	AA83A to F	A84F
36	AA17F to E	F18E	588	AA96D to E	D97E
37	AA17F to H	F18H	589	AA53L to F	L54F
38	AA17F to K	F18K	590	AA119K to L	K120L
39	AA17F to N	F18N	591	AA9I to Q	I10Q
40	AA17F to Q	F18Q	592	AA96D to A	D97A

41	AA17F to R	F18R	593	AA46L to W	L47W
42	AA47F to A	F48A	594	AA63Q to E	Q64E
43	AA47F to D	F48D	595	AA93D to F	D94F
44	AA47F to E	F48E	596	AA55I to R	I56R
45	AA47F to H	F48H	597	AA14N to L	N15L
46	AA47F to I	F48I	598	AA81V to E	V82E
47	AA47F to K	F48K	599	AA3L to W	L4W
48	AA47F to L	F48L	600	AA68D to A	D69A
49	AA47F to N	F48N	601	AA43K to R	K44R
50	AA47F to Q	F48Q	602	AA68D to E	D69E
51	AA47F to R	F48R	603	AA83A to Y	A84Y
52	AA47F to V	F48V	604	AA44W to V	W45V
53	AA57G to K	G58K	605	AA9I to K	I10K
54	AA77F to V	F78V	606	AA82G to N	G83N
55	AA6F to D	F7D	607	AA43K to N	K44N
56	AA98F to E	F99E	608	AA99V to Y	V100Y
57	AA98F to H	F99H	609	AA68D to Q	D69Q
58	AA98F to N	F99N	610	AA55I to F	I56F
59	AA98F to Q	F99Q	611	AA53L to R	L54R
60	AA98F to W	F99W	612	AA16V to K	V17K
61	AA98F to Y	F99Y	613	AA61Y to H	Y62H
62	AA107G to E	G108E	614	AA44W to R	W45R
63	AA107G to D	G108D	615	AA119K to R	K120R
64	AA107G to L	G108L	616	AA22I to L	I23L
65	AA107G to Q	G108Q	617	AA43K to E	K44E
66	AA107G to F	G108F	618	AA21A to E	A22E
67	AA107G to W	G108W	619	AA96D to R	D97R
68	AA107G to Y	G108Y	620	AA50K to H	K51H
69	AA42G to I	G43I	621	AA92G to A	G93A
70	AA42G to K	G43K	622	AA93D to N	D94N
71	AA42G to L	G43L	623	AA99V to I	V100I
72	AA42G to R	G43R	624	AA96D to K	D97K
73	AA42G to V	G43V	625	AA93D to H	D94H
74	AA42G to Y	G43Y	626	AA52I to D	I53D
75	AA57G to A	G58A	627	AA15H to F	H16F
76	AA57G to D	G58D	628	AA54S to T	S55T
77	AA57G to E	G58E	629	AA87A to F	A88F
78	AA57G to F	G58F	630	AA96D to Q	D97Q
79	AA57G to H	G58H	631	AA120Q to N	Q121N
80	AA57G to I	G58I	632	AA9I to N	I10N
81	AA57G to L	G58L	633	AA65R to Q	R66Q
82	AA57G to N	G58N	634	AA81V to K	V82K
83	AA57G to Q	G58Q	635	AA68D to K	D69K
84	AA57G to R	G58R	636	AA81V to Y	V82Y

85	AA57G to V	G58V		637	AA93D to Y	D94Y
86	AA57G to W	G58W		638	AA14N to Y	N15Y
87	AA57G to Y	G58Y		639	AA104V to K	V105K
88	AA60G to A	G61A		640	AA81V to F	V82F
89	AA60G to D	G61D		641	AA88E to Y	E89Y
90	AA60G to E	G61E		642	AA92G to Q	G93Q
91	AA60G to F	G61F		643	AA120Q to F	Q121F
92	AA60G to H	G61H		644	AA81V to Q	V82Q
93	AA60G to I	G61I		645	AA82G to H	G83H
94	AA60G to K	G61K		646	AA81V to H	V82H
95	AA60G to L	G61L		647	AA14N to F	N15F
96	AA60G to N	G61N		648	AA68D to H	D69H
97	AA60G to Q	G61Q		649	AA21A to H	A22H
98	AA60G to R	G61R		650	AA68D to L	D69L
99	AA60G to V	G61V		651	AA81V to D	V82D
100	AA60G to W	G61W		652	AA92G to E	G93E
101	AA60G to Y	G61Y		653	AA1P to H	P2H
102	AA71G to D	G72D		654	AA96D to W	D97W
103	AA71G to E	G72E		655	AA23Y to F	Y24F
104	AA9I to D	I10D		656	AA48I to K	I49K
105	AA9I to W	I10W		657	AA16V to H	V17H
106	AA9I to Y	I10Y		658	AA65R to K	R66K
107	AA22I to D	I23D		659	AA68D to R	D69R
108	AA22I to E	I23E		660	AA21A to K	A22K
109	AA22I to N	I23N		661	AA96D to L	D97L
110	AA39I to D	I40D		662	AA22I to V	I23V
111	AA39I to E	I40E		663	AA96D to V	D97V
112	AA39I to F	I40F		664	AA80L to V	L81V
113	AA39I to K	I40K		665	AA56R to K	R57K
114	AA39I to N	I40N		666	AA56R to V	R57V
115	AA39I to Q	I40Q		667	AA52I to H	I53H
116	AA39I to R	I40R		668	AA85E to D	E86D
117	AA39I to W	I40W		669	AA14N to Q	N15Q
118	AA39I to Y	I40Y		670	AA105R to L	R106L
119	AA48I to D	I49D		671	AA120Q to H	Q121H
120	AA48I to F	I49F		672	AA101Q to H	Q102H
121	AA48I to H	I49H		673	AA14N to R	N15R
122	AA48I to N	I49N		674	AA120Q to Y	Q121Y
123	AA48I to W	I49W		675	AA82G to F	G83F
124	AA48I to Y	I49Y		676	AA74R to Q	R75Q
125	AA7I to D	I8D		677	AA93D to Q	D94Q
126	AA7I to H	I8H		678	AA17F to I	F18I
127	AA7I to K	I8K		679	AA39I to L	I40L
128	AA7I to N	I8N		680	AA21A to R	A22R

129	AA7I to Q	I8Q	681	AA93D to E	D94E
130	AA7I to W	I8W	682	AA16V to D	V17D
131	AA7I to Y	I8Y	683	AA96D to N	D97N
132	AA10K to D	K11D	684	AA83A to D	A84D
133	AA10K to E	K11E	685	AA38S to M	S39M
134	AA50K to D	K51D	686	AA68D to V	D69V
135	AA50K to F	K51F	687	AA46L to N	L47N
136	AA100L to A	L101A	688	AA102K to R	K103R
137	AA100L to F	L101F	689	AA53L to W	L54W
138	AA100L to K	L101K	690	AA37L to E	L38E
139	AA100L to Q	L101Q	691	AA86V to I	V87I
140	AA100L to R	L101R	692	AA44W to Y	W45Y
141	AA100L to Y	L101Y	693	AA71G to A	G72A
142	AA111L to H	L112H	694	AA44W to I	W45I
143	AA111L to N	L112N	695	AA63Q to D	Q64D
144	AA111L to Q	L112Q	696	AA96D to H	D97H
145	AA111L to Y	L112Y	697	AA93D to A	D94A
146	AA37L to D	L38D	698	AA112Q to H	Q113H
147	AA37L to N	L38N	699	AA43K to Y	K44Y
148	AA3L to E	L4E	700	AA14N to I	N15I
149	AA49L to D	L50D	701	AA68D to Y	D69Y
150	AA49L to E	L50E	702	AA39I to H	I40H
151	AA49L to F	L50F	703	AA68D to I	D69I
152	AA49L to H	L50H	704	AA44W to F	W45F
153	AA49L to K	L50K	705	AA37L to K	L38K
154	AA49L to N	L50N	706	AA96D to I	D97I
155	AA49L to R	L50R	707	AA56R to W	R57W
156	AA49L to V	L50V	708	AA96D to F	D97F
157	AA49L to W	L50W	709	AA25K to A	K26A
158	AA49L to Y	L50Y	710	AA90S to T	S91T
159	AA11N to A	N12A	711	AA68D to F	D69F
160	AA11N to D	N12D	712	AA99V to L	V100L
161	AA11N to E	N12E	713	AA22I to K	I23K
162	AA11N to F	N12F	714	AA68D to W	D69W
163	AA11N to H	N12H	715	AA102K to Q	K103Q
164	AA11N to I	N12I	716	AA92G to D	G93D
165	AA11N to K	N12K	717	AA92G to K	G93K
166	AA11N to L	N12L	718	AA16V to Y	V17Y
167	AA11N to Q	N12Q	719	AA12N to H	N13H
168	AA11N to R	N12R	720	AA15H to Y	H16Y
169	AA11N to W	N12W	721	AA15H to L	H16L
170	AA11N to Y	N12Y	722	AA21A to F	A22F
171	AA12N to I	N13I	723	AA74R to K	R75K
172	AA12N to V	N13V	724	AA6F to N	F7N

173	AA12N to W	N13W		725	AA97G to Q	G98Q
174	AA103P to E	P104E		726	AA81V to R	V82R
175	AA103P to I	P104I		727	AA43K to A	K44A
176	AA33P to V	P34V		728	AA97G to E	G98E
177	AA33P to W	P34W		729	AA74R to A	R75A
178	AA33P to Y	P34Y		730	AA84D to A	D85A
179	AA35P to A	P36A		731	AA14N to V	N15V
180	AA35P to D	P36D		732	AA92G to R	G93R
181	AA35P to E	P36E		733	AA65R to N	R66N
182	AA35P to F	P36F		734	AA16V to R	V17R
183	AA35P to H	P36H		735	AA76T to S	T77S
184	AA35P to I	P36I		736	AA75V to I	V76I
185	AA35P to K	P36K		737	AA83A to W	A84W
186	AA35P to L	P36L		738	AA14N to W	N15W
187	AA35P to N	P36N		739	AA92G to H	G93H
188	AA35P to Q	P36Q		740	AA69G to E	G70E
189	AA35P to R	P36R		741	AA15H to I	H16I
190	AA35P to V	P36V		742	AA56R to E	R57E
191	AA35P to W	P36W		743	AA50K to V	K51V
192	AA35P to Y	P36Y		744	AA16V to W	V17W
193	AA41P to D	P42D		745	AA34E to A	E35A
194	AA41P to F	P42F		746	AA23Y to H	Y24H
195	AA41P to H	P42H		747	AA99V to K	V100K
196	AA41P to L	P42L		748	AA21A to Y	A22Y
197	AA41P to N	P42N		749	AA102K to E	K103E
198	AA41P to V	P42V		750	AA84D to Q	D85Q
199	AA41P to W	P42W		751	AA93D to L	D94L
200	AA41P to Y	P42Y		752	AA22I to F	I23F
201	AA95P to K	P96K		753	AA104V to R	V105R
202	AA95P to N	P96N		754	AA92G to Y	G93Y
203	AA112Q to D	Q113D		755	AA21A to N	A22N
204	AA36Q to F	Q37F		756	AA84D to N	D85N
205	AA36Q to W	Q37W		757	AA67G to D	G68D
206	AA36Q to Y	Q37Y		758	AA45D to N	D46N
207	AA105R to A	R106A		759	AA15H to E	H16E
208	AA105R to D	R106D		760	AA97G to H	G98H
209	AA105R to E	R106E		761	AA43K to D	K44D
210	AA105R to N	R106N		762	AA86V to K	V87K
211	AA105R to W	R106W		763	AA65R to A	R66A
212	AA109R to D	R110D		764	AA92G to N	G93N
213	AA109R to E	R110E		765	AA112Q to A	Q113A
214	AA109R to L	R110L		766	AA44W to L	W45L
215	AA109R to V	R110V		767	AA14N to H	N15H
216	AA109R to W	R110W		768	AA16V to F	V17F

217	AA109R to I	R110I	769	AA97G to Y	G98Y
218	AA19R to D	R20D	770	AA82G to A	G83A
219	AA19R to E	R20E	771	AA97G to R	G98R
220	AA19R to F	R20F	772	AA15H to K	H16K
221	AA19R to H	R20H	773	AA6F to A	F7A
222	AA19R to N	R20N	774	AA97G to F	G98F
223	AA19R to W	R20W	775	AA16V to Q	V17Q
224	AA19R to Y	R20Y	776	AA94L to I	L95I
225	AA56R to F	R57F	777	AA37L to H	L38H
226	AA56R to L	R57L	778	AA101Q to A	Q102A
227	AA91S to T	S92T	779	AA99V to R	V100R
228	AA104V to D	V105D	780	AA80L to I	L81I
229	AA62V to D	V63D	781	AA40S to T	S41T
230	AA62V to E	V63E	782	AA83A to N	A84N
231	AA62V to F	V63F	783	AA44W to N	W45N
232	AA62V to H	V63H	784	AA21A to D	A22D
233	AA62V to K	V63K	785	AA86V to Y	V87Y
234	AA62V to L	V63L	786	AA44W to D	W45D
235	AA62V to N	V63N	787	AA63Q to A	Q64A
236	AA62V to Q	V63Q	788	AA3L to R	L4R
237	AA62V to R	V63R	789	AA48I to Q	I49Q
238	AA62V to W	V63W	790	AA86V to Q	V87Q
239	AA62V to Y	V63Y	791	AA7I to F	I8F
240	AA72W to A	W73A	792	AA70P to A	P71A
241	AA72W to E	W73E	793	AA15H to W	H16W
242	AA72W to I	W73I	794	AA93D to K	D94K
243	AA72W to K	W73K	795	AA9I to L	I10L
244	AA72W to N	W73N	796	AA109R to K	R110K
245	AA72W to Q	W73Q	797	AA21A to W	A22W
246	AA72W to R	W73R	798	AA43K to F	K44F
247	AA23Y to D	Y24D	799	AA43K to V	K44V
248	AA23Y to L	Y24L	800	AA70P to R	P71R
249	AA61Y to A	Y62A	801	AA37L to Q	L38Q
250	AA61Y to D	Y62D	802	AA99V to H	V100H
251	AA61Y to E	Y62E	803	AA71G to H	G72H
252	AA61Y to K	Y62K	804	AA15H to R	H16R
253	AA61Y to L	Y62L	805	AA41P to I	P42I
254	AA61Y to N	Y62N	806	AA99V to Q	V100Q
255	AA61Y to R	Y62R	807	AA93D to R	D94R
256	AA64Y to D	Y65D	808	AA10K to Y	K11Y
257	AA63Q to W	Q64W	809	AA94L to V	L95V
258	AA64Y to A	Y65A	810	AA80L to Q	L81Q
259	AA64Y to E	Y65E	811	AA63Q to H	Q64H
260	AA64Y to F	Y65F	812	AA73V to L	V74L

261	AA64Y to H	Y65H	813	AA70P to N	P71N
262	AA64Y to I	Y65I	814	AA56R to I	R57I
263	AA64Y to K	Y65K	815	AA70P to K	P71K
264	AA64Y to L	Y65L	816	AA15H to N	H16N
265	AA64Y to N	Y65N	817	AA87A to V	A88V
266	AA64Y to Q	Y65Q	818	AA65R to D	R66D
267	AA64Y to R	Y65R	819	AA101Q to Y	Q102Y
268	AA64Y to V	Y65V	820	AA67G to A	G68A
269	AA65R to F	R66F	821	AA102K to N	K103N
270	AA65R to Y	R66Y	822	AA46L to E	L47E
271	AA66V to D	V67D	823	AA3L to N	L4N
272	AA66V to E	V67E	824	AA18T to S	T19S
273	AA66V to F	V67F	825	AA43K to W	K44W
274	AA66V to H	V67H	826	AA63Q to V	Q64V
275	AA66V to K	V67K	827	AA43K to L	K44L
276	AA66V to N	V67N	828	AA63Q to I	Q64I
277	AA66V to Q	V67Q	829	AA102K to D	K103D
278	AA66V to R	V67R	830	AA3L to Q	L4Q
279	AA66V to W	V67W	831	AA80L to T	L81T
280	AA66V to Y	V67Y	832	AA56R to A	R57A
281	AA67G to E	G68E	833	AA56R to D	R57D
282	AA67G to F	G68F	834	AA84D to K	D85K
283	AA67G to H	G68H	835	AA86V to L	V87L
284	AA67G to I	G68I	836	AA93D to V	D94V
285	AA67G to K	G68K	837	AA70P to Q	P71Q
286	AA67G to L	G68L	838	AA99V to W	V100W
287	AA67G to Q	G68Q	839	AA52I to Y	I53Y
288	AA67G to R	G68R	840	AA65R to V	R66V
289	AA67G to V	G68V	841	AA16V to N	V17N
290	AA67G to W	G68W	842	AA14N to K	N15K
291	AA67G to Y	G68Y	843	AA50K to L	K51L
292	AA69G to A	G70A	844	AA74R to V	R75V
293	AA69G to D	G70D	845	AA7I to E	I8E
294	AA69G to F	G70F	846	AA87A to L	A88L
295	AA69G to H	G70H	847	AA15H to Q	H16Q
296	AA69G to I	G70I	848	AA3L to Y	L4Y
297	AA69G to K	G70K	849	AA65R to E	R66E
298	AA69G to L	G70L	850	AA74R to L	R75L
299	AA69G to N	G70N	851	AA11N to V	N12V
300	AA69G to Q	G70Q	852	AA74R to I	R75I
301	AA69G to R	G70R	853	AA6F to W	F7W
302	AA69G to V	G70V	854	AA86V to E	V87E
303	AA69G to W	G70W	855	AA88E to F	E89F
304	AA69G to Y	G70Y	856	AA97G to K	G98K

305	AA70P to W	P71W	857	AA70P to H	P71H
306	AA71G to F	G72F	858	AA61Y to Q	Y62Q
307	AA71G to I	G72I	859	AA61Y to I	Y62I
308	AA71G to K	G72K	860	AA112Q to I	Q113I
309	AA71G to L	G72L	861	AA52I to F	I53F
310	AA71G to Q	G72Q	862	AA105R to Q	R106Q
311	AA71G to R	G72R	863	AA102K to H	K103H
312	AA71G to V	G72V	864	AA84D to V	D85V
313	AA72W to D	W73D	865	AA112Q to V	Q113V
314	AA72W to L	W73L	866	AA42G to H	G43H
315	AA72W to V	W73V	867	AA82G to Q	G83Q
316	AA73V to D	V74D	868	AA63Q to K	Q64K
317	AA73V to E	V74E	869	AA34E to Y	E35Y
318	AA73V to H	V74H	870	AA88E to A	E89A
319	AA73V to K	V74K	871	AA70P to D	P71D
320	AA73V to N	V74N	872	AA70P to Y	P71Y
321	AA73V to Q	V74Q	873	AA92G to W	G93W
322	AA73V to R	V74R	874	AA86V to H	V87H
323	AA73V to W	V74W	875	AA37L to W	L38W
324	AA73V to Y	V74Y	876	AA70P to F	P71F
325	AA74R to D	R75D	877	AA104V to Q	V105Q
326	AA74R to F	R75F	878	AA16V to E	V17E
327	AA74R to N	R75N	879	AA12N to Y	N13Y
328	AA74R to W	R75W	880	AA37L to F	L38F
329	AA74R to Y	R75Y	881	AA104V to Y	V105Y
330	AA75V to D	V76D	882	AA88E to Q	E89Q
331	AA75V to E	V76E	883	AA86V to F	V87F
332	AA75V to H	V76H	884	AA93D to I	D94I
333	AA75V to K	V76K	885	AA2D to H	D3H
334	AA75V to N	V76N	886	AA82G to V	G83V
335	AA75V to Q	V76Q	887	AA105R to F	R106F
336	AA75V to R	V76R	888	AA63Q to N	Q64N
337	AA75V to W	V76W	889	AA24S to T	S25T
338	AA75V to Y	V76Y	890	AA42G to N	G43N
339	AA77F to A	F78A	891	AA70P to V	P71V
340	AA77F to D	F78D	892	AA12N to F	N13F
341	AA77F to E	F78E	893	AA2D to N	D3N
342	AA77F to H	F78H	894	AA63Q to R	Q64R
343	AA77F to I	F78I	895	AA86V to R	V87R
344	AA77F to K	F78K	896	AA42G to A	G43A
345	AA77F to L	F78L	897	AA75V to L	V76L
346	AA77F to N	F78N	898	AA36Q to L	Q37L
347	AA77F to Q	F78Q	899	AA92G to L	G93L
348	AA77F to R	F78R	900	AA65R to L	R66L

349	AA80L to Y	L81Y		901	AA99V to E	V100E
350	AA81V to W	V82W		902	AA41P to Q	P42Q
351	AA82G to W	G83W		903	AA84D to R	D85R
352	AA84D to W	D85W		904	AA70P to I	P71I
353	AA85E to F	E86F		905	AA37L to R	L38R
354	AA85E to K	E86K		906	AA15H to V	H16V
355	AA85E to L	E86L		907	AA12N to R	N13R
356	AA85E to N	E86N		908	AA42G to Q	G43Q
357	AA85E to Q	E86Q		909	AA22I to H	I23H
358	AA85E to R	E86R		910	AA101Q to F	Q102F
359	AA85E to V	E86V		911	AA37L to Y	L38Y
360	AA85E to W	E86W		912	AA41P to A	P42A
361	AA85E to Y	E86Y		913	AA42G to F	G43F
362	AA86V to N	V87N		914	AA70P to E	P71E
363	AA86V to W	V87W		915	AA15H to A	H16A
364	AA87A to D	A88D		916	AA50K to Q	K51Q
365	AA87A to E	A88E		917	AA3L to K	L4K
366	AA87A to H	A88H		918	AA99V to N	V100N
367	AA87A to I	A88I		919	AA34E to K	E35K
368	AA87A to K	A88K		920	AA102K to V	K103V
369	AA87A to R	A88R		921	AA7I to V	I8V
370	AA88E to W	E89W		922	AA70P to L	P71L
371	AA89W to A	W90A		923	AA39I to V	I40V
372	AA89W to D	W90D		924	AA22I to R	I23R
373	AA89W to E	W90E		925	AA82G to I	G83I
374	AA89W to H	W90H		926	AA5S to T	S6T
375	AA89W to K	W90K		927	AA82G to L	G83L
376	AA89W to N	W90N		928	AA94L to E	L95E
377	AA89W to Q	W90Q		929	AA10K to F	K11F
378	AA89W to R	W90R		930	AA19R to L	R20L
379	AA92G to F	G93F		931	AA10K to H	K11H
380	AA92G to I	G93I		932	AA65R to H	R66H
381	AA92G to V	G93V		933	AA103P to V	P104V
382	AA93D to W	D94W		934	AA50K to Y	K51Y
383	AA94L to D	L95D		935	AA23Y to V	Y24V
384	AA94L to F	L95F		936	AA17F to V	F18V
385	AA94L to H	L95H		937	AA12N to Q	N13Q
386	AA94L to K	L95K		938	AA63Q to L	Q64L
387	AA94L to N	L95N		939	AA34E to L	E35L
388	AA94L to R	L95R		940	AA94L to A	L95A
389	AA94L to W	L95W		941	AA2D to Q	D3Q
390	AA94L to Y	L95Y		942	AA9I to F	I10F
391	AA95P to A	P96A		943	AA109R to Q	R110Q
392	AA95P to D	P96D		944	AA12N to K	N13K

393	AA95P to E	P96E	945	AA36Q to V	Q37V
394	AA95P to F	P96F	946	AA88E to D	E89D
395	AA95P to H	P96H	947	AA36Q to N	Q37N
396	AA95P to I	P96I	948	AA9I to H	I10H
397	AA95P to L	P96L	949	AA88E to H	E89H
398	AA95P to Q	P96Q	950	AA77F to W	F78W
399	AA95P to R	P96R	951	AA42G to E	G43E
400	AA95P to V	P96V	952	AA17F to W	F18W
401	AA95P to W	P96W	953	AA10K to L	K11L
402	AA95P to Y	P96Y	954	AA65R to W	R66W
403	AA97G to I	G98I	955	AA34E to Q	E35Q
404	AA97G to L	G98L	956	AA61Y to V	Y62V
405	AA97G to V	G98V	957	AA15H to D	H16D
406	AA97G to W	G98W	958	AA84D to L	D85L
407	AA98F to D	F99D	959	AA2D to V	D3V
408	AA98F to K	F99K	960	AA104V to F	V105F
409	AA98F to R	F99R	961	AA67G to N	G68N
410	AA100L to D	L101D	962	AA80L to H	L81H
411	AA100L to E	L101E	963	AA51D to N	D52N
412	AA100L to H	L101H	964	AA104V to E	V105E
413	AA100L to N	L101N	965	AA2D to Y	D3Y
414	AA100L to W	L101W	966	AA102K to Y	K103Y
415	AA103P to D	P104D	967	AA112Q to Y	Q113Y
416	AA103P to F	P104F	968	AA111L to F	L112F
417	AA103P to H	P104H	969	AA112Q to E	Q113E
418	AA103P to K	P104K	970	AA84D to I	D85I
419	AA103P to L	P104L	971	AA43K to I	K44I
420	AA103P to N	P104N	972	AA52I to W	I53W
421	AA103P to Q	P104Q	973	AA102K to I	K103I
422	AA103P to R	P104R	974	AA116E to L	E117L
423	AA103P to W	P104W	975	AA88E to K	E89K
424	AA103P to Y	P104Y	976	AA65R to I	R66I
425	AA107G to I	G108I	977	AA10K to A	K11A
426	AA107G to V	G108V	978	AA80L to W	L81W
427	AA110P to A	P111A	979	AA34E to F	E35F
428	AA110P to D	P111D	980	AA50K to W	K51W
429	AA110P to E	P111E	981	AA116E to Q	E117Q
430	AA110P to F	P111F	982	AA51D to Q	D52Q
431	AA110P to H	P111H	983	AA102K to L	K103L
432	AA110P to I	P111I	984	AA45D to H	D46H
433	AA110P to K	P111K	985	AA36Q to I	Q37I
434	AA110P to L	P111L	986	AA10K to W	K11W
435	AA110P to N	P111N	987	AA10K to Q	K11Q
436	AA110P to Q	P111Q	988	AA42G to W	G43W

437	AA110P to R	P111R	989	AA80L to D	L81D
438	AA110P to V	P111V	990	AA82G to K	G83K
439	AA110P to W	P111W	991	AA72W to F	W73F
440	AA110P to Y	P111Y	992	AA46L to D	L47D
441	AA111L to D	L112D	993	AA74R to H	R75H
442	AA111L to E	L112E	994	AA48I to E	I49E
443	AA111L to K	L112K	995	AA2D to A	D3A
444	AA111L to R	L112R	996	AA10K to N	K11N
445	AA111L to W	L112W	997	AA79S to T	S80T
446	AA113A to D	A114D	998	AA82G to R	G83R
447	AA113A to E	A114E	999	AA100L to I	L101I
448	AA113A to H	A114H	1000	AA23Y to A	Y24A
449	AA113A to K	A114K	1001	AA74R to E	R75E
450	AA113A to N	A114N	1002	AA2D to K	D3K
451	AA113A to Q	A114Q	1003	AA4S to T	S5T
452	AA113A to R	A114R	1004	AA20T to S	T21S
453	AA115F to D	F116D	1005	AA34E to R	E35R
454	AA115F to E	F116E	1006	AA104V to H	V105H
455	AA115F to H	F116H	1007	AA104V to N	V105N
456	AA115F to I	F116I	1008	AA88E to V	E89V
457	AA115F to K	F116K	1009	AA64Y to W	Y65W
458	AA115F to N	F116N	1010	AA88E to L	E89L
459	AA115F to Q	F116Q	1011	AA112Q to N	Q113N
460	AA115F to R	F116R	1012	AA80L to R	L81R
461	AA115F to V	F116V	1013	AA19R to V	R20V
462	AA117A to H	A118H	1014	AA2D to W	D3W
463	AA117A to K	A118K	1015	AA58T to S	T59S
464	AA117A to N	A118N	1016	AA87A to W	A88W
465	AA117A to Q	A118Q	1017	AA104V to W	V105W
466	AA117A to R	A118R	1018	AA72W to Y	W73Y
467	AA117A to W	A118W	1019	AA2D to R	D3R
468	AA117A to Y	A118Y	1020	AA12N to A	N13A
469	AA6F to I	F7I	1021	AA41P to E	P42E
470	AA98F to I	F99I	1022	AA100L to V	L101V
471	AA7I to R	I8R	1023	AA2D to I	D3I
472	AA17F to L	F18L	1024	AA75V to F	V76F
473	AA98F to V	F99V	1025	AA102K to W	K103W
474	AA6F to H	F7H	1026	AA2D to F	D3F
475	AA98F to L	F99L	1027	AA88E to N	E89N
476	AA105R to I	R106I	1028	AA105R to Y	R106Y
477	AA6F to V	F7V	1029	AA116E to A	E117A
478	AA6F to Y	F7Y	1030	AA34E to V	E35V
479	AA46L to F	L47F	1031	AA59S to T	S60T
480	AA101Q to L	Q102L	1032	AA22I to Q	I23Q

481	AA3L to H	L4H	1033	AA105R to H	R106H
482	AA119K to Q	K120Q	1034	AA50K to A	K51A
483	AA55I to D	I56D	1035	AA99V to D	V100D
484	AA80L to E	L81E	1036	AA12N to E	N13E
485	AA97G to D	G98D	1037	AA86V to D	V87D
486	AA6F to K	F7K	1038	AA47F to W	F48W
487	AA52I to K	I53K	1039	AA116E to H	E117H
488	AA119K to F	K120F	1040	AA88E to I	E89I
489	AA6F to Q	F7Q	1041	AA112Q to W	Q113W
490	AA46L to R	L47R	1042	AA89W to V	V90V
491	AA53L to D	L54D	1043	AA12N to L	N13L
492	AA6F to R	F7R	1044	AA34E to I	E35I
493	AA112Q to R	Q113R	1045	AA109R to H	R110H
494	AA61Y to W	Y62W	1046	AA84D to Y	D85Y
495	AA3L to F	L4F	1047	AA3L to D	L4D
496	AA46L to H	L47H	1048	AA101Q to W	Q102W
497	AA46L to Y	L47Y	1049	AA109R to N	R110N
498	AA36Q to A	Q37A	1050	AA41P to R	P42R
499	AA119K to V	K120V	1051	AA88E to R	E89R
500	AA50K to R	K51R	1052	AA116E to N	E117N
501	AA83A to Q	A84Q	1053	AA50K to N	K51N
502	AA46L to Q	L47Q	1054	AA89W to I	W90I
503	AA56R to H	R57H	1055	AA113A to I	A114I
504	AA46L to K	L47K	1056	AA112Q to F	Q113F
505	AA9I to V	I10V	1057	AA102K to F	K103F
506	AA44W to Q	W45Q	1058	AA116E to Y	E117Y
507	AA119K to I	K120I	1059	AA71G to N	G72N
508	AA53L to E	L54E	1060	AA94L to Q	L95Q
509	AA55I to N	I56N	1061	AA42P to D	P42D
510	AA13S to T	S14T	1062	AA23Y to Q	Y24Q
511	AA52I to N	I53N	1063	AA107G to R	G108R
512	AA105R to K	R106K	1064	AA107G to H	G108H
513	AA44W to E	W45E	1065	AA12N to D	N13D
514	AA119K to Y	K120Y	1066	AA87A to N	A88N
515	AA71G to W	G72W	1067	AA23Y to E	Y24E
516	AA87A to Y	A88Y	1068	AA36Q to H	Q37H
517	AA55I to Q	I56Q	1069	AA116E to K	E117K
518	AA53L to H	L54H	1070	AA49L to Q	L50Q
519	AA9I to R	I10R	1071	AA23Y to K	Y24K
520	AA105R to V	R106V	1072	AA80L to K	L81K
521	AA14N to D	N15D	1073	AA116E to R	E117R
522	AA119K to W	L120W	1074	AA116E to W	E117W
523	AA83A to R	A84R	1075	AA23Y to R	Y24R
524	AA10K to I	K11I	1076	AA36Q to D	Q37D

525	AA54S to M	S55M	1077	AA107G to A	G108A
526	AA113A to V	A114V	1078	AA116E to F	E117F
527	AA55I to E	I56E	1079	AA63Q to F	Q64F
528	AA112Q to K	Q113K	1080	AA23Y to I	Y24I
529	AA48I to R	I49R	1081	AA41P to K	P42K
530	AA52I to R	I53R	1082	AA80L to F	L81F
531	AA120Q to L	Q121L	1083	AA112Q to L	Q113L
532	AA83A to H	A84H	1084	AA63Q to Y	Q64Y
533	AA52I to Q	I53Q	1085	AA50K to E	K51E
534	AA96D to Y	D97Y	1086	AA80L to N	L81N
535	AA119K to H	K120H	1087	AA103P to A	P104A
536	AA53L to K	L54K	1088	AA89W to Y	W90Y
537	AA53L to N	L54N	1089	AA89W to L	W90L
538	AA43K to Q	K44Q	1090	AA19R to I	R20I
539	AA55I to Y	I56Y	1091	AA19R to Q	R20Q
540	AA56R to Y	R57Y	1092	AA107G to N	G108N
541	AA38S to T	S39T	1093	AA23Y to N	Y24N
542	AA120Q to I	Q121I	1094	AA8T to S	T9S
543	AA101Q to I	Q102I	1095	AA22I to W	I23W
544	AA52I to E	I53E	1096	AA72W to H	W73H
545	AA10K to V	K11V	1097	AA89W to F	W90F
546	AA120Q to V	Q121V	1098	AA107G to K	G108K
547	AA116E to I	E117I	1099	AA19R to A	R20A
548	AA43K to H	K44H	1100	AA109R to Y	R110Y
549	AA119K to N	K120N	1101	AA101Q to D	Q102D
550	AA53L to Y	L54Y	1102	AA85E to A	E86A
551	AA116E to V	E117V	1103	AA109R to F	R110F
552	AA23Y to W	Y24W	1104	AA48I to C	I49C
			1105	AA32S to C	S33C
			1106	AAY23 to C	Y24C
			1107	AAY61 to C	Y62C

[0602] All single site mutations of Table 8 can be introduced into the HTS-2 isoform of SEQ ID NO:141. All single site mutations of Table 8 can also have methionine missing at position 1. All single site mutations of Table 8 can be generated with or without a His-tag or other protein tag.

[0603] EXAMPLE 16: Additional HTS Variants Exhibit Sweet Taste in Tasting Assays

[0604] Table 9 includes additional HTS single site mutational variants which exhibit sweet taste. Variants are identified (column 2) with respect to the variation from SEQ ID NO:3 as well as based on amino acid numbering when the methionine at position 1 is absent. The presence or absence of methionine does not affect sweet taste of variants. The concentration at which sweet taste was detected is shown in column 3, each sample

volume was 100 μ L, thus between 40-60 μ g of protein was tested. Entries where the concentration is labeled with “*” indicate that not all taste testers indicated that the sample was sweet.

TABLE 9: Variants Exhibiting Sweet Taste			
Mutant No. as in Table 8	Amino Acid Substitution (no Met at position 1)	Amino Acid Substitution (Met present at position 1) Position with respect to SEQ ID NO:3	Sweetness and Concentration, mg/mL
1002	AA2D to K	D3K	0.60
893	AA2D to N	D3N	0.65
941	AA2D to Q	D3Q	0.62
495	AA3L to F	L4F	0.65
848	AA3L to Y	L4Y	0.62
469	AA6F to I	F6I	0.42
921	AA7I to V	I8V	0.84
795	AA9I to L	I10L	0.64
931	AA10K to H	K11H	0.77
937	AA12N to Q	N13Q	0.47
669	AA14N to Q	N14Q	0.75
804	AA15H to R	H16R	0.33
768	AA16V to F	V17F	0.53
472	AA17F to L	F18L	0.57
722	AA21A to F	A22F	0.48
616	AA22I to L	I23L	0.69
552	AA23Y to W	Y24W	0.68
27	AA34E to N	E35N	0.85
947	AA36Q to N	Q37N	0.73
880	AA37L to F	L38F	0.71
541	AA38S to T	S39T	0.61
679	AA39I to L	I40L	0.76
896	AA42G to A	G43A	0.23*
601	AA43K to R	K44R	0.42
692	AA44W to Y	W45Y	0.47

479	AA46L to F	L47F	0.51
46	AA47F to I	F48I	0.61*
134	AA50K to D	K51D	0.62
500	AA50K to R	K51R	0.33
18	AA51D to H	D52H	0.48
20	AA51D to K	D52K	0.40
487	AA52I to K	I53K	0.45
839	AA52I to Y	I53Y	0.48
628	AA54S to T	S55T	0.38
610	AA55I to F	I56F	0.46
494	AA61Y to W	Y62W	0.25
888	AA63Q to N	Q64N	0.25*
1009	AA64Y to W	Y65W	0.44
658	AA65R to K	R66K	0.47
273	AA66V to F	V67F	0.23*
820	AA67G to A	G68A	0.40*
602	AA68D to E	D69E	0.43
693	AA71G to A	G72A	0.40
245	AA72W to Q	W73Q	0.35*
583	AA73V to I	V74I	0.28
723	AA74R to K	R75K	0.41
664	AA80L to V	L81V	0.40
651	AA81V to D	V82D	0.40*
598	AA81V to E	V82E	0.40*
640	AA81V to F	V82F	0.40
770	AA82G to A	G83A	0.40*
645	AA82G to H	G83H	0.40
587	AA83A to F	A84F	0.40
756	AA84D to N	D85N	0.40
835	AA86V to L	V87L	0.40
946	AA88E to D	E89D	0.40

621	AA92G to A	G93A	0.35
681	AA93D to E	D94E	0.53
809	AA94L to V	L95V	0.41
588	AA96D to E	D97E	0.26
404	AA97G to L	G97L	0.40
470	AA98F to I	F99I	0.40
712	AA99V to L	V100L	0.40
1022	AA100L to V	L101V	0.40*
688	AA102K to R	K103R	0.40
933	AA103P to V	P104V	0.40
960	AA104V to F	V105F	0.40
512	AA105R to K	R106K	0.40
1077	AA107G to A	G108A	0.40
213	AA109R to E	R110E	0.31
796	AA109R to K	R110K	0.27
968	AA111L to F	L112F	0.33*
1011	AA112Q to N	Q113N	0.43
615	AA119K to R	K120R	0.40
631	AA120Q to N	Q121N	0.40*

[0605] All single site mutations of Table 9 can be introduced into the HTS-2 isoform of SEQ ID NO:141. All single site mutations of Table 9 can also have methionine missing at position 1. All single site mutations of Table 9 can be generated with or without a His-tag or other protein tag.

[0606] EXAMPLE 17: Additional HTS Variants Exhibiting Sweet Taste

[0607] Table 10 includes additional HTS single site mutational variants which exhibit sweet taste. Variants are identified (column 2) with respect to the variation from SEQ ID NO:3 as well as based on amino acid numbering when the methionine at position 1 is absent. The presence or absence of methionine does not affect sweet taste of variants.

[0608] For sweet tasting variants the following are preferred where amino acid numbering is based on SEQ ID NO:3:

[0609] Positions 20, 66 and 75 should maintain a positively charged side-chain;

[0610] Substitutions of aspartate or glutamate are not preferred at positions 18-21, 58-67, or 73-81;

[0611] Aspartates or glutamates should not be added in positions 1-4,26-29,53-57,82-86,109-111;

[0612] Substitutions of D85 and E86 are allowed, but substitution of D for E and E for D are not preferred;

[0613] Substitutions of non-polar side chains (F,G,I,P,T,V,W,Y) in 5-13,18-21,24-25,30-34,39-40,45-52,58-67,73-81,87-93 or 112-120 may not be for alpha-helix favoring residues (A,K,L,M) except that I can be substituted for L;

[0614] Prolines at positions 2, 36, 42, 71 and 96 should be maintained;

[0615] Prolines at positions 34 and 104 should only be substituted to amino acid that favor beta sheet formation (e.g., F,G,I,T,V,W,Y); and/or

[0616] Substitutions of aliphatic, aromatic, or neutral base residues (e.g., A,F,G,I,L,N,Q,W,V,Y) in positions 5-13,18-21,24-25,30-34,39-40,45-52,58-67,73-81,87-93 or 112-120 should not have a change in side chain volume greater than 50Å³.

Table 10: Additional Sweet Taste Variants				
Mutant No. (Table 8)	Variant ID Without Met01Table 10:	Amino Acid Substitution (Met present at position 1)	Amino Acid in SEQ ID NO:3	Variant Amino Acid
7	D2L	3	D	L
8	D45F	46	D	F
9	D45I	46	D	I
10	D45K	46	D	K
11	D45L	46	D	L
12	D45Q	46	D	Q
13	D45R	46	D	R
14	D45V	46	D	V
15	D45W	46	D	W
16	D45Y	46	D	Y
17	D51F	52	D	F
18	D51H	52	D	H
19	D51I	52	D	I
20	D51K	52	D	K
21	D51L	52	D	L
22	D51R	52	D	R
23	D51V	52	D	V
24	D51W	52	D	W
25	D51Y	52	D	Y
26	E34H	35	E	H
27	E34N	35	E	N
28	E34W	35	E	W
29	E85H	86	E	H
30	E85I	86	E	I
32	F115W	116	F	W
33	F115Y	116	F	Y
40	F17Q	18	F	Q
46	F47I	48	F	I
50	F47Q	48	F	Q
56	F98E	99	F	E
57	F98H	99	F	H

58	F98N	99	F	N
59	F98Q	99	F	Q
60	F98W	99	F	W
61	F98Y	99	F	Y
62	G107E	108	G	E
63	G107D	108	G	D
64	G107L	108	G	L
65	G107Q	108	G	Q
66	G107F	108	G	F
67	G107W	108	G	W
68	G107Y	108	G	Y
69	G42I	43	G	I
70	G42K	43	G	K
71	G42L	43	G	L
72	G42R	43	G	R
73	G42V	43	G	V
74	G42Y	43	G	Y
102	G71D	72	G	D
103	G71E	72	G	E
106	I9Y	10	I	Y
107	I22D	23	I	D
108	I22E	23	I	E
109	I22N	23	I	N
112	I39F	40	I	F
114	I39N	40	I	N
115	I39Q	40	I	Q
118	I39Y	40	I	Y
119	I48D	49	I	D
120	I48F	49	I	F
122	I48N	49	I	N
124	I48Y	49	I	Y
128	I7N	8	I	N
129	I7Q	8	I	Q
131	I7Y	8	I	Y
132	K10D	11	K	D
133	K10E	11	K	E
134	K50D	51	K	D
135	K50F	51	K	F
136	L100A	101	L	A
137	L100F	101	L	F
138	L100K	101	L	K
139	L100Q	101	L	Q
140	L100R	101	L	R
141	L100Y	101	L	Y
143	L111N	112	L	N
144	L111Q	112	L	Q
145	L111Y	112	L	Y
146	L37D	38	L	D
147	L37N	38	L	N
151	L49F	50	L	F
154	L49N	50	L	N
156	L49V	50	L	V
158	L49Y	50	L	Y
159	N11A	12	N	A

167	N11Q	12	N	Q
172	N12V	13	N	V
175	P103I	104	P	I
176	P33V	34	P	V
177	P33W	34	P	W
178	P33Y	34	P	Y
204	Q36F	37	Q	F
205	Q36W	37	Q	W
206	Q36Y	37	Q	Y
207	R105A	106	R	A
208	R105D	106	R	D
209	R105E	106	R	E
210	R105N	106	R	N
211	R105W	106	R	W
214	R109L	110	R	L
215	R109V	110	R	V
216	R109W	110	R	W
217	R109I	110	R	I
221	R19H	20	R	H
225	R56F	57	R	F
226	R56L	57	R	L
227	S91T	92	S	T
228	V104D	105	V	D
235	V62N	63	V	N
236	V62Q	63	V	Q
260	Y64F	65	Y	F
266	Y64Q	65	Y	Q
276	V66N	67	V	N
277	V66Q	67	V	Q
281	G67E	68	G	E
282	G67F	68	G	F
283	G67H	68	G	H
284	G67I	68	G	I
285	G67K	68	G	K
286	G67L	68	G	L
287	G67Q	68	G	Q
288	G67R	68	G	R
289	G67V	68	G	V
290	G67W	68	G	W
291	G67Y	68	G	Y
292	G69A	70	G	A
293	G69D	70	G	D
294	G69F	70	G	F
295	G69H	70	G	H
296	G69I	70	G	I
297	G69K	70	G	K
298	G69L	70	G	L
299	G69N	70	G	N
300	G69Q	70	G	Q
301	G69R	70	G	R
302	G69V	70	G	V
303	G69W	70	G	W
304	G69Y	70	G	Y
306	G71F	72	G	F

307	G71I	72	G	I
308	G71K	72	G	K
309	G71L	72	G	L
310	G71Q	72	G	Q
311	G71R	72	G	R
312	G71V	72	G	V
320	V73N	74	V	N
321	V73Q	74	V	Q
334	V75N	76	V	N
335	V75Q	76	V	Q
343	F77I	78	F	I
347	F77Q	78	F	Q
349	L80Y	81	L	Y
350	V81W	82	V	W
351	G82W	83	G	W
352	D84W	85	D	W
353	E85F	86	E	F
354	E85K	86	E	K
355	E85L	86	E	L
356	E85N	86	E	N
357	E85Q	86	E	Q
358	E85R	86	E	R
359	E85V	86	E	V
360	E85W	86	E	W
361	E85Y	86	E	Y
362	V86N	87	V	N
370	E88W	89	E	W
382	D93W	94	D	W
383	L94D	95	L	D
384	L94F	95	L	F
385	L94H	95	L	H
386	L94K	95	L	K
387	L94N	95	L	N
388	L94R	95	L	R
389	L94W	95	L	W
390	L94Y	95	L	Y
403	G97I	98	G	I
404	G97L	98	G	L
405	G97V	98	G	V
406	G97W	98	G	W
407	F98D	99	F	D
408	F98K	99	F	K
409	F98R	99	F	R
410	L100D	101	L	D
411	L100E	101	L	E
412	L100H	101	L	H
413	L100N	101	L	N
414	L100W	101	L	W
416	P103F	104	P	F
423	P103W	104	P	W
424	P103Y	104	P	Y
425	G107I	108	G	I
426	G107V	108	G	V
427	P110A	111	P	A

430	P110F	111	P	F
431	P110H	111	P	H
432	P110I	111	P	I
433	P110K	111	P	K
434	P110L	111	P	L
435	P110N	111	P	N
436	P110Q	111	P	Q
437	P110R	111	P	R
438	P110V	111	P	V
439	P110W	111	P	W
440	P110Y	111	P	Y
450	A113N	114	A	N
456	F115I	116	F	I
459	F115Q	116	F	Q
464	A117N	118	A	N
469	F6I	7	F	I
470	F98I	99	F	I
473	F98V	99	F	V
475	F98L	99	F	L
476	R105I	106	R	I
478	F6Y	7	F	Y
479	L46F	47	L	F
480	Q101L	102	Q	L
481	L3H	4	L	H
482	K119Q	120	K	Q
487	I52K	53	I	K
488	K119F	120	K	F
489	F6Q	7	F	Q
494	Y61W	62	Y	W
495	L3F	4	L	F
497	L46Y	47	L	Y
498	Q36A	37	Q	A
499	K119V	120	K	V
500	K50R	51	K	R
501	A83Q	84	A	Q
502	L46Q	47	L	Q
503	R56H	57	R	H
505	I9V	10	I	V
509	I55N	56	I	N
510	S13T	14	S	T
511	I52N	53	I	N
512	R105K	106	R	K
514	K119Y	120	K	Y
515	G71W	72	G	W
517	I55Q	56	I	Q
518	L53H	54	L	H
520	R105V	106	R	V
521	N14D	15	N	D
523	A83R	84	A	R
524	K10I	11	K	I
525	S54M	55	S	M
526	A113V	114	A	V
530	I52R	53	I	R
531	Q120L	121	Q	L

532	A83H	84	A	H
533	I52Q	53	I	Q
534	D96Y	97	D	Y
535	K119H	120	K	H
536	L53K	54	L	K
537	L53N	54	L	N
538	K43Q	44	K	Q
539	I55Y	56	I	Y
540	R56Y	57	R	Y
541	S38T	39	S	T
542	Q120I	121	Q	I
543	Q101I	102	Q	I
545	K10V	11	K	V
546	Q120V	121	Q	V
547	E116I	117	E	I
548	K43H	44	K	H
549	K119N	120	K	N
550	L53Y	54	L	Y
551	E116V	117	E	V
552	Y23W	24	Y	W
553	I55H	56	I	H
554	Q101R	102	Q	R
555	N14A	15	N	A
556	Q120R	121	Q	R
557	A21Q	22	A	Q
558	D68N	69	D	N
559	V81N	82	V	N
560	I55K	56	I	K
561	G97N	98	G	N
562	Q120K	121	Q	K
563	R56N	57	R	N
564	R56Q	57	R	Q
565	Y61F	62	Y	F
566	Q101V	102	Q	V
567	Q120W	121	Q	W
568	A83K	84	A	K
570	Q36R	37	Q	R
572	L53Q	54	L	Q
573	V99F	100	V	F
574	K50I	51	K	I
578	G71Y	72	G	Y
580	N14E	15	N	E
581	I55W	56	I	W
583	V73I	74	V	I
584	G82Y	83	G	Y
585	I22Y	23	I	Y
587	A83F	84	A	F
588	D96E	97	D	E
589	L53F	54	L	F
590	K119L	120	K	L
591	I9Q	10	I	Q
592	D96A	97	D	A
595	D93F	94	D	F
596	I55R	56	I	R

597	N14L	15	N	L
599	L3W	4	L	W
600	D68A	69	D	A
601	K43R	44	K	R
602	D68E	69	D	E
603	A83Y	84	A	Y
606	G82N	83	G	N
607	K43N	44	K	N
608	V99Y	100	V	Y
609	D68Q	69	D	Q
610	I55F	56	I	F
611	L53R	54	L	R
612	V16K	17	V	K
615	K119R	120	K	R
616	I22L	23	I	L
617	K43E	44	K	E
618	A21E	22	A	E
619	D96R	97	D	R
620	K50H	51	K	H
622	D93N	94	D	N
623	V99I	100	V	I
624	D96K	97	D	K
625	D93H	94	D	H
627	H15F	16	H	F
628	S54T	55	S	T
630	D96Q	97	D	Q
631	Q120N	121	Q	N
632	I9N	10	I	N
634	V81K	82	V	K
635	D68K	69	D	K
636	V81Y	82	V	Y
637	D93Y	94	D	Y
638	N14Y	15	N	Y
639	V104K	105	V	K
640	V81F	82	V	F
641	E88Y	89	E	Y
643	Q120F	121	Q	F
644	V81Q	82	V	Q
645	G82H	83	G	H
646	V81H	82	V	H
647	N14F	15	N	F
648	D68H	69	D	H
649	A21H	22	A	H
650	D68L	69	D	L
654	D96W	97	D	W
655	Y23F	24	Y	F
657	V16H	17	V	H
658	R65K	66	R	K
659	D68R	69	D	R
660	A21K	22	A	K
661	D96L	97	D	L
662	I22V	23	I	V
663	D96V	97	D	V
664	L80V	81	L	V

665	R56K	57	R	K
666	R56V	57	R	V
667	I52H	53	I	H
669	N14Q	15	N	Q
670	R105L	106	R	L
671	Q120H	121	Q	H
672	Q101H	102	Q	H
673	N14R	15	N	R
674	Q120Y	121	Q	Y
675	G82F	83	G	F
677	D93Q	94	D	Q
678	F17I	18	F	I
679	I39L	40	I	L
680	A21R	22	A	R
681	D93E	94	D	E
682	V16D	17	V	D
683	D96N	97	D	N
685	S38M	39	S	M
686	D68V	69	D	V
687	L46N	47	L	N
688	K102R	103	K	R
689	L53W	54	L	W
690	L37E	38	L	E
691	V86I	87	V	I
692	W44Y	45	W	Y
693	G71A	72	G	A
696	D96H	97	D	H
697	D93A	94	D	A
699	K43Y	44	K	Y
700	N14I	15	N	I
701	D68Y	69	D	Y
703	D68I	69	D	I
704	W44F	45	W	F
705	L37K	38	L	K
706	D96I	97	D	I
707	R56W	57	R	W
708	D96F	97	D	F
709	K25A	26	K	A
710	S90T	91	S	T
711	D68F	69	D	F
712	V99L	100	V	L
713	I22K	23	I	K
714	D68W	69	D	W
715	K102Q	103	K	Q
718	V16Y	17	V	Y
720	H15Y	16	H	Y
721	H15L	16	H	L
722	A21F	22	A	F
723	R74K	75	R	K
725	G97Q	98	G	Q
726	V81R	82	V	R
727	K43A	44	K	A
728	G97E	98	G	E
730	D84A	85	D	A

731	N14V	15	N	V
734	V16R	17	V	R
735	T76S	77	T	S
736	V75I	76	V	I
737	A83W	84	A	W
738	N14W	15	N	W
740	G69E	70	G	E
741	H15I	16	H	I
743	K50V	51	K	V
744	V16W	17	V	W
745	E34A	35	E	A
747	V99K	100	V	K
748	A21Y	22	A	Y
749	K102E	103	K	E
750	D84Q	85	D	Q
751	D93L	94	D	L
752	I22F	23	I	F
753	V104R	105	V	R
755	A21N	22	A	N
756	D84N	85	D	N
757	G67D	68	G	D
758	D45N	46	D	N
759	H15E	16	H	E
760	G97H	98	G	H
761	K43D	44	K	D
767	N14H	15	N	H
768	V16F	17	V	F
769	G97Y	98	G	Y
770	G82A	83	G	A
771	G97R	98	G	R
772	H15K	16	H	K
774	G97F	98	G	F
775	V16Q	17	V	Q
776	L94I	95	L	I
777	L37H	38	L	H
778	Q101A	102	Q	A
779	V99R	100	V	R
780	L80I	81	L	I
781	S40T	41	S	T
782	A83N	84	A	N
784	A21D	22	A	D
788	L3R	4	L	R
789	I48Q	49	I	Q
790	V86Q	87	V	Q
791	I7F	8	I	F
793	H15W	16	H	W
794	D93K	94	D	K
795	I9L	10	I	L
796	R109K	110	R	K
797	A21W	22	A	W
798	K43F	44	K	F
799	K43V	44	K	V
801	L37Q	38	L	Q
802	V99H	100	V	H

803	G71H	72	G	H
804	H15R	16	H	R
806	V99Q	100	V	Q
807	D93R	94	D	R
808	K10Y	11	K	Y
809	L94V	95	L	V
810	L80Q	81	L	Q
814	R56I	57	R	I
816	H15N	16	H	N
817	A87V	88	A	V
819	Q101Y	102	Q	Y
820	G67A	68	G	A
821	K102N	103	K	N
823	L3N	4	L	N
824	T18S	19	T	S
825	K43W	44	K	W
826	Q63V	64	Q	V
827	K43L	44	K	L
829	K102D	103	K	D
830	L3Q	4	L	Q
832	R56A	57	R	A
834	D84K	85	D	K
836	D93V	94	D	V
838	V99W	100	V	W
839	I52Y	53	I	Y
841	V16N	17	V	N
842	N14K	15	N	K
843	K50L	51	K	L
847	H15Q	16	H	Q
848	L3Y	4	L	Y
851	N11V	12	N	V
853	F6W	7	F	W
855	E88F	89	E	F
856	G97K	98	G	K
858	Y61Q	62	Y	Q
861	I52F	53	I	F
862	R105Q	106	R	Q
863	K102H	103	K	H
864	D84V	85	D	V
865	Q112V	113	Q	V
866	G42H	43	G	H
867	G82Q	83	G	Q
869	E34Y	35	E	Y
870	E88A	89	E	A
875	L37W	38	L	W
877	V104Q	105	V	Q
878	V16E	17	V	E
880	L37F	38	L	F
881	V104Y	105	V	Y
882	E88Q	89	E	Q
884	D93I	94	D	I
885	D2H	3	D	H
886	G82V	83	G	V
887	R105F	106	R	F

888	Q63N	64	Q	N
889	S24T	25	S	T
890	G42N	43	G	N
893	D2N	3	D	N
896	G42A	43	G	A
898	Q36L	37	Q	L
901	V99E	100	V	E
903	D84R	85	D	R
905	L37R	38	L	R
906	H15V	16	H	V
908	G42Q	43	G	Q
909	I22H	23	I	H
910	Q101F	102	Q	F
911	L37Y	38	L	Y
913	G42F	43	G	F
915	H15A	16	H	A
916	K50Q	51	K	Q
917	L3K	4	L	K
918	V99N	100	V	N
919	E34K	35	E	K
920	K102V	103	K	V
921	I7V	8	I	V
923	I39V	40	I	V
924	I22R	23	I	R
925	G82I	83	G	I
926	S5T	6	S	T
927	G82L	83	G	L
928	L94E	95	L	E
929	K10F	11	K	F
931	K10H	11	K	H
932	R65H	66	R	H
933	P103V	104	P	V
934	K50Y	51	K	Y
937	N12Q	13	N	Q
939	E34L	35	E	L
940	L94A	95	L	A
941	D2Q	3	D	Q
942	I9F	10	I	F
943	R109Q	110	R	Q
945	Q36V	37	Q	V
946	E88D	89	E	D
947	Q36N	37	Q	N
949	E88H	89	E	H
950	F77W	78	F	W
951	G42E	43	G	E
952	F17W	18	F	W
953	K10L	11	K	L
955	E34Q	35	E	Q
957	H15D	16	H	D
958	D84L	85	D	L
959	D2V	3	D	V
960	V104F	105	V	F
961	G67N	68	G	N
963	D51N	52	D	N

964	V104E	105	V	E
965	D2Y	3	D	Y
966	K102Y	103	K	Y
967	Q112Y	113	Q	Y
968	L111F	112	L	F
970	D84I	85	D	I
971	K43I	44	K	I
972	I52W	53	I	W
973	K102I	103	K	I
974	E116L	117	E	L
975	E88K	89	E	K
977	K10A	11	K	A
979	E34F	35	E	F
980	K50W	51	K	W
981	E116Q	117	E	Q
982	D51Q	52	D	Q
983	K102L	103	K	L
984	D45H	46	D	H
985	Q36I	37	Q	I
986	K10W	11	K	W
987	K10Q	11	K	Q
988	G42W	43	G	W
990	G82K	83	G	K
991	W72F	73	W	F
993	R74H	75	R	H
995	D2A	3	D	A
996	K10N	11	K	N
997	S79T	80	S	T
998	G82R	83	G	R
999	L100I	101	L	I
1002	D2K	3	D	K
1003	S4T	5	S	T
1004	T20S	21	T	S
1005	E34R	35	E	R
1006	V104H	105	V	H
1007	V104N	105	V	N
1008	E88V	89	E	V
1009	Y64W	65	Y	W
1010	E88L	89	E	L
1011	Q112N	113	Q	N
1014	D2W	3	D	W
1015	T58S	59	T	S
1017	V104W	105	V	W
1018	W72Y	73	W	Y
1019	D2R	3	D	R
1020	N12A	13	N	A
1022	L100V	101	L	V
1023	D2I	3	D	I
1025	K102W	103	K	W
1026	D2F	3	D	F
1027	E88N	89	E	N
1028	R105Y	106	R	Y
1029	E116A	117	E	A
1030	E34V	35	E	V

1031	S59T	60	S	T
1032	I22Q	23	I	Q
1033	R105H	106	R	H
1034	K50A	51	K	A
1035	V99D	100	V	D
1038	F47W	48	F	W
1039	E116H	117	E	H
1040	E88I	89	E	I
1044	E34I	35	E	I
1045	R109H	110	R	H
1046	D84Y	85	D	Y
1048	Q101W	102	Q	W
1049	R109N	110	R	N
1051	E88R	89	E	R
1052	E116N	117	E	N
1053	K50N	51	K	N
1056	Q112F	113	Q	F
1057	K102F	103	K	F
1058	E116Y	117	E	Y
1059	G71N	72	G	N
1060	L94Q	95	L	Q
1062	Y23Q	24	Y	Q
1063	G107R	108	G	R
1064	G107H	108	G	H
1066	A87N	88	A	N
1068	Q36H	37	Q	H
1069	E116K	117	E	K
1070	L49Q	50	L	Q
1073	E116R	117	E	R
1074	E116W	117	E	W
1076	Q36D	37	Q	D
1077	G107A	108	G	A
1078	E116F	117	E	F
1079	Q63F	64	Q	F
1082	L80F	81	L	F
1084	Q63Y	64	Q	Y
1085	K50E	51	K	E
1086	L80N	81	L	N
1088	W89Y	90	W	Y
1092	G107N	108	G	N
1094	T8S	9	T	S
1095	I22W	23	I	W
1097	W89F	90	W	F
1098	G107K	108	G	K
1100	R109Y	110	R	Y
1101	Q101D	102	Q	D
1102	E85A	86	E	A
1103	R109F	110	R	F

[0617] All single site mutations of Table 10 can be introduced into the HTS-2 isoform of SEQ ID NO:141. All single site mutations of Table 10 can also have methionine missing at position 1. All single site mutations of Table 10 can be generated with or without a His-tag or other protein tag.

[0618] EXAMPLE 18: HTS and Sucrose in a Citric Acid Solution (Sweetener Composition)

[0619] Sucrose is the standard for sweetness intensity perception in the food and beverage industry. The absolute reference scale for sweetness intensity is based on sucrose dissolved in water (% sucrose (w/v) in water) and demonstrates a linear relationship between concentration (sucrose w/v) and response (sweetness intensity). Other carbohydrate and polyol sweeteners exhibit a linear potency curve similar to sucrose at the following multipliers, Erythritol (0.7x), and Allulose (0.7x) (DuBois GE, Walters DE, Schiffman SS, Warwick ZS, Booth BJ, et al. (1991) Concentration-response relationships of sweeteners: a systematic study. In Sweeteners: Discovery, Molecular Design and Chemoreception, ed. DE Walters, FT Orthoefer, GE DuBois, pp. 261–276. Washington, DC: Am. Chem. Soc). High potency (HP) sweeteners exhibit hyperbolic curves. With regard to the following **Examples 18 - 38**, sucrose equivalent (SE) values of non-sucrose sweeteners were calculated from Dubois et al. (1991) and cross referenced with other studies (Wee, M., Tan, V., & Forde, C. (2018). A Comparison of Psychophysical Dose-Response Behaviour across 16 Sweeteners. *Nutrients*, 10(11), 1632).

[0620] The relationship between sweetness intensity and concentration for HP sweeteners has been demonstrated to be well modeled by the law of mass action, a hyperbolic function of the form $R = R_m C / (K_d + C)$, where R is the response in units of sucrose equivalence (SE) on a percentage (w/v) basis, R_m is the maximal response in SE units, K_d is the apparent sweetener/receptor dissociation constant, and C is the sweetener concentration in mg/L (DuBois GE, Walters DE, Schiffman SS, Warwick ZS, Booth BJ, et al. 1991. Concentration-response relationships of sweeteners: a systematic study. In Sweeteners: Discovery, Molecular Design and Chemoreception, ed. DE Walters, FT Orthoefer, GE DuBois, pp. 261–276. Washington, DC: Am. Chem. Soc).

[0621] An HTS sweetness concentration response curve was developed. These values were used to determine approximate sucrose equivalents for the following blending examples (**Examples 18 - 37**).

[0622] For **Examples 18 - 38**, HTS generated by expression in *Pichia pastoris* was utilized which has been determined to be a mixture of HTS-1 and HTS-2 (with no His-tags) of relative amounts of 10% HTS-1 to 90% HTS-2 by weight.

[0623] HTS ((0.0 mg/mL, 0.007 mg/mL, 0.015 mg/mL and 0.035 mg/mL) and sucrose (8% w/w, 6% w/w, 4% w/w, and 2% w/w (respectively)) were added to a citric acid solution. The sweetener mixture was compared to a control with only sucrose added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the sucrose control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 11, 75% comes from sucrose and 25% comes from HTS, 50% comes from sucrose and 50% comes from HTS, 25% comes from sucrose and 75% comes from HTS, respectively.

[0624] Table 11: HTS and Sucrose Mixtures in a Citric Acid Solution Compared to Controls

<u>Sample</u>	<u>Sucrose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Sour, astringent, harsh.
Sucrose Control	8%	0.0 mg/mL	Reminiscent of sweet dessert and ice wines, limoncello, fruit candy, apple juice.
75 Sucrose / 25 HTS : 75% total perceived sweetness from Sucrose and 25% perceived sweetness from HTS	6%	0.007 mg/mL	Less acidic, slightly enhanced sweetness, better balance, fruitier.
50 Sucrose / 50 HTS : 50% total perceived sweetness from Sucrose and 50% perceived sweetness from HTS	4%	0.015 mg/mL	Increased acidity up front, sweet aftertaste.
25 Sucrose / 75 HTS : 25% total perceived sweetness from Sucrose and 75% perceived sweetness from HTS	2%	0.035 mg/mL	Intensely sour up front, slow build to max sweetness intensity, minimal sweet aftertaste instead mostly sour.

[0625] EXAMPLE 19: HTS and Sucrose in a Lemon/Lime Soda (Sweetener Composition)

[0626] HTS ((0.0 mg/mL, 0.008 mg/mL, 0.023 mg/mL and 0.064 mg/mL) and sucrose (10% w/w, 7.5% w/w, 5% w/w, and 2.5% w/w (respectively)) were added to a lemon/lime soda. The sweetener mixture was compared to a control with only sucrose added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the sucrose control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 12, 75% comes from sucrose and 25% comes from HTS, 50% comes from sucrose and 50% comes from HTS, 25% comes from sucrose and 75% comes from HTS, respectively.

[0627] Table 12: HTS and Sucrose Mixtures in a Lemon/Lime Soda Compared to Controls

<u>Sample</u>	<u>Sucrose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Sour, bitter, astringent, harsh, citrus.
Sucrose Control	10%	0.0 mg/mL	Good, sweet to sour balance and pleasant citrus flavor. Reminiscent of commercial lemon sides carbonated soft drinks.
75 Sucrose /25 HTS	7.5%	0.008 mg/mL	Very similar to sucrose control, slightly lower sweet intensity up front but reaches same max sweetness intensity. Pleasant, sweet lemon lime aftertaste.

Sucrose 50/50 HTS	5%	0.023 mg/mL	Sour up front, sweetness intensity builds slowly and is present in aftertaste. Lacking mouthfeel and body.
Sucrose 25/75 HTS	2.5%	0.064 mg/mL	Harsh acidity up front, sweetness intensity builds around 10 seconds peaking near 30. Strong sweet aftertaste.

[0628] EXAMPLE 20: HTS and Sucrose in an Almond Milk (Sweetener Composition)

[0629] HTS ((0.0 mg/mL, 0.005 mg/mL, 0.007 mg/mL and 0.010 mg/mL) and sucrose (4% w/w, 3.0% w/w, 2% w/w, and 1.0% w/w (respectively)) were added to an unsweetened almond milk. The sweetener mixture was compared to a control with only sucrose added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 13, 75% comes from sucrose and 25% comes from HTS, 50% comes from sucrose and 50% comes from HTS, 25% comes from sucrose and 75% comes from HTS, respectively.

[0630] Table 13: HTS and Sucrose Mixtures in an Almond Milk Compared to Controls

<u>Sample</u>	<u>Sucrose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Nutty, creamy, low sweetness.
Sucrose Control	4%	0.0 mg/mL	Pleasantly sweetened almond milk, creamy mouthfeel.
Sucrose 75/25 HTS	3.0%	0.005 mg/mL	Similar max sweetness intensity, slight sweet aftertaste, full creamy mouthfeel.
Sucrose 50/50 HTS	2%	0.007 mg/mL	Lower initial sweetness intensity, but higher max sweetness intensity overall. Saltiness up front and sweet aftertaste.
Sucrose 25/75 HTS	1.0%	0.010 mg/mL	Increased almond flavor intensity. Lower max sweetness intensity but strong sweet aftertaste.

[0631] EXAMPLE 21: HTS and Sucrose in a Chocolate Spread (Sweetener Composition)

[0632] HTS ((0.0 mg/mL, 0.008 mg/mL, 0.023 mg/mL and 0.064 mg/mL) and sucrose (45% w/w, 33.8% w/w, 23% w/w, and 11.3% w/w (respectively)) were added to a chocolate spread. The sweetener mixture was compared to a control with only sucrose added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived

sweetness intensity in each of the experimental samples in Table 14, 75% comes from sucrose and 25% comes from HTS, 50% comes from sucrose and 50% comes from HTS, 25% comes from sucrose and 75% comes from HTS, respectively.

[0633] Table 14: HTS and Sucrose Mixtures in a Chocolate Spread Compared to Controls

<u>Sample</u>	<u>Sucrose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Intense cocoa and bitter, salty, astringent.
Sucrose Control	45%	0.0 mg/mL	Strong cocoa- sweet, bitter, salty, and roasted brown notes. Reminiscent of ice cream chocolate coating.
Sucrose 75/25 HTS	33.8%	0.008 mg/mL	Lower max sweetness intensity, higher salt intensity. Reminiscent of cacao nibs.
Sucrose 50/50 HTS	23%	0.023 mg/mL	Similar max sweetness intensity to sucrose control. Pleasant nutty and cocoa flavors perceived. Some sweet and bitter cocoa aftertaste
Sucrose 25/75 HTS	11.3%	0.064 mg/mL	Bitter cocoa and salt notes. Low max sweetness intensity but some sweet aftertaste.

[0634] EXAMPLE 22: HTS and Erythritol in a Citric Acid Solution (Sweetener Composition)

[0635] HTS ((0.0 mg/mL, 0.007 mg/mL, 0.015 mg/mL and 0.035 mg/mL) and erythritol (11.43% w/w, 8.57% w/w, 5.71% w/w, and 2.86% w/w (respectively)) were added to a citric acid solution. The sweetener mixture was compared to a control with only erythritol added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 15, 75% comes from erythritol and 25% comes from HTS, 50% comes from erythritol and 50% comes from HTS, 25% comes from erythritol and 75% comes from HTS, respectively.

[0636] Table 15: HTS and Erythritol Mixtures in a Citric Acid Solution Compared to Controls

<u>Sample</u>	<u>Erythritol w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Sour, astringent, harsh.
Erythritol Control	11.43%	0 mg/mL	Reduced sour intensity compared to sucrose examples, mouth cooling effect, metallic aftertaste.

Erythritol 75/25 HTS	8.57%	0.007 mg/mL	Increased initial sour intensity compared to control, increased astringency and chalky mouthfeel.
Erythritol 50/50 HTS	5.71%	0.015 mg/mL	Increased initial sour intensity compared to control. Mouth puckering, minimal sweetness linger or aftertaste.
Erythritol 25/75 HTS	2.86%	0.035 mg/mL	Increased initial sour intensity compared to control, astringency, sweet aftertaste.

[0637] EXAMPLE 23: HTS and Erythritol in a Lemon/Lime soda (Sweetener Composition)

[0638] HTS ((0.0 mg/mL, 0.008 mg/mL, 0.023 mg/mL and 0.064 mg/mL) and erythritol (13.30% w/w, 9.31% w/w, 6.90% w/w, and 3.10% w/w (respectively)) were added to a lemon/lime soda. The sweetener mixture was compared to a control with only erythritol added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 16, 75% comes from erythritol and 25% comes from HTS, 50% comes from erythritol and 50% comes from HTS, 25% comes from erythritol and 75% comes from HTS, respectively.

[0639] Table 16: HTS and Erythritol Mixtures in a Lemon/Lime Soda Compared to Controls

<u>Samples Name</u>	<u>Erythritol w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Sour, bitter, astringent, harsh, citrus.
Erythritol Control	13.30%	0.0 mg/mL	Reduced citrus flavor intensity. Strong mouth cooling and metallic aftertaste.
Erythritol 75/25 HTS	9.31%	0.008 mg/mL	Increased citrus flavor intensity compared to control. Similar max sweetness intensity. More intense and extended citrus aftertaste compared to control.
Erythritol 50/50 HTS	6.90%	0.023 mg/mL	Reduced max sweetness intensity compared to control. Increased citrus flavor intensity compared to control. Reminiscent of tonic water, lemon lime candy.
Erythritol 25/75 HTS	3.10%	0.064 mg/mL	Reduced initial and max sweetness intensity compared to control. Strong sweet aftertaste. Reminiscent of flavored sparkling water.

[0640] EXAMPLE 24: HTS and erythritol in an almond milk (Sweetener Composition)

[0641] HTS ((0.0 mg/mL, 0.005 mg/mL, 0.007 mg/mL and 0.010 mg/mL) and erythritol (5.71% w/w, 4.29% w/w, 2.86% w/w, and 1.43% w/w (respectively)) were added to an unsweetened almond milk. The sweetener mixture was compared to a control with only erythritol added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 17, 75% comes from erythritol and 25% comes from HTS, 50% comes from erythritol and 50% comes from HTS, 25% comes from erythritol and 75% comes from HTS, respectively.

[0642] Table 17: HTS and Erythritol Mixtures in an Almond Milk Compared to Controls

<u>Samples Name</u>	<u>Erythritol w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Nutty, creamy, low sweetness.
Erythritol Control	5.71%	0.0 mg/mL	Reduced salt and almond intensity compared to unsweetened control. Mouth cooling and metallic aftertaste.
Erythritol 75/25 HTS	4.29%	0.005 mg/mL	Increased initial sweetness intensity. Increased almond intensity. Reduced mouth cooling and metallic aftertaste. Sweet and nutty aftertaste.
Erythritol 50/50 HTS	2.86%	0.007 mg/mL	Increased almond intensity. Lower max sweetness intensity but strong sweet aftertaste. Reminiscent of true dairy rounded sweetness profile.
Erythritol 25/75 HTS	1.43%	0.010 mg/mL	Similar max sweetness intensity to control. Reduced mouth cooling and metallic intensity. Strong sweet aftertaste.

[0643] EXAMPLE 25: HTS and Erythritol in a Chocolate Spread (Sweetener Composition)

[0644] HTS ((0.0 mg/mL, 0.008 mg/mL, 0.023 mg/mL and 0.064 mg/mL) and erythritol (45% w/w, 33.8% w/w, 23% w/w, and 11.3% w/w (respectively)) were added to a chocolate spread. The sweetener mixture was compared to a control with only erythritol added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 18, 75% comes from erythritol and 25% comes from HTS, 50% comes from erythritol and 50% comes from HTS, 25% comes from erythritol and 75% comes from HTS, respectively.

[0645] Table 18: HTS and Erythritol Mixtures in a Chocolate Spread Compared to Controls

<u>Samples Name</u>	<u>Erythritol w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Intense cocoa and bitter, salty, astringent.

Erythritol Control	45%	0.0 mg/mL	Brief initial sweetness, mouth cooling, cocoa associated bitter.
Erythritol 75/25 HTS	33.8%	0.008 mg/mL	Increased nutty and salt intensity, reduced max sweetness intensity and mouth cooling.
Erythritol 50/50 HTS	23%	0.023 mg/mL	Increased salt and cocoa intensity. Lower max sweetness intensity compared to control. Increased salty and cocoa aftertaste.
Erythritol 25/75 HTS	11.3%	0.064 mg/mL	Reduced max sweetness intensity.

[0646] EXAMPLE 26: HTS and Sucralose in a Citric Acid Solution (Sweetener Composition)

[0647] HTS ((0.0 mg/mL, 0.007 mg/mL, 0.015 mg/mL and 0.035 mg/mL) and sucralose (0.0170% w/w, 0.0100% w/w, 0.0053% w/w, and 0.0023% w/w (respectively)) were added to a citric acid solution. The sweetener mixture was compared to a control with only sucralose added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 19, 75% comes from sucralose and 25% comes from HTS, 50% comes from sucralose and 50% comes from HTS, 25% comes from sucralose and 75% comes from HTS, respectively.

[0648] Table 19: HTS and Sucralose Mixtures in a Citric Acid Solution Compared to Controls

<u>Samples Name</u>	<u>Sucralose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Sour, astringent, harsh.
Sucralose Control	0.0170%	0.0 mg/mL	Intense initial sweetness that overpowers acidity. Citrus and fruit flavors. Strong sweet aftertaste.
Sucralose 75/25 HTS	0.0100%	0.007 mg/mL	Similar max sweet intensity as control. Reduced astringency compared to control. Sour aftertaste.
Sucralose 50/50 HTS	0.0053%	0.015 mg/mL	Increased sour intensity compared to control. Reduced max sweet intensity compared to control.
Sucralose 25/75 HTS	0.0023%	0.035 mg/mL	Reduced max sweet intensity compared to control. Strong sweet aftertaste.

[0649] EXAMPLE 27: HTS and Sucralose in a Lemon/Lime Soda (Sweetener Composition)

[0650] HTS ((0.0 mg/mL, 0.008 mg/mL, 0.023 mg/mL and 0.064 mg/mL) and sucralose (0.0387% w/w, 0.0247% w/w, 0.0096% w/w, and 0.0030% w/w (respectively)) were added to a lemon/lime soda. The sweetener mixture was compared to a control with only sucralose added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 20, 75% comes from sucralose and 25% comes from HTS, 50% comes from sucralose and 50% comes from HTS, 25% comes from sucralose and 75% comes from HTS, respectively.

[0651] Table 20: HTS and Sucralose Mixtures in a Lemon/Lime Soda Compared to Controls

<u>Samples Name</u>	<u>Sucralose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Sour, bitter, astringent, harsh, citrus.
Sucralose Control	0.0387%	0.0 mg/mL	Intense initial sourness. Strong sweet aftertaste.
Sucralose 75/25 HTS	0.0247%	0.008 mg/mL	Increased citrus flavor intensity compared to control. Enhanced sweetness profile, similar to sucrose control.
Sucralose 50/50 HTS	0.0096%	0.023 mg/mL	Intense initial sourness. Reduced max sweetness intensity compared to control. Strong sweet aftertaste.
Sucralose 25/75 HTS	0.0030%	0.064 mg/mL	Intense initial sourness. Reduced max sweetness intensity compared to control. Strong sweet aftertaste.

[0652] EXAMPLE 28: HTS and Sucralose in an Almond Milk (Sweetener Composition)

[0653] HTS ((0.0 mg/mL, 0.005 mg/mL, 0.007 mg/mL and 0.010 mg/mL) and sucralose (0.0053% w/w, 0.0037% w/w, 0.0023% w/w, and 0.0011% w/w (respectively)) were added to an unsweetened almond milk. The sweetener mixture was compared to a control with only sucralose added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 21, 75% comes from sucralose and 25% comes from HTS, 50% comes from sucralose and 50% comes from HTS, 25% comes from sucralose and 75% comes from HTS, respectively.

[0654] Table 21: HTS and Sucralose Mixtures in an Almond Milk Compared to Controls

<u>Sample Name</u>	<u>Sucralose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
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Unsweetened Control	NA	NA	Nutty, creamy, low sweetness.
Sucralose Control	0.0053%	0.0 mg/mL	Pleasantly sweetened almond milk. Little mouthfeel or body. Sweet aftertaste.
Sucralose 75/25 HTS	0.0037%	0.005 mg/mL	Increased mouthfeel and body compared to control. Enhanced sweetness profile similar to sucrose control. Sweet and nutty aftertaste.
Sucralose 50/50 HTS	0.0023%	0.007 mg/mL	Increase almond flavor intensity. Lower max sweetness intensity but strong sweet aftertaste.
Sucralose 25/75 HTS	0.0011%	0.010 mg/mL	Increased almond flavor intensity. Lower max sweetness intensity but strong sweet aftertaste.

[0655] EXAMPLE 29: HTS and Sucralose in a Chocolate Spread (Sweetener Composition)

[0656] HTS ((0.0 mg/mL, 0.008 mg/mL, 0.023 mg/mL and 0.064 mg/mL) and sucralose (0.0387% w/w, 0.0247% w/w, 0.0096% w/w, and 0.0030% w/w (respectively)) were added to a chocolate spread. The sweetener mixture was compared to a control with only sucralose added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 22, 75% comes from sucralose and 25% comes from HTS, 50% comes from sucralose and 50% comes from HTS, 25% comes from sucralose and 75% comes from HTS, respectively.

[0657] Table 22: HTS and Sucralose Mixtures in a Chocolate Spread Compared to Controls

<u>Samples Name</u>	<u>Sucralose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Intense cocoa and bitter, salty, astringent
Sucralose Control	0.0387%	0.0 mg/mL	Reduced cocoa flavor intensity compared to sucrose control, sweet aftertaste.
Sucralose 75/25 HTS	0.0247%	0.008 mg/mL	Increased cocoa flavor intensity. Sweet aftertaste.
Sucralose 50/50 HTS	0.0096%	0.023 mg/mL	Increased cocoa flavor intensity. Sweet aftertaste.

Sucralose 25/75 HTS	0.0030%	0.064 mg/mL	Increased cocoa flavor intensity. Sweet aftertaste.
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[0658] EXAMPLE 30: HTS and Stevia (Stevia Extract) in a Citric Acid Solution (Sweetener Composition)

[0659] HTS ((0.0 mg/mL, 0.007 mg/mL, 0.015 mg/mL and 0.035 mg/mL) and stevia (0.0500% w/w, 0.0190% w/w, 0.0085% w/w, and 0.0032% w/w (respectively)) were added to a citric acid solution. The sweetener mixture was compared to a control with only stevia added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 23, 75% comes from stevia and 25% comes from HTS, 50% comes from stevia and 50% comes from HTS, 25% comes from stevia and 75% comes from HTS, respectively.

[0660] Table 23: HTS and Stevia Mixtures in a Citric Acid Solution Compared to Controls

<u>Samples Name</u>	<u>Stevia (RA95) w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Sour, astringent, harsh.
Stevia RA95 Control	0.0500%	0.0 mg/mL	Similar max sweetness intensity to sucrose control. Bitter and metallic notes. Bitter and metallic aftertaste.
Stevia RA95 75/25 HTS	0.0190%	0.007 mg/mL	Increased citrus and fruit flavor intensity compared to control. Reduced maximum sweetness intensity and sourness intensity compared to control. Reduced bitter and metallic intensity and aftertaste.
Stevia RA95 50/50 HTS	0.0085%	0.015 mg/mL	Initial sourness. Reduced bitter and metallic intensity and aftertaste. Reduced maximum sweetness intensity compared to control.
Stevia RA95 25/75 HTS	0.0032%	0.035 mg/mL	Reduced max sweet intensity compared to control. Strong sweet aftertaste.

[0661] EXAMPLE 31: HTS and Stevia in a Lemon/Lime Soda (Sweetener Composition)

[0662] HTS ((0.0 mg/mL, 0.008 mg/mL, 0.023 mg/mL and 0.064 mg/mL) and stevia (0.0750% w/w, 0.0400% w/w, 0.0130% w/w, and 0.0042% w/w (respectively)) were added to a lemon/lime soda. The sweetener mixture was compared to a control with only stevia added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness

intensity in each of the experimental samples in Table 24, 75% comes from stevia and 25% comes from HTS, 50% comes from stevia and 50% comes from HTS, 25% comes from stevia and 75% comes from HTS, respectively.

[0663] Table 24: HTS and Stevia Mixtures in a Lemon/Lime Soda Compared to Controls

<u>Samples Name</u>	<u>Stevia (RA95) w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Sour, bitter, astringent, harsh, citrus.
Stevia RA95 Control	0.0750%	0.0 mg/mL	Intense initial sweetness. Intense bitter and metallic. Strong sweet aftertaste. Reduced citrus flavor intensity compared to control.
Stevia RA95 75/25 HTS	0.0400%	0.008 mg/mL	Increased citrus flavor intensity compared to control. Increased initial sourness. Reduced maximum sweetness intensity compared to control.
Stevia RA95 50/50 HTS	0.0130%	0.023 mg/mL	Increased initial sourness. Reduced maximum sweetness intensity compared to control. Sweet aftertaste.
Stevia RA95 25/75 HTS	0.0042%	0.064 mg/mL	Increased initial sourness. Reduced maximum sweetness intensity compared to control. Sweet aftertaste.

[0664] EXAMPLE 32: HTS and Stevia in an Almond Milk (Sweetener Composition)

[0665] HTS ((0.0 mg/mL, 0.005 mg/mL, 0.007 mg/mL and 0.010 mg/mL) and stevia (0.0085% w/w, 0.0055% w/w, 0.0032% w/w, and 0.0015% w/w (respectively)) were added to an unsweetened almond milk. The sweetener mixture was compared to a control with only stevia added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 25, 75% comes from stevia and 25% comes from HTS, 50% comes from stevia and 50% comes from HTS, 25% comes from stevia and 75% comes from HTS, respectively.

[0666] Table 25: HTS and Stevia Mixtures in an Almond Milk Compared to Controls

<u>Sample Name</u>	<u>Stevia (RA95) w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Nutty, creamy, low sweetness.
Stevia RA95 Control	0.0085%	0.0 mg/mL	Similar max sweetness intensity compared to sucrose control. Bitter and metallic notes. Increased grain flavor intensity. Sweet aftertaste.

Stevia RA95 75/25 HTS	0.0055%	0.005 mg/mL	Reduced bitter, grain, and metallic notes. Similar max sweetness intensity to control.
Stevia RA95 50/50 HTS	0.0032%	0.007 mg/mL	Increased almond flavor intensity. Similar max sweetness intensity to control. Increased sweet aftertaste compared to control.
Stevia RA95 25/75 HTS	0.0015%	0.010 mg/mL	Increased almond flavor intensity. Similar max sweetness intensity to control. Increased sweet aftertaste compared to control.

[0667] EXAMPLE 33: HTS and Stevia in a Chocolate Spread (Sweetener Composition)

[0668] HTS ((0.0 mg/mL, 0.008 mg/mL, 0.023 mg/mL and 0.064 mg/mL) and stevia (0.0750% w/w, 0.0400% w/w, 0.0130% w/w, and 0.0042% w/w (respectively)) were added to a chocolate spread. The sweetener mixture was compared to a control with only stevia added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the control for this matrix. Of total perceived sweetness intensity in each of the experimental samples in Table 26, 75% comes from stevia and 25% comes from HTS, 50% comes from stevia and 50% comes from HTS, 25% comes from stevia and 75% comes from HTS, respectively.

[0669] Table 26: HTS and Stevia Mixtures in a Chocolate Spread Compared to Controls

<u>Sample</u>	<u>Stevia RA95 w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Intense cocoa and bitter, salty, astringent.
Stevia RA95 Control	0.0750%	0.0 mg/mL	Initial cocoa notes up front then strong sweetness similar to sucrose control.
Stevia RA95 75/25 HTS	0.0400%	0.008 mg/mL	Increased initial sweetness compared to control. Increased initial cocoa and salt flavor intensity compared to control. Increased sweet aftertaste compared to control.
Stevia RA95 50/50 HTS	0.0130%	0.023 mg/mL	Increased initial cocoa and salt flavor intensity compared to control. Reduced max sweetness intensity compared to control.
Stevia RA95 25/75 HTS	0.0042%	0.064 mg/mL	Reduced max sweetness intensity compared to control. Strong sweet and salty aftertaste.

[0670] EXAMPLE 34: HTS and Sucrose (Flavor Modifying Composition)

[0671] HTS (0.004 mg/mL) and sucrose (10% w/w concentration) were added to a mock beverage matrix (citric acid, a flavor and carbonation). The mock beverage had a pH of about 3 and 0.25% w/w lemon/lime flavor. HTS and sucrose were added to hit a target of 10% w/w sucrose equivalent sweetness. The HTS/sucrose mixture was compared to an unsweetened control, an unsweetened control + 0.004 mg/mL HTS, and a sucrose sweetened control (10% w/w concentration of sucrose). See Table 27. The HTS/sucrose mixture increased the perception of the flavor as compared to the controls.

[0672] Table 27: HTS and Sucrose Mixtures Compared to Controls

<u>Sample</u>	<u>Sucrose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	0	Reduced citrus flavor intensity; sour, astringent, and harsh.
Unsweetened Control + HTS	NA	0.004 mg/mL	More body, mellowed mouthfeel, more citrus
Sucrose	10%	0	Good sweetness to acid ratio; quick onset of sweet followed by sour; pleasant citrus flavor
Sucrose + HTS	10%	0.004 mg/mL	Extended sweetness intensity, more body. Enhanced lime note from citrus blend

[0673] EXAMPLE 35: HTS and Erythritol (Flavor Modifying Composition)

[0674] HTS (0.004 mg/mL) and erythritol (13.3% w/w concentration) were added to a mock beverage matrix (citric acid, a flavor, and carbonation). The mock beverage had a pH of about 3 and 0.25% w/w lemon/lime flavor. HTS and erythritol were added to hit a target of 10% w/w sucrose equivalent sweetness. The HTS/erythritol mixture was compared to an unsweetened control, an unsweetened control + 0.004 mg/mL HTS, and an erythritol sweetened control (13.3% w/w concentration). See Table 28. The HTS/erythritol mixture increased the perception of the flavor as compared to the controls.

[0675] Table 28: HTS and Erythritol Mixtures in Beverage Samples Compared to Controls

<u>Sample</u>	<u>Erythritol w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	0	Reduced citrus flavor intensity; sour, astringent, and harsh.

Unsweetened Control + HTS	NA	0.004 mg/mL	More body, mellowed mouthfeel, more citrus
Erythritol	13.30%	0	More sour, bitter, mineral, mouth drying, less sweetness
Erythritol + HTS	13.30%	0.004 mg/mL	More citrus, less bitter, less astringency, more sweet than erythritol alone, better body

[0676] EXAMPLE 36: HTS and Sucralose (Flavor Modifying Composition)

[0677] HTS (0.004 mg/mL) and sucralose (0.0387% w/w concentration) were added to a mock beverage matrix (citric acid, a flavor, and carbonation). The mock beverage had a pH of about 3 and 0.25% w/w lemon/lime flavor. HTS and sucralose were added to hit a target of 10% w/w sucrose equivalent sweetness. The HTS/sucralose mixture was compared to an unsweetened control, an unsweetened control + 0.004 mg/mL HTS, and a sucralose sweetened control (0.0387% w/w concentration). See Table 29. The HTS/sucralose mixture increased the perception of the flavor as compared to the two controls.

[0678] Table 29: HTS and Sucralose Mixtures in Beverage Samples Compared to Controls

<u>Sample</u>	<u>Sucralose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	0	Reduced citrus flavor intensity; sour, astringent, and harsh.
Unsweetened Control + HTS	NA	0.004 mg/mL	More body, mellowed mouthfeel, more citrus
Sucralose	0.0387%	0	Sweet linger, little flavor carry over
Sucralose + HTS	0.0387%	0.004 mg/mL	Enhanced sweetness intensity, more body, lemon and lime extension, strong sweet linger but bright and fruity. Reduced artificial sweetness.

[0679] EXAMPLE 37: HTS and Stevia (Stevia Extract) (Flavor Modifying Composition)

[0680] HTS (0.004 mg/mL) and stevia (0.0395% w/w concentration) were added to a mock beverage matrix (citric acid, a flavor, and carbonation). The mock beverage had a pH of about 3 and 0.25% w/w lemon/lime flavor. HTS and stevia were added to hit a target of 10% w/w sucrose equivalent sweetness. The HTS/stevia mixture was compared to an unsweetened control, an unsweetened control

+ 0.004 mg/mL HTS, and a stevia sweetened control (0.0395% w/w concentration). See Table 30. The HTS/stevia mixture increased the perception of the flavor as compared to the two controls.

[0681] Table 30: HTS and Stevia Mixtures in Beverage Samples Compared to Controls

<u>Sample</u>	<u>Stevia w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	0	Reduced citrus flavor intensity; sour, astringent, and harsh.
Unsweetened Control + HTS	NA	0.004 mg/mL	More body, mellowed mouthfeel, more citrus
Stevia	0.0395%	0	Stevia gives sweetness with bitter and metallic off notes; no body, medicinal, astringent, lemon lime flavor
Stevia + HTS	0.0395%	0.004 mg/mL	Helped to reduce Stevia off note intensity, juicier fresher citrus character, and longer linger

[0682] EXAMPLE 38: HTS and Allulose in a Lemon/Lime Soda (Sweetener Composition)

[0683] HTS ((0.0 mg/mL and 0.023 mg/mL) and allulose (14.3% w/w and 7.14% w/w (respectively)) were added to a lemon/lime soda. The sweetener mixture was compared to a control with only allulose added to the matrix. The sweetener mix was expected to be as sweet or sweeter than the allulose control for this matrix. Of total perceived sweetness intensity in the experimental sample in Table 31, 50% comes from allulose and 50% comes from HTS.

[0684] Table 31: HTS and Allulose Mixture in a Lemon/Lime Soda Compared to Controls

<u>Sample</u>	<u>Allulose w/w Concentration</u>	<u>HTS Amount</u>	<u>Observations</u>
Unsweetened Control	NA	NA	Sour, bitter, astringent, harsh, citrus.
Allulose Control	14.3%	0.0 mg/mL	Muted citrus flavor and sweetness intensity. Metallic and astringent.
Allulose 50/50 HTS	7.14%	0.023 mg/mL	Enhanced citrus flavor intensity and max sweetness intensity.

INCORPORATION BY REFERENCE

[0685] The contents of all references (including literature references, issued patents, published patent applications, co-pending patent applications, and GenBank numbers) cited throughout this application

are hereby expressly incorporated herein in their entireties by reference. Unless otherwise defined, all technical and scientific terms used herein are accorded the meaning commonly known to one with ordinary skill in the art.

[0686] International patent application PCT/US2020/012955, filed 01/09/2020, published as WO2020/146650 on 07/16/2020 is incorporated by reference herein in its entirety at least to provide additional description, which is not inconsistent with the description herein, of sweetening composition comprising mycelia of an ascomycete or an aqueous extract thereof or comprising an aqueous extract of a fruiting body of an ascomycete, as well as uses of such composition to provide improve flavor to a product for oral administration.

[0687] International patent application PCT/US2021/039176, filed June 25, 2021, published as WO2021/263158 on December 30, 2021 is incorporated by reference herein in its entirety at least to provide additional description, which is not inconsistent with the description herein, of fungal sweet-taste modifying proteins and cDNA encoding such proteins. This patent application can also provide description of methods for isolating such cDNA and for isolating and expressing such proteins as well as description of sweetening compositions and methods to provide improved flavor to products for oral administration.

[0688] International patent application PCT/US2022/82443, filed December 27, 2022, published as WO2023/129938, on July 6, 2023, is also incorporated by reference herein in its entirety at least to provide additional description, which is not inconsistent with the description herein, of sweet protein mutants, including proteins, genes, cDNA encoding said proteins, and compositions thereof. The sequence listing of this published patent application is incorporated by reference herein in its entirety.

[0689] U.S. provisional application 63/444,185, filed February 8, 2023 is incorporated by reference herein in its entirety. If the disclosure of the '185 provisional application is inconsistent with that of the present application, the present application controls.

[0690] U.S. provisional application 63/524,794, filed July 3, 2023 is incorporated by reference herein in its entirety. If the disclosure of the '794 provisional application is inconsistent with that of the present application, the present application controls.

[0691] U.S. provisional application 63/541,591, filed September 29, 2023 is incorporated by reference herein in its entirety. If the disclosure of the '591 provisional application is inconsistent with that of the present application, the present application controls.

EQUIVALENTS

[0692] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

[0693] The use of the terms “a” and “an” and “the” and “at least one” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The use of the term “at least one” followed by a list of one or more items (for example, “at least one of A and B”) is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “comprises,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. The terms “consisting of” or “consists of” are construed as a close-ended term (i.e., excluding components or steps other than those listed). The terms “consisting essentially of” or “consists essentially of” allow for the inclusion of components or steps that are not essential to the function or activity of the product or method and do not materially affect the function or activity. Any recitation herein of the term “comprising”, particularly in a description of components of a composition, polynucleotide or polypeptide, is understood to encompass those compositions and methods "consisting essentially of and consisting of the recited components or elements.

[0694] When a group of materials, compositions, components or compounds is disclosed herein, it is understood that all individual members of those groups and all subgroups thereof are disclosed separately. Every formulation or combination of components described or exemplified herein can be used to practice the invention, unless otherwise stated. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. Additionally, the end points in a given range are to be included within the range. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0695] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become

apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0696] All publications referred to herein are incorporated herein to the extent not inconsistent herewith. Some references provided herein are incorporated by reference to provide details of additional uses of the invention. All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. References cited herein are incorporated by reference herein in their entirety to indicate the state of the art as of their filing date and it is intended that this information can be employed herein, if needed, to exclude specific embodiments that are in the prior art.

[0697] TABLE 31: TABLE OF SEQUENCES:

Sequence Number (ID): 1

Length: 1072

Molecule Type: DNA

> organism, *Mattirolomyces terfezioides*

Residues:

```

caagtcttta agtgttgatc gagcagtaga aatattaatc geccaattaa ttgctcttac 60
tctaggaaat gtagtactca agttggccct ctaagtcctt gattcagtgt cactttttct 120
ccittgacag taggctagat tctagctctt gtgtccgttt cttaaaatcc tgatatggca 180
ccittagctc agagcagaat accgtgtctc atacaccttg gctatgatcc gatatttaaa 240
tagagcaatc accctctggg atatcttccc catagctccc atgacctca aacattttct 300
aatcccctat accagccata ttacttgtct aagcacttca cattcttctt cacaatacga 360
aacctcctaa aatgcctgat ctctctctct tcattacgat taagaacaac tctaaccacg 420
tcttcactcg gacggcaatt tattctaagt atgccgctgt gcagtggagt cccgagccgc 480
aacttcaat ctctccgggc aaatgggatt tgtttatatt aaaggacatc ttgtctatcc 540
gtgggacttc gggatatgta caatatcggg ttggggatgg tcctggatgg gttagggtca 600
cctttcttc tctagtcggg gccgatgaag tggcagagtg gagctcaggt gacctacctg 660
atggctttgt tctccaaaaa ccagttcgca ctgggtccag gcctttgcag gcaacgttcg 720
aggctacaaa acagtaaaaga tcgatgatgc ctaatatgcc tcataaacac tgaccgcgct 780
gcacatggcc gcatgaatga taagggggat atcgatgatg atgggattag ttattggaat 840
ttcacaatg gacgtcggct tggatttaca ataactgttt catttgattt caaatattcc 900
tatttcttg ggtttttgta ttatctctct tcatcacgcc ttctgaggcc gtgggaagat 960
gaatatgtaa tcaaaagaag ttaggatatg catcatgtac agaaagtgga ccgcaacccc 1020
ttcagcgaaa tgtataaaag atgatatcta agacgcaaaa gcacattctc ag 1072

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Sequence Number (ID): 2

Length: 366

Molecule Type: DNA

> organism, *Mattirolomyces terfezioides*

Residues:

atgectgac tctctcctt cattacgatt aagaacaact ctaaccacgt cttcactcgg 60
 acggcaattt attctaagta tgccgctgtg cagtggagtc ccgagccgca actctcaatc 120
 tctcccgca aatgggattt gttatttta aaggacatct tgtctatccg tgggacttcg 180
 ggatatgtac aatatcgggt tgggatgggt cctggatggg ttagggcac ctttcttct 240
 ctatcgggg ccgatgaagt ggcagagtgg agctcaggtg acctacctga tggcttgg 300
 ctccaaaaac cagttcgac tgggtccagg ccttgcagg caacgttca ggctacaaaa 360
 cagtaa 366

Sequence Number (ID): 3

Length: 121

Molecule Type: AA

Features Location/Qualifiers:

> organism, *Mattirolomyces terfezioides*

Residues:

MPDLSSFITI KNNSNHVFTR TAIYSKYAAV QWSPEPQLSI SPGKWDLFIL KDILSIRGTS 60
 GYVQYRVGDG PGWVRVTFSS LVGADEVAEW SSGDLPDGFV LQKPVRTGSR
 PLQATFEATK Q 121

Sequence Number (ID): 4

Length: 384

Molecule Type: DNA

Features Location/Qualifiers:

> note, Modified coding sequence for *E. coli* expression for protein from
Mattirolomyces terfezioides with His-Tag

Residues:

atgccgatt tgagctcgtt cattactatt aagaataact ctaacctgt tttacgcgt 60
 accgcaatct attccaaata tgccgcagtc cagtggagcc cggaaccgca gctgagcatt 120
 agcccaggt aatgggacct gttcatcctg aaagacatcc tgagcatccg cggtagctcc 180
 ggctacgtgc agtaccgtgt cggcgacgggt ccgggctggg tgcgtgttac ctttagcagc 240
 ctgggtgggtg cggacgaagt tgctgagtgg agcagcgggt atctgccgga tggcttgg 300
 ctgcaaaagc ctgtccgcac cggtagccgt ccgctgcaag cgacgttca ggcgaccaag 360
 caacatcacc atcaccacca ctaa 384

Sequence Number (ID): 5

Length: 127

Molecule Type: AA

Features Location/Qualifiers:

> note, Synthetic polypeptide with His-tag

Residues:

MPDLSSFITI KNNSNHVFTR TAIYSKYAAV QWSPEPQLSI SPGKWDLFIL
 KDILSIRGTS 60
 GYVQYRVGDG PGWVRVTFSS LVGADEVAEW SSGDLPDGFV LQKPVRTGSR
 PLQATFEATK 120
 QHHHHHH 127

Sequence Number (ID): 6

Length: 366

Molecule Type: DNA

Features Location/Qualifiers:

> note, Modified coding sequence for *Saccharomyces cerevisiae* expression
 for protein from *Mattirolomyces terfezioides* to which His-Tag coding region is added

Residues:

atgcctgatt tatcaagttt tatcaccatt aaaaataact ctaacatgt ttctactaga 60
 acagccatct actcaaagta cgcagccgtc caatgggtccc cagaacctca gttgtctata 120
 tctccaggta aatgggatct ttcatctta aaagatattc taagtattag aggcatttct 180
 gggtacgtac agtatcgtgt tggatgatga cctgggtggg ttagagtaac attcagctca 240
 ttggttgggg ctgacgaagt ggctgagtgg tcctcagggtg acctcccaga tggcttcgtg 300
 ctgcaaaagc cagtcagaac tggatctaga ccattgcaag cgacattcga agcaacaaag 360
 caatga 366

Sequence Number (ID): 7

Length: 5

Molecule Type: AA

Features Location/Qualifiers:

> note, Amino Acid Linker

> note, May be repeated n times, where n is any integer

Residues:

GGGGS 5

Sequence Number (ID): 8

Length: 5

Molecule Type: AA

Features Location/Qualifiers:

> note, Amino Acid Linker

> note, May be repeated n times, where n is any integer

Residues:

EAAAK 5

Sequence Number (ID): 9

Length: 58

Molecule Type: DNA

Features Location/Qualifiers:

> note, Synthetic Adapter Sequence

Residues:

aatgatacgg cgaccaccga gatctacact ctttcctac acgacgtctt tccgatct 58

Sequence Number (ID): 10

Length: 58

Molecule Type: DNA

Features Location/Qualifiers:

> note, Synthetic Adapter Sequence

> note, insertion of i7 barcode

Residues:

gatcggaaga gcacacgtct gaactccagt cacnatctcg tatgccgtct tctgcttg 58

SEQ ID NO:11-SEQ ID NO:140 see: WO2023/129938 published July 6, 2023

Sequence Number (ID):141

Length: 121

Molecule Type: AA

Features Location/Qualifiers:

> organism, *Mattirolomyces terfezioides* (mature protein HTS-2 isoform,

SEQ ID NO:3 with methionine missing at position 1)

Residues:

PDLSSFITI KNNSNHVFTR TAIYSKYAAV QWSPEPQLSI SPGKWDLFIL KDILSIRGTS 60
 GYVQYRVGDG PGWVRVTFSS LVGADEVAEW SSGDLPDGFV LQKPVRTGSR
 PLQATFEATK Q 120

AMINO ACID SEQUENCE (SEQ ID NO:142) HTS-2 isoform with His-tag

PDLSSFITIK NNSNHVFTRT AIYSKYAAVQ WSPEPQLSIS PGKWDLFILK DILSIRGTSG 60
 YVQYRVGDGP GWVRVTFSSL VGADEVAEWS SSGDLPDGFVL QKPVRTGSRPL 110
 QATFEATKQH HHHHH 205

AMINO ACID SEQUENCE: (SEQ ID NO:143) Artificial (Double Mutant S33C_I49C)

MPDLSSFITI KNNSNHVFTR TAIYSKYAAV QWCPEPQLSI SPGKWDLFCL KDILSIRGTS
 GYVQYRVGDGPWVRVTFSS LVGADEVAEW SSGDLPDGFV LQKPVRTGSR PLQATFEATK
 QHHHHHH 121 aa + optional His₆ tag

REVERSE TRANSLATION of HTS variant of SEQ ID NO:143-DNA Sequence (SEQ ID NO:144)

atgccggatc ttagcagctt tattaccatt aaaaacaaca gcaacatgt gtttaccgc 60
 accgcgattt atagcaaata tgcggcgggtg cagtgggtgcc cggaaccgca gctgagcatt 120
 agccccgggca aatgggatct gtttgcctg aaagatatc ttagcattcg cggcaccagc 180
 ggctatgtgc agtatcgctg gggcgatggc ccgggctggg tgcgcgtgac cttagcagc 240
 ctggtgggcg cggatgaagt ggcggaatgg agcagcggcg atctgccgga tggctttgtg 300
 ctgcagaaac cgtgctgcac cggcagccgc ccgctgcagg cgaccttga agcgacaaa 360
 cag 363 (no taa and no His-tag)

AMINO ACID SEQUENCE; (SEQ ID NO:145) (Double mutant Y24C_Y62C)

MPDLSSFITI KNNSNHVFTR TAICSKYAAV QWSPEPQLSI SPGKWDLFIL KDILSIRGTS
 GCVQYRVGDG PGWVRVTFSS LVGADEVAEW SSGDLPDGFV LQKPVRTGSR PLQATFEATK
 Q (121 AA)

Amino Acid SEquence (SEQ ID NO:146) (Double Mutant S33C_I49C with methionine at position 1 missing)

PDLSSFITIK NNSNHVFTRT AIYSKYAAVQ WCPEPQLSIS PGKWDLFCLK DILSIRGTSG
 YVQYRVGDGP GWVRVTFSSL VGADEVAEWS SSGDLPDGFVL QKPVRTGSRP LQATFEATKQ (120
 AA)

Amino Acid SEquence (SEQ ID NO:147) (Double Mutant Y24C_Y62C with methionine at position 1 missing)

PDLSSFITIK NNSNHVFTRT AICSKYAAVQ WSPEPQLSIS PGKWDLFILK DILSIRGTSG
 CVQYRVGDGP GWVRVTFSSL VGADEVAEWS SSGDLPDGFVL QKPVRTGSRP LQATFEATKQ (120
 AA)

E. COLI CODON OPTIMIZED Sequence (SEQ ID NO:148) Artificial

atgccggacc tgccttctt taccactatc aaaaataact ctaacatgt ttaccaccgc 60
 acggcgatct acagcaaata cggcgggtg cagtgggtgc cggaaccgca gctgtctatc 120

agcccgggca aatgggacct gttctgtctg aaagatatcc tgagcatccg tggtagctct 180
ggttatgttc agtaccgtgt cggcgatggt cggggtggg ttcgcgttac cttcagctct 240
ctggttggcg cggacgaagt tgcggaatgg tcctctggcg acctgccaga tggtttcgtg 300
ctgcagaaac cggttcgtac tggctctcgt ccgctccagg ccactttcga agctacaaa 360
caa 363 (no taa and no His Tag)

EXEMPLARY SEQUENCE ENCODING (HIS)₆ (SEQ ID NO: 149)

caacatcaccatcaccaccac

The His-Tag encoding sequence (or one that is optimized for expression in a selected host) is inserted after the last amino acid codon in polynucleotide sequence and before stop codon taa.

THE CLAIMS

1. A polynucleotide encoding a Myd variant polypeptide selected from the group consisting of:
 - (a) a polypeptide selected from the group consisting of the polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10, as described relative to the polypeptide of SEQ ID NO:3 or a polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 where in addition the methionine at position 1 is absent;
 - (b) a polypeptide having at least 80% sequence identity to a polypeptide selected from the group consisting of the polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10, as described relative to the polypeptide of SEQ ID NO:3 or a polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 where in addition the methionine at position 1 is absent; and
 - (c) a polypeptide having an amino acid sequence that is modified by deletion, insertion, substitution, or addition of no more than 24 amino acids from a polypeptide selected from the group consisting of the polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10, as described relative to the polypeptide of SEQ ID NO:3 or a polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 where in addition the methionine at position 1 is absent;wherein the encoded polypeptide has a sweet-taste modulation activity or optionally has sweet taste and differs from the polypeptide of SEQ ID NO:3 and the polypeptide of SEQ ID NO:141.
2. The polynucleotide of claim 1, operably linked to one or more heterologous regulatory element.
3. The polynucleotide of claim 1 or 2, wherein the polynucleotide further encodes a protein/peptide tag, optionally an affinity tag or optionally a histidine tag.
4. The polynucleotide of any one of claims 1-3, wherein the polynucleotide encodes a polypeptide modified by deletion, insertion, substitution, or addition of no more than 12 amino acids from a polypeptide selected from the group consisting of polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 or a polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 where in addition the methionine at position 1 is absent.

5. The polynucleotide of any one of claims 1-4, wherein the polynucleotide encodes a polypeptide modified by deletion, insertion, substitution, or addition of no more than 6 amino acids from a polypeptide selected from the group consisting of polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 or a polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 where in addition the methionine at position 1 is absent.
6. The polynucleotide of any one of claims 1-5, wherein the encoded polypeptide has at least 90% sequence identity to a polypeptide selected from the group consisting of polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 or a polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 where in addition the methionine at position 1 is absent.
7. The polynucleotide of any one of claims 1-6, wherein the encoded polypeptide has at least 95% sequence identity to a polypeptide selected from the group consisting of polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 or a polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 where in addition the methionine at position 1 is absent.
8. The polynucleotide of any one of claims 1-7, wherein the encoded polypeptide does not contain a methionine at position 1 of its amino acid sequence and wherein the encoded polypeptide differs from the polypeptide of SEQ ID NO: 141
9. The polynucleotide of any one of claims 1-8, wherein the variant is a variant listed in Table 8 or a variant listed in Table 8 where in addition the methionine at position 1 is absent.
10. The polynucleotide of any one of claims 1-8, wherein the variant is a variant listed in Table 9 or a variant listed in Table 8 where in addition the methionine at position 1 is absent.
11. The polynucleotide of any one of claims 1-8, wherein the variant is a variant listed in Table 10 or a variant listed in Table 8 where in addition the methionine at position 1 is absent.
12. A polynucleotide of any one of claims 1-11 encoding a polypeptide selected from the group consisting of:
 - (a) a polypeptide selected from the group consisting of a polypeptide having an amino acid sequence of a variant listed in Table 8, a polypeptide thereof further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag, or a polypeptide thereof not having a methionine at amino acid position 1;

(b) a polypeptide having at least 80% sequence identity to a polypeptide selected from the group consisting of a polypeptide having an amino acid sequence of a variant listed in Table 8, a polypeptide thereof further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag; or a polypeptide thereof not having a methionine at amino acid position 1; and

(c) a polypeptide modified by deletion, insertion, substitution, or addition of no more than 24 amino acids from a polypeptide selected from the group consisting of a polypeptide having an amino acid sequence of a variant listed in Table 8, a polypeptide thereof further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag, or a polypeptide thereof not having a methionine at amino acid position 1;

wherein the polypeptide sequence is different from that of SEQ ID NO:3, SEQ ID NO:3 containing the protein/peptide tag, affinity tag or histidine tag and optionally is different from the polypeptide of SEQ ID NO:141.

13. A polynucleotide of any one of claims 1-11 encoding a polypeptide selected from the group consisting of:

(a) a polypeptide selected from the group consisting of a polypeptide having an amino acid sequence of a variant listed in Table 9, a polypeptide thereof further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag, or a polypeptide thereof not having a methionine at amino acid position 1;

(b) a polypeptide having at least 80% sequence identity to a polypeptide selected from the group consisting of a polypeptide having an amino acid sequence of a variant listed in Table 9, a polypeptide thereof further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag; or a polypeptide thereof not having a methionine at amino acid position 1; and

(c) a polypeptide modified by deletion, insertion, substitution, or addition of no more than 24 amino acids from a polypeptide selected from the group consisting of a polypeptide having an amino acid sequence of a variant listed in Table 9, a polypeptide thereof further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag, or a polypeptide thereof not having a methionine at amino acid position 1;

wherein the polypeptide sequence is different from that of SEQ ID NO:3, SEQ ID NO:3 containing the protein/peptide tag, affinity tag or histidine tag and optionally is different from the polypeptide of SEQ ID NO:141.

14. A polynucleotide of any one of claims 1-11 encoding a polypeptide selected from the group consisting of:

(a) a polypeptide selected from the group consisting of a polypeptide having an amino acid sequence of a variant listed in Table 8, a polypeptide thereof further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag, or a polypeptide thereof not having a methionine at amino acid position 1;

(b) a polypeptide having at least 80% sequence identity to a polypeptide selected from the group consisting of a polypeptide having an amino acid sequence of a variant listed in Table 8, a polypeptide thereof further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag; or a polypeptide thereof not having a methionine at amino acid position 1; and

(c) a polypeptide modified by deletion, insertion, substitution, or addition of no more than 24 amino acids from a polypeptide selected from the group consisting of a polypeptide having an amino acid sequence of a variant listed in Table 8, a polypeptide thereof further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag, or a polypeptide thereof not having a methionine at amino acid position 1;

wherein the polypeptide sequence is different from that of SEQ ID NO:3, SEQ ID NO:3 containing the protein/peptide tag, affinity tag or histidine tag and optionally is different from the polypeptide of SEQ ID NO:141.

15. The polynucleotide of any one of claims 1-14, wherein the encoded polypeptide does not include a mutation listed in Table 7.

16. The polynucleotide of any one of claims 1-14, wherein the encoded polypeptide does not include a mutation listed in Table 3.

17. The polynucleotide of any one of claims 1-14, wherein the encoded polypeptide does not include a mutation listed in Table 6.

18. The polynucleotide of any one of claims 12-17, operably linked to one or more heterologous regulatory element.

19. An expression cassette comprising the polynucleotide of any one of claims 1-18.

20. A vector comprising the polynucleotide of any one of claims 1-18.

21. A host cell transformed with the vector of claim 18.

22. A method of producing a protein having sweet-taste modulation activity (and particularly sweet taste) comprising:

culturing a host cell transformed with the vector of claim 18 in a medium under conditions for protein expression.

23. A method of producing a protein having sweet-taste modulation activity and/or having sweet taste comprising:

culturing a host cell transformed with a vector in a medium under conditions for protein expression, wherein the vector comprises an isolated polynucleotide encoding a polypeptide selected from the group consisting of:

- (a) a polypeptide selected from the group consisting of polypeptides having the amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 or a variant thereof wherein the methionine at position 1 is missing;
- (b) a polypeptide having at least 80% sequence identity to a polypeptide selected from the group consisting of polypeptides having the amino acid sequence of a variant listed in Table 8, Table 9 or Table 10 or a variant thereof wherein the methionine at position 1 is missing;
- and
- (c) a polypeptide modified by deletion, insertion, substitution, or addition of no more than 24 amino acids from a polypeptide selected from the group consisting of polypeptides having the amino acid sequence of a variant listed in Table 8, Table 9 or Table 10 or a variant thereof wherein the methionine at position 1 is missing;

wherein the encoded polypeptide has a sweet-taste modulation activity and optionally sweet taste and differs from polypeptide SEQ ID NO:3 and differs from the polypeptide of SEQ ID NO:141.

24. The method of claim 23, wherein the variant is a variant of Table 8.

25. The method of claim 23, wherein the variant is a variant of Table 9.

26. The method of claim 23, wherein the variant is a variant of Table 10.

27. The method of claim 23, wherein the variant is a variant other than a variant of Table 3, a variant other than a variant of Table 6 or other than a variant of Table 7.

28. The method of producing a protein of any one of claims 22-27, wherein the host cell is selected from the group consisting of *Escherichia coli*, *Klebsiella oxytoca*, *Anaerobiospirillum succiniciproducens*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Agrobacterium tumefaciens*, *Rhizobium etli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, *Gluconobacter oxydans*, *Zymomonas mobilis*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Streptomyces coelicolor*, *Clostridium acetobutylicum*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces*

marxianus, *Aspergillus terreus*, *Aspergillus niger*, *Pichia pastoris*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Yarrowia lipolytica*, *Candida albicans*, *Issatchenkia orientalis*, *Scheffersomyces stipitis*, *Yarrowia lipolytica*, *Ogataea polymorpha*, *Phaffia rhodozyma*, *Candida utilis*, *Arxula adeninivorans*, *Debaryomyces hansenii*, *Debaryomyces polymorphus*, *Schwanniomyces occidentalis*, *Bacillus megaterium*, *Trichoderma reesei*, *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium Longum*, *Carnobacterium divergens*, *Lactobacillus acidophilus*, *Lactobacillus amylolyticus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus alimentarius*, *Lactobacillus aviaries*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus collinoides*, *Lactobacillus coryniformis*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus dextrinicus*, *Lactobacillus diolivorans*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Lactobacillus hilgardii*, *Lactobacillus johnsonii*, *Lactobacillus kefiranoferiens*, *Lactobacillus kefir*, *Lactobacillus mucosae*, *Lactobacillus panis*, *Lactobacillus paracasei*, *Lactobacillus parafarraginis*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactobacillus salivarius*, *Lactobacillus Sanfranciscensis*, *Leuconostoc citreum*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Leuconostoc Pseudomesenteroides*, *Microbacterium imperial*, *Oenococcus oeni*, *Pasteuria nishizawae*, *Pediococcus acidilactici*, *Pediococcus parvulus*, *Pediococcus pentosaceus*, *Propionibacterium acidipropionic*, *Propionibacterium freudenreichii*, *Streptococcus thermophilus*, *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus flexus*, *Bacillus fusiformis*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus mojavensis*, *Bacillus paralicheniformis*, *Bacillus pumilus*, *Bacillus smithii*, *Bacillus subtilis*, *Bacillus vallismortis*, *Bacillus velezensis*, *Geobacillus stearothermophilus*, *Paenibacillus illinoisensis*, *Parageobacillus thermoglucosidasius*, *Gluconobacter oxydans*, *Komagataeibacter sucrofermentans*, *Xanthomonas campestris*, *Candida cylindracea*, *Cyberlindnera jadinii*, *Debaryomyces hansenii*, *Hanseniaspora uvarum*, *Khuyveromyces lactis*, *Khuyveromyces marxianus*, *Komagataella pastoris*, *Komagataella phaffii*, *Lindnera jadinii*, *Ogataea angusta*, *Saccharomyces bayanus*, *Schizosaccharomyces pombe*, *Wickerhamomyces anomalus*, *Xanthophyllomyces dendrorhous*, and *Zygosaccharomyces rouxii* cells.

29. The method of producing a protein of any one of claims 22-27, wherein the host cell is selected from the group consisting of gram-positive non-spore forming bacteria, gram-positive spore forming bacteria, gram negative bacteria, yeast, and protists/algae.

30. The method of producing a protein of any one of claims 22-27, wherein the host cell is a plant cell.
31. The method of producing a protein of any one of claims 22-27, wherein the host cell is a cell of a fungus other than *Mattiolomyces terfezioides*.
32. The method of producing a protein of any one of claims 22-27, wherein the host cell is a cell of *Eschericia coli*, *Saccharomyes cerevisiae*, a *Pichia pastoris* or a *Yarrowia lipolytica*.
33. The method of producing a non-naturally occurring mixture of the two isoforms of the Myd sweet protein which comprises recombinant expression of a nucleic acid coding sequence encoding SEQ ID NO:3 in a heterologous host.
34. The method of claim 33, wherein the nucleic acid coding sequence further encodes a protein tag.
35. The method of claim 33, wherein the nucleic acid encoding sequence further encodes a His-tag.
36. The method of any one of claims 33-35, wherein the heterologous host is selected from a strain of *E. coli*, a strain of *S. cerevisiae*, a strain of *Pichia pastoris*, a strain of *Trichoderma reesei* or a strain of *Yarrowia lipolytica*.
37. The method of any one of claims 33-36, wherein the mixture of sweet proteins is enriched in HST-1 or protein-tagged HST-1.
38. The method of any one of claims 33-36, wherein the mixture of sweet proteins is enriched in HST-2 or protein-tagged HST-2.
39. A polypeptide having sweet-taste modulation activity (particularly sweet taste) which comprises:
- (i) a polypeptide sequence selected from amino acid sequences of any variant listed in Table 8, Table 9 or Table 10 or a variant listed in Table 8, Table 9 or Table 10 which also has the methionine at position 1 missing;
 - (ii) a polypeptide sequence having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of amino acid sequences of any variant listed in Table 8; Table 9 or Table 10 or a variant listed in Table 8, Table 9 or Table 10 which also has the methionine at position 1 missing; or
 - (iii) a polypeptide sequence containing at least one modification relative to a polypeptide sequence selected from the group consisting of an amino acid sequence of a variant listed in Table 8, Table 9 or Table 10 or a variant listed in Table 8, Table 9 or Table 10 which also

has the methionine at position 1 missing, by deletion, insertion, substitution, or addition of no more than 24 amino acids,

wherein the polypeptide has sweet-taste modulation activity (particularly sweet taste) and:

(a) the polypeptide differs from the polypeptide of SEQ ID NO:3, and/or

(b) the polypeptide differs from the polypeptide of SEQ ID NO:141; and/or

(c) the polypeptide differs from the polypeptide of SEQ ID NO:3 or SEQ ID NO:141 further comprising a protein/peptide tag, optionally an affinity tag or optionally a histidine tag or optionally a (His)₆ tag.

40. The polypeptide of claim 39, further comprising a protein/peptide tag, optionally an affinity tag or optionally a histidine tag, or optionally a (His)₆ tag.

41. The polypeptide of claim 39 or 40, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:3 which is modified by at least 1 and up to 24 amino acid mutations as shown in Table 8, Table 9 or Table 10.

42. The polypeptide of claim 39 or 40, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:3 which is modified by at least 1 and up to 12 amino acid mutations as shown in Table 8, Table 9 or Table 10.

43. The polypeptide of claim 39-40, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:3 which is modified by at least 1 and up to 6 amino acid mutations as shown in Table 8, Table 9 or Table 10.

44. The polypeptide of claim 39-40, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:3 which is modified by at least one mutation, where the at least one mutation is selected from the mutations listed in Table 8, and where the polypeptide is further modified by one or more additional mutations, where the one or more additional mutations are selected from the mutations listed in Table 7.

45. The polypeptide of claim 39 or 40, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:3 which is modified by two or more mutations selected from the mutations listed in Table 8.

46. The polypeptide of claim 39 or 40, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:3 which is further modified by two or more additional mutations, where the two or more additional mutations are selected from the mutations listed in Table 7.

47. The polypeptide of claim 39 or 40, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:3 which is modified by two or more mutations selected from the mutations listed in Table 9.
48. The polypeptide of claim 39 or 40, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:3 which is modified by two or more mutations selected from the mutations listed in Table 10.
49. The polypeptide of claim 39 or 40, wherein the polypeptide comprises an amino acids sequence other than an amino acid sequence of a variant of Table 3 or Table 6.
50. The polypeptide of claim 39 or 40, wherein the polypeptide comprises an amino acids sequence other than an amino acid sequence of a variant of Table 7.
51. The polypeptide of claim 39 or 40, wherein the polypeptide comprises the amino acid of SEQ ID NO:3; or the amino acid sequence of SEQ ID NO:3 wherein the methionine at position 1 is absent and which further has one or more or two or more and up to 24 different mutations as listed in Table 9 or Table 10.
52. The polypeptide of claim 51, wherein the polypeptide further comprises a protein/peptide tag, optionally an affinity tag or optionally a histidine tag, or optionally a (His)₆ tag.
53. The polypeptide of any one of claims 39-52 which exhibits sweet taste.
54. The polypeptide of any one of claims 39-53, wherein the polypeptide is an isolated polypeptide or a purified polypeptide.
55. The polypeptide of any one of claims 39-54, wherein the polypeptide has at least 90%, at least 95% or at least 99% sequence identity to a polypeptide sequence selected from the group consisting of an amino acid sequence of any variant listed in Table 8, Table 9 or Table 10 or a variant listed in Table 8, Table 9 or Table 10 in which in addition the methionine at position 1 is missing.
56. The polypeptide of any one of claims 39-54, wherein the polypeptide has at least 90%, at least 95% or at least 99% sequence identity to a polypeptide sequence selected from the group consisting of an amino acid sequence of any variant listed in Table 9 or Table 10 or a variant listed in Table 8, Table 9 or Table 10 in which in addition the methionine at position 1 is missing.
57. The polypeptide of any one of claims 39-54, wherein the polypeptide has at least 90%, at least 95% or at least 99% sequence identity to a polypeptide sequence selected from the group consisting of an amino acid sequence of any variant listed in Table 9 or a variant listed in Table 8, Table 9 or Table 10 in which in addition the methionine at position 1 is missing.

58. The polypeptide of any one of claims 39-54, wherein the polypeptide has at least 90%, at least 95% or at least 99% sequence identity to a polypeptide sequence selected from the group consisting of an amino acid sequence of any variant listed in Table 10 or a variant listed in Table 10 in which in addition the methionine at position 1 is missing.
59. The polypeptide of any one of claims 39-58, wherein the polypeptide sequence does not contain an amino acid substitution as listed in Table 3 or Table 4.
60. The polypeptide of any one of claims 39-59, wherein the polypeptide further comprises a protein/peptide tag, optionally an affinity tag or optionally a histidine tag.
61. The polypeptide of any one of claims 39-60, wherein the polypeptide contains a histidine tag which is (His)₆.
62. A polypeptide having sweet taste which comprises the amino acid sequence of SEQ ID NO:3 or SEQ ID NO:141 with two or more mutations listed in Table 8, Table 9 or Table 10 and which also exhibits thermal stability greater than that of native (wild-type) HTS protein.
63. The polypeptide of claim 62, which comprises two mutations to introduce two cysteine amino acids such that the two cysteines can form a –S-S- bond in the polypeptide.
64. The polypeptide of claim 63, wherein the two mutations to introduce cysteines are at amino acids 20-70 (inclusive) of SEQ ID NO:3.
65. The polypeptide of claim 63, wherein the mutations to introduce cysteines are at any two of amino acids 24, 33, 49, and 62 of SEQ ID NO:3.
66. The polypeptide of claim 63, wherein the mutations to introduce cysteines are at amino acids 33 and 49 of SEQ ID NO:3.
67. The polypeptide of any one of claims 62-66, wherein the polypeptide does not have a methionine at position 1.
68. The polypeptide of any one of claims 62-67, wherein the polypeptide further comprises a protein tag, an affinity tag or optionally a histidine tag or optionally a (His)₆ tag.
69. The polypeptide of claim 62 wherein the polypeptide is selected from a polypeptide having amino acid sequence of SEQ ID No:143, SEQ ID NO:145; SEQ ID No:146 or SEQ ID NO:147.
70. A sweet protein having the amino acid sequence of SEQ ID NO:141 which is substantially free of a protein having the amino acid sequence of SEQ ID NO:3.
71. The sweet protein of claim 70 which comprises less than 1% of a protein having the amino acid sequence of SEQ ID NO:3.

72. A sweet protein having the amino acid sequence of SEQ ID NO:3 which is substantially free of a protein having the amino acid sequence of SEQ ID NO:141.
73. The sweet protein of claim 72 which comprises less than 1% of a protein having the amino acid sequence of SEQ ID NO:141.
74. A sweet protein which is a non-naturally occurring mixture of a sweet protein having the amino acid sequence of SEQ ID NO:3 and a sweet protein having the amino acid sequence of SEQ ID NO:141.
75. The sweet protein of claim 74 which is a mixture of a sweet protein having the amino acid sequence of SEQ ID NO:3 and a sweet protein having the amino acid sequence of SEQ ID NO:141 wherein the amount of the sweet protein having the sequence of SEQ ID NO:3 is greater than 60% by weight of the mixture.
76. The sweet protein of claim 74 which is a mixture of a sweet protein having the amino acid sequence of SEQ ID NO:3 and a sweet protein having the amino acid sequence of SEQ ID NO:141 wherein the amount of the sweet protein having the sequence of SEQ ID NO:141 is greater than 60% by weight of the mixture.
77. The sweet protein of claim 74 which is a mixture of a sweet protein having the amino acid sequence of SEQ ID NO:3 and a sweet protein having the amino acid sequence of SEQ ID NO:141 wherein the amount of the sweet protein having the sequence of SEQ ID NO:141 is 90% or more by weight of the mixture.
78. A composition, comprising a combination of:
- (a) a product for oral administration, wherein the product differs from *Mattirolomyces terfezioides* truffle, and
 - (b) a flavor modulating composition comprising one or more isolated polypeptide or one or more sweet protein of any one of claims 39-77, or one or more isolated polypeptide produced by expression of a polynucleotide of any one of claims 1-18 in a heterologous host cell;
- wherein the combination exhibits flavor modulation compared to the product for oral administration.
79. The composition of claim 78, wherein the flavor modulating composition is a sweetening composition and the combination has sweet taste or enhanced sweet taste compared to the product for oral administration.

80. The composition of claim 78 or 79, wherein the composition comprises a plurality of isolated polypeptides.
81. The composition of any one of claims 78-80, wherein the product for oral administration is a food product selected from the group consisting of baked goods; sweet bakery products, pre-made sweet bakery mixes for preparing sweet bakery products; pie fillings and other sweet fillings, gelatins and puddings; frozen desserts; yogurts; snack bars; bread products; pre-made bread mixes for preparing bread products; sauces, syrups and dressings; sweet spreads; confectionary products; and sweetened breakfast cereals.
82. The composition of any one of claims 78-81, wherein the product for oral administration is a food product which is warmed or heated prior to eating or which is served for consumption warm or hot.
83. The composition of claim 82, wherein at least one of the polypeptides exhibits enhanced thermostability compared to native HTS.
84. The composition of any one of claims 78-80, 82 or 83, wherein the product for oral administration is a beverage product selected from the group consisting of carbonated beverages; non-carbonated beverages; beverage concentrates, coffee-based beverages or tea-based beverages.
85. A method for modulating the taste of a product for oral administration, comprising:
combining a product for oral administration with an effective amount of one or more an isolated polypeptide having an at least 80% sequence identity to a polypeptide sequence selected from the group consisting of those amino acid sequences of a variant listed in Table 8, Table 9 or Table 10 or a variant listed in Table 8, Table 9 or Table 10 wherein the methionine at position 1 is missing or having at least 80% sequence identity to a polypeptide thereof or further having a protein/peptide tag, optionally an affinity tag or optionally a histidine tag or optionally a (His)₆ tag,
wherein the product for oral administration differs from *Mattirolomyces terfezioides* truffle, wherein the one or more isolated polypeptide is not the polypeptide of SEQ ID NO:3, the polypeptide of SEQ ID NO:141, or the polypeptide of SEQ ID NO:3 containing a protein/peptide tag, affinity tag or histidine tag and wherein the combination has flavor modulation compared to the product for oral administration.
86. The composition of claim 85, wherein at least one polypeptide has sweet taste and the composition has sweet taste or enhanced sweet taste compared to the product for oral administration.

87. The method of claim 85 or 86, wherein the product for oral administration is a food product selected from the group consisting of baked goods; sweet bakery products, pre-made sweet bakery mixes for preparing sweet bakery products; pie fillings and other sweet fillings, gelatins and puddings; frozen desserts; yogurts; snack bars; bread products; pre-made bread mixes for preparing bread products; sauces, syrups and dressings; sweet spreads; confectionary products; and sweetened breakfast cereals.
88. The method of any one of claims 85-87, wherein the product for oral administration is a food product which is warmed or heated prior to eating or which is served for consumption warm or hot.
89. The method of claim any one of claims 85, 86, or 88, wherein the product for oral administration is a beverage product selected from the group consisting of carbonated beverages; non-carbonated beverages; and beverage concentrates.
90. The method of claim 89, wherein the product for oral administration is a heated or warmed beverage product which is optionally consumed warm or hot, or is optionally a coffee or tea-based product.
91. A method for purifying a polypeptide having sweet-taste modulation activity comprising:
(a) conducting a hydrophobic interaction chromatography (HIC) on the polypeptide, and
(b) then conducting a size exclusion chromatography (SEC) on the polypeptide,
wherein the polypeptide comprises an amino acid sequence having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of an amino acid sequence of any variant listed in Table 8, Table 9 or Table 10, or an amino acid sequence having at least 80% sequence identity thereto, or wherein the polypeptide further comprises a protein/peptide tag, optionally an affinity tag or optionally a histidine tag or optionally a (His)₆ tag.
92. A polypeptide purified by the method of claim 91.
93. A polypeptide of any preceding claim which exhibits enhanced sweet taste modulation activity compared to the sweet protein having amino acid sequenc of SEQ ID NO:3.
94. The polypeptide of any preceding claim, wherein the polypeptide has enhanced thermal stability compared to the sweet protein having amino acid sequenc of SEQ ID NO:3.
95. The polypeptide of any preceding claim, wherein the polypeptide has enhanced sweet taste modulation activity and enhanced thermal stability compared to the sweet protein having amino acid sequenc of SEQ ID NO:3.

96. A polynucleotide encoding a polypeptide of claim 93 or 95.
97. An expression cassette or vector comprising the polynucleotide of claim 96.
98. A host cell transformed with the expression cassette or vector of claim 97.
99. A method of producing a protein having sweet-taste modulation activity comprising:
culturing a host cell transformed with the expression cassette or vector of claim 97 in a medium under conditions for protein expression.
100. A host cell expressing a polypeptide of any preceding claim.
101. The polypeptide of any preceding claim which is isolated.
102. The polynucleotide of any preceding claim which is isolated.
103. The polynucleotide of any preceding claim which is operable linked to a heterologous regulatory element.
104. The composition of any preceding claim, wherein at least one of the one or more isolated polypeptide exhibits enhanced sweet-taste modulation activity compared to the polypeptide of SEQ ID NO:3.
105. The composition of any preceding claim, wherein at least one of the one or more the isolated polypeptide exhibits enhanced thermal stability compared to the polypeptide of SEQ ID NO:3.
106. A polypeptide of any preceding claim, further comprising one or more derivatizations of the N-terminal amino acid, the C-terminus amino acid or an amino acid side chain of the polypeptide.
107. The polypeptide of claim 106, wherein the derivatization is selected from an addition to, a subtraction from, or substitution for an anime nitrogen of any of the following chemical moieties: acyl, acetyl (CH₃CO), formyl (HCO), glycosyl (C₆H₁₁O₆), hydroxyl (HO), methyl (CH₃), phosphatidyl (PO₄), phosphonyl (PO₂), sulfhydryl (SH), or sulfonyl (HSO₂).
108. The polypeptide of claim 106, wherein the derivatization is selected from an addition to, a subtraction from, or substitution for a delta-sulfur of a methionine of any of the following chemical moieties: acyl, acetyl (CH₃CO), formyl (HCO), glycosyl (C₆H₁₁O₆), hydroxyl (HO), methyl (CH₃), phosphatidyl (PO₄), phosphonyl (PO₂), sulfhydryl (SH), or sulfonyl (HSO₂).

109. The polypeptide of claim 106, wherein the derivatization is selected from acylation, acetylation, esterification, glycosylation, oxidation, methylation, reductive alkylation, phosphorylation, sulfurylation, or sulfonylation.
110. The polypeptide of claim 106, where the derivatization(s) are acylations of one or more different amino groups.
111. The polypeptide of claim 106, wherein the derivatization is acylation, and optionally is acetylation of the N-terminal amino acid.
112. The polypeptide of claim 106, wherein the derivatization is oxidation of the delta sulfur of a methionine.
113. The polypeptide of claim 106, wherein the derivatization is esterification of the C-terminal carboxyl (carboxylate) group.
114. The polypeptide of any one of claims 106-113, wherein the derivatization(s) is accomplished by chemical methods or *in vitro* enzymatic methods.
115. The polypeptide of any one of claims 106-113, wherein the derivatization(s) is accomplished by post-translational processing.
116. A sweetener composition comprising:
 - (a) one or more polypeptide, wherein the one or more polypeptide comprises:
 - (i) a polypeptide sequence selected from amino acid sequences of any variant listed in Table 8, Table 9 or Table 10;
 - (ii) a polypeptide sequence having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of amino acid sequences of any variant listed in Table 8; Table 9 or Table 10;
 - (iii) a polypeptide sequence containing at least one modification by deletion, insertion, substitution, or addition of no more than 24 amino acids, relative to a polypeptide sequence selected from the group consisting of an amino acid sequence of a variant listed in Table 8, Table 9 or Table 10; or
 - (iv) a polypeptide of any of (i)- (iii), wherein the methionine at position 1 is absent; and/or the polypeptide further comprises a protein/peptide tag, optionally an affinity tag or optionally a histidine tag or optionally a (His)₆ tag;
 - and
 - (b) at least one additional sweetener other than a Myd sweet protein.

117. The sweetener composition of claim 116 wherein the additional sweetener is selected from a steviol glycoside sweetener, a mogroside sweetener, sucrose, allulose, sucralose, a polyol, and high fructose corn syrup (HFCS).
118. The sweetener composition of claim 117, wherein the steviol glycoside sweetener is selected from Rebaudioside M ("Reb M"), RebM80, Rebaudioside D ("Reb D"), RebA95 and Rebaudioside A ("Reb A").
119. The sweetener composition of claim 117, wherein the mogroside sweetener is selected from siamenoside I and mogroside V.
120. The sweetener composition of claim 117, wherein the mogroside sweetener is mogroside V.
121. The sweetener composition of claim 118, wherein the Reb M has a purity of greater than about 95%.
122. The sweetener composition of any one of claims 116-121, wherein the polypeptide has the amino acid sequence of SEQ ID NO:3 or the amino acid sequence of SEQ ID NO:141.
123. A consumable or consumable product, optionally a beverage or beverage product, comprising the sweetener composition of any one of claims 116-122.
124. The consumable or consumable product, optionally the beverage or beverage product, of claim 123, wherein the polypeptide is present in an amount between about 1 ppm and about 50 ppm.
125. The consumable or consumable product, optionally the beverage or beverage product, of claim 123, wherein the polypeptide is present in an amount between about 1 ppm and about 40 ppm.
126. The consumable or consumable product, optionally the beverage or beverage product, of claim 123, wherein the polypeptide is present in an amount selected from an amount between about 1 ppm and about 30 ppm, about 1 ppm and about 25 ppm, about 1 ppm and about 20 ppm or about 1 ppm and about 15 ppm.
127. The consumable or consumable product, optionally the beverage or beverage product, of any one of claims 123-126, wherein the consumable or consumable product optionally the beverage or beverage product has at least one improved organoleptic property compared to a consumable or consumable product or beverage or beverage product that does not contain the

sweetener composition, wherein the organoleptic property is selected from the group consisting of aroma, flavor, basic taste (sweetness, sour, saltiness, bitterness or umami), aftertaste or linger, temporal profile, mouthfeel or a combination thereof.

128. The consumable or consumable product, optionally the beverage or beverage product, of claim 127, wherein the at least one improved organoleptic property is a reduction in bitterness, aftertaste or an improvement in mouthfeel.

129. The consumable or consumable product, optionally the beverage or beverage product, of claim 127, wherein the at least one improved organoleptic property is an improvement in mouthfeel and the amount of the polypeptide is between about 1 ppm and about 40 ppm.

130. The consumable or consumable product, optionally the beverage or beverage product, of any one of claims 123-129, which is a beverage or beverage product selected from a low-calorie or no-calorie beverage or beverage product.

131. The consumable or consumable product, optionally the beverage or beverage product, of any one of claims 123-129, which is a beverage or beverage product selected from the group consisting of cola, ginger-ale, soft drinks, root beer, fruit juice, fruit-flavored juice, vegetable juice, vegetable-flavored juice, sports drinks, energy drinks, plant protein beverage, near water drinks (e.g., water with natural or synthetic flavorants), tea type (e.g. black tea, green tea, red tea, oolong tea), coffee, cocoa drink, beverage containing milk components (e.g. milk beverages, coffee containing milk components, café au lait, milk tea, fruit milk beverages).

132. The consumable or consumable product, optionally the beverage or beverage product, of any one of claims 123-131, further comprising at least one organic acid additive salt that is a sodium, calcium, potassium, or magnesium salt of an organic acid.

133. The consumable or consumable product, optionally the beverage or beverage product of claim 132, wherein the organic acid is selected from the group consisting of citric acid, malic acid, tartaric acid, fumaric acid, lactic acid, alginic acid, ascorbic acid, benzoic acid and adipic acid.

134. The consumable or consumable product, optionally the beverage or beverage product, of any one of claims 123-133, which is a beverage or beverage product having at least one change in organoleptic property compared to a beverage or beverage product that does not contain the sweetener composition, wherein the organoleptic property is selected from the group consisting of aroma, flavor, basic taste (sweetness, sour, saltiness, bitterness or umami), aftertaste or linger, temporal profile, mouthfeel or a combination thereof.

135. A method of improving at least one organoleptic property of a consumable, or consumable product, optionally a beverage or beverage product, comprising adding a sweetener composition of any one of claims 116-122 to the consumable, or consumable product, optionally a beverage or beverage product thereby improving at least one organoleptic property thereof.

136. The method of claim 135, wherein the consumable, or consumable product, is a beverage or beverage product and the sweetener composition is added to a liquid matrix thereby providing a beverage or beverage product having at least one improved organoleptic property.

137. The method of claim 135 or 136, wherein the added sweetener of the sweetener composition is a steviol glycoside sweetener is selected from Rebaudioside M ("Reb M"), RebM80, Rebaudioside D ("Reb D"), Reb A95 and Rebaudioside A ("Reb A").

138. The method of any one of claims 135-137, wherein the improved organoleptic property is selected from the group consisting of aroma, flavor, basic taste, aftertaste or linger, temporal profile, mouthfeel or a combination thereof

139. The method of any one of claims 135-138, wherein the improved organoleptic property is a reduction in bitterness, aftertaste or an improvement in mouthfeel.

140. The method of any one of claims 135-139, wherein the improved organoleptic property is an improvement in mouthfeel and the amount of polypeptide present in the beverage or beverage product is an amount between about 1 ppm and about 40 ppm.

141. The method of any one of claims 135-140, wherein polypeptide is present in the consumable or consumable product optionally a beverage or beverage product in an amount between about 1 ppm and about 50 ppm.

142. The method of any one of claims 135-140, wherein the polypeptide is present in the the consumable or consumable product optionally a beverage or beverage product in an amount selected from an amount between about 1 ppm and about 40 ppm, about 1 ppm and about 30 ppm, about 1 ppm and about 25 ppm, about 1 ppm and about 20 ppm or about 1 ppm and about 15 ppm.

143. The method of any one of claims 135-142, wherein the added sweetener is a mogroside sweetener selected from siamenoside I and mogroside V.

144. A method of modifying/changing at least one organoleptic property of a beverage or beverage product, comprising adding a sweetener composition of any preceeding claim to a liquid

matrix thereby providing a beverage or beverage product having at least one modified/changed organoleptic property.

145. The method of claim 144, wherein the organoleptic property is selected from the group consisting of aroma, flavor, basic taste (sweetness, sour, saltiness, bitterness or umami), aftertaste or linger, temporal profile, mouthfeel or a combination thereof.

146. A sweetener composition comprising:

(a) one or more polypeptide, wherein the one or more polypeptide comprises:

(i) a polypeptide sequence selected from amino acid sequences of any variant listed in Table 8, Table 9 or Table 10;

(ii) a polypeptide sequence having at least 80% sequence identity to a polypeptide sequence selected from the group consisting of amino acid sequences of any variant listed in Table 8; Table 9 or Table 10;

(iii) a polypeptide sequence containing at least one modification by deletion, insertion, substitution, or addition of no more than 24 amino acids, relative to a polypeptide sequence selected from the group consisting of an amino acid sequence of a variant listed in Table 8, Table 9 or Table 10; or

(iv) a polypeptide of any of (i)- (iii), wherein the methionine at position 1 is absent; and/or the polypeptide further comprises a protein/peptide tag, optionally an affinity tag or optionally a histidine tag or optionally a (His)₆ tag;
and

(b) at least one additional component selected from sucrose, mannitol, citric acid, hypoxanthine, theophylline, leucine, and combinations thereof.

147. The composition of claim 146, wherein the product for oral administration is a food product selected from the group consisting of pie fillings and other sweet fillings, gelatins and puddings; yogurts; sauces, syrups and dressings; and sweet spreads.

148. The composition of claim 147, wherein the product for oral administration further comprises a low methoxyl pectin.