

BRAZZEIN VARIANT**TECHNICAL FIELD**

5 The present disclosure relates broadly to variants of brazzein.

BACKGROUND

10 Artificial sweeteners are currently widely used as sugar replacements in food and drinks in response to the dramatic rise in obesity and diabetes in recent decades. These artificial sweeteners provide a palatable sweet taste while reducing caloric consumption and preventing a rise in blood glucose level. However, recent data surrounding the detrimental side effects of consuming artificial sweeteners highlight the need for alternative sweeteners.

15 Sweet proteins from natural sources have the potential to replace these artificial sweeteners due to their low-risk safety profile and intensely sweet taste. So far, seven different sweet proteins – brazzein, thaumatin, monellin, neoculin, mabinlin, miraculin and pentadin have been discovered from plants located in tropical rainforests.

20 Originally isolated from the fruit of the west African plant, *Pentadiplandra brazzeana* Bailon, brazzein is the smallest known sweet protein comprising of 54 amino acids and possesses an intense sweetness that is 500 to 2000 times more than sucrose. Structurally, brazzein has two α -helices, three strands of antiparallel β -sheet with four disulfide bonds, and its termini are in proximity with each other.
25 This structure makes brazzein thermostable and enables it to bind to the sweet taste receptor TAS1R2/TAS1R3 to bring about sweet taste perception in humans. It has been proposed that this binding occurs via a wedge model which is based on the surface charge complementarity between brazzein and TAS1R2/TAS1R3. Furthermore, the sweetness of brazzein has been shown to be affected by the
30 intramolecular forces within brazzein.

 As the food industry is experiencing a significant transformation due to precision fermentation and advancements in protein engineering, there is a potential in revolutionizing the world's relationship with food. As a result, healthier and more sustainable food ingredients with improved functionalities and better nutritional value
35 are needed.

Therefore, there is a need to provide an alternative sweetener. There is a need to provide alternative brazzein variants that are sweet and remain stable during food processing.

5 SUMMARY

Lowering sugar consumption reduces the likelihood of developing obesity and other metabolic disorders. Currently, this is accomplished by artificial non-nutritive sweeteners, which have raised concerns regarding their safety and potential
10 links to various diseases. Recent research suggests that these sweeteners may increase the risk of cancer and cardiovascular diseases and can cause adverse health effects such as gastrointestinal problems. A safer alternative is to use sweet-tasting proteins like brazzein, which do not have any negative health effects. Brazzein is therefore a promising alternative to both sugar and artificial sweeteners.

15 Brazzein is a sweet protein that is 500 to 2000 times sweeter than sucrose. It has a specific structure with two alpha helices, three strands of antiparallel beta-sheet and four disulfide bonds. Brazzein binds to the sweet taste receptor TAS1R2/TAS1R3 to bring about sweet taste perception in humans and its sweetness is affected by the intramolecular forces within the protein.

20 In the present disclosure, optimization of brazzein for food applications and processing is provided. In particular, circular permutation was applied to brazzein to improve its properties, specifically its thermostability while retaining or improving its sweetness. The circular permutation performed in the present disclosure provides various alternative amino acid sequence and new termini. Some variants disclosed
25 herein had increased bioactivity and is sweeter than wild type brazzein.

In one aspect, there is provided a brazzein variant comprising a circular permutation brazzein.

In some examples, the circular permutation comprises a coupling of the N-terminus and the C-terminus of a (wild type) brazzein sequence.

30 In some examples, the coupling covalently linked the N-terminus and the C-terminus of a brazzein sequence with a linker.

In some examples, the variant comprises termini positions that differ from the wild type brazzein.

In some examples, the variant has substantially similar tertiary structure to wild
35 type brazzein.

In some examples, the variant comprises different intramolecular forces as compared to wild type brazzein.

In some examples, the linker is 2 to 500 amino acid residues in length, optionally the linker comprises small non-polar and/or polar amino acids. In some examples, the
5 linker is a globular protein.

In some examples, the circular permutation brazzein comprises a GGGS linker to bridge the N- and C-termini of the brazzein variant.

In some examples, a circular permutation at the amino acid position 7 to 55, or 7 to 30, or 53 to 55.

10 In some examples, one or more circular permutation at any one of the amino acid positions 2, 3, 5, 7, 8, 9, 23, 24, 29, 30, 31, 32, 33, 34, 35, 36, 37, 49, 50, 53, 54, and 55, optionally comprising one or more circular permutation at any one of the amino acid positions 30, 31, 32, 33, 34, 35, 36, 53, 54, and 55.

15 In some examples, a circular permutation at any one of the amino acid positions selected from the group consisting of K30, H31, A32, R33, S34, G35, E36, E53, and Y54.

In some examples, the brazzein variant comprises any one of the following sequences:

ID	Description	Sequence for Patent
CP	S14CP	SKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYP QDKCKKVYENYPV (SEQ ID NO: 13)
CP GS	S14CP/G S linker	SKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYP GSQDKCKKVYENYPV (SEQ ID NO: 14)
CP GG GS	S14CP/G GGGS linker	SKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYP GGGSQDKCKKVYENYPV (SEQ ID NO: 15)
K3C P		KCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPGGGSQ(SEQ ID NO: 2)

V7C P		VYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCI CDYCEYPGGGSQDKCKK (SEQ ID NO: 3)
K30 CP		KHARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYP VSKCQLANQCNYDCKLD (SEQ ID NO: 4)
H31 CP		HARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPV SKCQLANQCNYDCKLDK (SEQ ID NO: 16)
A32 CP		ARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVS KCQLANQCNYDCKLDK (SEQ ID NO: 5)
R33 CP		RSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSK CQLANQCNYDCKLDKHA (SEQ ID NO: 6)
S34 CP		SGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKC QLANQCNYDCKLDKHAR (SEQ ID NO: 7)
G35 CP		GECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQ LANQCNYDCKLDKHARS (SEQ ID NO: 17)
E36 CP		ECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQL ANQCNYDCKLDKHARSG (SEQ ID NO: 8)
E53 CP		EYPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDKHARS GECFYDEKRNLQCICDYC (SEQ ID NO: 9)
Y54 CP		YPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDKHARSG ECFYDEKRNLQCICDYCE (SEQ ID NO: 10)
P55 CP		PGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGE CFYDEKRNLQCICDYCEY (SEQ ID NO: 11)
WT		QDKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEK RNLQCICDYCEY (SEQ ID NO: 1)

In some examples, the brazzein variant may further comprise one or more modifications that improve protein stability and/or ability to confer sweeter taste, optionally further comprising one or more mutation/substitution selected from the group consisting of H31R, E36D, E41A, H31R/E36D, H31R/E41A, E36D/E41A, H31R/E36D/E41A, D29A, D29K, D29N, E41K, D2A, D2I, D2N, Q17A, Y54, and replacement of tyrosine residue at C-terminus, insertion of residues before the N-terminus.

In another aspect, there is provided a polynucleotide encoding the brazzein variant as described herein.

In yet another aspect, there is provided a vector comprising the polynucleotide as described herein.

In yet another aspect, there is provided a host cell comprising the vector of any one of the preceding claims, optionally the host cell is capable of facilitating (or facilitates) the formation of S-S bonds.

In yet another aspect, there is provided a method of producing a brazzein variant, comprising performing concatemerization of a brazzein, and/or providing overlapping-fragment of brazzein polynucleotide, and/or providing the polynucleotide as described herein.

In some examples, the method comprises modifying the expressed brazzein to incorporate 4 S-S bonds.

In yet another aspect, there is provided a method of increasing the sweetness of a food product, comprising providing the brazzein variant as described herein, and wherein the food product that undergoes harsh bioprocessing and production conditions.

In yet another aspect, there is provided a method of producing a food product, providing the brazzein variant as described herein, optionally bioprocessing the food product.

DETAILED DESCRIPTION

Based on this relationship between brazzein's structure and function, many studies have utilised amino acid addition, deletion, and substitution to alter the structure of brazzein and analyse the resulting effect on its sweetness. In the present disclosure, circular permutation (CP) in brazzein is utilised to generate variants of brazzein and screen for improved thermostability whilst retaining or improving its

intense sweet taste property. CP allows for the creation of protein variants with similar tertiary structure with the amino acids connected different and thus altered quaternary structures. When deployed in protein engineering, properties such as reduced proteolytic susceptibility, improved catalytic activity, improved substrate binding, or improved thermostability can be derived. The present disclosure may be the first where CP was applied to a sweet protein. This involved the rearrangement of brazzein's amino acid sequence such that the native N-terminus and C-terminus are covalently linked with a linker region, and new termini are formed at different positions using genetic means. Based on studies with other proteins, the inventors hypothesised that the overall tertiary structure of the circular permuted brazzein variants to remain relatively similar to wild type (WT) brazzein, but the intramolecular forces may be significantly altered to confer the altered protein with stability and better sweet taste receptor binding.

In view of the above, in one aspect, the present disclosure provides a brazzein variant comprising a circular permutation brazzein.

As used herein, the term "variant" refers to a variation of brazzein as disclosed herein that differs from brazzein known in the art (for example wild type brazzein or other brazzein variants/mutants that is free of circular permutation). In some examples, the brazzein variant of the present disclosure is a brazzein that does not occur naturally in nature (or is a synthetic or engineered brazzein). In some examples, the brazzein variant may be naturally occurring. In all examples, the circular permutation brazzein is capable of forming tertiary or quaternary structure that confers protein stability (for example thermostability or structural stability) and/or ability to bind to sweet taste receptors. In some examples, the tertiary or quaternary structure of the circular permuted/permutation brazzein may be substantially the same as the tertiary or quaternary structure of a wild type brazzein.

In some examples, the circular permutation connects the brazzein termini via a linker. In some examples, the circular permutation introduces new ends through the cleavage of an existing peptide bond. In some examples, circular permutation may perturb local tertiary structure and protein dynamics. As such, in some examples, circular permutation may introduce possible quaternary structure changes.

In some examples, the circular permutation comprises a coupling of the N-terminus and the C-terminus of a (wild type) brazzein sequence.

In some examples, the coupling comprises a linker.

In some examples, the coupling covalently linked the N-terminus and the C-terminus of a brazzein sequence with a linker.

In some examples, the variant comprises termini positions that differ from the wild type brazzein.

5 In some examples, the variant has substantially similar tertiary structure to wild type brazzein.

In some examples, the circular permutation does not result in any amino acid substitutions to the brazzein wild type sequence. In some examples, the circular permutation only comprises (or consist of) reorganization of the order of residues in the
10 brazzein (wild type) sequence.

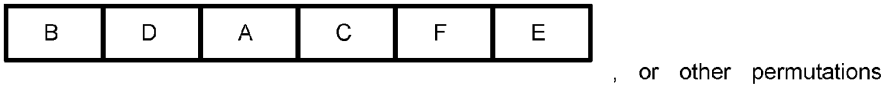
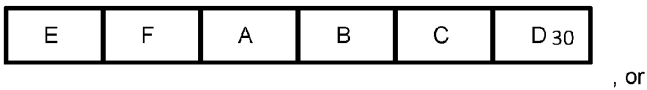
Without wishing to be bound by theory, it is believed that the introduction of circular permutation in a protein, such as brazzein, enables the relocation of protein building blocks. In some examples, the foldable variants may assume an overall structure that closely resembles the parent. In some examples, the variants may comprise local
15 perturbations near the old and/or new termini.

In some examples, circular permutations may reorganize the brazzein linear protein sequence through any one or more mechanistic models such as, but is not limited to, duplication, deletion, fission/fusion, and the like. That is, if the brazzein sequence is segmented into a plurality of segments, the circular permuted brazzein would comprise
20 segments that have different linear sequence organization from the non-circularly permuted brazzein.

For example, where a brazzein sequence comprises six segmented sequences as (I) below:



In some examples, the circular permuted brazzein may comprised reorganized segmented sequence as represented below (non-exhaustive representation):



35 thereof.

It would be appreciated by the person skilled in the art that the reorganization of brazzein linear protein sequence may occur in many possibilities that still allows the brazzein to have protein stability (for example thermostability) and/or ability to bind to sweet taste receptors. It is also understood that linear DNA sequence may lead to circular permutation protein through sortases, intein circularization, and the like.

In some examples, the variant comprises different intramolecular forces as compared to wild type brazzein.

In some examples, the circular permuted brazzein has improved stability (for example thermostability) and/or better sweet taste receptor binding.

In some examples, the linker is 2 to 500 or more amino acid residues in length, optionally the linker comprises small non-polar and/or polar amino acids.

In some examples, the linker may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, or more amino acid residues in length.

In some examples, the linker may be a flexible linker. In some examples, the linker may be a rigid linker.

In some examples, the linker is a globular protein. In some examples, the linker is a globular protein with N- and C-termini within 15 Å of each other.

In some examples, the linker may be at the N- and/or C-termini of the variant as disclosed herein. In some examples, the N- and C-terminal linker(s) may be modified to increase the solubility of the variant as described herein. In some examples, the variant may be modified to comprise a histidine tag (e.g. 6 His residues) to improve solubility.

In some examples, the linker may comprise small non-polar and/or polar amino acids such as, but is not limited to, glycine, serine, threonine, and the like.

In some examples, the linker may comprise varying blocks of linker repeats.

In some examples, the linker may be 2, 3, or 4 amino acid residues in length. In some examples, the linker may comprise the sequence GS, GGS, GGGS, and the like. In some examples, the linker may comprise the sequence GGGS. In some examples, the linker may be incorporated into the sequence as follows

QDKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLCICDY
CEYPPGGGS (SEQ ID NO: 12).

In some examples, the circular permutation brazzein comprises a GGGS linker to bridge the N- and C-termini of the brazzein variant.

In some examples, a circular permutation at the amino acid position 7 to 55, or 7 to 30, or 53 to 55.

In some examples, the amino acid position is determined from the brazzein sequence

QDKCKKYYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLCICDYCEYP
(SEQ ID NO: 1), where position 1 starts from the residue Q (glutamine).

5 In some examples, the brazzein variant may comprise one or more circular permutation at amino acid position 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, and 55. In some examples, the brazzein variant may comprise one (or more) circular permutation at amino acid positions
10 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, and 55.

In some examples, one or more circular permutation at any one of the amino acid positions 2, 3, 5, 7, 8, 9, 23, 24, 29, 30, 31, 32, 33, 34, 35, 36, 37, 49, 50, 53, 54, and
15 55.

In some examples, one or more circular permutation at any one of the amino acid positions 30, 31, 32, 33, 34, 35, 36, 53, 54, and 55.

In some examples, a circular permutation at any one of the amino acid positions selected from the group consisting of K30, H31, A32, R33, S34, G35, E36, E53, and
20 Y54.

In some examples, the linker may be found at amino acid position 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, or 55.

25 In some examples, the linker may be found at any one (or more) of the amino acid positions 2, 3, 5, 7, 8, 9, 23, 24, 29, 30, 31, 32, 33, 34, 35, 36, 37, 49, 50, 53, 54, or 55.

In some examples, the linker may be found at any one (or more) of the amino acid positions 30, 31, 32, 33, 34, 35, 36, 53, 54, or 55.

30 In some examples, the linker may be found at any one of the amino acid K30, H31, A32, R33, S34, G35, E36, E53, and Y54.

In some examples, the brazzein variant comprises any one of the following sequences:

ID	Description	Sequence for Patent
CP	S14CP	SKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPQDKCKKVYENYPV (SEQ ID NO: 13)
CP GS	S14CP/G S linker	SKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPGSQDKCKKVYENYPV (SEQ ID NO: 14)
CP GG GS	S14CP/G GGS linker	SKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPGGSQDKCKKVYENYPV (SEQ ID NO: 15)
K3C P		KCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPGGGSQ(SEQ ID NO: 2)
V7C P		VYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKK (SEQ ID NO: 3)
K30 CP		KHARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLD (SEQ ID NO: 4)
H31 CP		HARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDK (SEQ ID NO: 16)
A32 CP		ARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDK(SEQ ID NO: 5)
R33 CP		RSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDKHA (SEQ ID NO: 6)
S34 CP		SGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDKHAR (SEQ ID NO: 7)

G35 CP		GECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQ LANQCNYDCKLDKHARS(SEQ ID NO: 17)
E36 CP		ECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQL ANQCNYDCKLDKHARSG (SEQ ID NO: 8)
E53 CP		EYPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDKHARS GECFYDEKRNLQCICDYC(SEQ ID NO: 9)
Y54 CP		YPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDKHARSG ECFYDEKRNLQCICDYCE (SEQ ID NO: 10)
P55 CP		PGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGE CFYDEKRNLQCICDYCEY (SEQ ID NO: 11)
WT		QDKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEK RNLQCICDYCEYP (SEQ ID NO: 1)

In some examples, further comprising one or more modifications that improve protein stability (for example thermostability) and/or ability to confer sweeter taste (e.g. better binding to sweet taste receptors).

In some examples, further comprising one or more mutation/substitution selected from the group consisting of H31R, E36D, E41A, H31R/E36D, H31R/E41A, E36D/E41A, 5 H31R/E36D/E41A, D29A, D29K, D29N, E41K, D2A, D2I, D2N, Q17A, Y54,, replacement of tyrosine residue at C-terminus, insertion of residues before the N-terminus (such as two residues insertion of isoleucine, glycine, proline), substitutions of non-essential amino acid residues, and the like.

10 In another aspect, there is provided a polynucleotide encoding the brazzein variant as described herein.

In yet another aspect, there is provided a polynucleotide encoding a brazzein variant comprising a circular permutation brazzein.

15 In yet another aspect, there is provided a vector comprising the polynucleotide of as described herein.

In yet another aspect, there is provided a vector capable of expressing the brazzein variant as described herein.

As described herein, a "vector" is any molecule or composition that has the ability to carry a nucleic acid sequence into a suitable host cell where e.g., synthesis of the encoded polypeptide can take place. Typically, and preferably, a vector is a nucleic acid that has been engineered, using recombinant DNA techniques that are known in the art, to incorporate a desired nucleic acid sequence (e.g., a nucleic acid of the present disclosure). Expression vectors typically contain one or more of the following components (if they are not already provided by the nucleic acid molecules): a promoter, one or more enhancer sequences, an origin of replication, a transcriptional termination sequence, a complete intron sequence containing a donor and acceptor splice site, a leader sequence for secretion, a ribosome binding site, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element.

Vectors are typically selected to be functional in the host cell in which the vector will be used (the vector is compatible with the host cell machinery such that amplification of the gene and/or expression of the gene can occur. The vector as described herein may be an expression vector and/or a cloning vector.

In yet another aspect, there is provided a vector expressing the polypeptide as described herein. In some examples, the vector is selected from the group consisting of a plasmid, a viral particle, a phage, a baculovirus, a yeast plasmid, a lipid based vehicle, a polymer microsphere, a liposome, and a cell based vehicle, a colloidal gold particle, lipopolysaccharide, polypeptide, polysaccharide, a viral vehicle, an adenovirus, a retrovirus, a lentivirus, an adeno-associated viruses, a herpesvirus, a vaccinia virus, a foamy virus, a cytomegalovirus, a Semliki forest virus, a poxvirus, a pseudorabies virus, an RNA virus vector, a DNA virus vector and a vector derived from a combination of a plasmid and a phage DNA, further optionally wherein said polynucleotide is operatively linked to an expression control sequence(s) to direct peptide synthesis, even further optionally wherein the vector comprises one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells.

In yet another aspect, there is provided a host cell comprising the vector as described herein.

The term "host cell," as used herein, is intended to refer to a cell into which an expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either

mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

In some examples, the host cell is capable of facilitating (or facilitates) the formation of S-S bonds.

5 In yet another aspect, there is provided an expression system encoding and expressing the brazzein variant as described herein.

In yet another aspect, there is provided a method of producing or making a brazzein variant, comprising expressing the polynucleotide as described herein, and/or culturing the host cell as described herein.

10 In some examples, the host cell is capable of facilitating (or facilitates) the formation of S-S bonds.

In some examples, the method comprises modifying the expressed brazzein to incorporate a circular permutation.

15 In some examples, the method comprises modifying the expressed brazzein to incorporate 4 S-S bonds.

In yet another aspect, there is provided a method of producing the brazzein variant as described herein, comprising performing concatemerization of a brazzein, and/or providing overlapping-fragment of brazzein polynucleotide, and/or providing the polynucleotide of any one of the preceding claims.

20 In some examples, the brazzein variant as described herein may be produced by ordering the polynucleotides encoding the gene. In particular, the genes encoding for the brazzein may be manipulated by typical molecular biological techniques. In some examples, the brazzein variant as described herein may be produced by chemically circularizing the brazzein.

25 In some examples, the method further comprises the utilization of techniques (such as T7 shuffle) to incorporate 4 S-S bonds, and/or providing a host that facilitates the formation of S-S bonds.

In yet another aspect, there is provided a method of increasing the sweetness of a product, comprising providing the brazzein variant as described herein.

30 In some examples, the product is a food product, optionally food product that undergoes harsh bioprocessing and production conditions (e.g. high and prolonged heat treatments).

In yet another aspect, a method of producing a food product, providing the brazzein variant as described herein, optionally bioprocessing the food product.

In some examples, the brazzein variant as disclosed herein may undergo harsh bioprocessing and/or production conditions (e.g. high and prolonged heat treatments). In some examples, the brazzein variant as disclosed herein may undergo biomanufacturing processes including fermentation, and the like. Withstanding the conditions, it is believed that the brazzein variant as disclosed herein may retain its sweetness and/or heat stability.

Also disclosed are circular permutation brazzein variants as disclosed herein.

Also disclosed are methods of producing circular permutation brazzein as disclosed herein.

Also disclosed are methods and/or products as disclosed herein.

Additionally, when describing some embodiments, the disclosure may have disclosed a method and/or process as a particular sequence of steps. However, unless otherwise required, it will be appreciated that the method or process should not be limited to the particular sequence of steps disclosed. Other sequences of steps may be possible.

The particular order of the steps disclosed herein should not be construed as undue limitations. Unless otherwise required, a method and/or process disclosed herein should not be limited to the steps being carried out in the order written. The sequence of steps may be varied and still remain within the scope of the disclosure.

Furthermore, it will be appreciated that while the present disclosure provides embodiments having one or more of the features/characteristics discussed herein, one or more of these features/characteristics may also be disclaimed in other alternative embodiments and the present disclosure provides support for such disclaimers and these associated alternative embodiments.

DESCRIPTION OF FIGURES

Example embodiments of the disclosure will be better understood and readily apparent to one of ordinary skill in the art from the following discussions and if applicable, in conjunction with the figures. It should be appreciated that other modifications may be made without deviating from the scope of the invention. Example embodiments are not necessarily mutually exclusive as some may be combined with one or more embodiments to form new exemplary embodiments. The example embodiments should not be construed as limiting the scope of the disclosure.

Fig. 1 shows the sequence alignment of WT brazzein against selected CP variants. Sequences were aligned by clustal omega Clustal Omega (Sievers et al 2011)

and depicted by ESPript 3.0x (Robert & Gouet, 2014). Bold letters indicate identical amino acids between sequences and letters boxed in black indicate identical amino acids across all the sequences. Structural elements are given by chain A, brazzein structure (PDB: 4HE7)).

5 **Fig. 2** shows the comparison of non-heat-treated CP variant library. Magnitude of change in calcium mobilization for the CP variants were tested and compared against WT* brazzein in the sweet taste receptor luminescence assay. Variants with statistically significant positive changes over WT* are K30, E53 ($p \leq 0.05$), A32, S34, E36, Y54 ($p \leq 0.01$). Data presented here is interpolated and averaged from non-linear fits of
10 experimentally derived data of minimally two and maximally four replicates at a standardized protein concentration of 0.07 mg/mL. Error bars are standard deviation. WT* refers to WT with GGGS linker.

Fig. 3 shows the calcium mobilization of heat-treated WT brazzein and CP variants. Calcium mobilization of WT brazzein and CP variants in region 1 in the sweet
15 taste receptor luminescence assay. The response of 2 concentrations of thaumatin in the assay is shown for scale. Data is interpolated and averaged from non-linear fits of experimentally derived data at a single protein concentration point of 0.1 mg/mL. Data presented here are the average of minimally two and maximally four replicates. Error bars are standard deviation. Bonferroni's multiple comparisons test was employed to
20 determine statistical significance of CP variant against WT brazzein. Four asterisks (****) indicate $p \leq 0.0001$. Calcium mobilization of WT brazzein and CP variants in region 2 in the sweet taste receptor luminescence assay. Data is interpolated and averaged from non-linear fits of experimentally derived data at a single protein concentration point of 0.2 mg/mL. Data presented here are the average of minimally two and maximally four
25 replicates. Error bars are standard deviation. Bonferroni's multiple comparisons test was employed to determine statistical significance of CP variant against WT brazzein. Three asterisks (***) indicate $p \leq 0.001$. Two asterisks (**) indicate $p \leq 0.01$.

Fig. 4 shows the sweet taste receptor activity of brazzein clones containing termini linkers GS or GGGS when introduced to circular permutation variant S14. Data
30 is presented as an average of two run replicates and two assay runs were performed on separate days –Day 1 and Day 2.

Fig. 5 shows calcium mobilization of WT brazzein with and without GGGS linker. Data is interpolated and averaged from non-linear fits of experimentally derived data at protein concentration points of 24.5 and 0.78 mM. Data presented here are the average
35 of triplicates. Error bars are standard deviation.

Fig. 6 shows WT brazzein and CP variants were expressed in *E. coli*. Cells were heat-lysed and the lysates were purified by Ni-NTA affinity chromatography. The purified lysates were concentrated, buffer exchanged and run on a Novex 16 % Tricine gel with Tricine SDS Running buffer. The gel was stained with Coomassie blue and imaged. L: Novex pre-stained ladder, 1: K30CP, 2: H31CP, 3: A32CP, 4: R33CP, 5: S34CP, 6: G35CP, 7: E36CP and 8: WT brazzein.

Fig. 7 shows WT brazzein and CP variants were expressed in *E. coli*. Cells were heat-lysed and the lysates were purified by Ni-NTA affinity chromatography. The purified lysates were concentrated, buffer exchanged and run on a Novex 16 % Tricine gel with Tricine SDS Running buffer. The gel was stained with Coomassie blue and imaged. L: Novex pre-stained ladder, 1: E53CP, 2: Y54CP, 3: P55CP and 4: WT brazzein.

EXPERIMENTAL SECTION

Methods

BL21(DE3) *Escherichia coli* protein expression and purification

DNA sequences optimized for *E. coli* codons were synthesized in a pET24a(+) vector by Twist Biosciences, USA, for the constructs (Table 1). To start, single colonies of transformed BL21(DE3) *E. coli* (New England Biolabs, USA) were cultured overnight at 37 °C with shaking at 200 rpm in Lysogeny broth (LB) supplemented with 50 µg/mL kanamycin. The resulting cultures were then used to inoculate 300 mL of Terrific broth (TB) (Thermo Fisher Scientific, USA) with 50 µg/mL kanamycin and grown at 37 °C with shaking at 200 rpm until the optical density at 600nm (OD600) reached 0.4 – 0.6. At this point, 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression, followed by overnight incubation at 30 °C with shaking at 200 rpm. The resulting cultures were harvested by centrifugation at 8,000 g for 10 min at 4 °C, and the resulting pellets were freeze-thawed. For characterization of heat-treated proteins, the collected pellets were lysed by resuspending them in 50 mM sodium phosphate buffer (pH 7.4), 300 mM sodium chloride, 10 mM imidazole, and heating them at 95 °C for 10 min. The lysed cells were then centrifuged at 18,000 g for 20 min at 4 °C, and the resulting supernatant was incubated with PureCube 100 INDIGO Ni-Agarose resin (Cube Biotech, Germany) at room temperature for 1 h. The protein-bound resin was then washed with 50 mM sodium phosphate buffer (pH 7.4), 500 mM sodium chloride and 20 mM imidazole, and the bound protein was eluted with 50 mM sodium phosphate buffer

(pH 7.4), 500 mM sodium chloride and 500 mM imidazole. The eluate was buffer-exchanged and concentrated using Hank's Balanced Salt Solution (HBSS) with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.0.

5 Sweet taste receptor luminescence assay

HEK 293T cells (ATCC CRL-3216) were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, USA) supplemented with 10 % (v/v) heat-inactivated Fetal Bovine Serum (FBS; Biowest, France) and 1 % (v/v) penicillin-streptomycin (Gibco, Thermo Fisher Scientific, USA) at 37 °C in a
10 humidified atmosphere of 5 % CO₂.

HEK 293T cells were seeded at a density of 20,000 cells per well in white 384-well tissue culture plates (Greiner, Germany), coated with Poly-D-Lysine (PDL; Sigma-Aldrich, USA) at a final concentration of 1 mg/mL. After an overnight incubation, the cells were transfected with two plasmids containing the sweet taste receptor
15 (TAS1R2/TAS1R3), chimeric Gα16-gust44, and mitochondrial-targeted (mt)-clytin II genes at a ratio of 20 ng: 20 ng per well using ViaFect (Promega), using 3 µL of transfection agent for every 1 µg of plasmid used. After 6 h, the media was completely replaced with low-glucose DMEM (Gibco, Thermo Fisher Scientific, USA) supplemented with 10 % (v/v) dialysed FBS (Biowest, France) and 1 % (v/v) penicillin-streptomycin
20 (Gibco, Thermo Fisher Scientific, USA).

After an overnight incubation at standard cell culture conditions, the spent media in the wells with transfected cells was removed and 10 µL of this spent media was reintroduced into the wells, before 25 µL of coelenterazine F (AAT Bioquest, Inc., USA) was added to a final concentration of 10 µM, in assay buffer (1× HBSS assay buffer with
25 20 mM HEPES at pH 7.0). The assay plate was incubated for 4 h, at 27 °C in the dark. The test ligands were prepared to a 2.4 times concentration in assay buffer. Two reference sweeteners, sucralose, and the sweet protein thaumatin were used for comparison to the brazzein test samples (Joseph et al., 2019).

The assay was performed using the luminescence mode of the Fluorescent
30 Imaging Plate Reader (FLIPR TETRA, Molecular Devices, USA) controlled by the ScreenWorks software (version 4.0.0.30, Molecular Devices). During the run, a baseline read was captured for 10 s before 25 µL of test ligand was dispensed from the source plate into the assay plate. The kinetic data was acquired for an additional 100s. The response from each well was expressed as RLU relative light units (RLU) and calculated
35 using area under the curve (AUC) values. The data of the reported responses were

derived from at least two independent experiments, performed in duplicates, and were plotted using the four-parameter logarithmic regression equation using Prism 8 (GraphPad) software.

5 Results

Circular permutation

The distance between the D2 (N-terminus) and Y54 (C-terminus) of brazzein was estimated to be 15 Å, based on the previously solved three-dimensional crystal structure (PDB: 4HE7). As the termini of brazzein are not rigid, the inventors hypothesized that it is possible to use a flexible linker such as GGGS and GS, while retaining the functionality of brazzein to bind to the sweet taste receptor. A comparison of circular permuted variants with GGGS, GS and no linker was performed with the sweet taste receptor assay, where the GGGS linker resulted in a more sensitive and thus, potentially sweeter variant (Fig 4).

Based on the estimation of 15 Å, the inventors reasoned that the estimated length of the GGGS linker at 14 Å, assuming 3.5 Å per amino acid residue, was best able to maintain the relative positions of the N and C termini and thus best preserve the three-dimensional structure of the protein. Given the localized secondary structures in brazzein, the inventors expected to see hotspots that can better tolerate circular permutation (Fig 1). The inventors also believed that it is possible that a globular protein with N-, C- termini within 15 Å of each other would be able to be replaced by the linker (circular permutations <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6747460/>).

In the present disclosure, the recombinant protein construct encoding for wild type brazzein sequence is annotated as wild type (WT). From this starting construct, a total of 54 variants for the 54 positions were designed, constructed and expressed in *E. coli* (D2CP to P55CP). This is the complete combination of circular permutations without changing the linear order of the amino acids to preserve the local secondary structures in the protein.

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Characterization of CP variants

In the present disclosure, the inventors employed a cell-based taste receptor assay to systematically quantify the sweetness of the CP variants (Riedel et al., 2017). HEK 293T cells were engineered for heterologous expression of human sweet taste receptor (TAS1R2/TAS1R3) and their corresponding signaling components, allowing

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them to detect a broad range of known sweet substances, including brazzein (Chua et al., 2023). Through this method, the inventors measured the mobilization of calcium released by the activation of sweet taste receptors with sweet tasting substances such as sweet proteins like brazzein, carbohydrate-based sweeteners such as sucrose, and both naturally occurring and artificial sweetener molecules like stevioside and sucralose. The initial screen involved all 54 CP variants expressed in *E. coli*, which were compared to WT brazzein with the corresponding GGGS linker (WT*). Prior comparison of WT brazzein with and without GGGS linker on the C-terminus showed there is no detectable difference between the 2 proteins (Fig 5). The results revealed two regions with improved sweetness: region 1, positions 30 – 36, and region 2, positions 53 – 55, (Fig 2). Both of these regions lie outside of the alpha helices and beta folds and are likely to be hinged regions. In addition, a drop in potency was observed for CP variants around positions 13 – 22, 25 – 30 and 38 – 48 (Figure 2, regions a – c). These positions correlate to the amino acids that are involved in the secondary structure formation and thus by shifting the order, the secondary structure may have been disrupted. Interestingly, position 23 and 24 tolerated circular permutation, further showing that those residues may not contribute strongly to the local folding.

Table 1. Strains and plasmids used in this study for recombinant protein production.

Plasmid or strain	Relevant genotype
Plasmid	
pET-24(+) wild type brazzein	His-tagged wild type brazzein under control of T7 promoter; kanamycin resistance
pET-24(+) XYCP	XY refers to each amino acid residue in brazzein from D2 to P55; His-tagged circular permuted (CP) brazzein under control of T7 promoter; kanamycin resistance
Strain	
BL21(DE3) <i>E. coli</i>	Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible lac promoter; T7 RNA polymerase gene

Heat treated CP variants

To test the thermostability of the CP variants within regions 1 and 2, a heat treatment protocol was employed on samples of *E. coli*-expressed proteins, subjecting

them to 10 min heat at 95 °C. Observations from this heat treatment step were consistent with previous studies (Chua et al., 2023; Kalthoff, 2003), which have shown that heat lysis can improve yield and purity (Fig 6 and 7). The results showed that only E36CP exhibited a higher calcium mobilization compared to WT brazzein when subjected to heat treatment (Fig 3), retaining its observed increase in sweetness compared to WT brazzein. The results are consistent with previous findings which indicate that several amino acid residues, including E36 (Assadi-Porter, Maillet, et al., 2010), and the N and C-termini play crucial roles in determining the sweetness of a protein. Subsequently, we conclude that the circularization of the E36 junction can improve sweetness in our cell-based sweet taste receptor assay. In comparison, heat-treated CP variants within region 2 however, appear to have lost some sweet potency when compared against WT brazzein (Fig 3).

The present disclosure utilized protein engineering with CP to improve the properties of brazzein, specifically its sweet potency and thermostability. The strategy employed here retains the brazzein native core sequence. These CP variants can be combined with mutations described elsewhere which may have a synergistic effect in improving both sweetness and thermostability. The results here with the CP-generated brazzein variants revealed sites in brazzein that are important for its sweet potency similar to what was observed in previous studies (Assadi-Porter, Maillet, et al., 2010). However, while these variants showed promise in the initial sweet taste receptor assay comparison, most were not as robust as WT brazzein after thermal treatment. Nevertheless, the inventors have demonstrated for the first time, a single variant, E36CP, was both thermostable and retained its increased sweetness compared to WT brazzein after cooling. By thoroughly exploring the circular permutation space of brazzein, the inventors were also able to identify regions in which possible insertional mutagenesis may be possible. This would allow for the creation of fusion proteins that have similar or better sweet profile compared to brazzein. Thermostability could be further enhanced through fusing the protein with a thermostable protein.

The present disclosure have, therefore, explored the potential of the variants identified here in food applications and bioprocessing, including combinations of various mutations and economical production of recombinant sweet proteins.

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APPLICATIONS

20 Embodiments of brazzein variant as disclosed herein provides for sweeter than wild type brazzein. Advantageously, the brazzein variants as disclosed herein maintains this sweetness under heat treatment.

It will be appreciated by a person skilled in the art that other variations and/or modifications may be made to the embodiments disclosed herein without departing
25 from the spirit or scope of the disclosure as broadly described. For example, in the description herein, features of different exemplary embodiments may be mixed, combined, interchanged, incorporated, adopted, modified, included etc. or the like across different exemplary embodiments. The present embodiments are, therefore, to be considered in all respects to be illustrative and not restrictive.

30

CLAIMS

- 5 1. A brazzein variant comprising a circular permutation brazzein.
2. The brazzein variant of claim 1, wherein the circular permutation comprises a coupling of the N-terminus and the C-terminus of a (wild type) brazzein sequence.
- 10 3. The brazzein variant of claim 1 or 2, wherein the coupling covalently linked the N-terminus and the C-terminus of a brazzein sequence with a linker.
4. The brazzein variant of any one of the preceding claims, wherein the variant comprises termini positions that differ from the wild type brazzein.
- 15 5. The brazzein variant of any one of the preceding claims, wherein the variant has substantially similar tertiary structure to wild type brazzein.
6. The brazzein variant of any one of the preceding claims, wherein the variant comprises different intramolecular forces as compared to wild type brazzein.
- 20 7. The brazzein variant of any one of the preceding claims, wherein the linker is 2 to 500 amino acid residues in length, optionally the linker comprises small non-polar and/or polar amino acids, optionally the linker is a globular protein.
- 25 8. The brazzein variant of any one of the preceding claims, wherein the circular permutation brazzein comprises a GGGS linker to bridge the N- and C-termini of the brazzein variant.
- 30 9. The brazzein variant of any one of the preceding claims, comprising a circular permutation at the amino acid position 7 to 55, or 7 to 30, or 53 to 55.
10. The brazzein variant of any one of the preceding claims, comprising one or more circular permutation at any one of the amino acid positions 2, 3, 5, 7, 8, 9, 23, 24, 29, 30,
- 35 31, 32, 33, 34, 35, 36, 37, 49, 50, 53, 54, and 55, optionally comprising one or more

circular permutation at any one of the amino acid positions 30, 31, 32, 33, 34, 35, 36, 53, 54, and 55.

11. The brazzein variant of any one of the preceding claims, comprising a circular permutation at any one of the amino acid positions selected from the group consisting of K30, H31, A32, R33, S34, G35, E36, E53, and Y54.

12. The brazzein variant of any one of the preceding claims, wherein the brazzein variant comprises any one of the following sequences:

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ID	Description	Sequence for Patent
CP	S14CP	SKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPQDKCKKVYENYPV (SEQ ID NO: 13)
CP GS	S14CP/G S linker	SKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPGSQDKCKKVYENYPV (SEQ ID NO: 14)
CP GG GS	S14CP/G GGG linker	SKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPV (SEQ ID NO: 15)
K3C P		KCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPGGGSQ(SEQ ID NO: 2)
V7C P		VYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKK (SEQ ID NO: 3)
K30 CP		KHARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLD (SEQ ID NO: 4)
H31 CP		HARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQLANQCNYDCKLDK (SEQ ID NO: 16)

A32 CP		ARSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVS KCQLANQCNVDCKLDK(SEQ ID NO: 5)
R33 CP		RSGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSK CQLANQCNVDCKLDKHA (SEQ ID NO: 6)
S34 CP		SGECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKC QLANQCNVDCKLDKHAR (SEQ ID NO: 7)
G35 CP		GECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQ LANQCNVDCKLDKHARS(SEQ ID NO: 17)
E36 CP		ECFYDEKRNLQCICDYCEYPGGGSQDKCKKVYENYPVSKCQL ANQCNVDCKLDKHARSG (SEQ ID NO: 8)
E53 CP		EYPGGGSQDKCKKVYENYPVSKCQLANQCNVDCKLDKHARS GECFYDEKRNLQCICDYC(SEQ ID NO: 9)
Y54 CP		YPGGGSQDKCKKVYENYPVSKCQLANQCNVDCKLDKHARSG ECFYDEKRNLQCICDYCE (SEQ ID NO: 10)
P55 CP		PGGGSQDKCKKVYENYPVSKCQLANQCNVDCKLDKHARSGE CFYDEKRNLQCICDYCEY (SEQ ID NO: 11)
WT		QDKCKKVYENYPVSKCQLANQCNVDCKLDKHARSGECFYDEK RNLQCICDYCEYP (SEQ ID NO: 1)

13. The brazzein variant of any one of the preceding claims, further comprising one or more modifications that improve protein stability and/or ability to confer sweeter taste, optionally further comprising one or more mutation/substitution selected from the group consisting of
- 5 H31R, E36D, E41A, H31R/E36D, H31R/E41A, E36D/E41A, H31R/E36D/E41A, D29A, D29K, D29N, E41K, D2A, D2I, D2N, Q17A, Y54, and replacement of tyrosine residue at C-terminus, insertion of residues before the N-terminus.

14. A polynucleotide encoding the brazzein variant of any one of the preceding claims.
15. A vector comprising the polynucleotide of claim 14.
- 5 16. A host cell comprising the vector of any one of the preceding claims, optionally the host cell is capable of facilitating (or facilitates) the formation of S-S bonds.
- 10 17. A method of producing a brazzein variant, comprising performing concatemerization of a brazzein, and/or providing overlapping-fragment of brazzein polynucleotide, and/or providing the polynucleotide of claim 14.
- 15 18. The method of claim 17, wherein the method comprises modifying the expressed brazzein to incorporate 4 S-S bonds.
- 20 19. A method of increasing the sweetness of a food product, comprising providing the brazzein variant of any one of claims 1 to 13, and wherein the food product that undergoes harsh bioprocessing and production conditions.
- 25 20. A method of producing a food product, providing the brazzein variant of any one of claims 1 to 13, optionally bioprocessing the food product.