ENZYME METHOD

Field

The present disclosure relates to mutant inulosucrase enzymes. The mutant enzymes have improved properties compared to their wild-type equivalents when used to convert sucrose (e.g. dietary sucrose) to fructooligosaccharides such as inulin, especially *in vivo*. The invention thus relates to the enzymes per se, as well as to compositions comprising them, to their medical and non-medical uses, and to methods for the production. Related aspects are also provided.

Background

Sucrose is a disaccharide formed from glucose and fructose monomer units. Sucrose is commonly informally referred to as "sugar", reflecting the fact that fully refined table sugar comprises around 99.9% sucrose. Sucrose is produced naturally in plants such as sugar cane and sugar beet. Sucrose is commonly added to foodstuffs in order to increase the sweetness of such foods, and also as a preservative. The use of sucrose in foodstuffs, in particular baked goods, is typically considered to be important for satisfactory "mouthfeel" (texture etc). Sucrose is a major commodity with annual global production in the order of hundreds of millions of tonnes.

Once consumed by subjects, typically mammalian subjects, sucrose is typically metabolised into its component monomeric units (glucose and fructose) by enzymes such as sucrases, isomaltase glycoside hydrolases, and invertases, which are often found in (e.g.) the duodenum. The glucose and fructose units thus generated are rapidly absorbed into the bloodstream. Sucrose is a high energy compound yielding around 17 kJ/g.

The significant calorific value of sucrose means that health authorities around the world have recommended limits on daily consumption by subjects such as humans. For example, the UK National Health Service recommends that adults should not consume more than 30g of sugar a day. Recommended daily limits for children are lower, at approximately 19-24 g per day. It is also recommended that sugar should not exceed more than 5% of the total calories obtained from food and drink per day. Broadly similar guidelines are issued by other health authorities around the world. For example, the US *Dietary Guidelines for Americans 2015-2020* recommends limiting sugar intake for adults to around 200 calories (kcal) (ca. 50 g).

Despite these recommendations, typical daily sugar consumption is significantly in excess of the suggested levels. For example, the Dietary Guidelines for Americans

indicates that the average American adult consumes approximately 70 g of sugar per day, corresponding to an energy intake of around 270 kcal.

Excess sucrose consumption is problematic as it is associated with numerous health issues. For example, excess sucrose consumption is associated with metabolic syndrome, diabetes (particularly type 2 diabetes), non-alcoholic fatty liver disease, constipation and weight gain and obesity in adults and children.

In view of these issues, one strategy that is widely promoted is for subjects to simply reduce their overall sugar intake. This strategy can be successful where circumstances allow. However, for many adults the reality is that consuming high-sugar foods is a pleasurable and often unavoidable part of life. Low-sugar alternatives to high-sugar foods are often perceived as being less desirable, e.g. as being less satisfying. Furthermore, in many cases low-sugar options are simply not available, whether due to scarcity of supply in some regions or through social pressure to partake of high-sugar foods. In addition, for many people the need to consider the sugar levels of foodstuffs is a significant time and mental burden. In practice, such subjects tend to simply consume excess sugar levels leading to health problems as set out above.

These difficulties have been long recognised and various attempts have been made to address them by replacing sugar with substitutes. Most attempts have focussed on reducing sugar levels in commercially available foodstuffs. However, this can have significant adverse implications, in terms of increased production costs, reduced shelf-life requiring the need for artificial preservatives, which have been linked to health and taste issues, and a perceived worsening in taste. For example, the commonly-used artificial sweetener saccharin has been associated with a bitter aftertaste. These issues have led to a degree of consumer resistance to products containing artificial sweeteners.

In view of these difficulties, one approach that has been considered is to treat foodstuffs made using sucrose in order to reduce their calorific burden without the need for artificial sweeteners. One approach that has been described is the industrial treatment of sucrose-containing foods prior to their consumption with enzymes such as glycosyltransferases. These enzymes have been shown to convert sucrose to fructooligosacharides which cannot be metabolised by the body, and thus do not contribute to a subject's calorific intake.

In some cases, fructosyltransferases may be used to treat a foodstuff. More commonly, fructosyltransferases are used to generate fructooligosaccharides which may be added to recipes. The presence of added fructooligosacharides to a foodstuff may lead (or

be perceived to lead) to adverse effects, such as a worsened taste. In addition, "mouthfeel" is often perceived to be adversely affected by fructooligosaccharides generated in the pretreatment of food, as these can negatively affect the texture of the foodstuffs treated. These difficulties mean that even subjects seeking to make healthy choices often end up in practice consuming high-sucrose foods. These issues particularly affect foodstuffs treated with previously used glycosyltransferases where typically high concentrations of the enzyme are needed in order to achieve useful conversion efficiencies.

Accordingly, there is a need for new and/or improved methods of reducing the problems associated with excess sucrose consumption without compromising on taste or mouthfeel.

As described herein, the use of fructosyltransferase enzymes to convert sucrose to insoluble products that are not metabolised by the body has been described previously. Such enzymes, and compositions comprising them, are useful in therapeutic and non-therapeutic uses such as appetite suppression.

However, there remains a need for improved enzymes for reducing the problems associated with excess sucrose consumption without compromising on taste or mouthfeel. For example, there is a need for improved enzymes with increased catalytic (enzymatic) activity, improved stability (e.g. at low pH, such as at gastric pH) and/or improved resistance to degradation (e.g. degradation by proteases such as digestive proteases).

The present inventors have recognised the issues above. The present disclosure addresses some or all of such problems.

Summary

The present disclosure relates to a mutant inulosucrase enzyme. The mutant enzyme comprises a variant of the amino acid sequence shown in SEQ ID NO: 1, which is the amino acid sequence of the wild-type inulosucrase enzyme of gene inuGB from Lactobacillus gasseri DSM 20604.

The variant comprises a modification at one or more of the following positions: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, L316, S336, K339, K343, D384, D406, M422, T474, H489, W492, A510, and D522. In some embodiments the variant comprises a modification at one or more of V49, D57, A71, Q134, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K339, D406, M422, T474, H489, W492, T513, and D522. As explained herein in more detail, the inventors have surprisingly found that introducing amino acid modifications at these

positions in the amino acid sequence of SEQ ID NO: 1 is associated with significant improvements in the properties of the resulting enzyme. Typically, the enzymatic activity of the variant is improved significantly compared to the corresponding activity of the wild-type enzyme; the stability of the variant in the presence of low pH is typically enhanced significantly compared to the corresponding stability of the wild-type enzyme; and/or the ability of the variant to withstand degradation by digestive proteases is typically enhanced significantly compared to the corresponding stability of the wild-type enzyme. The variant is therefore particularly suitable for being administered to a subject, for example as an isolated enzyme, to catalyse the conversion of dietary sucrose to inulin *in vivo*. In more detail, the variant converts sucrose to inulin thus preventing or reducing generation of free fructose by *in vivo* metabolism of sucrose in the subject. The reduction or prevention of free fructose reduces or prevents fructose uptake by the subject. As explained below this is associated with significant health benefits.

Accordingly, the present disclosure provides a mutant inulosucrase enzyme comprising a variant of the amino acid sequence shown in SEQ ID NO: 1, wherein the variant comprises a modification at one or more of the following positions: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, L316, S336, K339, K343, D384, D406, M422, T474, H489, W492, A510, and D522. In some embodiments the variant comprises modifications at one or more of V49, D57, A71, Q134, N143, G152, T159, G172, K238, Q240, K270, L316, S336, K339, D406, M422, T474, H489, W492, T513, and D522. In some embodiments the modification is substitution with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine.

Also provided is a mutant inulosucrase enzyme comprising one or more modification selected from Q134Y, T513A, A71Y, T10P, V49Y, D57Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K339R, K339Y, K343S, D384A, D406I, M422Y, T474A, H489N, W492A, A510D, and D522P. In some embodiments the one or more modification is selected from V49Y, D57Y, A71Y, Q134Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K339R, K339Y, D406I, M422Y, T474A, H489N, W492A, T513A, and D522P.

In some embodiments the mutant inulosucrase enzyme provided herein has at least 70% identity to the amino acid sequence shown in SEQ ID NO: 1.

In some embodiments the mutant inulosucrase enzyme comprises a modification at one or more of: Q134, T513, A71, T10, V49, D57, G152, G172, K238, A309, L316, K339,

K343, S345, D384, D406, H489, and A510. In some embodiments the variant comprises one or more of Q134Y, T513A, A71Y, T10P, V49Y, D57Y, G152A, G172R, K238A, A309I, L316I, K339Y, K343S, S345A, D384A, D406I, H489N and A510D.

In some embodiments the mutant inulosucrase enzyme comprises a modification at one or more of: Q134, T513, A71, V49, D57, A309, K339, S345, D406, H489, and A510. In some embodiments the variant comprises one or more of Q134Y, T513A, A71Y, V49Y, D57Y, A309I, K339Y, S345A, D406I, H489N and A510D.

In some embodiments, the mutant inulosucrase enzyme comprises a modification at one or more of: V49, D57, A71, Q134, T159, G172, A309, L316, K339, S345, D406, M422, and T513. In some embodiments the variant comprises one or more of V49Y, D57Y, A71Y, Q134Y, T159R, G172R, A309I, L316I, K339Y, S345A, D406I, M422Y, and T513A.

In some embodiments the mutant inulosucrase enzyme comprises a modification at one or more of: D57, A71, Q134, N143, G152, A309, L316, K339, S345, D406, and T474. In some embodiments the variant comprises one or more of D57Y, A71Y, Q134Y, N143D, G152A, A309I, L316I, K339Y, S345A, D406I and T474A.

In some embodiments the mutant inulosucrase enzyme comprises a modification at one or more of: G172, L316, K339, S345, D406, M422, and T513. In some embodiments the variant comprises one or more of G172R, L316I, K339R, K339Y, S345A, D406I, M422Y and T513A.

In some embodiments the mutant inulosucrase enzyme comprises a modification at one or more of: D57, A71, G172, A309, L316, K339, S345, D406, and M422. In some embodiments the variant comprises one or more of D57Y, G172R, A71Y, A309I, L316I, K339Y, S345A, D406I and M422Y.

In some embodiments the mutant inulosucrase enzyme comprises a modification at one or more of: D57, G172, L316, D406, and M422. In some embodiments the variant comprises one or more of D57Y, G172R, L316I, D406I and M422Y.

In some embodiments the mutant inulosucrase enzyme comprises at least two, three, four, five, six, seven, eight, nine, ten or more of the following modifications: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K343, S345, K339, D384, D406, M422, T474, H489, W492, A510, and D522. In some embodiments the mutant inulosucrase enzyme comprises a modification at at least two, three, four, five, six, seven, eight, nine, ten or more of the following positions: V49,

D57, A71, Q134, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K339, S345, D406, M422, T474, H489, W492, T513, and D522.

In some embodiments the mutant inulosucrase enzyme comprises at least two, three, four, five, six, seven, eight, nine, ten or more of the following modifications: Q134Y, T513A, A71Y, T10P, V49Y, D57Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K343S, S345A, K339R, K339Y, D384A, D406I, M422Y, T474A, H489N, W492A, A510D, and D522P. In some embodiments the mutant inulosucrase enzyme comprises at least two, three, four, five, six, seven, eight, nine, ten or more of the following modifications: V49Y, D57Y, A71Y, Q134Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K339R, K339Y, S345A, D406I, M422Y, T474A, H489N, W492A, T513A, and D522P.

In some embodiments the mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 1 – 92, 303 – 345 and/or 400 – 418 of SEQ ID NO 1. In some embodiments said region corresponds to an alpha-helical region of the structure of the mutant inulosucrase enzyme. In some embodiments the variant comprises one or more certain specific combinations of modifications disclosed herein. In some embodiments the variant comprises any number and any combination of modifications and/or substitutions defined herein.

In some embodiments the variant is capable of catalysing transfructosylation. In some embodiments the variant is capable of catalysing transfructosylation under physiological conditions.

In some embodiments the mutant inulosucrase enzyme is expressed by or is obtainable by intracellular or extracellular expression from an organism of genus *Escherichia, Lactobacillus, Saccharomyces, Bacillus*, *Komagataella, Pichia*, *Trichoderma, Corynebacterium* or *Aspergillus*; preferably *E. coli, S. cerevisiae, B. subtilis, K. phaffii, P. pastoris, T. reesei, C. glutamicum, A. niger,* or *A. oryzae*.

In some embodiments the mutant inulosucrase enzyme is in isolated form.

Also provided is a polynucleotide which encodes a mutant inulosucrase enzyme as provided herein. Also provided is a vector comprising the polynucleotide. Also provided is a cell comprising the vector. In some embodiments the cell is a bacterial cell, a yeast cell, or a fungal cell. Also provided is a method of producing a mutant inulosucrase enzyme as described herein, comprising expressing said mutant inulosucrase enzyme from a cell as provided herein. In some embodiments the method comprises isolating said mutant inulosucrase enzyme from said cell.

Also provided is a food composition or foodstuff comprising a mutant inulosucrase enzyme as provided herein, and one or more carbohydrate, fat, lipid, protein, flavouring agent, or colouring agent. In some embodiments the food composition or foodstuff comprises sucrose.

Also provided is a nutraceutical composition comprising a mutant inulosucrase enzyme as provided herein, and one or more nutraceutically acceptable filler, stabilizing agent, colouring agent or flavouring agent; optionally wherein said composition is a dietary supplement. Also provided is a pharmaceutically acceptable composition comprising a mutant inulosucrase enzyme as provided herein, and one or more pharmaceutically acceptable carrier, excipient, or diluent. In some embodiments the composition is for oral administration. In some embodiments the composition (i) comprises an enteric coating and/or (ii) is formulated as a tablet, a troche, a lozenge, an aqueous or oily suspension, a dispersible powder or as granules.

Also provided is an *in vivo* method of reducing fructose and/or glucose uptake in a subject, the method comprising administering to the subject a mutant inulosucrase enzyme, food composition or foodstuff, nutraceutical or pharmaceutical composition as provided herein, and thereby converting sucrose to fructooligosaccharide (preferably inulin) *in vivo*. Also provided is an *in vivo* method of producing fructooligosaccharides (preferably inulin) in a subject *in vivo*, the method comprising administering to the subject a mutant inulosucrase enzyme, food composition or foodstuff, nutraceutical or pharmaceutical composition as provided herein, and thereby converting sucrose to fructooligosaccharide (preferably inulin) *in vivo*. Also provided is a method of suppressing a subject's appetite and/or increasing a subject's satiety, comprising administering to the subject a mutant inulosucrase enzyme, food composition or foodstuff, nutraceutical or pharmaceutical composition as provided herein.

In some embodiments the provided method is a non-therapeutic method. In some embodiments the provided method comprises orally administering the mutant inulosucrase enzyme to the subject. In some embodiments the mutant inulosucrase enzyme is administered as an isolated enzyme.

Also provided is a mutant inulosucrase enzyme, food composition or foodstuff, nutraceutical or pharmaceutical composition as provided herein, for use in reducing fructose uptake and producing fructooligosaccharide (preferably inulin) in a subject, wherein said use comprises administering to the subject the mutant inulosucrase enzyme and thereby converting sucrose to fructooligosaccharide (preferably inulin) *in vivo*.

In some embodiments said use comprises orally administering said mutant inulosucrase enzyme to said subject. In some embodiments of said use, said mutant inulosucrase enzyme is administered as an isolated enzyme.

In some embodiments administering the mutant inulosucrase enzyme to said subject suppresses the subject's appetite and/or increases the subject's satiety.

Also provided is a mutant inulosucrase enzyme, food composition or foodstuff, nutraceutical or pharmaceutical composition as provided herein, for use in *in vivo* administration to a subject.

Also provided is a mutant inulosucrase enzyme, food composition or foodstuff, nutraceutical or pharmaceutical composition as provided herein, for use in medicine.

Also provided is a mutant inulosucrase enzyme, food composition or foodstuff, nutraceutical or pharmaceutical composition as provided herein, for use in treating or preventing metabolic syndrome, diabetes, non-alcoholic fatty liver disease, constipation or obesity in a subject in need thereof.

In some embodiments said use comprises orally administering said mutant inulosucrase enzyme to said subject. In some embodiments said mutant inulosucrase enzyme is administered as an isolated enzyme.

Also provided is use a mutant inulosucrase enzyme, food composition or foodstuff, nutraceutical or pharmaceutical composition as provided herein, for (i) reducing fructose uptake in a subject; (ii) producing fructooligosaccharide (preferably inulin) in a subject; (iii) converting sucrose to fructooligosaccharide (preferably inulin) in a subject; suppressing a subject's appetite and/or increasing a subject's satiety.

Also provided is a method of increasing (i) *in vivo* transfructosylation activity, (ii) pH stability and/or (iii) protease resistance of an inulosucrase enzyme; by making in the inulosucrase enzyme one or more modifications as described herein; and thereby increasing (a) the *in vivo* transfructosylation activity of the inulosucrase enzyme, (b) the pH stability of the inulosucrase enzyme and/or (iii) the protease resistance of the inulosucrase enzyme.

Detailed Description

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. Of course, it is to be understood that not necessarily all aspects or advantages may be achieved

in accordance with any particular embodiment of the invention. Thus, for example those skilled in the art will recognize that the invention may be embodied or carried out in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other aspects or advantages as may be taught or suggested herein.

In addition as used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an enzyme molecule" includes two or more such molecules, reference to "a fructooligosaccharide" includes two or more such fructooligosaccharides and the like.

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

Definitions

The following terms or definitions are provided solely to aid in the understanding of the invention. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Press, Plainsview, New York (2012); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 114), John Wiley & Sons, New York (2016), for definitions and terms of the art. The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ± 20 % or ± 10 %, more preferably ± 5 %, even more preferably ± 1 %, and still more preferably ± 0.1 % from the specified value, as such variations are appropriate to perform the disclosed methods.

The term "amino acid" in the context of the present disclosure is used in its broadest sense and is meant to include organic compounds containing amine (NH_2) and carboxyl (COOH) functional groups, along with a side chain (e.g., a R group) specific to each amino acid. In some embodiments, the amino acids refer to naturally occurring L α-amino acids or residues. The commonly used one and three letter abbreviations for naturally occurring amino acids are used herein: A=Ala; C=Cys; D=Asp; E=Glu; F=Phe; G=Gly; H=His; I=Ile; K=Lys; L=Leu; M=Met; N=Asn; P=Pro; Q=Gln; R=Arg; S=Ser;

T=Thr; V=Val; W=Trp; and Y=Tyr (Lehninger, A. L., (1975) Biochemistry, 2d ed., pp. 71-92, Worth Publishers, New York). The general term “amino acid” further includes D-amino acids, retro-inverso amino acids as well as chemically modified amino acids such as amino acid analogues, naturally occurring amino acids that are not usually incorporated into proteins such as norleucine, and chemically synthesised compounds having properties known in the art to be characteristic of an amino acid, such as β-amino acids. For example, analogues or mimetics of phenylalanine or proline, which allow the same conformational restriction of the peptide compounds as do natural Phe or Pro, are included within the definition of amino acid. Such analogues and mimetics are referred to herein as "functional equivalents" of the respective amino acid. Other examples of amino acids are listed by Roberts and Vellaccio, The Peptides: Analysis, Synthesis, Biology, Gross and Meiehofer, eds., Vol. 5 p. 341, Academic Press, Inc., N.Y. 1983, which is incorporated herein by reference.

The terms “polypeptide”, and “peptide” are interchangeably used herein to refer to a polymer of amino acid residues and to variants and synthetic analogues of the same. Thus, these terms apply to amino acid polymers in which one or more amino acid residues is a synthetic non-naturally occurring amino acid, such as a chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally-occurring amino acid polymers. Polypeptides can also undergo maturation or post-translational modification processes that may include, but are not limited to: glycosylation, proteolytic cleavage, lipidization, signal peptide cleavage, propeptide cleavage, phosphorylation, and such like. A peptide can be made using recombinant techniques, e.g., through the expression of a recombinant or synthetic polynucleotide. A recombinantly produced peptide is typically substantially free of culture medium, e.g., culture medium represents less than about 20 %, more preferably less than about 10 %, and most preferably less than about 5 % of the volume of the protein preparation.

The term “protein” is used to describe a folded polypeptide having a secondary or tertiary structure. The protein may be composed of a single polypeptide, or may comprise multiple polypepties that are assembled to form a multimer. The multimer may be a homooligomer, or a heterooligmer. The protein may be a naturally occurring, or wild type protein, or a modified, or non-naturally, occurring protein. The protein may, for example, differ from a wild type protein by the addition, substitution or deletion of one or more amino acids.

A “variant” of a protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes having amino acid substitutions, deletions and/or insertions relative to the unmodified or wild-type protein in question and having similar or enhanced biological and functional activity as the unmodified protein from which they are derived.

The term "amino acid identity" as used herein refers to the extent that sequences are identical on an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical amino acid residue (e.g., Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

For all aspects and embodiments of the present invention, a “variant” typically has at least 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% complete sequence identity to the amino acid sequence of the corresponding wild-type protein. Sequence identity can also be to a fragment or portion of the full length polynucleotide or polypeptide. Hence, a sequence may have only 50 % overall sequence identity with a full length reference sequence, but a sequence of a particular region, domain or subunit could share 80 %, 90 %, 95%, 97%, 98% or as much as 99% or 100% sequence identity with the reference sequence.

The term “wild-type” refers to a gene or gene product isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the “normal” or “wild-type” form of the gene. In contrast, the term “modified”, “mutant” or “variant” refers to a gene or gene product that displays modifications in sequence (e.g., substitutions, truncations, or insertions), post-translational modifications and/or functional properties (e.g., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product. Methods for introducing or substituting naturally-occurring amino acids are well known in the art. For instance, methionine (M) may be substituted with arginine (R) by replacing the codon for methionine (ATG) with a codon for arginine (CGT) at the relevant position in a polynucleotide encoding the mutant protein. Methods for introducing or substituting non-

naturally-occurring amino acids are also well known in the art. For instance, non-naturally-occurring amino acids may be introduced by including synthetic aminoacyl-tRNAs in the IVTT system used to express the mutant protein. Alternatively, they may be introduced by expressing the mutant proteinin *E. coli* that are auxotrophic for specific amino acids in the presence of synthetic (i.e. non-naturally-occurring) analogues of those specific amino acids. They may also be produced by naked ligation if the mutant proteinis produced using partial peptide synthesis. Conservative substitutions replace amino acids with other amino acids of similar chemical structure, similar chemical properties or similar side-chain volume. The amino acids introduced may have similar polarity, hydrophilicity, hydrophobicity, basicity, acidity, neutrality or charge to the amino acids they replace. Alternatively, the conservative substitution may introduce another amino acid that is aromatic or aliphatic in the place of a pre-existing aromatic or aliphatic amino acid. Conservative amino acid changes are well-known in the art and may be selected in accordance with the properties of the 20 main amino acids as defined in Table 1 below. Where amino acids have similar polarity, this can also be determined by reference to the hydropathy scale for amino acid side chains in Table 2.

Table 1 - Chemical properties of amino acids

Ala	aliphatic, hydrophobic, neutral	Met	hydrophobic, neutral
Cys	polar, hydrophobic, neutral	Asn	polar, hydrophilic, neutral
Asp	polar, hydrophilic, charged (-)	Pro	hydrophobic, neutral
Glu	polar, hydrophilic, charged (-)	Gln	polar, hydrophilic, neutral
Phe	aromatic, hydrophobic, neutral	Arg	polar, hydrophilic, charged (+)
Gly	aliphatic, neutral	Ser	polar, hydrophilic, neutral
His	aromatic, polar, hydrophilic, charged (+)	Thr	polar, hydrophilic, neutral
Ile	aliphatic, hydrophobic, neutral	Val	aliphatic, hydrophobic, neutral
Lys	polar, hydrophilic, charged(+)	Trp	aromatic, hydrophobic, neutral
Leu	aliphatic, hydrophobic, neutral	Tyr	aromatic, polar, hydrophobic

Table 2 - Hydropathy scale

Side Chain	Hydropathy	Side Chain	Hydropathy
Ile	4.5	Trp	-0.9
Val	4.2	Tyr	-1.3
Leu	3.8	Pro	-1.6
Phe	2.8	His	-3.2
Cys	2.5	Glu	-3.5
Met	1.9	Gln	-3.5
Ala	1.8	Aps	-3.5
Gly	-0.4	Asn	-3.5
Thr	-0.7	Lys	-3.9
Ser	-0.8	Arg	-4.5

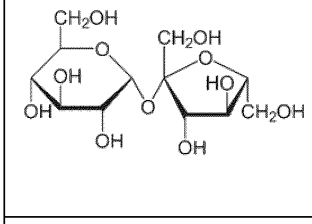
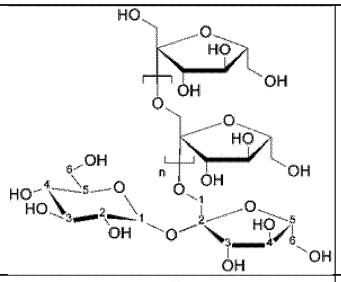
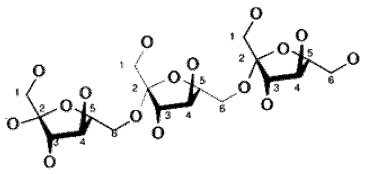
A mutant or modified protein or peptide can also be chemically modified in any way and at any site. A mutant or modified protein or peptide may be chemically modified by attachment of a molecule to one or more cysteines (cysteine linkage), attachment of a molecule to one or more lysines, attachment of a molecule to one or more non-natural amino acids, enzyme modification of an epitope or modification of a terminus. Suitable methods for carrying out such modifications are well-known in the art. The mutant or modified protein or peptide may be chemically modified by the attachment of any molecule. For instance, the mutant or modified protein or peptide may be chemically modified by attachment of a dye or a fluorophore.

Inulosucrase enzymes and their uses

Inulin is a naturally occurring fructan-type oligosaccharide first reported in 1804 by Rose as a carbohydrate isolated from *Inula helenum* (Elecampane). Later that century (1879) inulin was mentioned in the "Pharmacographia: a history of the principle drugs of vegetable origin met within Great Britain and British India" by Friedrich Flueckinger and Daniel Hanbury. Inulin is naturally found in high concentrations in Jerusalem artichoke, chicory root, garlic, asparagus root and to a lesser degree in onion, leek, banana and wheat (Kaur & Gupta 2002). As such, the average daily intake of inulin ranges between 1 and 10 g in a typical Western diet, wherein European intake (3 - 11 g) is higher than American (1 - 4 g) (Coussement 1999).

Inulin is composed of repeating β-D-fructosyl units linked by glycosidic bonds, and a chain nucleating α-D-glucosyl group. Short inulin chains (less than 10 fructose units) are also referred to as oligofructose. Inulin is distinguished from the compositionally similar oligosaccharide known as levan in the nature of the glycosidic bonds in the polymers: in

inulin, the bonds are (2→1), whereas in levan the bonds are (2→6). The relationship between these structures is shown below:

		
Sucrose	Inulin	Levan

As used herein the term "inulin" embraces short polymeric chains with a degree of polymerisation (DP) of at least 2, such as at least 4, e.g. at least 6, such as at least 8, e.g. at least 10.

Fructooligosaccharides such as inulin cannot be naturally metabolised by subjects such as mammals, e.g. humans. Accordingly, such fructooligosaccharides are often referred to as "low-calorie" or "calorie-free" dietary fibres.

The inventors have recognised that production of fructooligosaccharides such as inulin *in vivo* will reduce the amount of sucrose available for metabolism into its component monomers (glucose and fructose) by enzymes in the body such as sucrases, isomaltase glycoside hydrolases, and invertases. In other words, by reducing the concentration of the sucrose substrate for these enzymes, the production of free glucose and especially free fructose may be reduced. Because the concentration of free glucose and fructose in the body is decreased, the uptake of these molecules is reduced.

A further advantage of producing fructooligosaccharides such as inulin *in vivo* is that the fructooligosaccharides thereby produced are associated with their own health benefits apart from those arising from the reduction of sucrose. Thus, a synergistic advantage may arise from the administration of isolated fructosyltransferases, as dual benefits arise from both reducing sucrose levels and also increasing fructooligosaccharides (e.g. inulin) levels.

One further driver of health benefits arising from fructooligosaccharides such as inulin is a shift in the colonic microbiome. Unlike many dietary fibres, fermentation of fructooligosaccharides such as inulin is selective. Such fructooligosaccharides can be metabolised by genera associated with gut health including *lactobacilli*, *bifidobacteria* and *fusobacteria*, leading to their proliferation. This proliferation reduces the proportion of

pathogenic/opportunistic bacteria in the gut, such as certain strains of *E. coli*, *Clostridia* and *Candida*. Further benefits in terms of reduction of intrahepatocellular and intramyocellular lipids have been associated with fructooligosaccharides such as inulin.

Mutant inulosucrase enzymes

As explained in more detail below, fructosyltransferases are capable of catalysing the conversion of sucrose (typically present as a result of consumption of sugars in food) to fructooligosaccharides such as inulin and levan. Inulosucrases are enzymes which catalyse the conversion of sucrose (typically present as a result of consumption of sugars in food) to inulin. Levansucrases are enzymes which catalyse the conversion of sucrose (typically present as a result of consumption of sugars in food) to levan.

WO 2022/096864, the entire contents of which are incorporated by reference, discloses the use of isolated fructosyltransferase enzymes to reduce fructose uptake in a subject, as well as associated compositions. The compositions are useful in therapeutic and non-therapeutic uses such as appetite suppression.

Whilst the use of isolated fructosyltransferase enzymes as described in WO 2022/096864 has major advantages, issues remain. For example, it would be advantageous for the enzymes to have improved catalytic activity (especially improved fructooligosaccharide polymerisation activity, also referred to as transfructosylation activity). This could allow for more efficient conversion of sucrose to fructooligosaccharide leading to less sucrose being available for metabolism into its component monomers, and increased physiological benefit. Improved efficiency of conversion would reduce the amount of enzyme needed to effect conversion of a given amount of sucrose. The need to use lower amounts of enzyme to achieve the same benefit would reduce costs associated with enzyme expression and purification.

It would also be advantageous for the enzymes to have improved stability, especially in the presence of acidic (low pH). Improved stability at low pH would increase the amount of enzyme which remains intact following oral administration and which reaches the intestinal tract in a catalytically active form capable of converting sucrose to inulin. This again would reduce the amount of enzyme required to catalyse conversion of a given amount of sucrose to inulin, and would again reduce costs and improve efficiency.

It would further be advantageous for the enzymes to have improved resistance to degradation by digestive enzymes such as proteases. For example, proteases such as pepsin, trypsin and chymotrypsin are present in the gastric tract and may degrade enzymes

such as inulosucrases which may be administered orally. Improved resistance to proteases would increase the amount of enzyme which remains intact over time following oral administration and which is capable of converting sucrose to inulin in the gastric tract. This again would reduce the amount of enzyme required to catalyse conversion of a given amount of sucrose to inulin, and would again reduce costs and improve efficiency.

Recognising benefits such as those set out above, the inventors have sought to develop an improved inulosucrase enzyme.

Surprisingly, the inventors have found that the properties of the inulosucrase enzyme having the amino acid sequence of SEQ ID NO: 1 can be significantly improved by making one or more modifications in the amino acid sequence. The one or more modifications are described in more detail herein.

As explained below, the one or more modifications lead to improved properties of the inulosucrase enzyme. The variant that arises from making the one or more modifications typically exhibits a higher activity than the wild type enzyme; is typically more stable under low pH than the wild type enzyme, and/or is typically more resistant to proteases than the wild type enzyme.

Amino acid modifications

In some embodiments, provided herein is a mutant inulosucrase enzyme comprising a variant of the amino acid sequence shown in SEQ ID NO: 1. The provided mutant inulosucrase enzyme has beneficial properties which make it useful in various applications, including therapeutic applications and non-therapeutic applications.

In some embodiments the fructosyltransferase is an inulosucrase of EC class 2.4.1.9.

In some embodiments the variant has an increased transfructosylation activity under physiological conditions compared to the corresponding transfructosylation activity of the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has an increased *in vivo* transfructosylation activity compared to the *in vivo* transfructosylation activity of the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has an increased pH stability compared to the pH stability of the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has increased protease resistance compared to the protease resistance of the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has increased transfructosylation activity (e.g.

under physiological conditions and/or *in vivo*) and/or increased pH stability and/or increased protease resistance compared to the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has increased transfructosylation activity (e.g. under physiological conditions and/or *in vivo*) and increased pH stability and increased protease resistance compared to the protein having the amino acid sequence shown in SEQ ID NO: 1. These properties are discussed in more detail herein. In some embodiments the properties are linked: for example, in some embodiments increasing pH stability increases the transfructosylation activity of the enzyme under conditions of low pH. In some embodiments increasing protease resistance of the enzyme increases the transfructosylation activity of the enzyme in the presence of proteases.

In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 1 – 92, 303 – 345 and/or 400 – 418 of SEQ ID NO: 1. In some embodiments the region corresponding to positions 1 – 92, 303 – 345 and 400 – 418 of SEQ ID NO: 1 is or corresponds to an alpha-helical region of the structure of the mutant inulosucrase enzyme. According, in some embodiments the mutant inulosucrase enzyme comprises a modification in an alpha helical portion of the region corresponding to positions 1 – 92, 303 – 345 and/or 400 – 418 of SEQ ID NO: 1. Without being bound by theory, the inventors believe that modifications in this region may render the resulting protein more resistant to damage by low pH and/or more resistant to degradation (e.g. cleavage) by proteases and/or may improve the structural stability of the enzyme (which may in some embodiments allow for more efficient catalytic activity).

In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 10 – 90 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 20 – 90 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 30 – 85 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 40 – 80 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 45 – 75 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 49 – 71 of SEQ ID NO: 1.

In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 130 – 240 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 134 – 238 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 134 – 172 of SEQ ID NO: 1.

In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 300 – 360 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 305 – 355 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 310 – 350 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 309 – 345 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 315 – 340 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 316 – 339 of SEQ ID NO: 1.

In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 380 – 450 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 384 – 406 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 385 – 440 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 390 – 430 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 395 – 420 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 400 – 418 of SEQ ID NO: 1.

In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 480 – 520 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 485 – 515 of SEQ ID NO: 1. In some embodiments the provided mutant inulosucrase enzyme comprises a modification in a region corresponding to positions 489 – 513 of SEQ ID NO: 1.

In some embodiments, provided herein is a mutant inulosucrase enzyme comprising a variant of the amino acid sequence shown in SEQ ID NO: 1, wherein the variant comprises a modification at one or more of the following positions: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K339, K343, S345, D384, D406, M422, T474, H489, W492, A510, and D522. In some embodiments, provided herein is a mutant inulosucrase enzyme comprising a variant of the amino acid sequence shown in SEQ ID NO: 1, wherein the variant comprises a modification at one or more of the following positions: V49, D57, A71, Q134, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, S345, K339, D406, M422, T474, H489, W492, T513, and D522. As explained in more detail below, modification at these positions is associated with increased *in vivo* transfructosylation activity and/or increased pH stability and/or increased protease resistance compared to the protein having the amino acid sequence shown in SEQ ID NO: 1.

In some embodiments, provided herein is a mutant inulosucrase enzyme comprising a variant of the amino acid sequence shown in SEQ ID NO: 1, wherein the variant comprises a modification at one or more of the following positions: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, L316, S336, K339, K343, D384, D406, M422, T474, H489, W492, A510, and D522. In some embodiments, provided herein is a mutant inulosucrase enzyme comprising a variant of the amino acid sequence shown in SEQ ID NO: 1, wherein the variant comprises a modification at one or more of the following positions: V49, D57, A71, Q134, N143, G152, T159, G172, K238, Q240, K270, L316, S336, K339, D406, M422, T474, H489, W492, T513, and D522.

In some embodiments the modification is a chemical modification of the amino acid of the wild type enzyme at the relevant position. In some embodiments the modification is a modification of the amino acid of the wild type enzyme at the relevant position with a non-natural amino acid. In some embodiments the modification is a modification of the amino acid of the wild type enzyme at the relevant position with a different naturally-occurring (canonical) amino acid. In other words, in some embodiments the modification is an amino acid substitution. Those skilled in the art will appreciate that the substitution replaces an amino acid present in the wild-type amino acid sequence of SEQ ID NO: 1 with a different amino acid.

In some embodiments the modification is substitution with tyrosine (Y), alanine (A), isoleucine (I), proline (P), arginine (R), serine (S), asparagine (N) and aspartic acid (D). In some embodiments the modification is substitution with alanine (A), aspartic acid

(D), isoleucine (I), asparagine (N), proline (P), arginine (R) or tyrosine (Y). In some embodiments the modification is substitution with alanine, arginine, isoleucine or tyrosine. In some embodiments the modification is substitution with tyrosine (Y), alanine (A), or isoleucine (I).

In some embodiments the modification alters the charge at the modified position. In some embodiments the modification increases negative charge (or decreases positive charge) at the modified position (e.g. by substituting a positively charged amino acid for a negatively charged or neutral amino acid; or by substituting a neutral amino acid with a negatively charged amino acid). In some embodiments the modification increases positive charge (or decreases negative charge) at the modified position (e.g. by substituting a negatively charged amino acid for a positively charged or neutral amino acid; or by substituting a neutral amino acid with a positively charged amino acid).

In some embodiments the substitution alters aromatic character at the modified position. In some embodiments a non-aromatic amino acid is substituted for an aromatic amino acid. In some embodiments an aromatic amino acid is substituted for a non-aromatic amino acid. Aromatic amino acids may interact through delocalized electron pi systems.

In some embodiments the substitution alters the polarity and/or hydrogen-bonding properties of the modified position. In some embodiments a non-polar amino acid is substituted for a polar amino acid, e.g. an amino acid having a side chain comprising a hydroxy or amino group. In some embodiments a polar amino acid, e.g. an amino acid having a side chain comprising a hydroxy or amino group, is substituted for a non-polar amino acid.

In some embodiments the substitution alters the size and/or steric effect of the amino acid at the modified position. In some embodiments an amino acid having a small side chain is substituted for an amino acid having a larger side chain. In some embodiments an amino acid having a large side chain is substituted for an amino acid having a smaller side chain. The size of an amino acid can be expressed in terms of its van der Waals volume; van der Waals volumes of amino acids are well known in the art.

In some embodiments the substitution alters the hydrophobicity and/or hydrophilicity of the amino acid at the modified position. In some embodiments an amino acid having a hydrophobic side chain is substituted for an amino acid having a hydrophilic side chain. In some embodiments an amino acid having a hydrophilic side chain is substituted for an amino acid having a hydrophobic side chain. The hydrophilicity/hydrophobicity of an amino acid can be expressed in terms of its

hydropathy; hydropathy values for amino acids are well known in the art and values for various amino acids are set out in Table 2 above.

In some embodiments the modification is substitution of T10 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at T10, the modification is substitution with proline. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises T10P.

In some embodiments the modification is substitution of V49 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of V49 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at V49, the modification is substitution with tyrosine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises V49Y.

In some embodiments the modification is substitution of D57 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of D57 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at D57, the modification is substitution with tyrosine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises D57Y.

In some embodiments the modification is substitution of A71 with aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of A71 with aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at A71, the modification is substitution with tyrosine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises A71Y.

In some embodiments the modification is substitution of Q134 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of Q134 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at Q134, the modification is substitution with tyrosine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises Q134Y.

In some embodiments the modification is substitution of N143 with alanine, aspartic acid, isoleucine, proline, arginine, serine or tyrosine. In some embodiments the

modification is substitution of N143 with alanine, aspartic acid, isoleucine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at N143, the modification is substitution with aspartic acid. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises N143D.

In some embodiments the modification is substitution of G152 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of G152 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at G152, the modification is substitution with alanine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises G152A.

In some embodiments the modification is substitution of T159 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of T159 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at T159, the modification is substitution with arginine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises T159R.

In some embodiments the modification is substitution of G172 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of G172 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at G172, the modification is substitution with arginine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises G172R.

In some embodiments the modification is substitution of K238 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of K238 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at K238, the modification is substitution with alanine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises K238A.

In some embodiments the modification is substitution of Q240 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some

embodiments the modification is substitution of Q240 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at Q240, the modification is substitution with alanine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises Q240A. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at Q240, the mutant inulosucrase enzyme also comprises a modification at R566, such as R566Y.

In some embodiments the modification is substitution of K270 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of K270 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at V49, the modification is substitution with alanine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises K270A.

In some embodiments the modification is substitution of A309 with aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of A309 with aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments the mutant inulosucrase enzyme does not comprise a modification at A309. In some embodiments the mutant inulosucrase enzyme does not comprise a modification at A309 which is substitution with glutamic acid (E); thus, in some embodiments the mutant inulosucrase enzyme does not comprise A309E. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at A309, the modification is substitution with isoleucine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises A309I.

In some embodiments the modification is substitution of L316 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of L316 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at L316, the modification is substitution with isoleucine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises L316I.

In some embodiments the modification is substitution of S336 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of S336 with alanine, aspartic acid,

isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at S336, the modification is substitution with alanine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises S336A.

In some embodiments the modification is substitution of K339 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of K339 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at K339, the modification is substitution with tyrosine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises K339Y. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at K339, the modification is substitution with arginine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises K339R.

In some embodiments the modification is substitution of S345 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments the mutant inulosucrase enzyme does not comprise a modification at S345. In some embodiments the mutant inulosucrase enzyme does not comprise a modification at S345 which is substitution with alanine; i.e. in some embodiments the mutant inulosucrase enzyme does not comprise S345A. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at S345, the modification is substitution with alanine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises S345A.

In some embodiments the modification is substitution of K343 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at K343, the modification is substitution with serine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises K343S.

In some embodiments the modification is substitution of D384 with alanine, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at D384, the modification is substitution with alanine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises D384A.

In some embodiments the modification is substitution of D406 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of D406 with alanine, aspartic acid,

isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at D406, the modification is substitution with isoleucine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises D406I.

In some embodiments the modification is substitution of M422 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of M422 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at M422, the modification is substitution with tyrosine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises M422Y.

In some embodiments the modification is substitution of T474 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of T474 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at T474, the modification is substitution with alanine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises T474A.

In some embodiments the modification is substitution of H489 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of H489 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at H489, the modification is substitution with asparagine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises H489N.

In some embodiments the modification is substitution of W492 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of W492 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at W492, the modification is substitution with alanine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises W492A.

In some embodiments the modification is substitution of A510 with aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments, when

the mutant inulosucrase enzyme comprises a modification at A510, the modification is substitution with aspartic acid. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises A510D.

In some embodiments the modification is substitution of T513 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of T513 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at T513, the modification is substitution with alanine. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises T513A.

In some embodiments the modification is substitution of D522 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine, serine or tyrosine. In some embodiments the modification is substitution of D522 with alanine, aspartic acid, isoleucine, asparagine, proline, arginine or tyrosine. In some embodiments, when the mutant inulosucrase enzyme comprises a modification at D522, the modification is substitution with proline. Therefore, in some embodiments, the mutant inulosucrase enzyme comprises D522P.

In some embodiments the variant comprises a modification at at least two of the following positions: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K343, S345, K339, D384, D406, M422, T474, H489, W492, A510, and D522. In some embodiments the variant comprises a modification at at least two, three, four, five, six, seven, eight, nine, ten or more of the following positions: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K343, S345, K339, D384, D406, M422, T474, H489, W492, A510, and D522. In some embodiments the variant comprises a modification at from about 2 to about 10, such as from about 2 to about 8, e.g. from about 2 to about 6, e.g. from about 2 to about 4 of the following positions: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K343, S345, K339, D384, D406, M422, T474, H489, W492, A510, and D522. In some embodiments the variant comprises a modification at from about 3 to about 9, such as from about 4 to about 8, e.g. from about 5 to about 7, of the following positions: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K343, S345, K339, D384, D406, M422, T474, H489, W492, A510, and D522.

In some embodiments the variant comprises a modification at at least two of the following positions: V49, D57, A71, Q134, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K339, S345, D406, M422, T474, H489, W492, T513, and D522. In some embodiments the variant comprises a modification at at least two, three, four, five, six, seven, eight, nine, ten or more of the following positions: V49, D57, A71, Q134, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K339, S345, D406, M422, T474, H489, W492, T513, and D522. In some embodiments the variant comprises a modification at from about 2 to about 10, such as from about 2 to about 8, e.g. from about 2 to about 6, e.g. from about 2 to about 4 of the following positions: V49, D57, A71, Q134, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K339, S345, D406, M422, T474, H489, W492, T513, and D522. In some embodiments the variant comprises a modification at from about 2 to about 10, such as from about 3 to about 8, e.g. from about 4 to about 7, such as from about 5 to about 6 of the following positions: V49, D57, A71, Q134, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K339, S345, D406, M422, T474, H489, W492, T513, and D522.

In some embodiments the variant comprises a modification at one or more of: T10, V49, D57, A71, Q134, G152, G172, K238, A309, L316, K339, K343, S345, D384, D406, H489, A510, and T513.

In some embodiments the variant comprises a modification at one or more of: V49, D57, A71, Q134, A309, L316, K339, S345, D384, D406, H489, A510, and T513.

In some embodiments the variant comprises a modification at one or more of: V49, D57, A71, Q134, A309, K339, S345, D384, D406, H489, A510, and T513.

In some embodiments the variant comprises a modification at one or more of: V49, D57, A71, Q134, A309, K339, S345, D406, H489, A510, and T513.

In some embodiments the variant comprises a modification at three, four, five, six, seven, eight or nine, e.g. four, five, six, seven or eight of the following positions: Q134, T513, A71, T10, V49, D57, G152, G172, K238, A309, L316, K339, K343, S345, D384A, D406I, H489 and A510.

In some embodiments the variant comprises a modification at three, four, five, six, seven, eight or nine, e.g. four, five, six, seven or eight of the following positions: Q134, T513, A71, V49, D57, A309, L316, K339, S345, D384, D406, H489N and A510.

In some embodiments the variant comprises a modification at three, four, five, six, seven, eight or nine, e.g. four, five, six, seven or eight of the following positions: Q134, T513, A71, V49, D57, A309, K339, S345, D384, D406, H489 and A510.

In some embodiments the variant comprises a modification at three, four, five, six, seven, eight or nine, e.g. four, five, six, seven or eight of the following positions: Q134, T513, A71, V49, D57, A309, K339, S345, D406, H489 and A510.

In some embodiments the variant comprises a modification at one or more of: V49, D57, A71, Q134, T159, G172, A309, L316, K339, S345, D406, M422, and T513.

In some embodiments the variant comprises a modification at one or more of: D57, A71, Q134, N143, G152, A309, L316, K339, S345, D406, and T474.

In some embodiments the variant comprises a modification at one or more of: G172, L316, K339, S345, D406, M422, and T513.

In some embodiments the variant comprises a modification at one or more of: D57, A71, G172, A309, L316, K339, S345, D406, and M422

In some embodiments the variant comprises a modification at one or more of D57, G172, L316, D406, and M422.

In some embodiments the variant comprises a modification at D57. In some embodiments the variant comprises a modification at G172. In some embodiments the variant comprises a modification at L316. In some embodiments the variant comprises a modification at D406. In some embodiments the variant comprises a modification at M422. In some embodiments the variant comprises a modification at V49. In some embodiments the variant comprises a modification at A71. In some embodiments the variant comprises a modification at Q134. In some embodiments the variant comprises a modification at A309. In some embodiments the variant comprises a modification at K339. In some embodiments the variant comprises a modification at S345. In some embodiments the variant comprises a modification at D406. In some embodiments the variant comprises a modification at H489. In some embodiments the variant comprises a modification at A510. In some embodiments the variant comprises a modification at T513.

In some embodiments the variant comprises modifications at one of the following:

L316

D57

S345

A71

G172

D406

M422

T513

A309
V49
T159
Q134
K339
V49 / A309
L316 / M422
S345 / M422
G172 / L316
G172 / D406
D406 / M422
T159 / K339
S345 / D406
L316 / D57
A71 / K339
A71 / T513
S345 / G172
D57 / G172
D57 / S345
D57 / M422
L316 / D406 / M422
S345 / D406 / L316
S345 / G172 / L316
S345 / M422 / A71
D57 / G172 / M422
D57 / L316 / S345
D57 / D406 / T513
M422 / A309 / A71
S345 / K339 / T513
G172 / K339 / V49
G172 / D406 / A71
D57 / A71 / A309
A309 / T513 / Q134
V49 / T513 / L316

A309 / T159 / D406
K339 / S345 / M422
V49 / A309 / T159
D57 / K339 / T159
A71 / V49 / T513
K339 / G172 / M422
D57 / L316 / D406 / M422
D57 / G172 / L316 / M422
S345 / A309 / G172 / T513
D57 / L316 / G172 / K339
K339 / D406 / T513 / A71
A309 / V49 / Q134 / T159
G172 / D406 / M422 / S345
G172 / D406 / M422 / A71
K339 / D406 / S345 / V49
L316 / D57 / A309 / S345
T513 / A309 / A71 / S345
M422 / A309 / V49 / T513
T159 / K339 / A71 / V49
L316 / M422 / D406 / T513
A309 / L316 / D57 / T513
A71 / G172 / Q134 / V49
S345 / T159 / A71 / K339
S345 / D57 / V49 / A309
K339 / Q134 / D522 / H489
D57 / D406 / L316 / G172
D57 / G172 / L316 / D406 / M422
S345 / L316 / G172 / M422 / T159
V49 / D57 / T159 / G172 / L316
G172 / L316 / M422 / T513 / A71
S345 / A309 / D406 / K339 / A71
T513 / K339 / D406 / V49 / Q134
H489 / D522 / Q134 / D57 / A309
D57 / D406 / M422 / S345 / A71

A71 / A309 / T513 / S345 / Q134

S345 / D406 / T513 / A309 / G172

K339 / H489 / D522 / A71 / D57

L316 / S345 / K339 / T513 / V49

V49 / D57 / T159 / M422 / L316

G172 / A71 / M422 / T513 / A309

V49 / D57 / T159 / G172 / A309 / L316 / S345 / D406 / M422 / T513

V49 / D57 / G172 / A309 / L316 / S345 / D406 / M422 / T513

V49 / D57 / A71 / G172 / A309 / S345 / D406 / M422 / T513

V49 / G172 / K339 / L316 / S345 / D406 / M422 / T513

D57 / T159 / G172 / A309 / L316 / S345 / M422

V49 / D57 / T159 / G172 / A71 / M422 / T513

V49 / D57 / G172 / L316 / M422 / T513

V49 / T159 / G172 / L316 / M422 / T513

V49 / D57 / D522 / A309 / L316 / S345 / D406 / T513

V49 / D57 / T159 / G172 / A309 / L316 / S345 / D406 / T513

V49 / A71 / Q134 / K339 / D406 / T513

V49 / D57 / A71 / Q134 / A309 / T513

V49 / D57 / A71 / Q134 / A309 / D406 / T513

V49 / A71 / Q134 / A309 / D384 / D406 / A510 / T513

V49 / A71 / Q134 / A309 / T513

Q134 / A309 / S345 / H489 / T513

V49 / A71 / Q134 / A309 / S345 / T513

V49 / A71 / Q134 / A309 / S345 / H489 / T513

V49 / Q134 / A309 / K339 / S345 / D406 / H489 / T513

V49 / A71 / Q134 / A309 / K339 / S345 / D406 / H489 / T513

T10 / G152 / S345 / A510

Q134 / G172 / K238 / L316

A309 / K343 / D406

V49 / Q134 / A309 / A510 / T513

V49 / Q134 / D406 / A510 / T513

V49 / Q134 / D384 / D406 / A510 / T513

V49 / Q134 / S345 / D406 / A510 / T513

V49 / Q134 / L316 / D384 / D406 / A510 / T513

V49 / Q134 / S345 / D384 / D406 / A510 / T513

In some embodiments, provided herein is a mutant inulosucrase enzyme comprising one or more modification selected from T10A/D/I/N/P/R/S/Y); V49A/D/I/N/P/R/S/Y, D57A/I/N/P/R/S/Y, A71D/I/N/P/R/S/Y, Q134A/D/I/N/P/R/S/Y, N143A/D/I/P/R/S/Y, G152A/D/I/N/P/R/S/Y, T159A/D/I/N/P/R/S/Y, G172A/D/I/N/P/R/S/Y, K238A/D/I/N/P/R/S/Y, Q240A/D/I/N/P/R/S/Y, K270A/D/I/N/P/R/S/Y, A309D/I/N/P/R/S/Y, L316A/D/I/N/P/R/S/Y, S336A/D/I/N/P/R/Y, K343A/D/I/N/P/R/S/Y; D384A/I/N/P/R/S/Y; S345A/D/I/N/P/R/Y, K339A/D/I/N/P/R/S/Y, D406A/I/N/P/R/S/Y, M422A/D/I/N/P/R/S/Y, T474A/D/I/N/P/R/S/Y, H489A/D/I/N/P/R/S/Y, W492A/D/I/N/P/R/S/Y, T513A/D/I/N/P/R/S/Y, and D522A/I/N/P/R/Y.

In some embodiments, provided herein is a mutant inulosucrase enzyme comprising one or more modification selected from V49A/D/I/N/P/R/Y, D57A/I/N/P/R/Y, A71D/I/N/P/R/Y, Q134A/D/I/N/P/R/Y, N143A/D/I/P/R/Y, G152A/D/I/N/P/R/Y, T159A/D/I/N/P/R/Y, G172A/D/I/N/P/R/Y, K238A/D/I/N/P/R/Y, Q240A/D/I/N/P/R/Y, K270A/D/I/N/P/R/Y, A309D/I/N/P/R/Y, L316A/D/I/N/P/R/Y, S336A/D/I/N/P/R/Y, S345A/D/I/N/P/R/Y, K339A/D/I/N/P/R/Y, D406A/I/N/P/R/Y, M422A/D/I/N/P/R/Y, T474A/D/I/N/P/R/Y, H489A/D/I/N/P/R/Y, W492A/D/I/N/P/R/Y, T513A/D/I/N/P/R/Y, and D522A/I/N/P/R/Y.

In some embodiments, provided herein is a mutant inulosucrase enzyme comprising one or more modification selected from Q134Y, T513A, A71Y, T10P, V49Y, D57Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K339R, K339Y, K343S, S345A, D384A, D406I, M422Y, T474A, H489N, W492A, A510D, and D522P.

In some embodiments, provided herein is a mutant inulosucrase enzyme comprising one or more modification selected from V49Y, D57Y, A71Y, Q134Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, S345A, K339R, K339Y, D406I, M422Y, T474A, H489N, W492A, T513A, and D522P.

In some embodiments, provided herein is a mutant inulosucrase enzyme comprising one or more modification selected from V49Y, D57Y, A71Y, Q134Y, N143D,

G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K339R, K339Y, D406I, M422Y, T474A, H489N, W492A, T513A, and D522P.

In some embodiments the variant comprises at least two of the following modifications: Q134Y, T513A, A71Y, T10P, V49Y, D57Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K343S, S345A, K339R, K339Y, D384A, D406I, M422Y, T474A, H489N, W492A, A510D, and D522P. in some embodiments the variant comprises at least two, three, four, five, six, seven, eight, nine, ten or more of the following modifications: Q134Y, T513A, A71Y, T10P, V49Y, D57Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K343S, S345A, K339R, K339Y, D384A, D406I, M422Y, T474A, H489N, W492A, A510D, and D522P. In some embodiments the variant comprises from about 2 to about 10, such as from about 3 to about 9, such as from about 4 to about 8, e.g. from about 5 to about 7, of the following modifications: Q134Y, T513A, A71Y, T10P, V49Y, D57Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K343S, S345A, K339R, K339Y, D384A, D406I, M422Y, T474A, H489N, W492A, A510D, and D522P.

In some embodiments the variant comprises at least two of the following modifications: V49Y, D57Y, A71Y, Q134Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K339R, K339Y, S345A, D406I, M422Y, T474A, H489N, W492A, T513A, and D522P. In some embodiments the variant comprises at least two, three, four, five, six, seven, eight, nine, ten or more of the following modifications: V49Y, D57Y, A71Y, Q134Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K339R, K339Y, S345A, D406I, M422Y, T474A, H489N, W492A, T513A, and D522P. In some embodiments the variant comprises from about 2 to about 10, such as from about 2 to about 8, e.g. from about 2 to about 6, e.g. from about 2 to about 4 of the following modifications: V49Y, D57Y, A71Y, Q134Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K339R, K339Y, S345A, D406I, M422Y, T474A, H489N, W492A, T513A, and D522P. In some embodiments the variant comprises from about 2 to about 10, such as from about 3 to about 8, e.g. from about 4 to about 7, such as from about 5 to about 6 of the following modifications: V49Y, D57Y, A71Y, Q134Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K339R, K339Y, S345A, D406I, M422Y, T474A, H489N, W492A, T513A, and D522P.

In some embodiments the variant comprises a modification at one or more of: Q134Y, T513A, A71Y, T10P, V49Y, D57Y, G152A, G172R, K238A, A309I, L316I, K339Y, K343S, S345A, D384A, D406I, H489N and A510D.

In some embodiments the variant comprises a modification at one or more of: Q134Y, T513A, A71Y, V49Y, D57Y, A309I, L316I, K339Y, S345A, D384A, D406I, H489N and A510D.

In some embodiments the variant comprises a modification at one or more of: Q134Y, T513A, A71Y, V49Y, D57Y, A309I, K339Y, S345A, D384A, D406I, H489N and A510D.

In some embodiments the variant comprises a modification at one or more of: Q134Y, T513A, A71Y, V49Y, D57Y, A309I, K339Y, S345A, D406I, H489N and A510D.

In some embodiments the variant comprises three, four, five, six, seven, eight or nine, e.g. four, five, six, seven or eight of the following modifications: Q134Y, T513A, A71Y, T10P, V49Y, D57Y, G152A, G172R, K238A, A309I, L316I, K339Y, K343S, S345A, D384A, D406I, H489N and A510D.

In some embodiments the variant comprises three, four, five, six, seven, eight or nine, e.g. four, five, six, seven or eight of the following modifications: Q134Y, T513A, A71Y, V49Y, D57Y, A309I, L316I, K339Y, S345A, D384A, D406I, H489N and A510D.

In some embodiments the variant comprises three, four, five, six, seven, eight or nine, e.g. four, five, six, seven or eight of the following modifications: Q134Y, T513A, A71Y, V49Y, D57Y, A309I, K339Y, S345A, D384A, D406I, H489N and A510D.

In some embodiments the variant comprises three, four, five, six, seven, eight or nine, e.g. four, five, six, seven or eight of the following modifications: Q134Y, T513A, A71Y, V49Y, D57Y, A309I, K339Y, S345A, D406I, H489N and A510D.

In some embodiments the variant comprises one or more of V49Y, D57Y, A71Y, Q134Y, T159R, G172R, A309I, L316I, K339Y, S345A, D406I, M422Y, and T513A.

In some embodiments the variant comprises one or more of D57Y, A71Y, Q134Y, N143D, G152A, A309I, L316I, K339Y, S345A, D406I and T474A.

In some embodiments the variant comprises one or more of G172R, L316I, K339R, K339Y, S345A, D406I, M422Y and T513A.

In some embodiments the variant comprises one or more of D57Y, G172R, A71Y, A309I, L316I, K339Y, S345A, D406I and M422Y.

In some embodiments the variant comprises one or more of D57Y, G172R, L316I, D406I and M422Y.

In some embodiments the variant comprises D57Y. In some embodiments the variant comprises G172R. In some embodiments the variant comprises L316I. In some embodiments the variant comprises D406I. In some embodiments the variant comprises M422Y. In some embodiments the variant comprises V49Y. In some embodiments the variant comprises A71Y. In some embodiments the variant comprises Q134Y. In some embodiments the variant comprises A309I. In some embodiments the variant comprises K339Y. In some embodiments the variant comprises S345A. In some embodiments the variant comprises D406I. In some embodiments the variant comprises H489N. In some embodiments the variant comprises A510D. In some embodiments the variant comprises T513A.

In some embodiments the variant comprises one of the following:

L316I

D57Y

S345A

A71Y

G172R

D406I

M422Y

T513A

A309I

V49Y

T159R

Q134Y

K339Y

V49Y / A309I

L316I / M422Y

S345A / M422Y

G172R / L316I

G172R / D406I

D406I / M422Y

T159R / K339Y

S345A / D406I

L316I / D57Y

A71Y / K339Y

A71Y / T513A

S345A / G172R

D57Y / G172R

D57Y / S345A

D57Y / M422Y

L316I / D406I / M422Y

S345A / D406I / L316I

S345A / G172R / L316I

S345A / M422Y / A71Y

D57Y / G172R / M422Y

D57Y / L316I / S345A

D57Y / D406I / T513A

M422Y / A309I / A71Y

S345A / K339Y / T513A

G172R / K339Y / V49Y

G172R / D406I / A71Y

D57Y / A71Y / A309I

A309I / T513A / Q134Y

V49Y / T513A / L316I

A309I / T159R / D406I

K339Y / S345A / M422Y

V49Y / A309I / T159R

D57Y / K339Y / T159R

A71Y / V49Y / T513A

K339Y / G172R / M422Y

D57Y / L316I / D406I / M422Y

D57Y / G172R / L316I / M422Y

S345A / A309I / G172R / T513A

D57Y / L316I / G172R / K339Y

K339Y / D406I / T513A / A71Y

A309I / V49Y / Q134Y / T159R

G172R / D406I / M422Y / S345A

G172R / D406I / M422Y / A71Y

K339Y / D406I / S345A / V49Y

L316I / D57Y / A309I / S345A

T513A / A309I / A71Y / S345A

M422Y / A309I / V49Y / T513A

T159R / K339Y / A71Y / V49Y

L316I / M422Y / D406I / T513A

A309I / L316I / D57Y / T513A

A71Y / G172R / Q134Y / V49Y

S345A / T159R / A71Y / K339Y

S345A / D57Y / V49Y / A309I

K339Y / Q134Y / D522P / H489N

D57Y / D406I / L316I / G172R

D57Y / G172R / L316I / D406I / M422Y

S345A / L316I / G172R / M422Y / T159R

V49Y / D57Y / T159R / G172R / L316I

G172R / L316I / M422Y / T513A / A71Y

S345A / A309I / D406I / K339Y / A71Y

T513A / K339Y / D406I / V49Y / Q134Y

H489N / D522P / Q134Y / D57Y / A309I

D57Y / D406I / M422Y / S345A / A71Y

A71Y / A309I / T513A / S345A / Q134Y

S345A / D406I / T513A / A309I / G172R

K339Y / H489N / D522P / A71Y / D57Y

L316I / S345A / K339Y / T513A / V49Y

V49Y / D57Y / T159R / M422Y / L316I

G172R / A71Y / M422Y / T513A / A309I

V49Y / D57Y / T159R / G172R / A309I / L316I / S345A / D406I / M422Y / T513A

V49Y / D57Y / G172R / A309I / L316I / S345A / D406I / M422Y / T513A

V49Y / D57Y / A71Y / G172R / A309I / S345A / D406I / M422Y / T513A

V49Y / G172R / K339Y / L316I / S345A / D406I / M422Y / T513A

D57Y / T159R / G172R / A309I / L316I / S345A / M422Y

V49Y / D57Y / T159R / G172R / A71Y / M422Y / T513A

V49Y / D57Y / G172R / L316I / M422Y / T513A

V49Y / T159R / G172R / L316I / M422Y / T513A

V49Y / D57Y / D522P / A309I / L316I / S345A / D406I / T513A

V49Y / D57Y / T159R / G172R / A309I / L316I / S345A / D406I / T513A

V49Y / A71Y / Q134Y / K339Y / D406I / T513A

V49Y / D57Y / A71Y / Q134Y / A309I / T513A

V49Y / D57Y / A71Y / Q134Y / A309I / D406I / T513A

V49Y / A71Y / Q134Y / A309I / D384A / D406I / A510D / T513A

V49Y / A71Y / Q134Y / A309I / T513A

Q134Y / A309I / S345A / H489N / T513A

V49Y / A71Y / Q134Y / A309I / S345A / T513A

V49Y / A71Y / Q134Y / A309I / S345A / H489N / T513A

V49Y / Q134Y / A309I / K339Y / S345A / D406I / H489N / T513A

V49Y / A71Y / Q134Y / A309I / K339Y / S345A / D406I / H489N / T513A

T10P / G152A / S345A / A510D

Q134Y / G172R / K238A / L316I

A309I / K343S / D406I

V49Y / Q134Y / A309I / A510D / T513A

V49Y / Q134Y / D406I / A510D / T513A

V49Y / Q134Y / D384A / D406I / A510D / T513A

V49Y / Q134Y / S345A / D406I / A510D / T513A

V49Y / Q134Y / L316I / D384A / D406I / A510D / T513A

V49Y / Q134Y / S345A / D384A / D406I / A510D / T513A

In some embodiments the variant comprises any number and any combination of the modifications and/or substitutions defined herein.

In some embodiments the variant has at least 70% identity to the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the sequence identity is assessed over at least 70% of the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the identity is assessed over at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% of the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the sequence identity is assessed over the entire length of SEQ ID NO: 1.

Sequence homology or identity can be determined as described above, e.g. based on sequence alignment of the sequence at issue with a reference sequence (e.g. SEQ ID NO: 1).

In some embodiments sequence identity may be assessed over the region of the sequence shown in SEQ ID NO: 1 which comprises the active site of the protein.

The active site of a fructosyltransferase enzyme can be determined by any suitable means. The active site may be determined by X-ray crystallography, e.g. in the presence of a substrate. The active site may be determined by *in silico* homology modelling based on the experimentally or theoretically determined structures of similar enzymes, such as related fructosyltransferases. The active site may be determined by genetic studies e.g. by mutating or deleting portions of the enzyme and correlating the changes made with the activity of the resulting variant. Residues associated with the active sites of the polypeptides corresponding to SEQ ID NO: 1 are shown in grey/bold/bold&underlined in the sequence listing.

In some embodiments the variant has at least 70% homology or identity to SEQ ID NO: 1, wherein said homology or identity is assessed relative to the amino acid sequence from position 126 to 483 of SEQ ID NO: 1.

In some embodiments the variant has at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO: 1, wherein said identity is assessed relative to some or all of positions 126, 128, 129, 131, 153, 156, 157, 158, 159, 160, 161, 162, 163, 194, 195, 196, 197, 198, 213, 215,

221, 223, 225, 277, 278, 279, 280, 281, 282, 283, 298, 378, 379, 380, 381, 382, 383, 397, 399, 401, 402, 457, 458, 459, 476, 479, 480, 481, 482 and 483 of SEQ ID NO: 1. These residue are shown in grey/bold/bold&underlined in SEQ ID NO: 1.

In some embodiments the variant has at least 70% at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO: 1, wherein said identity is assessed relative to some or all of positions 128, 129, 153, 158, 159, 160, 161, 162, 194, 196, 197, 213, 223, 277, 278, 281, 282, 298, 379, 381, 382, 399, 402, 457, 458 and 480 of SEQ ID NO: 1. These residue are shown in bold/bold&underlined in SEQ ID NO: 1.

In some embodiments the variant has at least at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO: 1, wherein said identity is assessed relative to some or all of positions 128, 129, 153, 158, 159, 160, 162, 196, 197, 281, 282, 298, 379, 381, 399, 402, 457, 458 and 480 of SEQ ID NO: 1. These residue are shown in bold&underlined in SEQ ID NO: 1.

Without being bound by theory, the inventors believe that some or all of positions 126, 128, 129, 131, 153, 156, 157, 158, 159, 160, 161, 162, 163, 194, 195, 196, 197, 198, 213, 215, 221, 223, 225, 277, 278, 279, 280, 281, 282, 283, 298, 378, 379, 380, 381, 382, 383, 397, 399, 401, 402, 457, 458, 459, 476, 479, 480, 481, 482 and 483 are comprised in the active site of the protein of SEQ ID NO: 1.

Thus, in some embodiments the variant (i) comprises one or more modifications as described herein; (ii) has at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identity to the amino acid sequence shown in SEQ ID NO: 1 when the sequence identity is assessed over the entire length of the amino acid sequence of SEQ ID NO: 1; and (iii) has at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO: 1, wherein said identity is assessed relative to some or all of positions 126, 128, 129, 131, 153, 156, 157, 158, 159, 160, 161, 162, 163, 194, 195, 196, 197, 198, 213, 215, 221, 223, 225, 277, 278, 279, 280, 281, 282, 283, 298, 378, 379, 380, 381, 382, 383, 397, 399, 401, 402, 457, 458, 459, 476, 479, 480, 481, 482 and 483 of SEQ ID NO: 1; such as relative to some or all of positions 128, 129, 153, 158, 159, 160, 161, 162, 194, 196, 197, 213, 223, 277, 278, 281, 282, 298, 379, 381, 382, 399, 402, 457, 458 and 480 of SEQ ID NO: 1; e.g. relative to some or all of positions 128, 129, 153, 158, 159, 160, 162, 196, 197, 281, 282, 298, 379, 381, 399, 402, 457, 458 and 480 of SEQ ID NO: 1.

In some embodiments the variant is a variant of SEQ ID NO: 1 in which from about 1 to about 20, such as from about 1 to about 15, such as from about 1 to about 10, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids are modified e.g. by being substituted for a different amino acid. In some embodiments the modifications are modifications as described herein.

In some embodiments the variant is a variant of SEQ ID NO: 1 in which from about 1 to about 20, such as from about 1 to about 15, such as from about 1 to about 10, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in the region corresponding to positions 10 – 90, 130 – 240, 300 – 360, 380 – 450 and/or 480 – 520 of SEQ ID NO: 1 are modified e.g. by being substituted for a different amino acid. In some embodiments the modifications are modifications as described herein.

The mutant inulosucrase enzyme may be a functional fragment of the protein of SEQ ID NO: 1. As those skilled in the art will appreciate, fragments of amino acid sequences include deletion variants of such sequences wherein one or more, such as at least 1, 2, 5, 10, 15, 20, 30, 40, 50 or more amino acids are deleted. Deletion may occur at the C- terminus or N-terminus of the native sequence or within the native sequence. Typically, deletion of one or more amino acids does not influence the residues immediately surrounding the active site of an enzyme.

The mutant inulosucrase enzyme may be a derivative of the protein of the amino acid sequence of SEQ ID NO: 1. Derivatives of amino acid sequences include post-translationally modified sequences including sequences which are modified *in vivo* or *ex vivo*. Many different protein modifications are known to those skilled in the art and include modifications to introduce new functionalities to amino acid residues, modifications to protect reactive amino acid residues or modifications to couple amino acid residues to chemical moieties such as reactive functional groups on linkers.

Derivatives of amino acid sequences also include addition variants of such sequences wherein one or more, such as at least 1, 2, 5, 15, 10, 20, 30, 40, 50, 100, 200 or 300 amino acids are added or introduced into the native sequence. Addition may occur at any point relative to the native sequence. Typically, addition of one or more amino acids does not influence the residues immediately surrounding the active site of an enzyme.

The disclosed variants comprise one or more amino acid modifications relative to the amino acid sequence of SEQ ID NO: 1. Further modifications can be included. For example, native chemical ligation can be used to splice non-native amino acid sequences into partial native sequences to produce variants of native enzymes. Variants of amino

acid sequences include sequences carrying naturally occurring amino acids and/or unnatural amino acids. Variants, derivatives and functional fragments of the aforementioned amino acid sequences retain at least some of the activity/ functionality of the native/ wild-type sequence. Preferably, variants, derivatives and functional fragments of the aforementioned sequences have increased/ improved activity/ functionality when compared to the native/ wild-type sequence.

Typically, the mutant inulosucrase enzyme is soluble in aqueous solution. Solubility can be expressed as a GRAVY (Grand Average of Hydropathy) score which can be determined based on the amino acid sequence of the fructosyltransferase. Calculation of GRAVY scores is routine for those skilled in the art. The GRAVY value is typically calculated by adding the hydropathy value for each residue (Kyte and Doolittle; J Mol Biol 1982 157(1):105-32) and dividing by the length of the sequence. GRAVY scores can be easily determined using freely available software e.g. at https://www.bioinformatics.org/sms2/protein_gravy.html. Typically, the mutant inulosucrase enzyme provided herein has a solubility GRAVY score of -0.4 or more negative than -0.4, such as at most -0.5, e.g. at most -0.6. Typically the fructosyltransferase is an inulosucrase having a GRAVY score of -0.4 or more negative than -0.4. The protein having the amino acid sequence of SEQ ID NO: 1 has a GRAVY score determined using the tool accessible at https://www.bioinformatics.org/sms2/protein_gravy.html of -0.601.

The variant retains enzymatic (catalytic) activity. In some embodiments the catalytic activity is transfructosylation (e.g. formation of inulin from sucrose). In other words, the variant is a functional variant. As used herein, a functional variant is a variant comprising an amino acid sequence related to but different from that of the reference sequence (i.e. SEQ ID NO: 1) and which retains the ability to catalyse the production of one or more inulin from sucrose.

In some embodiments the variant is capable of catalysing transfructosylation. In some embodiments the variant is capable of catalysing the formation of inulin from sucrose. In some embodiments the variant is capable of hydrolysing inulin. In some embodiments the variant is capable of catalysing the addition of one or more fructose monomers to a fructooligosaccharide.

In some embodiments the variant is capable of catalysing such reactions under physiological conditions. In some embodiments physiological conditions comprise a pH of

from about 2 to about 10, such as from about 3 to about 8. In some embodiments physiological conditions comprise a temperature of from about 35 °C to about 40 °C, such as from about 36 °C to about 38 °C, such as about 37 °C. Typically the variants provided herein are active at body temperature (e.g. at about 37 °C) and at about pH 3 to about pH 9, such as from about pH 6 to about pH 8. The pH of the small intestine is typically from about pH 6 to about pH 8. The pH of the stomach is lower, typically below pH 4.

Often, the mutant inulosucrase enzyme has a high affinity for sucrose. Typically, the mutant inulosucrase enzyme has a Michaelis constant (K_M) for sucrose of less than 15.6 mM, such as less than 12 mM, e.g. less than 10 mM, such as less than 5 mM.

Typically, the variant is capable of converting sucrose to fructooligosaccharides such as inulin under low concentrations of sucrose. Low concentrations of sucrose are typically considered to favour sucrose hydrolysis (to fructose and glucose) over transfructosylation. However, typically the mutant inulosucrase enzymes provided herein are capable of maintaining a high ratio of transfructosylation compared to hydrolysis (i.e. a high T/H ratio) even under conditions of low sucrose concentration. For example, the variants provided herein are typically capable of converting sucrose to inulin with a T/H ratio of at least 0.05, such as at least 0.1, e.g. at least 0.2, such as at least 0.25 or at least 0.3 at a sucrose concentration of about 0.5% (e.g. about 0.5% w/w or w/v). The variants provided herein are typically capable of converting sucrose to inulin with a T/H ratio of at least 0.1, such as at least 0.2, e.g. at least 0.3, such as at least 0.4, e.g. at least 0.42 at a sucrose concentration of about 1% (e.g. about 1% w/w or w/v).

In some embodiments the variant has an improved enzymatic (catalytic) activity compared to the wild-type enzyme, i.e. compared to the activity of the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the enzymatic activity is transfructosylation activity. As used herein, the term transfructosylation activity refers to the enzymatic ability of the variant to catalyse the polymerisation of fructose and glucose monomers (particularly fructose monomers) to form fructooligosaccharides, particularly inulin.

In some embodiments the transfructosylation activity of the variant is increased relative to that of the protein having the amino acid sequence shown in SEQ ID NO: 1 by a factor of at least 2 (i.e. the variant has at least twice the transfructosylation activity of the protein having the amino acid sequence shown in SEQ ID NO: 1). In some embodiments the transfructosylation activity of the variant is increased relative to that of the protein

having the amino acid sequence shown in SEQ ID NO: 1 by a factor of at least 2, at least 2.5, at least 3, at least 3.5, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10.

Transfructosylation activity can be determined in any appropriate way. An example of assays useful to measure the activity of variants as provided herein is set out in herein. In some embodiments the activity of the variant (e.g. relative to the protein having the amino acid sequence of SEQ ID NO: 1) is determined by reacting the proteins with a known concentration of sucrose in a reaction solution and monitoring the concentration of sucrose in the reaction solution. In some embodiments the activity of the variant (e.g. relative to the protein having the amino acid sequence of SEQ ID NO: 1) is determined by reacting the proteins with a known concentration of sucrose in a reaction solution and monitoring the concentration of free glucose that appears in the reaction solution. In some embodiments the activity of the variant (e.g. relative to the protein having the amino acid sequence of SEQ ID NO: 1) is determined by reacting the proteins with a known concentration of sucrose in a reaction solution and monitoring the concentration of free fructose that appears in the reaction solution. In some embodiments the activity of the variant (e.g. relative to the protein having the amino acid sequence of SEQ ID NO: 1) is determined by reacting the proteins with a known concentration of sucrose in a reaction solution and monitoring the concentration of free fructose and free glucose that appears in the reaction solution, with the difference in the concentrations being identified with the concentration of the fructooligosaccharide thereby produced.

In some embodiments the concentration of sucrose is from about 50 mM to about 500 mM such as from about 100 mM to about 200 mM e.g. about 125 mM to about 150 mM. In some embodiments the reaction solution is a sodium phosphate buffer. In some embodiments the activity of the proteins is assessed at a pH of from about 6 to about 8, such as about pH 7. In some embodiments the activity of the proteins is assessed at a pH of from about pH 2.5 to about pH 3, such as 2.6, 2.7 or 2.8. In some embodiments the proteins are preincubated in the buffer at a low pH (e.g. from about pH 2 to about pH 4, such as from about pH 2.5 to about pH 3, e.g. about pH 2.6, 2.7 or 2.8). In some embodiments the preincubation (if used) is maintained for from about 5 minutes to about 30 minutes, such as for about 10 minutes/ in some embodiments the measurements are taken at about 37 °C. In some embodiments the variant has an improved activity compared to the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has an improved activity at a pH of from about pH 2.5 to about

pH 3, such as 2.6, 2.7 or 2.8 compared to the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has an improved activity at a pH of from about pH 6 to about pH 8, such as about pH 7 compared to the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has an improved activity at a pH of from about pH 2.5 to about pH 3, such as 2.6, 2.7 or 2.8 and at a pH of from about pH 6 to about pH 8, such as about pH compared to the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has an improved activity at a pH of from about pH 2.5 to about pH 3, such as 2.6, 2.7 or 2.8 relative to the activity at a pH of from about pH 6 to about pH 8, such as about pH, compared to the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the variant has a similar activity at a pH of from about pH 2.5 to about pH 3, such as 2.6, 2.7 or 2.8 and at a pH of from about pH 6 to about pH 8.

In some embodiments the variant has an improved pH stability compared to the wild-type enzyme, i.e. compared to the activity of the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the stability of the variant is improved at low pH compared to that of the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the stability of the variant is improved at a pH of from about pH 2 to about pH 4, such as from about pH 2.5 to about pH 3, e.g. about pH 2.6, 2.7 or 2.8, compared to that of the protein having the amino acid sequence shown in SEQ ID NO: 1. Many proteins are denatured by exposure to low pH, but the variants provided herein typically have improved resistance to low pH compared to the wild-type protein and are therefore denatured less (e.g. to a lesser extent or less permanently, or are not denatured) compared to the protein having the amino acid sequence shown in SEQ ID NO: 1.

Improved pH stability (e.g. improved stability when exposed to conditions of low pH) is useful because when orally administered to a subject a variant as provided herein will be exposed to stomach acid before reaching the intestines.

In some embodiments the stability of the variant under conditions of low pH (e.g. from about pH 2 to about pH 4, such as from about pH 2.5 to about pH 3, e.g. about pH 2.6, 2.7 or 2.8) is increased relative to that of the protein having the amino acid sequence shown in SEQ ID NO: 1 by a factor of at least 2 (i.e. the variant has at least twice the pH stability of the protein having the amino acid sequence shown in SEQ ID NO: 1). In some embodiments the pH stability of the variant is increased relative to that of the protein having the amino acid sequence shown in SEQ ID NO: 1 by a factor of at least 2, at least

2.5, at least 3, at least 3.5, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10.

Sometimes the activity of the variant at a pH of from about 1 to about 2 may at least 5%, such as at least 10%, e.g. at least 20%, e.g. at least 30%, e.g. at least 40%, e.g. at least 50%, e.g. at least 60%, e.g. at least 70%, , e.g. at least 80%, e.g. at least 90% or more of the maximum activity of the variant at a pH of from about 4 to about pH 9.

The stability of a variant to pH conditions (e.g. the stability of the variant in the presence of low pH conditions) can be determined in any appropriate way. For example, in one embodiment the structural stability of the variant can be monitored at a given pH using physical techniques such as circular dichroism. In other embodiments the stability of the variant can be inferred by determining the catalytic (enzymatic) properties of the variant after exposure to the pH conditions compared with those of the variant absent the exposure to the pH conditions.

An example of assays useful to determine the pH stability activity of variants as provided herein is set out in herein.

Improved pH stability can be determined by determining the enzymatic ability of the variant to catalyse the polymerisation of fructose and glucose monomers (particularly fructose monomers) to form fructooligosaccharides, particularly inulin, following exposure to the pH conditions (e.g. exposure to low pH), compared to the activity of the variant in the absence of such exposure to the pH conditions. For example, the enzymatic ability of the variant to catalyse the polymerisation of fructose and glucose monomers (particularly fructose monomers) to form fructooligosaccharides, particularly inulin, can be determined at a pH of from about pH 6 to about pH 8 (e.g. about pH 7) when the variant has been previously exposed to conditions of low pH (e.g. from about pH 2 to about pH 4, such as from about pH 2.5 to about pH 3, e.g. about pH 2.6, 2.7 or 2.8) and compared the corresponding activity when the variant has not been previously exposed to conditions of low pH.

In some embodiments the transfructosylation ability of the variant is determined as described above.

In some embodiments the variant has an improved protease stability compared to the wild-type enzyme, i.e. compared to the activity of the protein having the amino acid sequence shown in SEQ ID NO: 1. As used herein, the term “protease stability” is identified with the resistance of the variant to degradation (e.g. hydrolysis) by protease enzymes, such as by intestinal proteases.

In some embodiments the stability of the variant is improved in the presence of proteases (such as pepsin, trypsin and/or chymotrypsin) compared to that of the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the stability of the variant is improved in the presence of gastric concentrations of proteases compared to that of the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the stability of the variant is improved in the presence of intestinal concentrations of proteases compared to that of the protein having the amino acid sequence shown in SEQ ID NO: 1. In some embodiments the stability of the variant is improved in the presence of concentrations from about 100 μg/mL to about 2000 μg/mL of proteases such as pepsin. In some embodiments the stability of the variant is improved in the presence of physiological activity levels of proteases such as pepsin. In some embodiments such activity levels are defined in units/mL; for example one unit of pepsin may be defined as that which produces a ΔA_{280} of 0.001 per minute at pH 2.0 at 37 °C measured as trichloroacetic acid (TCA)-soluble products using hemoglobin as the substrate (https://www.sigmaaldrich.com/GB/en/technical-documents/protocol/protein-biology/enzyme-activity-assays/enzymatic-assay-of-pepsin; Ansen, J Gen Physiol (1938) 22 (1): 79–89). In some embodiments physiological activity levels of pepsin are from about 1000 to about 5000 U/mL, typically from about 1500 to about 2500 U/mL such as about 2000 U/mL.

Many proteins are degraded (e.g. hydrolysed) by exposure to proteases, but the variants provided herein typically have improved resistance to pepsin digestion compared to the wild-type protein and are therefore broken down less (e.g. to a lesser extent or less quickly, or are not broken down) compared to the protein having the amino acid sequence shown in SEQ ID NO: 1.

Improved stability to proteases (e.g. improved stability when exposed to proteases under physiological conditions) is useful because when orally administered to a subject a variant as provided herein will be exposed to proteases in the intestines.

In some embodiments the stability of the variant in the presence of proteases (e.g. in the presence of from about 100 μg/mL to about 2000 μg/mL pepsin, such as from about 500 μg/mL to about 1500 μg/mL, e.g. about 1000 μg/mL pepsin) is increased relative to that of the protein having the amino acid sequence shown in SEQ ID NO: 1 by a factor of at least 2 (i.e. the variant has at least twice the protease stability of the protein having the amino acid sequence shown in SEQ ID NO: 1). In some embodiments the protease stability of the variant is increased relative to that of the protein having the amino acid

sequence shown in SEQ ID NO: 1 by a factor of at least 2, at least 2.5, at least 3, at least 3.5, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9 or at least 10.

The protease stability of a variant can be determined in any appropriate way. For example, in one embodiment the mass of the variant can be monitored at a given protease concentration (or following exposure to a given protease concentration) using physical techniques such as gel electrophoresis (e.g. native or denaturing PAGE) or mass spectroscopy, which can be used to identify cleavage of proteins by proteases. In other embodiments the protease stability of the variant can be inferred by determining the catalytic (enzymatic) properties of the variant after exposure to proteases compared with those of the variant absent the exposure to the proteases.

An example of assays useful to determine the protease stability activity of variants as provided herein is set out in herein.

Improved protease stability can be determined by determining the enzymatic ability of the variant to catalyse the polymerisation of fructose and glucose monomers (particularly fructose monomers) to form fructooligosaccharides, particularly inulin, following exposure to the proteases, compared to the activity of the variant in the absence of such exposure to proteases. For example, the enzymatic ability of the variant to catalyse the polymerisation of fructose and glucose monomers (particularly fructose monomers) to form fructooligosaccharides, particularly inulin, can be determined in simulated gastric conditions (e.g. around pH 3 to about pH 4, e.g. around pH 3.2 and in the presence of about 0.5 to 1.5 mg/mL protease (e.g. pepsin), such as around 1 mg/mL pepsin) and compared to the corresponding activity when the variant is not exposed to the protease.

In some embodiments the transfructosylation ability of the variant is determined as described above.

The examples described in more detail herein provide examples of two activity assays that provide information about the enzymatic activity, pH stability and protease stability of the variants provided herein.

In one activity assay (*screen 1*), activity of the protein (e.g. the variant) is measured at pH 3.2 in the presence of 1 mg/mL pepsin. Improvements in activity relative to wild type protein (e.g. relative to the protein having the amino acid sequence of SEQ ID NO: 1) in this screen show that the variant has improvements in: (i) general enzymatic activity, overall or specifically at low pH, (ii) resilience to inactivation at low pH (denaturation), and/or (iii) resistance to digestion by pepsin.

In a second activity assay (*screen 2*), the protein (e.g. the variant) is pre-incubated at low pH (e.g. from about pH 2.5 to about pH 3, e.g. about pH 2.6, 2.7 or 2.8) or at neutral pH (around pH 7). Activity of the protein is then assessed in simulated intestinal conditions at pH 7. Improvements in activity relative to wild type protein (e.g. relative to the protein having the amino acid sequence of SEQ ID NO: 1) in this screen indicate: (i) generally better activity, (ii) improved resilience to low pH exposure, and/or (iii) better resistance to intestinal conditions.

For example, those skilled in the art will appreciate that these screens can be used to infer information about the variants provided herein. For example, if a variant shows little change in activity versus the wild-type after incubation at pH 7, but larger changes when incubated at low pH, this could indicate that the variant has improved pH stability. If a variant shows little change in activity versus the wild-type after incubation at low pH, but larger changes when incubated in the presence of proteases compared to in the absence of such proteases, this could indicate that the variant has improved protease stability.

The modifications provided herein thus improve the properties of the protein of SEQ ID NO: 1. Accordingly, also provided herein is a method of increasing (i) *in vivo* transfructosylation activity, (ii) pH stability and/or (iii) protease resistance of an inulosucrase enzyme; by making in the inulosucrase enzyme one or more modifications as described herein; and thereby increasing (a) the *in vivo* transfructosylation activity of the inulosucrase enzyme, (b) the pH stability of the inulosucrase enzyme and/or (iii) the protease resistance of the inulosucrase enzyme.

In some embodiments, the mutant inulosucrase enzyme is provided in isolated form. Isolated forms of the provided mutant inulosucrase enzymes are provided per se, as well as their uses in methods and applications provided herein. In some embodiments the mutant inulosucrase enzyme is used in the form of an isolated enzyme. In some embodiments the mutant inulosucrase enzyme is administered to a subject as an isolated enzyme.

As used herein, the term isolated refers to the enzyme being extra cellular. The enzyme is typically purified from a cellular host. An isolated enzyme as used herein is not provided within a bacterial or fungal host. Administration of a bacteria or fungus comprising a fructosyltransferase enzyme to an organism does not correspond to administration of the isolated enzyme to the organism. The term “isolated enzyme” thus

does not embrace whole cells such as bacterial or fungal cells. In some embodiments the inulosucrase enzyme used as described herein is an extracellular isolate.

Those skilled in the art will appreciate, however that the term "isolated enzyme" does not require that nothing apart from the enzyme is present. As explained in more detail herein, an "isolated enzyme" may be administered in the form of a nutraceutical or pharmaceutical composition. An "isolated enzyme" may be comprised in a food composition or foodstuff. Impurities may also be present. However, often no impurities are present, e.g. the enzyme is substantially purified. Often a composition comprising an isolated enzyme as defined herein may be substantially free or free of impurities such as host DNA (e.g. DNA from an organism such as a bacteria or yeast in which the enzyme may be expressed). As explained below in more detail, a nutraceutical composition typically comprises the isolated enzyme and one or more excipients, diluents, or other nutraceutically acceptable additive(s), such as fillers, stabilizing agents, colouring agents and flavouring agents. Pharmaceutical compositions typically comprise the isolated enzyme and one or more pharmaceutically acceptable carrier, excipient, diluent, or other pharmaceutically acceptable additive(s). A food composition or foodstuff comprising an isolated enzyme as described herein typically comprises the isolated enzyme and one or more carbohydrates, fats, lipids, proteins, flavouring agent, colouring agent, etc.

As explained above, the mutant inulosucrase enzyme provided herein is useful to convert sucrose (such as dietary sucrose) to fructooligosaccharides such as inulin.

Typically, the mutant inulosucrase enzyme is capable of catalysing the production of fructooligosaccharides (e.g. inulin) at least 2, such as at least 3, e.g. at least 4, e.g. at least 5, e.g. at least 10, such as at least 20, e.g. at least 30, e.g. at least 40, e.g. at least 50 monomer units in length. Thus, in some embodiments the methods and uses disclosed herein which comprise application of the provided mutant inulosucrase enzyme comprise production of fructooligosaccharides (e.g. inulin) at least 2, such as at least 3, e.g. at least 4, e.g. at least 5, e.g. at least 10, such as at least 20, e.g. at least 30, e.g. at least 40, e.g. at least 50 monomer units in length.

The fructooligosaccharide (e.g. inulin) is often of from 2 to 200 monomer units in length, such as from 5 to 100 monomer units, such as 10 to 80 monomer units, e.g. from 20 to 60 monomer units, such as from 30 to 50 monomer units in length.

Typically, the variant is suitable for being administered to a subject in the form of a nutraceutical or pharmaceutical composition, or in the form of a food composition or

foodstuff. Such compositions *per se* are also expressly provided herein. Nutraceutical and pharmaceutical compositions are described in more detail herein. Food compositions and foodstuffs are described in more detail herein. The variant, or a composition comprising such fructosyltransferase, is typically orally administered to the subject.

The mutant inulosucrase enzyme is a variant of SEQ ID NO: 1 which is the amino acid sequence of the fructosyltransferase of gene inuGB from *Lactobacillus gasseri* DSM 20604. The mutant inulosucrase enzyme may thus be described as being derived from *Lactobacillus gasseri.* Those skilled in the art will appreciate that references to a protein being derived from a given organism refers to the original host organism that natively expresses the protein at issue. References to a protein being "derived" from a specific organism does not mean that the protein is necessarily expressed in practice in such an organism. For example, expression organisms such as *E. coli* transformed with appropriate expression vectors are often used to express proteins natively produced by other organisms. Practitioners are referred to Sambrook et al., Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Press, Plainsview, New York (2012); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 114), John Wiley & Sons, New York (2016), for further discussion of the use of non-native expression systems in order to produce proteins. The source organism for the fructosyltransferase may be chosen based on desired characteristics of the sequence. Desired characteristics include activity of the fructosyltransferase, its stability in storage, its resistance to proteases, etc. Protease resistance can be determined as described in the examples. Exemplary expression systems are described in more detail herein.

Production of the variant

The mutant inulosucrase enzyme may be expressed intracellularly in a cellular host and isolated by being purified from the host. For example, an intracellular enzyme may be isolated via cell lysis followed by purification of the cell lysate.

Alternatively, the variant may be an extracellular enzyme, e.g. an enzyme that is expressed by an organism by secretion into the expression medium. Such enzymes may be isolated by purification of the expression medium without requiring cell lysis. Such enzymes may in some embodiments be prepared for use as disclosed herein by removing biomass from the expression medium. In some embodiments biomass is removed by centrifugation and/or filtration. In some embodiments a suitable organism for extracellular expression of an inulosucrase enzyme according to the present disclose is *Pichia pastoris*

(also known as *Komagataella phaffi*). A mutant inulosucrase enzyme may be expressed naturally in a cellular organism as an intracellular enzyme and be modified in order to be excreted from the cell as an extracellular enzyme. For example, the enzyme can be modified by deleting a cell wall anchor domain, e.g. at the C terminus of the protein sequence, in order to promote secretion into the expression medium.

A mutant inulosucrase enzyme may in some embodiments comprise a signal peptide. A signal peptide can be chosen or designed according to the expression system used to express the mutant inulosucrase enzyme. Not all expression systems require a signal peptide. Signal peptides and their amino acid sequences are well known in the art. Practitioners are directed to references such as Sambrook et al., Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Press, Plainsview, New York (2012); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 114), John Wiley & Sons, New York (2016). For example, in some embodiments a mutant inulosucrase enzyme as described herein can be expressed in an organism such as *Pichia pastoris* and the mutant inulosucrase enzyme may comprise a signal peptide for targeting the mutant inulosucrase enzyme to the growth medium. Known signal peptides for expression of proteins in *Pichia pastoris* include the secretion signal sequence from the *Saccharomyces cerevisiae* factor prepro peptide (Cregg *et al.*, Biotechnology 11(8), 905-910, 1993). In some embodiments the mutant inulosucrase enzyme is used with the signal peptide retained. Thus in some embodiments provided herein is mutant inulosucrase enzyme comprising a variant of SEQ ID NO: 1 as described herein, attached to a signal peptide, e.g. at the N terminus of the variant of SEQ ID NO: 1. In some embodiments a mutant inulosucrase enzyme is expressed with a signal peptide and the signal peptide is removed from the mutant inulosucrase enzyme before its use as described herein. The enzyme can be modified by deleting a signal peptide if present from the protein sequence.

A mutant inulosucrase enzyme may be expressed in any suitable organism. Protein expression is routine to those skilled in the art and is described in, for example, references such as Sambrook et al., Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Press, Plainsview, New York (2012); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 114), John Wiley & Sons, New York (2016).

Typically, a mutant inulosucrase enzyme as provided herein may be expressed in cells such as in bacterial cells, yeast cells, or insect cells. In some embodiments bacterial cells are used. In some embodiments the inulosucrase enzyme may be produced in yeast cells. Many bacteria are recognised as being GRAS (Generally Recognised as Safe) and

thus suitable for human consumption. Whilst the disclosed methods focus on the administration of isolated enzymes, the production of such enzymes from GRAS organisms is beneficial in conferring GRAS status on the resultant enzymes and their uses. Accordingly, in some embodiments the organisms used to express the mutant inulosucrase enzyme for use in the disclosed methods are suitable for human or animal consumption. Accordingly, in some embodiments the bacteria used to express the mutant inulosucrase enzyme for use in the disclosed methods is certified as being GRAS. When a bacterium is used to express the mutant inulosucrase enzyme, any suitable bacterium may be used. In some other embodiments the yeast used to express the mutant inulosucrase enzyme is certified as being GRAS. When a yeast is used to express the mutant inulosucrase enzyme, any suitable yeast may be used. For example, the enzyme is typically expressed by or is obtainable by expression from an organism of genus *Escherichia, Lactobacillus, Saccharomyces, Bacillus*, *Komagataella, Pichia*, *Trichoderma, Corynebacterium* or *Aspergillus;* preferably *E. coli, S. cerevisiae, B. subtilis, K. phaffii, P. pastoris, T. reesei, C. glutamicum, A. niger,* or *A. oryzae.* (The organism *P. pastoris* is sometimes referred to as *K. phaffii.*) For example, the enzyme is typically expressed by or is obtainable by expression from an organism of genus *Escherichia, Lactobacillus, Saccharomyces* or *Bacillus*, such as *Escherichia* or *Bacillus*, preferably *E. coli, S. cerevisiae* or *B. subtilis*. In some embodiments the enzyme is expressed by or is obtainable by expression from an organism of genus *Escherichia, Bacillus, Komagataella or Pichia, such as E. coli, B. subtilis, K. phaffii or P. pastoris*. Accordingly, in some embodiments the disclosed methods comprise expressing the enzyme in an organism of genus *Escherichia, Lactobacillus, Saccharomyces, Bacillus*, *Komagataella, Pichia*, *Trichoderma, Corynebacterium* or *Aspergillus*, such as *Escherichia, Bacillus, or Pichia*, isolating the enzyme and then administering the isolated enzyme to a subject. In some embodiments the disclosed methods comprise expressing the enzyme in *E. coli, S. cerevisiae, B. subtilis, K. phaffii, P. pastoris, T. reesei, C. glutamicum, A. niger,* or *A. oryzae*, such as *E. coli, B. subtilis, K. phaffii or P. pastoris*, isolating the enzyme and then administering the isolated enzyme to a subject. In some embodiments the disclosed methods comprise expressing the enzyme in an organism of genus *Escherichia, Lactobacillus* or *Bacillus*, such as *Escherichia* or *Bacillus*, preferably *E. coli* or *B. subtilis*, isolating the enzyme and then administering the isolated enzyme to a subject. In some embodiments the disclosed methods comprise expressing the enzyme in an organism of genus *Pichia*, such as preferably *P. pastoris*, isolating the enzyme (e.g. by removing biomass from the

fermentation liquor and optional further purification) then administering the isolated enzyme to a subject.

The variant may be expressed in any suitable GRAS organism such as a GRAS bacterium, yeast or fungus, such as a GRAS bacterium or yeast.

A mutant inulosucrase enzyme for use in the disclosed methods may be isolated by cell lysis if required. Cell lysis can be conducted using any suitable method. For example, cells can be physical lysed, e.g. using a French press, or by sonication in a suitable buffer. Such buffers are commercially available e.g. from Qiagen.

Impure enzyme solutions can be purified for use in the disclosed methods by any suitable means. Typically mutant inulosucrase enzymes can be purified using suitable chromatographic methods which are readily accessible to those skilled in the art. Suitable chromatographic methods include ion exchange chromatography (e.g. anion exchange or cation exchange chromatography), size exclusion chromatography, and/or hydrophobic interaction chromatography. Affinity chromatography may also be used. Any suitable affinity system can be used. For example, a fructosyltransferase enzyme may be tagged with a tag such as poly-histidine tag (e.g. HHHH, HHHHHH, or HHHHHHHH) and purified on a metal-containing column, e.g. a nickel or cobalt nitriloacetic acid column. Other purification tags include peptide tags such as Strep (WSHPQFEK), FLAG (DYKDDDDK), Human influenza hemagglutinin (HA) (YPYDVPDYA), Myc (EQKLISEED), and V5 (GKPIPNPLLGLDST), etc which may be purified using suitable columns. Purification tags may be cleavable or non-cleavable. The selection of suitable purification techniques is routine to those skilled in the art.

In yet another aspect, a mutant inulosucrase enzyme can be expressed in a cell free expression system. For example, an enzyme can be expressed by *in vitro* transcription/translation (IVTT) from a suitable expression plasmid. Kits for conducting IVTT are commercially available from suppliers such as New England Biolabs (NEB).

In some embodiments a mutant inulosucrase enzyme as described herein may be obtained by fermentation. In some embodiments a mutant inulosucrase enzyme may be obtained by fermentation from one or more microorganism. In some embodiments a mutant inulosucrase enzyme may be obtained by fermentation of one or more microorganisms of genus *Escherichia, Lactobacillus, Saccharomyces, Bacillus, Komagataella, Pichia, Trichoderma, Corynebacterium* or *Aspergillus*. In some embodiments a mutant inulosucrase enzyme may be obtained by fermentation of Pichia (e.g. P. pastoris). In some embodiments the inulosucrase enzyme may be obtained from

the fermentation medium following such fermentation. In some embodiments the inulosucrase enzyme may be obtained by removing biomass from the fermentation medium, e.g. by centrifugation and/or filtration. In some embodiments the inulosucrase enzyme may be obtained by concentrating the fermentation medium.

Nutraceutical compositions

As mentioned above, the mutant inulosucrase enzyme may be administered to a subject in the form of a nutraceutical composition. Such compositions *per se* are also expressly provided herein.

A nutraceutical composition as provided herein typically comprises a mutant inulosucrase enzyme as provided herein, and one or more nutraceutically acceptable filler, stabilizing agent, colouring agent or flavouring agent.

Suitable excipients for use in the nutraceutical composition include:

- fillers such as lactose, maltodextrin, sucrose, magnesium stearate, glucose, plant cellulose, calcium carbonate etc;
- stabilizers such as vitamin A, C, E, selenium, amino acids, methyl paraben, and propyl paraben;
- anti-adherents;
- binders such as lactose, sucrose, microcrystalline cellulose, malitol, sorbitol, xylitol, starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone;
- diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch;
- disintegrants such as starch, alginic acid, alginates or sodium starch glycolate;
- lubricants such as silica, talc, stearic acid, magnesium or calcium stearate and/or polyethylene glycols;
- dyestuffs and other colouring agents such as FD&C Blue No. 1 (brilliant blue FCF), FD&C Blue No. 2 (indigotine), FD&C Green No. 3 (fast green FCF), FD&C Red No. 40 (allura red AC), FD&C Red No. 3 (erythrosine), FD&C Yellow No. 5 (tartrazine), and FD&C Yellow No. 6 (sunset yellow);
- flavouring agents such as sweet almond oil, benzaldehyde, DL-menthol, ethyl acetate, ethyl vanillin, L-menthol, methyl salicylate, peppermint oil, peppermint spirit, and vanillin;
- effervescing mixtures;

- sweeteners; and
- wetting agents, such as lecithin, polysorbates, and laurylsulphates;

Any suitable combination of any of the aforementioned excipients can be used in the nutraceutical compositions provided and described in more detail herein. Such nutraceutical preparations may be manufactured in a known manner, for example, by means of mixing, granulating, tableting, sugar coating, or film coating processes.

Typically, a nutraceutical composition as described herein is formulated as a tablet, a troche, a lozenge, an aqueous or oily suspension, a dispersible powder or as granules. A powder may be obtained by e.g. lyophilisation.

Liquid dispersions for oral administration may be syrups, emulsions and suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol. Suspensions and emulsions may contain a carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. Syrups may be formulated to avoid the use of sucrose.

Choice of formulation of the nutraceutical composition is within the capability of those skilled in the art, and may depend on factors such as cultural, societal or commercial preferences, the end consumer of the product, any specific foodstuff targeted, etc.

Typically, a nutraceutical composition as described herein is suitable for oral administration to the subject. Thus, the methods disclosed herein which comprise the use of a nutraceutical composition as described herein typically comprise orally administering the nutraceutical composition to the subject.

Typically, the nutraceutical composition is intended to release the active agent (i.e. the mutant inulosucrase enzyme) in an appropriate part of the body, where it can be active in converting sucrose. For example, the nutraceutical composition may release the active mutant inulosucrase enzyme in the gastrointestinal tract, e.g. in the stomach or the small intestine. Accordingly, a nutraceutical composition as described herein may comprise an enteric coating. Any suitable enteric coating material known in the art can be used. Suitable materials include but are not limited to methyl acrylate-methacrylic acid copolymers; cellulose acetate phthalate (CAP); cellulose acetate succinate; hydroxypropyl methyl cellulose (HMPC) and hydroxypropyl methyl cellulose phthalate; hydroxypropyl methyl cellulose acetate succinate (hypromellose acetate succinate; HMPAS); polyvinyl acetate phthalate (PVAP); methyl methacrylate-methacrylic acid copolymers; shellac; cellulose acetate trimellitate; sodium alginate; zein and the like.

Typically, the nutraceutical composition is provided as a dietary supplement. The composition may be provided as a kit together with instructions for use. The composition may be provided in the form of a supplement to be taken before, with, or after consuming food.

Typically, the nutraceutical composition comprises only ingredients which are generally recognised as safe (GRAS). The components of the composition are typically food grade components.

The mutant inulosucrase enzyme is typically stable in the nutraceutical composition under appropriate storage conditions for extended periods of time. For example, the mutant inulosucrase enzyme may be stable for in excess of 1 day, 1 month, 1 year, etc, when stored under appropriate conditions. The necessary stability of the mutant inulosucrase enzyme can be determined based on its application and the form of the composition in which it is provided and can be controlled using methods known in the art, including the use of high purity reagents and storage under appropriate conditions.

Typically, a nutraceutical composition will contain up to 85 wt% of the mutant inulosucrase enzyme described herein. It may contain up to 50 wt%, up to 40 wt%, up to 30 wt%, up to 20 wt% or up to 10 wt% of the mutant inulosucrase enzyme.

Typically, a nutraceutical composition may contain a sufficient amount of the mutant inulosucrase enzyme provided herein to produce from about 1 to about 100 g, such as from about 2 g to about 50 g, e.g. from about 5 g to about 20 g such as about 10 g of inulin within about 0.5 to 5 hours, such as within about 1 to about 3 hours, e.g. within about 2 hours under physiological conditions. A nutraceutical composition thus may comprise from about 1 to about 1000 mg of the mutant inulosucrase enzyme, such as from about 10 to about 100 mg e.g. about 50 mg of the mutant inulosucrase enzyme per unit dose.

A nutraceutical composition may comprise from about 1 mg to about 100 mg such as from about 2 mg to about 50 mg e.g. from about 5 mg to about 20 mg such as from about 7 mg to about 15 mg, e.g. about 10 mg of the mutant inulosucrase enzyme per unit dose.

A nutraceutical composition may be capable of acting on from about 1% to about 100% (e.g. % w/w or % w/v) e.g. from about 1% to about 80%, such as from about 5% to about 50%, e.g. from about 10% to about 40%, e.g. from about 20 to about 30% of sucrose molecules available within about 1 minute to about 1 hour, e.g. within about 10 minutes to

about 45 minutes, such as within about 15 minutes to about 30 minutes under physiological conditions.

Those skilled in the art will appreciate that "available fructose" represents the fructose present in sucrose to be converted. As explained herein, each sucrose molecule comprises one glucose unit and one fructose unit such that conversion of 100% available fructose monomer units corresponds to incorporation of 50% monomer units of sucrose. Thus, to a first approximation (discounting the terminal glucose unit on the inulin generated by the mutant inulosucrase enzyme), conversion of 100% of all sucrose molecules present in a sample thus corresponds to 50% conversion of saccharide units in sucrose (100% conversion/incorporation of fructose units).

A nutraceutical composition may thus be capable of converting/incorporating from about 1% to about 100% e.g. from about 1% to about 80%, such as from about 5% to about 50%, e.g. from about 10% to about 40%, e.g. from about 20 to about 30% of available fructose into inulin within about 1 minute to about 1 hour, e.g. within about 10 minutes to about 45 minutes, such as within about 15 minutes to about 30 minutes under physiological conditions. In other words a nutraceutical composition as provided herein may be capable of converting/incorporating from about 1% to about 50% e.g. from about 1% to about 40%, such as from about 5% to about 30%, e.g. from about 10% to about 20%, of the saccharide units present in available sucrose into inulin within about 1 minute to about 1 hour, e.g. within about 10 minutes to about 45 minutes, such as within about 15 minutes to about 30 minutes under physiological conditions.

A nutraceutical composition may be administered to a subject at any suitable administration frequency. For example, a nutraceutical composition may be administered at least once per day, such as between about 1 and about 20 times a day, e.g. between about 1 and about 10 times a day, such as between 2 and 5 times a day, e.g. about 3 or 4 times a day.

Typically, a neutraceutical composition is used in non-therapeutic methods. Accordingly, provided herein is use of a neutraceutical composition as described herein in a method (e.g. a non-therapeutic method) of reducing fructose uptake; reducing formation of fructose via metabolism of sucrose; reducing glucose uptake and/or reducing formation of glucose via metabolism of sucrose; producing inulin; suppressing appetite; and/or increasing satiety in a subject. Also provided is a method (e.g. a non-therapeutic method) of reducing fructose uptake; reducing formation of fructose via metabolism of sucrose; reducing glucose uptake and/or reducing formation of glucose via metabolism of sucrose;

producing inulin; suppressing appetite; and/or increasing satiety in a subject, comprising administering a nutraceutical composition as described herein to the subject. Further provided is a neutraceutical composition as described herein for use in a method (e.g. a non-therapeutic method) of reducing fructose uptake; reducing formation of fructose via metabolism of sucrose; reducing glucose uptake and/or reducing formation of glucose via metabolism of sucrose; producing inulin; suppressing appetite; and/or increasing satiety in a subject. Still further provided is use of a mutant inulosucrase enzyme as described herein in the manufacture of a nutraceutical composition as described herein for the (typically non-therapeutic) reduction of fructose uptake; reduction of formation of fructose via metabolism of sucrose; reduction of glucose uptake and/or reduction of formation of glucose via metabolism of sucrose; production of inulin; suppression of appetite; and/or increase in satiety in a subject. Such methods and uses are described in more detail herein.

Pharmaceutical compositions

In another embodiment, the mutant inulosucrase enzyme is administered to a subject in the form of a pharmaceutical composition. Such compositions *per se* are also expressly provided herein.

A pharmaceutical composition as used herein typically comprises the mutant inulosucrase enzyme as described herein, and one or more pharmaceutical acceptable carrier, excipient or diluent.

Suitable components for such use in the pharmaceutical composition include:

- fillers such as lactose, sucrose, magnesium stearate, glucose, plant cellulose, calcium carbonate etc;
- stabilizers such as vitamin A, C, E, selenium, amino acids, methyl paraben, and propyl paraben;
- anti-adherents;
- binders such as lactose, sucrose, microcrystalline cellulose, malitol, sorbitol, xylitol, starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone;
- diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch;
- disintegrants such as starch, alginic acid, alginates or sodium starch glycolate;
- lubricants such as silica, talc, stearic acid, magnesium or calcium stearate and/or polyethylene glycols;

- dyestuffs and other colouring agents such as FD&C Blue No. 1 (brilliant blue FCF), FD&C Blue No. 2 (indigotine), FD&C Green No. 3 (fast green FCF), FD&C Red No. 40 (allura red AC), FD&C Red No. 3 (erythrosine), FD&C Yellow No. 5 (tartrazine), and FD&C Yellow No. 6 (sunset yellow);
- flavouring agents such as sweet almond oil, benzaldehyde, DL-menthol, ethyl acetate, ethyl vanillin, L-menthol, methyl salicylate, peppermint oil, peppermint spirit, and vanillin;
- effervescing mixtures;
- sweeteners; and
- wetting agents, such as lecithin, polysorbates, and laurylsulphates;

Any suitable combination of any of the aforementioned excipients can be used in the pharmaceutical compositions provided and described in more detail herein. Such pharmaceutical preparations may be manufactured in a known manner, for example, by means of mixing, granulating, tableting, sugar coating, or film coating processes.

Typically, a pharmaceutical composition as described herein is formulated as a tablet, a troche, a lozenge, an aqueous or oily suspension, a dispersible powder or as granules. A powder may be obtained by e.g. lyophilisation.

Liquid dispersions for oral administration may be syrups, emulsions and suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol. Suspensions and emulsions may contain a carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. Syrups may be formulated to avoid the use of sucrose.

Typically, a pharmaceutical composition as described herein is suitable for oral administration to the subject. Thus, the methods disclosed herein which comprise the use of a pharmaceutical composition as described herein typically comprise orally administering the pharmaceutical composition to the subject.

Typically, the pharmaceutical composition is intended to release the active agent (i.e. the mutant inulosucrase enzyme) in an appropriate part of the body, where it can be active in converting sucrose. Accordingly, a pharmaceutical composition as described herein may comprise an enteric coating. Any suitable enteric coating material known in the art can be used. Suitable materials include but are not limited to methyl acrylate-methacrylic acid copolymers; cellulose acetate phthalate (CAP); cellulose acetate succinate; hydroxypropyl methyl cellulose (HMPC) and hydroxypropyl methyl cellulose

phthalate; hydroxypropyl methyl cellulose acetate succinate (hypromellose acetate succinate; HMPAS); polyvinyl acetate phthalate (PVAP); methyl methacrylate-methacrylic acid copolymers; shellac; cellulose acetate trimellitate; sodium alginate; zein and the like.

The mutant inulosucrase enzyme is typically stable in the pharmaceutical composition under appropriate storage conditions for extended periods of time. For example, the mutant inulosucrase enzyme may be stable for in excess of 1 day, 1 month, 1 year, etc, when stored under appropriate conditions. The necessary stability of the mutant inulosucrase enzyme can be determined based on its application and the form of the composition in which it is provided and can be controlled using methods known in the art, including the use of high purity reagents and storage under appropriate conditions.

Preferred pharmaceutical compositions are sterile and pyrogen free.

Typically, a pharmaceutical composition will contain up to 85 wt% of the mutant inulosucrase enzyme described herein. It may contain up to 50 wt%, up to 40wt%, up to 30wt%, up to 20wt% or up to 10wt% of the mutant inulosucrase enzyme.

Typically, a pharmaceutical composition may contain a sufficient amount of the mutant inulosucrase enzyme to produce from about 1 to about 100 g, such as from about 2 g to about 50 g, e.g. from about 5 g to about 20 g such as about 10 g inulin within about 0.5 to 5 hours, such as within about 1 to about 3 hours, e.g. within about 2 hours under physiological conditions. A pharmaceutical composition thus may comprise from about 1 to about 10,000 mg of the mutant inulosucrase enzyme, such as from about 10 to about 1000 mg e.g. about 50 to 500 mg of the mutant inulosucrase enzyme per unit dose.

A pharmaceutical composition may comprise from about 1 mg to about 100 mg such as from about 2 mg to about 50 mg e.g. from about 5 mg to about 20 mg such as from about 7 mg to about 15 mg, e.g. about 10 mg of the mutant inulosucrase enzyme per unit dose.

A pharmaceutical composition may be capable of acting on from about 1% to about 100% (e.g. % w/w or % w/v) e.g. from about 1% to about 80%, such as from about 5% to about 50%, e.g. from about 10% to about 40%, e.g. from about 20 to about 30% of available (e.g. excess) sucrose within about 1 minute to about 1 hour, e.g. within about 10 minutes to about 45 minutes, such as within about 15 minutes to about 30 minutes under physiological conditions. A pharmaceutical composition may thus be capable of converting/incorporating from about 1% to about 100% e.g. from about 1% to about 80%, such as from about 5% to about 50%, e.g. from about 10% to about 40%, e.g. from about 20 to about 30% of available (e.g. excess) fructose into inulin within about 1 minute to

about 1 hour, e.g. within about 10 minutes to about 45 minutes, such as within about 15 minutes to about 30 minutes under physiological conditions. In other words a pharmaceutical composition as provided herein may be capable of converting/incorporating from about 1% to about 50% e.g. from about 1% to about 40%, such as from about 5% to about 30%, e.g. from about 10% to about 20%, of the saccharide units present in the available (e.g. excess) sucrose into inulin within about 1 minute to about 1 hour, e.g. within about 10 minutes to about 45 minutes, such as within about 15 minutes to about 30 minutes under physiological conditions.

A pharmaceutical composition may be administered to a subject at any suitable administration frequency. For example, a pharmaceutical composition may be administered at least once per day, such as between about 1 and about 20 times a day, e.g. between about 1 and about 10 times a day, such as between 2 and 5 times a day, e.g. about 3 or 4 times a day.

Also provided herein is a composition described herein, e.g. a pharmaceutical composition described herein, for use in medicine.

Typically, a pharmaceutical composition is used in therapeutic methods. Accordingly, provided herein is a pharmaceutical composition as described herein for use in a method (e.g. a therapeutic method) of reducing fructose uptake; reducing formation of fructose via metabolism of sucrose; reducing glucose uptake and/or reducing formation of glucose via metabolism of sucrose; producing inulin; suppressing appetite; and/or increasing satiety in a subject. Also provided is a method (e.g. a therapeutic method) of reducing fructose uptake; reducing formation of fructose via metabolism of sucrose; reducing glucose uptake and/or reducing formation of glucose via metabolism of sucrose; producing inulin; suppressing appetite; and/or increasing satiety in a subject, comprising administering a pharmaceutical composition as described herein to the subject. Still further provided is use of a mutant inulosucrase enzyme as described herein in the manufacture of a pharmaceutical composition as described herein for the (typically therapeutic) reduction of fructose uptake; reduction of formation of fructose via metabolism of sucrose; reduction of glucose uptake and/or reduction of formation of glucose via metabolism of sucrose; production of inulin; suppression of appetite; and/or increase in satiety in a subject. Such methods and uses are described in more detail herein.

Food compositions and foodstuffs

Also provided herein are food compositions comprising a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as described herein. Such food compositions are also referred to herein as foodstuffs. Such compositions may be administered to a subject in accordance with the methods and uses provided herein.

A food composition or foodstuff as described herein typically comprises a mutant inulosucrase enzyme as described herein, and one or more carbohydrates, fats, lipids, proteins, flavouring agents, colouring agent, etc.

Food compositions and foodstuffs described herein may comprise:

- sugar sources such as corn sugar, dextrose, fructose, glucose, high-fructose glucose syrup, honey, maple syrup, agave syrup, invert sugar, isoglucose, levulose, maltose, molasses, and sucrose;

- starch sources such as corn, cassava, sweet potato, wheat (e.g. as flour, e.g. in the form of bread or pasta), potato, sorghum, barley, rice, etc;

- fruits such as açaí, apple, apricot, avocado, banana, bilberry, blackberry, blackcurrant, blueberry, boysenberry, cherry, cloudberry, crab apple, cranberry, damson, date, dragonfruit, durian, elderberry, fig, goji berry, gooseberry, grape, grapefruit, guava, jackfruit, jujube, kiwifruit, kumquat, lemon, lime, loganberry, lychee, mango, melon, mulberry, nectarine, orange, clementine, mandarine, tangerine, papaya, passionfruit, pawpaw, peach, pear, persimmon, plantain, plum, pineapple, pomegranate, pomelo, quince, raspberry, redcurrant, satsuma, tamarind, yuzu etc;

- vegetables such as artichoke, aubergine, asparagus, bean sprouts, beans, chickpeas, lentils, peas, broccoli (calabrese), brussels sprouts, cabbage, cauliflower, celery, endive, fennel, greens such as bok choy, chard (beet greens), collard greens, kale, mustard greens, lettuce, mushrooms, okra, onions, chives, garlic, leek, shallot, scallion, peppers, rhubarb, beetroot, carrot, celeriac, taro, ginger, parsnip, rutabaga, radish, potato, sweet potato, yam, turnip, sweetcorn, squash, courgette, cucumber, tomato, watercress etc.

- nuts and seeds such as almonds, Brazil nuts, cashew nuts, hazelnuts, macadamias, pecans, pine nuts, pistachios, walnuts, peanuts, pumpkin seeds, flax seeds, sesame seeds, poppy seeds, sunflower seeds, psyllium seeds and chia seeds.

- fats and lipids such as vegetable fats (e.g. cocoa butter, corn oil, sunflower oil, soybean oil, cotton soil, peanut oil, olive oil, canola oil, pumpkin seed oil, safflower oil, grape seed oil, sesame oil bran oil, argan oil, palm oil, linseed oil, coconut oil) and animal fats (e.g. lard, tallow and butterfat, and fish oils such as cod liver oil and salmon oil);

- animal products such as meat, fish and eggs.

- dyestuffs and other colouring agents such as FD&C Blue No. 1 (brilliant blue FCF), FD&C Blue No. 2 (indigotine), FD&C Green No. 3 (fast green FCF), FD&C Red No. 40 (allura red AC), FD&C Red No. 3 (erythrosine), FD&C Yellow No. 5 (tartrazine), and FD&C Yellow No. 6 (sunset yellow);
- flavouring agents such as sweet almond oil, benzaldehyde, DL-menthol, ethyl acetate, ethyl vanillin, L-menthol, methyl salicylate, peppermint oil, peppermint spirit, and vanillin; and
- sweeteners such as allulose, acesulfame potassium, aspartame, cyclamate, mogrosides, saccharin, steviol glycosides (stevia), sucralose, and sugar alcohols.

Exemplary foodstuffs include confectionary such as chocolate, desserts such as ice cream, gelato, sorbet, yoghurt, cheesecake, flan, tarts etc; baked goods such as cakes, pastries and pies (both sweet and savoury), bread products, etc.

Preferably the foodstuff comprises sucrose.

Typically, a foodstuff as described herein is administered to a subject orally. Thus, the methods disclosed herein which comprise the use of a foodstuff as described herein typically comprise orally administering the foodstuff to the subject.

Typically, the foodstuff is formulated to release the active agent (i.e. the mutant inulosucrase enzyme) in an appropriate part of the body, where it can be active in converting sucrose. For example, the foodstuff may be formulated to release the active mutant inulosucrase enzyme in the gastrointestinal tract, e.g. in the small intestine, and/or in the stomach.

The foodstuff may be formulated such that the mutant inulosucrase enzyme comprised therein is prevented from acting on any sucrose in the foodstuff prior to the foodstuff being consumed. This may be achieved e.g. by encapsulating the enzyme such that it cannot contact the sucrose prior to the foodstuff being consumed; by physically separating the part of the foodstuff comprising the enzyme from the part of the foodstuff comprising sucrose, or by formulating the foodstuff to have a condition that is incompatible with significant sucrose conversion prior to the foodstuff being consumed. In some embodiments the foodstuff is formulated to have a low moisture (e.g. water) content, e.g. less than about 50% by weight, e.g. less than 40%, less than 30%, less than 20% or less than 10% by weight. Alternatively, the enzyme may be formulated or chosen such that it has low activity outside the body but high activity inside the body, e.g. by selecting or modifying the mutant inulosucrase enzyme to have a pH- or temperature- dependent

activity wherein the active pH or temperature is provided in the body, e.g. in the small intestine, but is not provided by the foodstuff prior to its consumption.

Typically, the food composition or foodstuff comprises only ingredients which are generally recognised as safe (GRAS).

The mutant inulosucrase enzyme is typically stable in the foodstuff under appropriate storage conditions for extended periods of time. For example, the mutant inulosucrase enzyme may be stable for in excess of 1 day, 1 month, 1 year, etc, when stored under appropriate conditions. Suitable conditions for the storage of the foodstuff may comprise temperatures such as -25 to -15 °C, such as -20 to -18 °C (e.g. for foodstuffs such as ice cream, gelato, sorbet, and other foodstuffs that are sold in frozen form); temperatures such as from about 0 to about 10 °C, such as from about 4 to about 7 °C (e.g. for foodstuffs such as yoghurt and chilled desserts that are sold in chilled form); or temperatures such as from about 15 to about 25 °C such as from about 18 to about 20 °C (e.g. for foodstuffs such as chocolate and baked goods e.g. cakes and confectionary that are typically sold at ambient temperature). Suitable conditions for the storage of the foodstuff include under aerobic conditions (e.g. in the presence of air) or anaerobic conditions (e.g. under an inert, e.g. nitrogen environment). Foodstuffs may be provided in the form of a tin, packet, box, pouch or any other suitable container.

The necessary stability of the mutant inulosucrase enzyme can be determined based on its application and the form of the composition in which it is provided and can be controlled using methods known in the art, including the use of high purity reagents and storage under appropriate conditions.

Typically, a foodstuff will contain up to 10 wt% of the mutant inulosucrase enzyme described herein. It may contain up to 5 wt%, up to 4 wt%, up to 3 wt%, up to 2 wt% or up to 1 wt% of the mutant inulosucrase enzyme.

Typically, a foodstuff may contain sufficient of the mutant inulosucrase enzyme to produce from about 1 to about 100 g, such as from about 2 g to about 50 g, e.g. from about 5 g to about 20 g such as about 10 g of inulin within about 0.5 to 5 hours, such as within about 1 to about 3 hours, e.g. within about 2 hours under physiological conditions.

A foodstuff thus may comprise from about 0.1 to about 1000 mg of the mutant inulosucrase enzyme, such as from about 1 to about 100 mg e.g. about 10 to about 50 mg of the mutant inulosucrase enzyme per serving. A foodstuff may comprise from about 1 mg to about 100 mg such as from about 2 mg to about 50 mg e.g. from about 5 mg to about

20 mg such as from about 7 mg to about 15 mg, e.g. about 10 mg of the mutant inulosucrase enzyme per serving.

A foodstuff may contain sufficient of the mutant inulosucrase enzyme to act on from about 1% to about 100% (e.g. % w/w or % w/v) e.g. from about 1% to about 80%, such as from about 5% to about 50%, e.g. from about 10% to about 40%, e.g. from about 20 to about 30% of available sucrose (e.g. of the sucrose molecules in the foodstuff) within about 1 minute to about 1 hour, e.g. within about 10 minutes to about 45 minutes, such as within about 15 minutes to about 30 minutes under physiological conditions. A foodstuff may thus contain sufficient of the mutant inulosucrase enzyme be capable of converting/incorporating from about 1% to about 100% e.g. from about 1% to about 80%, such as from about 5% to about 50%, e.g. from about 10% to about 40%, e.g. from about 20 to about 30% of available fructose (e.g. of available fructose in the foodstuff) into inulin within about 1 minute to about 1 hour, e.g. within about 10 minutes to about 45 minutes, such as within about 15 minutes to about 30 minutes under physiological conditions. In other words a foodstuff as provided herein may contain sufficient of the mutant inulosucrase enzyme to be capable of converting/incorporating from about 1% to about 50% e.g. from about 1% to about 40%, such as from about 5% to about 30%, e.g. from about 10% to about 20%, of the saccharide units present in the available sucrose (e.g. in the sucrose in the foodstuff) into inulin within about 1 minute to about 1 hour, e.g. within about 10 minutes to about 45 minutes, such as within about 15 minutes to about 30 minutes under physiological conditions.

A subject may consume a foodstuff as described herein between about 1 and about 10 times a day, such as between 2 and 5 times a day, e.g. about 3 or 4 times a day.

Typically, a foodstuff is consumed in a non-therapeutic context. Accordingly, provided herein is use of a foodstuff as described herein in a method (e.g. a non-therapeutic method) of reducing fructose uptake; reducing formation of fructose via metabolism of sucrose; reducing glucose uptake and/or reducing formation of glucose via metabolism of sucrose; producing inulin; suppressing appetite; and/or increasing satiety in a subject. Also provided is a method (e.g. a non-therapeutic method) of reducing fructose uptake; reducing formation of fructose via metabolism of sucrose; reducing glucose uptake and/or reducing formation of glucose via metabolism of sucrose; producing inulin; suppressing appetite; and/or increasing satiety in a subject, comprising administering a foodstuff as described herein to the subject. Further provided is a foodstuff as described herein for use in a method (e.g. a non-therapeutic method) of reducing fructose uptake;

reducing formation of fructose via metabolism of sucrose; reducing glucose uptake and/or reducing formation of glucose via metabolism of sucrose; producing inulin; suppressing appetite; and/or increasing satiety in a subject. Still further provided is use of a mutant inulosucrase enzyme as described herein for use in the manufacture of a foodstuff as described herein for the (typically non-therapeutic) reduction of fructose uptake; reduction of formation of fructose via metabolism of sucrose; reduction of glucose uptake and/or reduction of formation of glucose via metabolism of sucrose; production of inulin; suppression of appetite; and/or increase in satiety in a subject. Such methods and uses are described in more detail herein.

As those skilled in the art will appreciate, the mutant inulosucrase enzyme used in the disclosed methods is functional, i.e. it is capable of converting sucrose to inulin. A denatured enzyme is typically not functional. Thus, administration of a foodstuff to a subject wherein the foodstuff has been pretreated with an inulosucrase enzyme or an organism expressing an inulosucrase typically does not correspond to administration of an isolated mutant inulosucrase enzyme to the subject. In many such foodstuffs the enzyme with which the foodstuff has been pretreated is typically denatured or inactivated such that it is not functional, e.g. via heat treatment during cooking processes e.g. baking. This contrasts with embodiments of the present disclosure in which the enzyme is administered in a foodstuff such that it retains enzymatic activity *in vivo*, e.g. in the digestive system e.g. in the small intestine and/or the stomach.

As explained above, the mutant inulosucrase enzyme provided herein typically has improved pH stability. Accordingly, the mutant inulosucrase enzyme provided herein is also useful in treating acidic food and drinks (especially sucrose-containing foods and drinks), such as fruit products, for example fruit juices, purees, smoothies, concentrates, powders, portions (including fruit slices, whole fruit etc), essences, dried fruit, fruit based sodas, jams, jellies, etc. Typical fruits for treating using the mutant inulosucrase enzyme provided herein include lemons, limes, grapefruits, tangerines, oranges, apples, grapes, peaches, pomegranates, blueberries, pineapples and tomatoes. Other acidic food products include sodas (carbonated sweetened beverages) and alcoholic drinks (including wine, beer, cider, perry, spirits and liqueurs).

Accordingly, provided herein is the use of a mutant inulosucrase enzyme provided herein to convert sucrose to fructooligosaccharide (e.g. inulin) in a food or drink product (e.g. an acidic food or drink product as provided herein). Also provided is a food or drink

product (e.g. an acidic food or drink product as provided herein) comprising a mutant inulosucrase enzyme.

Uses

As described in more detail herein, the mutant inulosucrase enzyme provided herein and compositions comprising it (such as neutraceutical compositions, food compositions and/or pharmaceutical compositions comprising the mutant inulosucrase enzyme provided herein) are capable of converting sucrose to one or more fructooligosaccharides such as inulin. Herein, unless implied otherwise by the context, the term "inulin" embraces fructooligosaccharides with DP > 2. Accordingly in this section the term inulin and fructooligosaccharide can typically be used interchangeably, unless implied otherwise by the context.

Accordingly, various uses, methods and applications of the mutant inulosucrase enzyme and compositions comprising the mutant inulosucrase enzyme are provided. Unless implied otherwise by the context, such methods, uses and applications may be therapeutic or non-therapeutic as described herein. Thus in some embodiments the methods provided herein are non-therapeutic methods which do not comprise treatment of the human or animal body by therapy or surgery.

Provided herein therefore is a method comprising the administration of a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) to a subject. Also provided is a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) for use in *in vivo* administration to a subject.

In some embodiments provided herein is a method of reducing fructose and/or glucose uptake in a subject, comprising administering to the subject a mutant inulosucrase enzyme as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) and thereby converting sucrose to fructooligosaccharide (e.g. inulin) *in vivo*. In some embodiments the method is

an *in vivo* method. Such a method may be therapeutic or non-therapeutic as described herein. Such a method may be a non-therapeutic method which does not comprise treatment of the human or animal body by therapy or surgery. The methods typically comprise administering to the subject the mutant inulosucrase enzyme as an isolated enzyme and thereby converting sucrose to fructooligosaccharide (e.g. inulin) *in vivo*.

Also therefore provided herein is a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) for use in reducing fructose and/or glucose uptake in a subject, wherein said use comprises administering said mutant inulosucrase enzyme or composition comprising said mutant inulosucrase enzyme to said subject and thereby converting sucrose to fructooligosaccharide (e.g. inulin) *in vivo*.

Also provided is the use of a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) in the manufacture of an agent for reducing fructose and/or glucose uptake in a subject.

Typically the provided methods and uses are for reducing fructose uptake in a subject. The reduction in fructose uptake typically comprises production of fructooligosaccharide (e.g. inulin).

Accordingly, in some embodiments provided herein is a method of reducing fructose uptake in a subject, comprising administering to the subject a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) and thereby converting sucrose to inulin *in vivo*. In some embodiments fructose uptake is reduced by reducing the formation of fructose via metabolism of sucrose in a subject and producing inulin *in vivo*. Thus, in some embodiments the provided methods and uses comprise reducing the formation of fructose via metabolism of sucrose in a subject and producing inulin *in vivo* by administering to the subject a mutant inulosucrase enzyme as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein and thereby converting sucrose to inulin *in vivo*.

Such methods may be therapeutic or non-therapeutic as described herein. Such a method may be a non-therapeutic method which does not comprise treatment of the human or animal body by therapy or surgery.

Also provided herein is a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) for use in reducing fructose uptake in a subject, wherein said use comprises administering said mutant inulosucrase enzyme or composition comprising said mutant inulosucrase enzyme to said subject and thereby converting sucrose to inulin *in vivo*. The mutant inulosucrase enzyme or composition comprising the mutant inulosucrase enzyme may be for use in reducing fructose uptake and producing inulin *in vivo*. The mutant inulosucrase enzyme or composition comprising the mutant inulosucrase enzyme may be for use in reducing the formation of fructose via metabolism of sucrose in a subject and producing inulin *in vivo*.

Also provided is the use of a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) in the manufacture of an agent for reducing fructose uptake in a subject. The agent may be for reducing fructose uptake and producing inulin *in vivo*. The agent may be for reducing the formation of fructose via metabolism of sucrose in a subject and producing inulin *in vivo*.

The methods also typically involve reducing glucose production, although often at a lower level than the reduction of fructose production. For example, the initial monomer in inulin is typically glucose and thus free glucose levels are reduced by production of fructooligosaccharide (e.g. inulin).

Accordingly, in some embodiments provided herein is a method of reducing glucose uptake in a subject, comprising administering to the subject a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) and thereby converting sucrose to inulin *in vivo*. In some embodiments glucose uptake is reduced by reducing the formation of glucose via metabolism of sucrose in a subject and producing inulin *in vivo*. Thus, in some embodiments the provided

methods and uses comprise reducing the formation of glucose via metabolism of sucrose in a subject and producing inulin *in vivo* by administering to the subject a mutant inulosucrase enzyme as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein and thereby converting sucrose to inulin *in vivo*. Such methods may be therapeutic or non-therapeutic as described herein. Such a method may be a non-therapeutic method which does not comprise treatment of the human or animal body by therapy or surgery.

Also provided herein is a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) for use in reducing glucose uptake in a subject, wherein said use comprises administering said mutant inulosucrase enzyme or composition comprising said mutant inulosucrase enzyme to said subject and thereby converting sucrose to inulin *in vivo*. The mutant inulosucrase enzyme or composition comprising the mutant inulosucrase enzyme may be for use in reducing glucose uptake and producing inulin in vivo. The mutant inulosucrase enzyme or composition comprising the mutant inulosucrase enzyme may be for use in reducing the formation of glucose via metabolism of sucrose in a subject and producing inulin *in vivo*.

Also provided is the use of a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) in the manufacture of an agent for reducing glucose uptake in a subject. The agent may be for reducing glucose uptake and producing inulin *in vivo*. The agent may be for reducing the formation of glucose via metabolism of sucrose in a subject and producing inulin *in vivo*.

The beneficial properties of fructooligosaccharide (e.g. inulin) production using the disclosed mutant inulosucrase enzymes are described in more detail herein.

Accordingly, in some embodiments provided herein is an *in vivo* method of producing inulin *in vivo*, comprising administering to the subject a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein), and thereby converting sucrose to inulin *in vivo*. In some embodiments

inulin is produced from fructose and/or glucose monomers produced by sucrose metabolism *in vivo*. In some embodiments the production of inulin comprises reduction of fructose and/or glucose uptake by the subject. Also provided is a method of converting sucrose to inulin in a subject. Such methods may be therapeutic or non-therapeutic as described herein. Such a method may be a non-therapeutic method which does not comprise treatment of the human or animal body by therapy or surgery.

Also provided is a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) for use in producing inulin *in vivo* in a subject, wherein said use comprises administering said mutant inulosucrase enzyme or composition to the subject and thereby converting sucrose to inulin *in vivo*. In some embodiments the inulin is produced from fructose and/or glucose monomers produced by sucrose metabolism *in vivo*. In some embodiments the production of inulin comprises reduction of fructose and/or glucose uptake by the subject. Also provided is a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) for use in converting sucrose to inulin *in vivo*.

Also provided is use of a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) in the manufacture of an agent for producing inulin *in vivo*. In some embodiments the inulin is produced from fructose and/or glucose monomers produced by sucrose metabolism *in vivo*. In some embodiments the production of inulin comprises reduction of fructose and/or glucose uptake by the subject. In some embodiments the agent is for converting sucrose to inulin *in vivo*.

In the methods and uses provided herein, the mutant inulosucrase enzyme or composition is typically orally administered to the subject. Usually the mutant inulosucrase enzyme is administered (e.g. orally administered) to the subject as an isolated enzyme as described in more detail herein.

As will be apparent from the above discussion, the mutant inulosucrase enzyme provided herein (and compositions comprising the mutant inulosucrase enzyme) are useful in reducing fructose uptake and metabolism of sucrose to form glucose and fructose. As such, they can be used in controlling the energy taken up by a subject following consumption of food such as sugar.

In many embodiments described herein the mutant inulosucrase enzyme and compositions comprising the mutant inulosucrase enzyme are for use in an *in vivo* method of reducing fructose and/or glucose uptake in a subject. Typically such methods are non-therapeutic methods, which typically do not comprise treatment of the human or animal body by surgery or therapy.

However, the mutant inulosucrase enzyme provided herein and compositions comprising it also have other therapeutic and non-therapeutic uses. In some embodiments provided herein is a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) as provided herein or a composition comprising such a mutant inulosucrase enzyme as provided herein (such as a food composition or foodstuff provided herein, or a nutraceutical or pharmaceutical composition provided herein) for use in medicine.

In one aspect, administration of mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) can be used to suppress a subject's appetite and/or increase satiety. Exogenous inulin has previously been shown to have beneficial effects on weight management through appetite control (e.g. see Guess et al, Nutrition & Metabolism 12 36 (2015) accessible at https://doi.org/10.1186/s12986-015-0033-2). The inventors have recognised that similar beneficial effects will arise from the production of inulin *in vivo* in accordance with the methods provided herein. Without being bound by theory, one mechanism proposed for the suppression of appetite is the fructooligosaccharides-stimulated production of peptide YY. Peptide YY is also known as peptide tyrosine tyrosine, and is a short (36-amino acid) peptide released from cells in the ileum and colon in response to feeding. In the blood, gut, and other elements of periphery, PYY acts to reduce appetite; similarly, when injected directly into the central nervous system, PYY is also anorexigenic. (Woods S. C.; D'Alessio D. A. (2008). "Central control of body weight and appetite". J Clin Endocrinol Metab. 93 (11 Suppl 1): S37–50.)

Accordingly, provided herein is a method of suppressing a subject's appetite, comprising administering to the subject a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) or a composition comprising a mutant inulosucrase enzyme as provided herein. Also provided is a method of increasing a subject's satiety, comprising

administering to the subject a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) or a composition comprising a mutant inulosucrase enzyme as provided herein. Typically such methods are non-therapeutic methods. Also provided is a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) or a composition comprising a mutant inulosucrase enzyme as provided herein for use in suppressing a subject's appetite. A mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) or a composition comprising a mutant inulosucrase enzyme as provided herein for use in increasing a subject's satiety is also provided. Further provided is the use of a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) or a composition comprising a mutant inulosucrase enzyme as provided herein in the manufacture of an agent for suppressing a subject's appetite. The use of a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) or a composition comprising a mutant inulosucrase enzyme as provided herein in the manufacture of an agent for increasing a subject's satiety is also provided.

The mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) or a composition comprising a mutant inulosucrase enzyme as provided herein may be administered to a subject for cosmetic purposes. Such purposes may comprise the non-therapeutic administration of the mutant inulosucrase enzyme or composition to a subject desiring the improvement of their body appearance. For example, in one embodiment provided herein is a method (e.g. a non-therapeutic and/or cosmetic method) of improving the bodily appearance of a subject comprising orally administering to the subject a mutant inulosucrase enzyme (e.g. an isolated mutant inulosucrase enzyme) or a composition comprising a mutant inulosucrase enzyme as provided herein in such an amount to decrease the appetite and/or increase the satiety of the subject, and repeating said administration until a cosmetically-desirable loss of body weight has occurred. The composition used in such methods and uses may be a nutraceutical or pharmaceutical composition or a foodstuff as described herein. Typically, the mutant inulosucrase enzyme is administered as an isolated enzyme. Typically, the isolated fructosyltransferase or composition is administered to the subject orally.

In another aspect, administration of a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) can be used to treat or prevent metabolic syndrome. Metabolic syndrome is a clustering of at least three of the following five medical conditions: abdominal obesity, high blood pressure, high blood sugar, high serum triglycerides, and low serum high-density lipoprotein (HDL). Metabolic syndrome is

associated with the risk of developing cardiovascular disease and type 2 diabetes. Metabolic syndrome can be diagnosed by the presence of any one of diabetes mellitus, impaired glucose tolerance, impaired fasting glucose or insulin resistance, AND two of the following:

- Blood pressure ≥ 140/90 mmHg
- Dyslipidemia: triglycerides (TG) ≥ 1.695 mmol/L and HDL cholesterol ≤ 0.9 mmol/L (male), ≤ 1.0 mmol/L (female)
- Central obesity: waist:hip ratio > 0.90 (male); > 0.85 (female), or BMI > 30 kg/m^2
- Microalbuminuria: urinary albumin excretion ratio ≥ 20 µg/min or albumin:creatinine ratio ≥ 30 mg/g.

Excess sucrose consumption and metabolism has been associated with metabolic syndrome (e.g. see Malik et al, Diabetes Care 2010 33(11) 2477-2483). Without being bound by theory, it is believed that by reducing the concentration of sucrose available for metabolism, metabolic syndrome can be addressed by administration of isolated fructosyltransferase in accordance with the methods provided herein.

Accordingly, provided herein is a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme), for use in treating or preventing metabolic syndrome in a subject in need thereof. Also provided is a method of treating or preventing metabolic syndrome in a subject in need thereof, the method comprising administering a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) to the subject. Further provided is the use of a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) in the manufacture of a medicament for treating metabolic syndrome in a subject. The composition used in such methods and uses may be a nutraceutical or pharmaceutical composition described herein. Typically, the mutant inulosucrase enzyme is administered as an isolated enzyme. Typically, the mutant inulosucrase enzyme is administered to the subject orally.

When applied in non-therapeutic methods and uses, the fructosyltransferase may be administered to a subject who is not suffering from and/or is not at risk of suffering from metabolic syndrome (e.g. is not suffering from and/or is not at risk of suffering from abdominal obesity, high blood pressure (e.g. ≥ 140/90 mmHg), high blood sugar, high serum triglycerides (e.g. ≥ 1.695 mmol/L), low serum high-density lipoprotein (HDL) (e.g. ≤ 0.9 mmol/L (male), ≤ 1.0 mmol/L (female)), cardiovascular disease, type 2 diabetes, diabetes mellitus, impaired glucose tolerance, impaired fasting glucose or insulin

resistance, elevated blood pressure, dyslipidemia; central obesity (e.g. waist:hip ratio > 0.90 (male); > 0.85 (female), or BMI > 30 kg/m$^{2)}$ and/or microalbuminuria (e.g. urinary albumin excretion ratio ≥ 20 µg/min or albumin:creatinine ratio ≥ 30 mg/g)).

Provided herein is a method of maintaining the health of a healthy subject, comprising administering to the subject an isolated fructosyltransferase, optionally in the form of a composition as described herein. Also provided is the use of an isolated fructosyltransferase, optionally in the form of a composition described herein, for maintaining the health of a healthy individual.

As described above, fermentation of fructooligosaccharides such as inulin is selective and fructooligosaccharides can be metabolised by genera associated with gut health including *lactobacilli*, *bifidobacteria* and *fusobacteria*, leading to their beneficial proliferation. Accordingly, also provided herein is a method of improving a subject's microbiome, comprising administering to the subject a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) as provided herein. Also provided herein is a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) for use in improving a subject's microbiome. The improvement of the microbiome may comprise promoting the proliferation of bacteria such as *lactobacilli*, *bifidobacteria* and *fusobacteria*.

Excess sucrose consumption and metabolism has also been associated directly with obesity. A subject may be considered obese if they have a body mass index (BMI) (defined by dividing the subject's weight by the square of their height) in excess of 30 kg/m^2. A subject may be considered overweight if they have a BMI of between about 25 and 30 kg/m^2. Without being bound by theory, it is believed that by reducing the concentration of sucrose available for metabolism, obesity can be addressed by administration of a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) in accordance with the methods provided herein. As used herein, addressing or treating obesity may include addressing or treating a subject who has a BMI of in excess of 30 kg.m^2 or who has a BMI of between 25 and 30 kg/m^2.

Accordingly, provided herein is a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme), for use in treating or preventing obesity in a subject in need thereof. Also provided is a method of treating or preventing obesity in a subject in need thereof, the method comprising administering a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) to the subject. Further provided is the use of a mutant inulosucrase enzyme (or composition comprising a mutant

inulosucrase enzyme), in the manufacture of a medicament for treating obesity in a subject. The composition used in such methods and uses may be a nutraceutical or pharmaceutical composition described herein. Typically, the mutant inulosucrase enzyme is administered as an isolated enzyme. Typically, the isolated fructosyltransferase or composition is administered to the subject orally.

When applied in non-therapeutic methods and uses, the mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) may be administered to a subject who is not overweight and/or is not obese. For example, the fructosyltransferase may be administered in the non-therapeutic methods and uses provided herein to a subject with a BMI of less than about 30 kg/m^2, e.g. less than about 25 kg/m^2.

Diabetes is a further disorder associated with excess sucrose levels *in vivo*. Diabetes is commonly linked with insulin deficiency. Type 1 diabetes results from reduced insulin production by the pancreas due to loss of beta cells caused by autoimmune responses. Type 2 diabetes arises from insulin resistance. Gestational diabetes is a further form of diabetes. Without being bound by theory, it is believed that administering a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) in accordance with the methods provided herein can reduce sucrose levels *in vivo* and thus have beneficial effects in treating or preventing diabetes. Administering an isolated fructosyltransferase in accordance with the methods provided herein can also beneficially reduce glucose levels *in vivo* as described herein.

Accordingly, provided herein is a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme), for use in treating or preventing diabetes in a subject in need thereof. Also provided is a method of treating or preventing diabetes in a subject in need thereof, the method comprising administering a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) to the subject. Further provided is the use of a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme), in the manufacture of a medicament for treating diabetes in a subject. Often, the diabetes is type 2 diabetes. The composition used in such methods and uses may be a nutraceutical or pharmaceutical composition described herein. Typically, the mutant inulosucrase enzyme is administered as an isolated enzyme. Typically, the isolated fructosyltransferase or composition is administered to the subject orally.

When applied in non-therapeutic methods and uses, the mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) may be administered to a subject who is not suffering from and/or is not at risk of suffering from diabetes. The

mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) may be administered to a subject having a fasting blood glucose level of from about 4 mM to about 5.5 mM or about 6 mM and/or a post-prandial (e.g. 90 minutes post-prandial) blood glucose level of under about 7.8 mM. The mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) may not, in some embodiments, be administered to a subject with a fasting blood glucose level of 4-7 mM, e.g. more than about 6 mM, and/or a post-prandial (e.g. 90 minutes post-prandial) blood glucose level of more than 7.8 mM.

Still a further condition associated with excess sucrose levels *in vivo* is non-alcoholic fatty liver disease. High fructose levels from sucrose consumption promotes fat accumulation in the liver by stimulating de novo lipogenesis in the liver and reducing the beta-oxidation of fat. In addition, fructokinases rapidly metabolize fructose leading to decreased intracellular ATP levels in the liver, which may increase oxidative stress impairing protein synthesis and mitochondrial liver function. Administering a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) in accordance with the disclosed methods reduces fructose levels taken up by the body and thus can have beneficial effects in treating or preventing non-alcoholic fatty liver disease.

Accordingly, provided herein is a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme), for use in treating or preventing non-alcoholic fatty liver disease in a subject in need thereof. Also provided is a method of treating or preventing non-alcoholic fatty liver disease in a subject in need thereof, the method comprising administering a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) to the subject. Further provided is the use of a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) in the manufacture of a medicament for treating non-alcoholic fatty liver disease in a subject. The composition used in such methods and uses may be a nutraceutical or pharmaceutical composition described herein. Typically, the mutant inulosucrase enzyme is administered as an isolated enzyme. Typically, the isolated fructosyltransferase or composition is administered to the subject orally.

When applied in non-therapeutic methods and uses, the mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) may be administered to a subject who is not suffering from and/or is not at risk of suffering from non-alcoholic fatty liver disease.

Yet another condition amenable to treatment using a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) is constipation. Constipation is among the most common health impediments especially in elderly populations. Inulin is non-digestible by humans and its fermentation in the colon can lead to increased bacterial cell mass and a higher water content of digesta, which aids bowel function. Accordingly, administering a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) in accordance with the disclosed methods promotes inulin production and can thus have beneficial effects in treating or preventing constipation.

Accordingly, provided herein is a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme), for use in treating or preventing constipation in a subject in need thereof. Also provided is a method of treating or preventing constipation in a subject in need thereof, the method comprising administering a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) to the subject. Further provided is the use of a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme) in the manufacture of a medicament for treating constipation in a subject. The composition used in such methods and uses may be a nutraceutical or pharmaceutical composition described herein. Typically, the mutant inulosucrase enzyme is administered as an isolated enzyme. Typically, the isolated fructosyltransferase or composition is administered to the subject orally.

When applied in non-therapeutic methods and uses, the fructosyltransferase may be administered to a subject who is not suffering from and/or is not at risk of suffering from constipation.

The methods and uses provided herein (particularly the therapeutic methods and uses described herein) may comprise administering the mutant inulosucrase enzyme (or composition comprising the a mutant inulosucrase enzyme) together with one or more additional therapies or compositions. For example, the mutant inulosucrase enzyme or composition may be administered together with conventional therapies for treating obesity. Such agents include orlistat, lorcaserin, liraglutide, phentermine–topiramate, metformin and naltrexone–bupropion. Where separately formulated, the two agents may be administered simultaneously or separately. They may be provided in the form of a kit, optionally together with instructions for their administration.

Alternatively or additionally, the mutant inulosucrase enzyme or compositions provided herein may be administered to a subject who is or has been also treated surgically, e.g. via gastric banding. For example, the subject may have received

laparoscopic adjustable gastric banding, Roux-en-Y gastric bypass, vertical-sleeve gastrectomy, or biliopancreatic diversion.

As described herein, a mutant inulosucrase enzyme (or composition comprising a mutant inulosucrase enzyme), can be administered to any suitable subject.

In one aspect, the subject is a mammal, in particular a human. However, it may be non-human. Preferred non-human animals include, but are not limited to, primates, such as marmosets or monkeys, commercially farmed animals, such as horses, cows, sheep or pigs, and pets, such as dogs, cats, mice, rats, guinea pigs, ferrets, gerbils or hamsters.

A subject may be overweight or obese. For example, a human subject may have a BMI of in excess of 25 kg/m^2; in excess of 30 kg/m^2; or in excess of 35 kg/m^2. A subject may be male or female. A subject may be aged from about 10 to about 80, such as from about 16 or about 18 to about 65; such as from about 20 to about 60, e.g. from about 25 to about 55, such as from about 30 to about 50. A subject may be of any racial or genetic background.

An agent described herein can be administered to the subject in order to prevent the onset or reoccurrence of one or more pathological symptoms, e.g. symptoms of obesity or metabolic syndrome. This is prophylaxis. In this embodiment, the subject can be asymptomatic. The subject is typically one that is at risk of obesity or metabolic syndrome. A prophylactically effective amount of the agent or formulation is administered to such a subject. A prophylactically effective amount is an amount which prevents the onset of one or more symptoms of obesity or metabolic syndrome..

An agent described herein can be administered to the subject in order to treat one or more pathological symptoms, e.g. symptoms or obesity or metabolic syndrome. In this embodiment, the subject is typically symptomatic. A therapeutically effective amount of the agent or formulation is administered to such a subject. A therapeutically effective amount is an amount effective to ameliorate one or more symptoms of the disorder.

The agent (i.e. a mutant inulosucrase enzyme or composition comprising a mutant inulosucrase enzyme) may be administered in a variety of dosage forms. Usually, it is administered orally, for example as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules. Such formulations are described in more detail herein.

However, for some applications the agent may also be administered parenterally, whether subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. Solutions for inhalation, injection or infusion may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous,

isotonic saline solutions. Pharmaceutical compositions suitable for delivery by needleless injection, for example, transdermally, may also be used.

The agent may also be administered as a suppository.

The agent may in some circumstances be administered via inhalation. The agent may be formulated for inhaled (aerosolised) administration as a solution or suspension. The compound, composition or combination of the invention may be administered by a metered dose inhaler (MDI) or a nebulizer such as an electronic or jet nebulizer. Alternatively, the compound, composition or combination of the invention may be formulated for inhaled administration as a powdered drug, such formulations may be administered from a dry powder inhaler (DPI). When formulated for inhaled administration, the compound, composition or combination of the invention may be delivered in the form of particles which have a mass median aerodynamic diameter (MMAD) of from 1 to 100 μm, preferably from 1 to 50 μm, more preferably from 1 to 20 μm such as from 3 to 10 μm, e.g. from 4 to 6 μm. When the compound, composition or combination of the invention is delivered as a nebulized aerosol, the reference to particle diameters defines the MMAD of the droplets of the aerosol. The MMAD can be measured by any suitable technique such as laser diffraction.

In use, a therapeutically or prophylactically effective amount of the agent is administered to a subject. The dose may be determined according to various parameters, especially according to the agent used; the age, weight and condition of the subject to be treated; the route of administration; and the required regimen. A physician or dietician will be able to determine the required route of administration and dosage for any particular subject. A typical daily dose is from about 0.01 to 100 mg per kg, preferably from about 0.1 mg/kg to 50 mg/kg, e.g. from about 1 to 10 mg/kg of body weight, according to the activity of the specific agent or inhibitor, the age, weight and conditions of the subject to be treated, the type and severity of the disease and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

In both therapeutic and non-therapeutic methods and uses, the amount of the agent to be administered is sufficient to convert a physiologically useful amount of sucrose to inulin. For example, although the volume of the small intestine varies considerably between subjects, a typical volume is in the region of 150 to 250 mL, such as around 180 mL. Sufficient agent may be administered to result in a small intestinal concentration of around 10-100 μg/mL such as from about 20 to about 70 μg/mL e.g. about 50 μg/mL. For

example, a dose of from about 1 mg to about 100 mg such as from about 2 mg to about 50 mg e.g. from about 5 mg to about 20 mg such as about 10 mg may be administered.

Further aspects

In another aspect, also provided herein is a polynucleotide which encodes a mutant inulosucrase enzyme as provided herein. The polynucleotide is typically a DNA or an RNA polynucleotide, most typically a DNA polynucleotide.

Polynucleotide sequences may be derived and replicated using standard methods in the art. Chromosomal DNA encoding the wild-type protein of SEQ ID NO: 1 may be extracted from a host organism such as *Lactobacillus gasseri* DSM 20604. The gene encoding the protein may be amplified using PCR involving specific primers. The amplified sequence may then undergo site-directed mutagenesis. Suitable methods of site-directed mutagenesis are known in the art and include, for example, combine chain reaction. Polynucleotides encoding a construct of the invention can be made using well-known techniques, such as those described in Sambrook, J. and Russell, D. (2001). Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

The resulting polynucleotide sequence may then be incorporated into a recombinant replicable vector such as a cloning vector. The vector may be used to replicate the polynucleotide in a compatible host cell. Thus polynucleotide sequences may be made by introducing a polynucleotide into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells for cloning of polynucleotides are known in the art.

The polynucleotide sequence may be cloned into a suitable expression vector. Accordingly, also provided herein is a vector comprising the polynucleotide.

In an expression vector, the polynucleotide sequence is typically operably linked to a control sequence which is capable of providing for the expression of the coding sequence by the host cell. Such expression vectors can be used to express a pore subunit.

The term “operably linked” refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence “operably linked” to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the

control sequences. Multiple copies of the same or different polynucleotide sequences may be introduced into the vector.

The vectors may be, for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide sequence and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example a tetracycline resistance gene. Promoters and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. A T7, *trc, lac*, *ara* or λ_L promoter is typically used.

The expression vector may then be introduced into a suitable host cell. Thus, a mutant inulosucrase enzyme as provided herein can be produced by inserting a polynucleotide sequence into an expression vector, introducing the vector into a compatible host cell (e.g. a bacterial host cell), and growing the host cell under conditions which bring about expression of the polynucleotide sequence. The host cell typically expresses the pore subunit at a high level. Host cells transformed with a polynucleotide sequence will be chosen to be compatible with the expression vector used to transform the cell. The host cell is typically bacterial and preferably *Escherichia coli.* Any cell with a λ DE3 lysogen, for example C41 (DE3), BL21 (DE3), JM109 (DE3), B834 (DE3), TUNER, Origami and Origami B, can express a vector comprising the T7 promoter.

Accordingly, also provided herein is a cell comprising a vector as provided herein. In some embodiments the cell is a bacterial cell, a yeast cell, or a fungal cell.

Also provided is a method of producing a mutant inulosucrase enzyme as provided herein, comprising expressing said mutant inulosucrase enzyme from a cell as provided herein, and optionally isolating said mutant inulosucrase enzyme from said cell. In some embodiments the expression is intracellular expression. In some embodiments the expression is extracellular expression.

It is to be understood that although particular embodiments, specific configurations as well as materials and/or molecules, have been discussed herein for methods according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention. The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting the application. In particular, there are many assays for assessing formation of

fructooligosacharides, activity of enzymes, etc, and so a negative result in any specific assay is not determinative.

EXAMPLES

Example 1

This example describes the preparation of inulosucrase variants.

Construction of inulosucrase variant library

A library of DNA encoding variants of the inulosucrase having an amino acid sequence of SEQ ID NO. 1 was synthesised by methods known in the art. For each amino acid position in SEQ ID NO. 1, five separate variants were synthesised to represent a single amino acid substitution to alanine (A), aspartic acid (D), isoleucine (I), arginine (R) or tyrosine (Y).

The DNA constructs were cloned into an *Escherichia coli* expression vector (pAVE18) containing a N-terminal His-tag and C-terminal GFP11-tag (6xHis-inulosucrase-GFP11), and used to transform *E. coli* BL21 *lacIq* using standard techniques. A representative construct is shown in SEQ ID NO. 2. Next generation sequencing techniques known in the art were used to confirm that 96% of the intended variants were represented in the library.

Preparation of inulosucrase soluble lysate

A preculture of each variant was grown by inoculating single colonies of *E. coli* BL21 *lacIq* transformed with pAVE18 encoding inulosucrase variants into LB broth in a 96-well F-bottom microplate (MTP) and incubating for 16 h at 28 °C and 900 rpm. 5 µl of preculture was inoculated into 150 µl of autoinduction medium (ZYM-5052, Studier, *Protein production by auto-induction in high density shaking cultures*, Protein Expr. Purif. 41(1):207-34, 2005) containing 50 µg/mL kanamycin. The culture was grown for 26 h at 28 °C and 900 rpm. Cells were harvested by centrifugation for 15 min at 4,500 rpm. The supernatant was discarded and cell pellets were stored at -20 °C until further processing.

Cell pellets in the 96-well MTP were resuspended in 100 µL 10 mM potassium phosphate pH 7.0 containing 1 mg/mL lysozyme and incubated for 1 h at 37 °C and 900 rpm in a microplate shaker. The soluble lysate fraction (SLF) was separated from the

insoluble components by centrifugation for 30 min at 4,500 rpm and 4 °C. SLF was stored on ice until use.

Example 2

This example describes the screening of inulosucrase variants for improved activity in gastric conditions.

Inulosucrase activity test in simulated gastric conditions

The activity of the wild-type inulosucrase (SEQ ID NO. 1) and the library of variants of SEQ ID NO. 1 were tested in simulated gastric conditions (Brodkorb, A., Egger, L., Alminger, M. et al. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat Protoc **14**, 991–1014, 2019) with 125 mM sucrose and 1 mg/mL pepsin (P7012, Merck, ≥ 2,500 units/mg) at pH 3.2 and 37 °C. Each inulosucrase variant in the library was tested in triplicate and compared to the wild-type.

Soluble lysate fraction (SLF) was diluted 1:2 in 10 mM potassium phosphate pH 7.0. A 500 mM sucrose stock solution was prepared in 50 mM potassium phosphate pH 7.0 with 2.4 mM $CaCl_2$. A 1.6 mg/mL pepsin (P7012, Merck, ≥ 2,500 units/mg) stock solution was prepared in 80 mM sodium phosphate at pH 3.0 immediately before the assay. 50 µL of sucrose solution was mixed with 100 µL of pepsin solution in a 96-well F-bottom microtiter plate (MTP) and pre-warmed at 37 °C. To initiate the reaction, 25 µL of diluted SLF was added to the pre-warmed sucrose and pepsin solution. In the final reaction composition the pH was 3.2. Incubation was continued at 37 °C for 10 min before stopping the reaction by addition of 10 µL 5 M NaOH. The inactivated reaction was stored at -20 °C until further analysis.

Glucose/fructose quantification and unit definition

The sugar concentrations following the inulosucrase reaction were determined using the K-FRUGL assay kit (Megazyme, Ireland) and performed according to manufacturer protocol (chapter "E. Microplate assay procedure; D-Glucose and D-Fructose (Sequential)) in F-bottom MTPs at 20 °C with minor modifications.

The measurement is based on NADPH formation, measured by absorbance at 340 nm. The amount of NADPH formed is in stoichiometric proportion with the amount of D-glucose (1st reaction, A2-A1) and the total amount of D-glucose + D-fructose (2nd reaction, A3-A2).

Deviating from the supplier`s protocol, solutions were premixed in 3 mastermixes for reduced handling steps:

Mastermix 1 (MM1)	1	x
MilliQ	166	µL
Solution I (Buffer)	8	µL
Solution II ($NADP^+$/ATP)	10	µL

Mastermix 2 (MM2)

Suspension III (HK/G-6-PDH)	2	µL
Solution I (Buffer)	1	µL
Distilled water	17	µL

Mastermix 3 (MM3)

Suspension IV (PGI)	2	µL
Solution I (Buffer)	1	µL
Distilled water	17	µL

Inulosucrase reaction samples were diluted 1:10 in deionised water to sugar concentrations in the linear range of the assay kit (0.01 – 0.8 g/L). 0.2 mg/mL D-glucose and D-fructose solutions were used as standards. 184 µL of MM1 was aliquoted into a 96-well MTP. 10 µL of the provided standards or diluted reaction samples were added to respective wells. A1 (blank) was measured in a Clariostar (BMG Labtech) plate reader at 340 nm. Reaction 1 was started by addition of 20 µL MM2 and samples were incubated in a plate shaker at 600 rpm at 20 °C for 1 h. Absorbance was measured again at 340 nm (A2). Reaction 3 was started with the addition of 20 µL MM3. Samples were incubated in a plate shaker at 600 rpm at 20 °C for 30 min before measuring absorbance at 340 nm (A3).

Glucose and fructose concentrations were calculated according to the supplier's instructions (K-FRUGL, Megazyme, Ireland). One Unit (U) of activity is defined as the amount of enzyme that produces 1 µmol of sugar equivalent per minute under the conditions of the assay. The assay is based on a 10-minute conversion of 125 mM sucrose

in 50 mM potassium phosphate pH 7.0, including 1 mM $CaCl_2$ at 37 °C followed by inactivation by the addition of NaOH.

AU (total Activity Unit) is the amount of enzyme that produces 1 µmol of glucose per minute, indicating the total amount of sucrose converted.

HU (Hydrolytic Unit) is the amount of enzyme that produces 1 µmol of fructose per minute, indicating the amount of hydrolysed sucrose.

TU (Transfructsylation Unit) is obtained by subtracting HU from AU and indicates the amount of enzyme that transfructosylates 1 µmol of fructose per minute.

Preparation of GFP1-10

Split-green fluorescent protein (GFP) is a tool for detecting and analysing protein interactions, as described by Kamiyama *et al.* (Versatile protein tagging in cells with split fluorescent protein; Nature Communications volume 7, 11046 (2016). A small tag (GFP11, 24 amino acids) is derived from the eleventh β-strand of superfolder GFP. Superfolder GFP is reconstituted when GFP11 is in the presence of excess GFP1-1, resulting in detectable fluorescence. Here, the split-GFP system was used to quantify the concentration of inulosucrase variants fused to a C-terminal GFP-11 tag.

GFP1-10 was expressed from *E. coli* BL21 *lacIq* transformed with pAVE18_GFP1-10. Main cultures were inoculated to an initial OD_{600} of 0.05 in 500 mL LB medium with JA 603A antifoam reagent and 50 µg/mL kanamycin in 2.5 L ultra yield shake flasks. Cultures were grown at 37 °C and 250 rpm. When OD_{600} of 0.7 was reached, GFP1-10 expression was induced with 1 mM IPTG and cells were grown for another 24 h at 37 °C and 250 rpm. Cells were harvested by centrifugation at 4 °C in four lots of 450 mL in a Hermle Z447 K centrifuge. The cell pellets were frozen at -20 °C until cell lysis.

For lysis, the pellets were thawed on ice and resuspended in 25 – 40x volume (relative to the pellet weight) of TNG buffer (100 mM Tris, 100 mM NaCl, 10% (v/v) glycerol, pH 7.4). Cells were first incubated with 1 mg/mL lysozyme and 1% (v/v) triton X-100 detergent for 1 h at 37 °C, followed by sonication (Bandelin Sonopuls, TS425 L) for 45 min at 50% amplitude and a duty cycle of 15 s, while stirring on ice.

The lysis solution was centrifuged at 4000 *g* for 315 min at 4 °C. The supernatant was discarded, and the pellet containing the inclusion bodies was resuspended in an equal volume of TNG buffer. Sonication was repeated as above. After the second sonication, the pellet was washed three times in TNG buffer, while reducing the volume 50% per wash, by means of centrifugation at 4000 *g* at 4 °C for 10 min. The protein was denatured by adding

1 ml of denaturing buffer (9 M urea, 5 mM DTT) per 75 mg of inclusion bodies. The GFP1-10 protein was stored at -20 °C until use.

The quality of the refolded GFP1-10 affects the obtainable fluorescence signal intensity. Therefore, each produced batch of GFP1-10 was normalised. The GFP1-10 solution was diluted to in order obtain a signal intensity of 100,000 RFU a scale of 75% and gain 972 at 21 °C in the ClarioStar plate reader (BMG Labtech) with 1 mg/mL of wild-type inulosucrase tagged with GFP11.

Split-GFP assay for normalising enzyme concentration

20 µL of the soluble lysate fraction (SLF) was added to wells of a 96-well MTP. 180 µL of normalised GFP1-10 solution was added to start the reaction. The plates were incubated at 4 °C for 48 h and the signal was measured at an excitation wavelength 472-15 nm and emission wavelength 515-20 nm. The GFP signal was used to normalise observed inulosucrase activity from SLF by enzyme concentration.

Example 3

This example describes measurements of the activity of inulosucrase variants in simulated gastric conditions.

A subset of variants identified in the screen of the inulosucrase library were chosen for further analysis. Each variant was expressed as described in Example 1. Each variant was purified from 1.8 mL of culture using PureCube Ni-NTA MagBeads (55305, Cube Biotech) according to the manufacturer's instructions. 40 µL of bead suspension was used for each variant. Beads with bound inulosucrase variant were washed with 20 mM sodium phosphate pH 7.5, 500 mM NaCl, 20 mM imidazole. Proteins were eluted from beads in 100 µL 20 mM sodium phosphate pH 7.5, 500 mM NaCl, 500 mM imidazole. Eluted proteins were buffer exchanged into 10 mM potassium phosphate pH 7 using a Zeba Spin Desalting plate (89808, Thermo Fisher Scientific).

The enzyme concentration in buffer exchanged fractions was determined using absorbance at 280 nm and a molar extinction coefficient of 122730 $M^{-1}cm^{-1}$. Purified variants were tested for activity in simulated gastric conditions as described in Example 2 with the following modifications. Instead of SLF, 25 µL of 80 ng/µL inulosucrase was added to the pre-warmed reaction solution with sucrose and pepsin. The final concentration of inulosucrase in the reaction was 10 ng/µL. Where the concentration of purified buffer-exchanged inulosucrase variant was less than 80 ng/µL, 25 µL of the highest available

concentration was used and the observed specific activity was adjusted according to the lower enzyme concentration.

Each variant was re-sequenced with Sanger sequencing. The amino acid mutation of each variant was confirmed, with the exception of Q240A which also contained S566Y, and N560A which also contained N560Y.

The variants tested all displayed increased specific total activity, hydrolytic activity and transfructosylation activity compared to the wild-type inulosucrase (see Example 5). The ratio of total activity to hydrolytic or transfructosylation activity was generally not changed by the mutations.

Eight of the variants tested displayed between two and three-fold higher total activity than the wild-type. Seven variants improved the total activity more than 3.5-fold relative to the wild-type inulosucrase: L316I, D57Y, S345A, A71Y, D406I, A309I and K339Y. The activity of L316I and D57Y exceeded the linear range of the assay.

Example 4

This example describes measurements of the pH stability of inulosucrase variants.

The pH resilience of a subset of variants identified in the screen of the inulosucrase library was tested. Enzyme variants were exposed to acidic pH before activity was measured in simulated intestinal conditions, including bile salts and pancreatic proteases (Brodkorb, A., Egger, L., Alminger, M. et al. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat Protoc **14**, 991–1014 (2019)).

3.6 mL of culture of each variant was expressed as described in Example 1 and purified using PureCube Ni-NTA MagBeads as described above. Purified enzymes were eluted in 100 µL 20 mM sodium phosphate pH 7.5, 500 mM NaCl, 500 mM imidazole and buffer exchanged into 7 mM potassium phosphate pH 7.0 using a Zeba Spin Desalting plate (89808, Thermo Fisher Scientific).

Purified enzyme variants were pre-incubated in 10 mM sodium phosphate buffer at pH 2.6, 2.7, 2.8 or 7.0 and 37 °C for 12 min. 30 µL of pre-incubated enzyme variants were diluted by addition to 120 µL SIF- (6.8 mM KCl, 0.8 KH_2PO_4, 123.4 mM NaCl, 0.33 mM $MgCl_2$, 8.4 mM HCl). 30 µL of diluted pre-incubated samples were mixed with 120 µL SIF+ (final concentrations 0.6 mM $CaCl_2$, 10 mM bile salts [B8756, Merck], 125 mM sucrose, 10 mg/mL pancreatin [P7545, Merck]) and incubated for 30 min at 37 °C. The pH of SIF- and SIF+ buffers after mixing with the respective samples was pH 7.0. The reaction was stopped by heating to 95 °C for 10 min. Heat-inactivated reactions were

stored at -20 °C until further analysis. Activity was measured using the K-FRUGL assay kit (Megazyme, Ireland) was described above. The enzyme concentration in the reaction in SIF+ buffer was 3 – 7 ng/µL. Observed activity was normalised to 10 ng/µL.

The variants tested displayed similar or higher total and transfructosylation activity than the wild-type inulosucrase (SEQ ID NO. 1) after pre-incubation at acidic pH (2.6, 2.7 and 2.8) (see Example 5; values reported below are an average of readings at pH 2.6, 2.7 and 2.8). L316I and S345A showed approximately two-fold higher activity after incubation at acidic pH than SEQ ID NO. 1. D406I, M422Y and G172R displayed four to five-fold higher activity than SEQ ID NO. 1 after incubation at acidic pH. The data indicate that the variants displayed similar or improved resilience to low pH. The activity of variants was comparable to that of SEQ ID NO. 1 after incubation at pH 7, indicating that the stability and activity in simulated intestinal conditions was not affected by the respective mutation.

Taken together, the data in Example 3 (gastric activity) and Example 4 (pH resilience, intestinal activity) demonstrate that the variants tested show improved performance in gastric conditions typified by resistance to pepsin digestion and resilience to acid. In addition, the variants do not lose their stability to bile salts and pancreatin (including pancreatic proteases), and retain the transfructosylation to total activity ratio.

Example 5

A summary of some data obtained in the above examples is provided in the following tables 3 and 4, in which:

+ relative activity of >1x and ≤2x that of SEQ ID NO: 1

++ relative activity of between >2x and ≤3.5x that of SEQ ID NO: 1

+++ relative activity of between >3.5x and ≤5x that of SEQ ID NO: 1

++++ relative activity of ≥5x that of SEQ ID NO: 1

nd not determined

Table 3: Relative absolute activity (AU; see definition in Example 2)

Position	Exemplary Variant	Mutant	Relative absolute activity (screen 1; method as Example 2)	Relative absolute activity (screen 2; method as Example 4)
WT PROTEIN OF SEQ ID NO: 1			1	1
K45	Y	K45Y	+	nd
V49	Y	V49Y	++	+
D57	Y	D57Y	++++	+

Position	Exemplary Variant	Mutant	Relative absolute activity (screen 1; method as Example 2)	Relative absolute activity (screen 2; method as Example 4)
A71	Y	A71Y	+++	+
Q134	Y	Q134Y	++	nd
N143	D	N143D	++	nd
G152	A	G152A	++	nd
T159	R	T159R	++	+
G172	R	G172R	nd	+++
N175	D	N175D	+	nd
T201	A	T201A	+	nd
K238	A	K238A	++	nd
Q240	A	Q240A / S566Y	++	nd
H247	Y	H247Y	+	nd
K270	A	K270A	++	nd
T271	D	T271D	+	nd
A309	I	A309I	+++	+
L316	I	L316I	++++	++
S336	A	S336A	++	nd
K339	Y	K339Y	+++	++
K339	R	K339R	nd	++
K343	A	K343A	nd	+
S345	A	S345A	++++	++
D384	A	D384A	+	nd
D406	I	D406I	+++	++++
M422	Y	M422Y	nd	++++
T474	A	T474A	++	nd
V483	R	V483R	+	nd
H489	N	H489N	++	nd
W492	A	W492A	++	nd
V508	I	V508I	+	nd
T513	A	T513A	++	++
D522	P	D522P	++	nd
N560	A, Y	N560A / N560Y	+	nd

Table 4: Relative transfructosylation activity (TU; see definition in Example 2)

Position	Exemplary Variant	Mutant	Relative transfructosylation activity (screen 1; method as Example 2)	Relative transfructosylation activity (screen 2; method as Example 4)
WT PROTEIN OF SEQ ID NO: 1			1	1
V49	Y	V49Y	++	+
D57	Y	D57Y	++++	+
A71	Y	A71Y	+++	+

Position	Exemplary Variant	Mutant	Relative transfructosylation activity (screen 1; method as Example 2)	Relative transfructosylation activity (screen 2; method as Example 4)
Q134	Y	Q134Y	++	nd
N143	D	N143D	++	nd
G152	A	G152A	++	nd
G172	R	G172R	nd	+++
N175	D	N175D	+	nd
T201	A	T201A	+	nd
K238	A	K238A	++	nd
Q240	A	Q240A / S566Y	++	nd
H247	Y	H247Y	+	nd
T271	D	T271D	+	nd
L316	I	L316I	++++	++
S336	A	S336A	++	nd
K339	Y	K339Y	+++	++
K339	R	K339R	nd	++
K343	A	K343A	nd	+
S345	A	S345A	++++	++
D384	A	D384A	+	nd
D406	I	D406I	+++	++++
M422	Y	M422Y	nd	++++
T474	A	T474A	++	nd
V483	R	V483R	+	nd
H489	N	H489N	++	nd
W492	A	W492A	++	nd
N560	A, Y	N560A / N560Y	+	nd

Example 6

Cloning

Individual beneficial mutations identified in Examples 2-5 were rationally combined to generate a further set of variants of inulosucrase SEQ ID NO 1 with two to ten mutations. DNA coding for the genes of the further variants was codon optimised for expression in *Pichia pastoris* and synthesised using methods known in the art. Expression constructs were generated by assembly cloning using overlapping homologous sequences. The homologous sequences to the expression vector were introduced to the genes of the further variants using primers with overhangs coding for the homology region by polymerase chain reaction (PCR). Each expression construct included a carbon-source de-

repressible promoter and signal sequence targeting the protein to be secreted extracellularly. Chemically competent *E. coli* K12 Top10 F' cells were transformed with the assembled expression constructs. Positive transformants were selected on LB agar plates containing 25 mg/L Zeocin or 50 mg/L Kanamycin. Correct assembly of constructs was verified by Sanger sequencing.

Expression constructs were purified from *E. coli* and linearized with the restriction enzyme *Smi*I. The linearised construct was column purified and ~1 µg linearised DNA was electroporated into *P. pastoris* strains. Electrocompentent *P. pastoris* cells were prepared by methods known in the art. Transformants were selected on YPD agar plates supplemented with 100 mg/L Zeocin.

Protein expression

Inulosucrase variants were produced in 96-well deep well plates. 250 µl of buffered minimal glycerol 1% (BMG1, Table 5) was inoculated with a single fresh colony of transformed *P. pastoris*. Each variant was expressed in quadruplicate from different colonies. Cells were grown for 60 h at 28 °C, 320 RPM and 80% humidity. Cells were further induced by addition of 50 µl BMG2.5 (Table 5) 68, 84 and 92 h after inoculation. The inulosucrase-containing supernatant was harvested 108 h after inoculation by centrifugation at 4,000 RPM and 4 °C for 10 min. The supernatant from different clones of the same variant was pooled and frozen until further analysis.

Table 5: BMG recipe

Volume (mL)	Component
200	1M sodium phosphate pH 6.0
100	13.4% yeast nitrogen base with ammonium sulphate and without amino acids
2	0.2 mg/L biotin
100 (BMG1) 250 (BMG2.5)	10% glycerol
to 1 L	Autoclaved deionised water

Gastric activity

The activity of the wild type inulosucrase of SEQ ID NO 1 and the further variants was compared at pH 7 and at pH 2.6 in the presence of 1 mg/mL pepsin. 25 µl of undiluted inulosucrase-containing supernatant was mixed with 175 µl phosphate buffer at pH 2.6 or pH 7. The final reaction included 1 mg/mL pepsin (P7012, Merck, ≥2,500 units/mg) 125 mM sucrose and 0.6 mM $CaCl_2$. Reactions were incubated for 30 min at 37 °C before being stopped by addition of 10 µl 5 M NaOH. The glucose and fructose concentrations were measured using the K-FRUGL kit (Megazyme, Ireland) as described in Example 2. The assay was performed with three technical replicates.

The total and hydrolytic activity between neutral and simulated gastric conditions was compared by dividing the observed activity at pH 2.6 by the observed activity at pH 7. Relative to the wild type protein, the variants had increased relative activity at pH 2.6 indicative of their improved activity and stability in gastric conditions. The wild-type inulosucrase (SEQ ID NO 1) had ~31% total activity at pH 2.6 compared to pH 7, while some variants had similar activity in both conditions.

Results are shown in the following table 6 which compares the relative activity (relative total activity and relative hydrolytic activity) for each mutant at (i) pH 2.6 + 1 mg/mL pepsin; compared to (ii) pH 7;

in which:

+ relative activity at pH 2.6 + 1 mg/mL pepsin compared to activity at pH 7 is >32% (i.e. greater than WT protein) and <50%

++ relative activity at pH 2.6 + 1 mg/mL pepsin compared to activity at pH 7 is ≥50% and <75%

+++ relative activity at pH 2.6 + 1 mg/mL pepsin compared to activity at pH 7 is ≥75% and <100%

++++ relative activity at pH 2.6 + 1 mg/mL pepsin compared to activity at pH 7 is ≥100%

Table 6: Relative activity of mutants

Example	Mutant	Relative total activity	Relative hydrolytic activity
	WT PROTEIN OF SEQ ID NO: 1	~31%	~32%
6-01	V49Y, A309I	++	++
6-02	L316I, M422Y	++	+++
6-03	S345A, M422Y	++	+++

Example	Mutant	Relative total activity	Relative hydrolytic activity
6-04	G172R, L316I	++	++
6-05	G172R, D406I	++	+
6-06	D406I, M422Y	+++	+++
6-07	T159R, K339Y	+	+
6-08	L316I, D57Y	++	++
6-09	A71Y, K339Y	+	+
6-10	A71Y, T513A	++	++
6-11	S345A, G172R	+	+
6-12	D57Y, G172R	+	+
6-13	D57Y, S345A	++	++
6-14	D57Y, M422Y	++	++
6-15	L316I, D406I, M422Y	++	++
6-16	S345A, D406I, L316I	++	+
6-17	S345A, G172R, L316I	++	++
6-18	S345A, M422Y, A71Y	+++	+++
6-19	D57Y, G172R, M422Y	++	+
6-20	D57Y, L316I, S345A	++	++
6-21	D57Y, D406I, T513A	+++	++
6-22	M422Y, A309I, A71Y	++	+++
6-23	S345A, K339Y, T513A	++	+++
6-24	G172R, K339Y, V49Y	++	++
6-25	G172R, D406I, A71Y	++	++
6-26	D57Y, A71Y, A309I	+	++
6-27	A309I, T513A, Q134Y	+++	+++
6-28	V49Y, T513A, L316I	++	++++
6-29	A309I, T159R, D406I	++	++
6-30	K339Y, S345A, M422Y	++	+++
6-31	V49Y, A309I, T159R	++	+++
6-32	D57Y, K339Y, T159R	++	++
6-33	A71Y, V49Y, T513A	++	++
6-34	K339Y, G172R, M422Y	++	++
6-35	D57Y, L316I, D406I, M422Y	++	++
6-36	D57Y, G172R, L316I, M422Y	++	+
6-37	S345A, A309I, G172R, T513A	++++	++++
6-38	D57Y, L316I, G172R, K339Y	+	++
6-39	K339Y, D406I, T513A, A71Y	++	++++
6-40	A309I, V49Y, Q134Y, T159R	++	+++
6-41	G172R, D406I, M422Y, S345A	++	++++
6-42	G172R, D406I, M422Y, A71Y	++	++
6-43	L316I, D57Y, A309I, S345A	++	+++
6-44	M422Y, A309I, V49Y, T513A	+++	++++
6-45	T159R, K339Y, A71Y, V49Y	+	++
6-46	L316I, M422Y, D406I, T513A	++	+++
6-47	A309I, L316I, D57Y, T513A	++	++
6-48	A71Y, G172R, Q134Y, V49Y	++	+++

Example	Mutant	Relative total activity	Relative hydrolytic activity
6-49	S345A, T159R, A71Y, K339Y	+	+
6-50	S345A, D57Y, V49Y, A309I	+	++
6-51	D57Y, D406I, L316I, G172R	+	++
6-52	D57Y, G172R, L316I, D406I, M422Y	++	+++
6-53	S345A, L316I, G172R, M422Y, T159R	++	+++
6-54	V49Y, D57Y, T159R, G172R, L316I	++	+++
6-55	G172R, L316I, M422Y, T513A, A71Y	+++	+++
6-56	S345A, A309I, D406I, K339Y, A71Y	+	++
6-57	T513A, K339Y, D406I, V49Y, Q134Y	+++	+++
6-58	D57Y, D406I, M422Y, S345A, A71Y	+++	++++
6-59	A71Y, A309I, T513A, S345A, Q134Y	++++	++++
6-60	S345A, D406I, T513A, A309I, G172R	+++	+++
6-61	K339Y, H489N, D522P, A71Y, D57Y	++	++++
6-62	L316I, S345A, K339Y, T513A, V49Y	+++	+++
6-63	V49Y, D57Y, T159R, L316I, M422Y	++	+++
6-64	G172R, A71Y, M422Y, T513A, A309I	++	++++
6-65	V49Y, D57Y, T159R, G172R, A309I, L316I, S345A, D406I, M422Y, T513A	++++	++++
6-66	V49Y, D57Y, G172R, A309I, L316I, S345A, D406I, M422Y, T513A	++++	++++
6-67	V49Y, D57Y, A71Y, G172R, A309I, S345A, D406I, M422Y, T513A	+++	+++
6-68	V49Y, G172R, K339Y, L316I, S345A, D406I, M422Y, T513A	++++	+++
6-69	D57Y, T159R, G172R, A309I, L316I, S345A, M422Y	+++	+++
6-70	V49Y, D57Y, T159R, G172R, A71Y, M422Y, T513A	+++	++++
6-71	V49Y, D57Y, G172R, L316I, M422Y, T513A	+++	+++
6-72	V49Y, T159R, G172R, L316I, M422Y, T513A	+++	++++
6-73	V49Y, D57Y, D522P, A309I, L316I, S345A, D406I, T513A	+++	+++

Example 7

Cloning and protein expression

Further combinatorial variants (SEQ ID NOs 3 - 21) were designed and cloned for expression in *P. pastoris* as described in Example 6. Single colonies of transformants were inoculated into 45 mL BMG1 in 250 mL baffled flasks. Flasks were covered with cloth and incubated at 28 °C, 320 RPM and 80% humidity for 60 h. Cultures were further induced by addition of 0.25 mL 50% glycerol 68, 84 and 92 h after inoculation. The supernatant was harvested after 108 h of cultivation by centrifugation at 4,000 RPM and 4 °C for 10 min. The supernatant was concentrated on 30 kDa centrifugal filters and buffer exchanged into

10 mM potassium phosphate pH 7. Glycerol was added to 10% (v/v) before storing at -20 °C until further analysis.

Activity in simulated gastric conditions

The activity of the combinatorial variants SEQ ID NOs 3 - 21 was compared to the wild-type inulosucrase (SEQ ID NO 1) in simulated gastric conditions. 25 µl of 160 ng/µl inulosucrase was mixed with 175 µl reaction buffer containing 50 mM sodium phosphate pH 2.75. The final reaction contained 1 mg/mL pepsin (P7012, Merck, ≥2,500 units/mg) 125 mM sucrose and 0.6 mM $CaCl_2$. The reaction was incubated for 15 min at 37 °C before being stopped by addition of 10 µl 5 M NaOH. The glucose and fructose concentrations were measured using the K-FRUGL kit (Megazyme, Ireland) as described in Example 2. The assay was performed with three technical replicates. A supernatant sample of a *P. pastoris* strain with an empty vector (not containing an inulosucrase gene) was included as negative control.

All variants (SEQ ID NOs: 3-21) demonstrate substantially increased activity in simulated gastric conditions compared to the WT (SEQ ID NO: 1). The ratio of transfructosylation to total activity was broadly similar for all inulosucrases tested.

Results are shown in the following table 7; in which the measured quantity of free sugars (glucose or free fructose) was determined as follows:

\+ greater than WT protein and <4 mM

++ ≥4 mM and <12 mM

+++ ≥12 mM and <20 mM

++++ ≥20 mM

Table 7: Relative activity of mutants

Example	SEQ ID NO	Mutant	Glucose [mM]	Free fructose [mM]
	1	WT PROTEIN OF SEQ ID NO: 1	2.6	0.3
		EMPTY VECTOR CONTROL	1.2	-
7-1	3	V49Y, A71Y, Q134Y, K339Y, D406I, T513A	++++	+++
7-2	4	V49Y, D57Y, A71Y, Q134Y, A309I, T513A	++++	+++
7-3	5	V49Y, D57Y, A71Y, Q134Y, A309I, D406I, T513A	++++	+++

Example	SEQ ID NO	Mutant	Glucose [mM]	Free fructose [mM]
7-4	6	V49Y, A71Y, Q134Y, A309I, D384A, D406I, A510D, T513A	+++	++
7-5	7	V49Y, A71Y, Q134Y, A309I, T513A	++	++
7-6	8	Q134Y, A309I, S345A, H489N, T513A	++	++
7-7	9	V49Y, A71Y, Q134Y, A309I, S345A, T513A	+++	++
7-8	10	V49Y, A71Y, Q134Y, A309I, S345A, H489N, T513A	+++	++
7-9	11	V49Y, Q134Y, A309I, K339Y, S345A, D406I, H489N, T513A	+++	++
7-10	12	V49Y, A71Y, Q134Y, A309I, K339Y, S345A, D406I, H489N, T513A	+++	++
7-11	13	T10P, G152A, S345A, A510D	++	+
7-12	14	Q134Y, G172R, K238A, L316I	++	++
7-13	15	A309I, K343S, D406I	++	+
7-14	16	V49Y, Q134Y, A309I, A510D, T513A	++	+
7-15	17	V49Y, Q134Y, D406I, A510D, T513A	++	++
7-16	18	V49Y, Q134Y, D384A, D406I, A510D, T513A	++	+
7-17	19	V49Y, Q134Y, S345A, D406I, A510D, T513A	+++	++
7-18	20	V49Y, Q134Y, L316I, D384A, D406I, A510D, T513A	++	+
7-19	21	V49Y, Q134Y, S345A, D384A, D406I, A510D, T513A	++	++

Residual activity after exposure to gastric conditions

The resistance of inulosucrase variants (SEQ ID NOs 1, 3 - 21) to exposure to simulated gastric conditions was also tested. 40 ng/µl inulosucrase was incubated in 42 mM sodium phosphate pH 3.0 with 1 mg/mL pepsin for 0 or 15 min at 37 °C. Residual activity was measured by incubating 10 ng/µl of pre-incubated inulosucrase with 125 mM sucrose in 100 mM sodium phosphate pH 7 and 0.6 mM $CaCl_2$ at 37 °C for 15 min. The reaction was stopped by addition of 10 µl 5M NaOH. The glucose and fructose concentrations were measured using the K-FRUGL kit (Megazyme, Ireland) as described in Example 2. The assay was performed with three technical replicates. To determine the activity lost due to exposure to pH 3.0 and 1 mg/mL pepsin, the activity observed 15 min pre-incubation was divided by the residual activity after 0 min of pre-incubation (immediate neutralization).

All variants (SEQ ID NOs: 3-21) demonstrated substantially increased activity after 15 min in presence of pH 3.0 and 1 mg/mL pepsin compared to the WT (SEQ ID NO: 1.

Some variants retained ~40% of their activity after 15 min in simulated gastric conditions compared to the 0 min pre-incubation.

Results are shown in the following table 8; in which the relative activity (relative total activity or relative transfructosylation activity) after 15 minutes in simulated gastric conditions, compared to the activity of the same mutant with 0 minutes pre-incubation, was determined as follows:

+ greater than WT protein and ≤10%

++ >10% and ≤20%

+++ ≥20% and ≤40%

++++ >40%

Table 8: Relative residual activity of mutants

Example	SEQ ID NO	Mutant	Relative Total activity	Relative Trans-fructo-sylation activity
	1	WT PROTEIN OF SEQ ID NO: 1	negligible	
7-1	3	V49Y, A71Y, Q134Y, K339Y, D406I, T513A	++++	++++
7-2	4	V49Y, D57Y, A71Y, Q134Y, A309I, T513A	+++	+++
7-3	5	V49Y, D57Y, A71Y, Q134Y, A309I, D406I, T513A	+++	++++
7-4	6	V49Y, A71Y, Q134Y, A309I, D384A, D406I, A510D, T513A	++	++
7-5	7	V49Y, A71Y, Q134Y, A309I, T513A	++	++
7-6	8	Q134Y, A309I, S345A, H489N, T513A	++	++
7-7	9	V49Y, A71Y, Q134Y, A309I, S345A, T513A	++	++
7-8	10	V49Y, A71Y, Q134Y, A309I, S345A, H489N, T513A	++++	++++
7-9	11	V49Y, Q134Y, A309I, K339Y, S345A, D406I, H489N, T513A	++++	++++
7-10	12	V49Y, A71Y, Q134Y, A309I, K339Y, S345A, D406I, H489N, T513A	+++	+++
7-11	13	T10P, G152A, S345A, A510D	+	+
7-12	14	Q134Y, G172R, K238A, L316I	+	+
7-13	15	A309I, K343S, D406I	+++	++++
7-14	16	V49Y, Q134Y, A309I, A510D, T513A	+	+
7-15	17	V49Y, Q134Y, D406I, A510D, T513A	+++	+++
7-16	18	V49Y, Q134Y, D384A, D406I, A510D, T513A	+	+

Example	SEQ ID NO	Mutant	Relative Total activity	Relative Trans-fructo-sylation activity
7-17	19	V49Y, Q134Y, S345A, D406I, A510D, T513A	+++	+++
7-18	20	V49Y, Q134Y, L316I, D384A, D406I, A510D, T513A	+++	+++
7-19	21	V49Y, Q134Y, S345A, D384A, D406I, A510D, T513A	+++	+++

Details of the Sequence Listing

SEQ ID NO: 1 shows the amino acid sequence of the fructosyltransferase of gene inuGB from *Lactobacillus gasseri* DSM 20604. Some or all of the residues shown in grey/bold/bold&underlined are believed to be associated with the active site of the protein.

SEQ ID NO: 2 shows a representative construct used to express mutant inulosucrase enzymes described herein (see example 1). Features in SEQ ID NO: 2 are as follows

6xHis tag AA1 - AA9

Fructosyltransferase gene inuGB (SEQ ID NO: 1): AA10 – AA574

Linker AA575 - AA576

GFP11 tag AA577 – AA602

SEQ ID NO: 1

AVKQDEKAAT AVKANTEVKA NETSTKSASK DNKAELKGQI KDIVKESGVD TSKLTDDQIN 60
ELNKISFSKE AKSGTQLTYS DFKKIAKTLI EQDARYAVPF FNASKIKNMP AAKTLDAQTG 120
KVEDLEIWDS WPVQDAKTGY VSNWNGYQLV IGMMGVPNTN DNHIYLLYNK YGDNNFNNWK 180
NAGPIFGLGT PVIQQWSGSA TLNKDGSIQL YYTKVDTSDN NTNHQKIASA TVYLNLEKNQ 240
DKISIAHVDN DHIVFEGDGY HYQTYNQWKK TNKGADNIAM RDAHVIDDKD GNRYLVFEAS 300
TGTENYQGAD QIYQWLNYGG TNKDNLGDFL QILSNSDIKD RAKWSNAAIG IIKLNNDTKN 360
PGVEKVYTPL ISAPMVSDEI ERPDVVRLGN KYYLFAATRL NRGSNDDAWM AANKAVGDNV 420
AMIGYVSDNL THGYVPLNES GVVLTASVPA NWRTATYSYY AVPVEGRDDQ LLITSYITNR 480
GEVAGKGMHA TWAPSFLLQI NPDNTTTVLA KMTNQGDWIW DDSSENADMM GVLEKDAPNS 540
AALPGEWGKP VDWDLIGGYN LKPHQ 565

SEQ ID NO. 2

MHHHHHHASAVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGVDTSKLTDD
QINELNKISFSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVE
DLEIWDSWPVQDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLG
TPVIQQWSGSATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVF
EGDGYHYQTYNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGADQIYQWLNYGGT
NKDNLGDFLQILSNSDIKDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVR
LGNKYYLFAATRLNRGSNDDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWR
TATYSYYAVPVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLAKMTNQGDWI
WDDSSENADMMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ**SR**GSDGGSGGGSRDHMVLHE
YVNAAGIT

SEQ ID NO. 3

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEYKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT

YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGADQIYQWLNYGGTNKDNLGDFL
QILSNSDIYDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLAKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 4

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDYQINELNKIS
FSKEYKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNDDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLAKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 5

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDYQINELNKIS
FSKEYKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLAKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 6

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEYKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPAVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLDKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 7

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEYKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNDDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLAKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 8

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGVDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP

VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWANAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNDDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMNATWAPSFLLQINPDNTTTVLAKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 9

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEYKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWANAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNDDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLAKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 10

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEYKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWANAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNDDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMNATWAPSFLLQINPDNTTTVLAKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 11

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIYDRAKWANAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMNATWAPSFLLQINPDNTTTVLAKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 12

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEYKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIYDRAKWANAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMNATWAPSFLLQINPDNTTTVLAKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 13

AVKQDEKAAPAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGVDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VQDAKTGYVSNWNGYQLVIAMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGADQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWANAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNDDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLDKMTNQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 14

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGVDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYRDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEANQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGADQIYQWINYGGTNKDNLGDFL
QILSNSDIKDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNDDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLAKMTNQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 15

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGVDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VQDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRASWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLAKMTNQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 16

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGIDQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNDDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLDKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 17

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGADQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLDKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 18

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGADQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPAVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLDKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 19

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGADQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWANAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPDVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLDKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 20

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGADQIYQWINYGGTNKDNLGDFL
QILSNSDIKDRAKWSNAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPAVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLDKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

SEQ ID NO. 21

AVKQDEKAATAVKANTEVKANETSTKSASKDNKAELKGQIKDIVKESGYDTSKLTDDQINELNKIS
FSKEAKSGTQLTYSDFKKIAKTLIEQDARYAVPFFNASKIKNMPAAKTLDAQTGKVEDLEIWDSWP
VYDAKTGYVSNWNGYQLVIGMMGVPNTNDNHIYLLYNKYGDNNFNNWKNAGPIFGLGTPVIQQWSG
SATLNKDGSIQLYYTKVDTSDNNTNHQKIASATVYLNLEKNQDKISIAHVDNDHIVFEGDGYHYQT
YNQWKKTNKGADNIAMRDAHVIDDKDGNRYLVFEASTGTENYQGADQIYQWLNYGGTNKDNLGDFL
QILSNSDIKDRAKWANAAIGIIKLNNDTKNPGVEKVYTPLISAPMVSDEIERPAVVRLGNKYYLFA
ATRLNRGSNIDAWMAANKAVGDNVAMIGYVSDNLTHGYVPLNESGVVLTASVPANWRTATYSYYAV
PVEGRDDQLLITSYITNRGEVAGKGMHATWAPSFLLQINPDNTTTVLDKMANQGDWIWDDSSENAD
MMGVLEKDAPNSAALPGEWGKPVDWDLIGGYNLKPHQ

CLAIMS

1. A mutant inulosucrase enzyme comprising a variant of the amino acid sequence shown in SEQ ID NO: 1, wherein the variant comprises a modification at one or more of the following positions: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, L316, S336, K339, K343, D384, D406, M422, T474, H489, W492, A510, and D522.

2. A mutant inulosucrase enzyme according to claim 1, wherein the modification is substitution with tyrosine, alanine, aspartic acid, isoleucine, asparagine, proline, or arginine.

3. A mutant inulosucrase enzyme comprising one or more modification selected from Q134Y, T513A, A71Y, T10P, V49Y, D57Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K339R, K339Y, K343S, D384A, D406I, M422Y, T474A, H489N, W492A, A510D, and D522P.

4. A mutant inulosucrase enzyme according to any one of the preceding claims, wherein the variant has at least 70% identity to the amino acid sequence shown in SEQ ID NO: 1.

5. A mutant inulosucrase enzyme according to any one of the preceding claims, comprising a modification at one or more of: Q134, T513, A71, T10, V49, D57, G152, G172, K238, A309, L316, K339, K343, S345, D384, D406, H489, and A510;

preferably wherein the variant comprises one or more of Q134Y, T513A, A71Y, T10P, V49Y, D57Y, G152A, G172R, K238A, A309I, L316I, K339Y, K343S, S345A, D384A, D406I, H489N and A510D.

6. A mutant inulosucrase enzyme according to any one of the preceding claims, comprising a modification at one or more of: Q134, T513, A71, V49, D57, A309, K339, S345, D406, H489, and A510;

preferably wherein the variant comprises one or more of Q134Y, T513A, A71Y, V49Y, D57Y, A309I, K339Y, S345A, D406I, H489N and A510D.

7. A mutant inulosucrase enzyme according to any one of the preceding claims, comprising a modification at one or more of: V49, D57, A71, Q134, T159, G172, A309, L316, K339, S345, D406, M422, and T513;

preferably wherein the variant comprises one or more of V49Y, D57Y, A71Y, Q134Y, T159R, G172R, A309I, L316I, K339Y, S345A, D406I, M422Y, and T513A.

8. A mutant inulosucrase enzyme according to any one of the preceding claims, comprising a modification at one or more of: D57, A71, Q134, N143, G152, A309, L316, K339, S345, D406, and T474;

preferably wherein the variant comprises one or more of D57Y, A71Y, Q134Y, N143D, G152A, A309I, L316I, K339Y, S345A, D406I and T474A.

9. A mutant inulosucrase enzyme according to any one of the preceding claims, comprising a modification at one or more of: G172, L316, K339, S345, D406, M422, and T513;

preferably wherein the variant comprises one or more of G172R, L316I, K339R, K339Y, S345A, D406I, M422Y and T513A.

10. A mutant inulosucrase enzyme according to any one of the preceding claims, comprising a modification at one or more of: D57, A71, G172, A309, L316, K339, S345, D406, and M422;

preferably wherein the variant comprises one or more of D57Y, G172R, A71Y, A309I, L316I, K339Y, S345A, D406I and M422Y.

11. A mutant inulosucrase enzyme according to any one of the preceding claims, comprising a modification at one or more of: D57, G172, L316, D406, and M422;

preferably wherein the variant comprises one or more of D57Y, G172R, L316I, D406I and M422Y.

12. A mutant inulosucrase enzyme according to any one of the preceding claims, comprising a modification at at least two, three, four, five, six, seven, eight, nine, ten or more of the following positions: Q134, T513, A71, T10, V49, D57, N143, G152, T159, G172, K238, Q240, K270, A309, L316, S336, K343, S345, K339, D384, D406, M422, T474, H489, W492, A510, and D522.

13. A mutant inulosucrase enzyme according to any one of the preceding claims, comprising at least two, three, four, five, six, seven, eight, nine, ten or more of the following modifications: Q134Y, T513A, A71Y, T10P, V49Y, D57Y, N143D, G152A, T159R, G172R, K238A, Q240A, K270A, A309I, L316I, S336A, K343S, S345A, K339R, K339Y, D384A, D406I, M422Y, T474A, H489N, W492A, A510D, and D522P.

14. A mutant inulosucrase enzyme according to any one of the preceding claims, comprising a modification in a region corresponding to positions 1 – 92, 303 – 345 and/or 400 – 418 of SEQ ID NO 1, preferably wherein said region corresponds to an alpha-helical region of the structure of the mutant inulosucrase enzyme.

15. A mutant inulosucrase enzyme according to any one of the preceding claims, wherein the variant comprises modifications at one of the following:

L316
D57
S345
A71
G172
D406
M422
T513
A309
V49
T159
Q134
K339
V49 / A309
L316 / M422
S345 / M422
G172 / L316
G172 / D406
D406 / M422
T159 / K339
S345 / D406
L316 / D57

A71 / K339

A71 / T513

S345 / G172

D57 / G172

D57 / S345

D57 / M422

L316 / D406 / M422

S345 / D406 / L316

S345 / G172 / L316

S345 / M422 / A71

D57 / G172 / M422

D57 / L316 / S345

D57 / D406 / T513

M422 / A309 / A71

S345 / K339 / T513

G172 / K339 / V49

G172 / D406 / A71

D57 / A71 / A309

A309 / T513 / Q134

V49 / T513 / L316

A309 / T159 / D406

K339 / S345 / M422

V49 / A309 / T159

D57 / K339 / T159

A71 / V49 / T513

K339 / G172 / M422

D57 / L316 / D406 / M422

D57 / G172 / L316 / M422

S345 / A309 / G172 / T513

D57 / L316 / G172 / K339

K339 / D406 / T513 / A71

A309 / V49 / Q134 / T159

G172 / D406 / M422 / S345

G172 / D406 / M422 / A71

K339 / D406 / S345 / V49

L316 / D57 / A309 / S345

T513 / A309 / A71 / S345

M422 / A309 / V49 / T513

T159 / K339 / A71 / V49

L316 / M422 / D406 / T513

A309 / L316 / D57 / T513

A71 / G172 / Q134 / V49

S345 / T159 / A71 / K339

S345 / D57 / V49 / A309

K339 / Q134 / D522 / H489

D57 / D406 / L316 / G172

D57 / G172 / L316 / D406 / M422

S345 / L316 / G172 / M422 / T159

V49 / D57 / T159 / G172 / L316

G172 / L316 / M422 / T513 / A71

S345 / A309 / D406 / K339 / A71

T513 / K339 / D406 / V49 / Q134

H489 / D522 / Q134 / D57 / A309

D57 / D406 / M422 / S345 / A71

A71 / A309 / T513 / S345 / Q134

S345 / D406 / T513 / A309 / G172

K339 / H489 / D522 / A71 / D57

L316 / S345 / K339 / T513 / V49

V49 / D57 / T159 / M422 / L316

G172 / A71 / M422 / T513 / A309

V49 / D57 / T159 / G172 / A309 / L316 / S345 / D406 / M422 / T513

V49 / D57 / G172 / A309 / L316 / S345 / D406 / M422 / T513

V49 / D57 / A71 / G172 / A309 / S345 / D406 / M422 / T513

V49 / G172 / K339 / L316 / S345 / D406 / M422 / T513

D57 / T159 / G172 / A309 / L316 / S345 / M422

V49 / D57 / T159 / G172 / A71 / M422 / T513

V49 / D57 / G172 / L316 / M422 / T513

V49 / T159 / G172 / L316 / M422 / T513

V49 / D57 / D522 / A309 / L316 / S345 / D406 / T513

V49 / D57 / T159 / G172 / A309 / L316 / S345 / D406 / T513

V49 / A71 / Q134 / K339 / D406 / T513

V49 / D57 / A71 / Q134 / A309 / T513

V49 / D57 / A71 / Q134 / A309 / D406 / T513

V49 / A71 / Q134 / A309 / D384 / D406 / A510 / T513

V49 / A71 / Q134 / A309 / T513

Q134 / A309 / S345 / H489 / T513

V49 / A71 / Q134 / A309 / S345 / T513

V49 / A71 / Q134 / A309 / S345 / H489 / T513

V49 / Q134 / A309 / K339 / S345 / D406 / H489 / T513

V49 / A71 / Q134 / A309 / K339 / S345 / D406 / H489 / T513

T10 / G152 / S345 / A510

Q134 / G172 / K238 / L316

A309 / K343 / D406

V49 / Q134 / A309 / A510 / T513

V49 / Q134 / D406 / A510 / T513

V49 / Q134 / D384 / D406 / A510 / T513

V49 / Q134 / S345 / D406 / A510 / T513

V49 / Q134 / L316 / D384 / D406 / A510 / T513

V49 / Q134 / S345 / D384 / D406 / A510 / T513

16. A mutant inulosucrase enzyme according to any one of the preceding claims, wherein the variant comprises one of the following:

L316I

D57Y

S345A

A71Y

G172R

D406I

M422Y

T513A

A309I

V49Y

T159R

Q134Y

K339Y

V49Y / A309I

L316I / M422Y

S345A / M422Y

G172R / L316I

G172R / D406I

D406I / M422Y

T159R / K339Y

S345A / D406I

L316I / D57Y

A71Y / K339Y

A71Y / T513A

S345A / G172R

D57Y / G172R

D57Y / S345A

D57Y / M422Y

L316I / D406I / M422Y

S345A / D406I / L316I

S345A / G172R / L316I

S345A / M422Y / A71Y

D57Y / G172R / M422Y

D57Y / L316I / S345A

D57Y / D406I / T513A

M422Y / A309I / A71Y

S345A / K339Y / T513A

G172R / K339Y / V49Y

G172R / D406I / A71Y

D57Y / A71Y / A309I

A309I / T513A / Q134Y

V49Y / T513A / L316I

A309I / T159R / D406I

K339Y / S345A / M422Y

V49Y / A309I / T159R

D57Y / K339Y / T159R

A71Y / V49Y / T513A

K339Y / G172R / M422Y

D57Y / L316I / D406I / M422Y

D57Y / G172R / L316I / M422Y

S345A / A309I / G172R / T513A

D57Y / L316I / G172R / K339Y

K339Y / D406I / T513A / A71Y

A309I / V49Y / Q134Y / T159R

G172R / D406I / M422Y / S345A

G172R / D406I / M422Y / A71Y

K339Y / D406I / S345A / V49Y

L316I / D57Y / A309I / S345A

T513A / A309I / A71Y / S345A

M422Y / A309I / V49Y / T513A

T159R / K339Y / A71Y / V49Y

L316I / M422Y / D406I / T513A

A309I / L316I / D57Y / T513A

A71Y / G172R / Q134Y / V49Y

S345A / T159R / A71Y / K339Y

S345A / D57Y / V49Y / A309I

K339Y / Q134Y / D522P / H489N

D57Y / D406I / L316I / G172R

D57Y / G172R / L316I / D406I / M422Y

S345A / L316I / G172R / M422Y / T159R

V49Y / D57Y / T159R / G172R / L316I

G172R / L316I / M422Y / T513A / A71Y

S345A / A309I / D406I / K339Y / A71Y

T513A / K339Y / D406I / V49Y / Q134Y

H489N / D522P / Q134Y / D57Y / A309I

D57Y / D406I / M422Y / S345A / A71Y

A71Y / A309I / T513A / S345A / Q134Y

S345A / D406I / T513A / A309I / G172R

K339Y / H489N / D522P / A71Y / D57Y

L316I / S345A / K339Y / T513A / V49Y

V49Y / D57Y / T159R / M422Y / L316I

G172R / A71Y / M422Y / T513A / A309I

V49Y / D57Y / T159R / G172R / A309I / L316I / S345A / D406I / M422Y / T513A

V49Y / D57Y / G172R / A309I / L316I / S345A / D406I / M422Y / T513A

V49Y / D57Y / A71Y / G172R / A309I / S345A / D406I / M422Y / T513A

V49Y / G172R / K339Y / L316I / S345A / D406I / M422Y / T513A

D57Y / T159R / G172R / A309I / L316I / S345A / M422Y

V49Y / D57Y / T159R / G172R / A71Y / M422Y / T513A

V49Y / D57Y / G172R / L316I / M422Y / T513A

V49Y / T159R / G172R / L316I / M422Y / T513A

V49Y / D57Y / D522P / A309I / L316I / S345A / D406I / T513A

V49Y / D57Y / T159R / G172R / A309I / L316I / S345A / D406I / T513A

V49Y / A71Y / Q134Y / K339Y / D406I / T513A

V49Y / D57Y / A71Y / Q134Y / A309I / T513A

V49Y / D57Y / A71Y / Q134Y / A309I / D406I / T513A

V49Y / A71Y / Q134Y / A309I / D384A / D406I / A510D / T513A

V49Y / A71Y / Q134Y / A309I / T513A

Q134Y / A309I / S345A / H489N / T513A

V49Y / A71Y / Q134Y / A309I / S345A / T513A

V49Y / A71Y / Q134Y / A309I / S345A / H489N / T513A

V49Y / Q134Y / A309I / K339Y / S345A / D406I / H489N / T513A

V49Y / A71Y / Q134Y / A309I / K339Y / S345A / D406I / H489N / T513A

T10P / G152A / S345A / A510D

Q134Y / G172R / K238A / L316I

A309I / K343S / D406I

V49Y / Q134Y / A309I / A510D / T513A

V49Y / Q134Y / D406I / A510D / T513A

V49Y / Q134Y / D384A / D406I / A510D / T513A

V49Y / Q134Y / S345A / D406I / A510D / T513A

V49Y / Q134Y / L316I / D384A / D406I / A510D / T513A

V49Y / Q134Y / S345A / D384A / D406I / A510D / T513A

17. A mutant inulosucrase enzyme according to any one of the preceding claims, wherein the variant comprises any number and any combination of modifications and/or substitutions defined in any one of the preceding claims.

18. A mutant inulosucrase enzyme according to any one of the preceding claims, wherein the variant is capable of catalysing transfructosylation, preferably under physiological conditions.

19. A mutant inulosucrase enzyme according to any one of the preceding claims, wherein said enzyme is expressed by or is obtainable by intracellular or extracellular expression from an organism of genus *Escherichia, Lactobacillus, Saccharomyces, Bacillus*, *Komagataella, Pichia*, *Trichoderma, Corynebacterium* or *Aspergillus*; preferably *E. coli, S. cerevisiae, B. subtilis, K. phaffii, P. pastoris, T. reesei, C. glutamicum, A. niger,* or *A. oryzae*.

20. A mutant inulosucrase enzyme according to any one of the preceding claims in isolated form.

21. A polynucleotide which encodes a mutant inulosucrase enzyme according to any one of the preceding claims.

22. A vector comprising a polynucleotide according to claim 21.

23. A cell comprising a vector according to claim 21; preferably wherein said cell is a bacterial cell, a yeast cell, or a fungal cell.

24. A method of producing a mutant inulosucrase enzyme according to any one of claims 1 to 20, comprising expressing said mutant inulosucrase enzyme from a cell according to claim 23, and optionally isolating said mutant inulosucrase enzyme from said cell.

25. A food composition or foodstuff comprising a mutant inulosucrase enzyme according to any one of claims 1 to 20, and one or more carbohydrate, fat, lipid, protein, flavouring agent, or colouring agent.

26. A food composition or foodstuff according to claim 24, wherein said food composition or foodstuff comprises sucrose.

27. A nutraceutical composition comprising a mutant inulosucrase enzyme according to any one of claims 1 to 20, and one or more nutraceutically acceptable filler, stabilizing agent, colouring agent or flavouring agent; optionally wherein said composition is a dietary supplement.

28. A pharmaceutically acceptable composition comprising a mutant inulosucrase enzyme according to any one of claims 1 to 20, and one or more pharmaceutically acceptable carrier, excipient, or diluent.

29. A composition according to claim 27 or 28, wherein said composition is for oral administration; optionally wherein said composition (i) comprises an enteric coating and/or (ii) is formulated as a tablet, a troche, a lozenge, an aqueous or oily suspension, a dispersible powder or as granules.

30. An *in vivo* method of reducing fructose and/or glucose uptake in a subject, the method comprising administering to the subject a mutant inulosucrase enzyme according to any one of claims 1 to 20, a food composition or foodstuff according to claim 25 or 26, or a nutraceutical or pharmaceutical composition according to any one of claims 27 to 29, and thereby converting sucrose to fructooligosaccharide (preferably inulin) *in vivo*.

31. An *in vivo* method of producing fructooligosaccharides (preferably inulin) in a subject *in vivo*, the method comprising administering to the subject a mutant inulosucrase enzyme according to any one of claims 1 to 20, a food composition or foodstuff according

to claim 25 or 26, or a nutraceutical or pharmaceutical composition according to any one of claims 27 to 28, and thereby converting sucrose to fructooligosaccharide (preferably inulin) *in vivo*.

32. A method of suppressing a subject's appetite and/or increasing a subject's satiety, comprising administering to the subject a mutant inulosucrase enzyme according to any one of claims 1 to 20, a food composition or foodstuff according to claim 25 or 26, or a nutraceutical or pharmaceutical composition according to any one of claims 27 to 29.

33. A method according to any one of claims 30 to 32, wherein said method is a non-therapeutic method.

34. A method according to any one of claims 30 to 33, comprising orally administering the mutant inulosucrase enzyme to the subject, preferably wherein said mutant inulosucrase enzyme is administered as an isolated enzyme.

35. A mutant inulosucrase enzyme according to any one of claims 1 to 20, a food composition or foodstuff according to claim 25 or 26, or a nutraceutical or pharmaceutical composition according to any one of claims 27 to 28, for use in reducing fructose uptake and producing fructooligosaccharide (preferably inulin) in a subject, wherein said use comprises administering to the subject the mutant inulosucrase enzyme and thereby converting sucrose to fructooligosaccharide (preferably inulin) *in vivo*.

36. A mutant inulosucrase enzyme, food composition, foodstuff, nutraceutical composition or pharmaceutical composition for use according to claim 35, wherein said use comprises orally administering said mutant inulosucrase enzyme to said subject, preferably wherein said mutant inulosucrase enzyme is administered as an isolated enzyme.

37. A mutant inulosucrase enzyme, food composition, foodstuff, nutraceutical composition or pharmaceutical composition for use according to claim 35 or 36, or a method according to any one of claims 30 to 34, wherein administering the mutant inulosucrase enzyme to said subject suppresses the subject's appetite and/or increases the subject's satiety.

38. A mutant inulosucrase enzyme according to any one of claims 1 to 20, a food composition or foodstuff according to claim 25 or 26, or a nutraceutical or pharmaceutical composition according to any one of claims 27to 29, for use in *in vivo* administration to a subject.

39. A mutant inulosucrase enzyme according to any one of claims 1 to 20, a food composition or foodstuff according to claim 25 or 26, or a nutraceutical or pharmaceutical composition according to any one of claims 27to 29, for use in medicine.

40. A mutant inulosucrase enzyme according to any one of claims 1 to 20, a food composition or foodstuff according to claim 25 or 26, or a nutraceutical or pharmaceutical composition according to any one of claims 27 to 29, for use in treating or preventing metabolic syndrome, diabetes, non-alcoholic fatty liver disease, constipation or obesity in a subject in need thereof;

41. A mutant inulosucrase enzyme, food composition, foodstuff, nutraceutical composition or pharmaceutical composition for use according to any one of claims 38 to 40, wherein said use comprises orally administering said mutant inulosucrase enzyme to said subject, preferably wherein said mutant inulosucrase enzyme is administered as an isolated enzyme.

42. Use of a mutant inulosucrase enzyme according to any one of claims 1 to 20, a food composition or foodstuff according to claim 25 or 26, or a nutraceutical or pharmaceutical composition according to any one of claims 27 to 29 for (i) reducing fructose uptake in a subject; (ii) producing fructooligosaccharide (preferably inulin) in a subject; (iii) converting sucrose to fructooligosaccharide (preferably inulin) in a subject; suppressing a subject's appetite and/or increasing a subject's satiety.

43. A method of increasing (i) *in vivo* transfructosylation activity, (ii) pH stability and/or (iii) protease resistance of an inulosucrase enzyme; by making in the inulosucrase enzyme one or more modifications as described in any one of claims 1 to 17; and thereby increasing (a) the *in vivo* transfructosylation activity of the inulosucrase enzyme, (b) the pH stability of the inulosucrase enzyme and/or (iii) the protease resistance of the inulosucrase enzyme.