Glucose-oligosaccharides comprising (al?6) and (al?4) glycosidic bonds, use thereof , and methods for providing them

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(54) **Title**: GLUCO-OLIGOSACCHARIDES COMPRISING (α1→4) AND (α1→6) GLYCOSIDIC BONDS, USE THEREOF, AND METHODS FOR PROVIDING THEM

(57) **Abstract**: The invention relates to the field of poly- and oligosaccharides and their nutritional effects. In particular, it relates to the application of α-glucanotransferases in methods for preparing dietary fibers, including prebiotic oligosaccharides, and to novel oligosaccharides obtainable thereby. Provided is a method for producing a mixture of gluco-oligosaccharides having one or more consecutive (α1→6) glucosidic linkages and one or more consecutive (α1→4) glucosidic linkages, comprising contacting a poly- and/or oligosaccharide substrate comprising at least two (α1→4) linked D-glucose units with an ?-glucanotransferase capable of cleaving (α1→4) glucosidic linkages and making new (α1→4) and (α1→6) glucosidic linkages. Also provided are (isolated) gluco-oligosaccharides obtainable thereby, and their application in nutritional and cosmetic compositions.

Title: Gluco-oligosaccharides comprising (alpha 1→4) and (alpha 1→6) glycosidic bonds, use thereof, and methods for providing them

The invention relates to the field of poly- and oligosaccharides and their nutritional effects. In particular, it relates to the application of α-glucanotransferases in methods for preparing dietary fibers, including prebiotic oligosaccharides, and to novel oligosaccharides obtainable thereby.

The term 'dietary fibre' was first used in 1953 by Hipsley to describe the plant cell wall components of food. Today there are many definitions of fibre in use, but as yet there is no universally accepted definition. Generally, fibres are derived from carbohydrate sources that have a non-digestible component. Fibres are typically divided into two categories; the insoluble fibres such as wheat bran, resistant starch, hemicelluloses, lignin etc., and the soluble fibres, which can be further classified into two subdivisions: short chain length soluble fibres, including polydextrose, inulin and oligosaccharides, and long chain length soluble fibres including pectins, gums (guar, locust bean, carrageenan, xanthan) and ß-glucan (from oat or barley for example).

Prebiotics are dietary fibres, as they are not digested by human enzymes but fermented by the flora of the large intestine. Thus they increase biomass and frequency of defecation, thus having a positive effect on constipation and on the health of the mucosa of the large intestine. Prebiotic carbohydrates are naturally occurring and can be found in numerous foods, including asparagus, chicory, tomatoes and wheat, as well as being a natural component of breast milk.

The term prebiotic was first defined by Gibson and Roberfroid in 1995. However, the initial definition proved difficult to verify and since then the authors have further developed the concept proposing a new definition: "A prebiotic is a selectively fermented ingredient that allows specific changes both in the composition and/or activity in the gastrointestinal microflora that confers benefit upon host well-being and health" (Nutr Res Rev 2004; 17: 259-275). In order to qualify for prebiotic classification, an ingredient is therefore required to (i) resist digestion (gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption); (ii) be fermented by the gastrointestinal microbiota; and (iii) selectively stimulate the growth and/or activity of intestinal bacteria associated with health and well-being. The latter

criterion is the main distinguishing feature between a dietary fibre and a prebiotic. Prebiotics are generally recognised for their ability to alter the colonic microbiota, promoting a healthier composition and/or activity by increasing the prevalence of saccharolytic (carbohydrate fermenting) micro-organisms while reducing putrefactive (protein fermenting) micro-organisms.

Established non-digestible carbohydrates that fulfil the prebiotic criteria include fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), lactulose, inulin and polydextrose.

Polydextrose is a polysaccharide composed of randomly cross-linked glucose units with all types of glycosidic bonding. Litesse polydextrose is resistant to digestion due to its unique arrangement of glycosidic linkages. Molecularly, (α1→6) bonds predominate, but about 13% of the polymer has (α1→4) linkages, which can be hydrolyzed by enzymes in the human small intestine. It is fermented throughout the colon and is particularly efficient at mediating a prebiotic effect in the distal colon. Human intervention studies have demonstrated that Litesse polydextrose enhances both bifidobacteria and lactobacilli in a dose dependent manner.

Starch is a polysaccharide found commonly in green plants – those containing chlorophyll - as a means of storing energy. Starch forms an integral part of the multi-billion food ingredients market and is characterised by its complex and consolidated nature. Starch is an ideal example of an essential commodity with a wide array of industrial applications, which include paper and card-board making, fermentation, biofuels, biodegradable plastics and detergents, bio-pesticides, surfactants, polyurethane, resins, binders and solvents. However, it is the food industry that provides the largest market for starch and its derivatives.

Starch is either degraded completely in the small intestine to glucose and taken up in the blood or those parts that escape digestion end up in the large intestine where they serve as a general substrate for the colonic microbial flora. Starch and its derivatives in itself do not stimulate specific beneficial colon microbes. Thus, starch in itself is not a prebiotic compound. The partial solution to the problem is to degrade starch into the disaccharide maltose and then use a transglucosidase enzyme to convert the maltose into (α1→6)-linked isomalto-oligosaccharides (IMO) with a degree of polymerization of 2 to 4. These IMO products are, however, too short and are mostly degraded in the small intestine, thus not reaching the colon. That part of the IMO product that reaches the colon is quickly degraded in the proximal part of the

colon by the intestinal microflora and does not reach the distal part were more malign, protein degrading bacteria reside. To outcompete these malign bacteria by stimulating beneficial bacterial species, in particular bifidobacteria, longer isomalto-oligosaccharides are required.

Previously, various methods have been developed for chemical modification of malto-oligosaccharides (MOS) and starch (amylose, amylopectin). More recently, also various transglycosylase enzymes (cyclodextrin glucanotransferase, amylomaltase, starch branching enzyme) have been used for modification of starch (amylose, amylopectin).

The present invention provides further means and methods for the (enzymatic) modification of starch, starch derivatives and/or MOS of different chain length in order to change their functional properties and enhance their nutritional value.

It was surprisingly found that these aims can be met by the use of an α-glucanotransferase of the GTFB type of glucansucrases, member of glycoside hydrolase family GH70 [http://www.cazy.org]. Whereas glucansucrase enzymes catalyze conversion of sucrose into α-glucan poly- and oligosaccharides, it was previously reported that GTFB is not reactive with sucrose at all (Kralj 2004). It is disclosed herein that GTFB displays a high activity towards gluco-oligosaccharides comprising (α1→4) linked glucose residues, such as malto-oligosaccharides (MOS). GTFB catalyzes a disproportionating type of reaction, shortening one substrate molecule and elongating a second substrate molecule. Both products can be substrates again in the next reaction. GTFB activity can thus yield a series of linear gluco-oligosaccharides up to at least DP35. Structural analysis of the products has revealed that GTFB cleaves (α1→4) glucosidic bonds and makes new (α1→4) and (α1→6) bonds. It is the first example of an enzyme with this reaction and product specificity. Accordingly, this enzyme is designated as (1→4)-α-D-glucan: (1→4) (1→6)-α-D-glucan α-D-glucanotransferase or, alternatively, as α-glucanotransferase. Glucansucrase enzymes also use MOS but only as acceptor substrates in the presence of sucrose as donor substrate. This results in synthesis of a range of oligosaccharides, e.g. a maltose extended with a series of glucose units bound via (α1→6) linkages in case of dextransucrase. In case of glucansucrases, however, the (α1→4) linkages in MOS substrates are not cleaved and MOS are only used as acceptor substrate. This is a major difference with the GFTB enzyme that fails to act on sucrose and instead uses MOS as donor and acceptor substrates, cleaving the (α1→4) linkages, and introducing

via a disproportionation type of reaction new (α1→6) and (α1→4) linkages. The products that GTFB can synthesize from starch or starch derivatives contain relatively long isomalto-oligosaccharide (IMO) side chains, in particular IMO side chains with a degree of polymerization of 4 and higher. Part of the IMO-maltodextrin (IMO-MALT) is degraded in the small intestine, being less than what would have been degraded when unmodified starch/derivatives would have been consumed. This is because those parts of the maltodextrin that are close to the IMO part will not be degraded by the intestinal amylases, since amylases need a certain length of linear (α1→4) linked glucose residues to act on. IMO-MALT can therefore be considered as a partially resistant starch derivative giving less glucose production in the small intestine than unmodified maltodextrin would give. This is considered beneficial and contributes to a healthy life style (reduce the risk of developing obesity, type II diabetes, and heart and coronary diseases related to the overconsumption of quickly degradable starch/derivatives). The IMO-MALT part that passes unmodified into the colon will be further degraded by the residual microflora. The IMO part of the IMO-MALT containing (α1→6) linkages can act as a specific substrate for beneficial bifidobacteria, making IMO-MALT a prebiotic ingredient. IMO-MALT therefore has at least the following benefits:

1. partially resistant maltodextrin/starch, giving less glucose production and thereby contributing to prevention of obesity and type II diabetes
2. prebiotic effect stimulating beneficial gut bifidobacteria and thereby promote gut health

In a first embodiment, the invention relates to a method for producing a mixture of gluco-oligosaccharides having one or more (α1→6) glucosidic linkages and one or more (α1→4) glucosidic linkages, comprising contacting a poly- and/or oligosaccharide substrate comprising at its non-reducing end at least two (α1→4)-linked D-glucose units with an α-glucanotransferase enzyme capable of cleaving (α1→4) glucosidic linkages and making new (α1→4) and (α1→6) glucosidic linkages. Alternatively, or in addition, the α-glucanotransferase is capable of transferring a maltosyl-, a maltotriosyl- or a maltotetraosyl-unit to the substrate via a new (α1→6) glucosidic linkage.

It is advantageous, especially for application as dietary fibre, that the gluco-oligosaccharide product(s) are linear or contain linear stretches/moieties of primarily

(α1→4) and (α1→6) glucosidic linkages, rendering them resistant to enzymatic attack in the small intestine. Accordingly, the α-glucanotransferase preferably does not introduce (α1→6) branching points, (α1→2) nor (α1→3) linkages.

In a specific aspect, the α-glucanotransferase (GTFB) is a new member of the GH70 family of glucansucrases [http://www.cazy.org], or a functional homolog thereof having the specified enzymatic activity and substrate preference as described above. For example, the enzyme is selected from those shown in Table 2 or from the group consisting of GTFB from *Lactobacillus reuteri* 121, GTF106B from *Lactobacillus reuteri* TMW 1.106, GTML4 from *Lactobacillus reuteri* ML1 and GTFDSM from *Lactobacillus reuteri* DSM 20016^{A}, GTF from *Lactobacillus fermentum* ATCC 14931, all of which are known in the art both at the protein and nucleic acid level. See in particular Figure 2 and Table 2 herein below for accession numbers. Of course, natural or artificial homologs (mutant) of these known sequences can also be used, including genetically engineered variants displaying desirable properties with respect to thermal stability, substrate specificity, enzymatic activity and the like. In one embodiment, a GTFB homolog is used that shows at least 55%, preferably at least 60%, 75%, like at least 80%, 85%, or at least 90%, sequence identity at the amino acid level with GTFB from the GTFB(-like) enzymes listed in Table 2, or, preferably, with *Lactobacillus reuteri* 121 (AAU08014), GTF106B from *Lactobacillus reuteri* TMW 1.106 (ABP88725), GTML4 from *Lactobacillus reuteri* ML1 (AAU08003), GTFDSM from *Lactobacillus reuteri* DSM 20016^{A} (ABQ83597) or GTF from *Lactobacillus fermentum* ATCC 14931 (ZP_03945763). For example, a GTFB homolog is used that shows at least 55%, preferably at least 60%, 75%, like at least 80%, 85%, or at least 90%, sequence identity at the amino acid level with GTFB from *Lactobacillus reuteri* 121.

It is preferred that the enzyme shows at least 45%, more preferably at least 50%, sequence identity or at least 60% sequence identity at the amino acid level with the catalytic core of GTFB, the catalytic core being represented by the contiguous amino acid sequence W^{790}YRP....IVMNQ^{1484} as found in the protein sequence of GFTB of *L. reuteri* 121: GenBank accession number AAU08014 (protein code).

The GTFB homolog preferably comprises one or more of the following conserved amino acid residues, wherein the numbering corresponds to the position in GTFB of *Lactobacillus reuteri* 121 : Arg1013; Asp1015; Ala1017; Asn1019; Glu1053, Gly1054, Tyr1055, His1124, Asp1125, Gln1126, Arg1127, Lys1128, Asp1479; Ile1480,

Met1482, Asn1483, Gln1484. Preferably, at least the catalytic residues Asp1015, Glu1053 and Asp1125 are present. More preferably, all of these residues are present.

Four conserved regions have been identified in the catalytic domain of GTF enzymes. Previous protein engineering studies have demonstrated that amino acid residues located in conserved sequence region III and IV (see Figure 1 for a sequence alignment) control the product specificity of GTF enzymes regarding the glycosidic bond type formed (Hellmuth et al. Biochemistry (2008); Kralj et al. (2005) Biochemistry 44, 9206-9216; Kralj et al. (2006) FEBS J. 273, 3735-3742). Also region I and region II contain amino acid residues that contribute to enzyme activity and reaction specificity [Kralj et al. (2005); Swistowska et al. (2007) FEBS Lett. 581, 4036-4042.]. In a specific aspect, the enzyme comprises at least one of the following consensus sequences wherein the numbering corresponds to the amino acid position in GTFB (see Figure 1):

A) (conserved region II): F^{1009}DGFRVDAADNIDADVLDQ^{1027}
B) (conserved region III): H^{1048}L(S/V)YNEGYHSGAA^{1060}
C) (conserved region IV): W^{1118}SFVTNHDQRKN(L/V)I^{1131}
D) (conserved region I): G^{1473}LKVQED(I/L)VMNQ^{1484}

In one embodiment, the enzyme is a GTFA member from the glucansucrase group, for instance GTFA from *Lactobacillus reuteri* 121 (GenBank accession number AX306822 or AY697435 (GTF sequence + flanking sequences a.o. GTFB + transposases), that has been genetically engineered to obtain the unique "GTFB-like" substrate specificity and activity required for practicing a method of the present invention. The invention thus also relates to a genetically modified enzyme belonging to the *gtfA* type of glucansucrase enzymes comprising at least one of the mutations of Table 1, said enzyme being capable of cleaving (α1→4)glucosidic linkages and making new (α1→4) and (α1→6) glucosidic linkages and having a substrate preference for poly- and/or oligosaccharide substrates comprising (α1→4)-linked D-glucose units, in particular malto-oligosaccharides. The skilled person will understand that mutations equivalent to those mentioned in Table 1 can be introduced in GTFA enzyme homologues from other organisms. For example, GTF180 from *Lactobacillus reuteri* 180, GTFML1 from *Lactobacillus reuteri* ML1, DSRS from *Leuconostoc mesenteroides* B512-F, GTFD from *Streptococcus mutans* GS-5 (also see van Hijum et al. 2006). Preferably, multiple

mutations selected from Table 1 are introduced. In a specific embodiment, all positions shown in Table 1 are altered.

Table 1: Mutations for introducing GTFB-like (a-glucanotransferase) activity in a GTFA-like (glucansucrase) enzyme

Position#	**Mutation***	**Position#**	**Mutation***
981	L → V	1136	delete S
1026	P → A	1134-1136	NNS → QR
1062	D → G	1137	Q → K
1063	W → Y	1414	N → L
1064	N → H	1463	D → R, T or M
1062-1064	DWN → GYH	1510	W → I or L
1134	N → Q	1512	P → M
1135	N → R	1513	D → N

numbering corresponding to *Lactobacillus reuteri* 121 GTFA
* single-letter amino acid code

Also provided is the use of an enzyme capable of cleaving (α1→4) glucosidic linkages and making new (α1→4) and (α1→6) glucosidic linkages, and/or transferring a maltosyl-, a maltotriosyl- or a maltotetraosyl-unit making a new (α1→6) glucosidic linkage, in a method for producing starch derivatives, preferably (partially) indigestible starch derivatives. In one embodiment, the enzyme is a GTFB type of glucansucrase, for example selected from Table 2 or from the group consisting of GTFB from *Lactobacillus reuteri* 121, GTF106B from *Lactobacillus reuteri* TMW 1.106, GTML4 from *Lactobacillus reuteri* ML1, GTFDSM from *Lactobacillus reuteri* DSM 20016[A] or GTF from *Lactobacillus fermentum* ATCC 14931, or a natural or artificial homolog (mutant) thereof. Preferably, the enzyme is GTFB from *Lactobacillus reuteri* 121.

The person skilled in the art will be able to determine suitable process conditions for performing a method as provided herein by routine experimentation, such as

temperature, incubation time, pH, amount of enzyme, etc. A pH range of 4-5, preferably 4-4.5, can be used. In one embodiment, a temperature of at least 30°C, preferably 37°C is used. In another embodiment, , for instance in view of substrate properties and/or sterility, it may be desirable to work at a more elevated temperature, like at least 70°C, provided that the enzyme is sufficiently heat stable. The dry matter content of the reaction mixture can vary. In one embodiment, it is at least 10%, preferably at least 25%.

Various oligosaccharide or glucan substrates or substrate mixtures can be used in a method according to the invention, provided that they comprises poly- and/or oligosaccharides whose non-reducing end contains (α1→4) linked glucose residues. Preferably, said non-reducing end contains 3 or more consecutive (α1→4)-linked glucose residues. Linear substrates are preferred. Accordingly, also provided is a method for producing a mixture of linear gluco-oligosaccharides having one or more (α1→6) glucosidic linkages and one or more (α1→4) glucosidic linkages, comprising contacting, e.g. by incubating, a linear poly- and/or oligosaccharide substrate comprising at its non-reducing end at least two (α1→4)-linked D-glucose units with an α-glucanotransferase enzyme capable of cleaving (α1→4) glucosidic linkages and making new (α1→4) and (α1→6) glucosidic linkages.

Very good results are observed when the substrate has a degree of polymerization of at least 4, preferably at least 5, more preferably at least 6. The substrate is for instance selected from the group consisting of native starch, modified starch, starch-derivatives, malto-oligosaccharides, amylose, amylopectin, maltodextrins, (α1→4) glucans, reuteran, or combinations thereof. The term "starch derivative" as used herein refers to the product of native starch that has undergone one or more modifications, be it by physical and/or (bio)chemical means. Modifications include depolymerization, cross linking and substitution. The starch or starch derivative can originate from various plant sources, including potato, maize, tapioca or wheat. Some of the other raw materials include; rice, cassava, arrowroot, mung bean, peas, barley, oats, buckwheat, banana, sorghum and lentils. Starch (derivative) from potato, maize, tapioca or wheat is preferred.

In a specific aspect, a method of the invention uses amylomaltase (AMase)-treated starch (ATS), preferably potato starch, as substrate. ATS is commercially available from AVEBE (Veendam The Netherlands) under the trade name Etenia™.

A further specific embodiment employs reuteran as substrate, which is an α-glucan product of reuteransucrase activity and comprises (α1→4) and (α1→6) linkages. Also a mixture of reuteran and malto-oligosaccharides (MOS) yields very good results. Also provided is the treatment of a product obtainable by the incubation of starch, a starch derivative, maltodextrin or maltooligosaccharide with GTFB or GTFB-related enzyme with a hydrolytic enzyme that degrades alpha,1-4-*O*-glycosidic linkages such as alpha-amylase, beta-amylase, alpha-glucosidase, or maltogenic amylase. This provides a slow or non-digestible oligosaccharide/fiber.

As is exemplified herein below, a method of the invention as described above will typically yield a mixture of various linear gluco-oligosaccharides having one or more consecutive (α1→6) glucosidic linkages and one or more, preferably two or more, consecutive (α1→4) glucosidic linkages. For many industrial (e.g. nutritional) applications, the mixture can essentially be used as such and does not require further purification. However, if desired it is of course possible to isolate or remove one or more individual gluco-oligosaccharides from the mixture. To that end, various methods known in the art can be used, for example precipitation-fractionation or chromatography techniques. In one embodiment, a method of the invention comprises subjecting the mixture to size exclusion and/or anion exchange chromatography and isolating at least one gluco-oligosaccharide having one or more (α1→6) glucosidic linkages and one or more, preferably two or more, (α1→4) glucosidic linkages.

As said, an enzyme activity as disclosed herein can give rise to an oligosaccharide with a unique structure. Provided is a linear (i.e. non-branched) gluco-oligosaccharide of the general formula A-B, a glucan comprising such linear moiety or a mixture comprising different gluco-oligosaccharides / moieties of the general formula A-B, wherein the linkage between the moiety A and the moiety B is an (α1→6) glucosidic linkage and wherein B comprises at least two, preferably at least three, consecutive (α1→4) linked glucose residues. Preferably, only (α1→6) and (α1→4) glucosidic linkages are present. The linear moiety of the general formula A-B can be attached to any type of glucan (be it branched or unbranched), for example waxy amylopectin.

In one embodiment, the linear (i.e. unbranched) gluco-oligosaccharide of the general formula A-B, or the mixture comprising different gluco-oligosaccharides of the general formula A-B, characterized in that (i) the linkage between the moiety A and

the moiety B is an (α1→6) glucosidic linkage, (ii) moiety A comprises two or more consecutive (α1→6) glucosidic linkages, preferably wherein A comprises an isomalto-oligosaccharide with a degree of polymerization of at least 4 glucose residues and (iii) B comprises at least two, preferably at least three, consecutive (α1→4) linked glucose residues. For example, the A moiety consists of a series of consecutive (α1→6) linked glucose residues and the B moiety consists of a series of consecutive (α1→4) linked glucose residues.

In another embodiment, the A moiety comprises one or more consecutive (α1→4) glucosidic linkages, preferably wherein A comprises a malto-oligosaccharide with at least four (α1→4) linked glucose residues. Thus, a stretch of (α1→4) linked residues can be linked via an (α1→6) linkage to another stretch of (α1→4) linked residues.

Oligosaccharides of varying chain lengths are provided. In one embodiment, the gluco-oligosaccharide (moiety) has a degree of polymerization (DP) of at least 7 (DP≥7), preferably at least 10 (DP≥10), more preferably at least 15, up to about 50. Oligosaccharides with a length up to more than 30 residues have been observed according to MALDI-TOF-MS analysis. Typically, the relative amount of the high molecular mass products DP10-DP35 in a mixture is less than the amount of products DP<10. An exemplary mixture has an average degree of polymerization of at least 5, preferably at least 6, such as between 6 and 15.

When using malto-oligosaccharides (e.g. DP7, or DP6) as substrates, a series of linear gluco-oligosaccharides are produced, and often different structures of a given DP are observed. For instance, at least 4 DP8 structures were identified, each differing with respect to the number of (α1→6) and (α1→4) glucosidic linkages. See also Figure 6. Generally speaking, the ratio (α1→6) to (α1→4) glucosidic linkages and the structural diversity increases with increasing chain length.

In one aspect, at least 20%, preferably at least 25%, of the linkages is (α1→6). The ratio between (α1→6) and (α1→4) glucosidic linkages generally ranges between 20:80 and 90:10. For example, provided is a linear DP7 product with two consecutive (α1→6) linkages and four consecutive (α1→4) linkages; linear DP8 product with two consecutive (α1→6) linkages and five consecutive (α1→4) linkages; or a DP8 with three consecutive (α1→6) linkages and four consecutive (α1→4) linkages; a DP9 with five consecutive (α1→6) linkages and three consecutive (α1→4) linkages; a DP9 with

four consecutive (α1→6) linkages and four consecutive (α1→4) linkages; a DP10 with five consecutive (α1→6) linkages and four consecutive (α1→4) linkages (see Figure 6)

For application as nutritional ingredient that provides the consumer with a prebiotic fiber as well as a source of energy, the oligosaccharide (mixture) preferably comprises substantial amounts of both (α1→6) and (α1→4) glucosidic linkages. Therefore, in one embodiment the ratio between (α1→6) and (α1→4) glucosidic linkages is between 30:70 and 70:30.

A gluco-oligosaccharide or gluco-oligosaccharide mixture according to the invention has important industrial applications, in particular in nutritional and dietary compositions. Provided is a (human or animal) food product comprising a gluco-oligosaccharide or gluco-oligosaccharide (mixture) according to the invention. The food product can be a solid, semi-solid or liquid food product. The food product can be a conventional nutritional product or a dietetic product. It can be a ready-to-eat food item or a food product that requires further handling prior to consumption, like a bake-off bread product. Exemplary products include a dairy product, baby or infant formula, bakery product, pasta product, noodle product, confectionery product, liquid drink, sport drink, beverage and ice cream.

A further embodiment relates to the use of a gluco-oligosaccharide or gluco-oligosaccharide mixture according to the invention as food additive, for example as prebiotic fiber. Prebiotics can be used in multiple food applications from dairy through to bakery, confectionery and beverage applications. Due to their chemical and physical structure they tend to be highly soluble and have the ability to improve body, texture and mouth feel.

Another useful application relates to inhibiting enzymes of the alpha-amylase type, such as salivary and pancreatic amylases. These enzymes normally act on a (α1→4) malto-oligosaccharide chain with DP ranging from 4-6. It is hypothesized that the presence of (non-hydrolyzable) (α1→6) linkages in an oligosaccharide of the invention only results in enzyme binding but not in glucose release. Addition of such oligosaccharides would lower the rate of metabolism of (e.g. starch metabolism), thereby reducing the glycaemic index (GI) of a food product. A gluco-oligosaccharide (mixture) according to the invention can therefore also help to reduce caloric value and/or the glycaemic load of food products. It thus contributes to a low GI diet. This is of particular interest for human health in general as well as in specific metabolic diseases, including diabetes mellitus and obesity.

In a further embodiment, the gluco-oligosaccharide (mixture) finds its use in a therapeutical or cosmetic application, in particular for controlling a normal skin flora and promoting a healthy skin. The oligosaccharide can bring about a probiotic effect in that it can preferably be utilized selectively by saprophytic bacteria. For example, the oligosaccharide can promote the growth of beneficial skin bacteria (e.g. *Micrococcus kristinae*) compared to the growth of less desirable bacteria such as *Staphylococcus aureus* and *Corynebacterium xerosis*. Provided is a cosmetic composition comprising a gluco-oligosaccharide or gluco-oligosaccharide mixture according to the invention and a suitable carrier. It is also possible to employ the gluco-oligosaccharide (mixture) in a personal care item, for instance to include an absorbent article such as a disposal diaper, sanitary napkin, or the like which can reduce odor and dermatitis (rash) generated when such an absorbent article is worn.

LEGENDS TO THE FIGURES

Figure 1. Amino acid sequence [http://www.cazy.org] alignment of conserved regions (II, III, IV and I) in the catalytic domains of (A) (putative) α-glucanotransferase enzymes, (B) DSRE and DSRP, glucansucrase enzymes containing two catalytic domains (CD1 and CD2) and (C) dextran-, mutan-, alternan- and reuteransucrase enzymes of lactic acid bacteria. The seven strictly conserved amino acid residues (1-7), having important contributions to the -1 and +1 subsites in glucansucrase enzymes are also conserved in the α-glucanotransferase enzymes (shown underlined and in grey scale for GTFA and GTFB of *L. reuteri* 121). Amino acid numbering (italics) is according to GTF180 of *L. reuteri* 180. GTFB amino acid D1015 (putative nucleophilic residue) is shown in bold type.

Figure 2. Phylogenentic tree of GTFB-like proteins derived from ahylogenetic analysis of all 108 glycoside hydrolase family 70 protein sequences available in the Pfam database. See table 2 above for more details on the sequences in this cluster.

Figure 3. TLC analysis of the reaction products of 90 nM GTFB incubated for 13 h in 50 mM NaAc buffer pH 4.7, 1 mM $CaCl_2$ with 25 mM sucrose or 25 mM malto-oligosaccharides. St= standard, Suc, sucrose; G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose; Pol, polymer

Figure 4. Dionex analysis of the reaction products of 90 nM GTFB incubated for either 0, 1, 2 or 8 h in 50 mM NaAc buffer pH 4.7, 1 mM $CaCl_2$ with A) 25 mM maltohexaose or B) 25 mM maltoheptaose.

Figure 5. Dionex analysis of incubated substrate samples without enzyme (panels A) or with 90 nM GTFB (panels B) incubated overnight at 37 °C in 25 mM NaAc pH 4.7, 1 mM $CaCl_2$ with 0.25% amylose-V (abbreviated to AMV) alone as donor substrate and amylose-V with 25 mM glucose (G1) or 25 mM maltose (G2) as acceptor substrates.

Figure 6. Schematic representation of the various α-glucans in the product mixture of the incubation of malto-oligosaccharide DP7 with GTFB(-like) activity.

Figure 7. ^1H NMR spectrum of the product mixture following incubation of malto-oligosaccharide DP7 (100 mM) with GTFB (250 mM) for 120 h.

Figure 8. Possible mode of action of GTFB. Schematic representation of the reaction sequences occurring in the active site of GTFB type of enzymes. The donor and (acceptor) subsites of GTFB type of enzymes are mapped out based on the available 3D structural information of glucansucrase enzymes (with one donor (-1) sub site) and data obtained in the present study. Binding of G7 to subsites –1 and +1 to +6 results in cleavage of the α-1,4 glycosidic bond (G6 released, shown in grey), and formation of a (putative) **covalent intermediate** at subsite -1 (indicated with a grey line). Depending on the acceptor substrate used, **hydrolysis** (with water) or glycosyltransfer with an oligosaccharide acceptor (see below). The *Lb. reuteri* 121 GTFB enzyme also catalyzes a **disproportionation** reaction with maltooligosaccharides. Two molecules of maltoheptaose (G7) for instance are

converted into one G6 molecule and into a G8 product containing 8 glucose residues but with a newly synthesized α-1,6 glycosidic linkage at the non-reducing end.

EXPERIMENTAL SECTION

Introduction

Glucansucrase (GS) (or glucosyltransferase; GTF) enzymes (EC 2.4.1.5) of lactic acid bacteria (LAB) use sucrose to synthesize a diversity of α-glucans with (α1→6) [dextran, mainly found in *Leuconostoc*], (α1→3) [mutan, mainly found in *Streptococcus*], alternating (α1→3) and (α1→6) [alternan, only reported in *Leuconostoc mesenteroides*], (α1→4) [reuteran, by GTFA and GTFO from *Lactobacillus reuteri* strains] glucosidic bonds {Monchois, 1999; van Hijum, 2006; Arguello-Morales, 2000 ;Kralj, 2002 ;Kralj, 2005 }.

Lactobacillus reuteri 121 uses the glucansucrase GTFA and sucrose as substrate to synthesize a reuteran product with large amounts of (α1→4) glucosidic linkages. Upstream of this *gtfA* gene another putative glucansucrase gene was identified designated *gtfB*. Previously it has been shown that after cloning and expression of this gene the enzyme showed no activity on sucrose as substrate. Also in the genome of *L. reuteri* ML1 the putative catalytic and C-terminal domain of a gtfB homolog, gtfML4, was identified upstream of gtfML1 encoding a mutansucrase {Kralj, 2004}. In the recently elucidated genome sequence of *L. reuteri* DSM 20016 also a GTFB homolog could be identified (73% identity 85% similarity in 883 amino acids). Furthermore, also *L. reuteri* TMW1.106 contains besides a GTFA homolog (GTFA106) a GTFB homolog (GTFB106). This enzyme showed 92% identity and 95% similarity in 1383 amino acids with GTFB from *L. reuteri* 121. However, in contrast to GTFB, GTF106B showed low (after 27 h of incubation) hydrolyzing activity on sucrose {Kaditzky, 2008 }.

It is shown herein that GTFB has a disproportionation type and polymerizing type of activity on malto-oligosaccharides. The enzyme uses malto-oligosaccharides (containing only (α1→4) glucosidic linkages) as substrate to synthesize oligosaccharides up to a degree of polymerization (DP) of 35. During this elongation/polymerization process large numbers of (α1→6) glucosidic linkages (~32%) are introduced in the final product. Furthermore, we show that with a large amylose substrate (Amylose-V) as donor and smaller saccharides (glucose, maltose) as acceptor

also larger saccharides linked via (α1→4) glucosidic linkages are synthesized containing more than five glucose units. Detailed analysis of the product synthesized from maltoheptaose by methylation analysis and 1H NMR showed that up to 32% of (α1→6) glucosidic linkages were introduced in the final product. Although the primary structure of GTFB is similar to GH70 enzymes, including the permuted $(\beta/\alpha)_8$ barrel, its activity resembles more the GH13 α-amylase type of enzymes using malto-oligosaccharides as preferred substrate.

Materials and Methods

Bacterial strains, plasmids, media and growth conditions. *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, Calif.) was used as host for cloning purposes. Plasmids pET15b (Novagen, Madison, WI) was used for expression of the (mutant) *gtfB* genes in *E. coli* BL21 Star (DE3). (Invitrogen). *E. coli* strains were grown aerobically at 37 °C in LB medium {Ausubel, 1987}. *E. coli* strains containing recombinant plasmids were cultivated in LB medium with 100 μg ml^{-1} ampicillin. Agar plates were made by adding 1.5% agar to the LB medium.

Amino acid sequence alignment of GTFB from *L. reuteri*. Multiple amino acid sequence alignments of GTFB and known glucansucrases and putative α-glucanotransferases from lactic acid bacteria were made with the ClustalW interface in MEGA version 4 (www.megasoftware. net) with gap-opening and extension penalties of 10 and 0.2, respectively.

Molecular techniques. General procedures for gene cloning, *E. coli* DNA transformations, DNA manipulations, and agarose gel electrophoresis were as described {Sambrook, 1989}. Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the enzyme suppliers (New England Biolabs, Beverly, MA; Roche Biochemicals, Basel, Switzerland). Primers were obtained from Eurogentec, Seraing, Belgium. Sequencing was performed by GATC (Konstanz, Germany). DNA was amplified by PCR on a DNA Thermal Cycler PTC-200 (MJ Research, Waltham, Massachusetts) using *Pwo* DNA polymerase (Roche Biochemicals) or Expand High Fidelity polymerase (Fermentas). Plasmid DNA of *E. coli* was isolated using a Wizard Plus SV plasmid extraction kit (Sigma)

Construction of plasmids. Appropriate primer pairs and template DNA were used to create two different expression constructs with a C-terminal His-tag: for

the complete GTFB (1587 amino acids), constructed using three separate PCR reactions using the method previously described for GTFA from *Lb. reuteri* 121 (see below){Kralj, 2002}, and an N-terminally truncated variant (without N-terminal) variable region of GTFB (889 amino acids).

To facilitate future mutagenesis and nucleotide sequencing, *gtfB* was divided and cloned in three parts. The first of the two *Pst*I restriction sites (1385 bp, 1751 bp) was altered, using the megaprimer method {Sarkar, 1990} and the following primers: BpstIfor 5'-GTAAGTCGTTACTC**A**GCAGATGCTAATGG-3' containing a mutated *Pst*I restriction site (underlined, silent mutation by change of base shown in bold face), and, BpstI rev 5'-GGTCAGTAAATCCACCGTTATTAATTGG-3'. In a subsequent PCR reaction the amplified product (420 bp) was used as (reverse) primer together with Bfor: 5'-GCAATT*GTCGAC***CATGG**ATACAAATACTGGTGATCAGCAAACTGAACA-GG-3' containing *Sal*I (italics) and *Nco*I (bold) restriction sites. The resulting product of 1700 bp was digested with *Sal*I and *Pst*I and ligated in the corresponding sites of pBluescript II SK+, yielding pBSP1600. The amplified 420 bp product was also used as a forward primer together with BrevBamHI 5'-GGACTGTTATCACTATTATTATTTCCGGCC-3' 70 bp downstream of a *Bam*HI restriction site. The resulting product of (~1500 bp) was digested with *Pst*I and *Bam*HI and ligated in the corresponding sites of pBluescript II SK+, yielding pBPB1000. The third fragment was obtained using primers BforBamHI 5'-CGCTATGTAATTGAACAGAGTATTGCTGC-3' 200 bp downstream of a *Bam*HI restriction site and BRevHis 5'-CCTCCTT*TCTAGA***TCT**ATTA<u>GTGATGGTGATGGTGATG</u>GTTGTTAAAGTTTAATG AAATTGCAGTTGG-3' containing *Xba*I (italics) and *Bgl*I (bold) and a 6× histidine tag (underlined). The resulting product of 2300 bp was digested with *Bam*HI and *Xba*I and ligated in the corresponding sites of pBluescript II SK+, yielding pBBX2300. The complete gene was assembled as follows: pBPB1000 was digested with *Pst*I and *Bam*HI and the resulting fragment was ligated into pBSP1600 restricted with the same restriction enzymes yielding pBSB2600 (containing the first and second fragment). Subsequently, plasmid pBBX2300 was digested with *Bam*HI and *Sac*II (present on the plasmid, used instead of *Xba*I) and the fragment was ligated into pBSB2600 yielding pBSS4900 containing the full length *gtfB* gene. This plasmid was

digested with *Nco*I and *Bgl*II and the *gtfB* gene was ligated in the *Nco*I and *Bam*HI sites of pET15b, yielding pET15B-GTFB.

Expression and purification of GTFB. An overnight culture of *E. coli* BL21star (DE3) harbouring (mutant) GTFB {Kralj, 2004} was diluted 1/100. Cells were grown to OD_{600} 0.4 and induced with 0.2 mM IPTG, after 4 h of growth cells were harvested by centrifugation (10 min at 4 °C at 10,000 × *g*). Proteins were extracted by sonication and purified by Ni-NTA and anion exchange chromatography as described previously for the GTFA (reuteransucrase) from *Lactobacillus reuteri* 121 {Kralj, 2004}, with the following modification: for anion exchange chromatography a 1 ml Hi-trap™ Q HP colum was used (Ge Healthcare).

(i) pH and temperature optima. pH and temperature optima were determined by measuring qualitatively on TLC the amount of oligo- and polysaccharides synthesized from 25 mM maltotetraose after overnight incubation (data not shown).

(ii) Products synthesized from malto-oligosaccharides and other saccharides. *Single substrate incubations* 90 nM GTFB and 25 mM of sucrose (Acros), raffinose (Sigma), turanose (Sigma), palatinose (Sigma), panose (Sigma), 0,25 % Amylose-V (Avebe, Foxhol, The Netherlands), 0.25 % amylopectin, 25 mM isomaltopentaose, isomaltohexaose (sigma), malto-oligosaccharides with a different degree of polymerization (G2-G7) were incubated separately overnight in 25 mM NaAc pH 4.7 1 mM $CaCl_2$ at 37 °C and analysed by TLC. Products synthesized from G6 and G7 over time were analyzed by TLC and HPAEC.

Acceptor / donor studies. 90 nM GTFB and 25 mM of glucose and malto-oligosaccharides with a different degree of polymerization (G2-G7) were incubated overnight together with 0,25% amylose-V in 25 mM NaAc pH 4.7 1 mM $CaCl_2$ at 37 °C and analysed by TLC.

(i) Characterization of the oligosaccharides and polysaccharides produced from G7. Purified GTFB enzyme preparations (90 nM) were incubated for 7 days with 150 mM G7 (sigma), using the conditions described above under enzyme assays. Oligo- and polysaccharides produced by purified recombinant GTFB were

separated by precipitation with 96% ethanol (most of the larger saccharide product precipitates) {van Geel-Schutten, 1999}.

(ii) Methylation analysis. Oligo- and polysaccharides were permethylated using methyl iodide and dimesyl sodium ($CH_3SOCH_2^-$-Na^+) in DMSO at room temperature {Kralj, 2004}

RESULTS

Alignment of GTFB

GTFB is the first representative of a group of homologues enzymes identified in different Lactobacilli. Alignments of members of this novel group of enzymes with other glucansucrases showed similarities but also some characteristics differences. The three catalytic residues present (D1024, E1061 and D1133 GTFA *L. reuteri* 121 numbering used throughout unless indicated otherwise) in glucansucrases are also present in the group of α-glucanotransferases (D1015, E1053 and D1125 GTFB *L. reuteri* 121 numbering. Nevertheless, a large number of amino acid residues conserved in glucansucrase in region I, II, III and IV are absent in the α-glucanotransferase group of enzymes (Fig. 1). In region II (encompassing the putative nucleophilic residue) the conserved V1025 (Pro in GTFA and GTFO) is substituted by an alanine in the α-glucanotransferases. Region III, the region downstream of the putative acid/base catalyst E1061 is completely different between the glucansucrases and the α-glucanotransferases.

Nevertheless, a large number of amino acid residues conserved in glucansucrase in region I, II, III and IV are absent in the α-glucanotransferase group of enzymes (Fig. 1). In region II (encompassing the putative nucleophilic residue) the conserved P1025 (Pro in GTFA and GTFO, Val in most GTFs) is substituted by a alanine in the α-glucanotransferases. Region III, the region downstream of the putative acid/base catalyst E1061 is completely different between the glucansucrases and the α-glucanotransferases. The conserved tryptophan 1063 is substituted by a tyrosine residue in the α-glucanotransferases (Fig. 1). In region IV the GTFB homologues contain a gap immediately upstream of the location of the Q1137 residue and at the position of the conserved glutamine a lysine residue is present.

GTFB homologs

Gene and protein sequence databank searches showed several sequences that may have the same catalytic activity as GTFB. The info is based on a phylogenetic tree of all glycoside hydrolase family 70 members (108 sequences as available in the Pfam database on 27 April 2010). Also the phylogenetic tree is available from the Pfam server, see Figure 2.

Table 2. Glycoside hydrolase family 70 sequences from the Pfam database (http://pfam.sanger.ac.uk) with clear similarity to GTFB, apparent from the alignments and phylogenetic trees. Note that no. 9 is GTFB and that the numbers follow the order as seen in the phylogenetic tree of Figure 2.

	UniProt entry	**Microorganism**
1	B1YMN6[1]	Exiguobacterium sibiricum 255-15
2	C0X0D3	Lactobacillus fermentum ATCC 14931
3	C2F8B9	Lactobacillus reuteri MM4-1
4	C0YXW9	Lactobacillus reuteri MM2-3
5	A5VL73	Lactobacillus reuteri DSM 20016
6	B2G8K2	Lactobacillus reuteri JCM 1112
7	B7U9D3	Weissella confusa MBF8-1
8	A9Q0J0	Lactobacillus reuteri TMW1.106
9	**Q5SBM0**	**GTFB (Lactobacillus reuteri 121)**
10	Q5SBN1	Lactobacillus reuteri ML1
11	Q9R4L7[2]	Leuconostoc mesenteroides
12	B1YMN6[1]	Exiguobacterium sibiricum 255-15

[1] This sequence is listed twice in the table since two fragments of this sequence are in the tree.

[2] The apparent sequence similarity of number 11 is based on 20 amino acids only. This sequence is therefore ignored.

Of the nine GTFB-like sequences, the putative dextransucrase from *Lactobacillus reuteri* DSM 20016 (nr. 5 in the table) was cloned and expressed in *Escherichia coli*. The recombinant protein was purified by a combination of affinity and anion exchange chromatography. The purified protein showed GTFB-like activity when incubated with malto-oligosaccharides. The putative dextransucrase from *Lactobacillus reuteri* DSM 20016 showed no activity with sucrose, instead it uses maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose as substrate

producing a ladder of shorter and longer products. Proton-NMR analysis of the products demonstrated that α-1,6-glycosidic bonds were introduced, as also seen for the GTFB incubations. Moreover, the putative dextransucrase from *Lactobacillus reuteri* DSM 20016 also increased the percentage of α-1,6-glycosidic bonds in soluble potato starch of Sigma-Aldrich.

Cloning and expression of GTFB

The full length, N-terminal truncated version and putative nucleophilic mutant of GTFB were constructed and expressed successfully. Both the full length as well as the N-terminal truncated variant showed clear activity on malto-oligosacharides as measured by TLC (data not shown). The constructed truncated GTFB version (GTFB-ΔN) was not expressed as efficiently as the full length GTFB and therefore all experiments were performed using full length GTFB. To rule out any background activity emerging from *E. coli* itself, an empty pET15b plasmid was purified, and already after His-tag purification no activity on malto-oligosaccharides (G2-G7) was detected (data not shown). Furthermore, the purified full length D1015N (putative) nucleophilic mutant showed no activity on malto-oligosaccharides (G2-G7; data not shown).

Enzyme characteristics

The optimal activity for GTFB with maltotetraose as a substrate as determined qualitatively by TLC was at a temperature of 30-37 °C and a pH of 4-5 (data not shown). Combinations of different temperatures and pH buffers indicated optimal activity at a temperature of 37 °C and a pH of 4.7, which was used in all subsequent assays.

Donor substrates

Since it had already been shown that GTFB is not able to use sucrose as donor substrate {Kralj, 2004}, different sucrose analogues (turanose, palatinose) and raffinose were tested for activity. We were not able to detect activity on any of these substrates (data not shown). Also no activity was observed on isomalto-oligosaccharides (IG5 and IG6) substrates (data not shown). Activity on

oligosaccharides derived from a partially purified reuteran (GTFA) hydrolysate or panose was also not detected (data not shown). However, on linear malto-oligosaccharides clear activity was observed already after short incubation times. Especially on malto-oligosaccharides with a degree of polymerization of 4 and larger, different oligosaccharides were synthesized (Fig. 3). From a DP of 6 and larger, besides oligosaccharides also larger polymeric material started to accumulate. On amylose-V (Avebe, Foxhol, The Netherlands) also low activity (mainly G1 and G2 release) was observed (Fig. 5). On maltose alone virtually no activity was observed. However, when amylose-V was incubated simultaneously with glucose or maltose a range of oligosaccharides were synthesized (Fig. 5). Using amylose-V as donor and glucose as acceptor larger numbers of maltose were synthesized compared to incubation on amylose-V alone, indicating (α1→4) synthesizing capability. On amylose-V alone virtually no G3 was released. Incubation of amylose-V with maltose as acceptor clearly yielded panose and G3 indicating GTFB capability to besides panose (indicating (α1→6) synthesizing capability) also maltotriose was synthesized demonstrating the enzyme capability to synthesize (α1→4) glucosidic linkages.

Product characterization in time on G6 and G7

The first reaction products detectable on G6 were G1 (glucose) and G5 (maltopentaose) (Fig. 4). Also on G7 the first products released were G1 (glucose) and G6 (maltohexaose). Later in time on G6 also other malto-oligosaccharides such as G2, G3 and G4 appeared. Also unknown saccharides next to G7 and G8 were identified, which besides (α1→4) glucosidic linkages also must contain other linkages indicated by the shift in their retention time.

After incubation of maltoheptaose with GTFB for 120h, the 1D ^{1}H-NMR spectrum of the total product mixture (Fig. 7) indicated the presence of newly formed (α1→6) linkages by a broad signal at $\delta_{H\text{-}1}$ ~ 4.96. The (α1→4) signal is present at $\delta_{H\text{-}1}$ ~ 5.39. After 120 h of incubation, the ratio (α1→4):(α1→6) is 67:33 in the product mixture. MALDI-TOF MS analysis of the product mixture revealed the presence of compounds ranging from DP2 up to DP35 (*m/z* 365 - *m/z* 5711, $[M+Na]^{+}$).

In the reaction mixture obtained from incubation of MOS DP7 with recombinant GFTB, seventeen different structures (Figure 6), ranging from DP2-DP10, could be elucidated in detail by NMR spectroscopy. The elucidated structures constitute only a part of the total number of compounds that were formed. More high

molecular mass products are present, including polysaccharides. It is clear that the oligosaccharides smaller than DP7 must be stemming from hydrolysis activity of GTFB on the substrate [products containing (α1→4) only] as well as from hydrolysis activity on the formed oligosaccharides [products containing (α1→4) and (α1→6)]. It has to be noted that no structures were found having a 6-substituted reducing-end glucose residue. Until now, only one structure (DP8) was found having a (α1→6)-linked glucose residue elongated by successive (α1→4)-linked glucose residues. In the other cases, only (successive) (α1→6) elongation has occurred. All oligosaccharides have a 4-substituted glucose residue at the reducing end. However, in the 1D ^{1}H NMR spectrum (Fig. 7) of the total product mixture a trace of a terminal reducing -(1→)-D-Glc unit was found (H-1α at δ 5.240 and H-1ß at δ 4.669), but this unit was not found in the elucidated structures.

Thus, recombinant GTFB catalyzes the cleavage of only (α1→4) linkages and initiates formation of new (α1→4) and (α1→6) bonds. In this way, many products are formed, ranging from monosaccharide to polysaccharide (DP>30). Different structures for a single-molecular-mass product are possible, as is shown clearly for the formed DP7- and DP8-oligosaccharides(-alditols). Furthermore, it was observed that the amount of (α1→6) bonds compared to (α1→4) bonds increases with increasing chain length, to a maximum of 50:50. No 4,6- or other types of branching points are introduced. The fact that the recombinant GTFB enzyme showed similar activities on the free malto-oligosaccharides as well as on their reduced forms (malto-oligosaccharide-alditols), demonstrates a non-reducing end elongation mechanism.

Important conclusions which can be drawn from the above results are the following:

- no structures were found having a 6-substituted reducing-end glucose residue;
- GTFB catalyzes the cleavage of only (α1→4) linkages and initiates formation of new (α1→4) and (α1→6) bonds;
- the amount of (α1→6) bonds compared to (α1→4) bonds increases with increasing chain length, to a maximum of 50:50.
- no 4,6- or other types of branching points are introduced;
- GTFB has a non-reducing end elongation mechanism.

Introducing GTFB like activity in GTFA via protein engineering

Previous protein engineering studies have demonstrated that amino acid residues located in conserved sequence region III and IV (see figure 1 for a sequence alignment) control the product specificity of GTF enzymes regarding the glycosidic bonding type formed. Also region I and region II contain amino acid residues that contribute to enzyme activity and reaction specificity. The amino acid residues of the conserved sequence regions form part of the acceptor substrate binding region of GTF enzymes. In the polymerization reaction using sucrose as substrate these residues interact with the glucose (subsite-1) and fructose (subsite +1) moiety of sucrose. As GTFB utilizes maltoheptaose (and other malto-oligosaccharides) as substrate a glucose moiety will interact at the acceptor subsites in the GTFB enzyme. The unique substrate specificity of GTFB compared to the traditional GTFs is therefore most likely determined by differences at the acceptor subsites. Thus, to introduce GTFB-like activity in a traditional GTFA enzyme it is envisaged to substitute residues at the acceptor subsites, located in the regions I, II, III and IV, to resemble the sequence of the GTFB enzyme.

Furthermore, the 3D structure of GTF180 (Vujicic, PhD thesis University of Groningen) shows some additional residues interacting at subsites -1 and +1 that are likely important for the interconversion of reaction specificity of GTFs. The 981 L→V mutations is based on an interaction seen in the 3D structure of GTF180, where Leu981 has a Van Der Waals interaction with the fructosyl moiety of sucrose. In GTFB this position is occupied by a valine residue as well as in the other α-glucanotransferases GTFDSM, GTF106B, GTFML4. The 1463 D→R, T or M mutations are aimed at substituting the aspartate residue, which is highly conserved in glucansucrases, but not in GTFB and the related enzymes, and interacts with the glucose moiety at subsite -1 in GTF180 3D structure.

Additionally, the mutations may be combined in any manner to obtain a stronger effect in alteration of the reaction specificity GTF enzymes. The proposed mutations are as follows:

Region I

position W1510:W→I/L

position P1512:P→

position D1513:D→N

Region II

position 1026: P→A

Region III

position 1062: D→G

position 1063: W→Y

position 1064: N→H

position 1062-1064 DWN→GYH

Region IV

position 1134: N→Q

position 1135: N→R

position 1136: S→delete this residue

position 1134-1136: NNS→QR

position 1137:Q→K

3D structure

position 981: L→V

position 1414: N→L

position 1463: D→R,T or M

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Claims

1. A method for producing a mixture of gluco-oligosaccharides having one or more consecutive (α1→6) glucosidic linkages and one or more consecutive (α1→4) glucosidic linkages, comprising contacting a poly- and/or oligosaccharide substrate comprising at its non-reducing end at least two α-1→4-linked D-glucose units with an α-glucanotransferase enzyme capable of cleaving (α1→4)glucosidic linkages and making new (α1→4) and (α1→6) glucosidic linkages.

2. Method according to claim 1, wherein said α-glucanotransferase does not introduce (α1→6) branching points nor (α1→2) or (α1→3) linkages.

3. Method according to claim 1 or 2, wherein said α-glucanotransferase is a GTFB type of enzyme, or a functional homolog thereof having the specified enzymatic activity.

4. Method according to any one of the preceding claims, wherein said α-glucanotransferase is selected from the group consisting of GTFB from *Lactobacillus reuteri* 121, GTF106B from *Lactobacillus reuteri* TMW 1.106, GTML4 from *Lactobacillus reuteri* ML1, GTFDSM from *Lactobacillus reuteri* DSM 20016[A] and GTF from *Lactobacillus fermentum* ATCC 14931, or a homolog thereof showing at least 55% sequence identity.

5. Method according to any one of the preceding claims wherein said substrate has a degree of polymerization of at least 4, preferably at least 6.

6. Method according to any one of the preceding claims, wherein said substrate is selected from the group consisting of starch, waxy starch, high amylose starch, their derivatives, malto-oligosaccharides, amylose, amylopectin, maltodextrins, (α1→4) glucans, reuteran, or combinations thereof.

7. Method according to claim 6, wherein said starch, waxy starch, high amylose starch or starch derivative is derived from potato, maize, tapioca, pea, mung bean, rice or wheat.

8. Method according to claim 6 or 7, wherein said starch derivative is produced by treating starch, waxy starch or high amylose starch with amylomaltase/4-alpha-glucanotransferase or glycogen-branching enzyme.

9. Method according to any one of the preceding claims, further comprising the step of isolating from the mixture at least one gluco-oligosaccharides having one or more consecutive (α1→6) glucosidic linkages and one or more consecutive (α1→4) glucosidic linkages, preferably using precipitation-fractionation and/or chromatography techniques.

10. A mixture comprising distinct linear gluco-oligosaccharides having one or more consecutive (α1→6) glucosidic linkages and one or more, preferably two or more, consecutive (α1→4) glucosidic linkages, obtainable by a method according to any one of claims 1 to 8.

11. An isolated linear gluco-oligosaccharide having one or more consecutive (α1→6) glucosidic linkages and one or more, preferably two or more, consecutive (α1→4) glucosidic linkages, obtainable by a method according to claim 9.

12. A linear gluco-oligosaccharide or gluco-oligosaccharide moiety of the general formula A-B, or a mixture comprising different linear gluco-oligosaccharides or gluco-oligosaccharide moieties of the general formula A-B, wherein the linkage between A and B is an (α1→6) glucosidic linkage and wherein B comprises at least two, preferably at least three, consecutive (α1→4) linked glucose residues.

13. Gluco-oligosaccharide or gluco-oligosaccharide mixture according to claim 12, wherein the ratio between (α1→6) and (α1→4) glucosidic linkages ranges from 20:80 to 90:10, preferably at least from 30:70 to 70:30.

14. Gluco-oligosaccharide or gluco-oligosaccharide mixture according to claim 12 or 13, wherein A comprises two or more consecutive (α1→6) glucosidic linkages, preferably wherein A comprises an isomalto-oligosaccharide with a degree of polymerization of at least 4 glucose residues.

15. Gluco-oligosaccharide or gluco-oligosaccharide mixture according to claim 12 or 13, wherein A comprises one or more consecutive (α1→4) glucosidic linkages, preferably wherein A comprises a malto-oligosaccharide with a degree of polymerization of at least 4 glucose residues.

16. Gluco-oligosaccharide or gluco-oligosaccharide mixture according to any one of claims 12-15, wherein the (average) degree of polymerization is at least 7, preferably at least 10, more preferably at least 15.

17. Nutritional or cosmetic composition comprising a gluco-oligosaccharide or gluco-oligosaccharide mixture according to any one of claims 10-16, preferably wherein said nutritional composition is selected from the group consisting of a dairy product, baby or infant formula, bakery product, confectionery product, cereal bar, candy bar, pasta product, noodle product, liquid drink, sport drink, beverage and ice cream.

18. Use of a gluco-oligosaccharide or gluco-oligosaccharide mixture according to any one of claims 10-16 as a nutritional or cosmetic additive, in particular as prebiotic fiber.

19. A genetically modified enzyme belonging to the GTFA type of glucansucrase enzymes comprising at least one of the mutations of Table 1, said mutant enzyme being capable of cleaving (α1→4)glucosidic linkages and making new (α1→4) and (α1→6) glucosidic linkages and having a substrate preference for poly- and/or oligo-saccharide substrate comprising (α1→4)-linked D-glucose units, in particular malto-oligosaccharides.

20. Use of an enzyme capable of cleaving (α1→4) glucosidic linkages and making new (α1→4) and (α1→6) glucosidic linkages, and/or transferring a maltose,

maltotriose or maltotetraosyl-unit making a new (α1→6) glucosidic linkage, in a method for producing starch derivatives, preferably (partially) indigestible starch derivatives.

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Figure 1

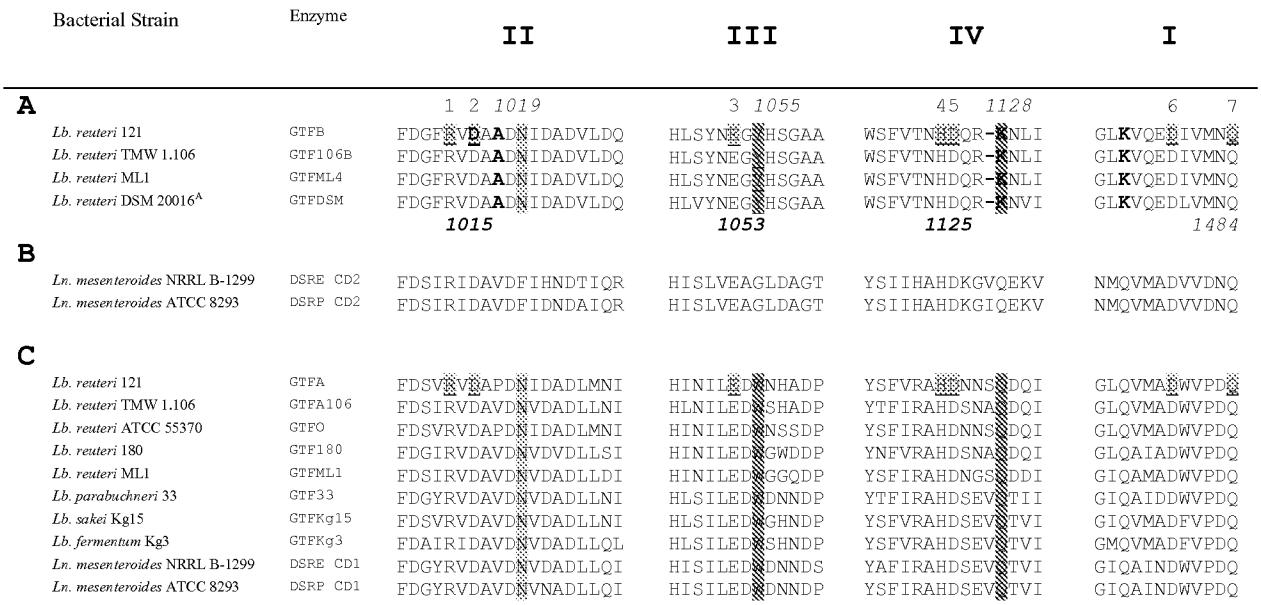

Figure 2

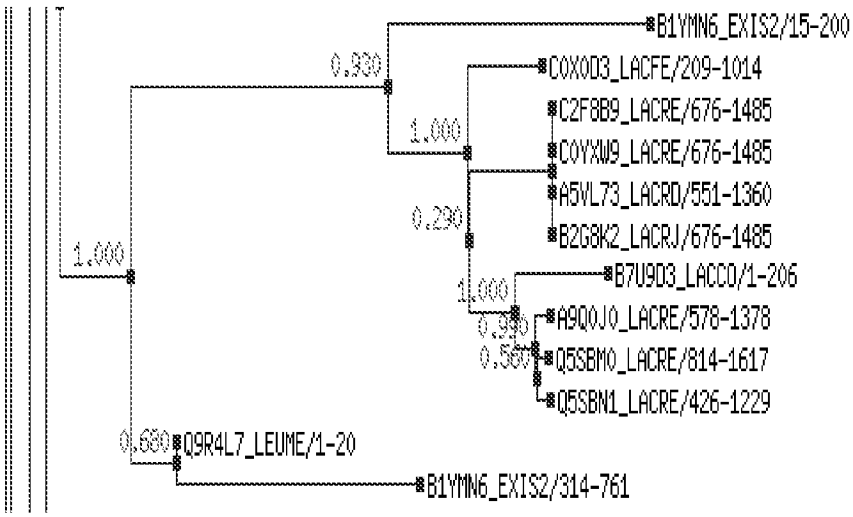
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Figure 3

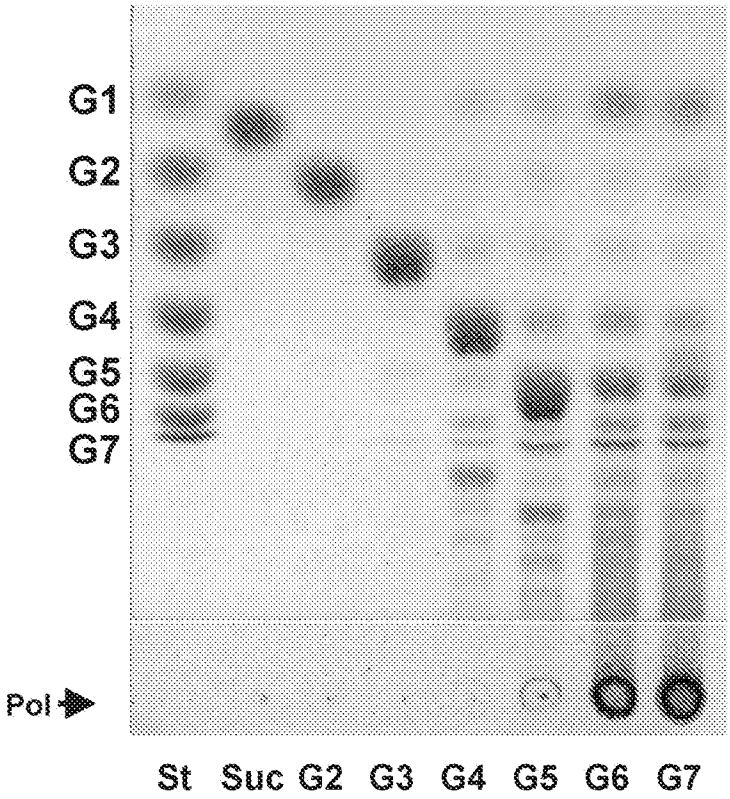


Figure 4

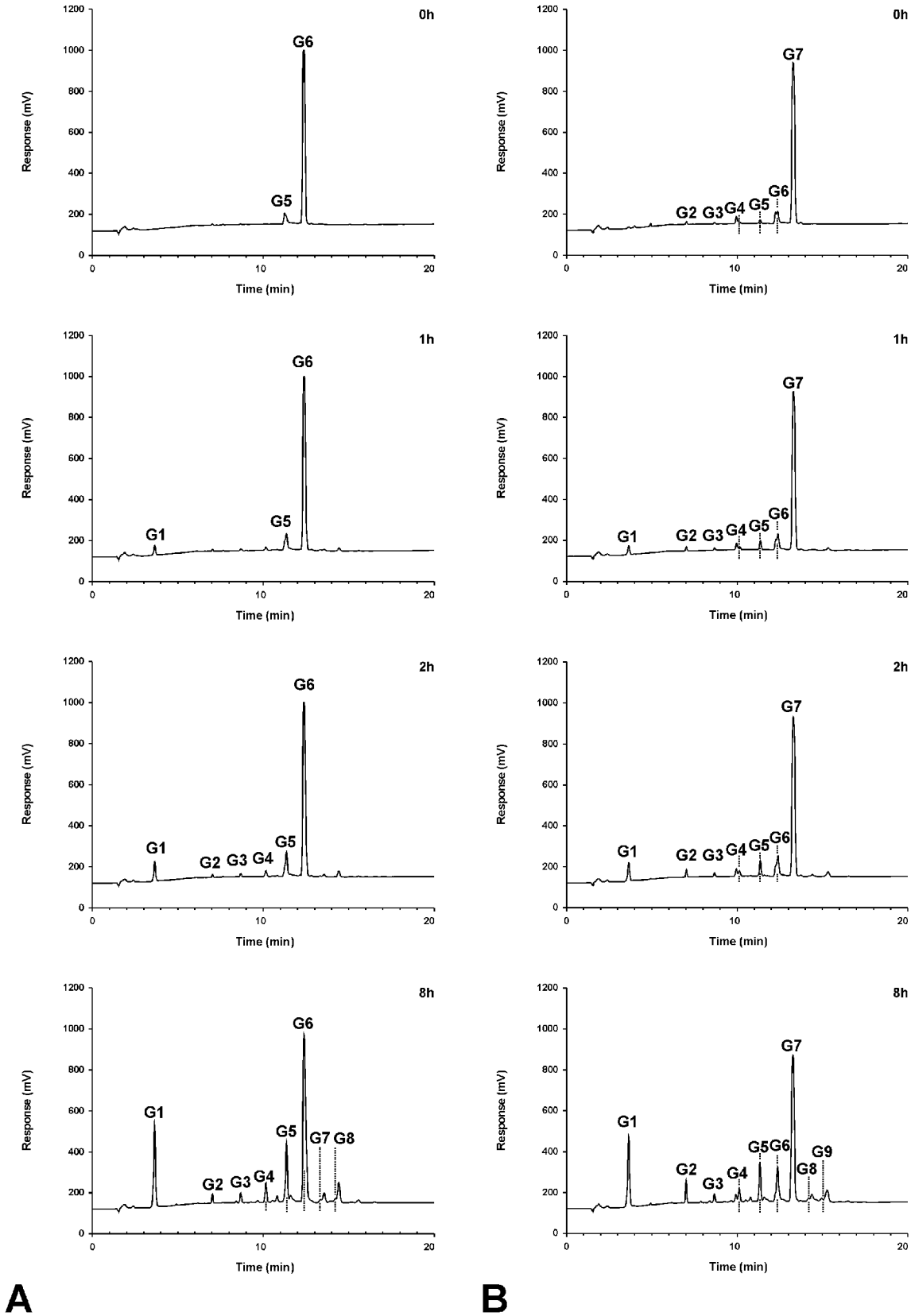


Figure 5

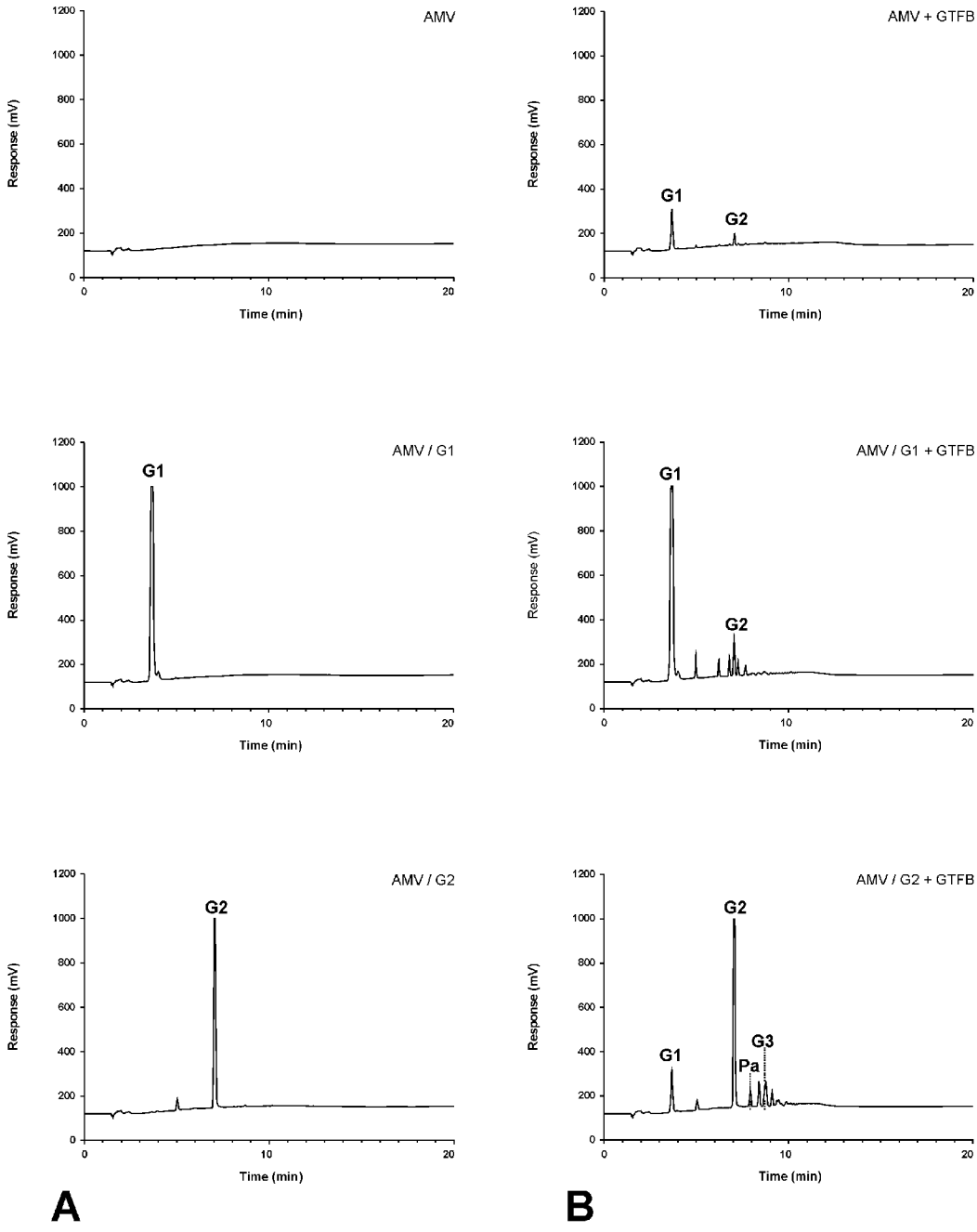
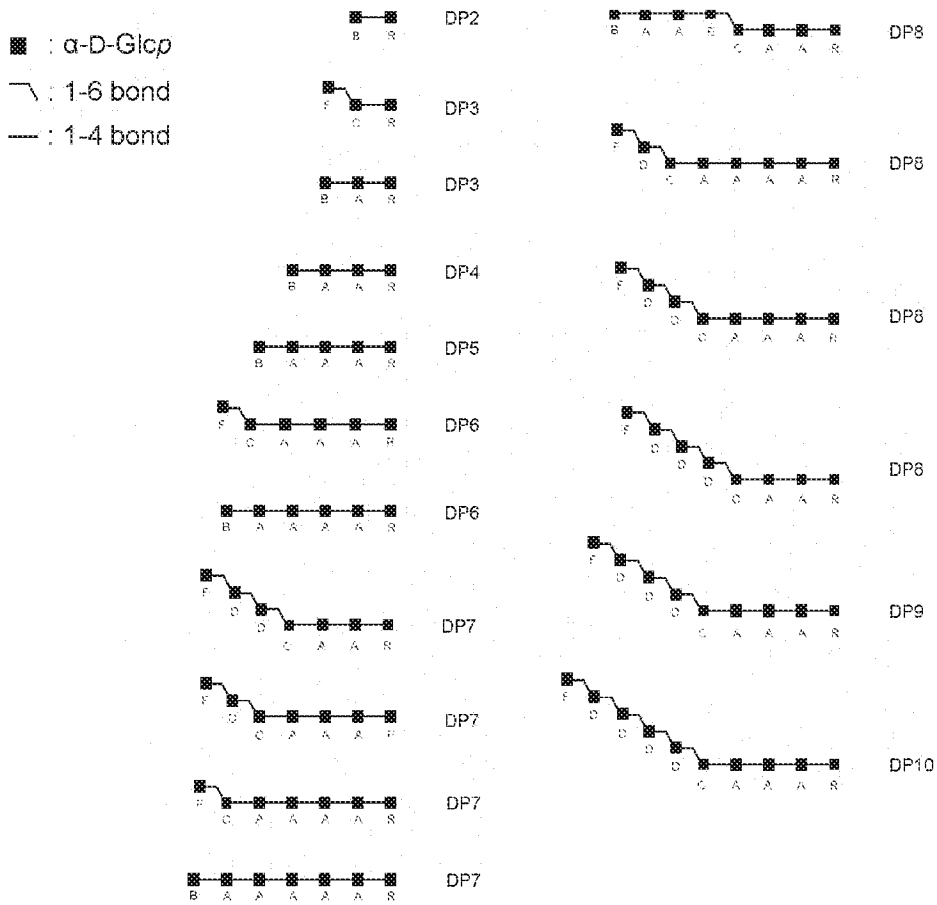

AMV
AMV + GTFB
AMV / G1
AMV / G1 + GTFB
AMV / G2
AMV / G2 + GTFB
Response (mV)
Time (min)
G1
G2
G3
Pa
A
B

Figure 6



A -(1→4)-α-D-Glcp-(1→4)-
B α-D-Glcp-(1→4)-
C -(1→6)-α-D-Glcp-(1→4)-
D -(1→6)-α-D-Glcp-(1→6)-
E -(1→4)-α-D-Glcp-(1→6)-
F α-D-Glcp-(1→6)-
Rα/β -(1→6)-D-Glcp
Rα/β -(1→4)-D-Glcp

Figure 7

A -(1→4)-α-D-Glcp-(1→4)-
B α-D-Glcp-(1→4)-
C -(1→6)-α-D-Glcp-(1→4)-
D -(1→6)-α-D-Glcp-(1→6)-
E -(1→4)-α-D-Glcp-(1→6)-
F α-D-Glcp-(1→6)-
Ra/β -(1→6)-D-Glcp
Ra/β -(1→4)-D-Glcp

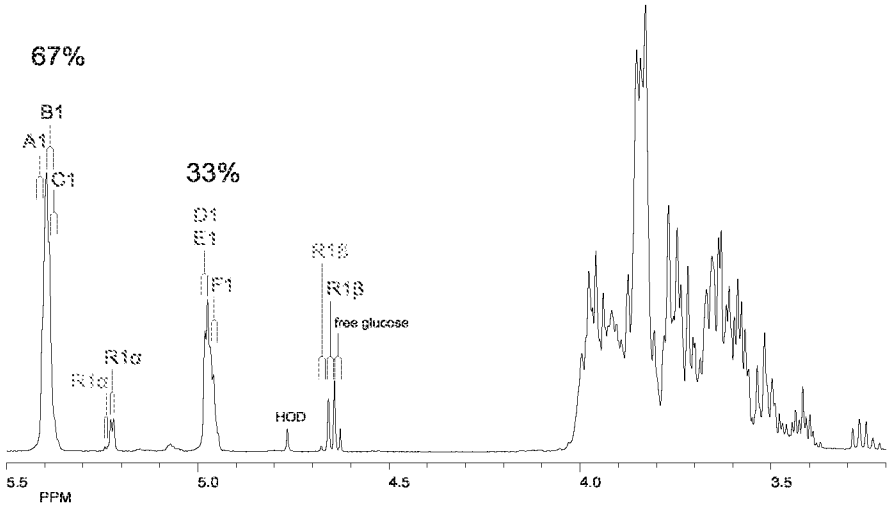
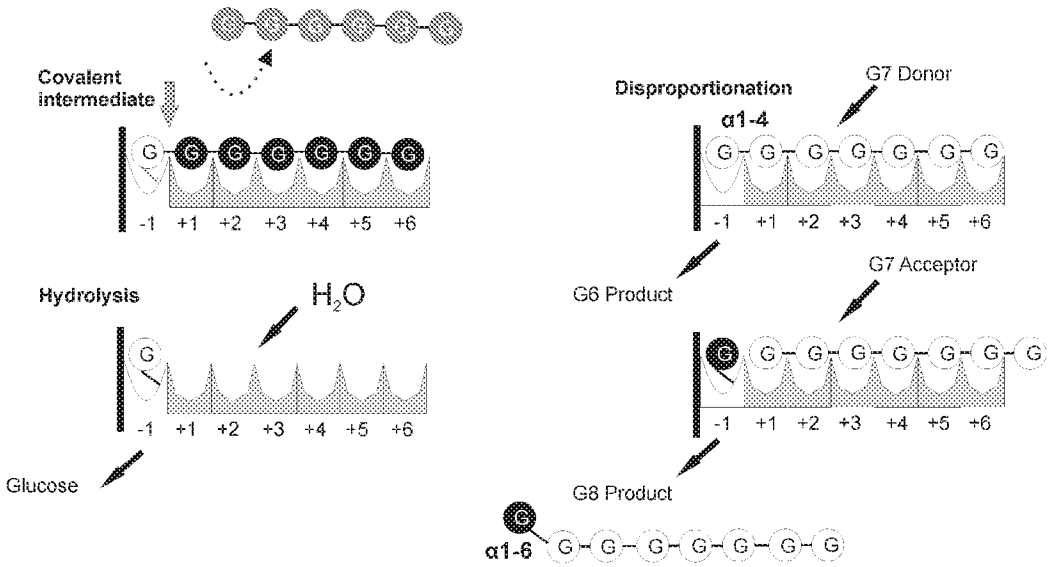


Figure 8


Covalent intermediate
-1 +1 +2 +3 +4 +5 +6
Hydrolysis
H_2O
Glucose
Disproportionation
α1-4
G7 Donor
G6 Product
G7 Acceptor
G8 Product
α1-6