Chapter 1

4,6-α-Glucanotransferases, starch-acting enzymes from lactic acid bacteria
Introduction

Starch, which exists as storage carbohydrate in seeds, roots, tubers and plants, is a major dietary carbohydrate in our life (1). It consists of α-glucan with (α1→4)-linked backbone chains and α1→6 branching points, in the form of amylose and amylpectin. Amylose is a roughly linear polysaccharide containing approximately 99% α1→4 and less than 1% α1→6 glycosidic linkages, while amylpectin has approximately 95% α1→4 and 5% α1→6 linkages (2). According to the distribution of amylose and amylpectin, starches are divided into three groups: waxy starch (<15% amylose), normal starch (16-35% amylose), and high-amylose starch (>35% amylose) (3).

Apart from the raw starches, starch derivatives produced by physical or chemical treatments are now widely used (4). For instance, pre-gelatinization improves the cold water dispersibility of starch, and thus can be applied in preparation of instant convenience food (5). Acid hydrolyzed starches have lower molecular mass and therefore lower viscosity; they are applied in food coatings and batter (4). Etherification and esterification can markedly alter the physiochemical properties of starches by introducing functional groups (6-9).

In addition, many starch acting enzymes that are active under industrial conditions such as high viscosity and high temperature have been commercialized and are now in use in a number of industrial processes (10, 11). This biological modification of starch draws a lot of attention from industry. Thermostable α-amylase enzymes of the glycoside hydrolase (GH) family 13 (EC 3.2.1.1) for instance are used for starch liquefaction in the syrup producing industry. They are also widely applied in the brewing industry to make products that partially replace the expensive malt, in the baking industry to improve the rheological properties of dough, in the paper industry to produce modified starch, and in the textile industry to remove starch (12-14). Glucoamylase (EC 3.2.1.3) also plays a vital role in the syrup producing industry, in the saccharification step following the liquefaction process (Fig. 1) (15). Moreover, isoamylase (EC 3.2.1.68) and pullulanase type I and II (EC 3.2.1.41), which hydrolyze α1→6 branching bonds within starch, constitute another important group classified as debranching enzymes (Fig. 1) (16-18).
Apart from these hydrolyzing enzymes, several glucanotransferases have also found industrial applications in starch conversion. These enzymes cleave α1→4 glycosidic bond of the donor molecule and transfer the cleaved glucose residue or aglycon segment to an acceptor molecule, forming a new glycosidic bond. Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) converts starch into cyclodextrins normally consisting of 6, 7, or 8 glucose residues (Fig. 1) (19). Because of the unique physiochemical properties of their rigid barrel structure, cyclodextrins are widely applied in the food, textile, cosmetic and pharmaceutical industry to improve the solubility and stability of guest molecules by partial or entire encapsulation (20, 21). 4-α-Glucan branching enzyme (EC 2.4.1.18) hydrolyzes an α1→4 bond within a chain and connects the non-reducing end to an α1→4-linked chain forming a branched α1→6 bond (Fig. 1). It plays an important role in amylopectin synthesis in plants along with starch synthases (22). 4-α-Glucanotransferase (4-α-GTase, EC 2.4.1.25) catalyzes the intermolecular disproportionation reaction by transferring a segment of α1→4 glucan to the non-reducing end of the acceptor α-glucan, forming a new α1→4 bond (Fig. 1). Following 4-α-GTase treatment, the modified starch is capable of forming gels with thermoreversible gelling property (23). Dextran dextrinase (DDase, EC 2.4.1.2) from *Gluconobacter oxydans* catalyzes the transfer of an α1→4 linked glucose unit from the non-reducing end of a donor to the non-reducing end of an acceptor molecule forming an α1→6 linkage. It is regarded as an interesting alternative for industrial dextran production from maltodextrins instead of sucrose (24). However, DDase cannot process unhydrolyzed starch (25).

4,6-α-Glucanotransferase enzymes (4,6-α-GTases, EC 2.4.1.B34 [http://www.brenda-enzymes.org/]) recently have been characterized (26). They predominately cleave an α1→4 bond from the non-reducing end of starch or maltodextrins and transfer the cleaved glucose unit to the non-reducing end of the acceptor mostly by formation of an α1→6 bond and occasionally an α1→4 bond, forming isomalto-/malto- oligosaccharides (IMMO) and polysaccharides (IMMP) (Fig. 1). Unlike DDase, 4,6-α-GTases do not have branching specificity on dextrin substrate and disproportion activity on isomaltooligosaccharides, but are able to process unhydrolyzed starch molecules such as amylose and side chains of amylopectin (Chapter 4), showing promising commercial value for the food industry (27, 28). Using protein blasts in the NCBI database
with the GtfB enzyme as a query, approx. 28 characterized and putative 4,6-α-GTases are retrieved. All of them are exclusively present in lactic acid bacteria. Interestingly, these 4,6-α-GTases do not belong to the well-known starch-acting GH families such as GH13, 57, or 77 but constitute a GH70 subfamily; family GH70 mainly consists of sucrose-acting glucansucrases (GS) (29-31).

**Fig. 1.** Schematic representation of the reactions of several starch-acting enzymes including α-amylase, glucoamylase, isoamylase/pullulanase, cyclodextrin glucanotransferase, branching enzyme, 4-α-glucanotransferase and 4,6-α-glucanotransferase.
Starch-acting enzymes in lactic acid bacteria (LAB)

Fermentation by lactic acid bacteria (LAB) is a popular approach in food processing to increase the nutritional value, and to improve the taste and flavor of food. LAB strains are widely distributed in fermented meals, beverages, sourdoughs, cooked rice, fish products and digestive tract of animals as well as plants (32-37). Some of the LAB strains, especially of the genus lactobacilli, are able to degrade and utilize starch as carbon- and energy source during fermentation, using a dedicated set of enzymes acting on starch (38). Several of these enzymes have been expressed heterologously and studied (3). By biochemical characterization of these enzymes, amylopullulanase, pullulanase and α-amylase were observed intracellularly and/or extracellularly in Lactobacilli such as *Lactobacillus amylophilus*, *Lactobacillus acidophilus*, *Lactobacillus cellobiosus*, *Lactobacillus plantarum*, *Leuconostoc lactis ssp. lactis* and *Streptococcus bovis* (39-44). These known LAB enzymes all catalyze starch-hydrolyzing reactions by cleaving the α1→4 bonds and/or α1→6 branching points.

Some LAB are known to produce extracellular polysaccharides (EPS) including homopolysaccharides (composed of glucose or fructose units, respectively) and heteropolysaccharides (composed of mainly glucose, fructose, rhamnose and some charged groups) (45-47). Genes involved in heteropolysaccharide synthesis are located either on plasmids or on the chromosome, and are organized in clusters. They carry genes involved in regulation, polymerization and chain length determination, biosynthesis of the repeating unit, polymerization and export (48, 49). Homopolysaccharides, such as β-fructans and α-glucans, are synthesized from sucrose by individual enzymes such as fructansucrases including inulosucrase (EC 2.4.1.9), levansucrase (EC 2.4.1.10), and GS including dextransucrase (EC 2.4.1.5) and alternansucrase (EC 2.4.1.140) (50-52). These GS convert sucrose into α-glucans with α1→2, α1→3, α1→4 or/and α1→6 glucosidic bonds and constitute family GH70 (47, 50, 52). In nature, GS are extracellular enzymes contributing to the EPS formation in host strains (53, 54). Interestingly, all reported GS have been exclusively isolated from LAB strains including *Streptococcus*, *Leuconostoc*, *Weissella*, and *Lactobacillus* (29, 55-57). The homo-exopolysaccharides (homo-EPS) synthesized by GS in LAB such as *Streptococcus mutans* play a clear role in occurrence of dental caries; GS three-
dimensional structures therefore are studied in order to design structure-based inhibitors for anti-caries drugs (55, 58).

Like GS, 4,6-α-GTases are also exclusively found in LAB strains. According to the primary structure of 4,6-α-GTases, they are classified into family GH70. The first 4,6-α-GTase representative is GtfB from Lactobacillus reuteri 121; it is also the first starch-acting glucanotransferase characterized from LAB strains. Besides gtfB, L. reuteri 121 also harbors genes encoding levansucrase, inulosucrase and reuteransucrase (29, 53, 59-61). Recently, more 4,6-α-GTases, such as GtfW from L. reuteri DSM20016 and GtfML4 from L. reuteri ML1, were characterized, thus expanding this subfamily of GH70 (28).

**GH70 and clan GH-H**

GH13, GH70 and GH77 constitute clan GH-H, also called the α-amylase superfamily, according to their protein sequences and the basic three-dimensional folds of their catalytic modules, rather than on their specificity of action (62-64). Of these families constituting clan GH-H, family GH13, including hydrolases, transglycosidases and isomerases, with around 30 known specificities, is most complex (www.cazy.org) (65, 66). Family GH77 is the least complex, all 25 characterized members are 4-α-glucanotransferases (4-α-GTases, amylomaltases). They are of similar specificity, cleaving an α1→4 bond and transferring a segment of the α1→4 linked chain to the non-reducing end of an acceptor substrate forming a new α1→4 bond (http://www.cazy.org/). (67-70). All members of clan GH-H harbor a basic (β/α)8-barrel, a TIM barrel, in the catalytic domain. The barrel consists of eight β-strands and eight α-helices that are alternately arranged (62). The C-terminal ends of β-strands join to the N-terminal ends of the following α-helices by irregular loops that also link the C-terminal ends of α-helices and the N-terminal ends of β-strands (Fig. 2A). In addition, the enzymes in clan GH-H catalyze reactions by a common mechanism that involves a nucleophilic aspartate residue at the C-terminal end of β-strand 4, an acid/base catalyst glutamic acid residue at the end of β-strand 5, and a putative transition state stabilizer aspartate residue located a few residues beyond the C-terminal end of β-strand 7.
For GH13 enzymes, loop 3 connecting the β-strand 3 to the helix 3 of the barrel is generally long compared to other loops, and appears to fold as an independent unit that even can be considered as a domain. In some cases, such as the branching enzymes, loop 3 is shorter and does not have the characteristic of a domain. Some of the GH13 members are more complex and have extra domains, e.g. an additional N-terminal domain in isoamylase (71) or domain D and E in CGTase (Fig. 3) (19, 72, 73). For CGTase, domain E is confirmed as starch binding domain; domain D is exclusively found in CGTases but its function has remained unclear (72).

Unlike GH13 enzymes, domain C is missing from GH77 amylomaltases. Another remarkable characteristic of amylomaltase structures is the presence of subdomain B2 that contains large α-helices (Fig. 3). This subdomain B2 is unique in GH77 enzymes. It only partially overlaps with the N-terminal domain of isoamylase (67). Additionally, the active-site cleft of amylomaltase is different from the open cleft of GH13 members as it is partially covered by a long extended loop (250s loop formed by residues 247-255 in subdomain B1 for Thermus aquaticus) (67). This loop is flexible, which may contribute to substrate binding and prevent the formation of small cyclic products because of the steric hindrance to the active site.

For GH70, three-dimensional structures of several GS have been elucidated, including Gtf180-ΔN from L. reuteri 180 (PDB: 3KLK), GtfA-ΔN from L. reuteri 121 (PDB: 4AMC), Gtf-SI from S. mutans (PDB: 3AIE) and ΔN123-GBD-CD2 of the (1→2) branching glucansucrase DSR-E from L. mesenteroides NRRL B-1299 (PDB: 3TTQ) (55, 74-76). The domain organization is similar to that of GH13 enzymes as the catalytic cores of all GS consist of domains A, B, and C. However, GS also contain two extra domains IV, V, and a variable N-terminus (Fig. 3). Interestingly, domains A, B, IV and V are all built up by two discontinuous segments of polypeptide chains. Only domain C is composed of a contiguous polypeptide. The polypeptide chain of the full glucansucrase follows an order of V, IV, B, A, C, A, B, IV, and V from the N- to C-terminus. Besides the domain organization, the characteristic (β/α)₈-barrel of clan GH-H in GH70 is also different from that of GH13 and GH77. A circular permutation of the (β/α)₈-barrel predicted by the primary structure was confirmed by the 3D structure
analysis (Fig. 2B). The barrel is initialized with the $\alpha$-helix that is equivalent to the $\alpha$-helix 3 of the GH13 or GH77 enzymes and ends with the $\beta$-strand equivalent to $\beta$-strand 3 of the GH13 or GH77 enzymes, following an order as shown in Fig. 2B.

**Fig. 2.** Schematic diagram of the (A) normal barrel of GH13 and GH77 and (B) the permuted barrel of family GH70; gray arrows represent $\beta$-strands and blue cylinders represent $\alpha$-helices. The order of helices is numbered. For GH70 proteins, this order is 34567812.
Fig. 3. Domain arrangements of clan GH-H proteins (A) human pancreatic α-amylase (PDB: 1HNY), (B) *Bacillus circulans* cyclodextrin glucanotransferase (CGTase) (PDB: 1CGT) of GH13, (C) *Lactobacillus reuteri* glucansucrase (PDB: 3KLK) of GH70 and (D) *Thermus aquaticus* amylomaltase (4-α-glucanotransferase) of GH77 (PDB: 1CWY).
4,6-α-Glucanotransferase and GH70

Family GH70 mainly consists of GS that are classified as dextranucrases, mutansucrases, alternansucrases, reuteransucrases, and branching dextranucrases, according to the linkage specificity of the products formed. Although the GH70 GS are structurally closely related to the members of GH13 and GH77, their substrate specificity is completely different. These GS enzymes are inactive on starches but active on sucrose, synthesizing oligo- and/or polysaccharides.

The occurrence of 4,6-α-GTases in family GH70 implies that all families of the GH-H clan in fact possess catalytic activity on starches and maltodextrins. This also provides insights into the evolutionary pathway between families GH13 and GH70. A phylogenetic tree based on the alignment of the catalytic core amino acid sequences of GS (http://www.cazy.org) and 4,6-α-GTases is shown in Fig. 4. Putative 4,6-α-GTases such as Gtf106B were identified in the non-redundant protein sequence database by a protein blast search (http://www.ncbi.nlm.nih.gov). The maximum-likelihood tree shows that the 4,6-α-GTases are more closely related to the GS from L. reuteri compared to those from Leuconostoc and Streptococcus. However, unlike GS, 4,6-α-GTases are completely inactive on sucrose, with the exception of Gtf106B that has faint activity which is detectable after 27 h incubation (77).

These GS from different species clearly cluster within their host genera of Streptococcus, Lactobacillus and Leuconostoc (78-84). Along with the time of occurrence and the order of specific events, these clusters may indicate that the gtf genes from Streptococcus were acquired from other genera like Lactobacillus via horizontal gene transfer (85). By the acquisition of Gtfs, Streptococcus mutans became capable to form EPS, resulting in the formation of cariogenic dental biofilms (86). However, the characterized and putative 4,6-α-GTases are only found within the family Lactobacillaceae including Lactobacillus and Pediococcus, and so far no 4,6-α-GTase gene has been found in Streptococcus, Leuconostoc and other genera of LAB.

Alignment of the conserved sequences of the characterized 4,6-α-GTases, GtfB, GtfML4 and GtfW, with those of typical GS showed not only similarities but also characteristic differences in their primary structures (Fig. 5). The three catalytic
residues (the nucleophilic aspartate, the acid/base glutamate and the transition state stabilizing aspartate) constituting the catalytic site in GS are also present in 4,6-α-GTases, e.g. D1015, E1053 and D1125 in GtfB of *L. reuteri* 121 (26, 58). They are in loops following β-strands β4, β5 and β7, in homology regions II, III, and IV of GS respectively (Fig. 2). Mutating D1015 or E1053 in GtfB resulted in loss of activity (26), indicating that 4,6-α-GTases and GS have the same catalytic residues. In view of such similarities it also appears likely that the reactions catalyzed by 4,6-α-GTases and GS share a similar double-displacement mechanism (Fig. 6). The glutamic acid residue at β-strand 5 donates a proton to the glycosidic oxygen, resulting in bond cleavage. The cleaved glucosyl unit or aglycon segment leaves the active site forming an intermediate. The glucose residue is stabilized by hydrogen bonding involving the aspartic acid that is located after β-strand 7 and the hydroxyl groups of the glucose. Then a covalent β-bond between C1 of the glucose and the nucleophilic aspartate at β-strand 4 forms in order to further stabilize the intermediate. In the second half of the reaction, the glutamate at β-strand 5 is reprotonated by removing a proton from the acceptor substrate. The covalently bonded glucosyl moiety at the donor subsite is transferred to an acceptor substrate with retention of the α-anomeric configuration forming a new α-bond. Besides the similarities, a large number of amino acid residues conserved in regions I, II, III, and IV of GS are different in the 4,6-α-GTase enzymes, especially the residues in region I, and IV that contribute to the -1, +1, and +2 donor/acceptor substrate binding subsites (Fig. 5). These differences in amino acid residues may contribute to the differences in substrate specificity of 4,6-α-GTases and GS.
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Fig. 4. Unrooted phylogenetic tree of characterized GH70 enzymes and some putative 4,6-α-GTases. Alignments and dendrogram construction were carried out using the catalytic core of all enzymes. Each sequence is labeled with the corresponding bacterial strain and enzyme names. The characterized ones are indicated in red frame.

Fig. 5. Sequence alignment of conserved sequences (Motifs II, III, IV, and I) in the catalytic domains of GS and 4,6-α-glucanotransferases in GH70. The catalytic residues (filled triangles) and several conserved amino acid residues (shielded with red color) in subsites -1, +1, and +2 are indicated.
Fig. 6. Reaction mechanism of GS and 4,6-α-glucanotransferase enzymes. Asp represents the nucleophilic aspartate residue and Glu represents the general acid/base residue.
Expression and production of 4,6-α-GTases

In order to improve the expression and production of 4,6-α-GTases, diverse methods previously reported for DDases, 4-α-GTases, and GS, which are either specificity- or structurally-related to 4,6-α-GTases, may be applied. According to previous reports, these enzymes mainly were produced in two different ways including whole-cell fermentation and heterologous expression.

In 1950s, Hehre et al. reported an enzyme which is capable of synthesizing dextran from maltoheptaose, or the hydrolysates of amylose, amylopectin and glycogen (87). The enzyme cleaved α1→4 bonds and transferred the glucose units to another (acceptor) chain introducing α1→6 linkages, and was named dextran dextrinase (DDase) (25). The distribution of these enzymes thusfar is limited to acetic acid bacteria such as *Acetobacter capsulatum*, *Gluconobacter oxydans* (24, 88-93). Unfortunately, to date no corresponding gene and protein sequences have been reported yet. The production of DDases was done in the original strain, i.e. *G. oxydans* (91, 94). Methods for the improvement of DDase production in *G. oxydans* by adding polyhydric alcohols such as mannitol, sorbitol and glycerol as substrates have been patented (95). The production of intracellular DDase of *G. oxydans* ATCC 11894 can also be enhanced by adding various carbon and nitrogen sources, such as glycerol and peptone (91). The yield of DDase in *G. oxydans* M5 has been improved by optimizing groups of medium constituents using a four-factor five-level central composite design. The optimization resulted in a 17-fold increase of yield of DDase compared to the original conditions (94).

For 4-α-GTases, little research has been done on the production improvement via whole-cell fermentation. Genes encoding 4-α-GTases have been successfully cloned from their original strains; these enzymes were preferably expressed in *E.coli* or *Bacillus subtilis* host strains. However, their heterologous expression to some extent is still a challenge. For instance, the *Thermococcus litoralis* 4-α-GTase tends to aggregate in inclusion bodies when expressed in *E. coli* as the corresponding gene is rich in AGA and AGG codons encoding arginine. Simultaneous expression of tRNA<sub>AGA</sub>, tRNA<sub>AGG</sub> and molecular chaperon GroEL resulted in an increase of both production and solubility of 4-α-GTase (96). Alternatively, codon optimization improved the expression yield of *Thermus*
thermophilus 4-α-GTase in *E. coli* 100-fold (97). In addition, the *Thermus scotoductus* 4-α-GTase gene has been expressed successfully in *B. subtilis*, and is produced extracellularly. To improve the expression, a dual promotor system was used, resulting in an increase of the yield by approx. 12-fold compared to the single promotor system (98).

For GS which are structurally related to 4,6-α-GTases, both whole-cell fermentation in their original host strain and heterologous expression in *E. coli* have been reported. In the whole cell fermentation, approaches including central composite design, response surface methodology, fed batch fermentation, with pH control, are used to enhance the production of dextranucrase in *Leuconostoc* strains (99-105). However, over the years more and more GS genes have been cloned and expressed in *E. coli* (106-109). In recent years, such studies mainly focussed on improving the heterologous expression level of GS. By dropping the induction temperature from 37 °C to 15°C, the yield of dextranucrase LcDS from *Leuconostoc citreum* HJ-P4 in *E. coli* sharply increased by 330-fold (108).

When changing the *E. coli* host strain/inducer system for the expression of dextranucrase DexT from *Lc. citreum* KM20 its activity was enhanced 12 fold under optimized conditions including induction OD$_{600}$ and concentration of inducer (110). Also, addition of 0.005% (w/v) calcium ions in the fermentation medium increased production of dextranucrase of *Lc. mesenteroides* PCSIR-4 by 2.5-fold (111). Also deletion of peptide segments from full-length GS has been applied, at either the N- or C-terminus, without significantly influencing its enzymatic activity. For example, the alternansucrase from *Leuconostoc mesenteroides* NRRL B-1355 (2057 amino acids) in *E. coli* is poorly soluble, instable, and thus hard to express. By truncation of the C-terminal APY repeats but retaining four CW-like repeats, the protein became more soluble and less degradable than the full-length alternansucrase (112). For the GtfR from *Sterptococcus oralis*, a deletion of 261 amino acids in N-terminus resulted in a 50-fold increase of yield compared to the original glucanotransferase (113). Moreover, partial gene deletion has been widely used in recombinant expression of other GS, such as Gtf180 from *L. reuteri* 180, GtfA from *L. reuteri* 121, and DSRS from *Lc. mesenteroides* NRRL B-512F (29, 74, 114, 115).
4,6-α-GTases, such as GtfB, GtfW and GtfML4, have been heterologously expressed in *E. coli* (26, 28). But the soluble protein expression levels are disappointingly low. High expression levels of GtfB resulted in accumulation (of at least partly active enzyme) in inclusion bodies (Chapter 2) (116). To extract the functional GtfB protein from inclusion bodies, conventional denaturing and refolding steps resulted in a recovery of 65% of the hydrolytic activity of GtfB compared to the soluble enzyme. Also the (non-classical) inclusion bodies (ncIBs) themselves were used as an alternative approach for preparation of active enzyme. The ncIB GtfB only had approximately 10% of the hydrolytic activity of soluble GtfB, but showed a much higher thermostability than soluble GtfB. In view of the high yield and the low preparation cost, as well as the structural stability, ncIB GtfB protein potentially may be used in industrial applications. In the next step the soluble expression of active GtfB enzyme was considerably improved by gene deletion of its N-terminally variable region without influencing the enzymatic activity compared to the full-length version. Truncation of 733 amino acids in the N-terminous resulted in a yield of more than 40 mg pure GtfB-ΔN protein from one-liter *E. coli* culture, which is 75-fold higher than that of full-length GtfB based on molar calculation (Chapter 3) (117).

**Assays for measurement of 4,6-α-GTase activity**

Proper activity assays for 4,6-α-GTase enzymes are essential for their biochemical characterization. In view of the complexity of the 4,6-α-GTase catalyzed hydrolysis and transglycosylation reactions, proper, specific and accurate assays are difficult to establish. However, the available assays for DDases and 4-α-GTases that catalyze somewhat similar reactions as 4,6-α-GTases provided leads, also because they all transfer glucose moieties from the non-reducing end of the donor substrate to the non-reducing end of the acceptor substrate.

The dinitrosalicylic acid (DNS) assay for reducing sugars, conventionally used for glucose measurement, has been adopted for estimating the activity of DDases. However, the poor sensitivity of DNS and interference by unreacted maltodextrins in the reaction mixture hindered its application (118). As an alternative, a viscosity build-up assay was tested based on the change of rheological properties from maltodextrins to dextran. But it was also hampered by
the complex non-Newtonian and time-dependent flow behavior of the maltodextrin/dextran mixture (118). Another assay, which uses maltose as a standard substrate, based on discrete transglycosylation reactions was successfully applied (119). Generated panose \([\alpha-D-glucose-(1,6)-\alpha-glucose-(1,4)-\alpha-glucose]\) was demonstrated to be a valid indicator for transglycosylation activity. An assay mimicking maltose utilization but based on \(p\)-nitrophenyl-\(\alpha\)-D-glucopyranoside (NPG) was also reliable (118). Use of NPG is more preferable than the maltose assay because it measures both transglycosylation activity and hydrolysis activity.

Several assays have been developed for characterization of 4-\(\alpha\)-GTases. For determining the hydrolysis activity, maltotriose was used as substrate and the released glucose was measured by the glucose oxidase enzyme method. The rate of glucose release was then defined as the hydrolysis activity (120, 121). The disproportionation activity was estimated by measuring the intensity of iodine staining of non-denaturing polyacrylamide electrophoresis gels containing glycogen (122). Lugol’s method was also adapted by monitoring the iodine-staining capacity of products during the conversion of amylose in the presence of maltose as acceptor (123). The iodine-staining capacity can be directly determined by measuring the optical density change (124). However, because 4-\(\alpha\)-GTases cleave \(\alpha1\rightarrow4\) bonds and form new \(\alpha1\rightarrow4\) bonds, the iodine staining method is less quantitative (120).

The maltose assay has been used for determining the kinetic parameters of 4,6-\(\alpha\)-GTase GtfW from \textit{L. reuteri} DSM20016 (28). However, this assay has significant limitations with other 4,6-\(\alpha\)-GTases such as GtfB which has detectable activity on longer maltooligosaccharides but has faint or virtually undetectable activity on maltose. To solve this problem, a combination of assays was developed using amylose as substrate and measuring both iodine staining and D-glucose (GOPOD) release to determine both transglycosylation and hydrolysis activities (Chapter 3) (117). The iodine staining assay determines the total activity of 4,6-\(\alpha\)-GTases and is based on the principle that iodine can be trapped by linear \(1\rightarrow4\)-\(\alpha\)-D-glucans (i.e. amylose) but not by linear \(1\rightarrow6\)-\(\alpha\)-D-glucans (125, 126). Hydrolysis activity results in release of glucose from the amylose substrate, which also impairs the complex capacity of amylose with iodine atoms. The decrease of the
complex capacity of amylose thus reflects total activity. Hydrolysis activity of 4,6-α-GTases is measured with the D-glucose assay kit (GOPOD). The transglycosylation activity was subsequently calculated by subtracting the hydrolysis activity from total activity (Fig. 7). These assays with amylose as substrate thus allow direct determination of total and hydrolysis activities, and calculation of the transglycosylation activities of 4,6-α-GTases.

Fig 7. Schematic diagrams of the complexes of iodine with amylose and with the amylose derived products of the 4,6-α-GTase activities. Iodine ions are shown as (short chains of) blue circles.

**Biological role of 4,6-α-GTases in LAB**

As described above, GS usually contribute to homo-EPS formation in their host LAB strains. These secreted GS enzymes are anchored to the cell wall, producing EPS in the presence of sucrose. Depending on the GS, the resulting EPS can be divided into dextran that mainly consists of α1→6 linked glucose units, mutan with mostly α1→3 linkages, alternan with strictly alternating α1→6 and α1→3 linkages, and reuteran with α1→4 and α1→6 linkages. The linkage specificities confer different physiochemical properties to the EPS, e.g. mutan from *S. mutans*
contains a relatively high percentage of α1→3 linkages and is water-insoluble, while dextran is rich in α1→6 linkages and is water-soluble. The insoluble mutan may contribute to the formation of dental caries because it adheres to teeth and traps oral bacteria, food debris and salivary components (127-129). EPS is also a major fraction of the extracellular polymeric substances that form the scaffold for the three-dimensional architecture of the biofilm (130). The EPS in extracellular polymeric substances may contribute to other cellular functions (Table 1) in bacterial biofilms.

Table 1. Roles ascribed to exopolysaccharides in biofilms (130, 131).

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<thead>
<tr>
<th>Biofilm Function</th>
<th>Functional Relevance of EPS to Biofilms</th>
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<tr>
<td>Adhesion</td>
<td>providing the initial steps in the colonization of surfaces</td>
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<tr>
<td>Bacterial cell aggregation</td>
<td>establishing bridges between cells to temporarily immobilize bacterial cell populations and developing high cell densities, and cell–cell recognition</td>
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<tr>
<td>Retention of water</td>
<td>maintaining a hydrated microenvironment around biofilms, leading to the survival of cells in water-deficient environments</td>
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<tr>
<td>Cohesion of biofilms</td>
<td>forming a hydrated polymer network to mediate the mechanical stability of biofilms and determine biofilm architecture, as well as allowing cell-cell communication</td>
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<tr>
<td>Nutrient source</td>
<td>serving as source of carbon containing compounds</td>
</tr>
<tr>
<td>Protective barrier</td>
<td>conferring resistance to non-specific and specific hosts during infection, confer tolerance to various antimicrobial agents</td>
</tr>
<tr>
<td>Sorption of organic compounds and inorganic ions</td>
<td>mediating the accumulation of nutrients from the environment, sorption of xenobiotics and recalcitrant materials, promote polysaccharide gel formation resulting in ion exchange, mineral formation and the accumulation of toxic metal ions</td>
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<tr>
<td>Enzyme binding</td>
<td>stabilizing and accumulating extracellular enzymes</td>
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<tr>
<td>Sink for excess energy</td>
<td>storing excess carbon under unbalanced carbon to nitrogen ratios</td>
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The strains carrying GS encoding genes are typically identified by the appearance of slimy colonies on solid media or viscous solutions in liquid media when grown on sucrose as carbon source (132, 133). Gene inactivation studies also revealed that the slimy colony morphology resulted from GS activity in wild-type LAB strains (132, 133). Besides GS, also the presence of fructansucrases producing β-fructans from sucrose results in the formation of slimy colonies. GS and fructansucrase enzymes can be further distinguished using raffinose (α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) as a carbon source: fructansucrase enzymes are able to utilize raffinose to produce EPS, but GS are not (54, 134). Using this method, many α-glucan producing bacterial strains have been identified from various sources, such as fruit and vegetables, sourdoughs, dairy, mammal intestine, etc (135-137).
Insights in the biological roles of 4,6-α-GTases in LAB strains were obtained when growing \textit{L. reuteri} 121 strain 35-5 (that lacks fructan formation and carries both the \textit{gtfA} and the \textit{gtfB} genes) on agar plates with maltodextrins (5% w/v, dextrose equivalent 13-17) as carbon source (Chapter 4). Surprisingly, slimy colony morphology (Fig. 8A) was observed (Chapter 6). The structure of the EPS extracted by ethanol precipitation was analyzed by NMR (Fig. 8B), showing that the percentage of α1→6 linkages increased dramatically compared to the substrate maltodextrins. GtfB has been expressed in \textit{E. coli} and characterized as an enzyme converting maltodextrins into isomalto/malto-oligosaccharides and polysaccharides, with α1→4 and α1→6 linkages (Chapter 4) (26, 27). Moreover, a periodic acid-Schiff (PAS) staining experiment confirmed that the GtfB enzyme contributes to the EPS formation \textit{in situ} (Chapters 4 and 5). Therefore, GtfB enzyme contributes to the homo-EPS formation in \textit{L. reuteri} 35-5 using starches or maltodextrins as substrates.

\textbf{Fig. 8.} (A) Colonies of \textit{L. reuteri} 121 strain 35-5 grown on MRS agar containing 5% glucose or maltodextrins (dextrose equivalent 13-17). Slime formation indicates the production of EPS from maltodextrins but not from glucose. (B) NMR spectra of the substrate maltodextrins and the corresponding EPS from \textit{L. reuteri} 35-5.
**Application of 4,6-α-GTases**

4,6-α-GTases raise the interest of the starch industry because of their capacity of converting starch or maltodextrins into isomalto/malto-polysaccharides (IMMP) with high percentages of α1→6 linkages (138, 139). These modified starch products are novel soluble dietary fibers. Dietary fibers positively contribute to the human health. Based on the physiochemical properties, they are divided into insoluble dietary fibers such as resistant starch, wheat bran, hemicelluloses etc., and soluble dietary fibers such as inulin, pectin, gums, and polydextrose (140). In food applications, the addition of small quantities of dietary fibers may significantly affect the texture, taste and mouth feel of the food products. Based on their physiochemical properties and health effects, these dietary fibers are applied in various food products, e.g. the soluble dietary fibers can be added in juice, viscous liquid and gel products. Some soluble dietary fibers also have many health benefits: reduction of the cholesterol level and thus lowering the risk of heart diseases as the soluble fiber attaches to the cholesterol particles, removing these from the body (141, 142); control of sugar absorption, providing protection against diabetes (143); assisting in keeping the body weight (144); stimulating healthy bowel movements (145). However, the production of soluble dietary fibers is costly because most soluble dietary fibers currently are extracted from foods such as oatmeal, nuts, beans, blueberries and apples (146). Therefore, 4,6-α-GTases producing soluble dietary fiber from cheap starches are of great commercial potential (139).

Of all the 4,6-α-GTases, GtfB from *L. reuteri* 121 has been studied in most detail using various starches as substrates. The IMMP products consist of linear α1→6-linked glucan chains that are attached to the non-reducing ends of starch fragments (Fig. 9). Comparison of thirty different starches and maltodextrins showed that the substrates with long and linear α1→4-linked glucan chains are most efficient for the production of products with a high percentage of α1→6 linkages (138). The percentage of α1→6 linkages even reached up to 92% when using amylose V as substrate. IMMP is a novel polysaccharide different from dextran although both are rich in α1→6 linkages. Firstly, dextrans are large polymers which are synthesized by dextranucrases using sucrose as substrate, while IMMP is produced by 4,6-α-GTase using starch as substrate. Secondly, the sizes of dextrans produced by glucansucrases (>10 MDa) (53) are much larger.
than of IMMP (10-1000 kDa) (Chapter 4) (28). Thirdly, dextran contains branches that are absent in IMMP. The in vitro experimental results revealed that IMMP, or more precisely the IMMP fraction rich in α1→6 linkage, is indigestible in the small intestine and consumed in the large intestine (138). Moreover, preliminary degradation tests using fecal samples from a single adult showed that the addition of IMMP resulted in an increased formation of short fatty acids acetate and propionate, indicating that IMMP may have a positive effect on human health (138). Conclusively, IMMP are novel soluble dietary fibers that may contribute to the health of the human body.

![Fig. 9. Schematic diagram of the 4,6-α-GTase-modified starch (amylopectin) produced by 4,6-α-GTases.](image)

These IMMP polysaccharide- and oligosaccharide products may act as prebiotic substrates (138, 139) defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” (147, 148). In the microflora, Lactobacilli and Bifidobacteria are usual target genera for identifying prebiotics. The known prebiotics including inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), xylo-oligosaccharides (XOS), arabinoxylo-oligosaccharides (AXOS), transgalacto-oligosaccharides (TGOS) are extracted from plants sometimes followed by enzymatic hydrolysis, or produced by chemical or enzymatic synthesis (149). IMMP produced by the GtfB enzyme is a soluble dietary fiber that partially escapes digestion in the upper part of the gastrointestinal tract and small intestine. Thus, it has potential to be a prebiotic that selectively boosts growth of some selected bacteria in the large intestine.
intestine. In this thesis, the EPS produced by *L. reuteri* 121 were used as carbon sources to stimulate the growth of some typical probiotic Bifidobacteria that secrete amylpullulanases (Chapter 4).

Although the physiochemical properties of oligo-/polysaccharides produced by 4,6-α-GTase from maltodextrins or starches have not been studied, the products synthesized by DDase with similar structures are comparable. The studies of these products may provide us with more thoughts of the application of 4,6-α-GTase. Thus, apart from acting as a dietary fiber, the IMMP may be applied in food as cryostabilizer, fat substitute, or low-calorie bulking agent for sweeteners (25). Moreover, as shown in Fig. 9, because of the changes in the external chains of starch, 4,6-α-GTase treatment may influence the rheological properties of starches, such as the viscous modulus, elastic modulus, and retrogradation properties, expanding the application scope of these enzymatically modified starches.

**Scope of this thesis**

4,6-α-GTases are newly identified glucanotransferases, producing soluble dietary fibers, isomalto-/malto-polysaccharides (IMMP) from starch and maltodextrins. The *in vivo* biological roles of 4,6-α-GTase remained to be explored, also in view of their limited distribution in LAB and the fact that the closely related GS clearly contribute to the formation of EPS from sucrose. 4,6-α-GTases potentially are of great commercial value as the starch substrates are cheap and widely available compared to the normal sources of soluble dietary fiber. However, industrial application is still a challenge because 4,6-α-GTases are only available in relatively minor amounts. There is also a general lack of reliable 4,6-α-GTase enzyme activity assays. These topics are addressed in this PhD thesis, also focusing on elucidation of the 4,6-α-GTase reaction mechanism by 3D structural protein analysis and mutagenesis.

Chapter 1 reviews the current knowledge of 4,6-α-GTases in comparison with other specificity- or structurally-related enzymes including members in clan GH-H and DDases.

In chapter 2, active 4,6-α-GTase GtfB protein was prepared from inclusion bodies as it is highly expressed in the heterologous host *E. coli*, but largely
accumulates in inclusion bodies. Following conventional denaturing, refolding of GtfB protein resulted in partial recovery of GtfB (hydrolysis) activity, but this required a set of complicated steps. The non-classical inclusion body (ncIB) GtfB enzyme directly extracted from IBs was much more thermostable than the soluble and refolded GtfB enzymes. In view of its high yield, low preparation cost and structural stability, ncIB GtfB protein thus provides a promising option for industrial applications.

In chapter 3, the soluble expression of GtfB was further improved by N-terminal truncation of the full-length protein, resulting in strong enhancement of the soluble expression level of fully active GtfB-ΔN (approx. 75 fold compared to full length wild type GtfB) in E. coli. In addition, quantitative assays based on amylose V as substrate are described, allowing accurate determination of both hydrolysis (minor) activity and total activity, and calculation of the transglycosylation (major) activity of these 4,6-α-GTase enzymes, such as GtfB and GtfW. Using these assays, the biochemical properties of GtfB-ΔN were characterized in detail, including determination of kinetic parameters and acceptor substrate specificity. Moreover, the GtfB-ΔN enzyme displayed high conversion yields at relatively high substrate concentrations, a promising feature for industrial applications.

Chapter 4 reports the in vivo homo-exopolysaccharide (homo-EPS) synthesis by LAB strains with starch as substrate. L. reuteri 121 modifies amylose and the side chain of amylopectin molecules, introducing α1→6 linkages, producing soluble dietary fiber that is structurally similar to the IMMP product synthesized in vitro by the GtfB enzyme. This study provides insights into the in vivo role of 4,6-α-GTase in LAB strains. The IMMP-like EPS produced in vivo can be used as carbon source to stimulate the growth of some typical probiotic Bifidobacterium strains, suggesting that these EPS have potential prebiotic activity and may contribute to the application of probiotic L. reuteri 121 in food and feed (synbiotics).

In chapter 5, a synergistic action of 4,6-α-GTase GtfB and GS GtfA in L. reuteri 35-5 was studied when both starch and sucrose substrates were present. The EPS produced by L. reuteri 35-5 in the presence of sucrose and starch is different from the EPS derived from either of the single substrates in both size and structure. In
*vitro* incubations with both GtfA and GtfB and sucrose plus starch further confirmed that both enzymes initially synthesized new oligosaccharides structurally different from those derived from either of the single substrates. This *in vivo* EPS formation from multiple dietary carbohydrates most likely reflects EPS formation in the oral cavity, potentially also affecting dental plaque formation.

In **Chapter 6**, the GtfB-ΔNΔV protein was successfully crystallized and its high resolution structure solved. It has an overall domain organization resembling GH70 glucansucrases, and an active site architecture reminiscent of GH13 α-amylases. A tunnel-like binding groove allows for a dual mode of action for 4,6-α-GTases involving both exo-specific and endo-specific cleavage/transfer reactions, and explains why GtfB only processes the external (amylose) branches of starch substrates. The GtfB-ΔNΔV crystal structure clearly supports the view that the 4,6-α-GTase subfamily is an evolutionary intermediate between the family GH70 and GH13 enzymes. The structural observations also support the phylogenetic and genomic analyses, revealing a close relation between 4,6-α-GTases and GS, and suggesting that gene duplication as well as horizontal gene transfer events occurred during evolution. We propose a common ancestor for the two enzyme functionalities; loop mutations near the active site likely resulted in either a different substrate specificity (in GS) or in an (α1→6) specific transglycosylation activity (in 4,6-α-GTases). Thus, the evolution of bacterial species like *L. reuteri* 121, using either their 4,6-α-GTase or GS to synthesize related types of α-glucan with (α1→4) and (α1→6) linkages, may have been driven by the availability of fermentable substrates such as starch and (later) sucrose in the oral cavity.

**Chapter 7** summarizes and discusses the results reported in this thesis and proposes topics for future research.


Chapter 1


Chapter 1

*Lactobacillus reuteri* wild-type strain and by mutant strains. Appl Environ Microbiol 65:3008-3014.


