Characterization of 4,6-α-glucanotransferase enzymes and their functional role in *Lactobacillus reuteri*
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Chapter 7

Summary and thesis discussion
Starch is one of the major carbohydrates on earth and is the main dietary carbohydrate in our life. Apart from the raw starches, many starch derivatives produced via physical, chemical, and enzymatic treatments are abundantly applied in food, paper, and textile industries because of their distinctive properties. Among these approaches, the enzymatic treatment has advantages such as low energy consumption, low cost, high controllability, high productivity. Therefore, more and more starch-acting enzymes including hydrolases and transglycosidases are introduced in food and pharmaceutical manufacturing processes to produce glucose and fructose syrup, cyclodextrins, thermoreversible gels or glycogen, or to improve the flour and dough for baking, etc.

4,6-α-Glucanotransferases (4,6-α-GTases) are recently reported enzymes that predominately cleave an α1→4 bond from the non-reducing ends of starch or maltodextrins and transfer the cleaved glucose unit to the non-reducing ends of the acceptor substrate, mostly introducing an α1→6 bond and occasionally an α1→4 bond, forming isomalto-/malto-oligosaccharides (IMMO) and polysaccharides (IMMP). Unlike other starch hydrolyzing enzymes, which are general members of Glycoside Hydrolase family (GH) 13, 57, or 77, 4,6-α-GTases are classified in a subfamily of GH70; family GH70 is mainly composed of glucansucrases (GS). The GS including dextran sucrase, mutan sucrase, reuter ansucrase, and alternansucrase, are active on sucrose, producing α-glucans with various linkage types such as α1→2, α1→3, α1→4, and α1→6 linkages. Like GS, 4,6-α-GTases are exclusively found in lactic acid bacteria (LAB). However, 4,6-α-GTases are completely inactive on sucrose but active on starch. Based on the primary structure analysis, the 4,6-α-GTases are homologous to GS. Both types of enzymes possess a circularly permutated (α/β)$_8$-barrel acting as catalytic core, and the same domain organization consisting of a variable N-terminal region, domains A, B, C, and extra domains IV and V (Chapter 1).

**Production and characterization of 4,6-α-GTases**

The IMMP produced by 4,6-α-GTases is of commercial potential as it can serve as soluble dietary fiber and potentially as a prebiotic. The 4,6-α-GTases-modified starches also may have special rheological properties, and thus may find applications in the preparation of novel and valuable food products. However, the introduction of 4,6-α-GTases in industrial processes is still a challenge.
GtfB of *Lactobacillus reuteri* 121 is the first described representative of 4,6-α-GTases. The IMMP produced by GtfB has been well investigated. The yield of soluble full-length GtfB enzyme expressed in *Escherichia coli* is poor and cannot meet the demand of downstream research and industrial production. A large quantity of GtfB protein accumulates in inclusion bodies (Chapter 2). The traditional denaturing-refolding procedures resulted in a high recovery of activity even in double distilled H2O. Moreover, the inclusion bodies themselves also display some enzymatic activity without any need for further processing. They therefore are regarded as non-classical inclusion bodies (ncIBs), which are known to retain activity due to partial preservation of the native protein structure. The native, refolded, and ncIB GtfB enzymes all produce IMMP with similar size distribution. In addition, ncIB GtfB protein clearly has a higher thermostability. Active GtfB protein from inclusion bodies in the form of refolded or ncIB GtfB enzyme may thus find industrial application in synthesis of IMMP modified starch. However, the expression level of soluble enzymes, which is preferable for industrial production, has remained unsatisfactorily low. The LAB GS are homologous to 4,6-α-GTase enzymes, and deletion of (parts of) their N-terminal and/or C-terminal domains successfully enhanced the yield of soluble active GS enzyme. GtfB differs from GS in lacking the C-terminal polypeptide segment of domain V. Notably, truncation of the variable N-terminal region (1-733 amino acids) resulted in a dramatic increase of the yield of soluble GtfBΔN enzyme (approx. 75 fold compared to full length GtfB) without influencing the activity of the enzyme and products formed (Chapter 3).

Development of proper activity assays for the characterization of 4,6-α-GTases is vital for their further biochemical characterization and development of industrial applications. Existing assays do not allow determination of the specific hydrolysis and transglycosylation activities nor a kinetic analysis. Therefore, we developed a new assay derived from the traditional amylose-iodine staining assay (Chapter 3). Based on the theory of steric hindrance, the assay allows accurate determination of both hydrolysis and total activity, and calculation of the transglycosylation activity of 4,6-α-GTases. With this assay, the biochemical properties of 4,6-α-GTases have been characterized in detail, including determination of hydrolysis ($H$) and transglycosylation ($T$) activities, kinetic parameters, and acceptor
substrate specificity. Based on the developed assay, a ratio of $T/H=4.3:1$ for GtfB-$\Delta N$ was obtained under the optimal conditions.

**Crystal structure of GtfB**

Although the structure of IMMP produced by GtfB has been characterized, the catalytic mechanism and structure-function relationships of 4,6-$\alpha$-GTases remained largely unclear. **Chapter 6** reports the high resolution crystal structure of a truncated but fully active fragment of GtfB (GtfB-$\Delta N\Delta V$) from *L. reuteri* 121. GtfB-$\Delta N\Delta V$ displays a GS-like domain arrangement; however unlike GS, the active site surprisingly features a long $\alpha$-amylase-like binding groove with multiple donor subsites in a tunnel. The presence of this tunnel, formed by three loops unique to 4,6-$\alpha$-GTases, explains why GtfB processes only the linear external chains of starch, thus preferring high-amylose starches as substrate. By combining structural information from an enzyme-donor substrate complex with analysis of acceptor specificity we can now fully explain the observed product spectrum of 4,6-$\alpha$-GTases with a new mechanism, in which the enzyme transfers not only single, but also multiple glucosyl units to acceptor substrates. In addition, the GtfB-$\Delta N\Delta V$ crystal structure clearly supports the idea that the 4,6-$\alpha$-GTase GH70 subfamily is an evolutionary intermediate between $\alpha$-amylase and glucansucrase enzymes.

**Biological role of 4,6-$\alpha$-GTases in Lactobacillus reuteri**

GS more widely exist in LAB strains and are known to contribute to the formation of exopolysaccharides (EPS), which play a role in the biofilm formation. In the oral cavity, the produced biofilm containing insoluble EPS enhances the adhesion of bacteria to tooth surfaces and consequently stimulates dental caries formation. EPS may also have other functionalities for the strain such as retention of water, nutrient source, protective barrier, sorption of organic compounds, immobilization of enzymes, and so on. As 4,6-$\alpha$-GTase is able to produce polysaccharide from maltodextrins or starch instead of sucrose *in vitro*, the question was raised whether 4,6-$\alpha$-GTase also contributes to the *in vivo* EPS formation in their host LAB (**Chapter 4**). In the model strain *L. reuteri* 121, the *gtfB* gene was found to be essential for EPS formation when the strain was grown on maltodextrins or starch as carbon source. *L. reuteri* 121 can modify both amylose and external chains of amylopectin in starch by converting $\alpha 1\rightarrow 4$
linkages into $\alpha_{1\rightarrow6}$ linkages. The formed EPS have the same assembling segments as IMMP produced \textit{in vitro} by GtfB. In addition, the EPS can be used as carbon and energy source by some probiotic \textit{Bifidobacterium} strains, revealing that the EPS are potentially prebiotic. Therefore, the \textit{L. reuteri} strains that can produce dietary fiber and potential prebiotics may find applications in the fermented food industry (as synbiotics).

In the oral cavity, \textit{Lactobacillus} and \textit{Streptococcus} strains are ubiquitous bacteria and have been proven to produce EPS from sucrose through their glucansucrase. Sucrose and starch are major carbohydrates in the modern diet and their cariogenicity has been widely investigated. Although the cariogenicity of starch alone is a matter of debate, uptake of a combination of starch and sucrose is known to enhance cariogenicity by influencing the polysaccharide formation in biofilm. The mechanism is not fully understood. The discovery of the tandem glucansucrase GtfA encoding gene \textit{gtfA} and 4,6-$\alpha$-GTase GtfB encoding gene \textit{gtfB} in \textit{L. reuteri} strains may provide new insights with regard to cariogenicity through biofilm formation. Both \textit{in vivo} and \textit{in vitro} experimental results indicated a synergistic action of 4,6-$\alpha$-GTase GtfB and GS GtfA in the presence of both starch and sucrose (Chapter 5). Therefore, a new pathway of EPS formation from multiple dietary carbohydrates in LAB strains was found and may contribute to the research on dental caries.

\textbf{Opportunities and Challenges}

4,6-$\alpha$-GTases producing soluble dietary fiber and potential prebiotics from starch or maltodextrins are likely to draw strong attention from the food industry including bakery, beverage, sausage, and so on. The produced IMMP with certain percentages of $\alpha_{1\rightarrow4}$ and $\alpha_{1\rightarrow6}$ linkages may also function to stabilize protein during lyophilization, to enhance the solubility of proteins and drug molecules, to substitute fat, to replace starch as low-calorie bulking agent. In addition, 4,6-$\alpha$-GTase-modified starches may have typical rheological properties and thus be used in many aspects. The elucidation of the three-dimensional structure of GtfB-$\Delta$N$\Delta$V will support the further engineering of this protein, e.g. aiming to change its substrate specificity or to synthesize products with different linkage specificity. Moreover, the discovery of \textit{Lactobacillus} strains carrying 4,6-$\alpha$-GTase encoding genes opens up new prospective for the application of these strains in food
fermentations. In addition to the regularly produced nutrients, such as short chain fatty acids, these *Lactobacillus* strains synthesize soluble dietary fiber and potential prebiotics by converting starch that is abundantly present in fermented foods, such as sourdough, soy bean sauce.

However, many questions still challenge the application of 4,6-α-GTase. Firstly, how 4,6-α-GTase exactly modifies starch especially amylopectin is unclear. One possibility is that the α1→4-linked side chains of amylopectin are converted into α1→6-linked chains, which are still located at the outside of amylopectin molecules. The other possibility is that the α1→4-linked side chains of amylopectin are cleaved and formed separate α1→6-linked chains. Secondly, the mechanism and location for IMMP degradation in the human intestinal tract remains to be investigated. Thirdly, the thermostability and activity of 4,6-α-GTase remain to be improved by enzyme engineering. For this purpose, computational design is preferable. Moreover, for application of *Lactobacillus* strains that possess 4,6-α-GTases, improvement of their expression levels and enhancement of their activities under fermentation conditions are challenges. But such challenges constitute the charm of research on 4,6-α-GTases.