Chapter 6

Summary and Perspectives

Microbial β-galactosidase enzymes are widely used as biocatalysts in industry to produce prebiotic galactooligosaccharides (GOS) from lactose. GOS are added in infant formula to mimic the molecular size and prebiotic benefits of human oligosaccharides (hMOS) [1], [2], [3]. GOS are oligosaccharides that consist of a number of galactose units linked to a terminal glucose or galactose residue via different glycosidic bonds, with degrees of polymerization (DP) from 2 to 10 units [4]. Various β-galactosidases have been characterized and their GOS products identified. In recent years our research group has optimized GOS structural characterization [5], resulting in detailed analysis and comparison of 7 commercial GOS product mixtures [6]. The GOS composition of these mixtures varies strongly, in size and in structure, including variations in linkage specificity. It has remained unclear what enzyme structural features determine GOS diversity and linkage specificity (**Chapter 1**). To study this in more detail we first made a careful comparison of three commercially used β-galactosidase enzymes, from *B. circulans*, *K. lactis*, and *A. oryzae* (**Chapter 2**). It turned out that the β-galactosidase from *B. circulans* has the highest GOS yield and synthesizes the largest variety in GOS structures. Then structure-based site-directed mutagenesis was applied to the *B. circulans* β-galactosidase to clarify the functions of individual amino acid residues in the active site, followed by the structural determination of their product specificity (**Chapters 3 & 4**). In the last part, lactulose was used as substrate for the *B. circulans* wild-type and mutant enzymes to compare the lactulose and lactose product specificity, and to broaden the application of β-galactosidases (**Chapter 5**).

Comparison of three commercial β-galactosidases

β-Galactosidases from different origins are used to synthesize GOS mixtures from lactose, especially the enzymes from the bacterium *B. circulans*, the yeast *K. lactis*, and the mould *A. oryzae*. These three enzymes have been characterized previously regarding their GOS yield, the optimal reaction pH, and optimal

reaction temperature. In **chapter 2**, we reported a detailed comparative study of the product profiles of these three enzymes from lactose. We identified 21, 12, and 11 major GOS structures as products from the β-galactosidases from *B. circulans*, *K. lactis*, and *A. oryzae*, respectively. GOS produced by the *B. circulans* β-galactosidase is a mixture of (β1→4), (β1→2), (β1→3), (β1→6) linked structures, among which the most abundant structures have (β1→4) linkages. The (β1→4) elongation of lactose (4`GalLac) was the first synthesized structure, with lactose as both donor and acceptor substrate. The structures with other linkages were synthesized later, and were then elongated with further (β1→4) linked galactose units, forming a variety of GOS structures. The data show that β-galactosidase from *B. circulans* clearly has a strong preference for this (β1→4) linkage type. The *K. lactis* β-galactosidase mostly forms GOS with (β1→6) linkages. The structure [β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc*p*] was first formed using lactose as both donor and acceptor substrate. Then some non-lactose disaccharides were formed, and subsequently elongated with (β1→6) linkages. The β-galactosidase from *A. oryzae* also has a preference for synthesizing GOS with (β1→6) linkages, with [β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc*p*] as the major structure.

Also the maximum GOS yields were clearly different, namely 48.3±1.2%, 34.9±1.8%, and 19.5±2.2% for the β-galactosidases from *B. circulans*, *K. lactis*, and *A. oryzae*, respectively. The maximum yields were achieved at 8, 6, and 8 h of incubation, respectively. The kinetic changes of the major structures produced by these three enzymes showed that the GOS composition thus strongly depends on the enzyme source and is dynamically related to the reaction time. However, the determinants for these differences in GOS composition remained unclear.

Previous studies have speculated that the galactose and glucose produced by the β-galactosidase enzymes are inhibitors for the transgalactosylation reaction. Our experimental results showed that the effects of these two monosaccharides are

dependent on the enzyme source, and are not necessarily inhibitors. The β-galactosidases from *B. circulans* and *K. lactis* used both galactose and glucose as acceptor substrates in the transgalactosylation reaction. Glucose also could be used as acceptor substrate by the *A. oryzae* β-galactosidase, while galactose was a clear inhibitor for its transgalactosylation reaction [7].

Structural basis for product specificity of the β-galactosidase from *B. circulans*

GOS composition may vary in linkage type, in size (DP) and branching, but it has remained unclear what protein structural features determine this product specificity. Guided by the three dimensional structure of the *B. circulans* β-galactosidase, we identified residue Arg484 in the active site of its C-terminally truncated BgaD-D variant, and subjected it to site saturation mutagenesis (**Chapter 3**). The results showed that all the mutations at this position altered the product linkage specificity, and resulted in a clearly different GOS product specificity. NMR analysis of the GOS mixture produced by Arg484Ser revealed that it contained a combination of both (β1→3) and (β1→4) linkages, while the GOS mixture produced by the wild-type enzyme was dominated by (β1→4) linkages. It is also noticeable that the yield of the trisaccharide β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p* produced by mutants R484S and R484H increased 50 times compared to that of the wild-type enzyme. NMR analysis showed that 10 novel GOS structures were produced by the Arg484Ser mutant, thus greatly enriching the total number of known (from 60 to 70) GOS structures. Although the mutant enzymes had a clearly altered GOS linkage specificity, the GOS yield did not change significantly. These results indicate that residue R484 is crucial for the linkage specificity of BgaD-D. This is the first study showing that β-galactosidase enzyme engineering results in an altered GOS linkage specificity and product mixture [8].

Structure-function relationship of residues in the active site of β-galactosidase from *B. circulans*

The crystal structure of a C-terminally truncated version of β-galactosidase from *B. circulans* (BgaD-D) has been elucidated in detail [9]. However, the roles of amino acid residues in the active site have remained unknown. In **chapter 4**, based on the structural alignment of BgaD-D with BgaA from *Streptococcus pneumoniae*, and the complex of BgaA with LacNAc, we identified 8 active site amino acid residues (Arg185, Asp481, Lys487, Tyr511, Trp570, Trp593, Glu601, and Phe616) in BgaD-D, which can be grouped in four sets based on their precise location.

The first group includes Arg185 and Glu601 which are located in the -1 substrate binding subsite (+n represents the non-reducing end and -n the reducing end, with cleavage taking place between the +1 and -1 subsites [10]) of BgaD-D. All mutants of Arg185 and Glu601 lost detectable enzyme activity. These two residues are strongly conserved in sequence alignments of GH2 β-galactosidases. Combined with the structural alignment with BgaA, we conclude that these two non-catalytic residues are essential for enzyme activity and may be involved in binding and positioning of the substrate.

The second group contains only one residue, Tyr511. The OH-group of Tyr511 has a hydrogen-bond interaction with the nearby nucleophile Glu532, it may donate its proton to the nucleophile to assist in catalysis. None of the obtained mutants were active thus confirming that Tyr511 is essential for enzyme activity.

The third group includes three aromatic residues: Trp570, located near the +1 subsite, and Trp593 and Phe616 that are located near the -1 subsite. These three residues form an aromatic pocket shaping the substrate binding site. The mutations at these residues greatly reduced the enzyme activity, especially the non-aromatic substitutions. Analysis of their GOS profiles showed that mutations

at these sites changed the linkage preference and enhanced the percentage of small oligosaccharides. Besides, Trp570 is essential for determining a relatively high ratio of transgalactosylation and hydrolysis activity, suggesting that it is involved in the selection of the acceptor substrate, either water or carbohydrates. Similarly, Trp999, an aromatic residue located at the +1 subsite of *E. coli* LacZ, is also a key residue for the selection of acceptor substrates and to ensure high yields of transgalactosylation products [11].

The last group is Asp481 and Lys487. Together with Arg484, they are located near the +1 subsite. Mutations at Asp481 reduced the enzyme activity dramatically. The GOS product profiles of Asp481 and Lys487 mutants changed strongly compared to that of the wild-type enzyme, resembling the changes seen with Arg484 mutants (**Chapter 3**). Considering that Asp481 is close to the acid/base catalyst Glu447, mutations at this site may affect the orientation of Glu447, thus influencing the catalysis and substrate binding. Lys487 is relatively far away from the +1 subsite and the activity of the mutants derived is relatively high compared to the mutants at the Asp481 site. Some of the Asp481 and Lys487 mutants showed a similar GOS profile as the wild-type enzyme, while others showed a similar GOS profile to that of Arg484 mutants. Mutations in Lys487 may affect the micro-environment of the +1 subsite, thus affecting the GOS profile and linkage specificity.

Chapter 4 thus gives a detailed biochemical characterization and product profile analysis of mutant enzymes with changed amino acid residues in the active site of BgaD-D of *B. circulans*. The data showed that these residues play crucial roles in binding and positioning of the substrate, and thus in determining the enzyme activity and product profile [12].

Synthesis of oligosaccharides derived from lactulose by wild-type and R484H mutant β-galactosidases from *B. circulans* and their utilization for growth by Bifidobacteria

Oligosaccharides derived from lactulose (β-D-Gal*p*-(1→4)-D-Fru) are drawing more and more attention nowadays because of their strong resistance to gut digestion, and the need to discover novel prebiotics. Studies on the structures and functions of lactulose derived oligosaccharides (OsLu) are very limited. In **chapter 5**, we report the synthesis of OsLu using the wild-type and R484H mutant BgaD-D β-galactosidases from *B. circulans*, and the separation and identification of their structures. The probiotic bacteria *Bifidobacterium dentium* and *Bifidobacterium breve* strains were tested for their ability to grow and use these purified OsLu and TS0903 GOS (Vivinal GOS minus mono- and disaccharides) as carbon sources.

In total, 8 OsLu structures were identified by the NMR analysis, and 5 of them represent totally new structures, which greatly enriched the number of known OsLu structures. The wild-type BgaD-D enzyme had the highest product yield of 202.9±2.3 g/L in incubations with 15 U/g lactulose, using 60% (w/w) lactulose (60 ^{o}C, for 8 h). The R484H mutant enzyme had a highest product yield of 197.7±5.4 g/L in incubations with 15 U/g lactulose, using 60% (w/w) lactulose (60 ^{o}C, for 16 h). Both Bifidobacteria only consumed the newly discovered β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Fru structure when incubated with this OsLu mixture. When growing on TS0903 GOS, *B. dentium* had a strong preference for oligosaccharides with a lower DP (DP3) while *B. breve* had a preference for longer oligosaccharides (DP ≥ 4).

This study reports a detailed analysis of the synthesis of OsLu using a (mutant) β-galactosidase enzyme and their utilization as growth substrates by Bifidobacteria. Considering the very limited structures of OsLu reported previously and the

general lack of growth tests of OsLu with probiotic bacteria, this study gave important new insights into the structures and utilization of OsLu.

Conclusions

This thesis reported the biochemical characterization of β-galactosidase enzymes and their products. Firstly, a detailed analysis of the GOS products synthesized by three commercial β-galactosidases from *B. circulans*, *K. lactis*, and *A. oryzae*. Their yields, linkage specificity, and diversity of GOS products were very different from each other. Among them, β-galactosidase from *B. circulans* had the highest GOS yield and product diversity. Then, protein structure-based mutagenesis was applied to β-galactosidase from *B. circulans* to investigate the roles of specific amino acid residues in the active site, putative determinants of GOS linkage specificity, which had not been studied previously. The roles of these amino acid residues in enzyme functions, activity, and linkage specificity, were studied in detail. The residues in the -1 subsite are essential for the enzyme activity and substrate binding. The aromatic residues in the active site form an aromatic pocket that can shape the binding of the acceptor substrate, changing the linkage ratio of the GOS products. Besides, mutations in the Trp570 aromatic residue in the +1 subsite changed the transgalactosylation/hydrolysis ratio of the enzyme, suggesting that it is involved in the selection of acceptor substrate, either water or carbohydrates, essential for a relatively high transgalactosylation activity. The residues located near the +1 subsite are essential for the linkage specificity of the GOS products, mutations at these positions yielded a GOS mixture with altered linkage specificity. This is the first study showing that enzyme engineering changed the linkage specificity of β-galactosidase enzymes. Considering the sequence similarity between β-galactosidases, mutations in these residues also may be applied to other β-galactosidase enzymes, to change their linkage specificity or the transgalactosylation/hydrolysis ratio. GOS mixtures with altered linkage specificity may have new functionalities which can be used

as prebiotics in the future. The ultimate goal is to engineer β-galactosidase to produce GOS with specific structures and desired functions. Our study provided important insights into the structure-function relationships of β-galactosidase enzymes. Finally, lactulose was used as substrate for both the wild-type and mutant β-galactosidase enzymes from *B. circulans*. This resulted in the synthesis and structural characterization of 5 new oligosaccharides from lactulose. Growth tests with Bifidobacteria suggested that the OsLu mixture are promising new prebiotics.

β-Galactosidases are interesting enzymes and widely used as biocatalysts in industry. Further process engineering and enzyme engineering studies are likely to yield β-galactosidases that can produce tailor-made oligosaccharides with desired functions.

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