Exploring the glucosylation potential of glucansucrases

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Chapter 8

Summary and future prospects

Part 1: Dealing with the limitations of glucansucrases

The introduction of a glycosyl moiety can influence the physicochemical and biological properties of organic molecules such as anti-oxidants, antibiotics, and flavors[6]. The chemical synthesis of glycosides is characterized by multistep routes generating lots of waste. One-step enzymatic glycosylation, thereby taking advantage of the high specificity of enzymes, is preferred as 5-fold less waste is produced compared to chemical glycosylation[17]. Among all carbohydrate-active enzymes that can be applied as glycosylation biocatalyst, glucansucrases have received a considerable part of the attention due to their broad acceptor substrate specificity and use of inexpensive sucrose as donor substrate.

Glucansucrases are glycoside hydrolase enzymes (GH70) originating from Gram-positive lactic acid bacteria. They catalyze the conversion of sucrose into α-glucan polysaccharides, linking the α-D-glucopyranosyl units by (α1→2), (α1→3), (α1→4), or (α1→6) bonds, depending on the enzyme specificity[73,74]. In addition, they are able to transfer glucosyl groups to a wide array of carbohydrate and non-carbohydrate acceptor molecules, catalyzing the so-called acceptor reaction[75]. To this date, most research on the acceptor reaction of glucansucrases has focused on the discovery of new acceptor substrate specificities[111-113,120-131]. Less progress has been made on the detailed structural characterization of products obtained, or the improvement of glucansucrases as industrial glycosylation biocatalyst, neither by reaction –nor enzyme engineering strategies.

Glucansucrases display a number of drawbacks, in particular if the acceptor reaction is targeted. First of all, the main glucansucrase catalyzed reaction is the synthesis of α-glucan polysaccharides from sucrose. It strongly impedes the efficient glycosylation of alternative acceptor substrates and complicates downstream processing of the glycosylated products. **Chapter 2** explored the potential of the N-terminally truncated glucansucrase Gtf180 from *Lactobacillus reuteri* 180 (Gtf180-ΔN)[80] and derived mutants[99,100] as glycosylation

biocatalysts[110]. Three Gtf180-ΔN mutants (L938F, L981A and N1029M) were selected from a mutant library on the basis of their impaired α-glucan synthesis. Analysis of the glycosylation of the model acceptor substrate catechol by these mutants revealed that this apparent imperfection resulted in a substantial increase in monoglycosylation yield. Also several other phenolic and alcoholic compounds were more efficiently converted into glycosylated products by the selected mutants. For example, the resorcinol conversion degree of the L981A variant tripled compared to the WT enzyme, reaching a substantial 53%. Further analysis showed that these mutants possess a higher affinity for the model acceptor substrate catechol but a lower affinity for its mono-α-D-glucoside product, explaining the improved monoglycosylation yields. An explanation of how mutagenesis of residues L938, L981 and N1029 impaired α-glucan synthesis was provided by analyzing the available high resolution 3D crystal structure of the Gtf180-ΔN protein. On the downside, the k_{cat} of the best performing mutant (L981A) displayed a 4-fold decrease compared to the WT enzyme. In addition, the glycosylation of larger non-carbohydrate acceptor substrates such as resveratrol or quercetin remained problematic, partly due to the low water solubility of these compounds. The L981A mutant could nevertheless serve as template for further mutational engineering of the Gtf180-ΔN enzyme, targeting the improved glycosylation of specific acceptor substrates.

A second drawback related to the use of glucansucrases is their relatively low operational stability at high temperatures and in systems containing high acceptor substrate concentrations and cosolvents[110,120,125]. Immobilization of Gtf180-ΔN on mesoporous silica particles enhanced its activity at temperatures above 50 °C and high concentrations of DMSO, conditions detrimental for the free enzyme (**Chapter 3**). Covalent cross-linking of Gtf180-ΔN with glutaraldehyde resulted in an undesired side-effect: less catechol was converted by the immobilized enzyme compared to the free enzyme, in favor of sucrose hydrolysis. Stabilizing Gtf180-ΔN by the developed immobilization protocol nevertheless allowed its incubation in 20% DMSO systems containing several poorly soluble acceptor substrates such as gallic acid and (+)-catechin, resulting in their improved glycosylation.

Part 2: Glycosylation of steviol glycosides

The steviol glycosides extracted from the leaves of the plant *Stevia rebaudiana* were approved for use as high-intensity sweetener (HIS) in food products by the European Commission in 2011[43]. Stevia extract consists mainly of the steviol glycosides stevioside and rebaudioside A (RebA), however, they display a bitter (after)taste which prevents the creation of zero-calorie stevia soft drinks and a complete sugar substitution in other food products[46]. Solving this taste issue could therefore greatly expand stevia sales and enforce its position on the HIS market. The correlation between the structure of steviol glycosides and their taste quality is still not fully understood, however, it is clear that the latter depends on the number, location and configuration of the glycosyl moieties[8]. Hence, the enzymatic glycosylation of steviol glycosides has been proposed as effective tool to improve their sensory properties[50-70].

Screening of our in-house collection of glucansucrases revealed that only Gtf180-ΔN from *L. reuteri* 180 is able to glycosylate RebA, displaying a RebA conversion of roughly 50% (**Chapter 4**). As this is insufficient for industrial applications, the ability of several Gtf180-ΔN mutants to glycosylate RebA was explored, revealing that the Q1140E mutant is the most promising variant. Structural analysis of the products showed that both enzymes exclusively glycosylate RebA at the Glc(β1→C-19 residue, with the formation of an (α1→6) linkage. Docking of RebA in the enzyme's active site provided an explanation for these results: only the steviol C-19 β-D-glucosyl moiety is available for glycosylation. Several previous studies already demonstrated the importance of an adequate C-13/C-19-regiospecificity for the taste quality of the glycosylated products. For example, (α1→4)-glycosylation of stevioside and rubusoside at the C-13 steviol position yielded products with improved intensity and quality of sweetness, whereas (α1→4)-glycosylation at the C-19 position resulted in an increased bitterness[52-54]. Alternatively, (α1→6)-glycosylation at the C-19 site of stevioside improved its taste profile[60].

Optimizing the reaction conditions of the Gtf180-ΔN-Q1140E-catalyzed glycosylation of RebA by response surface methodology (RSM) yet again identified the (low) remaining α-glucan synthesis as an important impediment to obtain high conversion degrees and high product concentrations. Nevertheless, a highly productive process with a RebA conversion of 95% and a production of 115 g/L glycosylated product (RebA-G) within 3 h was achieved at optimal reaction conditions. Development of a fed-batch reaction with continuous addition of relatively low sucrose levels further improved the product yield to 270 g/L by adequately further suppressing α-glucan synthesis. Sucrose acts as primer for α-glucan synthesis[103,104,135]; a constant excess of RebA relative to sucrose therefore favored the glycosylation of RebA. The continuous addition of sucrose ensured that enough donor substrate was available to drive the reaction. On the downside, 10 times more enzyme had to be supplied to the reaction mixture in order to obtain a 95% conversion within 3 h, which substantially adds to the production costs. Additionally, performing the fed-batch reaction required the addition of 34% more sucrose compared to performing the glycosylation in batch mode. Altogether, the batch reaction is thus preferred over the fed-batch reaction as performed in this work.

Sensory analysis of RebA and the glycosylated RebA products by a trained panel showed that RebA-G has a superior taste profile, displaying a significant reduction in bitterness compared to RebA. Glycosylation of RebA at the Glc(β1→C-19 residue with the introduction of an (α1→6) linkage is thus a very appropriate method to improve its sensory properties. Whether the off-flavors are sufficiently reduced to allow the production of zero-calorie soft drinks, jams, yoghurts, etc. is currently unknown. Answering this question requires the analysis of the sensory properties of the end product, including for example the bulking agent. In addition, the physicochemical properties of the ingredients, such as viscosity, density, etc. play an important role for the taste quality of the end product.

Stevioside is the most prevalent steviol glycoside (5-10% of leaf dry weight) and is perceived by consumers as more bitter than RebA (2-4% of leaf dry weight).

This explains why all commercial stevia products are high-purity variants of RebA, while stevioside is considered as an undesired side-product. Improvement of the sensory properties of stevioside by means of its glycosylation consequently offers an opportunity to valorize this “waste” stream. Incubation of stevioside with Gtf180-ΔN-Q1140E indeed resulted in its glycosylation, although at a lower efficiency than RebA glycosylation (as determined by RSM): 50 g/L glycosylated stevioside product (Stev-G) was produced with a 95% conversion, using 5 times more sucrose than needed for RebA glycosylation (**Chapter 5**). Elucidation of the glycosylated stevioside product structures revealed that, in contrast to RebA glycosylation, stevioside was not exclusively glycosylated at the C-19 site; minor products were also glycosylated at the C-13 site. Similarly to RebA glycosylation, the main product was glycosylated at the C-19 site, with the introduction of an (α1→6) linkage (Stev-G1). Remarkably, the most prevalent diglycosylated product contained an (α1→4) linkage and not the expected (α1→3) –or (α1→6) linkages, reflecting the regular linkage specificity of Gtf180[82].

Sensory analysis of the glycosylated stevioside products showed a significant reduction in bitterness compared to stevioside. The trained panel perceived Stev-G1 as sweet as stevioside but also as significantly less bitter. The large proportion of multiglycosylated products in Stev-G (67.5%) was translated into a significantly reduced sweetness, in contrast to RebA-G (22.3% multiglycosylated products) which maintained the sweetness of RebA. Interestingly, a double dose of Stev-G displayed a sweetness level similar to that of stevioside whereas bitterness remained equally suppressed. Comparison of the sensory properties of RebA-G and Stev-G identified the former as the superior product.

As the glycosylation of RebA and stevioside with Gtf180-ΔN-Q1140E showed great potential at laboratory scale, an efficient overall process at kg scale, including downstream processing, was developed (**Chapter 6**). Ultimately, adsorption of the glycosylated product with removal of sucrose, fructose, glucose and α-glucan oligo –and polysaccharides was applied as main purification step. An estimation of the production costs demonstrated the main strength of the process: the biocatalyst represents a minor part of the total cost, which is mostly

determined by the cost of the acceptor substrate, i.e. RebA or stevioside. A major cost reduction of 30% can consequently be achieved by using low-grade stevia extract as acceptor substrate instead of the more costly high-purity steviol glycosides. An additional advantage is that the side-product stevioside is valorized, representing an extra economic profit. If taste quality is priority, RebA is preferred as acceptor substrate as it displays a superior taste profile.

Gtf180-ΔN-Q1140E also displayed activity towards neohesperidin dihydrochalcone (NHDC), even though a lower conversion degree (64%) was obtained (**Chapter 7**). Use of 5-10 ppm NHDC with RebA is known to result in a decreased bitterness perception. Unfortunately, this is accompanied by an unpleasant lingering sensation, caused by NHDC[247]. Its glycosylation by Gtf180-ΔN-Q1140E resulted in a decreased perception of the off-flavors by the trained panel. In general, the bitterness suppressing effect of (glycosylated) NHDC on RebA was limited; RebA glycosylation reduced its bitterness much more effectively. Very recently, NHDC was attributed with potent anti-oxidant, hepatoprotective and prebiotic properties[260-264]. NHDC's low water solubility[265], restricting its application as nutraceutical, was overcome by its glycosylation, while its anti-oxidant potential remained very high.

Conclusions

This thesis explored the potential of Gtf180-ΔN from *L. reuteri* 180 and derived mutants to glycosylate a wide range of alternative acceptor substrates. It was shown that the Q1140E mutant is particularly suited to glycosylate steviol glycosides. High conversion degrees and product yields were obtained, and the glycosylated products displayed a superior taste profile compared to RebA and stevioside. The developed processes consequently hold excellent potential to be implemented at industrial scale.

The Q1140E mutant was also able to glycosylate other glycosides, such as NHDC, although at a lower efficiency than the glycosylation of steviol glycosides.

This is another illustration of the broad carbohydrate acceptor substrate specificity of Gtf180-ΔN and derived mutants. The high water solubility of carbohydrates forms an additional advantage for their Gtf180-ΔN catalyzed glycosylation, thus avoiding the use of inhibitory cosolvents in the reaction mixture.

The glycosylation of non-carbohydrate acceptor substrates was achieved with more difficulty, partly due to their low water solubility and their inhibition of Gtf180-ΔN. α-Glucan synthesis was identified as important impediment for the glycosylation of these compounds, however, its suppression by mutational engineering only partially solved the problem. In other words, specific mutants will need to be constructed to further improve the Gtf180-ΔN glycosylation potential, similarly to the approach followed for the glycosylation of steviol glycosides by the Q1140E mutant of Gtf180-ΔN.

In conclusion, the glucansucrase enzyme Gtf180-ΔN holds considerable potential as glycosylation biocatalyst, in particular for the glycosylation of carbohydrate acceptor substrates. The development of industrial processes will first and foremost depend on the construction of adequate mutants displaying high conversion degrees, and secondly, on the optimization of the reaction conditions, thereby suppressing α-glucan synthesis from sucrose as much as possible.