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Exploring the glycosylation potential of glucanases

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Chapter 4

Glucansucrase (mutant) enzymes from *Lactobacillus reuteri* 180 efficiently transglucosylate *Stevia* component rebaudioside A, resulting in a superior taste

This chapter is submitted for publication as:

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[#] these authors contributed equally to this work

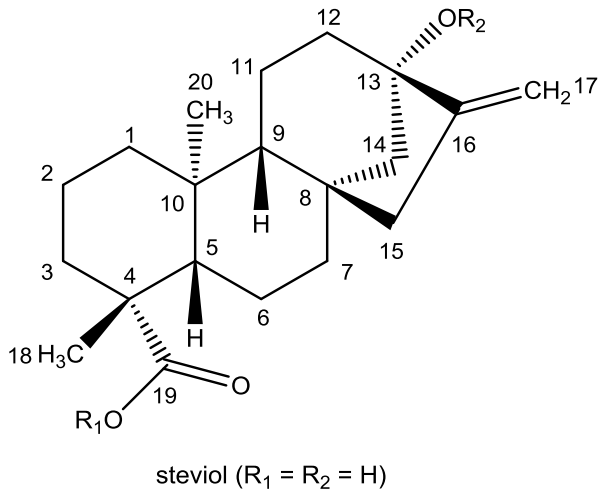
Abstract

Steviol glycosides from the leaves of the plant *Stevia rebaudiana* are high-potency natural sweeteners but suffer from a lingering bitterness. The *Lactobacillus reuteri* 180 wild-type glucansucrase Gtf180- Δ N, and in particular its Q1140E-mutant, efficiently α -glucosylated rebaudioside A (RebA), using sucrose as donor substrate. Structural analysis of the products by MALDI-TOF mass spectrometry, methylation analysis and NMR spectroscopy showed that both enzymes exclusively glucosylate the Glc(β 1 \rightarrow C-19 residue of RebA, with the initial formation of an (α 1 \rightarrow 6) linkage. Docking of RebA in the active site of the enzyme revealed that only the steviol C-19 β -D-glucosyl moiety is available for glucosylation. Response surface methodology was applied to optimize the Gtf180- Δ N-Q1140E-catalyzed α -glucosylation of RebA, resulting in a highly productive process with a RebA conversion of 95% and a production of 115 g/L α -glucosylated products within 3 h. Development of a fed-batch reaction further improved the product yield to 270 g/L by suppressing α -glucan synthesis. Sensory analysis by a trained panel revealed that glucosylated RebA products have a superior taste profile compared to RebA, showing a significant reduction in bitterness. The Gtf180- Δ N-Q1140E glucansucrase mutant enzyme thus is an efficient biocatalyst for generating α -glucosylated RebA variants with improved edulcorant/organoleptic properties.

1. Introduction

The world-wide increasing incidence of obesity, diabetes type II, cardiovascular diseases, and dental caries leads to an increased consumer demand for food products and beverages without high-calorie sugars⁴⁶. Steviol glycosides are excellent natural alternatives for sucrose and synthetic sweeteners¹⁸⁵⁻¹⁸⁹. These non-calorie compounds are extracted from the leaves of the herb plant *Stevia rebaudiana* BERTONI, a rhizomatous perennial shrub belonging to the Asteraceae [Compositae] family^{190,191}. Stevioside (~5-20% w/w of dried leaves) and rebaudioside A (RebA) (~2-5% w/w of dried leaves) are the most abundant steviol glycosides, followed in lower concentrations by rebaudioside B, C, D, E, F, M, steviolbioside, rubusoside and dulcoside A (Figure 1). Stevioside and RebA taste about 300 times sweeter than sucrose (0.4% aqueous solution). Steviol glycosides are approved as food additives in the USA since 2009 and they are on the European market (E 960, European Index) since December 2011⁴³. The main drawback for their more successful commercialization as sweeteners however, is a lingering bitter aftertaste of several steviol glycosides, experienced by about half of the human population due to a genetic basis of taste perception^{46,192}.

Structurally, steviol glycosides have *ent*-13-hydroxykaur-16-en-19-oic acid as aglycon, also called steviol (Figure 1)^{191,193}. The presence and composition of the different carbohydrate moieties at the C-19-carboxylic acid group (R_1) and at the C-13-*tert*-hydroxyl group (R_2) of steviol have a relationship with the sweetness as well as with the quality of taste of the steviol glycosides⁵². Sweetness increases and bitterness perception decreases with the total number of glycosyl residues⁴⁶. It has to be noted however, that the correlations bitterness/structure and sweetness/structure of steviol glycosides are still not fully understood, particularly in combination with the interactions with the human taste receptors^{46,192,194,195}.



Steviol glycoside	R_1 (C-19)	R_2 (C-13)
Stevioside	Glc(β 1 \rightarrow)	Glc(β 1 \rightarrow 2)Glc(β 1 \rightarrow)
Steviolbioside	H	Glc(β 1 \rightarrow 2)Glc(β 1 \rightarrow)
Rebaudioside A	Glc(β 1 \rightarrow)	Glc(β 1 \rightarrow 2)[Glc(β 1 \rightarrow 3)]Glc(β 1 \rightarrow)
Rebaudioside B	H	Glc(β 1 \rightarrow 2)[Glc(β 1 \rightarrow 3)]Glc(β 1 \rightarrow)
Rebaudioside C	Glc(β 1 \rightarrow)	Rha(α 1 \rightarrow 2)[Glc(β 1 \rightarrow 3)]Glc(β 1 \rightarrow)
Rebaudioside D	Glc(β 1 \rightarrow 2)Glc(β 1 \rightarrow)	Glc(β 1 \rightarrow 2)[Glc(β 1 \rightarrow 3)]Glc(β 1 \rightarrow)
Rebaudioside E	Glc(β 1 \rightarrow 2)Glc(β 1 \rightarrow)	Glc(β 1 \rightarrow 2)Glc(β 1 \rightarrow)
Rebaudioside F	Glc(β 1 \rightarrow)	Xyl(β 1 \rightarrow 2)[Glc(β 1 \rightarrow 3)]Glc(β 1 \rightarrow)
Rebaudioside M	Glc(β 1 \rightarrow 2)[Glc(β 1 \rightarrow 3)]Glc(β 1 \rightarrow)	Glc(β 1 \rightarrow 2)[Glc(β 1 \rightarrow 3)]Glc(β 1 \rightarrow)
Rubusoside	Glc(β 1 \rightarrow)	Glc(β 1 \rightarrow)
Dulcoside A	Glc(β 1 \rightarrow)	Rha(α 1 \rightarrow 2)Glc(β 1 \rightarrow)

Figure 1. Structures of major steviol glycosides, occurring in the leaves of *Stevia rebaudiana*. Glucose (Glc), xylose (Xyl) and rhamnose (Rha) occur in the pyranose ring form. Glc and Xyl have D configuration and Rha L configuration.

To improve the taste of steviol glycosides, especially for food applications, various (enzymatic) modifications of the carbohydrate moieties of steviol glycosides have been reported, mainly using cyclodextrin glycosyltransferase

(CGTase), α - and β -glucosidase, α - and β -galactosidase and β -fructosidase transglycosylation and β -glycosyltransferase glycosylation systems as biocatalysts⁸. In the context of our study, the reports on trans- α -glucosylation are of interest. CGTases are attractive enzymes, which catalyze coupling and disproportionation reactions, transferring glucose residues from starch or cyclodextrins to acceptor molecules, yielding Glc(α 1 \rightarrow 4) extensions. Although often high yields are obtained with steviol glycosides, CGTases have poor steviol C-13/C-19 site regiospecificity, producing mixtures of steviol glycoside derivatives with mostly (α 1 \rightarrow 4)-glucosylation at both carbohydrate moieties⁵¹. Several early studies have shown that both mono- and di-(α 1 \rightarrow 4)-glucosylation of the carbohydrate moiety at the steviol C-13 site of stevioside and rubusoside gave products with a remarkable improvement in both intensity and quality of sweetness. However, (α 1 \rightarrow 4)-glucosylation of the Glc(β 1 \rightarrow residue at the steviol C-19 site resulted in an increased bitter aftertaste and a lower sweetness intensity^{52-54,196}. α -Glucosylation of stevioside using Biozyme L (β -amylase preparation, probably contaminated with an α -glucosidase) and maltose as donor substrate resulted in a product with a decreased sweetness, but a remarkable improvement in the quality of taste [Glc(α 1 \rightarrow 6) residue attached at the Glc(β 1 \rightarrow C-19 residue], a product with a much lower sweetness [Glc(α 1 \rightarrow 6) residue attached to the terminal Glc(β 1 \rightarrow 2) residue of the β -sophorosyl-C-13 unit] and a product with a bitter taste [Glc(α 1 \rightarrow 3) residue attached to the terminal Glc(β 1 \rightarrow 2) residue of the β -sophorosyl-C-13 unit]⁶⁰.

In order to obtain steviol glycoside derivatives with improved organoleptic properties, we studied the α -glucosylation potential of mutant glucansucrase enzymes of the generally recognized as safe (GRAS) bacterium *Lactobacillus reuteri* 180 on RebA. Glucansucrases (EC 2.1.4.5; glycosyltransferases, Gtfs) are extracellular enzymes catalyzing the synthesis of α -D-glucan polymers from the donor substrate sucrose, thereby introducing different ratios of glycosidic linkages [(α 1 \rightarrow 2), (α 1 \rightarrow 3), (α 1 \rightarrow 4), (α 1 \rightarrow 6)] in their glucan products, depending on the enzyme specificities^{75,162}.

Recently, we have shown that the wild-type Gtf180- Δ N (N-terminally truncated) glucansucrase enzyme of *L. reuteri* 180 was able to glucosylate the steviol glycoside RebA, using sucrose as glucosyl donor substrate⁶⁹. About 55% of RebA was glucosylated with up to eight α -D-glucosyl units attached (RebA-G). The formed RebA derivatives only had elongations at the steviol C-19 β -D-glucosyl moiety, mainly with alternating (α 1 \rightarrow 6)- and (α 1 \rightarrow 3)-linked glucopyranose residues, starting with an (α 1 \rightarrow 6) linkage (RebA-G1). In the present study, we have screened our in-house collection of mutated Gtf180- Δ N glucansucrase enzymes for mutants with a better RebA glucosylating activity than the wild-type Gtf180- Δ N enzyme. One mutant was selected for more detailed studies and its biochemical characteristics and product profile were compared to wild-type Gtf180- Δ N. Glucosylated RebA products were isolated by flash chromatography and their structures were elucidated using MALDI-TOF mass spectrometry, methylation analysis and 1D/2D $^1\text{H}/^{13}\text{C}$ NMR spectroscopy. Furthermore, docking experiments with RebA and the available high-resolution 3D structure of Gtf180⁸³ were carried out to evaluate the experimental data. Response surface methodology was applied to optimize the reaction conditions of RebA glucosylation with the selected mutant. Finally, sensory evaluations were performed to determine the taste attributes of the novel α -glucosylated RebA derivatives.

2. Materials and methods

2.1. Glucansucrase enzymes

Gtf180- Δ N is the 117-kDa N-terminally truncated (741 residues) fragment of the wild-type Gtf180 full-length protein, derived from *L. reuteri* strain 180⁸⁰. In Gtf180- Δ N- Δ V both the N-terminal variable domain and the N-terminal domain V fragment (corresponding to the first 793 N-terminal amino acids), and the C-terminal domain V fragment (corresponding to the last 136 C-terminal amino acids) have been deleted⁸⁸. The glucansucrase Gtf180- Δ N mutant enzymes were

constructed using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA)^{80,88,97-100,102,197} (Table SI).

2.2. Standard reaction buffer

All enzymatic reactions were performed at 37 °C in 25 mM sodium acetate (pH 4.7), containing 1 mM CaCl₂.

2.3. Enzyme activity assays

Enzyme activity assays were performed at 37 °C in reaction buffer with 100 mM sucrose. Samples of 100 µL were taken every min over a period of 8 min and immediately inactivated with 20 µL 1 M NaOH. The glucose and fructose concentrations were enzymatically determined by monitoring the reduction of NADP with the hexokinase and glucose-6-phosphate dehydrogenase/phosphoglucose isomerase assays (Roche Nederland BV, Woerden, The Netherlands)¹⁶⁷. Determination of the release of glucose and fructose from sucrose allowed calculation of the total activity of the glucansucrase enzymes¹⁶⁶. One unit (U) of enzyme is defined as the amount of enzyme required for producing 1 µmol fructose per min in reaction buffer, containing 100 mM sucrose, at 37 °C.

2.4. Screening of (mutant) glucansucrases for α-glucosylation of RebA

In an initial screening, six Gtf180-ΔN-derived mutants were compared to wild-type Gtf180-ΔN by analyzing reaction products with high-performance liquid chromatography (HPLC). Then, an additional 76 Gtf180-ΔN mutants from our collection (Table SII) were screened and analyzed using thin-layer chromatography (TLC). Incubations of 3 h were performed in reaction buffer, containing ~1 mg/mL enzyme, 50 mM RebA (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) and 1 M sucrose (for HPLC analysis) or 0.2 M sucrose (for TLC analysis). For HPLC analysis, 10 µL of the incubation mixture was diluted in 250 µL 80% methanol and centrifuged for 2 min at 15,000 × g. Then, 40 µL of the

upper phase was injected on a Luna 10 μm NH_2 column (250 \times 4.6 mm; Phenomenex, Utrecht, The Netherlands). Separation was obtained at a flow-rate of 1 mL/min under gradient elution conditions (solvent A = acetonitrile; solvent B = 0.025% acetic acid in H_2O), starting with a 2-min isocratic step of 70% solvent A followed by a linear gradient from 70 to 55% solvent A over 9 min and a final 3-min washing step of 20% solvent A. HPLC analyses were performed using an UltiMate 3000 chromatography system, equipped with a VWD-3000 UV-vis detector (ThermoFisher Scientific, Amsterdam, The Netherlands; monitoring at 210 nm). For TLC analysis, 1 μL of the enzymatic reaction mixtures was spotted on TLC sheets (Kieselgel 60 F254, 20 \times 20 cm; Merck, Darmstadt, Germany), which were developed in *n*-butanol/acetic acid/water (2:1:1, v/v/v). After drying of the sheets, the bands were visualized by orcinol/sulfuric acid staining.

2.5. Quantitative preparation of α -glucosylated RebA products

Incubations of 84 mM RebA (Tereos PureCircle Solutions, Lille, France; 97% purity, HPLC grade) were performed in 50 mL reaction buffer with 282 mM sucrose donor substrate, using 5 U/mL Gtf180- ΔN -Q1140E enzyme, during 3 h. Fractionations of RebA-G were carried out by flash chromatography using a Reveleris X2 flash chromatography system (Büchi Labortechnik AG, Flawil, Switzerland) with a Reveleris C18 cartridge (12 g, 40 μm) with water (solvent A) and acetonitrile (solvent B) as the mobile phase (30 mL/min). The following gradient elution was used: 95% solvent A (0-2 min), 95-50% solvent A (2-20 min), 50-95% solvent B (20-22 min), 95% solvent B (22-25 min). The collected fractions were evaporated *in vacuo* and subsequently freeze dried to remove the residual water.

2.6. Design of response surface methodology experiment

Response surface methodology¹⁹⁸ was applied to optimize the glucosylation of RebA. A Box-Behnken design¹⁹⁹ was generated implementing RebA concentration (mM), sucrose/RebA ratio and agitation rate (rpm) as factors. For

each of them low (-1) and high (+1) level values were assigned as follows: RebA concentration (50 mM) and (200 mM), sucrose/RebA ratio (1:1) and (4:1), agitation rate (0 rpm) and (200 rpm). The addition of 5 U/mL Gtf180-ΔN-Q1140E enzyme ensured a steady-state was reached within 3 h of incubation. The experimental design was generated and analyzed using JMP software (release 12)²⁰⁰ and consisted of 15 experiments carried out at 50 mL scale in shake flasks, continuously mixed by shaking (Table SIII). Results were analyzed with HPLC (see below). The response surface analysis module of JMP software was applied to fit the following second order polynomial equation:

$$\hat{Y} = \beta_0 + \sum_{i=1}^I \beta_i X_i + \sum_{i=1}^I \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j$$

where \hat{Y} is the predicted response, I is the number of factors (3 in this study), β_0 is the model constant, β_i is the linear coefficient associated to factor X_i , β_{ii} is the quadratic coefficient associated to factor X_i^2 and β_{ij} is the interaction coefficient between factors X_i and X_j . X_i represents the factor variable in coded form:

$$X_{c,i} = \frac{[X_i - (low + high)/2]}{(high - low)/2}$$

with $1 \leq i \leq I$, where $X_{c,i}$ is the coded variable.

For the HPLC analysis of the RebA and glucosylated RebA products, an Agilent ZORBAX Eclipse Plus C18 column (100 × 4.6 mm, 3.5 μm) was used with water (solvent A) and acetonitrile (solvent B) as the mobile phase. The flow rate and temperature were set at 1.0 mL/min and 40 °C, respectively. The following gradient elution was used: 5-95% solvent B (0-25 min), 95% solvent B (25-27 min), 95-5% solvent B (27-30 min) and again 95% of solvent A (30-35 min). Detection was achieved with an ELS detector (evaporation temperature, 90 °C; nebulization temperature, 70 °C; gas flow rate, 1.6 SLM). Calibration of the obtained peaks for RebA and mono-α-glucosylated RebA (RebA-G1) was

accomplished using the corresponding standard curves. In this context, the concentration of multi- α -glucosylated product (RebA-G) at a specific time was calculated as the initial RebA concentration minus the RebA concentration at that time. Multi-glycosylated RebA product lacking RebA-G1 (defined as RebA-G2+) was equal to RebA-G minus RebA-G1.

2.7. Methylation analysis, mass spectrometry and NMR spectroscopy

For details of methylation analysis, gas-liquid chromatography – electron impact mass spectrometry (GLC-EIMS), matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and 1D/2D $^1\text{H}/^{13}\text{C}$ (TOCSY, ROESY, HSQC) NMR spectroscopy, see ref⁶⁹.

2.8. Molecular docking of RebA in the active site of Gtf180- ΔN and Gtf180- $\Delta\text{N-Q1140E}$

The X-ray crystal structure of Gtf180- ΔN complexed with maltose (PDB code: 3KLL⁸³) was used for docking by using LeadIT 2.1.8 from BiosolveIT²⁰¹. The acceptor binding site was defined by manual selection and included the following amino acid residues: 935–941, 944, 964–968, 975–983, 985, 1023–1032, 1035, 1061–1069, 1082–1093, 1096, 1111, 1129–1142, 1144, 1145, 1155, 1183, 1202, 1204, 1407, 1409, 1411, 1412, 1443, 1446, 1456–1458, 1463–1466, 1504–1509, 1511, 1526, 1527, and the water molecules numbered 7, 15, 45, 83, 106, 144, 145, 172, 401, 432–436, 466, 469, 470, 527, 572, 605, 648, 666, which have at least two interactions. The crystal structure of rebaudioside A.4H₂O.1CH₃OH was taken from the literature (CSD entry: DAWCEL)²⁰², and the water and methanol were removed. The default settings were used for docking, except for the following features: the docking strategy was chosen to be driven by Entropy (Single Interaction Scan), a hard enzyme was used (maximum allowed overlap volume: 2 Å) and the maximum number of solutions per fragmentation was increased to 400. In total, 30 poses were generated with LeadIT, using the scoring function HYDE in SeeSAR²⁰³, ranked according to their estimated affinity; finally, poses with torsions flagged in red were removed. Visual inspection of the

residual poses and deletion of unreliable poses resulted in a trustable set of poses. The model of mutant Q1140E, which was used for the docking experiment, was built in PyMOL²⁰⁴ and the rotamer showing the smallest number of steric clashes was chosen.

2.9. Sensory analysis

Panellists were selected on basis of their performance on basic taste recognitions, ability to ascertain degrees of differences for specific taste stimuli at different concentrations and repeatability²⁰⁵, as verified by triangle tests and Wald sequential analysis²⁰⁶. Following the selection, the panel was trained over a 6-month period. In a first session, the panellists had to taste RebA solution at 10% (w/w) sucrose equivalent level. Their own vocabulary was used to describe taste and off-tastes as well as after tastes. In the second session, the following attributes were selected from the first session and from literature²⁰⁷⁻²⁰⁹: sweetness, liquorice, astringency and bitterness. In the following months, training sessions were alternated with discussion sessions to agree on scoring of sweetness, off-tastes, aftertastes and lingering on a 15 point hedonic scale, and evaluation protocol.

The actual Quantitative Descriptive Analysis (QDA) was performed in individual tasting booths at the UGent Sensolab (Belgium) by the trained panel (9 persons). The fixed evaluation protocol with standardized vocabulary was applied. Firstly, taste (sweetness, liquorice, astringency and bitterness) was evaluated by swirling the sample in the mouth for 5 sec after which the sample was expectorated. Secondly, aftertaste was evaluated 10 sec after swallowing the solution. Next, lingering based on the maximum taste intensity was rated 1 min later. Sucrose reference solutions (5%, 7.5% and 10% sucrose scoring 5, 7.5 and 10, respectively) were provided. Water (Spa Reine) and plain crackers were used as palate cleansers between sampling. All samples were evaluated in duplicate.

Statistical analyses were performed with SPSS 23 (SPSS Inc., Chicago, USA). All tests were done at a significance level of 0.05. One-Way ANOVA was used to

investigate any significant difference between the solutions. Testing for equal variances was executed with the Modified Levene Test. When conditions for equal variance were fulfilled, the Tukey test²¹⁰ was used to determine differences between samples. In case variances were not equal, the Games-Howell test was performed²¹¹.

Three different glucosylated products were examined: multi- α -glucosylated product, containing residual RebA (RebA-G), mono- α -glucosylated product (RebA-G1) and RebA-G lacking RebA and RebA-G1 (RebA-G2+). Note that RebA-G, as defined here, contains a very minor amount of residual RebA, in contrast to the RebA-G as quantified by HPLC analysis.

3. Results

3.1. Screening of wild-type Gtf180- Δ N and mutant glucansucrase enzymes for α -glucosylation of RebA

The RebA glucosylation activity of 82 mutant enzymes of Gtf180- Δ N (Tables SI and SII) was compared to that of the Gtf180- Δ N wild-type enzyme. To this end, 50 mM RebA was incubated for 3 h with ~1 mg/mL of each enzyme in reaction buffer, containing either 0.2 M sucrose (for TLC product analysis) or 1 M sucrose (for HPLC product analysis). It has to be noted that the *in vivo* and most important activity of glucansucrases is the synthesis of α -D-glucan polymers and oligosaccharides from the donor substrate sucrose⁷⁵. During the transglucosylation reaction with acceptor substrates such as steviol glycosides, the formation of α -gluco-oligo/polysaccharides is observed as a side-reaction, occurring to a varying extent depending on the specific (mutant) glucansucrase. The TLC (Figure S1) and HPLC (Figure S2) profiles obtained after the different enzyme incubations with RebA and sucrose clearly showed that most of the Gtf180- Δ N mutants could α -glucosylate RebA in similar amounts (based on spot intensity) as the wild-type Gtf180- Δ N enzyme (TLC lane 77). Mutant A978P (TLC lane 64) and mutants Q1140E and S1137Y (Figure S2), however, converted

more RebA than the wild-type enzyme, as can be seen by a smaller amount of residual RebA after the 3 h incubation. Some mutants, i.e. L981A (TLC lane 31), W1065L (TLC lane 71) and W1065Q (TLC lane 72), converted comparable amounts of RebA as the wild-type enzyme, but showed almost no polymerization activity, as indicated by the absence of the α -gluco-oligo/polysaccharide tails on TLC. However, previously it has been shown that the mutations L981A, W1065L and W1065Q had a dramatic effect on the overall enzyme activity, resulting in a 93, 87 and 93% decrease at 100 mM sucrose, respectively^{99,102}. Of all mutants tested, Q1140E showed the highest RebA conversion (Figure S2), and was therefore chosen for further studies, and compared to the wild-type Gtf180- Δ N enzyme. Mutant Gtf180- Δ N-Q1140E contains a single amino acid substitution (from a glutamine to a glutamate) close to the transition-state-stabilizing residue D1136⁹⁷.

3.2. Analytical details of α -glucosylated RebA products prepared with Gtf180- Δ N-Q1140E

For structural analysis purposes, a large-scale preparation of α -glucosylated RebA products was performed using 84 mM RebA, 282 mM sucrose and 5 U/mL Gtf180- Δ N-Q1140E enzyme (pH 4.7, 3 h, 37 °C). The applied incubation conditions, shown to be optimal, were taken from the Box-Behnken experimental design study, as described in the section “Optimization of the synthesis of α -glucosylated RebA” (see below). The used commercial RebA substrate is of high purity, as indicated by its ¹H NMR spectrum (Figure S3; for the assignment of the resonances, see ref⁶⁹), MALDI-TOF mass spectrum (Figure S4) and methylation analysis (Table SIV). Contamination with other steviol glycosides was not detected, which is also of importance for the sensory analysis.

MALDI-TOF-MS analysis of the total RebA-incubation mixture (RebA-G) showed a series of quasi-molecular ions $[M+Na]^+$, revealing remaining RebA (m/z 989.7) and extensions of RebA with one (major peak, m/z 1152.9) up to eight glucose residues (m/z 2287.9) (Figure S4). The ¹H NMR spectrum of RebA-G (Figure 2A) showed the typical steviol core signal pattern as seen for RebA (Figure S3).

Besides the four β -anomeric ^1H signals related to RebA (for structure, see Figure 3; Glc1, δ 5.425; Glc2, δ 4.700; Glc3, δ 4.801; Glc4, δ 4.700), one additional α -anomeric ^1H signal of similar intensity at δ 4.870 (Glc5) stemming from mono- α -glucosylated RebA was observed, together with very minor α -anomeric signals (marked with * in Figure 2A) at δ 5.42 and δ 5.27, and a H-5 signal at δ 4.10, reflecting the presence of higher α -glucosylated RebA products (<10%).

Flash chromatography fractionations were carried out to separate mono- α -glucosylated RebA (major fraction RebA-G1; MALDI-TOF-MS analysis: $[\text{M}+\text{Na}]^+$, m/z 1152.9; Figure S4) from higher α -glucosylated RebA products (pooled very minor fractions RebA-G2+) and residual RebA. The ^1H NMR spectrum of RebA-G1 (Figure 2B) is identical to that reported recently for RebA-G1, prepared with the wild-type Gtf180- ΔN enzyme⁶⁹. The very minor pool of higher α -glucosylated RebA fractions, RebA-G2+, was not used for further structural analysis. In summary, it can be concluded that the mutant Gtf180- ΔN -Q1140E enzyme synthesizes as dominant product (77.7% in RebA-G) the same mono- α -glucosylated RebA derivative RebA-G1 as shown for the wild-type Gtf180- ΔN enzyme, i.e. a product with a specific elongation of the steviol C-19 β -D-Glcp moiety of RebA with an α -D-Glcp-(1 \rightarrow 6) residue (Figure 3). Taking into account the structural data of the α -glucosylated RebA products isolated in the wild-type Gtf180- ΔN /RebA/sucrose incubation study⁶⁹, combined with the above-mentioned extra minor signals in the ^1H NMR spectrum of RebA-G (Figure 2A), it can be concluded that also in the case of the Gtf180- ΔN -Q1140E/RebA/sucrose incubation, RebA-G1 is further extended at the C-19 site with mainly alternating (α 1 \rightarrow 3)- and (α 1 \rightarrow 6)-linked D-Glcp residues. The methylation analysis data of RebA-G and RebA-G1, presented in Table SIV, support the NMR data.

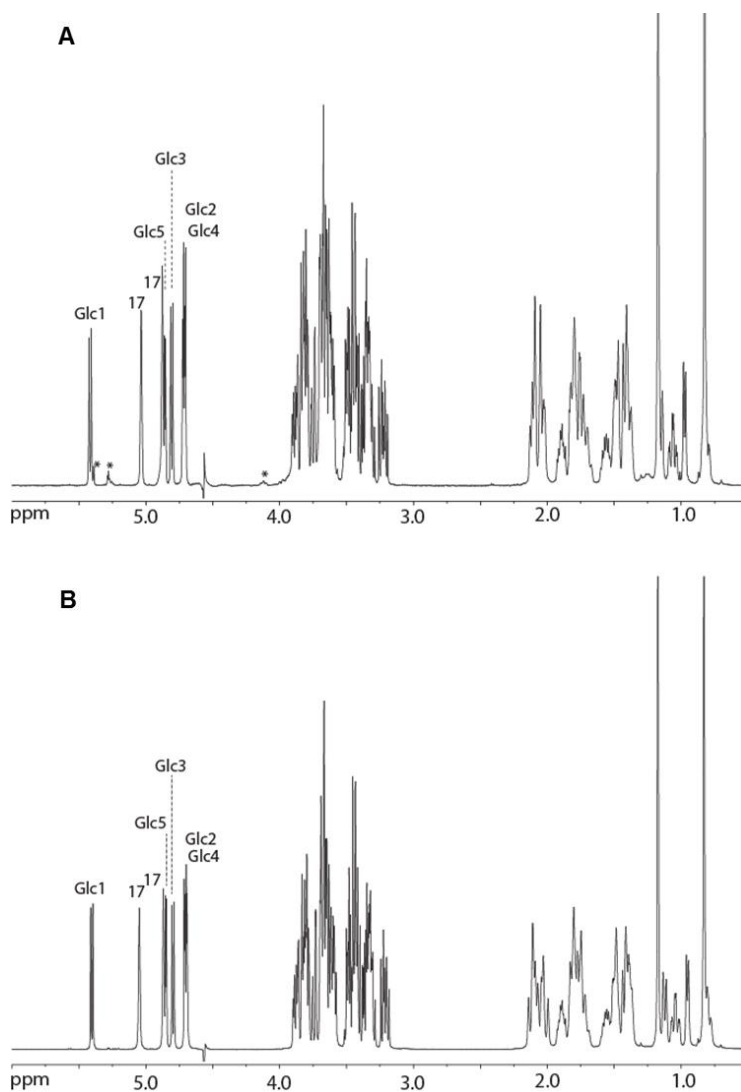


Figure 2. 500-MHz ^1H NMR spectra of (A) RebA-G and (B) RebA-G1, recorded in D_2O at 310 K. The positions of the anomeric protons of the glucose residues (see Figure 3) are indicated, as well as the steviol C-17 protons in the anomeric region. Products were synthesized using the mutant Gtf180- ΔN -Q1140E enzyme. Spectrum B is identical to that of RebA-G1, prepared with the wild-type Gtf180- ΔN enzyme, and recently published with a complete assignment of resonances⁶⁹. * signals stemming from higher α -glucosylated RebA products. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225).

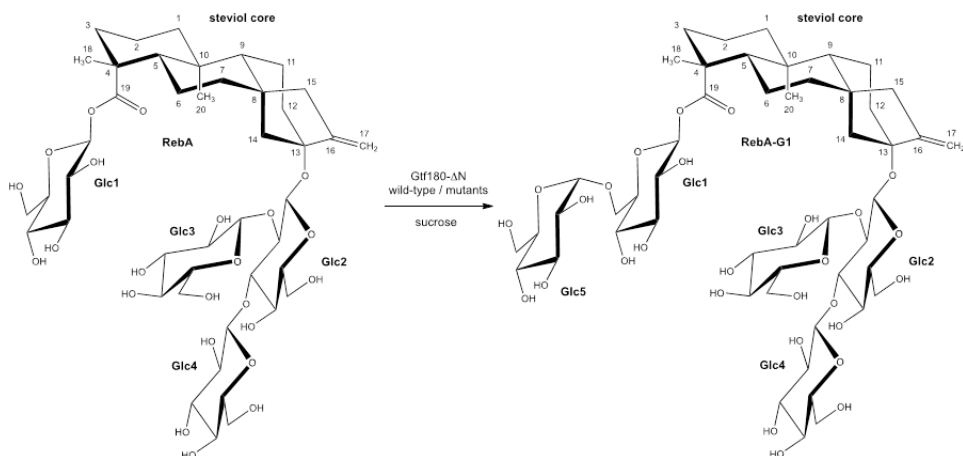


Figure 3. Major reaction product RebA-G1, obtained from the incubation of RebA with wild-type Gtf180-ΔN and mutant Gtf180-ΔN-Q1140E enzymes, in the presence of sucrose.

3.3. Optimization of the synthesis of α -glucosylated RebA

Batch reaction. RebA is only sparingly soluble in water (< 10 mM) at room temperature, however, it readily forms supersaturated solutions in water on simple stirring²¹². It was observed that up to 200 mM RebA could be dissolved at 37 °C before it started precipitating at 90 min. Hence, an efficient conversion of 200 mM RebA into glucosylated products has to be achieved within 90 min in order to prevent a suboptimal glucosylation yield caused by precipitation of RebA. Important factors for an optimal conversion of RebA into α -glucosylated RebA (RebA-G) are the RebA concentration, the ratio of donor substrate sucrose over acceptor substrate RebA (D/A ratio) and the agitation speed.

A response surface methodology (RSM) using a Box-Behnken experimental design was performed considering three factors: X_1 , RebA concentration (mM); X_2 , D/A ratio; X_3 , agitation speed (rpm). The addition of 5 U/mL Gtf180-ΔN-Q1140E enzyme ensured that a steady state in RebA conversion was obtained well before precipitation could occur for the highest RebA concentration (90 min). The results of the Box-Behnken experimental design are summarized in Table SIII. The analysis of variance (ANOVA) showed R^2 values of 98.8%, 78.0% and

99.3% for RebA conversion degrees (%), RebA-G1/RebA-G ratio (%) and amount of RebA-G synthesized (mM), respectively. The effects of the factors were analyzed applying the response surface contour plots (Figure 4).

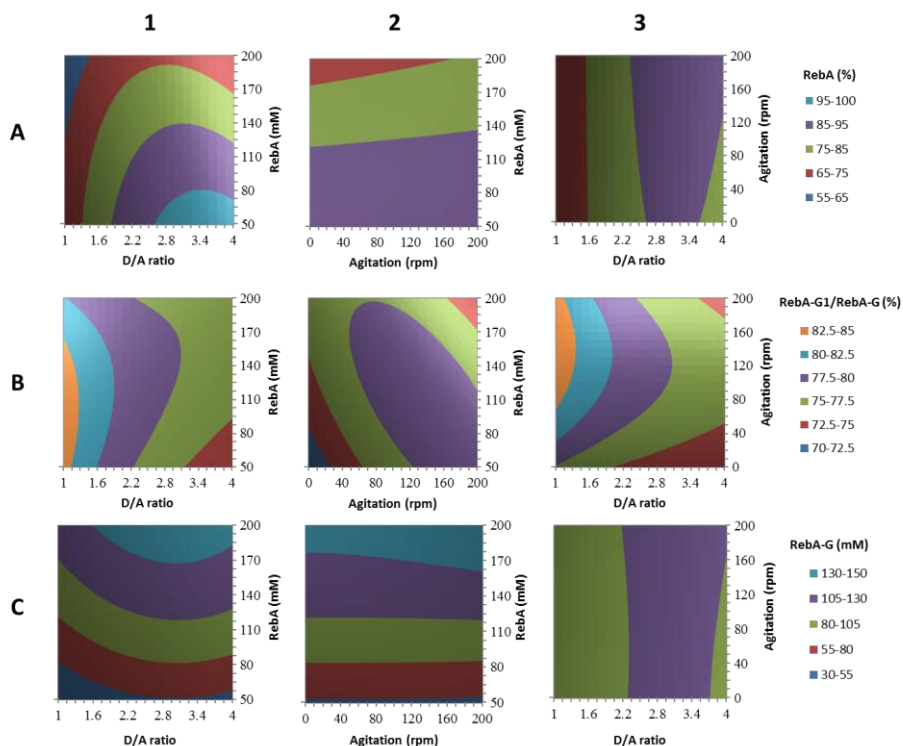


Figure 4. Response surface methodology contour plots of RebA glucosylation by Gtf180- Δ N-Q1140E, showing the effects of RebA concentration (mM), D/A ratio (ratio of donor substrate sucrose over acceptor substrate RebA) and agitation (rpm) on (A) RebA conversion degree (%); (B) RebA-G1/RebA-G ratio (%); (C) RebA-G synthesized (mM).

In summary, RebA conversion degrees decreased with increasing RebA concentrations, independent of the sucrose concentration (Figure 4A1). The RebA conversion degrees displayed an optimum at a D/A ratio of 2.5-3.5, depending on the RebA concentration (Figures 4A1-A3). Increasing the D/A ratio initially resulted in improved RebA conversion degrees, reflecting that more sucrose was available to drive the reaction. A further increase of the D/A ratio resulted in less RebA glucosylation in favor of more α -glucan synthesis. Agitation

had only a slight effect on RebA conversion degrees and amount of RebA-G synthesized (Figures 4A2-C2). Agitation influenced the RebA-G1/RebA-G ratio more strongly: the highest ratios were observed at an agitation speed of approximately 185 rpm (Figures 4B2-B3). Furthermore, low D/A ratios favored high RebA-G1/RebA-G ratios, since less donor substrate sucrose was available to glucosylate RebA-G1 into multi-glucosylated products (Figure 4B1).

The resulting Box-Behnken model (Table SIII) was subsequently used for the optimization of the reaction conditions. An efficient conversion of RebA into RebA-G yielding a maximal amount of RebA-G (at least 95%) was aimed for. The model predicted the synthesis of 80 mM RebA-G in case the following conditions were applied: 5 U/mL Gtf180- Δ N-Q1140E, 84 mM RebA, 282 mM sucrose and 185 rpm. The validation test resulted in the synthesis of 79 mM (or 115 g/L) RebA-G with a RebA-G1/RebA-G ratio of 77.7% (Figure 5A), which was in very good agreement with the prediction. Applying identical conditions for the glucosylation of RebA with wild-type Gtf180- Δ N resulted only in the conversion of 49.7% RebA, yielding 42 mM RebA-G with a RebA-G1/RebA-G ratio of 54.4% (Figure 5B). Hence, the Q1140E-mutant not only converted more RebA into RebA-G than wild-type Gtf180- Δ N (94.5% vs. 49.7%), its RebA-G consisted mostly of mono- α -glucosylated product RebA-G1 (77.7% vs. 54.4%). Compared to wild-type (45.6%), Q1140E produced less RebA-G2+ (22.3%), which is the minor fraction of the RebA glucosides with 2 and more glucose units.

Fed-batch reaction. The main remaining bottleneck for RebA glucosylation with Gtf180- Δ N-Q1140E is the synthesis of α -glucans at high sucrose concentrations, preventing complete RebA glucosylation at high RebA concentrations (Figure 4A1). This issue was addressed by performing a fed-batch reaction, in which sucrose was added to the reaction in fixed intervals of 20 min in order to keep the sucrose concentration low and hence suppress α -glucan formation as much as possible. The addition of 50 U/mL enzyme ensured complete usage of sucrose within 20 min and complete conversion of RebA within 3 h. Figure 5C represents RebA glucosylation at 200 mM RebA and an average sucrose concentration of 50 mM (fluctuating between 0-100 mM). In comparison to the batch reaction (200

mM RebA, 570 mM sucrose; Figure 5D), the RebA fed-batch conversion (Figure 5C) increased from 76.4% to 94.1%, attributed to a further suppressed α -glucan synthesis. The product yield consequently increased to 188 mM (or 270 g/L) RebA-G (Figure 5C).

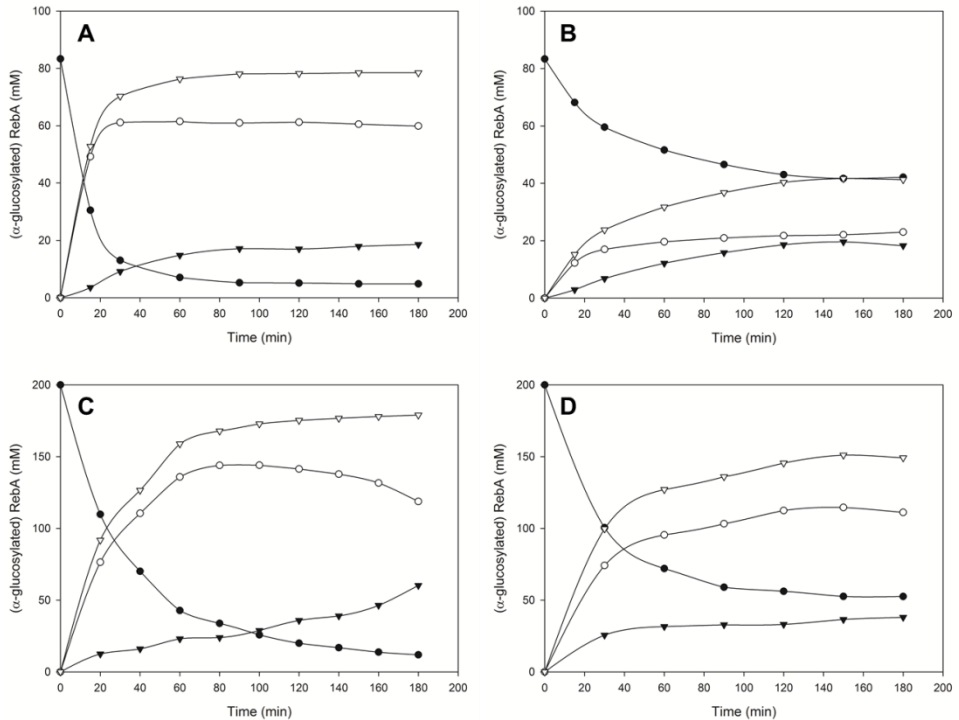


Figure 5. Time course of RebA glucosylation by (A) Gtf180-ΔN-Q1140E at optimal batch conditions (84 mM RebA; 282 mM sucrose; 5 U/mL), (B) Gtf180-ΔN at optimal batch conditions (84 mM RebA; 282 mM sucrose; 5 U/mL), (C) Gtf180-ΔN-Q1140E at optimal fed-batch conditions (200 mM RebA; 50 mM sucrose; 50 U/mL) and (D) Gtf180-ΔN-Q1140E at batch conditions (200 mM RebA; 570 mM sucrose; 5 U/mL). ● RebA, ○ RebA-G1, ▼ RebA-G2+, ▽ RebA-G. For the definition of these products, see Methods “Design of response surface methodology experiment”.

3.4. Docking of RebA in the active site of wild-type Gtf180- Δ N and mutant Q1140E

To gain further insight into the α -glucosylation of RebA by the Gtf180- Δ N wild-type enzyme and its mutant Gtf180- Δ N-Q1140E, *in silico* docking studies²⁰¹ were performed. To this end, the crystal structure of RebA as reported by ref²⁰² was used. Docking of RebA into the wild-type Gtf180- Δ N active site (X-ray crystal structure of a complex of Gtf180- Δ N with maltose; PDB code: 3KLL⁸³) afforded the pose as depicted in Figure 6A, showing that only the steviol C-19 β -D-glucosyl moiety (Glc1 in Figure 3) of RebA is available for glucosylation, especially at the HO-6 group, due to its orientation. Hydrogen bonding of Glc1 HO-6 with the catalytic residue D1136 possibly supports deprotonation in the glycosylation step (Figure 6C). A π - π -stacking interaction with W1065 as well as hydrogen bonding of Glc1 HO-4 and HO-3 to the backbone of D1136 was observed and appears to hold the Glc1 residue in place.

Docking of RebA into the active site of the mutant Gtf180- Δ N-Q1140E afforded the pose as depicted in Figure 6B. The binding of the steviol C-19 β -D-glucosyl moiety (Glc1 in Figure 3) is identical to the wild-type enzyme, but fewer hydrogen bonds of the steviol C-13 trisaccharide moiety (Glc2, Glc3 and Glc4 in Figure 3) were observed (Figure 6D). The modified binding pocket, resulting from the mutation, does not influence the binding of the steviol C-19 β -D-glucosyl unit, given that the mutation is not in direct proximity of the monosaccharide. The experimental observation that mutant Q1140E leads to more efficient α -glucosylation could be explained by the possibility that deprotonation of Glc1 HO-6 is aided by a water-mediated hydrogen bond between Glc1 HO-6 and E1140 as depicted in Figure 6B. The finding that the Q1140E mutant shows mostly mono-glucosylation instead of oligo-glucosylation is currently difficult to explain on the basis of these docking results.

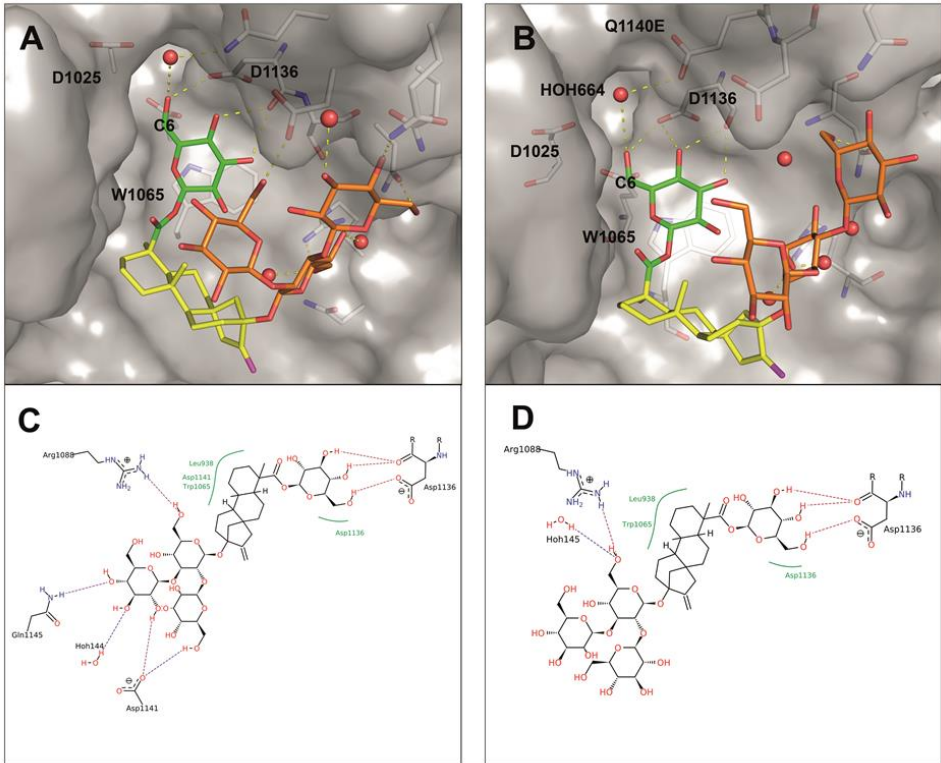


Figure 6. Docking poses of RebA into wild-type Gtf180- Δ N (PDB: 3KLL; **A** and **C**) and mutant Q1140E (**B** and **D**). The steviol part is indicated in yellow, the Glc1(β 1 \rightarrow residue at the steviol C-19 site in green and the Glc3(β 1 \rightarrow 2)[Glc4(β 1 \rightarrow 3)]Glc2(β 1 \rightarrow trisaccharide at the steviol C-13 site in orange. For monosaccharide coding system, see Figure 3.

3.5. Sensory analysis of glucosylated RebA products

A sensory analysis of aqueous solutions sweetened with RebA and glucosylated RebA products, prepared with the mutant Gtf180- Δ N-Q1140E enzyme, was performed by a trained panel, evaluating 9 different taste attributes. Three different glucosylated products were examined: multi- α -glucosylated product, containing residual RebA (RebA-G), mono- α -glucosylated product (RebA-G1) and RebA-G lacking RebA and RebA-G1 (RebA-G2+). The mean scores of the attributes of the sweetened water solutions are shown in Figure 7.

The glucosylated RebA products were all significantly less bitter than RebA, displaying almost no bitterness at all. Equally important, RebA-G and RebA-G1 retained the very high sweetness inherent to RebA. In contrast, RebA-G2+ was significantly less liquorice, astringent and lingering than RebA but also significantly less sweet, retaining only half of the RebA sweetness. The sensory analysis also revealed that RebA-G and RebA-G1 have very similar taste profiles, both combining a very high sweetness with a very low bitterness and other off-flavors. So, the small amounts of RebA and RebA-G2+ in RebA-G apparently do not influence the taste profile, when compared with RebA-G1. Therefore, RebA-G is the preferred product for commercialization, since it can be produced more economically, not requiring further purification and separation steps.

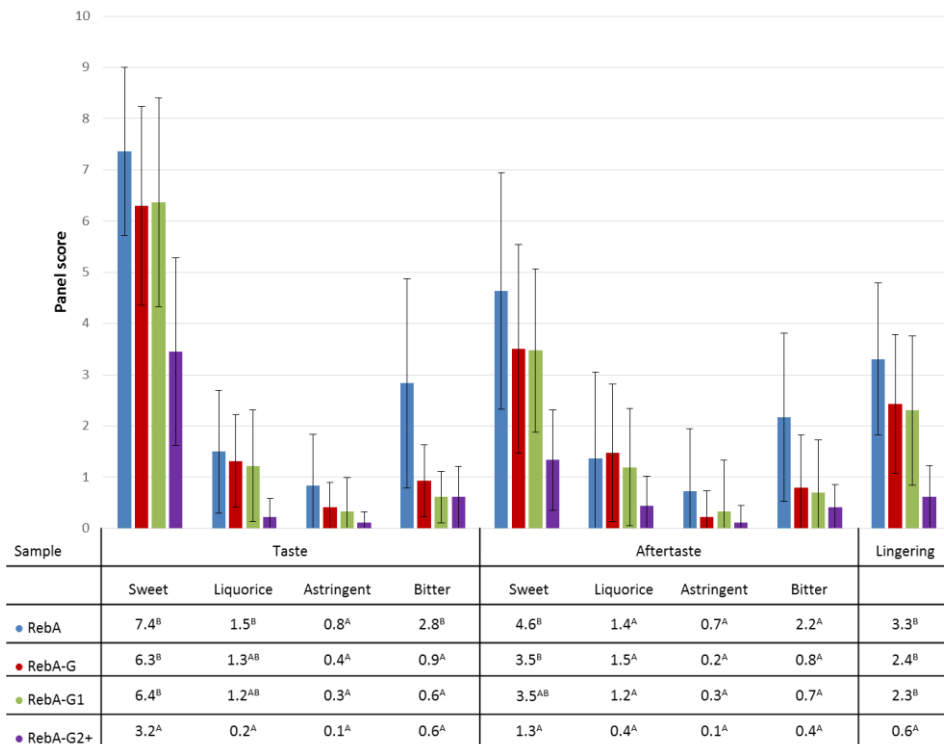


Figure 7. Sensory analysis of RebA, RebA-G, RebA-G1 and RebA-G2+. ^{A,B}: different letters indicate significant differences ($p < 0.05$) between solutions following one-way ANOVA and post-hoc test.

4. Discussion

The *Stevia rebaudiana* plant is a major source of high-potency natural sweeteners (steviol glycosides) for the growing natural food market of the future¹⁸⁶, however, due to their slight bitterness and unpleasant and lingering aftertaste, large-scale application of steviol glycosides is still hampered. In the past, several attempts have been made to improve the quality and sweet taste of steviol glycosides by modifying the carbohydrate moieties at the C-13 *tert*-hydroxyl and the C-19 carboxylic acid functions of steviol via transglycosylation reactions⁸.

In the present study, we have successfully used the glucansucrase mutant enzyme Gtf180- Δ N-Q1140E from *Lactobacillus reuteri* 180 (with sucrose as donor substrate) to glucosylate the steviol glycoside RebA, an important *Stevia* component. In a screening of 82 mutant enzymes of Gtf180- Δ N, mutant Q1140E was selected for further studies and compared to the wild-type Gtf180- Δ N enzyme. At optimal conditions, mutant Q1140E achieved ~95% RebA conversion into mainly mono- α -glucosylated RebA product RebA-G1 (Figure 3), compared to only 55% conversion by the wild-type enzyme. Under batch conditions, a high product yield of 115 g/L RebA-G (79 mM) was obtained within 3 h (from 84 mM RebA), applying only 5 U/mL of the Q1140E mutant enzyme. The product yield could even be enhanced to 270 g/L RebA-G (188 mM; from 200 mM RebA) by adopting a fed-batch reaction with stepwise addition of sucrose. This reduced availability of sucrose effectively suppressed the formation of α -gluco-oligo/polysaccharides. Instead, sucrose was mainly used as donor substrate for RebA glycosylation by the Q1140E enzyme, yielding more RebA α -glucosides.

The Gtf180- Δ N-Q1140E mutant glucosylated RebA specifically at the steviol C-19 position, introducing a Glc(α 1 \rightarrow 6) residue at the ester-linked Glc(β 1 \rightarrow residue. This finding is in line with the present knowledge about the wild-type Gtf180- Δ N enzyme, which also specifically elongates the C-19 glucose residue with mainly alternating (α 1 \rightarrow 6)- and (α 1 \rightarrow 3)-linked glucose units⁶⁹. In contrast to

the modified steviol glycosides prepared by incubation with CGTases, thereby introducing only (α 1 \rightarrow 4)-linked glucose residues, the (α 1 \rightarrow 3) and (α 1 \rightarrow 6) linkages are resistant to hydrolysis by the human amylolytic enzymes in saliva, which may prolong the sweet taste in the mouth.

Molecular docking studies were performed to gain insight into the glucosylation mechanism of RebA at the molecular level and to elucidate how a single amino acid change in Gtf180- Δ N, namely Q1140E, significantly improved RebA conversion. Docking of RebA into the active site of the Gtf180- Δ N wild-type enzyme indicated that only its steviol C-19-ester-linked Glc(β 1 \rightarrow residue is available for glycosylation. The Q1140E mutation is predicted not to affect the orientation and position of RebA in the active site, supporting the experimental observation that both enzymes specifically α -glucosylate RebA at the Glc1(β 1 \rightarrow C-19 residue, and not at the Glc3(β 1 \rightarrow 2)[Glc4(β 1 \rightarrow 3)]Glc2(β 1 \rightarrow C-13 trisaccharide (Figure 3). Furthermore, the docking results showed that Glc1 HO-6 and not Glc1 HO-3 of the Glc1(β 1 \rightarrow C-19 residue of RebA is prominently available for glucosylation. This is in agreement with the experimental results that both wild-type and mutant Gtf180- Δ N enzymes attach the first Glc residue (Glc5) exclusively via an (α 1 \rightarrow 6)-linkage. A faster and more efficient glucosylation of RebA was obtained by replacement of glutamine with the more negatively charged glutamate at position 1140 (mutant Q1140E). Conceivably, deprotonation of Glc1 HO-6 is aided by a water-mediated hydrogen bond between Glc1 HO-6 and glutamate, which is absent with glutamine at position 1140 (Figure 6). The finding that Q1140E shows mostly mono-glucosylation is currently difficult to explain on basis of the docking results. Elucidation of the Q1140E mutant protein 3D structure, followed by a comparison of the Gtf180- Δ N wild-type and mutant Q1140E structures, ideally in complex with RebA, may shed more light on the observed differences in RebA glucosylation.

An important finding in our study was that the multi-glucosylated RebA product (RebA-G) had a significantly reduced bitterness compared to RebA. This improved steviol glycoside product mixture thus displays appealing sensory properties and is likely to find application as a functional food ingredient. This

study also shows that Gtf180- Δ N-Q1140E is a very efficient catalyst for α -glucosylation of steviol glycosides.

5. Supplementary information

Table SI. Survey of wild-type glucansucrase Gtf180- Δ N of *L. reuteri* 180, and mutants derived, evaluated in this study. The 3-letter code DHT represents mutating amino acid residues D1085, R1088, and N1089 to D, H, and T, respectively. The same is valid for the other mutants shown with their 3-letter codes.

Mutation	Feature	Ref
Gtf180- Δ N	wild-type; N-terminally truncated	80
Gtf180- Δ N- Δ V	domain V deletion mutant	88
PNS (V1027P:S1137N:A1139S)	triple mutant	197
Q1140E/A/H, S1137Y	near transition state stabilizing residue D1136	97
L940G/M/C/A/S/E/F/W	near acceptor subsite +1	98
L938A/S/F/K/M, A978F/S/G/L/P/Y, L981A/N/K, D1028Y/W/L/K/G/N, N1029Y/R/G/P/T/M	near acceptor subsite +1	99
DHT, NRL, VKG, YTS, ETL, AAA, MYM, FFF, DED, LLL, D1085Y/V/A/E/H/L/Q, R1088E/W/T/N/G/H/K, N1089Y/G/S/L/R/D/P	near acceptor subsite +2	100
W1065F/K/L/Q/G/T/E/F	near acceptor subsite +1 and +2	102
Δ V L938N	L938 mutation in Gtf180- Δ N- Δ V	unpublished
Δ V L940E/F	L940 mutations in Gtf180- Δ N- Δ V	88

Table SII. Gtf180- Δ N mutants, screened for their RebA α -glucosylation potential with sucrose as donor substrate. Numbers shown here correspond to the TLC profiles (Figure S1). The 3-letter code DHT represents mutating amino acid residues D1085, R1088, and N1089 to D, H, and T, respectively. The same is valid for the other mutants shown with their 3-letter codes.

1.	DHT	21.	N1089D	41.	D1085A	61.	A978S
2.	NRL	22.	N1089P	42.	D1085E	62.	A978G
3.	VKG	23.	L940G	43.	D1085H	63.	A978L
4.	YTS	24.	L940M	44.	D1085L	64.	A978P
5.	ETL	25.	L940C	45.	D1085Q	65.	A978Y
6.	AAA	26.	L940A	46.	R1088H	66.	Δ V L938N
7.	MYM	27.	L940S	47.	R1088K	67.	Δ V L940E
8.	FFF	28.	L940E	48.	D1028Y	68.	Δ V L940F
9.	DED	29.	L940F	49.	D1028W	69.	W1065F
10.	LLL	30.	L940W	50.	D1028L	70.	W1065K
11.	R1088E	31.	L981A	51.	D1028K	71.	W1065L
12.	R1088W	32.	L981N	52.	D1028G	72.	W1065Q
13.	R1088T	33.	L981K	53.	D1028N	73.	W1065G
14.	R1088N	34.	L938A	54.	N1029Y	74.	W1065T
15.	R1088G	35.	L938S	55.	N1029R	75.	W1065E
16.	N1089Y	36.	L938F	56.	N1029G	76.	W1065F
17.	N1089G	37.	L938K	57.	N1029P	77.	Gtf180- Δ N
18.	N1089S	38.	L938M	58.	N1029T	-	no enzyme
19.	N1089L	39.	D1085Y	59.	N1029M		
20.	N1089R	40.	D1085V	60.	A978F		

Table SIII. Box-Behnken experimental design and results for the variables studied. Second-degree polynomial equation with coefficients of each factor is given for RebA conversion (%), RebA-G1/RebA-G (%) and amount of RebA-G synthesized (mM). X_1 , RebA concentration (mM); X_2 , D/A ratio; X_3 , agitation speed (rpm).

Pattern	X_1	X_2	X_3	RebA conversion (%)	RebA-G1/RebA-G (%)	RebA-G (mM)	
1	+0+	200	2.5	200	73.8	76.3	147.6
2	0-+	125	1	200	64.3	83.4	80.6
3	-0+	50	2.5	200	94.1	77.2	47.1
4	0--	125	1	0	63.4	80	79.3
5	0	200	4	100	66.6	74.8	133.2
6	-0-	50	2.5	0	97	67.7	48.6
7	--0	50	4	100	96.5	75.8	48.3
8	0+-	125	4	0	82.7	72.6	103.4
9	--0	50	1	100	67.4	85	33.8
10	0	125	2.5	100	85.5	78.9	106.9
11	+ -0	200	1	100	60.7	75.6	121.4
12	0	125	2.5	100	85.4	78	106.8
13	0	125	2.5	100	85.4	78.5	106.8
14	0++	125	4	200	88	72.2	110
15	+0-	200	2.5	0	69.1	77	138.2

$$\text{RebA conversion} = 85.4333 - 10.6000X_1 + 9.7500X_2 + 1.000X_3 - 5.8000X_1X_2 + 1.9000X_1X_3 + 1.1000X_2X_3 - 1.8667X_1^2 - 10.7667X_2^2 - 0.0667X_3^2.$$

$$\text{RebA-G1/RebA-G} = 78.4333 - 0.2375X_1 - 3.5375X_2 + 1.5250X_3 + 2.1250X_1X_2 - 2.5500X_1X_3 - 0.8500X_2X_3 - 1.6292X_1^2 + 0.9708X_2^2 - 2.2542X_3^2.$$

$$\text{RebA-G} = 106.8333 + 45.3250X_1 + 9.9750X_2 + 1.9750X_3 - 0.6750X_1X_2 + 2.7250X_1X_3 + 1.3250X_2X_3 - 10.3042X_1^2 - 12.3542X_2^2 - 1.1542X_3^2.$$

Table SIV. Methylation analysis of the carbohydrate moieties in RebA and α -glucosylated RebA products RebA-G and RebA-G1.

Alditol acetate	R_t^a	Structural feature	Peak area (%)		
			RebA	RebA-G	RebA-G1
2,3,4,6-Hex ^b	1.00	Glc $p(1\rightarrow$	74	58	61
2,4,6-Hex	1.16	$\rightarrow3)Glc$ $p(1\rightarrow$	-	3	-
3,4,6-Hex	1.18	$\rightarrow4)Glc$ $p(1\rightarrow$	-	tr ^c	tr ^c
2,3,4-Hex	1.22	$\rightarrow6)Glc$ $p(1\rightarrow$	-	17	18
4,6-Hex	1.32	$\rightarrow2,3)Glc$ $p(1\rightarrow$	26	20	21
2,4-Hex	1.39	$\rightarrow3,6)Glc$ $p(1\rightarrow$	-	2	-

^a R_t , retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (1.00) on GLC.

^b 2,3,4,6-Hex = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol-1-*d*. etc.

^c tr = trace (<2%).

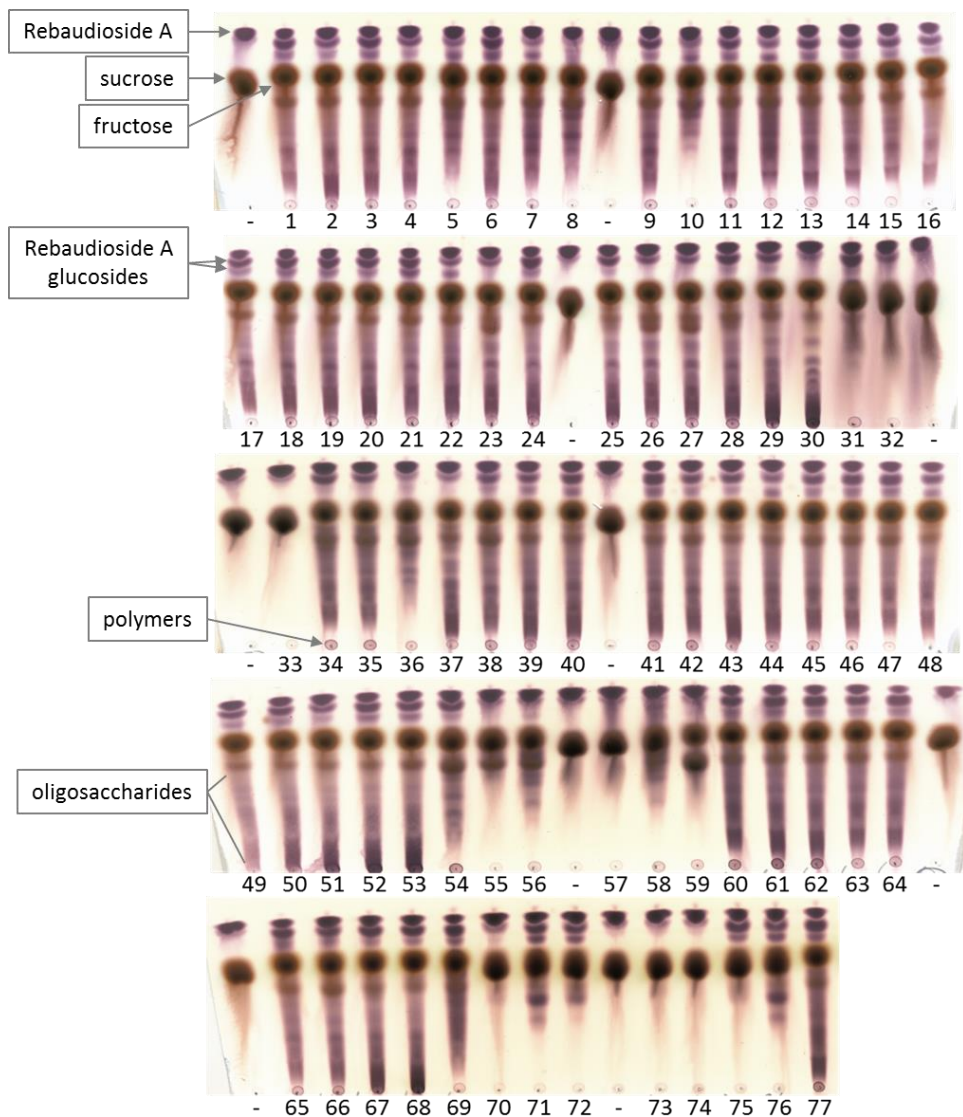


Figure S1. TLC substrate/product profiles after a 3 h incubation at 37 °C of a buffer solution (pH 4.7) containing 50 mM RebA, 0.2 M sucrose and ~1 mg/mL wild-type Gtf180- Δ N (lane 77) or Gtf180- Δ N mutant enzymes.

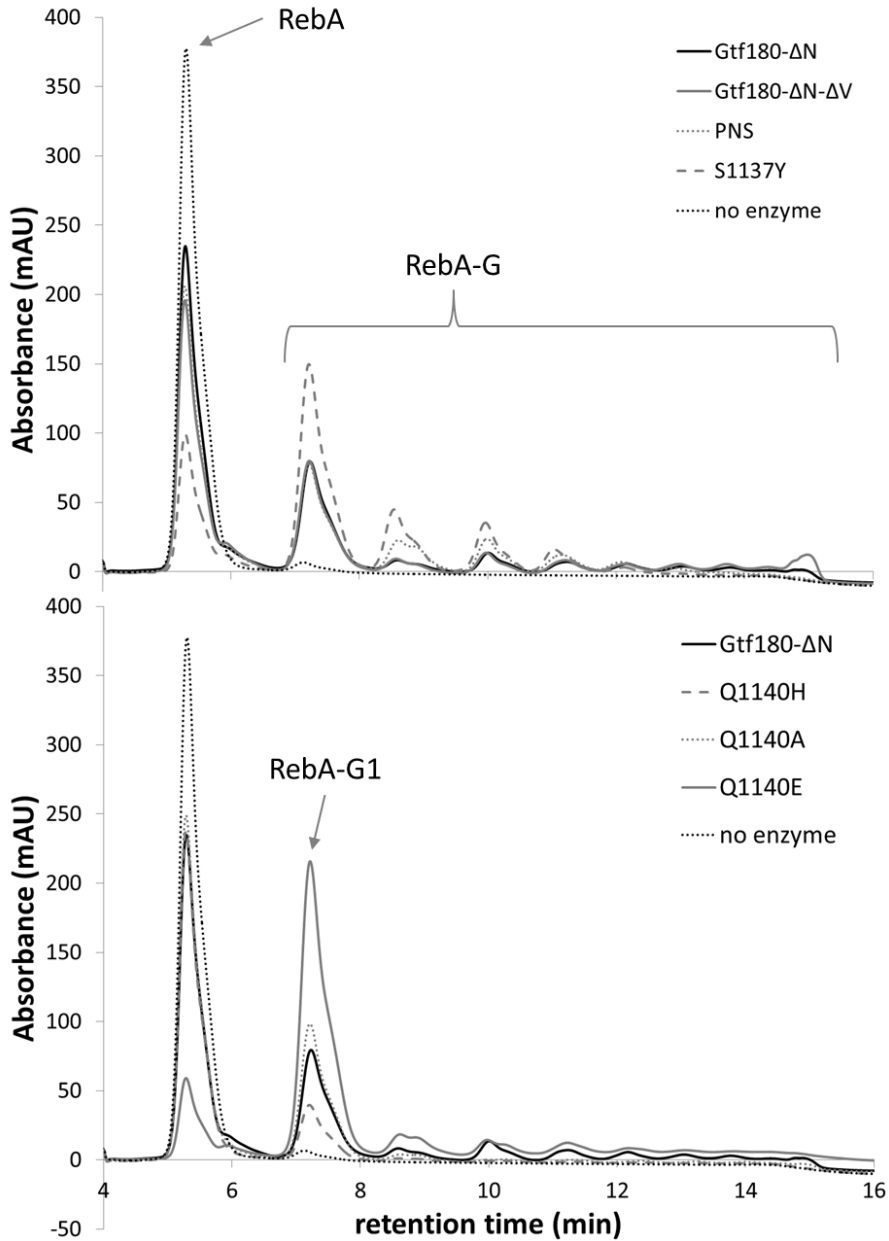


Figure S2. HPLC substrate/product profiles after a 3 h incubation at 37 °C of a buffer solution (pH 4.7) containing 50 mM RebA, 1 M sucrose and ~1 mg/mL wild-type Gtf180-ΔN, wild-type Gtf180-ΔN-ΔV or Gtf180-ΔN mutant enzymes. RebA-G: total amount of α-glucosylated RebA product. RebA-G1: mono-α-glucosylated RebA.

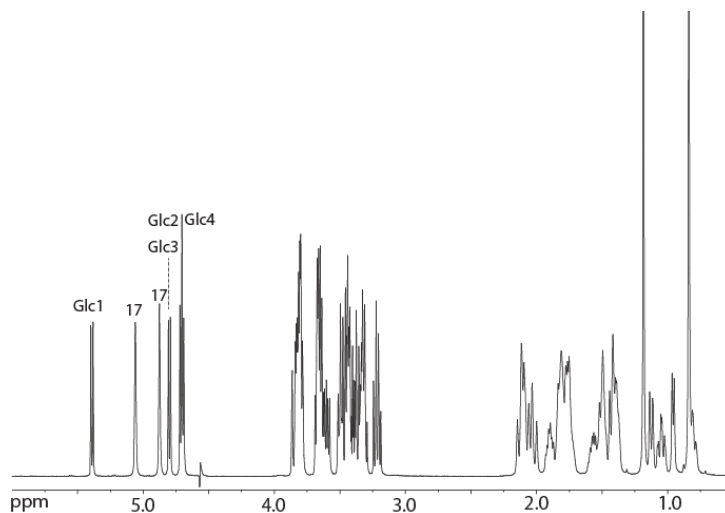


Figure S3. 500-MHz ^1H NMR spectrum of commercial RebA, supplied by Tereos PureCircle, recorded in D_2O at 310 K. The positions of the anomeric protons of the glucose residues (Figure 3) are indicated, as well as the steviol C-17 protons in the anomeric region. The spectrum is identical to that of commercial RebA, supplied by Aldrich-Sigma Chemie, and recently published with a complete assignment of resonances⁶⁹.

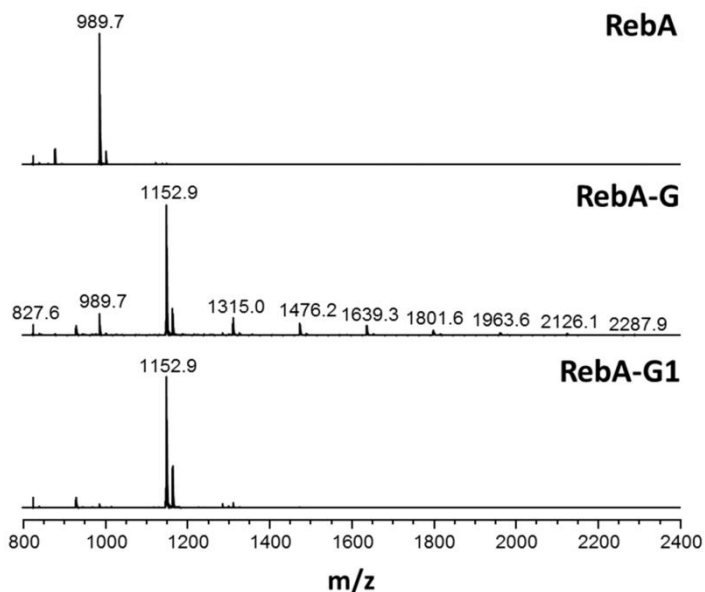


Figure S4. MALDI-TOF mass spectra of RebA, RebA-G and RebA-G1.

