Chapter 2

Gluco-oligomers initially formed by the reuteransucrase enzyme of *Lactobacillus reuteri* 121 incubated with sucrose and malto-oligosaccharides

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Abstract

The probiotic bacterium *Lactobacillus reuteri* 121 produces a complex, branched (1→4, 1→6)-α-D-glucan as extracellular polysaccharide (reuteran) from sucrose, using a single glucansucrase/glucosyltransferase enzyme (GTFA, reuteransucrase). To gain insight into the reaction/product specificity of the GTFA enzyme and the mechanism of reuteran formation, incubations with sucrose and/or a series of malto-oligosaccharides (DP2-DP6) were followed in time. The structures of the initially formed products, isolated via high-performance anion-exchange chromatography, were analyzed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry and 1D/2D $^{1}H/^{13}C$ NMR spectroscopy. Incubations with sucrose only, acting as both donor and acceptor, resulted in elongation of sucrose with glucose units via alternating (α1→4) and (α1→6) linkages, yielding linear gluco-oligosaccharides up to at least DP~12. Simultaneously with the ensemble of oligosaccharides, polymeric material was formed early on, suggesting that alternan fragments longer than DP~12 have higher affinity with the GTFA enzyme and are quickly extended, yielding high-molecular-mass branched reuteran (4×10^{7} Da). Malto-oligosaccharides (DP2-DP6) in the absence of sucrose turned out to be poor substrates. Incubations of GTFA with sucrose plus malto-oligosaccharides as substrates resulted in preferential elongation of malto-oligosaccharides (acceptors) with glucose units from sucrose (donor). This apparently reflects the higher affinity of GTFA for malto-oligosaccharides compared to sucrose. In accordance with the GTFA specificity, most prominent products were oligosaccharides with an (α1→4)/(α1→6) alternating structure.

Introduction

In an earlier screening of a large collection of probiotic *Lactobacillus* strains for their possible exopolysaccharide (EPS) production, we have shown that *Lactobacillus reuteri* strain 121 cells produce both a glucan (EPS121) and a fructan on sucrose (α-D-Glc*p*-(1↔2)-β-D-Fru*f*) (69). The water-soluble glucan was identified as a high-molecular-mass (3.5 x 10^6 Da) branched α-D-glucan with terminal, 4-substituted, 6-substituted, and 4, 6-disubstituted Glc units. The fructan turned out to be a low-molecular-mass (1.5 x 10^5 Da) (2→6)-β-D-fructofuranan or levan (33). Interestingly, from chemostat cultures of *L. reuteri* strain 121, a spontaneous mutant *L. reuteri* strain 35-5 was isolated that lacked all levansucrase activity, but still possessed glucansucrase (glucosyltransferase / GTF; EC 2.4.1.5) activity and produced the wild-type α-D-glucan (EPS35-5 = EPS121) in amounts up to 10 g/L (33).

Further studies with *L. reuteri* strain 121 resulted in isolation of the *gtfA* and *gtfB* genes, encoding the GTFA and GTFB enzymes, respectively (72,79). The GTFA enzyme is responsible for α-D-glucan biosynthesis from sucrose (72). It belongs to Glycoside Hydrolase family 70 (GH70), a group of bacterial glucansucrase or glucosyltransferase enzymes (192). Glucansucrase enzymes differ in reaction/product specificity, synthesizing high-molecular-mass α-D-glucans with different (ratios of) glycosidic linkages and degree of branching, depending on the bacterial source of the enzyme (71). The mechanistic and structural features determining these differences in glucansucrase reaction/product specificity are still not fully understood, and little is known about the products synthesized early in time (28,29,88).

Also the GTFB type of enzymes is classified in family GH70. Recently, we have reported that the GTFB enzyme of *L. reuteri* strain 121 is inactive with sucrose but instead has hydrolysis/transferase activity on malto-oligosaccharides (MOS) and starch-like polysaccharides (79,81,193). The elongated linear gluco-oligomers formed contain besides (α1→4) also a high percentage of (α1→6) glycosidic linkages. In view of its reaction/product specificity, the GTFB enzyme was classified as a 4,6-α-glucanotransferase, the first example of such an enzyme activity in family GH70, representing a new subfamily (79).

Recombinant GTFA purified from *E. coli*, and the culture supernatants of *L. reuteri* strain 35-5, synthesized virtual identical α-D-glucan polymers (reuteran = EPS35-5 = EPS121) when incubated overnight with sucrose (according to ^{1}H NMR analysis). Additionally, a detailed molecular (construction of site-directed and deletion mutants) and biochemical (main reactions catalyzed by wild-type and mutant enzymes) analysis of the GTFA enzyme, called reuteransucrase, has been reported (70). Recently, also the crystal structure of functional GTFA-ΔN, a 118-kDa fragment of GTFA comprising residues 745-1763 and including the catalytic domain, was determined at 3.6 Å resolution by molecular replacement (103). Detailed structural analysis of the reuteran product formed from sucrose by the recombinant glucansucrase GTFA enzyme yielded a (1→4, 1→6)-α-D-glucan, with no repeating units present. The constructed composite model is presented in Fig. 1. It should be noted that the reuteran structure showed a large number of alternating (α1→4)/(α1→6) linkages (73).

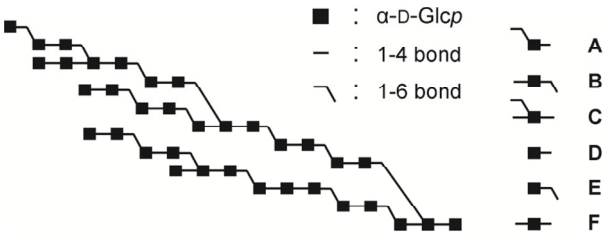

Figure 1. Composite structure of the exopolysaccharide reuteran (EPS35-5/EPS121) of *Lactobacillus reuteri* 121 (73).

When exploring the activity of the recombinant GTFA enzyme with the MOS series DP2-DP7, only DP4 (maltotetraose) gave some clear higher-molecular-mass bands on thin-layer chromatography (TLC) (S. Kralj, unpublished data). However, recombinant GTFA enzyme incubated with equimolar mixtures of sucrose and maltose for 60 h resulted in formation of panose (major), maltotriose (minor), and two unknown products (minor) (demonstrated by high-performance anion-exchange chromatography on CarboPac PA-1). When using equimolar amounts of sucrose and isomaltose, low amounts of isomaltotriose and isomaltotetraose were seen, together with two unknown products (70).

To gain a better understanding of the activity of the recombinant GTFA enzyme, especially during the first hours of *in vitro* incubation with sucrose, the present

study focused specifically on the structural analysis of the initially formed oligosaccharides en route to the polysaccharide reuteran. Furthermore, product mixtures from incubation of MOS with GTFA in the absence and presence of sucrose were analyzed. In all cases, the glycan products were analyzed by high-pH anion-exchange chromatography (HPAEC), matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and 1D/2D $^{1}H/^{13}C$ nuclear magnetic resonance (NMR) spectroscopy (TOCSY, HSQC, ROESY).

Materials and methods

Preparation and isolation of recombinant GTFA enzyme

The recombinant N-terminally truncated GTFA enzyme was prepared and isolated as previously described (70).

Incubation of sucrose and malto-oligosaccharides with GTFA

Before incubation, commercially available malto-oligosaccharides (DP2 – DP6) were purified via size-exclusion chromatography on Bio-Gel P-2 (DP2 - DP4) or via high-pH anion-exchange chromatography on CarboPac PA-1 (DP5 and DP6). Solutions of sucrose (50 mM) and the purified malto-oligosaccharides (100 mM) were individually incubated with 50 nM GTFA in 25 mM sodium acetate/1 mM $CaCl_2$, pH 4.7, at 37°C. The progress of the reactions was followed by analyzing aliquots of the incubation mixtures by TLC, MALDI-TOF-MS, and HPAEC-PAD. Furthermore, mixtures of malto-oligosacharides (DP2 – DP6, 100 mM), individually together with sucrose (100 mM) were incubated in a similar way. Comparable incubations without the addition of GTFA showed no product formation.

Isolation and purification of product oligosaccharides

Product mixtures of oligosaccharides obtained after incubation were fractionated by high-pH anion-exchange chromatography (HPAEC) on a Dionex DX500 workstation (Dionex, Amsterdam, The Netherlands), equipped with a CarboPac PA-1 column (250 x 9 mm; Dionex) and an ED40 pulsed amperometric detector (PAD), using a linear gradient of 0 – 500 mM sodium acetate in 100 mM NaOH (3 mL/min) or isocratic conditions of 100 mM sodium acetate in 100 mM NaOH

(3 mL/min). Collected fractions were immediately neutralized with 4 M acetic acid, desalted on CarboGraph SPE columns (Alltech, Breda, The Netherlands) using acetonitrile:water = 1:3 as eluent, and lyophilized. Polysaccharide material (DP>14) was isolated in the void volume on a Bio-Gel P-2 column (90 x 1 cm), eluted with 10 mM NH_4HCO_3 at a flow rate of 12 mL/h.

Thin-layer chromatography

Samples were spotted in 1-cm lines on TLC sheets (Merck Kieselgel 60 F254, 20 x 20 cm), which were developed with n-butanol:acetic acid:water = 2:1:1. Bands were visualized by orcinol/sulfuric acid staining and compared with a simultaneous run of standard oligosaccharides.

Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry

MALDI-TOF-MS experiments were performed on an Axima^{TM} mass spectrometer (Shimadzu Kratos Inc., Manchester, UK) equipped with a nitrogen laser (337 nm, 3 ns pulse width). Positive-ion mode spectra were recorded using the reflector mode at a resolution of 5000 Full Width at Half Maximum (FWHM) and delayed extraction (450 ns). The accelerating voltage was 19 kV with a grid voltage of 75.2%; the mirror voltage ratio was 1.12, and the acquisition mass range was 200-6000 Da. Samples were prepared by mixing on the target 0.5 μL sample solutions with 0.5 μL aqueous 10% 2,5-dihydroxybenzoic acid as matrix solution.

NMR spectroscopy

Resolution-enhanced 1D/2D 500-MHz ^{1}H NMR spectra were recorded in D_2O on a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) or a Varian Inova Spectrometer (NMR Center, University of Groningen) at probe temperatures of 300/310 K. Before analysis, samples were exchanged twice in D_2O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL D_2O. Suppression of the HOD signal was achieved by applying a WEFT (water eliminated Fourier transform) pulse sequence for 1D experiments and by a pre-saturation of 1 s during the relaxation delay in 2D experiments. The 2D TOCSY spectra were recorded using an MLEV-17 (composite pulse devised by M. Levitt) (194) mixing sequence with spin-lock

times of 20-200 ms. The 2D ROESY spectra were recorded using standard Bruker XWINNMR software with a mixing time of 200 ms. The carrier frequency was set at the downfield edge of the spectrum in order to minimize TOCSY transfer during spin-locking. Natural abundance 2D ^{13}C-^{1}H HSQC experiments (^{1}H frequency 500.0821 MHz, ^{13}C frequency 125.7552 MHz) were recorded without decoupling during acquisition of the ^{1}H Free Induction Decay (FID). Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation for 1D spectra or by multiplication with a squared-bell function phase shifted by $\pi/(2.3)$ for 2D spectra, and when necessary, a fifth order polynomial baseline correction was performed. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).

Results

Gluco-oligosaccharides initially formed by GTFA from sucrose

To identify the initially formed oligosaccharides, precursors of reuteran, 50 mM sucrose (Suc) was incubated at 37°C with 50 nM recombinant GTFA in 25 mM sodium acetate buffer/1 mM $CaCl_2$, pH 4.7. The progress of the reaction in time was followed by TLC (data not shown). The formation of lower- and higher-molecular-mass products than the substrate Suc indicated hydrolysis/transferase activity, demonstrating that the GTFA enzyme is active in the absence of an exogenous primer.

The HPAEC profiles of the oligosaccharide reaction products formed from Suc at t = 0, 1, 3, and 6 h are depicted in Fig. 2. Evaluation of these profiles clearly indicated that the $\alpha 1 \leftrightarrow 2\beta$ linkage of Suc as donor was split, yielding fructose (Fru) and glucose (Glc). Fru accumulated during the reaction, however, Glc remained low, because it was used mainly for the transfer to Suc and its formed gluco-elongations as acceptors, yielding an ensemble of non-reducing gluco-oligosaccharides of increasing DP (as determined by MALDI-TOF-MS analysis) with fructose at the end. Complementary analysis performed by size-exclusion chromatography revealed the presence of two major populations of products, increasing simultaneously during the reaction. A peak with polymeric material eluted from 20 to 24 min and a peak corresponding to oligosaccharides eluted

from 30 to 32 min. The polymer formed after 15 min of incubation was estimated to be already larger than 2 x 10^7 Da (data not shown).

To get insight into the structures of the formed oligosaccharides in the reaction mixtures, the HPAEC fractions 2-11 were isolated and studied by MALDI-TOF-MS and NMR spectroscopy (^{1}H, TOCSY, ^{1}H-^{13}C HSQC, and ROESY). In the assignment of the various NMR signals, our earlier developed structural-reporter-group concept for α-D-glucans played an important role (73,195). Analysis of the peaks after a 1-h incubation showed that Glc is used to elongate Suc with an (α1→4)- (compound 3) or an (α1→6)- (compound 2) linked Glc unit. Later in time (t = 3 h, 6 h), more products are formed by elongation of Suc-containing oligosaccharides, whereby the systematic build-up of the structures is impressive. Successive (1→4) elongation could be observed only up to DP4 (compounds 3 and 5), but otherwise the products contained alternating (1→6)/(1→4) linkages (compounds 2 → 4 → 5′ → 6 → 7 → 8 → 9 → 10 → 11). Due to the decrease in PAD response with increasing DP of products, together with their low abundance, it was not possible to detect the formation of higher-molecular-mass material by HPAEC. The minor amounts of two non-Fru-containing compounds (the tri- and tetrasaccharide marked with an asterix in Fig. 2, t = 6 h) most likely are hydrolysis products of initially formed larger oligosaccharides. It should be noted that Suc was not chemically hydrolyzed in the incubation buffer after 6 h at 37°C. Also, enzymatic cleavage of the glycosidic bond in the Suc fragment of higher oligosaccharides is highly improbable. Therefore, the presence of these products indicate that GTFA has a minor endo-(α1→4)-glycosidase activity, although it cannot be completely excluded that Glc units are transferred for a very low percentage onto free Glc as an acceptor. Finally, only a minor amount of leucrose (α-D-Glc*p*-(1→5)-D-Fru; <2%) was found, indicating that fructose is not a good acceptor substrate for GTFA.

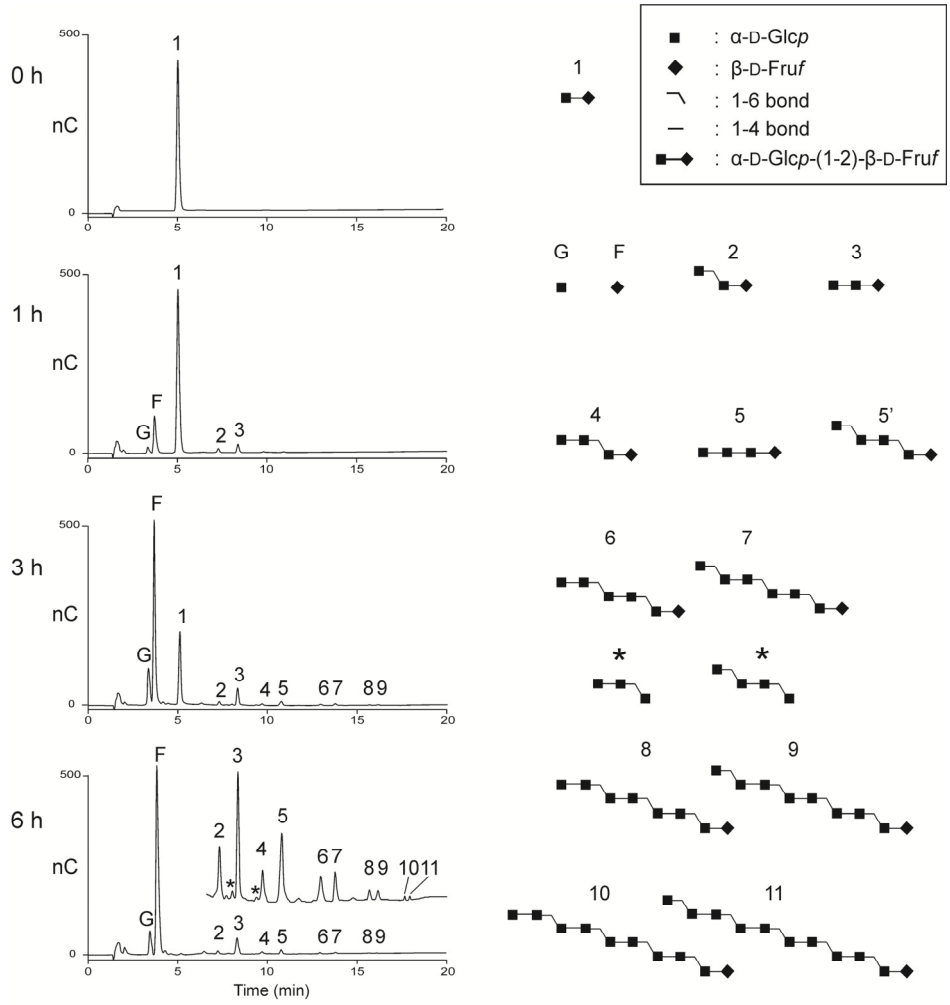

Figure 2. HPAEC-PAD profile (0 to 500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from sucrose by incubation with GTFA enzyme at t = 0, 1, 3, and 6 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included. G = glucose; F = fructose. Two non-Fru-containing compounds are marked with an asterix.

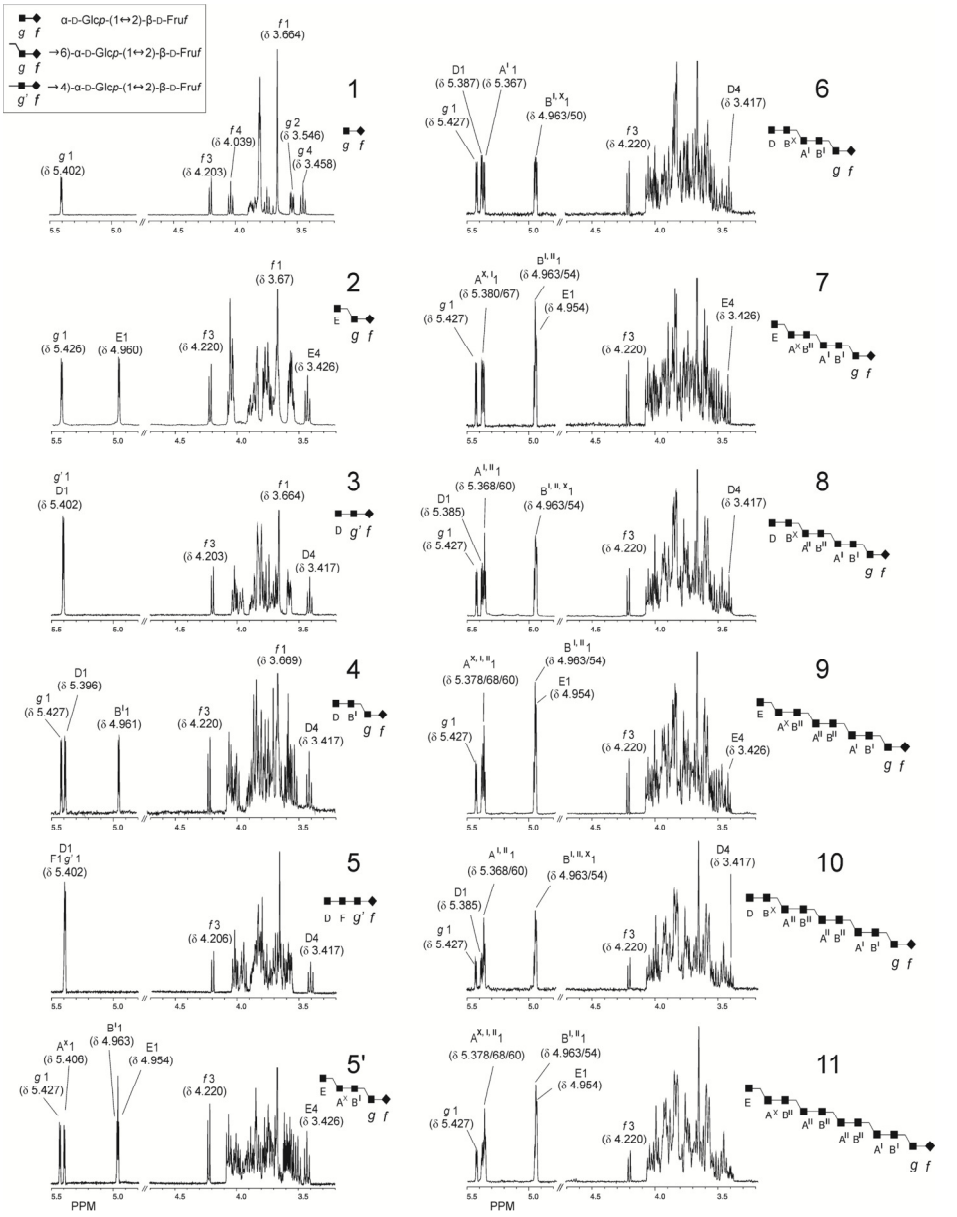

Figure 3. ^{1}H NMR spectra (D_2O, 300K) of compounds 1-11 (Fig. 2) found in the oligosaccharide mixture after incubation of sucrose with GTFA enzyme for 6 h (pH 4.7, 37°C).

The 1D ^{1}H NMR spectra of compounds 1 – 11, including some exact chemical shift values, are depicted in Fig. 3. For further NMR data, see Table 1. An adequate denotation system for the constituent residues was necessary (see Scheme I), since there are small variations of some of the chemical shift values of the different Glc residues, depending on their position in the oligosaccharide structure.

Scheme I. Monosaccharide coding system for the discussed oligosaccharides.

Residue	Position in the oligosaccharide sequence
g-f	sucrose of which glucose (***g***) is substituted at O-6
g'-f	sucrose of which glucose (***g'***) is substituted at O-4
$\mathbf{A^{I}}$	-(1→6)-α-D-Glc*p*-(1→4)- **A** residue, most close to sucrose
$\mathbf{A^{II,III}}$	more remote -(1→6)-α-D-Glc*p*-(1→4)- **A** residues
$\mathbf{A^{X}}$	sub-terminal -(1→6)-α-D-Glc*p*-(1→4)- **A** residue
$\mathbf{B^{I}}$	-(1→4)-α-D-Glc*p*-(1→6)- **B** residue, most close to sucrose
$\mathbf{B^{II,III}}$	more remote -(1→4)-α-D-Glc*p*-(1→6)- **B** residues
$\mathbf{B^{X}}$	sub-terminal -(1→4)-α-D-Glc*p*-(1→6)- **B** residue
D	terminal α-D-Glc*p*-(1→4)- residue
E	terminal α-D-Glc*p*-(1→6)- residue

As an example for the rationalization behind the interpretation of the various ^{1}H and ^{13}C NMR assignments, the TOCSY, ROESY, and HSQC spectra of one of the reaction products, Glc_5Suc (compound 7; *m/z* 1175.3, $[M+Na]^+$ according to MALDI-TOF-MS), are shown in Fig. 4. Typical chemical shift values for the Suc fragment (Glc, ***g***; Fru, ***f***) are δ 5.427, stemming from Glc ***g*** H-1, and δ 4.220, stemming from Fru ***f*** H-3. The ^{1}H chemical shifts of the anomeric signals around δ 5.37 and 4.95 indicate the presence of (α1→4) and (α1→6) linkages, respectively, between Glc residues (195). Starting from the anomeric signals of the Glc residues $\mathbf{A^{X}}$, $\mathbf{A^{I}}$, $\mathbf{B^{I}}$, $\mathbf{B^{II}}$, and **E** in the 2D ^{1}H-^{1}H TOCSY spectra (20, 50, 100, and 200 ms), and additional data from the HSQC and ROESY experiments, all chemical shifts of the non-anomeric protons of the differently substituted Glc residues could be determined (Table 1). Although the anomeric signals of **E** (H-1, δ 4.954) and $\mathbf{B^{I}/B^{II}}$ (H-1, δ 4.963/4.954) strongly overlap, the differences in chemical shift of their H-3, H-4, H-5, H-6 signals could be deduced from the

TOCSY built-up series of mixing times (20, 50, 100, and 200 ms; Fig. 4, 200 ms). The set of chemical shifts of **E** H-2, H-3, H-4, H-5, H-6a/b at δ 3.56, 3.74, 3.426, 3.75, 3.85/3.77, respectively, corresponds with that of a terminal α-D-Glc*p*-(1→6)- unit. The set of chemical shifts of $\mathbf{B^I/B^{II}}$ H-2, H-3, $H\text{-}4^I/H\text{-}4^{II}$, H-5, H6a/b at δ 3.60, 4.01, $3.63^I/3.65^{II}$, 3.85, 3.87/3.83, respectively, corresponds with that of internal -(1→4)-α-D-Glc*p*-(1→6)- units. The small differences in chemical shift between $\mathbf{B^I}$ H-1 and $\mathbf{B^{II}}$ H-1 and between $\mathbf{B^I}$ H-4 and $\mathbf{B^{II}}$ H-4 are due to the proximity of $\mathbf{B^I}$ to the Suc fragment (ROESY data, see below). The sets of chemical shifts of $\mathbf{A^X}$ and $\mathbf{A^I}$ reflect the presence of internal -(1→6)-α-D-Glc*p*-(1→4)- units ($\mathbf{A^X/A^I}$: H-1, δ 5.380/5.367; H-2, δ 3.59; H-3, δ 3.69; $H\text{-}4^X/H\text{-}4^I$, δ 3.49/3.46; H-5, δ 3.94; $H6a^X/b^X$ and $H6a^I/b^I$, δ $3.73^X/3.98^X$ and δ $3.77^I/3.94^I$). In this case, the small differences in chemical shift for H-1, H-4, and H6a/b are due to the proximity of $\mathbf{A^I}$ to the Suc fragment (ROESY data, see below). The set of chemical shifts of ***g*** H-2, H-3, H-4, H-5, H-6a/b at δ 3.57, 3.74, 3.52, 4.05, 3.69/4.02, respectively, corresponds with a 6-O-substituted ***g*** residue [-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*]. The TOCSY ***f*** H-3 track (δ 4.220), revealed cross-peaks with ***f*** H-4, ***f*** H-5 and ***f*** H-6a/b at δ 4.05, 3.90, and 3.85/3.78, respectively, in agreement with literature data (196).

The sequence of the five Glc residues followed directly from the ROESY experiments, revealing inter-residual cross-peaks between **E** H-1 and $\mathbf{A^X}$ H-6a ($\mathbf{E}1\rightarrow6\mathbf{A^X}$), $\mathbf{A^X}$ H-1 and $\mathbf{B^{II}}$ H-4 ($\mathbf{A^X}1\rightarrow4\mathbf{B^{II}}$), $\mathbf{B^{II}}$ H-1 and $\mathbf{A^I}$ H-6a ($\mathbf{B^{II}}1\rightarrow6\mathbf{A^I}$), $\mathbf{A^I}$ H-1 and $\mathbf{B^I}$ H-4 ($\mathbf{A^I}1\rightarrow4\mathbf{B^I}$), $\mathbf{B^I}$ H-1 and ***g*** H-6a ($\mathbf{B^I}1\rightarrow6\boldsymbol{g}$). The 4- and 6-substitution of the residues $\mathbf{B^I/B^{II}}$ and $\mathbf{A^X/A^I}/\boldsymbol{g}$, respectively, were further supported by their downfield C-4 and C-6 chemical shifts, when compared with terminal Glc (deduced from ^{13}C-^{1}H HSQC measurements): $\mathbf{B^I/B^{II}}$ C-4 at δ 78.5 and $\mathbf{A^X/A^I}/\boldsymbol{g}$ C-6 at δ 66.5/66.9.

In conclusion, the NMR chemical shift data, together with the peak areas of the anomeric signals and the molecular mass (1152 Da), revealed the complete structure of the oligosaccharide, being a linear heptasaccharide consisting of sucrose elongated by five glucose residues with alternating (α1→6)/(α1→4) glycosidic linkages: α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*.

Table 1. ^{1}H and ^{13}C chemical shifts[a] (D_2O, 300K) of saccharide residues present in linear oligomers (DP≥ 4) formed by incubation of sucrose with GTFA enzyme of *Lactobacillus reuteri* 121.

Residue	H-1a C-1	H-1b	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b
g -(1→6)-α-D-Glc*p*- (1↔2)-β-D-Fru*f*	5.427 92.9	-	3.57 71.8	3.74 73.4	3.52 70.5	4.05 72.3	3.69 66.9	4.02
g' -(1→4)-α-D-Glc*p*- (1↔2)-β-D-Fru*f*	5.402 93.1	-	3.58 72.2	4.02 75.1	3.69 78.0	3.96 72.1	3.85 61.5	3.76
f **[*g*→*f*]** -(1↔2)-β-D-Fru*f*	3.67 62.5	3.67	- 104.5	4.220 77.3	4.05 74.9	3.90 82.3	3.85 63.5	3.78
f **[*g'*→*f*]** -(1↔2)-β-D-Fru*f*	3.66 62.5	3.66	- 104.5	4.20 77.3	4.02 74.9	3.89 82.3	3.86 63.5	3.80
A[X,I,II] -(1→6)-α-D-Glc*p*- (1→4)-	5.38^{X}/ $37^{I}/36^{II}$ 100.7	-	3.59 72.4	3.69 74.3	3.49^{X}/ $46^{I,II}$ 70.5	3.94 72.4	3.73^{X}/ $77^{I,II}$ 66.5	3.98^{X}/ $94^{I,II}$
B[I,II,X] -(1→4)-α-D-Glc*p*- (1→6)-	4.96^{I}/9 $5^{II,X\ b}$ 98.9	-	3.60 72.5	4.01 74.4	3.63^{I}/6 $5^{II,X}$ 78.5	3.85 71.3	3.87 61.5	3.83
D α-D-Glc*p*-(1→4)-	5.40/3 9[b] 100.7	-	3.59 72.7	3.67 73.8	3.417 70.3	3.73 73.7	3.84 61.5	3.75
E α-D-Glc*p*-(1→6)-	4.954 98.8	-	3.56 72.4	3.74 72.8	3.426 70.5	3.75 72.3	3.85 61.5	3.77

[a] In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C) [b]Average chemical shifts values of the anomeric protons (see exact values: ^{1}H NMR spectra, Fig. 3)

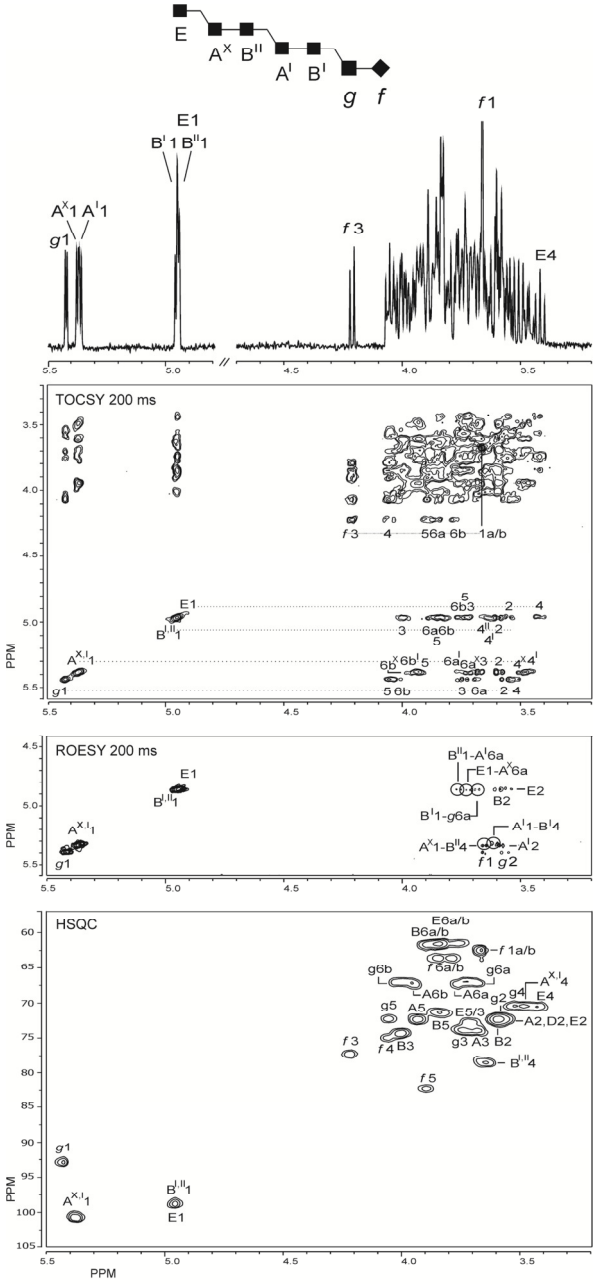

Figure 4. 1D ^{1}H NMR, TOCSY (200 ms), ROESY (200 ms), and HSQC spectra of α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f* (compound 7 in Fig. 2).

The data show that during the first 6 h of incubation with sucrose, GTFA mainly produces linear oligosaccharides with alternating (1→6)/(1→4) linkages; no branched oligosaccharides were formed. However, the consecutively formed polymeric material starting from DP14 on, clearly contained →4,6)-branched Glc residues, as indicated by ^{1}H-NMR signals at δ 5.33. The degree of branching may increase during the further growth of the glucan chain. Up to DP20, the presence of the Suc fragment in the structures could be observed in the NMR spectra by the small signal at δ 4.220 stemming from Fru*f* H-3. The polysaccharide material at 72 h of incubation was isolated via size-exclusion chromatography on Bio-Gel P2 by collecting the void-volume fraction. The 1D ^{1}H NMR spectrum (Fig. 5) of this fraction was identical to the spectrum of reuteran, containing 14% of →4,6)-branching, for which the composite model is depicted in Fig. 1 (73). Note that the NMR signals of Suc/Fru are no longer observable in the NMR spectrum of the polysaccharide.

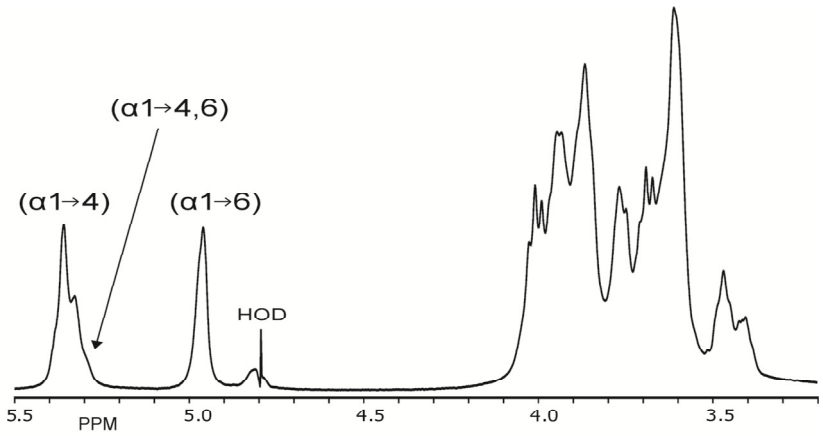

Figure 5. ^{1}H NMR spectrum of the void-volume Bio-Gel P-2 polysaccharide fraction, obtained after 72 h incubation of sucrose with GTFA enzyme. The spectrum is identical to the ^{1}H NMR spectrum of the native polysaccharide produced by *L. reuteri* 121 (73).

Products of the GTFA enzyme from malto-oligosaccharide substrates in the absence of sucrose

To generate more information about the earlier observed activity of recombinant GTFA with malto-oligosaccharides (MOSs), a detailed structural analysis of products formed was carried out. To this end, 100 mM solutions of MOS (DP2-DP6) were individually incubated for different times up to 72 h at 37°C with 50

nM recombinant GTFA in 25 mM sodium acetate buffer/1 mM $CaCl_2$, pH 4.7. TLC analysis (data not shown) of the various incubation mixtures revealed that only for MOS DP>3, the GTFA enzyme clearly possessed hydrolysis/transferase activity. GTFA converted only minor amounts of maltose and maltotriose. For MOS DP4-6, the yield of product formation was higher but still less than observed during the incubation of sucrose together with maltose (see below) under the same conditions and incubation time. GTFA thus has relatively minor activity with these MOS substrates.

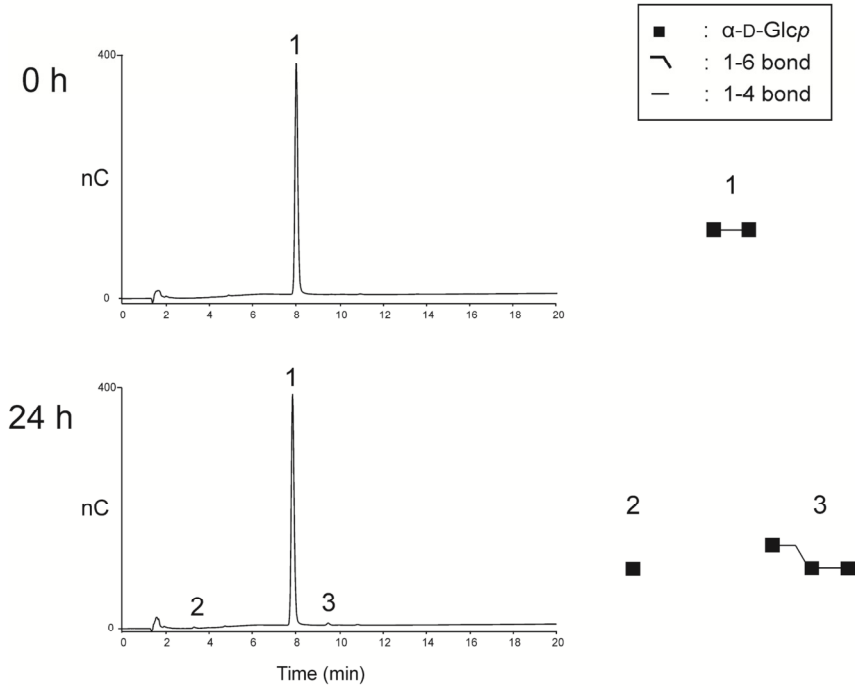

Figure 6. HPAEC-PAD profile (0 to 500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from maltose (MOS DP2) with GTFA enzyme at t = 0 and 24 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included.

Incubation of maltose (MOS DP2) with the GTFA enzyme for 24 h on a semi-preparative scale, followed by HPAEC profiling, product isolation, MALDI-TOF-MS and NMR analysis, showed only minimal formation of glucose and panose [α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*] (compound 3) (Fig. 6), indicating that GTFA is poorly hydrolyzing maltose. HPAEC-PAD profiling of

the incubations of GTFA with MOS DP3 to DP6 over periods up to 30 h gave more insight into the type of products formed. The chromatograms of the incubations of DP3 and DP4 at relevant incubation times are shown in Supplementary data, Fig. S1 and Fig. 7, respectively. Most of the fractions isolated were analyzed by MALDI-TOF-MS and 1D/2D NMR spectroscopy (including TOCSY, ^{1}H-^{13}C HSQC, and ROESY experiments). For general ^{1}H and ^{13}C NMR data, see Tables 2 and 3. The established structures are included in the various figures.

Table 2. ^{1}H and ^{13}C chemical shifts[a] (D_2O, 310K) of Glc residues present in -2,4)-substituted-oligomers formed by incubation of MOSs (with and without sucrose) with GTFA enzyme of *Lactobacillus reuteri* 121.

Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
	C-1	C-2	C-3	C-4	C-5	C-6	
A	5.433	3.58	3.68	3.50	3.90	3.74	3.98
-(1→6)-α-D-Glc*p*-(1→4)-	100.5	72.4	73.3	70.2	71.1	66.7	
D	5.45/40[b]	3.59	3.69	3.43	3.73	3.85	3.77
α-D-Glc*p*-(1→4)-	100.6	72.5	73.8	70.2	73.6	61.3	
E	4.958	3.56	3.74	3.43	3.72	3.85	3.76
α-D-Glc*p*-(1→6)-	99.0	72.3	73.9	70.3	72.6	61.2	
F	5.432	3.61	3.96	3.65	3.85	3.89	3.82
-(1→4)-α-D-Glc*p*-(1→4)-	100.0	72.3	74.1	77.7	72.0	61.3	
Rα	5.44[b]	3.68	4.09	3.71	3.97	3.87	3.83
-(1→2,4)-α-D-Glc*p*	90.1	76.5	72.5	77.5	70.6	61.2	
Rβ	4.809	3.43	3.87	3.68	3.60	3.94	3.77
-(1→2,4)-β-D-Glc*p*	97.0	79.5	75.8	77.5	75.3	61.7	
G_α	5.099	3.56	3.78	3.45	3.91	3.85	3.80
α-D-Glc*p*-(1→2)-	97.2	72.3	73.6	70.3	72.7	61.2	
G_β	5.40/38[b]	3.55	3.75	3.45	3.99	3.82	3.79
α-D-Glc*p*-(1→2)-	98.6	72.3	73.6	70.3	72.5	61.2	

[a] In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C) [b]Average chemical shifts values of the anomeric protons (see exact values: ^{1}H NMR spectra, Supplementary Material, Fig. S4)

Table 3. ^{1}H and ^{13}C chemical shifts[a] (D_2O, 300K) of Glc residues present in oligomers (DP≥ 4) formed by incubation of MOS (with and without sucrose) with GTFA enzyme of *Lactobacillus reuteri* 121.

Residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b
A^{I,II,III,X} -(1→6)-α-D-Glc*p*- (1→4)-	5.40^{X}/38^{I,III}/36^{II} [b] 101.0	3.59 72.7	3.69 74.1	3.49^{X}/46^{I,II,III} 70.4	3.94 72.4	3.73^{X}/77^{I,II,III} 66.9^{X}/67.3^{I,II,III}	3.98^{X}/94^{I,II,III} 66.9^{X}/67.3^{I,II,III}
B^{II,X} -(1→4)-α-D-Glc*p*- (1→6)-	4.98/95^{II,X} [b] 99.0	3.60 72.2	4.00 74.4	3.65 78.5	3.85 71.4	3.89 61.5	3.82
C -(1→4,6)-α-D- Glc*p*-(1→4)-	5.36[b] -	3.65 -	3.97 -	3.67 -	4.01 -	3.83 -	4.03
D α-D-Glc*p*-(1→4)-	5.39/38/34[b] 99.0	3.59 72.5	3.67 73.9	3.42 70.5	3.74 73.9	3.84 61.5	3.75
E α-D-Glc*p*-(1→6)-	4.99/95[b] 99.0	3.56 72.5	3.75 72.7	3.43 70.5	3.75 72.4	3.85 61.5	3.77
F -(1→4)-α-D-Glc*p*- (1→4)-	5.39[b] 100.7	3.62 72.5	3.96 74.3	3.66 77.8	3.84 72.2	3.88 61.5	3.81
J -(1→6)-α-D-Glc*p*- (1→6)-	4.955 98.8	3.58 72.3	3.70 74.3	3.51 70.6	3.91 71.2	3.76 66.7	3.98
Rα -(1→4)-α-D-Glc*p*	5.22/23[b] 92.9	3.56 72.3	3.97 74.2	3.61 78.5	3.95 71.0	3.88 61.5	3.83
Rβ -(1→4)-β-D-Glc*p*	4.650 96.8	3.27 75.0	3.77 77.2	3.62 78.5	3.61 75.6	3.94 61.9	3.80

[a] In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C) [b]Average chemical shifts values of the anomeric protons (see exact values: ^{1}H NMR spectra, Supplementary Material, Fig. S2, Fig. S3)

Next to the hydrolysis products Glc (compound 2) and maltose (compound 3), the transfer product (compound 4) formed from maltotriose (MOS DP3) at t = 6 h is maltotetraose (MOS DP4), the result of an (α1→4) → (α1→4) transfer of a single α-D-Glc*p* unit (Supplementary data, Fig. S1). After 24 h, an (α1→4) → (α1→6) linkage transfer of a single α-D-Glc*p* unit to the non-reducing end and an (α1→4) → (α1→2) linkage transfer of a single α-D-Glc*p* unit to the reducing end of maltotriose was observed. Furthermore, panose (compound 5) was detected,

showing that formed maltose also acts as an acceptor for an (α1→6) elongation, in agreement with the direct incubation of maltose with GTFA.

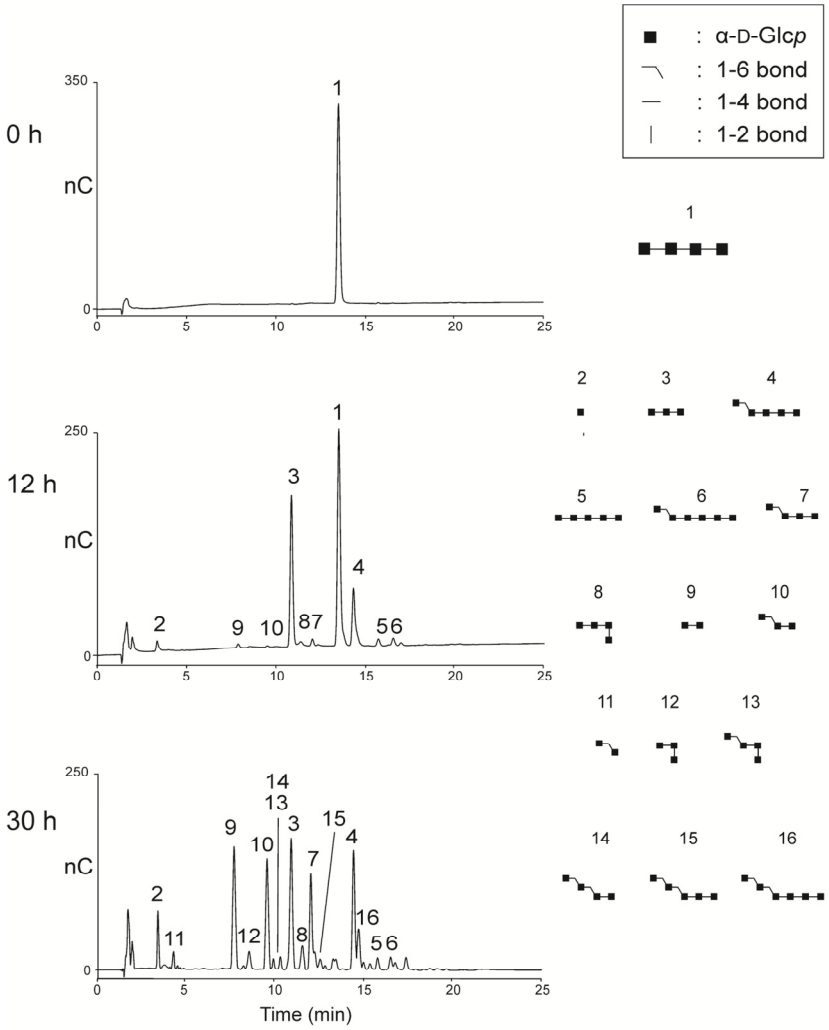

Figure 7. HPAEC-PAD profile (0 to 500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from maltotetraose (MOS DP4) with GTFA enzyme at t = 0, 12, and 30 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included.

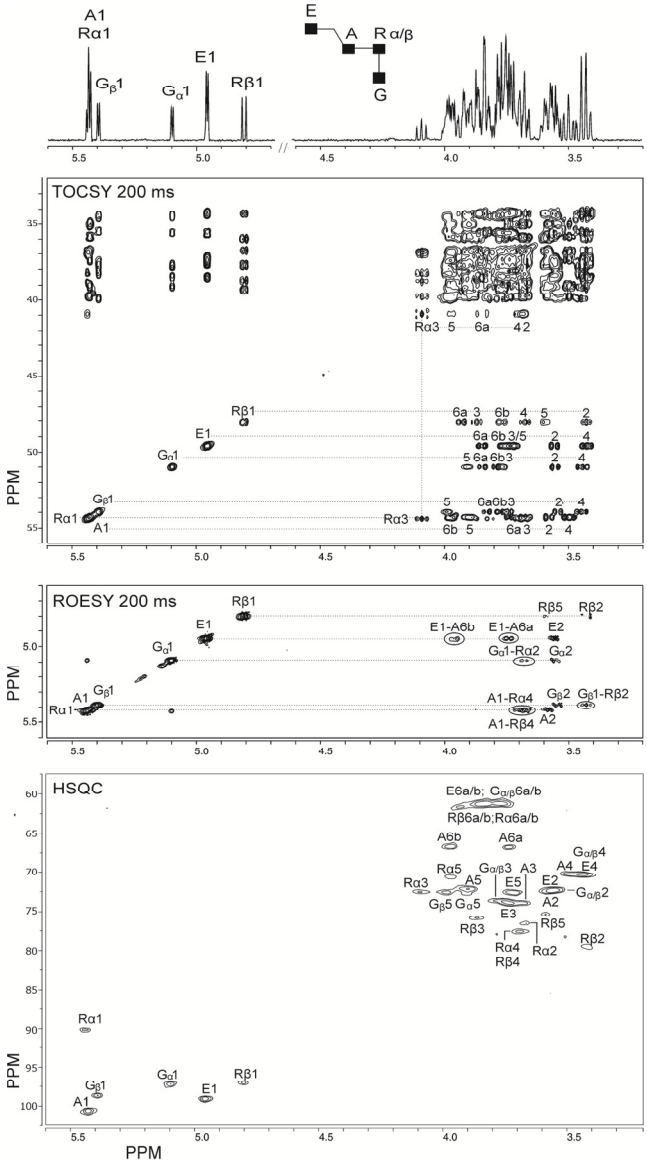

Figure 8. 1D ^{1}H NMR, TOCSY (200 ms), ROESY (200 ms), and HSQC spectra of α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-[α-D-Glc*p*-(1→2)]-α/β-D-Glc*p* (compound 13 in Fig. 7).

Incubation of maltotetraose (MOS DP4) with GTFA yielded, after 30 h, an interesting ensemble of products (MALDI-TOF-MS showed DP2 to DP6; $[M+Na]^{+}$, *m/z* 365.2 to *m/z* 1013.1); the major higher-molecular-mass product at t = 12 h comprised an (α1→4) → (α1→6) linkage transfer of a single α-D-Glc*p*

unit to the non-reducing end of maltotetraose (compound 4 in Fig. 7). As can be seen from the sixteen isolated products characterized (Fig. 7), GTFA displays hydrolysis (compounds 2, 3, and 9), as well as disproportionation activity, namely elongations with α-D-Glc*p*-(1→6) (compounds 4, 6, 7, 10, and 11) and α-D-Glc*p*-(1→4) (compound 5) units at the non-reducing end, as well as elongations with a single α-D-Glc*p*-(1→2) unit at the reducing end (compounds 8, 12, and 13). After prolonged incubation small amounts of higher-molecular-mass isomalto-/malto-oligomers were detected (compounds 14, 15, and 16), a type of structure that has been found earlier as products from incubations of MOS with GTFB, although with longer sequences of (1→6)-linked α-D-Glc*p* units (81). GTFA thus also has (minor) 4,6-α-glucanotransferase activity. Note that after 30 h, the original donor substrate maltotetraose (compound 1) had nearly disappeared. Higher polymeric material could not be detected: only oligosaccharide products (up to DP10) were observed. The 1D ^{1}H NMR spectra of compounds 4 and 16 (Fig. 7), including some exact chemical shift values, are depicted in Supplementary data, Fig. S2.

To illustrate the structure determination of oligosaccharides with a (1→2)-linked α-D-Glc*p* residue in a reducing end →2,4)-D-Glc*p* situation, the NMR analysis of compound 13 (Fig. 7) (tetrasaccharide; MALDI-TOF-MS *m/z* 689.3, $[M+Na]^{+}$) is presented here in more detail. The 1D ^{1}H NMR spectrum of compound 13, together with the TOCSY (200 ms), ROESY (200 ms), and HSQC spectra, are depicted in Fig. 8, and the various ^{1}H and ^{13}C NMR data are presented in Table 2.

The 1D ^{1}H NMR spectrum of compound 13 showed six anomeric signals, which were used as starting points for the assignment of the chemical shifts of all non-anomeric protons in the 2D TOCSY spectrum (TOCSY spectra with spin-lock times 20, 50, 100, and 200 ms were used to acquire all assignments). The H-1 signals at δ 5.441 (**R**α1) and 4.809 (**R**β1) are in agreement with a reducing -(1→2,4)-D-Glc*p* **R** unit [http://www.casper.organ.su.se/casper/; (197)]. The splitting of the **G** H-1 doublet into two doublets ($\mathbf{G}_{\alpha}$ H-1, δ 5.099 and $\mathbf{G}_{\beta}$ H-1, δ 5.387) corresponds with the long-range effect induced by the α- and β-anomeric forms of residue **R** (195). The anomeric signals at δ 5.433 and 4.958 reflect the presence of an internal -(1→6)-α-D-Glc*p*-(1→4)- unit and a terminal α-D-Glc*p*-(1→6)- unit, respectively (81). In the 2D ROESY spectrum, strong inter-residual

couplings were observed between $\mathbf{G}_{\alpha}$ H-1 and **R**α H-2, $\mathbf{G}_{\beta}$ H-1 and **R**β H-2, **A** H-1 and **R**α H-4, **A** H-1 and **R**β H-4, and **E** H-1 and **A** H-6a/b, in accordance with the structure α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-[α-D-Glc*p*-(1→2)-]D-Glc*p*. The various substitution patterns are confirmed by their ^{13}C NMR data, deduced from the ^{13}C-^{1}H HSQC spectrum: **R**α (C-2, δ 76.5; C-4, δ 77.5), **R**β (C-2, δ 79.5; C-4, δ 77.5), **A** (C-6, δ 66.7) (81,195,198).

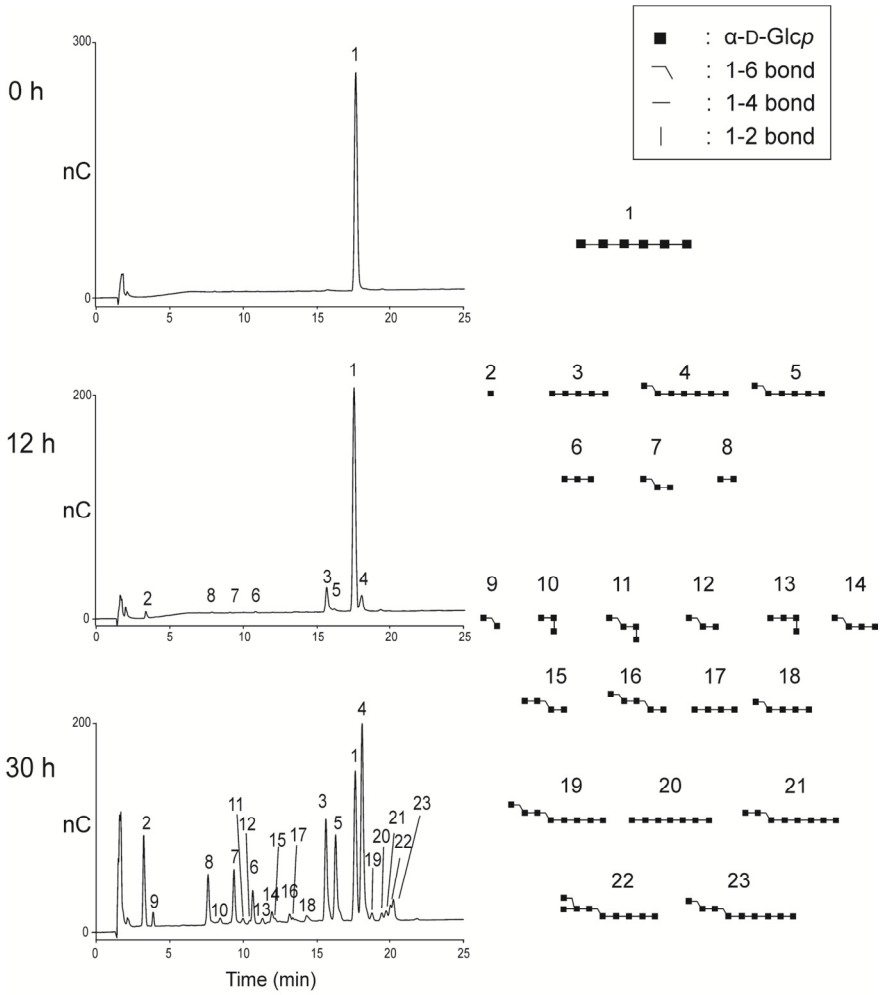

Figure 9. HPAEC-PAD profile (0 to 500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from maltohexaose (MOS DP6) with GTFA enzyme at t = 0, 12, and 30 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included.

Incubation of maltohexaose (MOS DP6) with GTFA yielded, after 30 h, twenty-three isolated products (Fig. 9) (MALDI-TOF-MS: DP2 to DP9; $[M+Na]^+$, *m/z* 365.3 to *m/z* 1499.2), which were subjected to detailed structural analysis. The major compounds comprised structures 2 (Glc), 8 (maltose), 7 (panose), 6 (maltotriose), 3 (maltopentaose), 5 (maltopentaose elongated with an (1→6)-linked α-D-Glc*p* unit), 1 (maltohexaose), and 4 (maltohexaose elongated with an (1→6)-linked α-D-Glc*p* unit), indicating hydrolysis activity and an (α1→4) → (α1→6) linkage transfer of a single α-D-Glc*p* unit. Besides similar structures as seen for MOS DP4, including relatively minor products with an (1→2)-linked α-D-Glc*p* unit (compounds 10, 11, and 13), also a minor 4,6-branched structure was identified (compound 22). Interestingly, in this case also minor structures with alternating (α1→6)/(α1→4) linkages were detected (for NMR details, see Table 3). Linear sequences of (1→6)-linked α-D-Glc*p* units, as seen in the incubation of MOS DP4 with GTFA, were not observed with MOS DP6. 1D ^{1}H NMR spectra of compounds 19, 21, 22, and 23 (Fig. 9), including some exact chemical shift values, are depicted in Supplementary data, Fig. S2.

Products of the GTFA enzyme incubated with malto-oligosaccharides plus sucrose as substrates

Previously, it has been observed that the presence of both maltose and sucrose influences the GTFA-catalyzed spectrum of generated oligosaccharides, when compared with sucrose alone as substrate (70). For a more detailed structural analysis, solutions of 100 mM sucrose and 100 mM MOS (DP2 - DP6) were each incubated with 50 nM recombinant GTFA in 25 mM sodium acetate buffer/1 mM $CaCl_2$, pH 4.7, at 37°C. The reaction was followed in time by TLC screening (data not shown).

Inspection of the HPAEC profiles of products formed after 1 h, 3 h, and 6 h incubation of sucrose and maltose with GTFA (Fig. 10) showed the formation of a series of products up to DP7, and after 6 h, the complete disappearance of Suc. Prolonged incubation resulted in an increase in molecular size of the products, but the amounts were very low (according to TLC and MALDI-TOF-MS screening), and these were not studied in more detail. Most of the products formed within 6 h were isolated and investigated by MALDI-TOF-MS and 1D/2D NMR

spectroscopy. Surprisingly, the majority of the initially formed compounds did not stem from elongations of Suc, but from extensions of maltose by Glc residues. Apparently, GTFA cleaves Suc and the Glc units generated are preferentially used to elongate maltose instead of sucrose. Only traces of Suc elongations, i.e., two trisaccharides (marked with an asterisk, Fig. 10) were detected. Fructose and panose (compound 3 in Fig. 10) were found as the most abundant products. The (α1→4)/(α1→6) alternating build-up of the structures of the minor oligosaccharides 5, 6, 11, and 12 in Fig. 10 is similar to the Suc-containing structures found for the sucrose incubation with GTFA (Fig. 2). In contrast to the "sucrose-only" incubation, just a minor amount of polymeric material (DP>20 according to MALDI-TOF-MS) was detected after prolonged incubations, and now a →4,6)-branched structure was already seen after 6 h of incubation (structure 5′, Fig. 10). Also in this case, the three structures with (1→2)-linked α-D-Glc*p*, earlier found in the GTFA incubations with maltotriose (only one of the three), maltotetraose and maltohexaose (Supplementary data, Fig. S1; Fig. 7 and 9), were detected. Compound 9 (Fig. 10) was also traced in the incubation of maltotetraose with GTFA (compound 14 in Fig. 7), showing the possibilities of synthesizing linear sequences of (1→6)-linked α-D-Glc*p* units. For a survey of the ^{1}H and ^{13}C NMR data of the various compounds identified, see Table 2 [→2,4)-branched oligosaccharide products] and Table 3 [alternating (α1→4) and (α1→6) linkages]. For a survey of some typical 1D ^{1}H NMR spectra (compounds 5, 5′, 6, 7, 8, 10, 11, and 12 in Fig. 10), including some exact chemical shift values, see the Supplementary data, Fig. S3 and S4.

Incubation of GTFA with Suc/MOS DP3-6 resulted in transferase activity in each case, yielding similar products (data not shown) as described for the Suc/MOS DP2 experiment, confirming the GTFA preference for Glc transfer to MOS acceptors above transfer to sucrose. This clearly reflects a higher affinity of GTFA for MOS than for sucrose as acceptor substrate.

Discussion

Detailed characterization of the initially formed oligosaccharides in the biosynthesis of reuteran (EPS121, EPS35-5) from sucrose with the *L. reuteri* GTFA enzyme as catalyst clearly shows that it possesses mainly transferase activity. Under the applied conditions, GTFA transfers glucose residues from

donor sucrose to acceptor sucrose and to the growing glucan chain attached to a sucrose. Such a reaction was suggested earlier in a study on the mechanism of a *Leuconostoc mesenteroides* NRRL B-512F dextranase and a *L. mesenteroides* NRRL B-1355 alternansucrase, whereby product oligosaccharides of unknown structure were found, of which methylation analysis and NMR spectroscopy showed the presence of fructose or sucrose located at the oligosaccharide extremity (88).

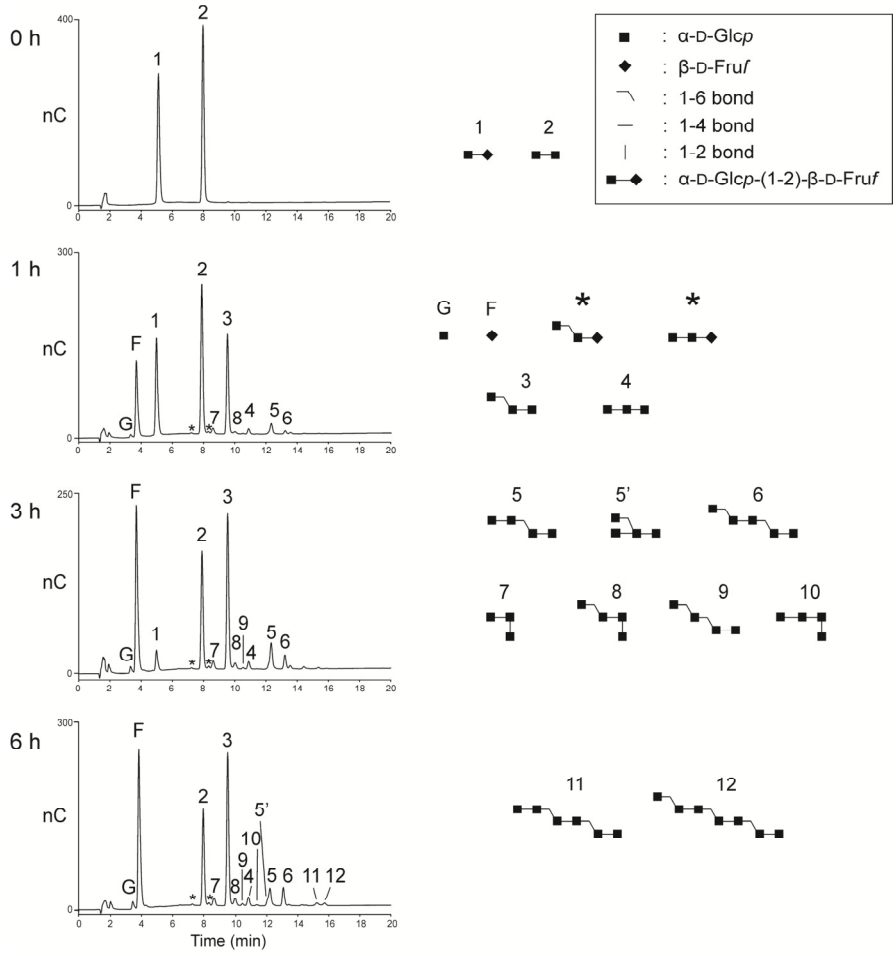

Figure 10. HPAEC-PAD profile (0 to 500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from sucrose + maltose (MOS DP2) with GTFA enzyme at t = 0, 1, 3, and 6 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included. G = glucose; F = fructose. Two Fru-containing compounds are marked with an asterix.

Incubation of sucrose with the GTFA enzyme resulted initially in synthesis of oligosaccharides that were all linear. The transferred Glc residues were mainly attached in alternating (α1→6)/(α1→4) linkages, forming a Suc-containing oligo-/polysaccharide; no linear (α1→6) sequences were found. This observation provides important new insights in the reaction specificity of the GTFA reuteransucrase enzyme. Apparently, the GTFA reaction mechanism seems to dictate alternate binding of the growing linear oligosaccharide acceptor substrates in two different ways in or near the active site, resulting in alternate formation of (α1→6) and (α1→4) linkages. Our ongoing studies aim to identify these different binding possibilities. After growth of the oligomer up to DP14, →4,6)-branching occurred, and increased during further growth of the polymer (DP>20) to an ultimate 14% branching content. Currently, experiments are in progress to investigate the effects of different sucrose concentrations on the synthesis of these oligo/polysaccharides, and to obtain further information about the branching reaction occurring during prolonged incubation. Also the GTFA structural features determining the molecular mass of its EPS formed remain to be elucidated.

Replacement of sucrose by malto-oligosaccharides (MOSs, DP2-DP6) in the incubation with GTFA showed that the latter compounds were relatively poor substrates, and very low amounts of only relatively short oligosaccharides (< DP10) were generated in time; no polymer formation was seen and the major reaction was hydrolysis. The established structures showed besides (α1→4) linkages, also elongations at the non-reducing end with (1→6)-linked α-D-Glc*p* and at the reducing end with (1→2)-linked α-D-Glc*p* residues. It should be noted that (α1→2) linkages have not been found in the detailed structural analysis of reuteran (EPS121, EPS35-5) (73). So, this minor (α1→2)-glucosylation activity of GTFA is only seen when it is incubated with relatively high concentrations of MOS, containing a reducing Glc residue. Recently, the 3D structure of an (α1→2)-branching glucansucrase, isolated from a *Leuconostoc mesenteroides* strain, transferring Glc units from sucrose onto gluco-oligosaccharides, has been reported (47,199).

Incubations of GTFA with MOS plus equimolar amounts of sucrose revealed that the enzyme initially uses sucrose as glucose donor and MOS as acceptor

substrates. Previous studies have shown that maltose is the most effective acceptor for an alternansucrase enzyme of a *L. mesenteroides* strain (200).

The GTFA enzyme clearly forms novel and interesting (alternan) oligosaccharide mixtures. These compounds will be further evaluated for their prebiotic properties, and ability to reduce the blood glucose levels after food consumption (29,193,201,202).

Funding

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Abbreviations

DP, degree of polymerization; EPS, exopolysaccharide; GTFA, glucosyltransferase A from *L. reuteri* 121; HPAEC-PAD, high-pH anion-exchange chromatography - pulsed amperometric detection; HSQC, ^{1}H detected heteronuclear single quantum coherence spectroscopy; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MLEV, composite pulse devised by M. Levitt; MOS, malto-oligosaccharides; NMR, nuclear magnetic resonance; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy; WEFT, water eliminated Fourier transform.

Supplemental data

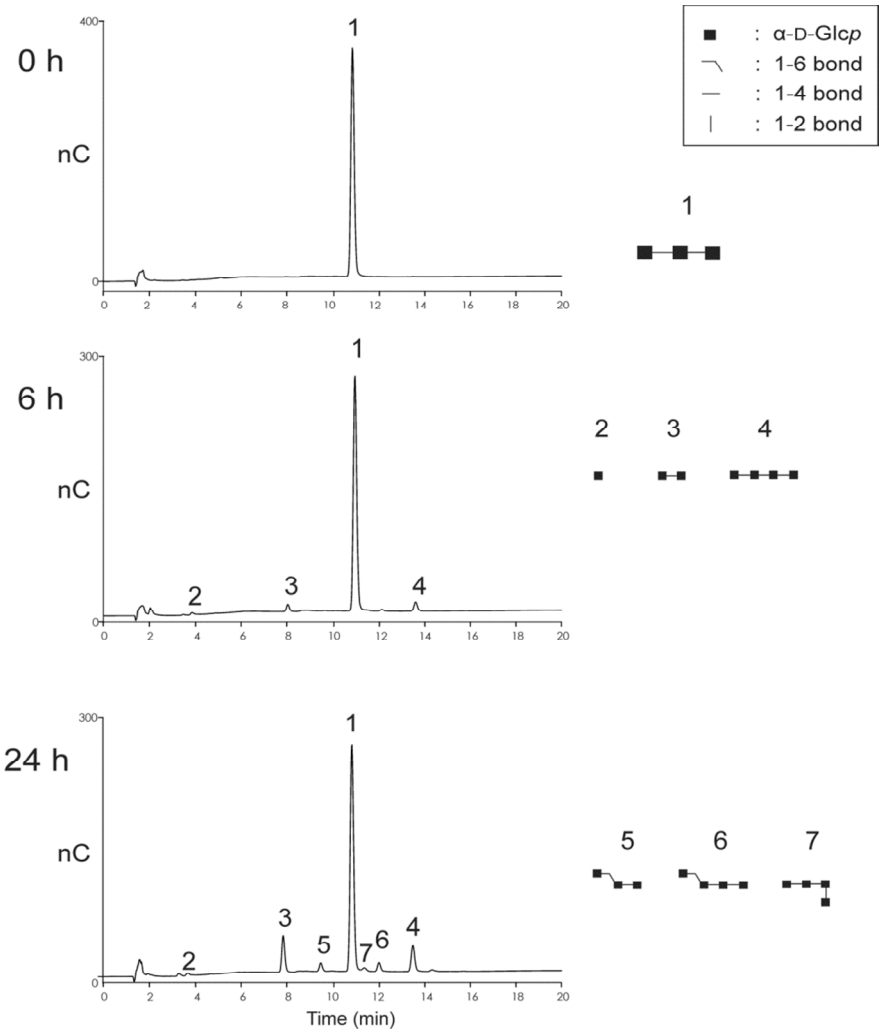

Figure S1. HPAEC-PAD profile (0 to 500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from maltotriose (MOS DP3) with GTFA enzyme at t = 0, 6 and 24 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included.

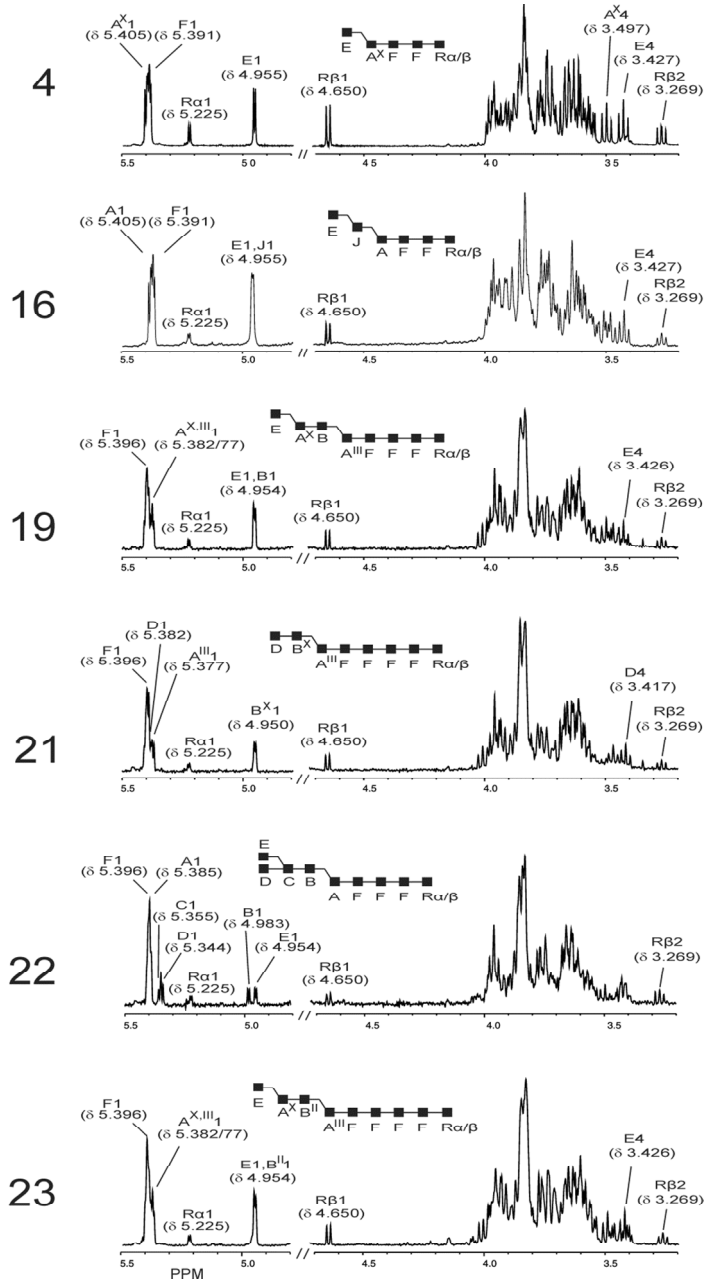

Figure S2. Selection of ^{1}H NMR spectra (300K) of the products found in the generated oligosaccharide mixtures after the incubation of maltotetraose (structure 4, 16) and maltohexaose (structure 19, 21, 22, 23) with GTFA enzyme, without sucrose, for 30 h at 37°C and pH 4.7.

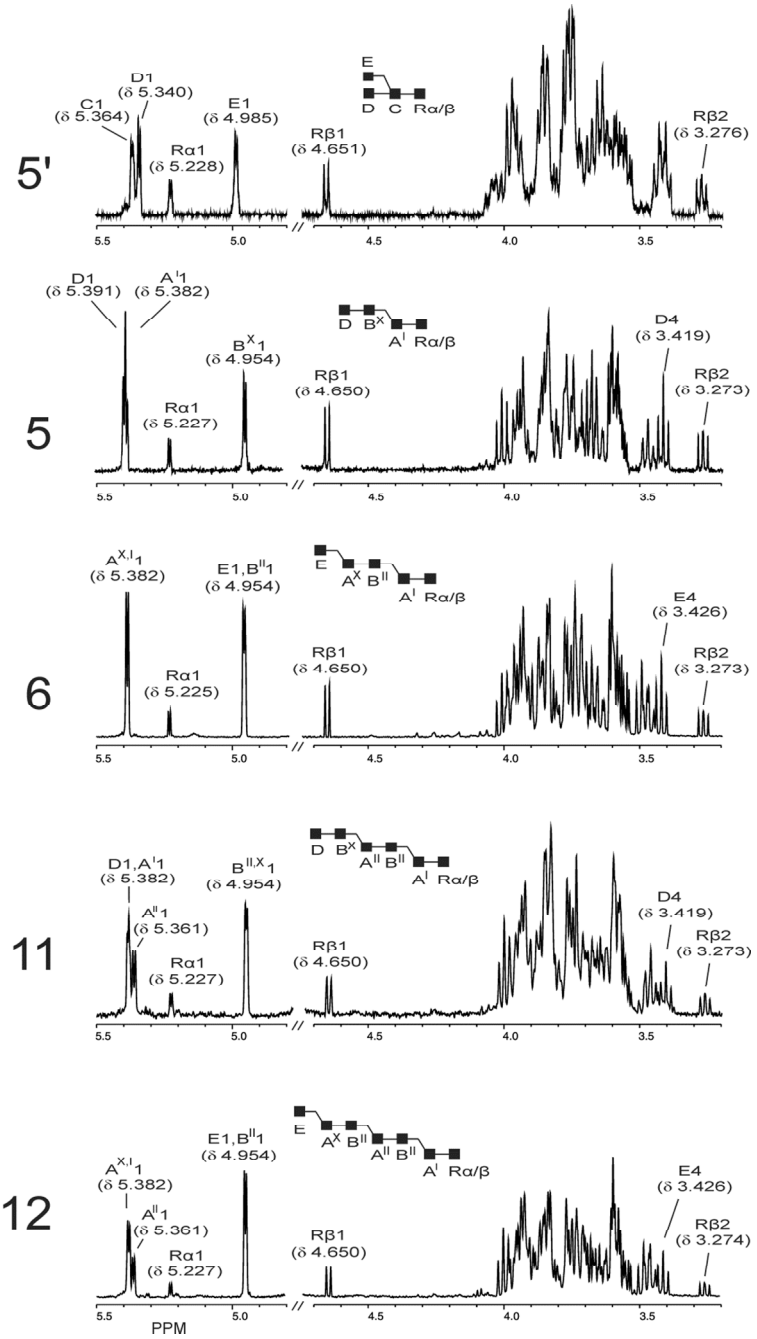

Figure S3. [1]H NMR spectra (300K) of generated oligosaccharides after the incubation of sucrose + maltose with GTFA enzyme for 72 h at 37°C and pH 4.7.

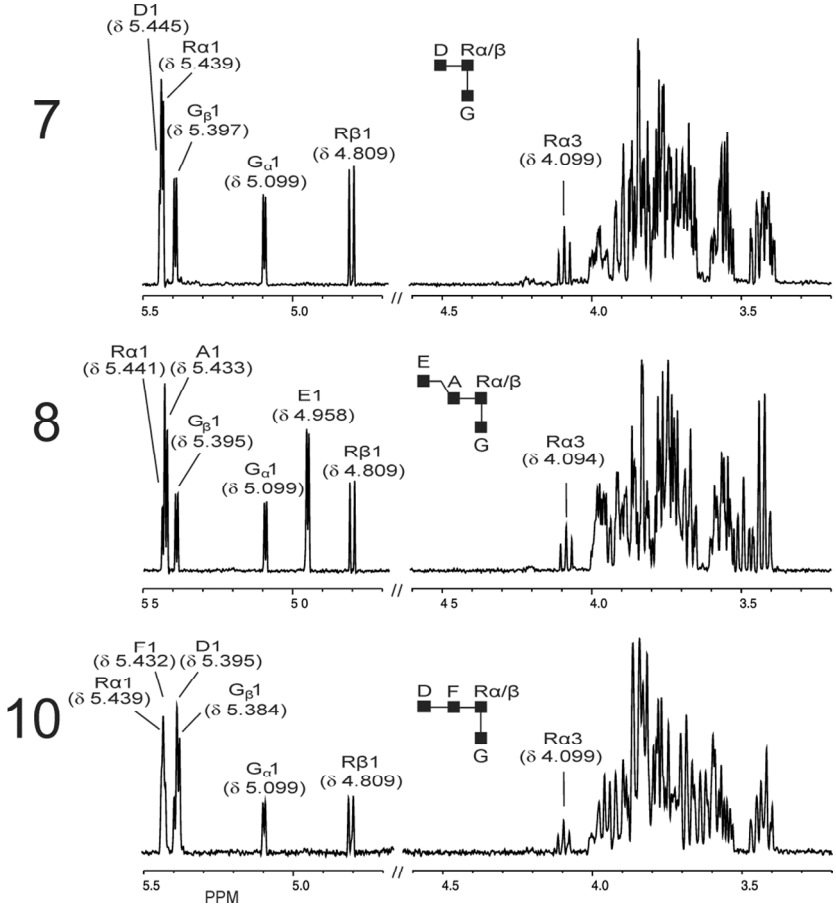

Figure S4. [1]H NMR spectra (310K) of generated oligosaccharides after the incubation of sucrose + maltose with GTFA enzyme for 72 h at 37°C and pH 4.7.