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Structural Characterization of Bioengineered α-D-Glucans Produced by Mutant Glucansucrase GTF180 Enzymes of *Lactobacillus reuteri* Strain 180

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Mutagenesis of specific amino acid residues of the glucansucrase (GTF180) enzyme from *Lactobacillus reuteri* strain 180 yielded 12 mutant enzymes that produced modified exopolysaccharides (mEPSs) from sucrose. Ethanol-precipitated and purified mEPSs were subjected to linkage analysis, Smith degradation analysis, and 1D/2D ^{1}H NMR spectroscopy. Comparison of the results with structural data of the previously described wild type **EPS180** and triple mutant **mEPS-PNNS** revealed a broad variation of structural elements between mEPS molecules. The amount of (α1→3) linkages varied from 14−43%, the amount of (α1→4) linkages (not present in the wild type) from 0−12%, and the amount of (α1→6) linkages from 51−86%. The average molecular weight (M_w) ranged from 9.4 to 32.3 MDa and the degree of branching varied from 8−20%. Using a previously established ^{1}H NMR structural-reporter-group concept, composite models, that include all identified structural features, were formulated for all mEPS molecules. Variations in the mEPS structures strongly affected the physical properties of the mEPSs.

Introduction

Exopolysaccharides (EPSs) produced by lactic acid bacteria (LAB) are regularly used in the food industry[1−4] as their physical properties influence viscosity and mouth-feel of dairy products. Many of the EPSs produced by LAB strains have been subjected to structural analysis, leading to postulated structure−function relationships.[5−7] The next challenge in the food technology process is the design of engineered EPSs with improved physical properties.[8−11] Because the majority of the studied EPSs from LAB are repeating-unit-composed heteropolysaccharides,[12] the modifications investigated are mostly in the ensemble of glycosyltransferase enzymes, using nucleotide activated sugars as substrate, that produce these repeating units.[8−10]

Structures of nonrepeating-unit-composed homopolysaccharide products of glucansucrase enzymes have not been extensively analyzed. Glucansucrase enzymes of lactic acid bacteria use sucrose to synthesize a diversity of α-D-glucans, i.e. dextrans with (α1→6) linkages, or with a majority of (α1→6) linkages and (α1→2), (α1→3), and/or (α1→4) branches (mainly found in *Leuconostoc*),[13−15] mutan with a majority of (α1→3) linkages (found in *Streptococcus*),[16] alternan with alternating (α1→3) and (α1→6) linkages (only reported in *L. mesenteroides*),[17] and reuteran being a highly branched structure with mainly (α1→4) linkages (found in *Lactobacillus reuteri*).[18]

Recently, two glucansucrase enzymes have been described in *L. reuteri*, converting sucrose into large, heavily branched α-D-glucans.[19,20] The structures of two of these α-D-glucans, that is, **EPS180** and **EPS35-5**, have been extensively analyzed.[21,22] Modifications in amino acid residues (N1134/N1135/S1136) adjacent to the catalytic D1133 (putative transition state stabilizing residue) of the reuteransucrase (GTFA) enzyme, responsible for the synthesis of **EPS35-5**, were shown to introduce changes into the glycosidic linkage patterns of the EPS products of the mutant GTFA enzymes. Subsequent introduction of modifications of P1026V and I1029V, close to the catalytic D1024 (nucleophile) increased the (α1→6) specificity of GTFA.[11] Glucansucrase enzymes modified in the above-mentioned tripeptide sequence (usually a conserved SEV sequence) immediately following the putative transition state stabilizing residue have also been reported for the dextransucrase enzyme of *L. mesenteroides* NRRL B-512F and the alternansucrase enzyme of *L. mesenteroides* NRRL B-1335.[23]

Here, based on linkage analysis, Smith degradation analysis, and 1D/2D ^{1}H NMR spectroscopy making use of a recently established ^{1}H NMR structural-reporter-group concept,[24] we formulate a series of composite models, that include all identified structural features of the mutant EPSs (mEPSs), produced by genetically engineered glucansucrase GTF180 enzymes using sucrose as a substrate (wild type is responsible for the synthesis of **EPS180**). Modifications in GTF180 were made in residues S1137/N1138/A1139, equivalent to GTFA (N1134/N1135/S1136), and in a fourth residue (Q1140), which has not been targeted before. Mutations were selected based on sequences observed in other glucansucrase enzymes, that is, reuteransucrase (NNS), mutansucrase (NNV), alternansucrase (YDA), and dextransucrase (SEV). Single and double mutant variants of these sequences were introduced. For comparison, the structural data of a recently

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analyzed mEPS produced by a triple mutant of the glucansucrase GTF180 enzyme (**mEPS-PNNS**)[25] are included.

Experimental Section

Bacterial Strains, Plasmids, Media, and Growth Conditions. *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, CA) was used as host for cloning purposes, and plasmid pET15b (Novagen, Madison, WI) for expression of the different (mutant) *gtf* genes in *E. coli* BL21 Star (DE3) (Invitrogen). *E. coli* strains were grown aerobically at 37 °C in LB medium. *E. coli* strains containing recombinant plasmids were cultivated in LB medium with 100 μg/mL ampicillin. Agar plates were made by adding 1.5% agar to the LB medium.

Cloning and Mutagenesis. To facilitate future manipulation the N-terminally truncated GTF180 gene,[19] obtained using PCR with primers 180expCF 5′-GATGCAT*GAGCTC***CCATGG**GCATTAACG-GCCAACAATATTATATTGACCC-3′, containing *Sac*I (Italics) and *Nco*I (bold) restriction sites and 180expR 5′-ATATCGAT*GGGC-CCC***GGATCC**TATTAGTGATGGTGATGGTGATGTTTTTGGCCG-TTTAAATCACCAGGTTTTAATGG-3′, containing *Apa*I (italics) and *Bam*HI (bold) restriction sites and a C-terminal His-tag (underlined),[19] was digested with *Sac*I/*Apa*I, and cloned in the corresponding sites of pBluescript II SK+, yielding plasmid pBGTF180-ΔN. Furthermore, two unique restrictions (*Sal*I, 2802bp; *Xho*I, 3606bp) were introduced using the QuikChange site-directed mutagenesis kit (Stratagene; La Jolla, CA) and the following primers 5′-GGAATAAGGATAGT-GAAAATGTCGA**C**TACGGTGGTTTGC-3′ 5′-ATAAAGATTCAGT-TC**C**TCGA**G**TTTACTATGGAGACC-3′, and the complementary reverse primers, containing an introduced *Sal*I and *Xho*I site, respectively (underlined, silent mutation by change of base shown in bold face), yielding plasmid pBGTF180-ΔN-SX. This plasmid was digested with *Nco*I and *Bam*HI and ligated in the corresponding sites of pET15b yielding p15GTF180-ΔN-SX. pBGTF180-ΔN-SX was used for site-directed mutagenesis, sequencing and rapid exchange (using *Nco*I and *Xho*I restriction sites) with p15GTF180-ΔN-SX.

The various single and double mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and appropriate primer pairs to introduce mutations in pBGTF180-ΔN-SX. After successful mutagenesis (confirmed by restriction analysis and/or nucleotide sequencing), pBGTF180-ΔN-SX (containing mutation) was digested with *Nco*I and *Xho*I and ligated in the corresponding sites of p15GTF180-ΔN-SX.

Purification of GTF180 Mutant Proteins. GTF180 mutant proteins were produced and Ni-NTA purified as described previously.[26]

In Vitro Glucan Production by Mutant GTF180 Enzymes. Polymers were produced by incubation of mutant enzyme preparations with 146 mM sucrose for 7 days, using the conditions described previously,[26] and addition of 1% Tween 80 and 0.02% sodium azide. Glucans produced were isolated by precipitation with ethanol as described previously.[27]

Linkage Analysis. Polysaccharide samples were permethylated using CH_3I and solid NaOH in DMSO, as described earlier.[28] After hydrolysis with 2 M TFA (2 h, 120 °C), the partially methylated monosaccharides were reduced with $NaBD_4$ (2 h, room temperature). Conventional workup, involving neutralization with HOAc and removal of boric acid by coevaporation with MeOH, followed by acetylation with 1:1 acetic anhydride:pyridine (3 h, 120 °C), yielded mixtures of partially methylated alditol acetates, which were analyzed by GLC-EI-MS.[29,30]

Smith Degradation. A sample of **mEPS-YDA** (10 mg) was incubated with 2 mL of 50 mM sodium periodate in 0.1 M NaOAc (pH 4.3) for 96 h at 4 °C in the dark. Then, the excess of periodate was destroyed by addition of 0.1 mL ethylene glycol. The oxidized polysaccharide solution was dialyzed against tap water (24 h, room temperature), treated with excess $NaBH_4$ (18 h, room temperature), and subsequently neutralized with 4 M HOAc.[31] After coevaporation of boric acid with MeOH, the residue was hydrolyzed with 90% HCOOH (30 min, 90 °C). Finally, the solution was concentrated under a stream of N_2, and the products were analyzed by HPAEC-PAD.

HPAEC-PAD. High-pH anion-exchange chromatography (HPAEC) was performed on a Dionex DX500 workstation, equipped with an ED40 pulsed amperometric detection (PAD) system. A triple-pulse amperometric waveform (E_1 0.1 V, E_2 0.7 V, E_3 −0.1 V) was used for detection with the gold electrode.[32] Analytical separations were performed on a CarboPac PA-100 column (250 × 4 mm; Dionex), using a linear gradient of 0−200 mM NaOAc in 100 mM NaOH (1 mL/min).

Mass Spectrometry. GLC-EI-MS was performed on a Fisons Instruments GC 8060/MD 800 system (Interscience BV; Breda, The Netherlands) equipped with an AT-1 column (30 m × 0.25 mm, Alltech Associates Inc., Illinois, U.S.A.), using a temperature gradient (140−240 at 4 °C/min).[29]

NMR Spectroscopy. ^{1}H NMR spectra were recorded on a Bruker DRX500 spectrometer (Bijvoet Center, Department of NMR spectroscopy) at a probe temperature of 300 K. Samples were exchanged once with 99.9 atom% D_2O (Cambridge Isotope Laboratories, Inc.; Andover, MA), lyophilized, and dissolved in 650 μL of D_2O. ^{1}H chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225). 1D ^{1}H NMR spectra were recorded with a spectral width of 5000 Hz in 16 k complex data sets and zero filled to 32 k. A WEFT pulse sequence was applied to suppress the HOD signal.[33] When necessary, a fifth order polynomial baseline correction was applied. 2D ^{1}H−^{1}H TOCSY spectra were recorded using MLEV17 mixing sequences with spin-lock times of 10, 30, 60, 120, and 150 ms. The spin-lock field strength corresponded to a 90° pulse width of about 28 μs at 13 dB. The spectral width in 2D TOCSY experiments was 4006 Hz at 500 MHz in each dimension. 400−1024 spectra of 2k data points with 8−32 scans per t_1 increment were recorded. 2D NMR spectroscopic data were analyzed by applying a sinus multiplication window and zero filling to spectra of 4 k by 1 k dimensions. A Fourier transform was applied, and where necessary, a fifth to fifteenth order polynomial baseline function was applied. All NMR data were processed using in-house developed software (J.A. van Kuik, Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University).

HPSEC-MALLS. The molecular weights of the glucans were determined by high-performance size-exclusion chromatography (HPSEC) coupled online with a multi angle laser light scattering (MALLS) and differential refractive index detection (RI, Schambeck SDF). The HPSEC system consisted of an isocratic pump, an injection valve, a guard column and a set of two SEC columns in series (Shodex SB806MHQ and TSK gel 6000PW). A Dawn-DSP-F (Wyatt Technology; St. Barbara, CA) laser photometer HeNe (λ = 632.8 nm), equipped with a K5 flow cell, and thermostatted by a Peltier heating system, was used as MALLS detector. Samples (3 mg) were dissolved under shaking in 10 mL of 0.1 M Na_2SO_4 for 16 h at room temperature, followed by heating and vortexing (15 min, 100 °C). Then, they were filtered through a 0.45 μm filter (MILLEX) and the injection volume was 220 μL. Na_2SO_4 (0.1 M) was used as eluent at a flow rate of 0.8 mL/min. Pullulan and dextran samples with Mw ranging from 4×10^4 to 2×10^6 Da were used as standards. Determinations were performed in duplicate.

Results

Mutant Construction, Enzyme Expression, Purification, and Glucan Synthesis. The glucansucrase GTF180 enzyme has a unique S1137/N1138/A1139 (SNA) sequence following the putative transition state stabilizing residue, followed by a fully conserved Q1140.[34] This region differs from the consensus sequence SEVQ, conserved in many glucansucrase enzymes from *Streptococcus*, *Leuconostoc*, and *Lactobacillus* species.[13,34] To study the effect on the **EPS180** structure this GTF180 region was mutated, substituting the unique tripeptide SNA for (1) the conserved SEV sequence, found in many other glucansucrase enzymes;[34] (2) the conserved NNS sequence, found in the reuteransucrase enzymes GTFA from *L. reuteri* 121,[11] and

Table 1. Polymer Linkage Type and Alignment of Amino Acid Sequences of Various (Mutant) Glucansucrase Enzymes with GTF180 Wild Type and Mutant Variants Derived[a]

enzyme	main α-linkages in glucan	amino acid sequence around transition-state stabilizer D1136 in GTF 180	
GTFA	1→4/1→6	1126	YSFVRAH DNNSQDQI
GTFA′	1→6	1126	YSFVRAH D*SEV*QDQI
GTFO	1→4	1126	YSFVRAH DNNSQDQI
GTFML1	1→3/1→6	1125	YSFIRAH DNGSQDDI
GTFJ	1→3	605	YVFIRAH DNNVQDII
ASR	1→6/1→3	760	YSFVRAH DYDAQDPI
GTF180	1→6/1→3	1129	YNFVRAH DSNAQDQI
A1139S			YNFVRAH DSN*S*QDQI
A1139L			YNFVRAH DSN*L*QDQI
N1138D			YNFVRAH DS*D*AQDQI
Q1140E			YNFVRAH DSNA*E*DQI
Q1140A			YNFVRAH DSNA*A*DQI
Q1140H			YNFVRAH DSNA*H*DQI
S1137N/A1139V			YNFVRAH D*N*N*V*QDQI
N1138E/A1139V			YNFVRAH DS*EV*QDQI
S1137N			YNFVRAH D*N*NAQDQI
S1137Y			YNFVRAH D*Y*NAQDQI
S1137N/A1139S			YNFVRAH D*N*N*S*QDQI
S1137Y/N1138D			YNFVRAH D*YD*AQDQI

[a] GTFA: *Lactobacillus reuteri* 121; GTFA′: mutant GTFA; GTFO: *Lactobacillus reuteri* ATCC 55730; GTFML1: *Lactobacillus reuteri* ML1; GTFJ: *Streptococcus salivarius* ATCC 25975; ASR: *Leuconostoc mesenteroides* NRRL B-1355; GTF180: *Lactobacillus reuteri* 180; GTF180 variants: mutants constructed in this study. Mutations are italicized, and the putative transition state stabilizer is shown underlined.

GTFO from *L. reuteri* ATCC 55730;[18] (3) NNA, SNS, and SNL sequences as single mutations; (4) the NNV sequence, present in the mutansucrase GTFJ enzyme of *S. salivarius* ATCC 25975;[35] (5) the unique YDA motif, found in the alternansucrase enzyme of *L. mesenteroides* NRRL B-1355,[17] as well as the single mutant variant sequences YNA and SDA. For an overview of alignments of these enzymes as well as the constructed mutants, see Table 1.

The completely conserved glutamine residue Q1140 has not been targeted before in any glucansucrase enzyme. This residue, directly following the tripeptide sequence was therefore also mutated: Q1140A (small), Q1140E (similar size, different side chain), and Q1140H (basic side chain).

A total of 12 mutant genes were constructed, expressed in *E. coli* BL21 Star (DE3), and the recombinant enzymes were purified. The mutant enzymes were used to produce mEPSs by incubating with sucrose (146 mM; 37 °C, 7 days). All mutant enzymes were able to synthesize sufficient polysaccharide material for a detailed structural analysis.

Analysis of Mutant GTF180 Exopolysaccharides (mEPSs). Using a combination of methylation analysis and 1D ^{1}H NMR spectroscopy (structural-reporter-group concept),[24] structural elements have been identified for the 12 mutant EPS structures. Surveys of the methylation analysis data are presented in Table 2, whereas ^{1}H NMR data are summarized in Tables 3 and 4. For comparison, the earlier data of wild type **EPS180**[21] and triple mutant **mEPS-PNNS**[25] are included in these tables. The various 1D ^{1}H NMR spectra reflected only the presence of α-D-Glc*p* residues in all mEPSs. The H-1 signal of (-)α-D-Glc*p*-(1→4)- (For Glc residues at semidefined places in the structure (-)α-D-Glc*p*-(1→*x*)- or -(1→*x*)-α-D-Glc*p*(-) is used. When the structural context of the residue is precisely known, this is indicated as follows: -(1→*x*)-α-D-Glc*p*-(1→*y*)- describing an *x*-substituted residue with a (1→*y*) linkage. In case of a nonreducing terminal residue α-D-Glc*p*-(1→*x*)- is used, a reducing terminal residue is indicated with -(1→*x*)-D-Glc*p*.) units can be observed between δ 5.41−5.33,[22,24,25] while (-)α-D-Glc*p*-(1→3)- H-1 can vary between δ 5.39−5.32,[21,24,25] causing possible overlap between anomeric signals. In this paper the surface area under the peak between δ 5.41 and 5.39 (when present) always corresponds with the amount of 4-substituted glucopyranose residues as determined by methylation analysis. This means that in these mEPSs there is a clear separation between the signals from (α1→3)-linked and (α1→4)-linked glucose residues. The peak at δ ∼4.96 is a typical structural reporter for (-)α-D-Glc*p*-(1→6)- units (δ-range between 4.99 and 4.95 ppm).[21,22,24,25]

2D ^{1}H−^{1}H TOCSY experiments with increasing mixing times (10, 30, 60, 120, and 150 ms; 60 and 120 ms are shown in figures as examples) were interpreted, where available, to unravel the structural elements present in the mEPSs. Starting from the anomeric signals in the TOCSY spectrum with 10 ms mixing time, cross-peaks are observed with H-2 signals. In each incremental step subsequent ^{1}H signals arise on the anomeric track and can be assigned to H-3, H-4, H-5, H-6a, and H-6b. Previous analysis of di- and trisaccharide standards revealed clear chemical-shift patterns for specific -(1→*x*)-α-D-Glc*p*-(1→*y*)- residues.[24] Making use of structural-reporter-group signals and knowledge of these chemical-shift patterns, the mEPS structures were analyzed.

S1137N (mEPS-NNA). Methylation analysis of **mEPS-NNA** revealed the presence of terminal, 3-substituted, 4-substituted, 6-substituted, and 3,6-disubstituted glucopyranose residues in a molar percentage of 12, 26, 3, 47, and 12% (Table 2). Integration of the anomeric signals in the 1D ^{1}H NMR spectrum (Figure 1) showed 35% (α1→3)-linked glucose ($\delta_{H\text{-}1}$ ∼5.35), 4% (α1→4)-linked glucose ($\delta_{H\text{-}1}$ ∼5.40), and 61% (α1→6)-linked glucose ($\delta_{H\text{-}1}$ ∼4.96) residues (Table 3), which is in accordance with the methylation analysis data.

The structural-reporter-group concept identified the peaks at $\delta_{H\text{-}5}$ 4.20 and 4.12 as markers for -(1→6)-α-D-Glc*p*-(1→3)- and -(1→4)-α-D-Glc*p*-(1→3)- units, respectively.[21,25] Integration of these peaks in the 1D ^{1}H NMR spectrum revealed that these moieties were present at 28 and 4%, respectively, in **mEPS-NNA**.

On the (α1→3)-anomeric track in the 60 ms TOCSY spectrum (Figure 1) of **mEPS-NNA** $\delta_{H\text{-}3}$ 4.01 was observed, confirming the occurrence of -(1→4)-α-D-Glc*p*-(1→3)- units (compare **a3** in **mEPS-PNNS**).[25] On the same track in the TOCSY spectrum the structural reporter $\delta_{H\text{-}4}$ 3.45 was detected, indicating the occurrence of α-D-Glc*p*-(1→3)- units (compare with residue B in compounds **5a**, **9a**, and **10** in ref 25). On the (α1→3)-anomeric track in the 120 ms TOCSY spectrum (Figure 1) the H-5 signal at δ 4.20 confirmed the presence of -(1→6)-α-D-Glc*p*-(1→3)- units.[21]

On the (α1→4) anomeric track in the 120 ms TOCSY spectrum a cross-peak at $\delta_{H\text{-}4}$ 3.42 is present, indicating the occurrence of α-D-Glc*p*-(1→4)- units.[22,24] The specific values $\delta_{H\text{-}4}$ 3.50 and $\delta_{H\text{-}6b}$ 4.00 for -(1→6)-α-D-Glc*p*-(1→4)- units, and $\delta_{H\text{-}3}$ 3.96 for -(1→4)-α-D-Glc*p*-(1→4)- units are not observed in the TOCSY spectra, making the occurrence of such elements highly unlikely. If present, they occur in negligible amounts.

On the (α1→6)-anomeric track in the 60 ms TOCSY spectrum a cross-peak at $\delta_{H\text{-}4}$ 3.42 occurred, indicating the presence of α-D-Glc*p*-(1→6)- units. In the 60 ms TOCSY spectrum the $\delta_{H\text{-}3}$ signal at 4.01 ppm, specific for -(1→4)-α-D-Glc*p*-(1→6)- units is not observed. On the same track in the 120 ms TOCSY spectrum the unique $\delta_{H\text{-}3}$ ∼3.87 signal (not marked in the spectrum), indicating the presence of -(1→3)-α-D-Glc*p*-(1→6)- and/or -(1→3,6)-α-D-Glc*p*-(1→6)- units, is

Table 2. Methylation Analysis Data of **EPS180** and mEPSs[a]

EPS	Glc*p*(1→	→3)Glc*p*(1→	→4)Glc*p*(1→	→6)Glc*p*(1→	→3,6)Glc*p*(1→
wild type[21]	12 ± 0.8[b]	24 ± 0.6		52 ± 1.3	12 ± 1.0
mEPS-PNNS[25]	18 ± 1.3	10 ± 1.5	12 ± 1.3	42 ± 0.8	18 ± 1.0
mEPS-SNS	12 ± 1.0	24 ± 0.8		51 ± 1.5	13 ± 1.3
mEPS-SNL	14 ± 0.8	23 ± 1.3		47 ± 1.3	16 ± 0.6
mEPS-SDA	10 ± 0.8	24 ± 0.6		56 ± 0.8	10 ± 0.6
mEPS-SNAE	12 ± 0.5	16 ± 0.6	2 ± 0.6	52 ± 1.3	18 ± 0.6
mEPS-SNAA	11 ± 0.3	6 ± 0.6		69 ± 0.6	14 ± 0.6
mEPS-SNAH	8 ± 0.3	8 ± 0.3		76 ± 1.6	8 ± 0.3
mEPS-NNV	11 ± 1.0	30 ± 1.3	2 ± 0.6	46 ± 1.3	11 ± 1.3
mEPS-SEV	13 ± 1.3	24 ± 1.8		48 ± 1.0	15 ± 1.5
mEPS-NNA	12 ± 0.6	26 ± 0.3	3 ± 0.3	47 ± 1.5	12 ± 0.8
mEPS-YNA	18 ± 1.8	21 ± 1.0	4 ± 0.8	39 ± 1.3	18 ± 2.0
mEPS-NNS	15 ± 0.8	27 ± 1.3	4 ± 1.0	39 ± 0.8	15 ± 1.0
mEPS-YDA	19 ± 1.3	23 ± 1.5	7 ± 0.8	31 ± 1.3	20 ± 1.5

[a] Linkage distribution data are shown in molar percentages based on GLC intensities. [b] Standard deviations are based on methylation analysis data acquired in triplicate on polysaccharide products of two separate expressions of each mutant GTF180-ΔN enzyme; data for wild type[21] and **mEPS-PNNS**[25] have been discussed previously.

Table 3. Surface Area Ratios of Peaks in the 500 MHz 1D ^{1}H NMR Spectra of **EPS180** and mEPSs Recorded at 300 K in D_2O[a]

EPS	(α1→3) $\delta_{H\text{-}1}$ 5.35	(α1→4) $\delta_{H\text{-}1}$ 5.40	(α1→6) $\delta_{H\text{-}1}$ 4.96	6G^3[b] $\delta_{H\text{-}5}$ 4.20	4G^3[c] $\delta_{H\text{-}5}$ 4.12
wild type[21]	31	0	69	31	0
mEPS-PNNS[25]	28	12	60	23	7
mEPS-SNS	34	0	66	34	0
mEPS-SNL	38	0	62	31	0
mEPS-SDA	35	<1	65	31	<1
mEPS-SNAE	29	3	68	24	3
mEPS-SNAA	16	0	84	11	0
mEPS-SNAH	14	0	86	12	0
mEPS-NNV	40	2	58	35	2
mEPS-SEV	34	1	65	29	1
mEPS-NNA	35	4	61	28	4
mEPS-YNA	36	6	58	28	6
mEPS-NNS	37	4	59	30	6
mEPS-YDA	40	8	52	21	8

[a] (columns 2−4) Ratios of H-1 signals of (α1→3)-, (α1→4)-, and (α1→6)-linked Glc*p* residues; (columns 5 and 6) ratios of H-5 signals of -(1→6)-α-D-Glc*p*-(1→3)- and -(1→4)-α-D-Glc*p*-(1→3)- units. Ratios are shown in percentages relative to the total amount of glucose residues. [b] 6G^3 represents -(1→6)-α-D-Glc*p*-(1→3)- units. [c] 4G^3 represents -(1→4)-α-D-Glc*p*-(1→3)- units.

detected. The absence of these $\delta_{H\text{-}3}$ resonances on the (α1→3)- and (α1→4)-anomeric tracks indicates that -(1→3)-α-D-Glc*p*-(1→3)- and -(1→3)-α-D-Glc*p*-(1→4)- units do probably not occur.[21,25] On the (α1→6)-anomeric track in the 120 ms spectrum $\delta_{H\text{-}4}$ 3.51, indicative of -(1→6)-α-D-Glcp-(1→6)- units, is observed, confirming the presence of this structural element.

Q1140H (mEPS-SNAH). Methylation analysis of **mEPS-SNAH** indicated the occurrence of terminal, 3-substituted, 6-substituted, and 3,6-disubstituted glucopyranose residues in a molar percentage of 8, 8, 76, and 8% (Table 2). In the 1D ^{1}H NMR spectrum (Figure 2) 14% (α1→3)-linked glucose ($\delta_{H\text{-}1}$ ∼5.35) and 86% (α1→6)-linked glucose ($\delta_{H\text{-}1}$ ∼4.96) residues were observed (Table 3), which fits with the methylation analysis data. The surface area of $\delta_{H\text{-}5}$ 4.20 (structural reporter for -(1→6)-α-D-Glc*p*-(1→3)- units)[21] corresponded with 12% of all residues in **mEPS-SNAH**.

On the (α1→3)-anomeric track in the 60 ms TOCSY spectrum (Figure 2) $\delta_{H\text{-}4}$ 3.45 was observed, indicating the occurrence of terminal α-D-Glc*p*-(1→3)- units. In the 120 ms TOCSY spectrum the H-5 signal at δ 4.20 is detected, confirming the occurrence of -(1→6)-α-D-Glc*p*-(1→3)- units.[21] The resonance at $\delta_{H\text{-}3}$ 3.90, specific for a -(1→3)-α-D-Glc*p*-(1→3)- unit was not seen on this track, which means that this element probably does not occur.

Table 4. Interpretation of ^{1}H NMR Spectra[a]

	(α1→3) $\delta_{H\text{-}1}$ 5.35				(α1→4) $\delta_{H\text{-}1}$ 5.40			(α1→6) $\delta_{H\text{-}1}$ 4.96			
EPS	G^3 $\delta_{H\text{-}4}$ 3.45	4G^3 $\delta_{H\text{-}3}$ 4.01	4G^3 $\delta_{H\text{-}5}$ 4.12	6G^3 $\delta_{H\text{-}5}$ 4.20	G^4 $\delta_{H\text{-}4}$ 3.41	4G^4 $\delta_{H\text{-}3}$ 3.96	6G^4 $\delta_{H\text{-}4}$ 3.50	G^6 $\delta_{H\text{-}4}$ 3.42	3G^6 $\delta_{H\text{-}3}$ 3.85	4G^6 $\delta_{H\text{-}3}$ 4.01	6G^6 $\delta_{H\text{-}4}$ 3.51
Wt[21]	−	−	−	++	−	−	−	+	+	−	+
PNNS[25]	+	+	++	++	+	+/−	+	+	+	+/−	+
SNS	-	−	−	++	−	−	−	+	+	−	+
SNL	+	−	−	++	−	−	−	+	+	−	+
SDA	+	−	−	++	−	−	−	+	+	−	+
SNAE	+	+	+/−	++	+	−	−	+	+	−	+
SNAA	+	−	−	++	−	−	−	+	+	−	+
SNAH	+	−	−	++	−	−	−	+	+	−	+
NNV	+	−	−	++	+/−	−	−	+	+	−	+
SEV	+	+/−	+/−	++	+	−	−	+	+	−	+
NNA	+	+	++	++	+	−	−	+	+	−	+
YNA	+	+	++	++	+	n.d.	n.d.	+	−	n.d.	n.d.
NNS	+	+	+	++	+	−	−	+	+	−	+
YDA	n.d.	n.d.	++	++	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Structural-reporter-group signals and the building blocks they represent are shown for **EPS180** and each mEPS. Note: (−) indicates that this structural-reporter-group signal is not observed in any of the data collected; (+/−) indicates that the signal is not observed in the spectra shown in the included figure (Figures 1−3; Figures A−I, Supporting Information), but was detected in low intensity in 1D cross-sections; (+) indicates that the cross-peak is clearly observed; (++) indicates that the structural-reporter-group signal was observed and could be quantified by integration in the 1D ^{1}H NMR spectrum; (n.d.) indicates that the presence or absence of the structural-reporter group could not be determined. G^3 represents α-D-Glc*p*-(1→3)- units, 4G^3 stands for -(1→4)-α-D-Glc*p*-(1→3)- units, and so on.

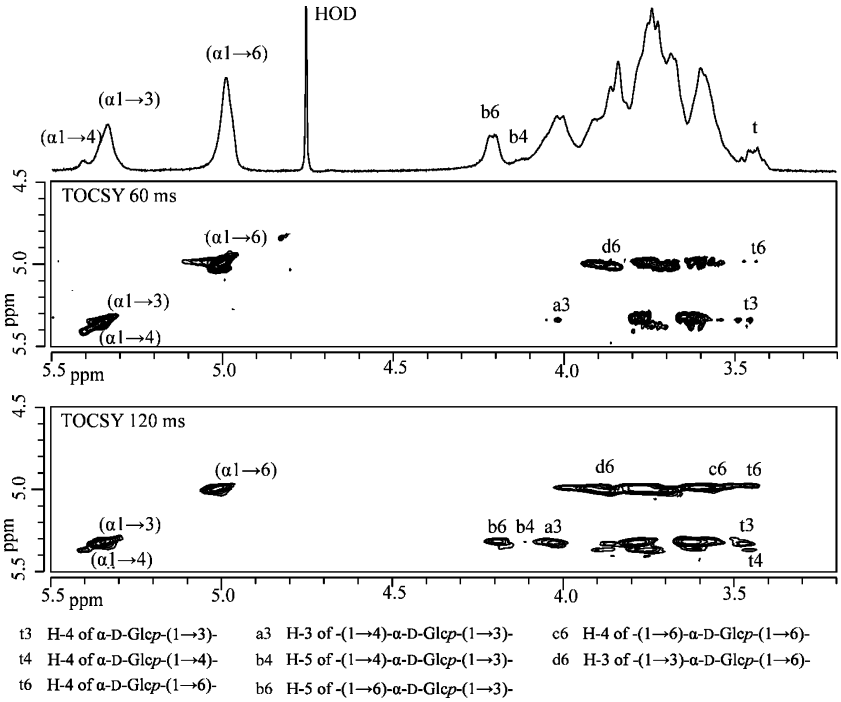


Figure 1. 1D ^{1}H NMR spectrum 500 MHz and 2D ^{1}H−^{1}H TOCSY spectra (top, mixing time 60 ms; bottom, 120 ms) of **mEPS-NNA**, recorded at 300 K in D_2O. Anomeric signals in the TOCSY spectra have been indicated on the diagonal. In the horizontal tracks structural-reporter-group signals have been indicated with labels; legends of the labels are included in the figure.

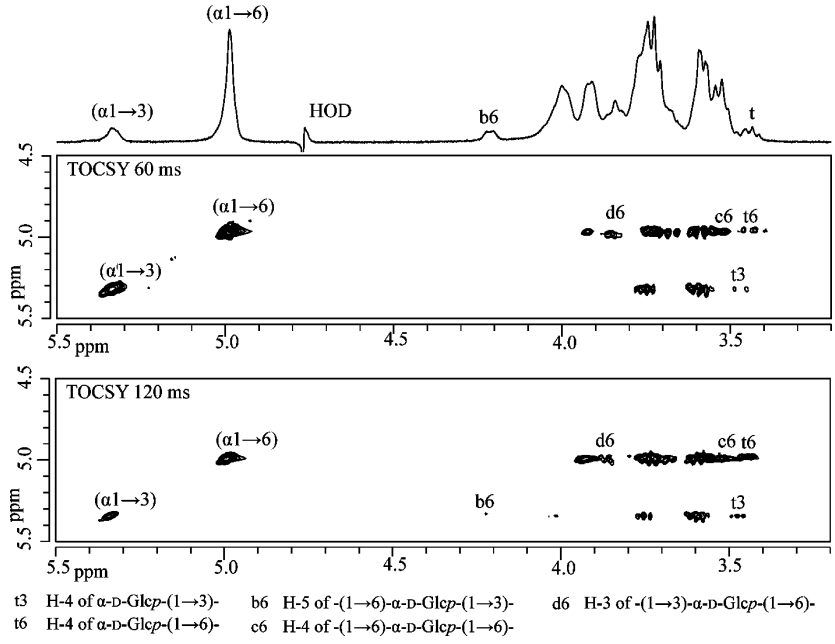


Figure 2. 1D ^{1}H NMR spectrum 500 MHz and 2D ^{1}H−^{1}H TOCSY spectra (top, mixing time 60 ms; bottom, 120 ms) of **mEPS-SNAH**, recorded at 300 K in D_2O. For details of the coding system, see Figure 1.

On the (α1→6)-anomeric track in the 60 ms TOCSY spectrum H-4 signals were observed at δ 3.50 and 3.42, indicating the occurrence of -(1→6)-α-D-Glc*p*-(1→6)- and α-D-Glc*p*-(1→6)- units, respectively. The occurrence of the former was further supported by the observation of $\delta_{H\text{-}5}$ 3.90 on the same track in the 120 ms TOCSY spectrum.[21,24] The H-3 resonance at δ ~3.87 (not marked in the spectrum) revealed the presence of -(1→3,6)-α-D-Glc*p*-(1→6)- and/or -(1→3)-α-D-Glc*p*-(1→6)- units.[21]

A1139L (mEPS-SNL). Methylation analysis of **mEPS-SNL** showed the presence of terminal, 3-substituted, 6-substituted, and 3,6-disubstituted glucopyranose residues in a molar percentage of 14, 23, 47, and 16% (Table 2). Integration of the anomeric peaks in the 1D ^{1}H NMR spectrum (Figure 3) showed 38% (α1→3)-linked glucose ($\delta_{H\text{-}1}$ ~5.35) and 62% (α1→6)-linked glucose ($\delta_{H\text{-}1}$ ~4.96) residues, matching the linkage analysis data (Table 3). A small signal seemed to be present at $\delta_{H\text{-}1}$ 5.40, suggesting the occurrence of (α1→4)-linked glucose residues. However, in the case that this small peak really reflects such residues, the amount is <0.5% of the residues in **mEPS-SNL** (see also the absence in the methylation analysis). Therefore, this observation was neglected for the formulation of the composite model (see Discussion and Conclusions).

On the H-1 track of the (-)α-D-Glc*p*-(1→3)- units in the 60 ms TOCSY spectrum (Figure 3) two H-4 signals were observed at δ 3.51 and 3.45, indicating the occurrence of -(1→6)-α-D-

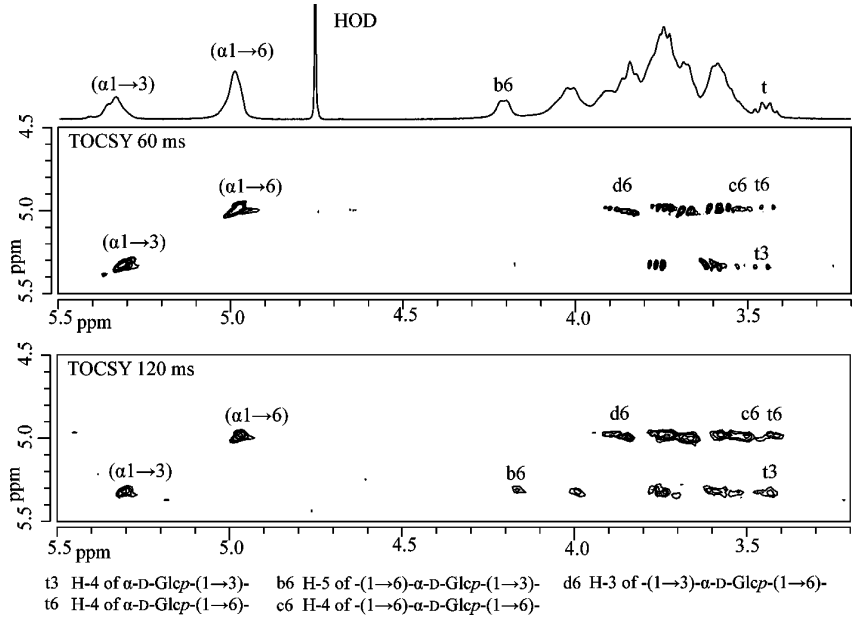


Figure 3. 1D ^{1}H NMR spectrum 500 MHz and 2D ^{1}H−^{1}H TOCSY spectra (top, mixing time 60 ms; bottom, 120 ms) of **mEPS-SNL**, recorded at 300 K in D_2O. For details of the coding system, see Figure 1.

Table 5. Average Molecular Weights (M_w), Number Average Molecular Weights (M_n), and Polydispersity (M_w/M_n) of **EPS180** and mEPSs are Shown, as Determined by HPSEC-MALLS, as wells as Percentages Relative to Wild Type **EPS180** M_w

EPS	M_w (MDa)	M_n (MDa)	M_w/M_n	relative M_w (%)
wild type **EPS180**	32.3	32.0	1.01	100
mEPS-PNNS	9.4	8.1	1.15	29
mEPS-SNS	30.5	29.9	1.02	94
mEPS-SNL	19.3	18.7	1.03	60
mEPS-SNAE	16.2	15.9	1.02	50
mEPS-SNAA	23.9	23.6	1.01	74
mEPS-SEV	21.2	20.8	1.02	66
mEPS-NNA	24.2	23.7	1.02	75
mEPS-YNA	12.5	11.5	1.09	39
mEPS-NNS	20.0	19.7	1.02	62
mEPS-YDA	10.6	9.3	1.14	33

Glc*p*-(1→3)- and α-D-Glc*p*-(1→3)- units, respectively.[21,24] The occurrence of -(1→6)-α-D-Glc*p*-(1→3)- units is further supported by the detection of $\delta_{H\text{-}5}$ 4.20 in the 120 ms TOCSY spectrum on the same track.[21] Integration of the $\delta_{H\text{-}5}$ signal at 4.20 ppm in the 1D ^{1}H NMR spectrum revealed that 31% of all residues are -(1→6)-α-D-Glc*p*-(1→3)- units.

On the (α1→6)-anomeric track in the 60 ms TOCSY spectrum the $\delta_{H\text{-}4}$ signals at 3.50 and 3.42 ppm reflected the occurrence of -(1→6)-α-D-Glc*p*-(1→6)- and α-D-Glc*p*-(1→6)- units. The H-3 signal at δ ~3.87 (not marked in the spectrum) corresponds with the presence of -(1→3)-α-D-Glc*p*-(1→6)- and/or -(1→3,6)-α-D-Glc*p*-(1→6)- units.[21]

Other mEPSs. In a similar fashion as described for **mEPS-NNA**, **mEPS-SNAH**, and **mEPS-SNL**, the other mEPSs were analyzed, and the relevant data are presented in Tables 2−4. The 1D/2D ^{1}H NMR spectra are available as Supporting Information.

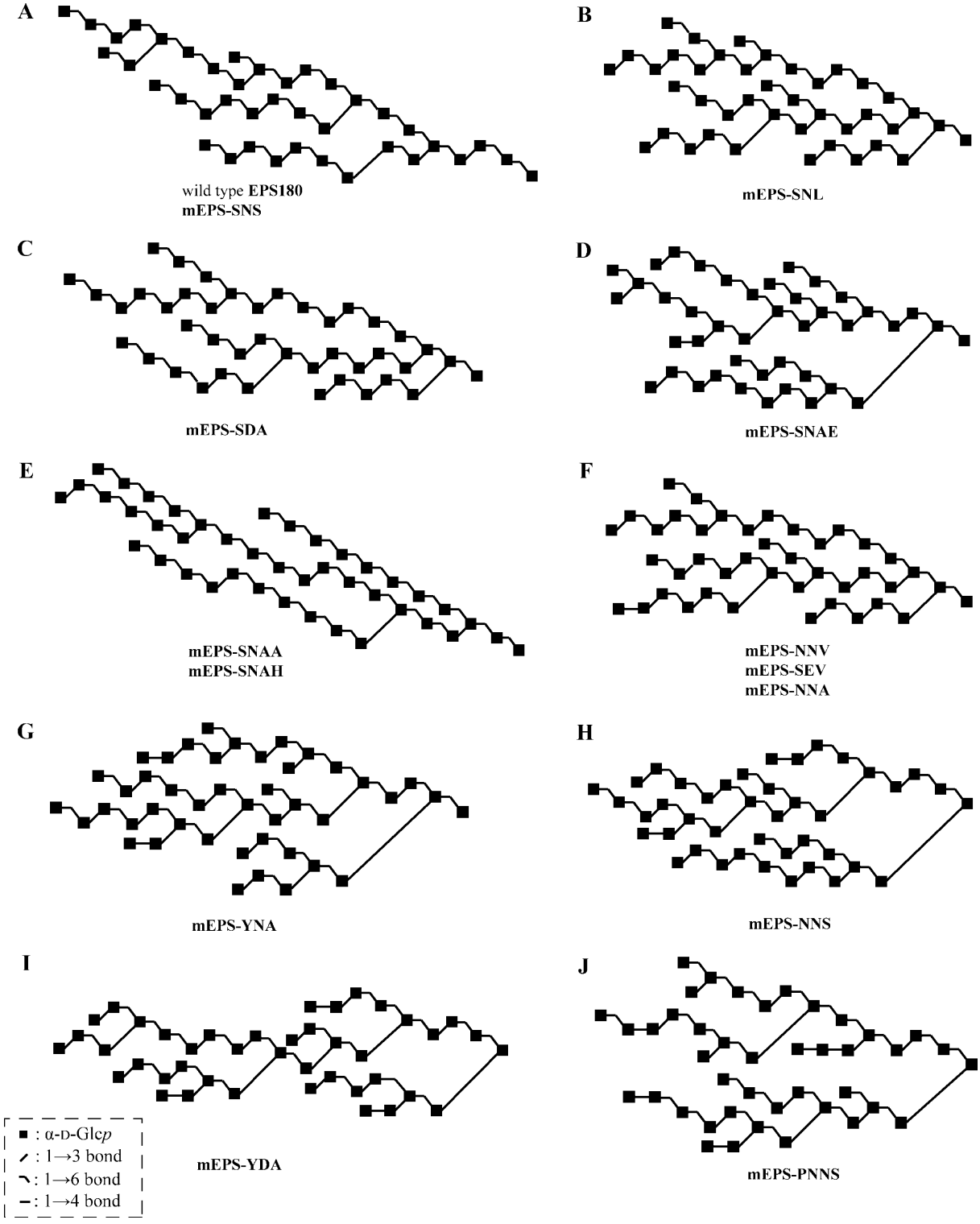
All ^{1}H NMR data of **mEPS-SNS** (Figure A, Supporting Information) match those of wild type **EPS180**.[21] In the 1D ^{1}H NMR spectrum of **mEPS-SDA** (Figure B, Supporting Information) a small signal at $\delta_{H\text{-}1}$ 5.40 was observed, corresponding with the occurrence of ~0.5% of (α1→4)-linked glucose residues. On the (α1→4)-anomeric track in the 60 ms TOCSY spectrum weak H-2 and H-3 signals at δ 3.58 and 3.70, respectively, were observed. These values fit with the occurrence of α-D-Glc*p*-(1→4)- or -(1→6)-α-D-Glc*p*-(1→4)- units.[22,25] Because no further data are available, no distinction between these two types could be made. On the (α1→4)-anomeric track in the 120 ms TOCSY spectrum of **mEPS-SNAE** (Figure C, Supporting Information) a weak $\delta_{H\text{-}5}$ 4.12 was observed, indicating the occurrence of -(1→4)-α-D-Glc*p*-(1→3)- units. In the 1D ^{1}H NMR spectrum this signal is not clear, however, the surface area determined for that region is sufficient to correspond with all (α1→4)-linked residues. For **mEPS-SNAA** (Figure D, Supporting Information), **mEPS-NNV** (Figure E, Supporting Information), and **mEPS-SEV** (Figure F, Supporting Information) no special remarks need to be made. The 1D ^{1}H NMR spectra of saturated solutions of **mEPS-YNA** (Figure G, Supporting Information), **mEPS-NNS** (Figure H, Supporting Information), and **mEPS-YDA** (Figure I, Supporting Information) showed a low signal-to-noise ratio, due to the lower solubility of these mEPSs. For **mEPS-YNA** and **mEPS-NNS** TOCSY spectra could be recorded with mixing times up to 60 ms. For **mEPS-YDA** only a 1D ^{1}H NMR spectrum could be recorded.

Smith Degradation. Previous studies of wild type **EPS180** and **mEPS-PNNS** revealed that only single (α1→3) bridges occurred in these polysaccharides.[21,25] Because **mEPS-YDA** represented one of the highest amounts of (α1→3)-linked glucose residues (43%), it is one of the most likely mEPSs to deviate from this structural limitation. Therefore, **mEPS-YDA** was subjected to Smith degradation to investigate the degree of polymerization of (α1→3) glycosidic bonds. Possible products include α-D-Glc*p*-(1→1)-Gro, [α-D-Glc*p*-(1→3)-]$_n$α-D-Glc*p*-(1→1)-Gro, and, due to overhydrolysis, Gro, D-Glc, and [α-D-Glc*p*-(1→3)-]$_n$D-Glc.

HPAEC analysis on CarboPac PA-100 (data not shown) of the residue revealed two major peaks that were identified as Gro (R_t 2.3 min) and α-D-Glc*p*-(1→1)-Gro (R_t 6.2 min).[21,25] Because α-D-Glc*p*-(1→3)-D-Glc*p* under the same conditions has an R_t value >10 min, the presence of [α-D-Glc*p*-(1→3)-]$_n$α-D-Glc*p*-(1→1)-Gro with $n = 1$ or higher could be excluded.[21,25]

The absence of larger structures than glucosyl-glycerol indicates that the **mEPS-YDA** structure does not contain two or more consecutive (α1→3) linkages. Because two mEPSs with

Scheme 1. Composite Models that Include All Identified Structural Features of (A) Wild Type **EPS180**[21] and **mEPS-SNS**, (B) **mEPS-SNL**, (C) **mEPS-SDA**, (D) **mEPS-SNAE**, (E) **mEPS-SNAA** and **mEPS-SNAH**, (F) **mEPS-NNV**, **mEPS-SEV** and **mEPS-NNA**, (G) **mEPS-YNA**, (H) **mEPS-NNS**, (I) **mEPS-YDA**, and (J) **mEPS-PNNS**[25]



significant changes in structure (i.e., **mEPS-YDA** and **mEPS-PNNS**[25]) show this feature, similar to the wild type **EPS180**,[21] it is probable that this holds true for all mEPS structures.

Solubility of mEPSs. Different solubilities were observed between the different polysaccharides analyzed. For instance, wild type **EPS180** could be effortlessly dissolved in 1−2 mg/mL concentrations, and at length in ∼10 mg/mL concentrations by vortexing and heating; samples of **mEPS-SNAA**, **mEPS-SNAE**, **mEPS-SNAH**, and **mEPS-NNV** could be dissolved readily in ∼10 mg/mL concentrations; samples of **mEPS-YNA** and **mEPS-PNNS** required effort and mild heating to dissolve at 1−2 mg/mL concentrations, and **mEPS-YDA** left an undissolved residue even when 0.1 mg was taken up in 1 mL of water.

Average Molecular Weights. Using HPSEC-MALLS, with standard mixtures of linear dextrans and pullulans for calibration, the size of a selection of the mEPSs was determined. The wild type **EPS180** was taken as a reference, set to an average

Table 6. Percentages of the Different Building Blocks Determined by Combining All Structural Data for the Wild Type **EPS180** and the Various mEPSs[a]

EPS	G^3	$4G^3$	$6G^3$	G^4	$4G^4$	$6G^4$	G^6	$3G^6$	$4G^6$	$6G^6$	$3,6G^6$
Wt[21]	0	0	36	0	0	0	12	24	0	16	12
PNNS[25]	4	7	17	6	3	3	8	10	2	22	18
SNS	0	1	31	1	0	0	12	24	0	19	13
SNL	7	0	31	0	0	0	8	23	0	16	15
SDA	3	1	31	1	0	0	6	25	0	23	10
SNAE	6	2	24	2	0	0	10	14	0	24	18
SNAA	6	0	12	0	0	0	6	6	0	58	12
SNAH	3	0	13	0	0	0	5	8	0	63	8
NNV	4	2	33	2	0	0	5	28	0	15	11
SEV	5	1	32	1	0	0	8	24	0	15	14
NNA	3	3	32	3	0	0	6	26	0	15	12
YNA	6	4	29	4	0	0	8	21	0	10	18
NNS	4	4	28	4	0	0	7	21	0	17	15
YDA	11	8	21	8	0	0	1	22	0	9	20

[a] Note: G^3 represents α-D-Glc*p*-(1→3)- units, $4G^3$ represents -(1→4)-α-D-Glc*p*-(1→3)- units, and so on.

molecular weight of 100%, and the average molecular weights of the mEPSs were expressed in percentages of that of the reference (Table 5).

The wild type **EPS180** and **mEPS-SNS**, having similar methylation and ^{1}H NMR analysis data, have also comparable average molecular weights. The average molecular weights of **mEPS-SNAA** and **mEPS-NNA** are decreased to 74 and 75%, respectively. The readily soluble **mEPS-SNL** and **mEPS-SEV** and the poorly soluble **mEPS-NNS** have similar average molecular weights at 60, 66, and 62%, respectively, of that of **EPS180**. The highly soluble **mEPS-SNAE** polysaccharide has an average molecular weight of 50% of that of **EPS180**. Finally, the mEPSs with the most reduced average molecular weight are the very poorly soluble **mEPS-YNA**, **mEPS-YDA**, and **mEPS-PNNS**, with 39, 33, and 29%, respectively, of that of **EPS180**. Whereas most mEPSs have a low polydispersity index, **mEPS-YNA**, **mEPS-YDA**, and **mEPS-PNNS** have significantly higher polydispersity indices at 1.09, 1.14, and 1.15, respectively.

Although the data for **mEPS-YNA**, **mEPS-YDA**, and **mEPS-PNNS** suggest a correlation between solubility and average molecular weight, the relatively low molecular weight (50%) for the highly soluble **mEPS-SNAE** and the relatively high molecular weight (62%) for the poorly soluble **mEPS-NNS** indicate that no clear conclusions can be made yet.

Discussion and Conclusions

Discussion. As described recently,[24] based on the ^{1}H/^{13}C NMR data of a series of model α-D-gluco-oligosaccharides, an ^{1}H NMR structural-reporter-group concept could be developed that was used in the characterization of fragments derived from bacterial α-D-glucans produced by native (GTF180,[21] GTFA[22]) and mutant (triple mutant of GTF180[25]) glucansucrase enzymes from *L. reuteri* strains. The developed and further expanded concept turned out to be also applicable on the polysaccharide level, and composite models, that include all identified structural features, were formulated for **EPS180**,[21] **EPS35-5**,[22] and **mEPS-PNNS**,[25] whereby the methylation analysis of each of the EPSs dictate the structural boundaries of the models. Making use of the established building blocks as generated by the structural-reporter-group ^{1}H NMR library, and taking into account the composite models of **EPS180** and **mEPS-PNNS**, Scheme 1 surveys composite models for the 12 α-D-glucan mEPSs produced by the different mutant glucansucrase GTF180 enzymes, which are based on the quantification of the different building blocks present, as shown in Table 6.

Starting from the integrations of the anomeric signals in the 1D ^{1}H NMR spectra, the amounts of (α1→3)-linked, (α1→4)-linked, and (α1→6)-linked residues are found (Table 3). Also the amounts of -(1→6)-α-D-Glc*p*-(1→3)- and -(1→4)-α-D-Glc*p*-(1→3)- units can be deduced from the 1D ^{1}H NMR spectra via the integration of their respective H-5 signals (Table 3). Because two consecutive (α1→3) linkages do not occur in any of the mEPSs, as evidenced from TOCSY measurements and Smith degradation analysis, there are only three types of (α1→3)-linked glucose residues possible, that is, the two internal units mentioned above and terminal α-D-Glc*p*-(1→3)- units. Therefore, the amount of α-D-Glc*p*-(1→3)- can be determined from the difference in surface area between the (α1→3) H-1 signal at δ ~5.35 and the H-5 signals at δ 4.20 and 4.12.

To determine the amounts of α-D-Glc*p*-(1→4)- and α-D-Glc*p*-(1→6)- units in the mEPSs, use was made of the methylation analysis data and the $\delta_{H\text{-}4}$ signals at 3.41−3.45 ppm on the (α1→3)-, (α1→4)-, and (α1→6)-anomeric tracks in the TOCSY spectra. The total amount of terminal residues follows directly from the methylation analysis (Table 2). Evaluation of the $\delta_{H\text{-}4}$ signals showed that all (α1→4)-linked glucose residues are, with the exception of **mEPS-PNNS** (Scheme 1J),[25] in fact terminal residues; and they occur only in α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)- sequences. Having determined the amounts of terminal α-D-Glc*p*-(1→3)- and α-D-Glc*p*-(1→4)- units, the amounts of terminal α-D-Glc*p*-(1→6)- units can be calculated via subtraction from the total amount of terminal residues.

As revealed by methylation analysis, all mEPSs contain only 3,6-branch points. The amount of the 3,6-branching (and terminal) residues follows directly from the integration of the GLC peaks. The 2D NMR spectra showed that none of the (-)α-D-Glc*p*-(1→4)- units was 3-substituted, which means that all 3,6-disubstituted residues are in fact -(1→3,6)-α-D-Glc*p*-(1→6)- units. In a similar fashion the amount of 3-substituted residues is obtained from the methylation analysis data, and for the reasons mentioned above all 3-substituted residues are in fact -(1→3)-α-D-Glc*p*-(1→6)- units.

These data leave only one type of building block for the mEPSs, except for **mEPS-YDA** and **mEPS-SDA**, undetermined, that is, the -(1→6)-α-D-Glc*p*-(1→6)- units. The latter can be deduced from the relative percentages of all the other building blocks, and verified by the difference between the total amount of 6-substituted glucose residues (methylation analysis) and the amount of -(1→6)-α-D-Glc*p*-(1→3)- units ($\delta_{H\text{-}5}$ 4.20 surface area in the 1D ^{1}H NMR spectrum).

Where the previously described **mEPS-PNNS** contained 11 different building blocks,[25] the 12 mEPSs described here contain between five and eight different building blocks. In the case of **mEPS-SDA** it was not possible to determine whether the (-)α-D-Glc*p*-(1→4)- units were situated terminally or as internal -(1→6)-α-D-Glc*p*-(1→4)- units as well. Because all other similar mEPSs that contain (α1→4)-linked glucose residues showed only terminal α-D-Glc*p*-(1→4)- residues, it seems likely that this is also the case for **mEPS-SDA**, therefore, the data shown in Table 6 assumes only terminal α-D-Glc*p*-(1→4)- units to occur (not included in Scheme 1). For **mEPS-YDA** it was most difficult determining the existing building blocks, because 2D NMR spectroscopy was not possible due to the particularly low solubility of **mEPS-YDA**. For the latter polysaccharide it is possible that all (α1→4)-linked residues are terminal, however, it could also resemble the **mEPS-PNNS** structure,[25] where some -(1→6)-α-D-Glc*p*-(1→4)- units occur, but also -(1→4)-α-D-Glc*p*-(1→6)- units are found. Because all (α1→4)-linked glucoses in **mEPS-YDA** are situated in (-)α-D-Glc*p*-(1→4)-α-D-Glc*p*-

(1→3)- sequences (Table 3), it seems likely that **mEPS-YDA** resembles the other mEPSs, leading to a structure with almost no terminal α-D-Glc*p*-(1→6)- units (Scheme 1).

Conclusions. Analysis of the EPS products (mEPSs) of mutant GTF180 enzymes revealed that minor amino acid modifications in a specific protein region can have large effects on the structure of the glucan product (see also ref 36). The amount of branching could be varied from 8% in **mEPS-SNAH** to 20% in **mEPS-YDA**. The amount of (α1→6)-linked glucose residues varied from 52% in **mEPS-YDA** to 86% in **mEPS-SNAH**, while the amount of (α1→3)-linked glucose residues varied from 16% in **mEPS-SNAH** to 43% in **mEPS-YDA**. The amount of (α1→4)-linked glucose residues (not present in wild type **EPS180**,[21] and 12% in **mEPS-PNNS**[25]) occurred up to 8% in **mEPS-YDA**. Some mEPSs were difficult to dissolve (**mEPS-YDA**, **mEPS-YNA**, and **mEPS-NNS**), resulting in difficult recording of NMR spectra. All the mEPSs showed the presence of a mix of terminal α-D-Glc*p*-(1→3)-, α-D-Glc*p*-(1→6)-, and α-D-Glc*p*-(1→4)- units, while the wild type **EPS180** contained only terminal α-D-Glc*p*-(1→6)- residues. Despite all the structural changes in the mutant EPSs, one structural element of the original **EPS180** remained: all mEPSs appear to contain single (α1→3) bridges.

The presented results show that a wide range of products, with differences in linkage distribution, average molecular weight, and solubility, can be made through minor modifications in a single glucansucrase enzyme.

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Supporting Information Available. 1D ^{1}H NMR spectra and the relevant parts of the 2D ^{1}H−^{1}H TOCSY spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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