Structural analysis of bioengineered alpha-D-glucan produced by a triple mutant of the glucansucrase GTF180 enzyme from Lactobacillus reuteri strain 180
van Leeuwen, Sander S.; Kralj, Slavko; Gerwig, Gerrit J.; Dijkhuizen, Lubbert; Kamerling, Johannis P.

Published in:
Biomacromolecules

DOI:
10.1021/bm800410w

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2008

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Structural Analysis of Bioengineered α-D-Glucan Produced by a Triple Mutant of the Glucansucrase GTF180 Enzyme from Lactobacillus reuteri Strain 180: Generation of (α1→4) Linkages in a Native (1→3)(1→6)-α-D-Glucan

Sander S. van Leeuwen,† Slavko Kralj,‡,§ Gerrit J. Gerwig,† Lubbert Dijkhuizen,‡,§ and Johannis P. Kamerling*†

Department of Bio-Organic Chemistry, Bijvoet Center, Utrecht University, 3584 CH Utrecht, The Netherlands, Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9751 NN Haren, The Netherlands, and Centre for Carbohydrate Bioprocessing, TNO - University of Groningen, 9750 AA Haren, The Netherlands

Received April 16, 2008; Revised Manuscript Received May 15, 2008

Site-directed mutagenesis of the glucansucrase gtf180 gene from Lactobacillus reuteri strain 180 was used to transform the active site region. The α-D-glucan (mEPS-PNNS) produced by the triple mutant V1027P:S1137N:A1139S differed in structure from that of the wild-type α-D-glucan (EPS180). Besides (α1→3) and (α1→6) linkages, as present in EPS180, mEPS-PNNS also contained (α1→4) linkages. Linkage analysis, periodate oxidation, and 1D/2D 1H NMR spectroscopy of the intact mEPS-PNNS, as well as MS and NMR analysis of oligosaccharides obtained by partial acid hydrolysis of mEPS-PNNS afforded a composite model, which includes all identified structural features.

Introduction

Lactic acid bacteria (LAB), like Lactobacilli, excrete exopolysaccharides (EPSs) into their surroundings. Exopolysaccharides have been found as adhesives,1 participants in certain cellular recognition processes,2 and as slime forming agents for protection against dehydration, phagocytosis, or toxins.3 The physical properties that are important for their in vivo functions also make these polysaccharides suitable for the food and dairy industry. Bacterial exopolysaccharides have been used as thickeners, stabilizers, and gelling agents.3 To improve these properties, attention has been paid to the engineering of polysaccharide structures via chemical4 or enzymatic derivatizations5,6 and by genetic modification of source micro-organisms.7,8

Recently, a family of glucansucrases was discovered in Lactobacillus reuteri, which converts sucrose into large, heavily branched α-D-glucans. Structural analysis of the homopolysaccharides produced by the glucansucrases GTF180 and GTFA revealed highly complex structures, and composite models have been proposed.10,11 Previous studies on the site-directed mutagenesis near the catalytic Asp1133 (putative transition state stabilizing residue) of GTFA have shown that specific amino acid mutations in this glucansucrase gave rise to large changes in linkage distribution compared to the wild-type polysaccharide (EPS180). Besides the native (α1→3) and (α1→6) linkages, as present in EPS180, a significant amount of (α1→4) linkages is introduced by this mutant enzyme. Here, we report a detailed structural analysis of mEPS-PNNS, identifying the structural elements and their quantities, and finally postulating a composite model, that includes all identified structural features.

Experimental Section

Mutant Construction, Enzyme Expression, Purification, and Glucan Synthesis. The triple mutant V1027P:S1137N:A1139S was generated using The QuickChange site-directed mutagenesis kit (Stratagene, LaJolla, CA) and appropriate primer pairs to introduce mutations in pIGTF180-AN.13 After successful mutagenesis (confirmed by nucleotide sequencing), GTF180-AN (V1027P:S1137N:A1139S) was overexpressed in E. coli BL21star (DE3) and the bioengineered polysaccharide mEPS-PNNS was produced by incubation of the His-tag purified enzyme with 146 mM sucrose, containing 1% Tween 80 and 0.02% sodium azide for 7 days.14 The mEPS-PNNS produced was isolated by precipitation with ethanol, as described previously.15

Methylation Analysis. Polysaccharide samples were permethylated using CH3I and solid NaOH in DMSO, as described earlier.16 After hydrolysis with 2 M TFA (2 h, 120 °C), partially methylated monosaccharide mixtures were reduced with NaBD4 (2 h, room temperature). Conventional workup involving neutralization with HOAc and removal of boric acid by coevaporation with MeOH, followed by acetylation with acetic anhydride-pyridine (1:1 v/v, 3 h, 120 °C), yielded mixtures of partially methylated alditol acetates, which were analyzed by GLC-EI-MS.17,18

Partial Acid Hydrolysis. A sample of mEPS-PNNS (800 mg) was treated with 0.5 M TFA (2 mL) for 30 min at 90 °C. After centrifugation (1500 g, 5 min), the supernatant was collected, and the pellet was treated again with 0.5 M TFA under the same conditions. This procedure was repeated 10 times. Each supernatant was investigated by 1D 1H NMR spectroscopy. Subsequently, the supernatant samples were pooled, profiled on CarboPac PA-100 and separated on Bio-Gel P-2 (400 × 15 mm, BioRad), eluted with 25 mM NH4HCO3; 1.2 mL fractions were collected at a flow rate of 11.5 mL/h. Fractions were tested for the
presence of carbohydrates by a TLC spot-test with orcinol-H$_2$SO$_4$ staining. Carbohydrate-containing fractions were analyzed by MALDITOF-MS.

**Smith Degradation.** A sample of mEPS-PNNS (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 0.2 mL of ethylene glycol. The oxidized polysaccharide solution was dialysed against tap water (24 h, room temperature), treated with excess NaBH$_4$ (18 h, room temperature), and subsequently neutralized with 4 M HOAc. 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 GLC-EI-MS and 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. Analytical separations were performed on a CarboPac PA-100 column (250 × 4 mm, Dionex), using a linear gradient of 0–300 mM NaOAc in 100 mM NaOH (1 mL/min). Samples were fractionated on a CarboPac PA-100 column (250 × 9 mm, Dionex), using a linear gradient of 0–300 mM NaOAc in 100 mM NaOH (4 mL/min) or isocratic conditions of 100 mM NaOAc in 100 mM NaOH (4 mL/min). Collected fractions were immediately neutralized with 4 M HOAc, desalted on CarboGraph SPE columns (150 mg graphitized carbon, Alltech) using acetonitrile, lyophilized.

**Mass Spectrometry.** GLC-EI-MS was performed on a Fisons Instruments GC 8060/ MD 800 system (Interscience BV; Breda, The Netherlands) using acetonitrile and 4 M HOAc, desalted on CarboPac PA-100, yielding fractions containing mostly fragments with DP4 in the dark. Then the excess of periodate was destroyed by addition of 0.2 mL of ethylene glycol. The oxidized polysaccharide solution was dialysed against tap water (24 h, room temperature), treated with excess NaBH$_4$ (18 h, room temperature), and subsequently neutralized with 4 M HOAc. 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 GLC-EI-MS and HPAEC-PAD.

**Composition of mEPS-PNNS.** Methylation analysis of mEPS-PNNS showed the presence of terminal, 3-, 4-, and 6-substituted, and 3,6-disubstituted glucopyranose in a molar percentage of 18, 10, 12, 42, and 18% (Table 1). 1D $^1$H NMR spectroscopy of intact mEPS-PNNS (Figure 1A) indicated that the proton patterns all resembled that of intact mEPS-PNNS. Integration of the anomic signals revealed 60% (α(1→6)-linked D-Glc (δ$_{H-1}$ ~4.96)), 28% (α(1→3)-linked D-Glc (δ$_{H-1}$ ~5.33)), and 12% (α(1→4)-linked D-Glc (δ$_{H-1}$ ~3.59) residues, which is in agreement with the overall substitution pattern found by methylation analysis. It should be noted that the low solubility of mEPS-PNNS influenced the quality of the 1D $^1$H NMR spectrum, whereas the broader peaks, compared to the 1D $^1$H NMR spectrum of wild-type EPS180, may be the result of a more structural diversity.

**Partial Acid Hydrolysis.** A sample of mEPS-PNNS (800 mg) was incubated 10 times with 0.5 M TFA (30 min, 90 °C), with intermediate centrifugation and collection of supernatant. Analysis of the 10 supernatant samples by 1D $^1$H NMR spectroscopy showed that the proton patterns all resembled that of intact mEPS-PNNS (Figure 1A), suggesting that all the structural elements present in mEPS-PNNS are also present in the hydrolsates. In each case, the linkage distribution, as determined from the 1D $^1$H NMR spectra of the 10 batches, amounts to 59% (α(1→6) linkages, 28% (α(1→3) linkages, and 13% (α(1→4) linkages. This indicates that, in contrast with wild-type EPS180, all three linkage types are equally susceptible to acid hydrolysis under the conditions selected. For further investigations, the 10 batches were pooled.

HPAEC profiling of the pool on CarboPac PA-100 using a linear gradient showed free glucose (fraction I) as the major component (Figure 2A). To obtain suitable fractions for further analysis, a prefractionation was performed on Bio-Gel P-2, yielding 16 overlapping fractions with a broad distribution of fragment sizes (data not shown). On the guidance of MALDI-TOF-MS analysis, the fractions were combined in four subpools as follows: fractions containing fragments with DP ≤ 3 were combined in subpool I, fractions containing mostly fragments with DP4–DP7 in subpool II, fractions containing mostly fragments with DP8–DP11 in subpool III, and fractions containing fragments with DP > 11 in subpool IV. Using a linear gradient subpools I and II were further separated on CarboPac PA-100, yielding fractions 1 to 6 and 7 to 11, respectively (Figure 2B). Subpools III and IV, containing fragments too large for a complete $^1$H NMR analysis were not separated.

**Fraction 2.** The retention time of fraction 2 on CarboPac PA-100, as well as the 1D $^1$H NMR spectrum (data not shown) were in agreement with the presence of isomaltose, that is, α-D-Glc(1→6)-D-Glc (Scheme 1). A sample of mEPS-PNNS (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 0.2 mL of ethylene glycol. The oxidized polysaccharide solution was dialysed against tap water (24 h, room temperature), treated with excess NaBH$_4$ (18 h, room temperature), and subsequently neutralized with 4 M HOAc. 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 GLC-EI-MS and HPAEC-PAD.

**Results**

Table 1. Methylation Analysis Data of mEPS-PNNS Compared with Wild-Type EPS180

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>mEPS-PNNS</th>
<th>EPS180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glcp(1→6)</td>
<td>18 ± 1.3$^a$</td>
<td>12 ± 0.8</td>
</tr>
<tr>
<td>Glcp(1→3)</td>
<td>10 ± 1.5</td>
<td>24 ± 0.6</td>
</tr>
<tr>
<td>Glcp(1→4)</td>
<td>12 ± 1.3</td>
<td>12 ± 1.3</td>
</tr>
<tr>
<td>Glcp(1→2)</td>
<td>42 ± 0.8</td>
<td>52 ± 1.3</td>
</tr>
<tr>
<td>Glcp(1→3,6)</td>
<td>18 ± 1.0</td>
<td>12 ± 1.0</td>
</tr>
</tbody>
</table>

$^a$ Substitution pattern data are shown in molar percentages based on GLC intensities. $^b$ Standard deviations have been calculated from multiple methylation analyses of mEPS-PNNS and EPS180 batches. Multiple batches of these EPSs were produced by using mutant and wild-type enzymes, stemming from two separate gene expressions per enzyme.

**Fraction 3.** The MALDI-TOF mass spectrum of fraction 3 revealed an [M + Na]$^+$ pseudomolecular ion at m/z 527, corresponding with Hex$_3$. The 1D $^1$H NMR spectrum (data not shown) matched that of isomaltotriose, that is, α-D-Glc(1→6)-α-D-Glc(1→6)-D-Glc (Scheme 1).
Fraction 4. MALDI-TOF-MS analysis of fraction 4 showed an [M + Na]+ pseudomolecular ion at m/z 527, corresponding with Hex3. However, the separation profile on CarboPac PA-100 (Figure 2B) showed two overlapping peaks. Therefore, fraction 4 was further separated on CarboPac PA-100 under isocratic conditions (Figure 2C), and fractions 4a and 4b were isolated. The 1D 1H NMR spectrum of compound 4a (data not shown) corresponded with that of maltose, that is, R-D-Glc-(1→4)-D-Glc. The 1D 1H NMR spectrum of compound 4b (data not shown), corresponding with Hex3 (MALDI-TOF-MS), indicated the presence of panose, that is, R-D-Glc-(1→6)-R-D-Glc-(1→4)-D-Glc (Scheme 1). The presence of R-D-Glc-(1→6)-D-Glc unit in a -(1→3)-α-D-Glc-(1→6)-D-Glc sequence (library data: -(1→3)-α-D-Glc-(1→6)-D-Glc, δ 5.246–5.249 and 4.676–4.680; -(1→4/6)-α-D-Glc-(1→6)-D-Glc, δ 5.240–5.241 and 4.667–4.672). The splitting of A H-1 is due to the influence of the α/β configuration of the reducing residue R. The B H-1 signal at δ

Figure 1. 1D 1H NMR (500 MHz) spectra of (A) mEPS-PNNS and (B) mEPS-PNNS subpool IV, recorded at 300 K in D2O.

Figure 2. (A) HPAEC profile of mEPS-PNNS partial acid hydrolysate on CarboPac PA-100, using a linear gradient; (B) HPAEC profiles of Bio-Gel P-2 subpools I and II on CarboPac PA-100, using a linear gradient; and (C) HPAEC profiles of HPAEC fractions 4, 5, and 8–11 on CarboPac PA-100, using isocratic conditions. For experimental details, see Experimental Section.
Scheme 1. Structures of Oligosaccharide Fragments Obtained by Partial Acid Hydrolysis of mEPS-PNNS

The 1D $^1$H NMR spectrum of Hex$_4$ 9a (Figure 4) showed six anemic signals at $\delta$ 5.535 (B$_9$ H-1, $^3$J$_{1,2}$ 3.8 Hz), 5.343 (B$_9$ H-1, $^3$J$_{1,2}$ 3.8 Hz), 5.249 (Ra H-1, $^3$J$_{1,2}$ 3.8 Hz), 4.975 (E H-1, $^3$J$_{1,2}$ 3.8 Hz), 4.965 (D H-1, $^3$J$_{1,2}$ 3.8 Hz), and 4.683 (Rf H-1, $^3$J$_{1,2}$ 7.6 Hz). 2D $^1$H–$^1$H COSY measurements (Figure 4/60 ms) delivered most of the $\delta$ values of the nonanumeric protons (Table 2). The chemical shift positions of Ra and Rf H-1 at $\delta$ 5.249 and 4.683, respectively, correspond with the occurrence of a (1→6)-d-GlcP unit in a (1→3)-α-d-GlcP-(1→6)-d-GlcP sequence. This is further corroborated by the Rf H-2 signal at $\delta$ 3.258, indicating that residue R is not 3- or 4-substituted. The split B H-1 signal at $\delta$ 5.353/5.343 fits best with the occurrence of an (-α-d-GlcP-(1→3)- unit. The set of B H-4 and H-5 at $\delta$ 3.45 and 4.00, respectively, identified residue B as a terminal α-d-GlcP-(1→3)-unit (see nigerose residue B and nigerotriose residue C in ref 22). Residue D H-1 at $\delta$ 4.965 is indicative of an (-α-d-GlcP-(1→6)- unit, and together with D H-4 at $\delta$ 3.43, a terminal α-d-GlcP-(1→6)-unit is demonstrated. The occurrence of two terminal units indicates a branched structure for 9a. Finally, the E H-1 signal at $\delta$ 4.975 revealed the occurrence of an (-α-d-GlcP-(1→6)- unit. Because residue R is not 3-substituted, the remaining internal residue E has to be a 3,6-disubstituted unit, yielding the sequence of 9a: B$_1$-[3-D]E$_1$-6R, that is, α-d-GlcP-([1→3]-α-d-GlcP-([1→6]-[α-d-GlcP-([1→6]-d-GlcP (Scheme 1).

Figure 10. MALDI-TOF-MS analysis of fraction 10 revealed an [M + Na$^+$] pseudomolecular ion at $m/z$ 1013, corresponding with Hex$_6$. The 1D $^1$H NMR spectrum (Figure 5) showed anemic signals at $\delta$ 5.36 (B$_9$ H-1), 5.34 (B$_9$ H-1), 5.246 (Ra H-1), 4.96–4.98 (C$_{ll}$H-1, D H-1, E H-1), and 4.679 (Rf H-1, $^3$J$_{1,2}$ 7.8 Hz). The chemical shift values of the nonanumeric protons (Table 2) were obtained from 2D $^1$H–$^1$H COSY measurements (Figure 5/60 ms). The Ra and Rf H-1 $\delta$ values at 5.246 and 4.679 ppm, respectively, correspond with the presence of a (1→6)-d-GlcP unit in a (1→3)-α-d-GlcP-(1→6)-d-GlcP sequence. The Rf H-2 signal at $\delta$ 3.259, indicating that residue R is not 3- or 4-substituted (library data: Rf in nigerose $\delta_{1,2}$ 3.32 and Rf in maltose $\delta_{1,2}$ 3.272), supports this conclusion. The set of B H-1, H-2, H-3, and H-4 values corresponds with that of 9a, revealing the occurrence of a terminal α-d-GlcP-([1→3]-) unit. The presence of the set of D H-1 and H-4 at $\delta$ ~4.97 and 3.42, respectively, indicates the presence of a terminal α-d-GlcP-([1→6]-) unit. The presence of two terminal units implicates a branched structure for compound 10 (Hex$_6$). Because the H-1 signal at $\delta$ 4.96–4.98, typical for (-α-d-GlcP-(1→6)- residues, corresponds to four protons and residue R is not 3-substituted, the structure has to contain an internal 3,6-disubstituted residue E (compare with 9a). Then, the remaining two residues can only be internal -α-d-GlcP-(1→6)– units, denoted residue C$^1$ and C$^2$. Because the B H-1 signal is split, due to the influence of the reducing residue α/β configuration, the most probable sequence for compound 10 is D$_1$-[6C$^1$]-1→6C$^1$-[6]-[B$_1$]-3E$_1$-6R, that is, α-d-GlcP-(1→6)-α-d-GlcP-(1→6)-α-d-GlcP-(1→3)-α-d-GlcP-(1→6)-d-GlcP (Scheme 1).

Fraction 11. MALDI-TOF-MS analysis of fraction 11 showed [M + Na$^+$] pseudomolecular ions at $m/z$ 851 and 1013, corresponding with Hex$_5$ and Hex$_5$, respectively. Fraction 11 was further separated on CarboPac PA-100, under isocratic conditions (Figure 2C), yielding one major fraction 11a (Hex$_5$, MALDI-TOF-MS).
Figure 3. 1D $^1$H NMR spectra (500 MHz) of (A) fraction 5a and (B) fraction 11a, recorded at 300 K in D$_2$O. Structural-reporter-group signals that could be distinguished are indicated in the spectra. Labels correspond with those used in Table 2 and Scheme 1.

Table 2. $^1$H Chemical Shifts of $\alpha$-Glycopyranose Residues of Oligosaccharide Fragments Obtained by Partial Acid Hydrolysis of mEPS-PNNS$^a$

<table>
<thead>
<tr>
<th>residue</th>
<th>8a</th>
<th>9a</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra-1</td>
<td>5.249</td>
<td>5.249</td>
<td>5.246</td>
</tr>
<tr>
<td>Ra-2</td>
<td>3.54</td>
<td>3.54</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ra-3</td>
<td>3.71</td>
<td>3.71</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ra-4</td>
<td>3.51</td>
<td>3.52</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ra-5</td>
<td>4.00</td>
<td>4.01</td>
<td>n.d.</td>
</tr>
<tr>
<td>R/B-1</td>
<td>4.680</td>
<td>4.683</td>
<td>4.679</td>
</tr>
<tr>
<td>R/B-2</td>
<td>3.258</td>
<td>3.258</td>
<td>3.259</td>
</tr>
<tr>
<td>R/B-3</td>
<td>3.49</td>
<td>3.49</td>
<td>n.d.</td>
</tr>
<tr>
<td>R/B-4</td>
<td>3.52</td>
<td>3.51</td>
<td>n.d.</td>
</tr>
<tr>
<td>R/B-5</td>
<td>3.65</td>
<td>3.65</td>
<td>n.d.</td>
</tr>
<tr>
<td>A-1</td>
<td>4.958</td>
<td>4.958</td>
<td>4.958</td>
</tr>
<tr>
<td>A-2</td>
<td>3.65</td>
<td>3.65</td>
<td>3.65</td>
</tr>
<tr>
<td>A-3</td>
<td>3.84</td>
<td>3.84</td>
<td>3.84</td>
</tr>
<tr>
<td>A-4</td>
<td>3.67</td>
<td>3.67</td>
<td>3.67</td>
</tr>
<tr>
<td>B-1</td>
<td>5.345/336</td>
<td>5.353/343</td>
<td>5.36/34</td>
</tr>
<tr>
<td>B-2</td>
<td>3.58</td>
<td>3.57</td>
<td>3.57</td>
</tr>
<tr>
<td>B-3</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
</tr>
<tr>
<td>B-4</td>
<td>3.51</td>
<td>3.45</td>
<td>3.45</td>
</tr>
<tr>
<td>B-5</td>
<td>4.20</td>
<td>4.00</td>
<td>n.d.</td>
</tr>
<tr>
<td>C-1</td>
<td>~4.97</td>
<td>~4.97</td>
<td>~4.97</td>
</tr>
<tr>
<td>C-2</td>
<td>3.57</td>
<td>3.57</td>
<td>3.57</td>
</tr>
<tr>
<td>C-3</td>
<td>3.74</td>
<td>3.74</td>
<td>3.74</td>
</tr>
<tr>
<td>D-1</td>
<td>4.965</td>
<td>4.965</td>
<td>4.965</td>
</tr>
<tr>
<td>D-2</td>
<td>3.55</td>
<td>3.55</td>
<td>3.55</td>
</tr>
<tr>
<td>D-3</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
</tr>
<tr>
<td>D-4</td>
<td>3.42</td>
<td>3.43</td>
<td>3.42</td>
</tr>
<tr>
<td>D-5</td>
<td>n.d.</td>
<td>3.76</td>
<td>3.76</td>
</tr>
<tr>
<td>E-1</td>
<td>4.975</td>
<td>4.975</td>
<td>4.975</td>
</tr>
<tr>
<td>E-2</td>
<td>3.65</td>
<td>3.65</td>
<td>3.65</td>
</tr>
<tr>
<td>E-3</td>
<td>3.88</td>
<td>3.88</td>
<td>3.88</td>
</tr>
<tr>
<td>E-4</td>
<td>3.76</td>
<td>3.76</td>
<td>n.d.</td>
</tr>
<tr>
<td>E-5</td>
<td>3.92</td>
<td>3.92</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*a* Residue labels correspond to those used in Scheme 1.

The 1D $^1$H NMR spectrum of fraction 11a (Figure 3B) showed anomeric signals at $\delta$ 5.410 (F H-1, $^3$J$_{1,2}$ 3.6 Hz; $\{\alpha\rightarrow4\}$ linkage), 5.35–5.36 (B H-1; $\{\alpha\rightarrow3\}$ linkage), 5.247 (Ra H-1), 4.97–4.99 (D H-1, E H-1; $\{\alpha\rightarrow6\}$ linkages), and 4.681 (Rf H-1, $^3$J$_{1,2}$ 7.6 Hz). The H-1$\alpha$ and H-1$\beta$ chemical shifts of the reducing residue Ra at $\delta$ 5.247 and 4.681, respectively, correspond with the occurrence of a $\{(1\rightarrow6)\alpha\rightarrow\beta\}$-Glcp unit in a $\{(1\rightarrow3)\alpha\rightarrow\beta\}$-Glcp-$(1\rightarrow6)$-Glcp sequence. The occurrence of the signal at $\delta$H$_{\alpha}$, 3.42 (dd, 2 H), being indicative for an $\alpha\rightarrow\beta$-Glcp-$(1\rightarrow\alpha\}$- unit, reflects a branched structure. As an H-5 signal at $\delta$ 4.20 is missing, the occurrence of a $\{(1\rightarrow6)\alpha\rightarrow\beta\}$-Glcp-$(1\rightarrow3)$- unit can be excluded. However, a signal at $\delta$ 4.12 is observed with a characteristic peak-shape of an H-5 signal, significantly downfield from H-5 of an $\alpha\rightarrow\beta$-Glcp-$(1\rightarrow3)$- unit (see also residue B in 9a), which can be explained by the effect of a 4-substitution on an $\alpha\rightarrow\beta$-Glcp-$(1\rightarrow3)$- unit ($\Delta\delta_{H,5}$ +0.10–0.14 ppm, ref 22), leading to the presence of a $\{(1\rightarrow4)\alpha\rightarrow\beta\}$-Glcp-B-$(1\rightarrow3)$- unit. This observation adds a new structural-reporter-group signal to the concept. These data lead to a structural element F1$\rightarrow$4B1$\rightarrow$3E1$\rightarrow$6R.

The final residue D has to be positioned to cause a branched structure, which can only occur at residue E, resulting in an F1$\rightarrow$4B1$\rightarrow$3[D1$\rightarrow$6]E1$\rightarrow$6R sequence for compound 11a, that is, $\alpha\rightarrow\beta$-Glcp-$(1\rightarrow4)\alpha\rightarrow\beta$-Glcp-$(1\rightarrow3)$-[$\alpha\rightarrow\beta$-Glcp-$(1\rightarrow6)$-]$_n$-$\beta$-Glc-$(1\rightarrow6)$-d-Glc (Scheme 1).

**Smith Degradation.** To investigate the degree of polymerization of $\{(\alpha\rightarrow3)\}$ glycosidic bonds, mEPS-PNNS was subjected to a Smith degradation, comprising a periodate oxidation, followed by reduction with NaBH$_4$, and mild acid hydrolysis with formic acid. The formed products were analyzed by GLC-EI-MS and HPAEC-PAD. In view of the linkage analysis of mEPS-PNNS (see above), $\alpha\rightarrow\beta$-Glcp-$(1\rightarrow1)$-Gro and $\{\alpha\rightarrow\beta$-Glcp-$(1\rightarrow3)\}$_n$\alpha\rightarrow\beta$-Glc-$(1\rightarrow1)$-Gro, but also, due to excessive hydrolysis, Gro, $\alpha$-Glc, and $\{\alpha\rightarrow\beta$-Glc-$(1\rightarrow3)$-$_n$-$\beta$-Glc can be expected.

GLC-EI-MS data (not shown) of the trimethylsilylated residue showed three major products: Gro, $\alpha$-Glc, and $\alpha\rightarrow\beta$-Glc-$(1\rightarrow1)$-Gro. Typical fragments for $\alpha\rightarrow\beta$-Glc-$(1\rightarrow1)$-Gro are m/z 451 (aa$_1$), 361 (aa$_2$), 271 (aa$_3$), 214 and 204 for the Glc moiety, and m/z 219 (ba$_1$) and 337 (ab$_1$) for the Gro moiety.

HPAEC analysis on CarboPac PA-100 (data not shown) of the residue revealed two major peaks that could be related to Gro (R$_g$ 2.2 min) and $\alpha$-Glc-$(1\rightarrow1)$-Gro (R$_g$ 6.2 min), which was identified by its elution position, being slightly later than $\beta$-Glc (R$_g$ 5.6 min). Because $\alpha\rightarrow\beta$-Glc-$(1\rightarrow3)$-d-Glc under the same conditions has an R$_g$ value >10 min, the presence of $\{\alpha\rightarrow\beta$-Glc-$(1\rightarrow3)$-$_n$-$\alpha\rightarrow\beta$-Glc-$(1\rightarrow1)$-Gro with n = 1 or higher could be excluded.

The absence of structures larger than glucosyl-glycerol indicates that the mEPS-PNNS structure does not contain two or more consecutive ($\alpha\rightarrow3$) linkages, similar to the wild-type EPS180.

**2D NMR Analysis of mEPS-PNNS Using Subpool IV.** Intact mEPS-PNNS does not dissolve in suitable amounts to allow 2D NMR analysis. As the $^1$H NMR spectrum of subpool IV, isolated from a partial hydrolysate of mEPS-PNNS and containing fragments of DP >11, is similar to that of intact mEPS-PNNS (Figure 1A,B), this subpool was subjected to 2D NMR analysis. Especially, the larger oligosaccharides in subpool IV are suitable to represent all the structural elements that are present in the full length polysaccharide and do not have interfering signals from reducing residues. 2D $^1$H–$^1$H TOCSY experiments with increasing mixing times (10, 30, 60, 120, and 150 ms) were interpreted (Figure 6; 40 and 120 ms) to unravel the structural elements present in mEPS-PNNS. In several cases, use is made of the $^1$H NMR data.
collected from the oligosaccharides obtained by partial acid hydrolysis of mEPS-PNNS, as well as from data reported in refs 10, 11, and 22.

So far (refs 11 and 22, this study), depending on the microenvironment of the unit, the δ value of the H-1 signal of a (R1f4)-D-GlcP-(1→4) unit varies between 5.41 and 5.33 ppm. In a similar way, the δ value of the H-1 signal of a (R1f3)-D-GlcP-(1→3) unit was found to lie between 5.39 and 5.32 ppm (refs 10 and 22, this study). This means that the anomeric signal of (α1→4)-linked residues could overlap with the anomeric...
signal of (α1→3)-linked residues. In the 1D 1H NMR spectrum of subpool IV (Figure 1B), the H-1 signal between δ 5.41 and 5.39 has a surface area matching the amount of (α1→4)-linked residues, as derived from the substitution pattern determined by methylation analysis, and can therefore be considered as the (α1→4)-anomeric signal. This means that the peak between δ 5.39 and 5.32 corresponds with (α1→3)-linked residues. The peak at δ 4.96 is a typical structural reporter for (α1→6)-linked residues, as derived from the substitution pattern determined by MS and 1H NMR analysis of oligosaccharides obtained in nigerotriose22 suggests that (α1→3)-α-D-Glc(α1→4)- units do not occur in mEPS-PNNS.

On the (α1→6)-α-D-Glc(α1→6) H-1 track in the 30 ms TOCSY spectrum (data not shown) δH,3-values were observed at 3.75, 3.85, and 4.00 ppm, respectively. The first value corresponds with the occurrence of (α1→6)-α-D-Glc(α1→6) and/or (α1→6)-α-D-Glc(α1→6) units,22 the second indicates the occurrence of (α1→3)-α-D-Glc(α1→6) and/or (α1→3,6)-α-D-Glc(α1→6) units,10 and the third is indicative for the presence of (α1→4)-α-D-Glc(α1→6) units (compare residue B in compounds 3a, 5a, 6a, and 7a in ref 11). The occurrence of terminal α-D-Glc(α1→6) is further supported by the presence of an H-4 resonance at δ 3.43 on the (α1→6)-anomeric track. The (α1→6)-α-D-Glc(α1→6) moiety was shown to occur by the presence of δH,4 3.50 on the same track.10,22

Discussion and Conclusions

Building blocks and their quantities were determined by combining data obtained from methylation analysis and Smith degradation analysis of intact mEPS-PNNS, from 1H NMR spectroscopy of intact mEPS-PNNS and its subpool IV, as well as from MS and 1H NMR analysis of oligosaccharides obtained by partial acid hydrolysis of mEPS-PNNS.

mEPS-PNNS turned out to contain 28% (α1→3)-linked α-D-Glc(B). From integration of the H-5 signals of (α1→4)-α-D-Glc(α1→6)-anomeric signal. This means that the peak between δ 5.39 and 5.32 corresponds with (α1→3)-linked residues. The peak at δ 4.96 is a typical structural reporter for (α1→6)-linked residues, as derived from the substitution pattern determined by MS and 1H NMR analysis of oligosaccharides obtained in nigerotriose22 suggests that (α1→3)-α-D-Glc(α1→4)- units do not occur in mEPS-PNNS.

On the (α1→6)-α-D-Glc(α1→6) H-1 track in the 30 ms TOCSY spectrum (data not shown) δH,3-values were observed at 3.75, 3.85, and 4.00 ppm, respectively. The first value corresponds with the occurrence of (α1→6)-α-D-Glc(α1→6) and/or (α1→6)-α-D-Glc(α1→6) units,22 the second indicates the occurrence of (α1→3)-α-D-Glc(α1→6) and/or (α1→3,6)-α-D-Glc(α1→6) units,10 and the third is indicative for the presence of (α1→4)-α-D-Glc(α1→6) units (compare residue B in compounds 3a, 5a, 6a, and 7a in ref 11). The occurrence of terminal α-D-Glc(α1→6) is further supported by the presence of an H-4 resonance at δ 3.43 on the (α1→6)-anomeric track. The (α1→6)-α-D-Glc(α1→6) moiety was shown to occur by the presence of δH,4 3.50 on the same track.10,22

Discussion and Conclusions

Building blocks and their quantities were determined by combining data obtained from methylation analysis and Smith degradation analysis of intact mEPS-PNNS, from 1H NMR spectroscopy of intact mEPS-PNNS and its subpool IV, as well as from MS and 1H NMR analysis of oligosaccharides obtained by partial acid hydrolysis of mEPS-PNNS.

mEPS-PNNS turned out to contain 28% (α1→3)-linked α-D-Glc(B). From integration of the H-5 signals of (α1→4)-α-D-Glc(α1→6)-anomeric signal. This means that the peak between δ 5.39 and 5.32 corresponds with (α1→3)-linked residues. The peak at δ 4.96 is a typical structural reporter for (α1→6)-linked residues, as derived from the substitution pattern determined by MS and 1H NMR analysis of oligosaccharides obtained in nigerotriose22 suggests that (α1→3)-α-D-Glc(α1→4)- units do not occur in mEPS-PNNS.

Discussion and Conclusions

Building blocks and their quantities were determined by combining data obtained from methylation analysis and Smith degradation analysis of intact mEPS-PNNS, from 1H NMR spectroscopy of intact mEPS-PNNS and its subpool IV, as well as from MS and 1H NMR analysis of oligosaccharides obtained by partial acid hydrolysis of mEPS-PNNS.

mEPS-PNNS turned out to contain 28% (α1→3)-linked α-D-Glc(B). From integration of the H-5 signals of (α1→4)-α-D-Glc(α1→6)-anomeric signal. This means that the peak between δ 5.39 and 5.32 corresponds with (α1→3)-linked residues. The peak at δ 4.96 is a typical structural reporter for (α1→6)-linked residues, as derived from the substitution pattern determined by MS and 1H NMR analysis of oligosaccharides obtained in nigerotriose22 suggests that (α1→3)-α-D-Glc(α1→4)- units do not occur in mEPS-PNNS.

Discussion and Conclusions

Building blocks and their quantities were determined by combining data obtained from methylation analysis and Smith degradation analysis of intact mEPS-PNNS, from 1H NMR spectroscopy of intact mEPS-PNNS and its subpool IV, as well as from MS and 1H NMR analysis of oligosaccharides obtained by partial acid hydrolysis of mEPS-PNNS.

mEPS-PNNS turned out to contain 28% (α1→3)-linked α-D-Glc(B). From integration of the H-5 signals of (α1→4)-α-D-Glc(α1→6)-anomeric signal. This means that the peak between δ 5.39 and 5.32 corresponds with (α1→3)-linked residues. The peak at δ 4.96 is a typical structural reporter for (α1→6)-linked residues, as derived from the substitution pattern determined by MS and 1H NMR analysis of oligosaccharides obtained in nigerotriose22 suggests that (α1→3)-α-D-Glc(α1→4)- units do not occur in mEPS-PNNS.

Discussion and Conclusions

Building blocks and their quantities were determined by combining data obtained from methylation analysis and Smith degradation analysis of intact mEPS-PNNS, from 1H NMR spectroscopy of intact mEPS-PNNS and its subpool IV, as well as from MS and 1H NMR analysis of oligosaccharides obtained by partial acid hydrolysis of mEPS-PNNS.

mEPS-PNNS turned out to contain 28% (α1→3)-linked α-D-Glc(B). From integration of the H-5 signals of (α1→4)-α-D-Glc(α1→6)-anomeric signal. This means that the peak between δ 5.39 and 5.32 corresponds with (α1→3)-linked residues. The peak at δ 4.96 is a typical structural reporter for (α1→6)-linked residues, as derived from the substitution pattern determined by MS and 1H NMR analysis of oligosaccharides obtained in nigerotriose22 suggests that (α1→3)-α-D-Glc(α1→4)- units do not occur in mEPS-PNNS.

Discussion and Conclusions

Building blocks and their quantities were determined by combining data obtained from methylation analysis and Smith degradation analysis of intact mEPS-PNNS, from 1H NMR spectroscopy of intact mEPS-PNNS and its subpool IV, as well as from MS and 1H NMR analysis of oligosaccharides obtained by partial acid hydrolysis of mEPS-PNNS.

mEPS-PNNS turned out to contain 28% (α1→3)-linked α-D-Glc(B). From integration of the H-5 signals of (α1→4)-α-D-Glc(α1→6)-anomeric signal. This means that the peak between δ 5.39 and 5.32 corresponds with (α1→3)-linked residues. The peak at δ 4.96 is a typical structural reporter for (α1→6)-linked residues, as derived from the substitution pattern determined by MS and 1H NMR analysis of oligosaccharides obtained in nigerotriose22 suggests that (α1→3)-α-D-Glc(α1→4)- units do not occur in mEPS-PNNS.

Discussion and Conclusions

Building blocks and their quantities were determined by combining data obtained from methylation analysis and Smith degradation analysis of intact mEPS-PNNS, from 1H NMR spectroscopy of intact mEPS-PNNS and its subpool IV, as well as from MS and 1H NMR analysis of oligosaccharides obtained by partial acid hydrolysis of mEPS-PNNS.

mEPS-PNNS turned out to contain 28% (α1→3)-linked α-D-Glc(B). From integration of the H-5 signals of (α1→4)-α-D-Glc(α1→6)-anomeric signal. This means that the peak between δ 5.39 and 5.32 corresponds with (α1→3)-linked residues. The peak at δ 4.96 is a typical structural reporter for (α1→6)-linked residues, as derived from the substitution pattern determined by MS and 1H NMR analysis of oligosaccharides obtained in nigerotriose22 suggests that (α1→3)-α-D-Glc(α1→4)- units do not occur in mEPS-PNNS.

Discussion and Conclusions

Building blocks and their quantities were determined by combining data obtained from methylation analysis and Smith degradation analysis of intact mEPS-PNNS, from 1H NMR spectroscopy of intact mEPS-PNNS and its subpool IV, as well as from MS and 1H NMR analysis of oligosaccharides obtained by partial acid hydrolysis of mEPS-PNNS.

mEPS-PNNS turned out to contain 28% (α1→3)-linked α-D-Glc(B). From integration of the H-5 signals of (α1→4)-α-D-Glc(α1→6)-anomeric signal. This means that the peak between δ 5.39 and 5.32 corresponds with (α1→3)-linked residues. The peak at δ 4.96 is a typical structural reporter for (α1→6)-linked residues, as derived from the substitution pattern determined by MS and 1H NMR analysis of oligosaccharides obtained in nigerotriose22 suggests that (α1→3)-α-D-Glc(α1→4)- units do not occur in mEPS-PNNS.
than the wild-type structure puts a limit on the maximum length of the (PNNS EPS180 (1→4))-unit (B; δ 4.20) and -(1→4)-α-D-Glcp-(1→3)- (B; δ 4.12) in the 1D 1H NMR spectrum, the occurrence of these building blocks was quantified at 17 and 7%, respectively (Table 3). This leaves 4% for the remaining α-D-Glcp-(1→3)-unit (B; Table 3).

The original structural limitation of single (α1→3) bridges in EPS180 also exists in mEPS-PNNS. Because -(α-D-Glcp-(1→4))-units were not 3-substituted, all 3-substituted Glcp residues (10%) are in fact -(1→3)-α-D-Glcp-(1→6)- (A). Furthermore, methylation analysis indicated 18% 3,6-disubstituted Glcp, which means 18% of all residues are -(1→3,6)-α-D-Glcp-(1→6)- (E; Table 3).

In accordance with 18% branching, 18% terminal residues also do occur. As mentioned above, 4% of these residues were α-D-Glcp-(1→3)-units (B). Because the H-4 resonance at δ 3.42–3.43, indicative for terminal residues,10,11,22 on the (α1→6)-anomeric track was stronger than on the (α1→4)-anomeric track, the occurrence of α-D-Glcp-(1→6)- (D) and α-D-Glcp-(1→4)- (F) will be around 8 and 6%, respectively.

With a total of 12% (α1→4)-linked residues (FfH) and the estimated 6% for α-D-Glcp-(1→4)-units (F), an amount of 6% is left for -(1→4)-α-D-Glcp-(1→4)- (H) and -(1→6)-α-D-Glcp-(1→4)- (Hf). M ethylation analysis showed 12% 4-substituted Glcp. Taking into account that already 7% -(1→4)-α-D-Glcp-(1→3)-units (Bf) were assigned (see above), 5% is left for both -(1→4)-α-D-Glcp-(1→4)- units (Hf) and -(1→4)-α-D-Glcp-(1→6)- units (H). Based on TOCSY cross-peak intensities, the amounts -(1→4)-α-D-Glcp-(1→4)- (H-3), -(1→6)-α-D-Glcp-(1→4)- (H-4), and -(1→4)-α-D-Glcp-(1→6)- (H-3) units will be about 3, 3, and 2%, respectively (Table 3).

With the building blocks that could be determined from the available 1H NMR and methylation analysis data, only one possible building block is left undetermined, that is, -(1→6)-α-D-Glcp-(1→6)- units (Cf), which can then be determined indirectly (22%) from the methylation analysis.

When the building block quantities and the sequences found for the different oligosaccharides are used, a composite model that includes all established structural features can be formulated as depicted in Scheme 2. For comparison, the earlier formulated composite model of wild-type EPS18010 is included in Scheme 2, whereas the percentages of building blocks are given in Table 3.

The composite model shows a more complex structure for mEPS-PNNS compared to wild-type EPS180. The presence of (α1→4)-di- and trisaccharide elements shows that the amino acid mutations in the GTF180 enzyme have significantly changed its structural selectivity. However, the original structural limitation of single (α1→3) bridges still exists. The presence of (α1→6)-linked elements up to five residues in a row (compound 10) indicates that the isomalto-oligosaccharide basis of the structure also still exists. The maximum length of (α1→6)-linked stretches was not determined in wild-type EPS180 and also cannot be determined in the case of mEPS-PNNS. The different linkage distribution in the mEPS-PNNS structure puts a limit on the maximum length of the (α1→6)-linked elements, however, it must be shorter than that in wild-type EPS180. The mEPS-PNNS polysaccharide is less soluble than the wild-type EPS180. The lower solubility is probably the result of a more rigid structure, since (α1→6) linkages are flexible, whereas (α1→3) and (α1→4) are more rigid.26 Increased rigidity of the structure may also cause the higher resistance to acid hydrolysis that was observed.

Acknowledgment. We thank the Ministry of Economic Affairs (Senter Novem; Bioprimer/project EETK 01129) for financial support.

References and Notes


BM800410W