Carbohydrate Polymers 337 (2024) 122164

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Detailed structural characterization of five water-insoluble α-glucans produced by glucansucrases from *Streptococcus* spp.

Luise Ernst [a], Celine Schulz [a], Albrecht Petzold [b], Thomas Thurn-Albrecht [b], Kay Saalwächter [c], Daniel Wefers [a,*]

[a] Institute of Chemistry, Food Chemistry, Martin Luther University Halle-Wittenberg, 06120 Halle (Saale), Germany
[b] Institute of Physics, Experimental Polymer Physics, Martin Luther University Halle-Wittenberg, 06120 Halle (Saale), Germany
[c] Institute of Physics, NMR, Martin Luther University Halle-Wittenberg, 06120 Halle (Saale), Germany

ARTICLE INFO

Keywords:
Mutan
Dextran
HPSEC-RI
XRD
^{13}C MAS NMR spectroscopy
Methylation analysis

ABSTRACT

Water-insoluble α-glucans synthesized from sucrose by glucansucrases from *Streptococcus* spp. are essential in dental plaque and caries formation. Because limited information is available on the fine structure of these biopolymers, we analyzed the structures of unmodified glucans produced by five recombinant *Streptococcus* (*S.*) *mutans* DSM 20523 and *S. salivarius* DSM 20560 glucansucrases in detail. A combination of methylation analysis, *endo*-dextranase and *endo*-mutanase hydrolyses, and HPSEC-RI was used. Furthermore, crystal-like regions were analyzed by using XRD and ^{13}C MAS NMR spectroscopy. Our results showed that the glucan structures were highly diverse: Two glucans with 1,3- and 1,6-linkages were characterized in detail besides an almost exclusively 1,3-linked and a linear 1,6-linked glucan. Furthermore, one glucan contained 1,3-, 1,4-, and 1,6-linkages and thus had an unusual, not yet described structure. It was demonstrated that the glucans had a varying structural architecture by using partial enzymatic hydrolyses. Furthermore, crystal-like regions formed by 1,3-glucopyranose units were observed for the two 1,3- and 1,6-linked glucans and the linear 1,3-linked glucan. 1,6-linked regions were mobile and not involved in the crystal-like areas. Altogether, our results broaden the knowledge of the structure of water-insoluble α-glucans from *Streptococcus* spp.

1. Introduction

The structure of bacterial α-glucans is highly complex because they are composed of different backbone linkages (1,3-, 1,4-, and 1,6-linked D-glucopyranoses (Glc*p*)) as well as side chains at positions *O*2, *O*3, *O*4, or *O*6 (depending on the backbone). α-Glucans are often grouped into dextrans, which contain >50 % of 1,6-linkages, and mutans, which contain >50 % of 1,3-linkages (Li, Wang, Meng, Dijkhuizen, & Liu, 2020; Monsan et al., 2001). Mutans are usually considered to be water-insoluble polysaccharides, which is most likely caused by consecutive α-1,3-linkages (Aires, Koo, Sassaki, Iacomini, & Cury, 2010; Ernst, Offermann, Werner, & Wefers, 2024; Hare, Svensson, & Walker, 1978; Inoue, Yakushiji, Katsuki, Kudo, & Koga, 1988; Inoue, Yakushiji, & Takehara, 1982). However, completely linear 1,6-linked α-glucans may be water-insoluble, too (He et al., 2020; Padmanabhan, Kim, Pak, & Sim, 2003; Pittrof, Kaufhold, Fischer, & Wefers, 2021). Furthermore, α-glucans with a high portion of 1,6-linkages and only minor amounts of 1,3-linkages can be insoluble in water (Ernst et al., 2024; Fels, Jakob, Vogel, & Wefers, 2018). Consequently, water-insoluble α-glucans are a group of polysaccharides with a high structural heterogeneity.

α-Glucans are synthesized from sucrose by glucansucrases (glycoside hydrolase family 70). These homologous enzymes are secreted by several lactic acid bacteria from the genera *Streptococcus* (*S.*), *Lactobacillus, Weissella,* and *Leuconostoc* and use the glucose moiety from sucrose to form polymeric glucans (Hoshino, Fujiwara, & Kawabata, 2012; Leemhuis et al., 2013; Meng et al., 2016; Xu, Yang, Niu, Wang, & Wang,

Abbreviations: PCR, polymerase chain reaction; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; MS, mass spectrometry; TFA, trifluoroacetic acid; HPSEC-RI, high-performance size exclusion chromatography coupled with refractive index detection; DMSO, dimethyl sulfoxide; Glc*p*, glucopyranose; XRD, X-ray diffraction; MAS, magic angle spinning; NMR, nuclear magnetic resonance; CP, cross polarization; DP, direct polarization; GTF, glucosyltransferase.

* Corresponding author at: Martin Luther University Halle-Wittenberg, Institute of Chemistry – Division of Food Chemistry, Kurt-Mothes-Straße 2, 06120 Halle (Saale), Germany.
E-mail address: daniel.wefers@chemie.uni-halle.de (D. Wefers).

https://doi.org/10.1016/j.carbpol.2024.122164
Received 15 January 2024; Received in revised form 11 April 2024; Accepted 12 April 2024
Available online 14 April 2024

2018; Yu, Qian, Ge, & Du, 2022). In the mouth cavity, *S. mutans* and *S. salivarius* are of particular importance, because these organisms are involved in caries formation (Bowen & Koo, 2011; Drucker, Shakespeare, & Green, 1984; Takahashi & Nyvad, 2008). Sucrose, which naturally occurs in our diet, is known to be a virulence factor for dental caries. This is based not only on the fermentation of sucrose to lactic acid, but also on its utilization for α-glucan synthesis (Forssten, Björklund, & Ouwehand, 2010; Leme, Koo, Bellato, Bedi, & Cury, 2006; Minah & Loesche, 1977). In particular, water-insoluble α-glucans enhance adherence and accumulation of acidogenic bacteria and are an essential component of the dental biofilm (plaque), which allows bacteria to establish an acidic environment resulting in tooth enamel demineralization (Koo, Falsetta, & Klein, 2013). To understand the role of water-insoluble α-glucans in dental caries promotion and in developing prevention methods, a detailed investigation of the molecular structure of these polysaccharides is crucial.

The important structural characteristics of glucans include the linkage types, side chain positions, and side chain lengths. Furthermore, some water-insoluble α-glucans form crystalline regions. Crystallinity can influence enzyme accessibility and thus impede enzymatic hydrolysis which is a tool considered for caries prevention. Ogawa, Yui, Okamura, and Misaki (1994) analyzed the 1,3-linked sections of two α-glucans of *S. salivarius* and *mutans* which were prepared by mild Smith degradation or dextranase hydrolysis. They found that the two glucans contained two different crystal polymorphs, one of them included one water molecule per glucose unit. This water molecule was removed by drying which resulted in the other polymorph.

S. salivarius ATCC 25975 is known to encode two glucansucrases, glucosyltransferase J (GTF-J) and GTF-L, which produce water-insoluble glucans. The glucans of GTF-J were described as mainly 1,3-linked, whereas 1,3- and 1,6-linkages were observed for the glucans produced by GTF-L (Kusumi et al., 2023; Simpson, Cheetham, Giffard, & Jacques, 1995). Kobayashi et al. (2017) investigated the solely α-1,3-linked glucans formed by glucansucrase GTF-J. They confirmed different crystallinity of the hydrated and dehydrated glucans and the existence of one water molecule per glucose unit in the hydrated form. Furthermore, they found a decrease in crystallinity due to drying. Generally, dehydration and hydration are known to induce crystal transitions in polysaccharides (Kobayashi, Kimura, Heux, & Wada, 2013; Kobayashi, Kimura, Togawa, Wada, & Kuga, 2010). *S. salivarius* also encodes for other glucansucrases such as GTF-K, but these enzymes were described to produce water-soluble glucans (Simpson et al., 1995). The cariogenic potential, glucansucrases, and glucans of *S. mutans* were extensively studied in the past (Bowen & Koo, 2011; Hare et al., 1978; Hayacibara et al., 2004; Kopec, Vacca-Smith, & Bowen, 1997; Kuramitsu & Wondrack, 1983; Lin, Chen, Zhou, & Li, 2021; Wiater, Pleszczyńska, Próchniak, & Szczodrak, 2012). This organism was described to produce glucansucrases GTF-I (GTF-B) and GTF-SI (GTF-C) which synthesize (partially) water-insoluble glucans as well as GTF-S (GTF-D) which synthesizes water-soluble glucans (Hanada & Kuramitsu, 1988, 1989). Glucans of GTF-I were described as mainly 1,3-linked, whereas 1,3- and 1,6-linkages were described for glucans of GTF-SI (Hare et al., 1978; Hayacibara et al., 2004; Kopec et al., 1997). However, glucan structures were mostly only investigated by using methylation analysis and by comparing dextranase and mutanase susceptibility. Data on the crystallinity of mixed-linkage α-glucans from *Streptococcus* spp. are not available.

Consequently, a study on the comparative analysis of the fine structures and crystallinity of different water-insoluble α-glucans produced by glucansucrases from *Streptococcus* spp. is not available, despite the important role of these glucans in the human mouth cavity. Therefore, we selected five homologous glucansucrases with different amino acid sequences from *S. mutans* DSM 20523 (NCTC 10449) and *S. salivarius* DSM 20560 (NCTC 8618) for the production and subsequent characterization of glucans. Our hypothesis was that water-insoluble, native α-glucans produced by these five glucansucrases vary in terms of their structural architecture and crystallinity. Thus, glucans were investigated by using methylation analysis, different chromatographic approaches in combination with partial enzymatic hydrolyses, X-ray diffraction (XRD), and ^{13}C magic angle spinning nuclear magnetic resonance (MAS NMR) spectroscopy.

2. Experimental

2.1. Materials

If not stated otherwise, all chemicals used were of "p.a." grade or better and were purchased from Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Thermo Fisher Scientific (Waltham, MA USA), VWR (Darmstadt, Germany), and Grüssing GmbH (Filsum, Germany). *Endo*-dextranase (EC 3.2.1.11) from *Chaetomium* sp., 8000 U/mL, was purchased from Megazyme (Bray, Ireland) and *endo*-mutanase SSAL4105 (EC 3.2.1.59) from *S. salivarius* was recombinantly produced as described by Ernst et al. (2024). Genomic DNA of *S. mutans* DSM 20523 and *S. salivarius* DSM 20560 was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany).

2.2. Production of recombinant glucansucrases

Molecular cloning, heterologous expression, and protein isolation of glucansucrases (SMUT1101 and SMUT1102 from *S. mutans* DSM 20523 as well as SSAL4540, SSAL4545, and SSAL4550 from *S. salivarius* DSM 20560) were performed as described previously (Ernst et al., 2024; Münkel et al., 2019). Briefly, encoding genes were amplified from genomic DNA by polymerase chain reaction (PCR) using a Phusion High-Fidelity PCR kit (Thermo Fisher Scientific, Waltham, USA). The primer pairs shown in Table 1 (synthesized by Integrated DNA Technologies (Coralville, IA, USA)) were used. Signal peptides (predicted by using the SignalP 5.0 web tool) were not amplified. Specific overhangs were inserted at both ends of the genes to allow ligase-independent cloning into the pLIC-SGC1 vector (Addgene plasmid no. 39187). For ligation independent cloning, the vector and PCR products were digested with T4 DNA polymerase (Thermo Fisher Scientific), and vector-gene adducts were transformed to 5α-competent *E. coli* cells (High efficiency, NEB, USA). The correct plasmid sequence was confirmed by Sanger sequencing (Eurofins GATC Biotech, Konstanz, Germany). For gene expression, the plasmids were transformed into One Shot BL21 Star *E. coli* (DE3, Invitrogen, USA) and cells were grown in LB medium supplemented with 100 μg ampicillin/L overnight at 37 °C and 225 rpm. The protein production was induced by the addition of isopropyl-β-D-thiogalactopyranoside (final concentration 0.1 mM). After cell lysis by sonication, cell debris was removed by centrifugation (30 min, 4 °C, 14,000 g), and recombinant proteins were isolated by immobilized metal affinity chromatography using a HisPur Ni-NTA resin (Thermo Fisher Scientific). Proteins were eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 100 mM imidazole, pH 7.5) and the protein concentration was calculated by absorption measurement at 280 nm (calculation of the extinction coefficient by using the amino acid sequence and the ProtParam tool). The obtained solutions were used for glucan synthesis. Sequence comparison of glucansucrases with previously described glucansucrases (Table S1) was performed with the multiple sequence alignment tools Clustal Omega and ESPript 3.0 (Robert & Gouet, 2014; Sievers & Higgins, 2014).

2.3. Glucan synthesis and monosaccharide analysis

For glucan synthesis, appropriate volumes of glucansucrase solutions (Section 2.2) containing 5 mg of protein were added to 100 mL of a 0.5 M sucrose solution (including 10 mM $CaCl_2$ and 0.05 vol-% ProClin, pH 5.9). After static incubation at 37 °C for 48 h, glucans were recovered by centrifugation at 4 °C (30 min at 10595 rcf). Subsequently, glucans were

Table 1
Genomic DNA and PCR primers used for molecular cloning of glucansucrases.

Genomic DNA	Locus tag	Primer sequence forward (5′ to 3′)	Primer sequence reverse (5′ to 3′)
S. mutans DSM 20523 (NCTC 10449)	SMUT1102	TACTTCCAATCCATGGATTCTAATGAATCGAAATCCCAAATTTCTAATGATTCTAATAC	TATCCACCTTTACTGTTAGTTAATCCGAACTCGTTCTCCAGAG
	SMUT1101	TACTTCCAATCCATGGATTCACTGACGACCGTCAACAGG	TATCCACCTTTACTGTTAAAATCTAAAGAAATTGTCAAAGAATCCATTACGAGAGTGTC
S. salivarius DSM 20560 (NCTC 8618)	SSAL4550	TACTTCCAATCCATGGAAGAAATAAATAATACTAATGGATCACCTTCAACAACTACAGTC	TATCCACCTTTACTGTTAATTTGACACGTATCCTTTTCCTTGAGCG
	SSAL4545	TACTTCCAATCCATGGATGAAACTCAAGATAAGATAGTAACTCAACCAAATTTAGATAC	TATCCACCTTTACTGTTAGTTTAGCACTCTAGGTGGGTAAGC
	SSAL4540	TACTTCCAATCCATGGATGAAACAACTCAACCAGTTGCCTCTACG	TATCCACCTTTACTGTTATTTATCAAAATCAGCATAACCATTTTCATCGAAGAAGACAG

resuspended in ultrapure water (100 mL) and centrifuged (30 min at 10595 rcf). This washing step was repeated twice and wet (paste-like) glucans were preserved by adding 10 μL of a 50 mg/mL NaN_3 solution (final concentration 0.5 mg/mL). Partial enzymatic hydrolyses (Section 2.4), XRD (Section 2.7), and ^{13}C MAS NMR spectroscopy (Section 2.8) were performed by using the undried polysaccharides, whereas an aliquot was lyophilized for methylation analysis (Section 2.6), HPSEC-RI (Section 2.5), and monosaccharide analysis. The latter was performed as described by Ernst et al. (2024) for water-insoluble homoexopolysaccharides. In brief, glucans were hydrolyzed by using trifluoroacetic acid (TFA) in two batches with two different conditions (2 M TFA, 60 min, 121 °C; 1 M TFA, 30 min, 70 °C), and the released monosaccharides were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

2.4. Partial enzymatic hydrolyses and oligosaccharide analysis

About 1 mg of undried glucans was suspended in 500 μL of ultrapure water and used for *endo*-dextranase hydrolysis. After the addition of *endo*-dextranase (5 U/mg glucans), the mixture was incubated for 24 h at 40 °C and 200 rpm. For *endo*-mutanase hydrolysis, 90 μg of protein were added to 1 mg of undried glucans in 100 μL ultrapure water and incubated for 24 h at 37 °C and 200 rpm. Dextranase and mutanase were inactivated by heating to 95 °C for 10 min. Insoluble material was removed by centrifugation and lyophilized. The supernatant was used for HPAEC-PAD coupled with mass spectrometric detection (HPAEC-PAD/MS) on an ICS-6000 system equipped with a LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific). Separation was achieved by using a CarboPac PA200 column (250 × 3 mm i.d., 5.5 μm particle size, Thermo Fisher Scientific). The column temperature was 30 °C and the PAD temperature was 25 °C. For separation, the following gradient composed of four eluents (A: ultrapure water, B: 10 mM NaOH, C: 200 mM NaOH, D: 200 mM NaOH +500 mM sodium acetate) was used at a flow rate of 0.4 mL/min: column equilibration with 100 % B 20 min before injection; 0–10 min: Isocratic 100 % B; 10–20 min: Linear gradient to 50 % C + 50 % A; 20–45 min: Linear gradient to 50 % A, 30 % C + 20 % D; 45–55 min: Linear gradient to 50 % A + 50 % D; 55–65 min: Linear gradient to 100 % D; 65–80 min: Isocratic 100 % D; 80–95 min: Isocratic 100 % C. A post column split allowed simultaneous PAD and MS analysis. Prior to MS analysis, desalting of the eluent was achieved by an AERS 500e suppressor (4 mm, Thermo Fisher Scientific). To facilitate ionization by electrospray ionization, 500 μM LiCl was added at a flow rate of 0.05 mL/min (AXP-MS pump, Thermo Fisher Scientific). The source temperature was 300 °C and lithium adducts of oligosaccharides were detected in positive mode. Peaks in the chromatograms of dextranase hydrolysates were assigned by using the oligosaccharides characterized by Münkel and Wefers (2019).

2.5. High-performance size exclusion chromatography coupled with refractive index detection (HPSEC-RI)

For HPSEC-RI analysis of glucans and water-insoluble residues after *endo*-dextranase and *endo*-mutanase hydrolyses (Section 2.4), the lyophilized samples were solubilized by adding 300 μL of dimethyl sulfoxide (DMSO). After shaking at ambient temperature overnight, about 2 mg of $LiCl_{(s)}$ were added (resulting LiCl concentration: 100 mM) and the samples were again shaken overnight (60 °C, 1500 rpm). Glucans and water-insoluble hydrolysis products of SSAL4545, SMUT1101, and SMUT1102 were dissolved by this procedure and analyzed. SSAL4540 and SSAL4550 were not solubilized, even after a second addition of $LiCl_{(s)}$ and shaking. For chromatographic analysis, DMSO with 100 mM LiCl was used as eluent at a flow rate of 0.2 mL/min on an HPLC-RI system (P6.1L, AS6.1 L, RID 2.1 L, Knauer, Germany) equipped with a GRAM column (300 × 8 mm, particle size 10 μm) (PSS, Germany). Column temperature was 80 °C and RI temperature was 35 °C. The molecular weight of the glucan samples was estimated by using

standard dextrans with known molecular weight (dextran blue (~ 2000 kDa), 670 kDa, 270 kDa and 12 kDa, Sigma Aldrich, Germany; 64.3 kDa, PSS, Germany).

2.6. Glycosidic linkage analysis

Analysis of glycosidic linkages was carried out by methylation analysis: Lyophilized glucans were derivatized and analyzed as described in detail by Ernst, Werner, and Wefers (2023). Briefly, samples were swollen in DMSO overnight followed by an ultrasonic treatment. $NaOH_{(s)}$ was added, and the samples were again sonicated before adding methyl iodide. Methylation was repeated once, and the partially methylated polysaccharides were hydrolyzed by 2 M TFA for 90 min at 121 °C. Partially methylated monosaccharides were reduced with $NaBD_4$ and acetylated by using 1-methylimidazole and acetic anhydride. Partially methylated alditol acetates were separated by gas chromatography, identified by their mass spectra (Fig. S4), and quantified by flame ionization detection by using the response factors described by Sweet, Shapiro, and Albersheim (1975).

2.7. Wide angle X-ray diffraction (XRD)

Undried glucans were prepared on a sample holder covered with amorphous tape. This setting allowed keeping a relative humidity of 100 % for at least 16 h. A spatula tip of each sample was used; however, the exact glucan amount is unknown due to the wet state of the samples. An EMPYREAN (Malvern Panalytical) diffractometer operating in Bragg–Brentano geometry using a programmable divergence slit and Cu-Kα-radiation was used for diffraction measurements.

2.8. ^{13}C Magic angle spinning nuclear magnetic resonance (MAS NMR) spectroscopy

The ^{13}C cross-polarization (CP) and direct polarization (DP) MAS spectra of undried glucans were obtained on a Bruker Avance spectrometer with the proton resonance frequency 400 MHz. The samples were packed in 4 mm rotors and span at a MAS rate of 10 kHz and at ambient temperature. The experimental parameters were: CP contact time 1.5 ms, ^{1}H 90-degree pulse 3.6 μs, ^{13}C 90-degree pulse 4 μs. The ^{13}C signal was recorded during 35 ms with 65 kHz SPINAL proton decoupling. Repetition delay was 3 s and 10 s for CP and DP spectra, respectively. The number of signal accumulations for different samples was 10,000 to 40,000.

3. Results and discussion

For comparative investigation of fine structures and crystallinity of different water-insoluble α-glucans, five homologous glucansucrases from *Streptococcus* spp. were cloned and heterologously expressed: SMUT1101 and SMUT1102 from *S. mutans* DSM 20523 as well as SSAL4540, SSAL4545, and SSAL4550 from *S. salivarius* DSM 20560. Glucansucrases SMUT1101 and SMUT1102 showed high sequence identities (>95 %) to the two *S. mutans* glucansucrases which produce insoluble glucans, GTF-I and GTF-SI (Table S1, Fig. S1 & S2). Furthermore, glucansucrases SSAL4545 (GTF-J) and SSAL4550 (GTF-K) from *S. salivarius* were selected for comparison because they were described to produce linear 1,3- and 1,6-linked glucans, respectively. Whereas linear 1,3-linked glucans are water-insoluble and may be crystalline, 1,6-linked glucans can at least in part be water-insoluble. Furthermore, glucansucrase SSAL4540 was investigated because its amino acid sequence showed similar conserved regions to those of known, water-insoluble glucan synthesizing glucansucrases of *S. salivarius* and *mutans*, but only shared a low degree of identity (< 60 %) with the other enzymes. Consequently, this enzyme may synthesize glucans with an unusual structure. Water-insoluble polysaccharides were successfully synthesized from sucrose by using the five recombinant glucansucrases. The paste-like undried polysaccharides were recovered by centrifugation and used for further analysis (after drying for some analyses, see Sections 2.4–2.8). Monosaccharide analysis of the obtained polysaccharides confirmed that they were solely composed of glucose. In addition, glucansucrases SSAL4540 and SSAL4550 synthesized in part water-soluble glucans resulting in lower yields of water-insoluble glucans. However, the water-soluble fraction was not of interest in this study.

3.1. Glycosidic linkages

The portions of glycosidic linkages in the water-insoluble glucans were determined by methylation analysis (Fig. 1). Low portions of artifacts from the sample workup such as fully substituted glucopyranose were detected in some chromatograms (Fig. S3). The formation of these compounds is caused by solubilization issues of the water-insoluble glucans in DMSO. Partial undermethylation may have some influence on the portions of the glycosidic linkages, however, due to the low abundance of the artifacts, the results can still be used to assess the structural composition. The glucans showed varying backbone linkages, but all samples contained 1,3,6-linked Glc*p* units which correspond to branched backbone units. Glucans produced by SMUT1101 and SMUT1102 were composed of 1,3- and 1,6-linked Glc*p* units in comparable portions: SMUT1101 glucans contained 55 % 1,3-linkages and 32 % 1,6-linkages, while SMUT1102 glucans contained 48 % 1,3-linkages and 38 % 1,6-linkages. Glucans containing mainly 1,3- and 1,6-linkages in different portions have also been described for GTF-I (GTF-B) and GTF-SI (GTF-C) of *S. mutans* GS-5 (Aires et al., 2010; Hayacibara et al., 2004; Kopec et al., 1997).

Glucans produced by SSAL4545 were mainly 1,3-linked with minor portions of 1,6-linkages (< 1 %), whereas glucans produced by SSAL4550 were mainly 1,6-linked with minor portions of 1,3-linkages (< 5 %). Simpson et al. (1995) found that GTF-J (97 % identity to SSAL4545) synthesized 1,3-linked glucans and GTF-K (96 % identity to SSAL4550) synthesized 1,6-linked glucans. However, it cannot be excluded that the minor portions of 1,3,6-linkages (and 1,6-/1,3-linkages) are artifacts which were formed during the sample workup. Furthermore, Simpson et al. (1995) described the 1,6-linked glucans of

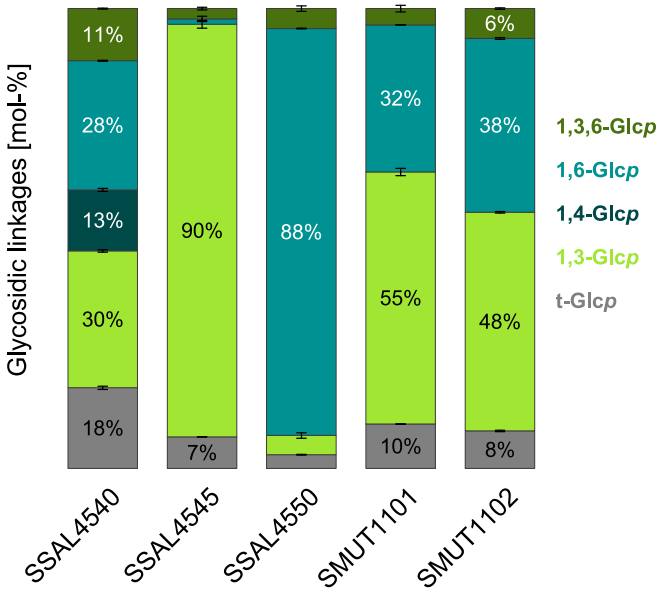

Fig. 1. Glycosidic linkages of glucans produced by glucansucrases SSAL4540, SSAL4545, SSAL4550, SMUT1101, and SMUT1102. Glycosidic linkages were determined by methylation analysis and all analyses were performed in duplicate. t: terminal, Glc*p*: glucopyranose, numbers indicate the position of substitution in the monosaccharide unit.

GTF-K to be water-soluble, but (partial) water-insolubility of the 1,6-linked glucans from SSAL4550 could be a result of higher linearity and/or higher molecular weight.

Linkages of SSAL4540 glucans were very diverse: Besides 30 % 1,3-linkages, 13 % 1,4- and 28 % 1,6-linkages were present. A few studies found low portions (< 5 %) of 1,4-linked Glc*p* units in glucans from *Streptococcus* spp. (Kopec et al., 1997; Tsumuraya & Misaki, 1979). However, these low amounts might be due to artifacts in methylation analysis and Smith degradation. Only Hayacibara et al. (2004) found a high portion of 1,4-linkages (16–25 %) in glucan produced by glucansucrase GTF-C from *S. mutans* WHB410. There is no previous report of mixed-linkage glucans containing a relevant portion of 1,4-linkages produced by glucansucrases of *S. salivarius*. Overall, the results indicated a high structural diversity of water-insoluble glucans produced by the five glucansucrases.

3.2. Partial enzymatic hydrolyses

To obtain information on fine structures, glucans were hydrolyzed by *endo*-dextranase and *endo*-mutanase. The resulting water-soluble products were analyzed by HPAEC-PAD/MS. The chromatograms obtained from *endo*-dextranase hydrolyses are shown in Fig. 2. *Endo*-dextranase hydrolyzes sections of linear, α-1,6-linked Glc*p* units and liberates glucose, isomaltose as well as previously described branched isomalto-oligosaccharides (Münkel et al., 2019; Pittrof et al., 2021). The chromatogram of the *endo*-dextranase hydrolysate of glucans produced by SSAL4550 contained three peaks which resulted from glucose, isomaltose, and isomaltotriose. These results suggested that SSAL4550 glucans were solely 1,6-linked because no branched oligosaccharides were detected. Consequently, the small portions of 1,3- and 1,3,6-linkages detected by methylation analysis (Section 3.1) were most likely artifacts from the sample workup. *Endo*-dextranase did not liberate any products from the glucans of SSAL4540 and SSAL4545. This supported the finding that glucans produced by SSAL4545 were exclusively 1,3-linked and that minor portions of 1,6-linkages determined by methylation analysis were artifacts (Section 3.1). In contrast, some hydrolysis could have been expected from glucans produced by SSAL4540, because they contained about 30 % 1,6-linkages (Section 3.1). The fact that these polysaccharides were not digested by *endo*-dextranase indicated that 1,6-linked Glc*p* units were not present in consecutive blocks. Chromatograms of *endo*-dextranase hydrolysates of glucans produced by SMUT1101 and SMUT1102 contained peaks resulting from isomaltose, isomaltotriose, and *O*3-branched isomalto-oligosaccharides. The released oligosaccharides demonstrated that SMUT1101 and SMUT1102 glucans contained consecutive 1,6-linked sections which were branched with mono-, di-, and oligomeric side chains at position *O*3. Relative peak intensities of SMUT1101 and SMUT1102 glucan hydrolysates varied, indicating a varying structural architecture.

A recombinant *endo*-mutanase was used to hydrolyze sections of α-1,3-linked Glc*p* units. The chromatogram of the *endo*-mutanase hydrolysate of glucans produced by SSAL4550 (Fig. 3) did not contain any peaks which indicated that these glucans cannot be hydrolyzed by *endo*-mutanase. This result was in accordance with the observation that these glucans were exclusively composed of 1,6-linkages. The chromatograms of the four remaining glucans produced by SSAL4540, SSAL4545, SMUT1101, and SMUT1102 showed peaks resulting from linear, 1,3-linked di−/oligosaccharides (nigerose, nigerotriose and nigerotetraose) which were identified by using standard compounds. The detection of these oligosaccharides confirmed the occurrence of consecutive α-1,3-linkages. Furthermore, unknown oligosaccharides which showed an *m/z* corresponding to gluco-oligosaccharides with a degree of polymerization from 3 to 7 were detected. The presence of gluco-oligosaccharides was also confirmed by analyzing the monosaccharide composition of the oligosaccharide-containing supernatant by HPAEC-PAD after TFA hydrolysis. Because the retention times of these compounds did not correspond to α-1,3-, α-1,4-, or α-1,6-linked standard oligosaccharides, the detected peaks can be assigned to mixed-linkage or branched gluco-oligosaccharides. Furthermore, the oligosaccharide profiles (the occurrence and relative intensities of the oligosaccharide-derived peaks) suggested structural differences between the analyzed glucans. For example, the hydrolysate of glucans produced by SSAL4540 showed several different pentasaccharides which were not present in the other samples. The liberation of these oligosaccharides could be a result of the 1,3-, 1,4-, and 1,6-linkages present in these glucans. SSAL4545, SMUT1101, and SMUT1102 glucans showed comparable oligosaccharide profiles, however, different relative peak intensities indicated varying portions of oligosaccharides for glucans produced by SMUT1101 and SMUT1102. This again confirmed the different fine structures of these two polysaccharides. The liberation of mixed-linkage or branched oligosaccharides from SSAL4545 glucans confirmed the presence of 1,3,6-linked Glc*p* units in these glucans (Section 3.1).

Enzymatic hydrolysis was used to investigate structures of water-insoluble glucans produced by different glucansucrases from *Streptococcus* spp. in previous studies (Hare et al., 1978; Kopec et al., 1997; Pearce, Walker, Slodki, & Schuerch, 1990; Vacca-Smith, Venkitaraman, Quivey Jr, & Bowen, 1996). However, merely Hare et al. (1978) found unknown, presumably mixed-linkage oligosaccharides in addition to isomalto- and nigero-oligosaccharides after *endo*-dextranase as well as simultaneous *endo*-dextranase and *endo*-mutanase hydrolysis of

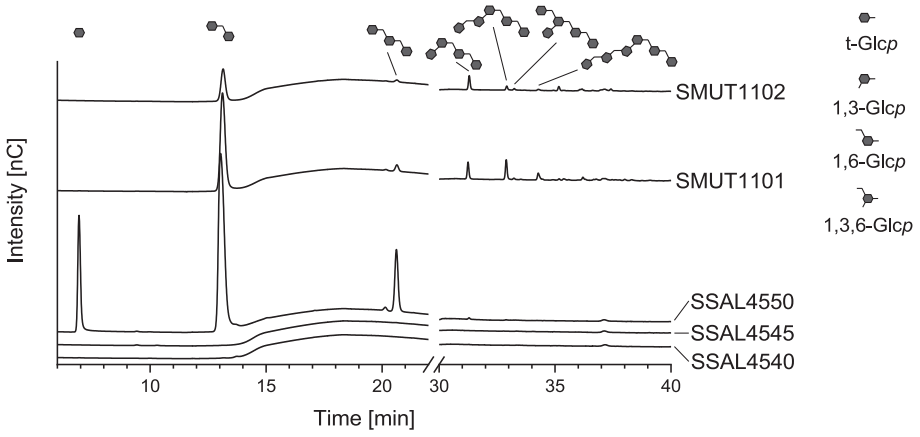

Fig. 2. HPAEC-PAD chromatograms of oligosaccharides released from glucans synthesized by glucansucrases SSAL4540, SSAL4545, SSAL4550, SMUT1101 and SMUT1102 by using *endo*-dextranase. Chromatograms are shown in stacked format and peaks were assigned according to Münkel et al. (2019) and Münkel and Wefers (2019).

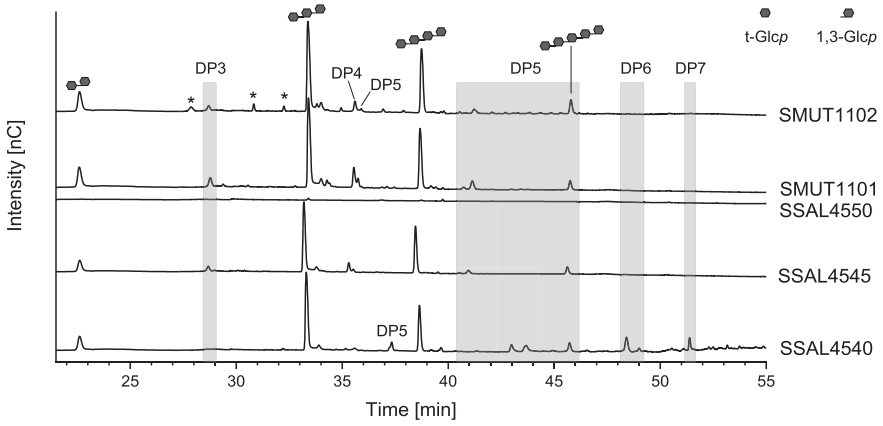

Fig. 3. HPAEC-PAD chromatograms of oligosaccharides released from glucans synthesized by glucansucrases SSAL4540, SSAL4545, SSAL4550, SMUT1101 and SMUT1102 by using *endo*-mutanase. Chromatograms are shown in stacked format and peak assignment resulted from standard compounds (nigero-oligosaccharides). The degree of polymerization (DP) of the unknown oligosaccharides was determined by mass spectrometry. Peaks marked with asterisks (*) are isomalto-oligosaccharides and were also present in SMUT1102 glucans without enzyme addition. Slight retention time shifts between 30 and 40 min were caused by different concentrations of buffer salts from mutanase solutions.

enzymatically synthesized glucans from *Streptococcus* spp. Nevertheless, their paper chromatographic approach could not provide further information on the identity of these oligosaccharides. The identification of *O*3-branched isomalto-oligosaccharides released by *endo*-dextranase hydrolysis and the release of different mixed-linkage or branched oligosaccharides by *endo*-mutanase hydrolysis from water-insoluble glucans from *Streptococcus* spp. is first reported herein.

3.3. High-performance size exclusion chromatography

Water-insoluble glucans produced by SSAL4545, SMUT1101, and SMUT1102 as well as their water-insoluble residues after *endo*-dextranase and *endo*-mutanase hydrolyses were analyzed by HPSEC-RI (Fig. 4). Glucans produced by SSAL4540 and SSAL4550 could not be dissolved in DMSO/100 mM LiCl, so size exclusion chromatography was not possible.

The elugram of untreated glucans produced by SMUT1102 showed an intense peak at low elution volumes, indicating that these polysaccharides had a high molecular weight of at least 2000 kDa (dextran blue). *Endo*-dextranase hydrolysis reduced the elution volume of the SMUT1102 glucans significantly and the main fraction of the insoluble residue eluted between 64.3 and 12 kDa. *Endo*-mutanase hydrolysis led to water-insoluble products with a broad molecular weight distribution: The high molecular weight fraction was reduced in size and a new fraction with a lower molecular weight (between 64.3 and 12 kDa) was formed. Therefore, *endo*-mutanase hydrolysis not only resulted in water-soluble oligosaccharides (Section 3.2) but also in water-insoluble glucans with a lower molecular weight. The incomplete hydrolysis might be

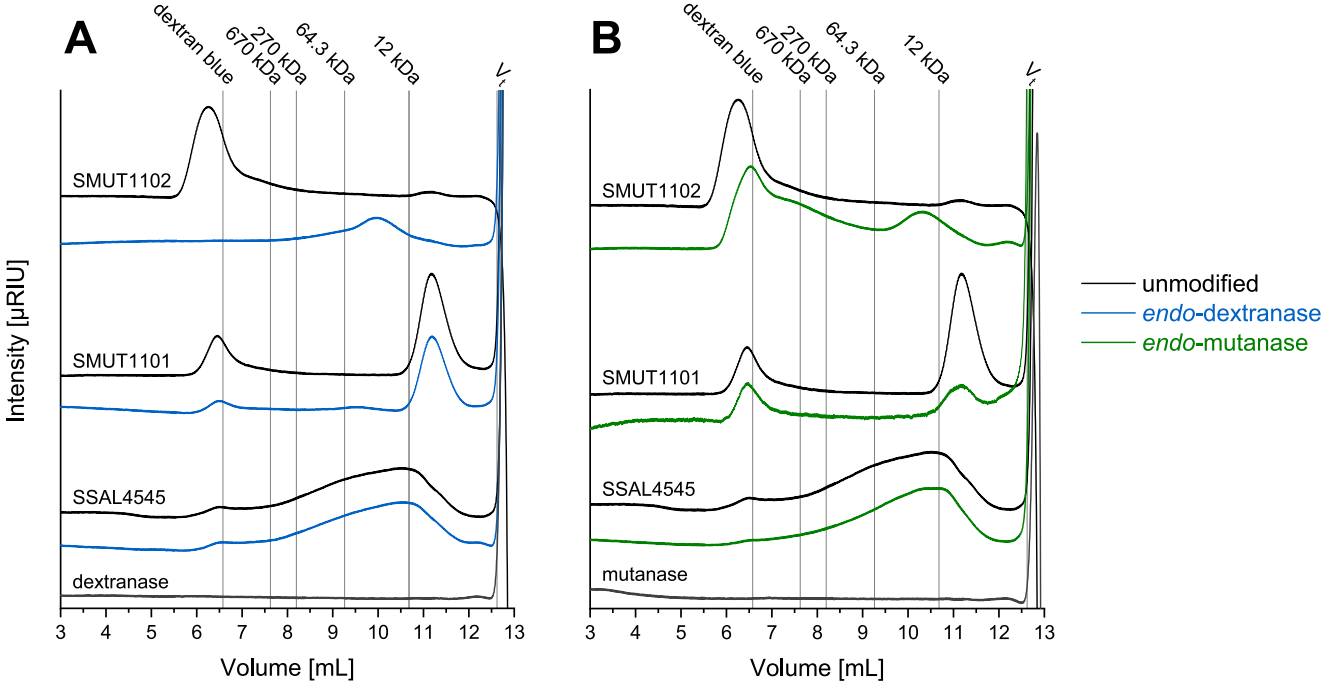

Fig. 4. HPSEC-RI elugrams of glucans produced by SSAL4545, SMUT1101, and SMUT1102 as well as their water-insoluble residues after *endo*-dextranase (A) and *endo*-mutanase (B) hydrolyses. Elugrams are shown in stacked format and peak maxima of size standards with known molecular weight are indicated by reference lines (V_t was determined by using glucose). The concentrations of injected samples differed because varying amounts of undried glucans were used for enzymatic hydrolyses (Section 2.4). Elugrams of both enzymes without glucan are displayed for comparison.

a result of branched 1,3-linked areas. However, the comparably small molecular weight shift after *endo*-mutanase hydrolysis as well as the elugrams from *endo*-dextranase hydrolysis suggested that 1,6-linked blocks had a high molecular weight, whereas 1,3-linked blocks were rather small. Therefore, our results allowed the conclusion that glucans of SMUT1102 were most likely block copolymers of 1,3- and 1,6-linked blocks.

Glucans produced by SMUT1101 contained two fractions, one at low elution volumes (below 12 kDa) and one at high elution volumes (ca. 2000 kDa). Hydrolysis with *endo*-dextranase reduced the intensity of the early-eluting peak and led to the occurrence of a peak resulting from smaller products (between 670 kDa and 12 kDa). The incomplete hydrolysis of the early-eluting, high molecular weight fraction demonstrated that SMUT1101 and SMUT1102 had different fine structures which was in good agreement with the results from the other analyses. *Endo*-mutanase hydrolyzed the low molecular weight fraction because its peak intensity decreased relative to the high molecular weight fraction. In conclusion, the high molecular weight fraction of SMUT1101 glucans contained a high portion of consecutive 1,6-linkages and the low molecular weight fraction contained a high portion of consecutive 1,3-linkages. However, both glucan molecular weight fractions were incompletely hydrolyzed, indicating that both also contained other backbone linkages or were branched.

Glucans produced by SSAL4545 exhibited a broad size distribution (ranging from ca. 2000 kDa to lower than 12 kDa) with the highest intensity at 12 kDa. Neither *endo*-dextranase nor *endo*-mutanase hydrolysis changed the elution pattern which indicated that the release of water-soluble products by *endo*-mutanase (Section 3.2) did not influence the molecular weight of the remaining water-insoluble fractions and only a small part of the glucans was hydrolyzed. Because the SSAL4545 glucans were (almost) exclusively 1,3-linked, this result was quite surprising. However, the incomplete hydrolysis could also result from limited enzymatic accessibility of the glucans.

Overall, glucans of glucansucrases SMUT1102, SMUT1101, and SSAL4545 showed varying molecular sizes and size distributions. Despite high portions of 1,3- and partly 1,6-linkages, glucans were not completely hydrolyzed to oligosaccharides by *endo*-mutanase or *endo*-dextranase. This might be due to low enzymatic accessibility, small sizes of the differently linked blocks and/or due to ramifications within the blocks of consecutive 1,3- or 1,6-linkages.

3.4. X-ray diffraction of undried glucans

For information on crystalline areas of the native glucans, they were used in an undried state to avoid changes in the crystalline structure during drying. Undried glucans were prepared on a sample holder covered with tape to avoid drying during the XRD measurement. Resulting diffractograms (Fig. 5) were compared with regards to peaks emerging from the amorphous background/halo, which indicate crystalline structures, and diffraction angles which depend on the unit cell structure. The diffractograms of glucans produced by SMUT1102 and SSAL4550 showed no peaks which indicated that these polysaccharides were largely amorphous or at least did not contain large enough ordered crystalline regions. Diffractograms of glucans produced by SSAL4540, SSAL4545, and SMUT1101 exhibited several peaks. Peak positions of glucans SMUT1101 and SSAL4545 were comparable to those described by Kobayashi et al. (2017) for the linear 1,3-linked glucans produced by GTF-J of *S. salivarius* ATCC25975. In contrast, glucans produced by SSAL4540 exhibited peaks at different angles, thus this sample contained another crystal polymorph than glucans produced by SMUT1101 and SSAL4545. This indicated that SSAL4540 glucans were structurally different from SMUT1101 and SSAL4545 glucans. However, the signals observed for SMUT1101 and SSAL4540 glucans indicated that mixed-linkage glucans from *Streptococcus* spp. may also contain crystalline areas. This finding was confirmed by ^{13}C MAS NMR spectroscopy for SMUT1101 glucans but not for SSAL4540 glucans (see Section 3.5). In the literature, indications of crystallinity in mixed-linkage glucans have only been found for water-insoluble glucans produced by a glucansucrase from *Leuconostoc citreum* (Wangpaiboon et al., 2020). The crystal structures of the polymorphs remain to be elucidated.

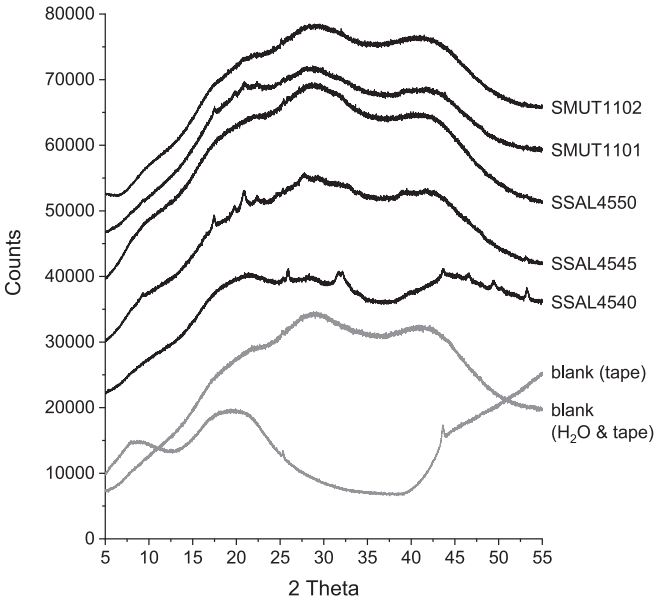

Fig. 5. X-ray diffractograms of glucans produced by SSAL4540, SAL4545, SSAL4550, SMUT1101, and SMUT1102 in an undried state (never dried or frozen). Sample diffractograms are shown in stacked format (+15,000 counts shift for SSAL4540; + 8000 counts shift for all other glucans) together with unshifted blank measurements (sample holder with tape as well as sample holder with tape and water, prepared as for the samples).

3.5. ^{13}C MAS NMR spectroscopy

In addition to XRD, ^{13}C MAS NMR spectra of the undried glucans were recorded to obtain information on potentially crystalline regions. The short-CP spectra (contact time 1.5 ms) of glucans produced by SSAL4545, SMUT1102, and SMUT1101 are shown in Fig. 6. The signals observed in the spectrum of glucans produced by SSAL4545 were assigned to 1,3-linked Glc*p* units by comparing their chemical shifts to literature data (Kobayashi et al., 2010; Kobayashi et al., 2017; Poulhazan, Arnold, Warschawski, & Marcotte, 2018). The rather sharp peaks

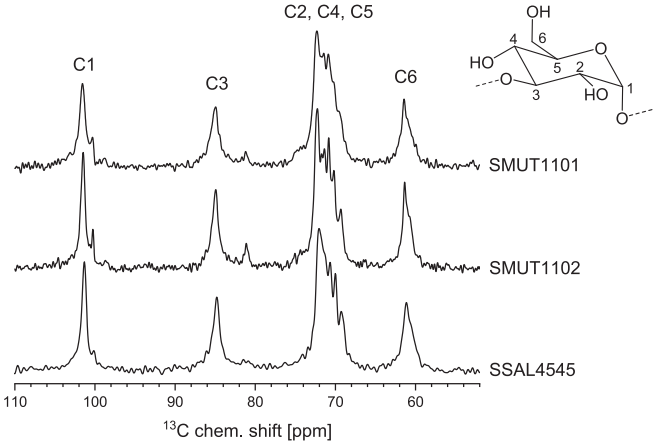

Fig. 6. ^{13}C MAS CP NMR spectra of undried glucans produced by glucansucrases SSAL4545, SMUT1101, and SMUT1102. Spectra are shown in stacked format. Peaks were assigned by comparison to literature (Kobayashi et al., 2010; Kobayashi et al., 2017; Poulhazan et al., 2018).

confirmed that 1,3-linked Glc*p* units form at least crystal-like (rather than glassy amorphous) regions. The spectra of glucans produced by SMUT1101 and SMUT1102 showed additional peaks at 81.1 ppm and 100.3 ppm which might arise from 1,3,6-Glc*p* units within the 1,3-linked sections. Both glucans contained 1,6-Glc*p* units in addition to 1,3-Glc*p* units, but no signal resulting from 1,6-Glc*p* units was found in the spectra (C6: ~ 65 ppm, C3: ~ 74 ppm; McIntyre & Vogel, 1991; Pidoux, Brooker, Colquhoun, & Morris, 1990). However, peaks of 1,6-Glc*p* units appeared in the corresponding DP spectra. Exemplarily, the CP and DP spectra of glucans from SMUT1101 are depicted in Fig. 7. This confirmed that 1,6-Glc*p* units were located in the mobile regions of the glucans. Overall, SMUT1101 and SMUT1102 glucans formed crystalline and/or crystal-like regions. These regions were exclusively formed by consecutive 1,3-linkages while 1,6-linked, dextran-like blocks remained mobile. The absence of peaks in the X-ray diffractogram of glucans produced by SMUT1102 might be due to small size crystal-like regions. To the best of our knowledge, mixed-linkage α-glucans have not been analyzed by ^{13}C MAS NMR spectroscopy before.

In contrast to DP spectra, CP NMR spectra of SSAL4540 glucans contained no peak which indicated, that these glucans had only mobile, hydrated residues and did not have sections with well-defined, crystal-like local packing environments. This was in contrast to the diffractograms from XRD analysis showing peaks that presumably resulted from crystalline glucan structures. This result suggested that the peaks observed in the X-ray diffractograms might not originate from glucans but from impurities. However, crystallization of glucansucrases was unlikely, as these large enzymes are difficult to crystallize (Molina, Cioci, Moulis, Séverac, & Remaud-Siméon, 2021). Here, further studies on the crystalline structure of mixed-linkage water-insoluble glucans should be performed. Spectra of SSAL4550 glucans did not show signals resulting from polysaccharides which is presumably due to low yields. However, no crystallinity is expected in the 1,6-linked SSAL4550 glucans because this linkage type was not found in the crystal-like regions of glucans SMUT1101 and SMUT1102.

3.6. Overall structure of the individual glucans

The overall structures of glucans are shown schematically in Fig. S5.

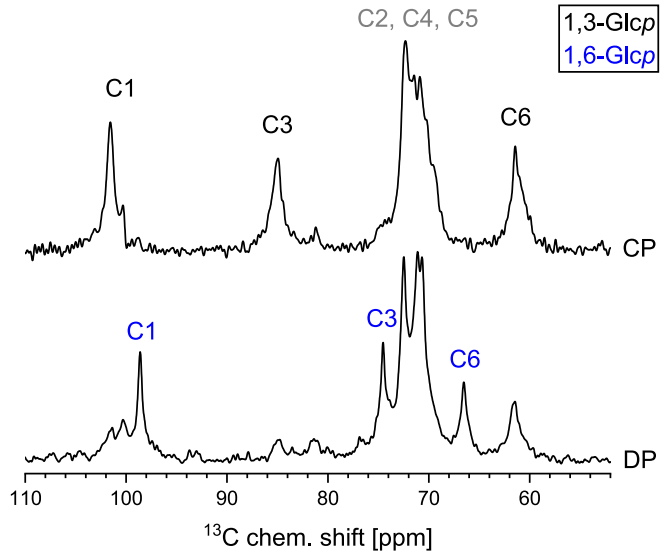

Fig. 7. ^{13}C MAS CP and DP NMR spectra of undried glucans produced by glucansucrase SMUT1101. Peaks were assigned to carbon atoms of Glc*p* units by comparison to literature (McIntyre & Vogel, 1991; Pidoux, Ruiter, Brooker, Colquhoun, & Morris, 1990; Kobayashi et al., 2010; Kobayashi et al., 2017; Poulhazan et al., 2018). Spectra are shown in stacked format. CP: cross polarization, DP: direct polarization, Glc*p*: glucopyranose.

Glucans produced by SMUT1101 and SMUT1102 contained comparable portions of 1,3- and 1,6-linked Glc*p* units as well as 1,3,6-linked Glc*p* units. Nevertheless, *endo*-dextranase and *endo*-mutanase hydrolyses revealed different fine structures: SMUT1101 glucans contained a high molecular weight fraction with high portions of consecutive α-1,6-linkages and a comparatively low molecular weight fraction with a high portion of consecutive 1,3-linkages. The results from XRD and ^{13}C MAS NMR spectroscopy indicated crystalline regions of 1,3-linked Glc*p* units. Partial enzymatic hydrolyses showed that SMUT1102 glucans contained long consecutive α-1,6-linked blocks and short α-1,3-linked blocks. The X-ray diffractogram of these high molecular weight glucans did not show any peaks, thus, large crystalline regions were absent. However, ^{13}C MAS NMR spectra demonstrated that 1,3-linked Glc*p* units were part of crystal-like regions while 1,6-linked areas were mobile.

Glucans produced by SSAL4550 were identified as linear dextrans which were water-insoluble but showed no indications of crystallinity.

Glucans produced by SSAL4545 were almost exclusively composed of 1,3-linked Glc*p* units, which formed crystalline regions according to XRD and ^{13}C MAS NMR spectroscopy.

SSAL4540 synthesized glucans which contained 1,3-, 1,4- and 1,6-linkages. Larger blocks with consecutive 1,6-linkages were most likely absent, because *endo*-dextranase did not release any oligosaccharides. Analogously, there were no consecutive 1,4-linkages because α-amylase did not hydrolyze the glucans (data not shown). However, *endo*-mutanase demonstrated that some consecutive 1,3-linkages were present. The X-ray diffractogram yielded some peaks which differed from those observed in the diffractograms of SSAL4545 and SMUT1101 glucans and the literature. However, the absence of signals in the CP ^{13}C MAS NMR spectrum indicated that the signals might not originate from the polysaccharides. Furthermore, SSAL4540 glucans were not solubilized in DMSO/100 mM LiCl. Therefore, the unusual structural composition led to varying physicochemical properties.

4. Conclusion

Our results demonstrated that the water-insoluble glucans formed by the five glucansucrases from *S. mutans* DSM 20523 and *S. salivarius* DSM 20560 have a high structural diversity and, thus, confirm our hypothesis. Glucans produced by glucansucrases SMUT1101 and SMUT1102 contained similar portions of 1,6- and 1,3-linkages, but clearly differed in their fine structure (distribution of linkage types, oligosaccharide profile, molecular weight). Both polymers showed crystal-like regions which are formed by consecutive 1,3-linkages. To our knowledge, there has been no literature report on the crystal-like structural elements in mixed-linkage water-insoluble α-glucans produced by glucansucrases from *Streptococcus* spp. In addition, completely linear, non-crystalline dextrans and mainly 1,3-linked mutans were produced by using the glucansucrases SSAL4550 and SSAL4545, respectively. The latter glucans were (in part) crystalline, which was in accordance with previously described linear 1,3-linked glucans (Kobayashi et al., 2017; Ogawa et al., 1994). The existence of water-insoluble α-glucans (glucansucrase SSAL4540) with 1,6-, 1,3-, and 1,4-linkages within the backbone was also demonstrated for the first time. Furthermore, we can conclude that the fine structures of the mixed-linkage glucans are very diverse and that the formation of crystalline or crystal-like areas is dependent on the molecular structure. Altogether, the combination of several analytical methods yielded detailed information on the fine structures of the glucans investigated.

CRediT authorship contribution statement

Luise Ernst: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Celine Schulz:** Investigation. **Albrecht Petzold:** Writing – review & editing, Investigation, Formal analysis. **Thomas Thurn-Albrecht:** Writing – review & editing, Resources. **Kay Saalwächter:** Writing – review & editing,

Resources. **Daniel Wefers:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

The authors are very grateful to Alexey Krushelnitsky (Institute of Physics, NMR, Martin Luther University Halle-Wittenberg) for NMR measurements and technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2024.122164.

References

Aires, C. P., Koo, H., Sassaki, G. L., Iacomini, M., & Cury, J. A. (2010). A procedure for characterizing glucans synthesized by purified enzymes of cariogenic *Streptococcus mutans*. *International Journal of Biological Macromolecules, 46*, 551–554.

Bowen, W. H., & Koo, H. (2011). Biology of *Streptococcus mutans*-derived glucosyltransferases: Role in extracellular matrix formation of cariogenic biofilms. *Caries Research, 45*, 69–86.

Drucker, D. B., Shakespeare, A. P., & Green, R. M. (1984). The production of dental plaque and caries by the bacterium *Streptococcus salivarius* in gnotobiotic WAG/RIJ rats. *Archives of Oral Biology, 29*, 437–443.

Ernst, L., Offermann, H., Werner, A., & Wefers, D. (2024). Comprehensive structural characterization of water-soluble and water-insoluble homoexopolysaccharides from seven lactic acid bacteria. *Carbohydrate Polymers, 323*, Article 121417.

Ernst, L., Werner, A., & Wefers, D. (2023). Influence of ultrasonication and hydrolysis conditions in methylation analysis of bacterial homoexopolysaccharides. *Carbohydrate Polymers, 308*, Article 120643.

Fels, L., Jakob, F., Vogel, R. F., & Wefers, D. (2018). Structural characterization of the exopolysaccharides from water kefir. *Carbohydrate Polymers, 189*, 296–303.

Forssten, S. D., Björklund, M., & Ouwehand, A. C. (2010). *Streptococcus mutans*, caries and simulation models. *Nutrients, 2*, 290–298.

Hanada, N., & Kuramitsu, H. K. (1988). Isolation and characterization of the *Streptococcus mutans* gtfC gene, coding for synthesis of both soluble and insoluble glucans. *Infection and Immunity, 56*, 1999–2005.

Hanada, N., & Kuramitsu, H. K. (1989). Isolation and characterization of the *Streptococcus mutans* gtfD gene, coding for primer-dependent soluble glucan synthesis. *Infection and Immunity, 57*, 2079–2085.

Hare, M. D., Svensson, S., & Walker, G. J. (1978). Characterization of the extracellular, water-insoluble α-D-glucans of oral *streptococci* by methylation analysis, and by enzymic synthesis and degradation. *Carbohydrate Research, 66*, 245–264.

Hayacibara, M. F., Koo, H., Vacca-Smith, A. M., Kopec, L. K., Scott-Anne, K., Cury, J. A., & Bowen, W. H. (2004). The influence of mutanase and dextranase on the production and structure of glucans synthesized by streptococcal glucosyltransferases. *Carbohydrate Research, 339*, 2127–2137.

He, Q., Kobayashi, K., Kusumi, R., Kimura, S., Enomoto, Y., Yoshida, M., … Wada, M. (2020). In vitro synthesis of branchless linear (1→ 6)-α-D-glucan by glucosyltransferase K: Mechanical and swelling properties of its hydrogels crosslinked with diglycidyl ethers. *ACS Omega, 5*, 31272–31280.

Hoshino, T., Fujiwara, T., & Kawabata, S. (2012). Evolution of cariogenic character in *Streptococcus mutans*: Horizontal transmission of glycosyl hydrolase family 70 genes. *Scientific Reports, 2*, 518.

Inoue, M., Yakushiji, T., Katsuki, M., Kudo, N., & Koga, T. (1988). Reduction of the adherence of *Streptococcus sobrinus* insoluble alpha-D-glucan by *endo*-(1,3)- α -D-glucanase. *Carbohydrate Research, 182*, 277–286.

Inoue, M., Yakushiji, T., & Takehara, T. (1982). Ultrastructural changes produced in the oral bacterium *Streptococcus mutans* glucans by the action of *endo*- α −1,3-glucanase. *Archives of Oral Biology, 27*, 753–757.

Kobayashi, K., Hasegawa, T., Kusumi, R., Kimura, S., Yoshida, M., Sugiyama, J., & Wada, M. (2017). Characterization of crystalline linear (1→3)-α-d-glucan synthesized in vitro. *Carbohydrate Polymers, 177*, 341–346.

Kobayashi, K., Kimura, S., Heux, L., & Wada, M. (2013). Crystal transition between hydrate and anhydrous (1→3)-β-D-xylan from *Penicillus dumetosus*. *Carbohydrate Polymers, 97*, 105–110.

Kobayashi, K., Kimura, S., Togawa, E., Wada, M., & Kuga, S. (2010). Crystal transition of paramylon with dehydration and hydration. *Carbohydrate Polymers, 80*, 491–497.

Koo, H., Falsetta, M. L., & Klein, M. I. (2013). The exopolysaccharide matrix: A virulence determinant of cariogenic biofilm. *Journal of Dental Research, 92*, 1065–1073.

Kopec, L. K., Vacca-Smith, A. M., & Bowen, W. H. (1997). Structural aspects of glucans formed in solution and on the surface of hydroxyapatite. *Glycobiology, 7*, 929–934.

Kuramitsu, H. K., & Wondrack, L. (1983). Insoluble glucan synthesis by *Streptococcus mutans* serotype c strains. *Infection and Immunity, 42*, 763–770.

Kusumi, R., Asai, S., He, Q., Kobayashi, K., Kimura, S., Yoshida, M., & Wada, M. (2023). α-D-(1→ 3)-graft-(1→ 6)-glucan: Comb-like polysaccharide synthesized *in vitro* with α-1, 3/1, 6-glucosyltransferase L from *Streptococcus salivarius*. *Carbohydrate Research, 534*, Article 108969.

Leemhuis, H., Pijning, T., Dobruchowska, J. M., van Leeuwen, S. S., Kralj, S., Dijkstra, B. W., & Dijkhuizen, L. (2013). Glucansucrases: Three-dimensional structures, reactions, mechanism, α-glucan analysis and their implications in biotechnology and food applications. *Journal of Biotechnology, 163*, 250–272.

Leme, A. P., Koo, H., Bellato, C. M., Bedi, G., & Cury, J. A. (2006). The role of sucrose in cariogenic dental biofilm formation - new insight. *Journal of Dental Research, 85*, 878–887.

Li, X., Wang, X., Meng, X., Dijkhuizen, L., & Liu, W. (2020). Structures, physico-chemical properties, production and (potential) applications of sucrose-derived α-D-glucans synthesized by glucansucrases. *Carbohydrate Polymers, 249*, Article 116818.

Lin, Y., Chen, J., Zhou, X., & Li, Y. (2021). Inhibition of *Streptococcus mutans* biofilm formation by strategies targeting the metabolism of exopolysaccharides. *Critical Reviews in Microbiology, 47*, 667–677.

McIntyre, D. D., & Vogel, H. J. (1991). Nuclear magnetic resonance studies of homopolysaccharides related to starch. *Starch-Starke, 43*, 69–76.

Meng, X., Gangoiti, J., Bai, Y., Pijning, T., Van Leeuwen, S. S., & Dijkhuizen, L. (2016). Structure-function relationships of family GH70 glucansucrase and 4,6-a-glucano-transferase enzymes, and their evolutionary relationships with family GH13 enzymes. *Cellular and Molecular Life Sciences, 73*, 2681–2706.

Minah, G. E., & Loesche, W. J. (1977). Sucrose metabolism by prominent members of the flora isolated from cariogenic and non-cariogenic dental plaques. *Infection and Immunity, 17*, 55–61.

Molina, M., Cioci, G., Moulis, C., Séverac, E., & Remaud-Siméon, M. (2021). Bacterial α-glucan and branching sucrases from GH70 family: Discovery, structure–function relationship studies and engineering. *Microorganisms, 9*, 1607.

Monsan, P., Bozonnet, S., Albenne, C., Joucla, G., Willemot, R.-M., & Remaud-Siméon, M. (2001). Homopolysaccharides from lactic acid bacteria. *International Dairy Journal, 11*, 675–685.

Münkel, F., Bechtner, J., Eckel, V., Fischer, A., Herbi, F., Jakob, F., & Wefers, D. (2019). Detailed structural characterization of glucans produced by glucansucrases from *Leuconostoc citreum* TMW 2.1194. *Journal of Agricultural and Food Chemistry, 67*, 6856–6866.

Münkel, F., & Wefers, D. (2019). Fine structures of different dextrans assessed by isolation and characterization of endo-dextranase liberated isomalto-oligosaccharides. *Carbohydrate Polymers, 215*, 296–306.

Ogawa, K., Yui, T., Okamura, K., & Misaki, A. (1994). Crystalline features of streptococcal (l→3)- α -D-glucans of human saliva. *Bioscience, Biotechnology, and Biochemistry, 58*, 1326–1327.

Padmanabhan, P. A., Kim, D.-S., Pak, D., & Sim, S. J. (2003). Rheology and gelation of water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523. *Carbohydrate Polymers, 53*, 459–468.

Pearce, B. J., Walker, G. J., Slodki, M. E., & Schuerch, C. (1990). Enzymic and methylation analysis of dextrans and 1,3-a-D-glucans. *Carbohydrate Research, 203*, 229–246.

Pidoux, M., Ruiter, G. A. de, Brooker, B. E., Colquhoun, I. J., & Morris, V. J. (1990). Microscopic and chemical studies of a gelling polysaccharide from *Lactobacillus hilgardii*. Carbohydrate Polymers, 13, 351–362.

Pittrof, S. L., Kaufhold, L., Fischer, A., & Wefers, D. (2021). Products released from structurally different dextrans by bacterial and fungal dextranases. *Foods, 10*.

Poulhazan, A., Arnold, A. A., Warschawski, D. E., & Marcotte, I. (2018). Unambiguous ex situ and in cell 2D ^{13}C solid-state NMR characterization of starch and its constituents. *International Journal of Molecular Sciences, 19*, 3817.

Robert, X., & Gouet, P. (2014). Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Research, 42*, W320–W324.

Sievers, F., & Higgins, D. G. (2014). Clustal omega. *Current Protocols in Bioinformatics, 48*, 3–13.

Simpson, C. L., Cheetham, N. W. H., Giffard, P. M., & Jacques, N. A. (1995). Four glucosyltransferases, GtfJ, GtfK, GtfL and GtfM, from *Streptococcus salivarius* ATCC 25975. *Microbiology, 141*, 1451–1460.

Sweet, D. P., Shapiro, R. H., & Albersheim, P. (1975). Quantitative analysis by various g. l.c. response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydrate Research, 40*, 217–225.

Takahashi, N., & Nyvad, B. (2008). Caries ecology revisited: Microbial dynamics and the caries process. *Caries Research, 42*, 409–418.

Tsumuraya, Y., & Misaki, A. (1979). Structure of the water-insoluble α-D-glucan of *Streptococcus salivarius* HHT. *Carbohydrate Research, 74*, 217–225.

Vacca-Smith, A. M., Venkitaraman, A. R., Quivey, R. G., Jr., & Bowen, W. H. (1996). Interactions of streptococcal glucosyltransferases with α-amylase and starch on the surface of saliva-coated hydroxyapatite. *Archives of Oral Biology, 41*, 291–298.

Wangpaiboon, K., Waiyaseesang, N., Panpetch, P., Charoenwongpaiboon, T., Nepogodiev, S. A., Ekgasit, S., … Pichayangkura, R. (2020). Characterisation of insoluble α-1,3-/α-1,6 mixed linkage glucan produced in addition to soluble α-1,6-linked dextran by glucansucrase (DEX-N) from *Leuconostoc citreum* ABK-1. *International Journal of Biological Macromolecules, 152*, 473–482.

Wiater, A., Pleszczyńska, M., Próchniak, K., & Szczodrak, J. (2012). Structural diversity of streptococcal mutans synthesized under different culture and environmental conditions and its effect on mutanase synthesis. *Molecules, 17*, 11800–11815.

Xu, R. R., Yang, W. D., Niu, K. X., Wang, B., & Wang, W. M. (2018). An update on the evolution of glucosyltransferase (Gtf) genes in *Streptococcus*. *Frontiers in Microbiology, 9*, 2979.

Yu, L., Qian, Z., Ge, J., & Du, R. (2022). Glucansucrase produced by lactic acid bacteria: Structure, properties, and applications. *Fermentation, 8*, 629.