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Influence of ultrasonication and hydrolysis conditions in methylation analysis of bacterial homoexopolysaccharides

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ABSTRACT

Homoexopolysaccharides (HoEPS) such as α-glucans and β-fructans are synthesized by lactic and acetic acid bacteria. Methylation analysis is an important and well-established tool for the structural analysis of these polysaccharides, however, multiple steps are required for polysaccharide derivatization. Because ultrasonication during methylation and the conditions during acid hydrolysis may influence the results, we investigated their role in the analysis of selected bacterial HoEPS. The results reveal that ultrasonication is crucial for water insoluble α-glucan to swell/disperse and deprotonate prior to methylation whereas it is not necessary for water soluble HoEPS (dextran and levan). Complete hydrolysis of permethylated α-glucans requires 2 M trifluoroacetic acid (TFA) for 60/90 min at 121 °C while levan is hydrolyzed in 1 M TFA for 30 min at 70 °C. Nevertheless, levan was also detectable after hydrolysis in 2 M TFA at 121 °C. Thus, these conditions can be used to analyze a levan/dextran mixture. However, size exclusion chromatography of permethylated and hydrolyzed levan showed degradation and condensation reactions at harsher hydrolysis conditions. Application of reductive hydrolysis with 4-methylmorpholine-borane and TFA did not lead to improved results. Overall, our results demonstrate that conditions used for methylation analysis have to be adjusted for the analysis of different bacterial HoEPS.

1. Introduction

The most important bacterial homoexopolysaccharides (HoEPS) are α-glucans and β-fructans which are enzymatically synthesized by glucansucrases and fructansucrases from sucrose (Leemhuis et al., 2013; Meng et al., 2016; van Hijum et al., 2006; Xu et al., 2019). The most common α-glucan is dextran, which is composed of a backbone of 1,6-linked D-glucopyranoses (Glc*p*) with ramifications at different positions (Monsan et al., 2001; Zannini et al., 2016). Usually dextran is water soluble, however, completely linear α-1,6-linked glucan is water insoluble (Padmanabhan et al., 2003; Pittrof et al., 2021). Furthermore, glucans containing sequences of α-1,3-linked Glc*p*, so-called mutans, are also found to be water insoluble (Côté & Leathers, 2009). β-D-Fructans are mostly water soluble and classified into levan (2,6-linked fructofuranoses (Fru*f*)) and inulin (1,2-linked Fru*f*) (Zannini et al., 2016). Levan and inulin may also be branched at position *O*1 or position *O*6, respectively. To understand the physicochemical properties of bacterial HoEPS, it is important to elucidate their molecular structure.

One part of a complete polysaccharide characterization is the examination of glycosidic linkages by using methylation analysis, which is based on the analysis of partially methylated alditol acetates (PMAAs) by gas chromatography (GC). Polysaccharides are converted into PMAAs by methylation, hydrolysis, reduction with concomitant deuterium labelling of the anomeric carbon atoms, and acetylation. Methylation analysis has its origins in the alkylation procedures of Purdie and Irvine (1903) and Haworth (1915), which were improved by Hakamori (1964) and optimized by Ciucanu and Kerek (1984) in terms of higher permethylation yields. Identification of PMAAs by mass spectrometry (MS) after GC separation was introduced by Björndal et al. (1967) and a one-tube derivatization procedure was developed by Harris et al. (1984). Today, methylation analysis is still a valuable method for the identification and semiquantitative determination of glycosidic linkages (Pettolino et al., 2012; Sims et al., 2018).

A complete permethylation of the analyzed polysaccharides is a

Abbreviations: HoEPS, homoexopolysaccharides; PMAA, partially methylated alditol acetate; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; MMB, 4-methylmorpholine-borane; RI, refractive index; UV, ultraviolet; HPSEC, high performance size exclusion chromatography; Fru*f*, fructofuranose; Glc*p*, glucopyranose; MS, mass spectrometry; GC, gas chromatography; FID, flame ionization detector; HPLC, high performance liquid chromatography..

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crucial step to obtain meaningful results. Available methylation procedures are diverse and depend on the type of polysaccharide (Ciucanu, 2006; Hanisch, 1994; Jay, 1996). Methylation according to Ciucanu and Kerek (1984) is most commonly used for uncharged polysaccharides. This procedure involves the addition of powdered $NaOH_{(s)}$ and methyl iodide to a solution of the polysaccharide sample in DMSO. To overcome carbohydrate oxidation during methylation, treatment of the polysaccharide with $NaOH_{(s)}$ prior to addition of methyl iodide is important (Needs & Selvendran, 1993). Many authors use ultrasonic treatment for better swelling/dissolution of the polysaccharide and better dispersion of $NaOH_{(s)}$ in DMSO (Fels et al., 2018; Needs & Selvendran, 1993; Nunes & Coimbra, 2001; Pettolino et al., 2012; Wangpaiboon et al., 2020). Unfortunately, ultrasonication is also known to induce polysaccharide degradation and a concomitant decrease of molecular weight (Basedow & Ebert, 1975; Cui & Zhu, 2021; Huang et al., 2015; Kardos & Luche, 2001).

Another critical step in methylation analysis is acid hydrolysis of the extracted and dried permethylated polysaccharides. Several protocols for cleavage of permethylated polysaccharide into partially methylated monosaccharides exist, amongst others formolysis, reductive hydrolysis, and hydrolysis in sulfuric acid or trifluoroacetic acid (TFA) (Hanisch, 1994; Jay, 1996). Hydrolysis in diluted TFA is widely used, because it offers an important practical advantage: TFA can be removed by evaporation prior to reduction with $NaBD_4$ which requires alkaline conditions (Biermann, 1988; Pettolino et al., 2012). By using 1-methylimidazole as a catalyst, the subsequent acetylation of the partially methylated alditols can then be achieved without additional transfer or evaporation steps (Blakeney et al., 1983). Polysaccharide hydrolysis is supposed to cleave all glycosidic linkages without degradation of the liberated monosaccharides. However, the rates of hydrolysis and degradation depend on the type of ring form (pyranose or furanose) as well as the type of glycosidic linkages (e.g. α- or β-glycosidic linkages). Thus, optimal conditions for hydrolysis vary depending on the polysaccharide.

For bacterial HoEPS, different hydrolysis procedures are described in the literature. Permethylated fructans were hydrolyzed with 1 M TFA for 30 min at 70 °C or 2 M TFA for 30 min at 60 °C (Carpita et al., 1991; Pollock et al., 1979) whereas permethylated glucans were hydrolyzed in 2 M TFA for 1,5–2 h at 121 °C, in 2 M TFA for 1 h at 125 °C or 2,5 M TFA for 4 h at 100 °C (Fels et al., 2018; Kralj et al., 2004; Maina et al., 2011; Wangpaiboon et al., 2018). An alternative procedure for the hydrolysis of labile monosaccharides is reductive acid hydrolysis, which can be achieved by the addition of a reducing agent such as 4-methylmorpholine-borane (MMB) to TFA hydrolysis (Garegg et al., 1988; Stevenson & Furneaux, 1991).

Based on the above considerations, we hypothesized that the results obtained from methylation analysis of bacterial HoEPS depend on the conditions used during derivatization. However, a systematic investigation of the influence of different sonication and hydrolysis procedures has not been conducted yet. Therefore, we applied different experimental conditions for methylation analysis of selected bacterial HoEPS and evaluated which conditions are suitable for their analysis.

2. Experimental

2.1. Materials

Levan was produced by fermentation of a sucrose solution with *Gluconobacter albidus* TMW 2.1191 and isolated as described by Hundschell et al. (2020). Water insoluble glucan was synthesized by glucansucrase Lc1215 from *Leuconostoc citreum* TMW 2.1194 (Münkel et al., 2019). Dextran was produced by dextransucrase from *Limosilactobacillus reuteri* TMW 1.106 (Münkel et al., 2020). The 50:50 (w/w)-mixture of levan and dextran was obtained by solubilizing 10 mg of each polysaccharide in ultrapure water, mixing and subsequent lyophilization. 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) and Grüssing GmbH (Filsum, Germany).

2.2. Methylation analysis

The basic protocol for methylation analysis was based on the methylation procedure described by Ciucanu and Kerek (1984), whereas hydrolysis, reduction, and acetylation were based on the procedures described by Albersheim et al. (1967); Carpita et al. (1991) and Blakeney et al. (1983). The procedure was slightly modified as described previously (Fels et al., 2018; Nunes & Coimbra, 2001) and carried out in glass culture tubes with PTFE lined screw caps. Unless stated otherwise, all steps and incubations were carried out under air. About 1 mg of each sample was swollen in 2 mL of dimethyl sulfoxide (DMSO) overnight and subsequently sonicated for 30 min (sonication 1) in an ultrasonic bath (35 kHz, 80 W; Sonorex RK 100H, Brandelin, Berlin, Germany). Ice cubes were occasionally added to the ultrasonic bath to avoid warming of the samples. After sonication, approximately 100 mg of dry $NaOH_{(s)}$ were freshly ground under argon atmosphere and added to the sample solutions/suspensions. Samples were sonicated for 90 min (sonication 2) as described above and subsequently incubated at room temperature for 90 min. Methylation was performed by adding 1 mL of methyl iodide. The reaction mixture was sonicated in the ultrasonic bath for 30 min (sonication 3) and subsequently incubated at room temperature for 30 min. Permethylated polysaccharides were extracted by adding 3 mL of dichloromethane and 5 mL of 0.1 M sodium thiosulfate. The organic layer was washed twice with ultrapure water. The solvent was evaporated and the samples were dried in a vacuum oven at 40 °C overnight. To ensure complete methylation, the methylation procedure was repeated once. The permethylated polysaccharides were hydrolyzed by adding 2 mL of 2 M TFA for 90 min at 121 °C (glucans) and 1 M TFA for 30 min at 70 °C (levan). TFA was removed by evaporation at 60 °C and 20 mg $NaBD_4$ in 2 M aqueous NH_3 (0.3 mL) were added to the residue. The sample was incubated at room temperature for 1 h. Subsequently, the reaction was stopped by adding glacial acetic acid (0.1 mL). For acetylation 3 mL of acetic anhydride and 450 µL of 1-methylimidazole were added under ice-cooling and samples were incubated for 30 min at room temperature. After addition of 3 mL of ultrapure water, PMAAs were extracted into 5 mL of dichloromethane and washed three times with ultrapure water. Residual water was removed by freezing at −20 °C overnight and the organic phase was analyzed as described in Section 2.4.

This standard protocol was varied regarding the ultrasonic treatments and hydrolysis conditions. Different combinations of the three ultrasonic treatments shown in Fig. 1 were used. Either all three sonications were applied or sonications 1 and 2, sonication 2, sonication 3 or

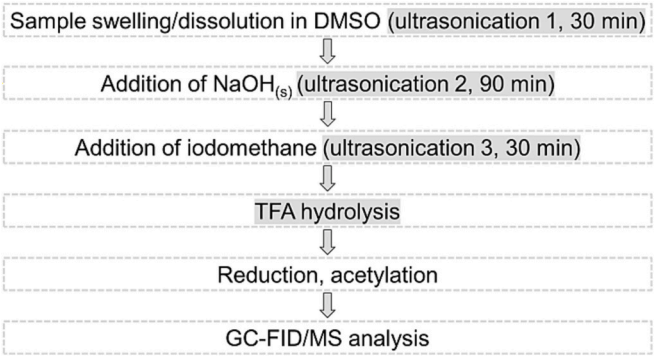

Fig. 1. Schematic derivatization protocol for methylation analysis. Parameters which were varied in this study are marked in gray. DMSO: dimethyl sulfoxide, TFA: trifluoroacetic acid, GC: gas chromatography, FID: flame ionization detector, MS: mass spectrometry.

all sonications steps were omitted. When sonication 1 was omitted, samples were vigorously shaken at about 3000 rpm. When sonication 2 of the sample was replaced, freshly ground $NaOH_{(s)}$ was added to 1 mL of DMSO in a separate tube and sonicated for 90 min. The obtained slurry was added to the sample which was dissolved/swollen in 1 mL instead of 2 mL of DMSO. Afterwards the sample with added NaOH dispersion was incubated at room temperature for 90 min. Sonication 3 was replaced by incubating at room temperature for the same duration. To investigate the influence of different hydrolysis conditions, TFA concentration, duration and temperature were varied: 1 M TFA was used at 70 °C for 30 min or at 121 °C for 90 min; 2 M TFA was used at 121 °C for 60 or 90 min; 3 M TFA at 121 °C for 90 min was solely used to hydrolyze the mixture of dextran and levan. All analyses were performed in duplicate.

2.3. Reductive hydrolysis of levan

Samples were methylated as described in the initial protocol and reductively hydrolyzed according to Stevenson and Furneaux (1991): A 80 mg/mL solution of MMB in ultrapure water was freshly prepared (the solution was heated to 50 °C to dissolve MMB). Subsequently, 50 μL of MMB solution and 0.2 mL of TFA were added to the dry methylated polysaccharides. The concentration of the added TFA solution was varied to achieve different final concentrations (0.5 and 1 M TFA). The samples were heated for 5 min to 70 °C or 80 °C (depending on the subsequent hydrolysis at 70 °C or 121 °C, respectively). After cooling to room temperature another 50 μL of MMB solution were added and samples were hydrolyzed for 30 or 60 min at 70 °C or for 90 min at 121 °C. Samples were cooled to room temperature and 100 μL of MMB solution were added. The solvent was removed by evaporation at 50 °C and 150 μL of 2 M NH_3 followed by $NaBD_4$ (20 mg in 0.3 mL 2 M NH_3) were added to ensure complete reduction. After incubating the samples for 1 h at room temperature, acetylation and PMAA extraction were performed as described in Section 2.2.

2.4. Gas chromatographic analysis

PMAAs in dichloromethane were analyzed by GC–MS and GC-FID. GC–MS (HP6890 + 5973, Agilent, Santa Clara, CA, USA) equipped with a VF5-MS column (30 m × 0.25 mm i.d., film thickness 0.25 μm, Agilent) with helium as carrier gas (constant flow at 1 mL/min) was used. Injector temperature was 220 °C and splitless injection was used. The following temperature program was applied: 80 °C held for 2 min; increased with 10 °C/min to 220 °C, held for 25 min; ramped at 40 °C/min to 260 °C, held for 5 min. The transfer line was held at 270 °C and mass spectra were recorded at 70 eV electron impact in full scan. GC (HP5890, Agilent) coupled to FID (HP5850, Agilent) was run under the same conditions as described for GC–MS except that the initial oven temperature was 140 °C (for 1 min). FID temperature was 240 °C. Identification of the PMAAs was based on their characteristic fragmentation patterns, which were interpreted by comparison to literature data (Carpita et al., 1989; CCRC – Complex Carbohydrate Research Center, 2022; Sassaki et al., 2005; Sassaki & Souza, 2013). Relative quantification of the PMAAs was carried out by using the molar response factors described by Sweet et al. (1975).

2.5. High performance size exclusion chromatography of methylated and hydrolyzed levan

Levan and fructose were methylated as described in Section 2.2 with three sonications. Hydrolysis was carried out by using different TFA-concentrations, durations, and temperatures (1 M TFA at 70 °C for 10, 20 and 30 min as well as 2 M TFA at 121 °C for 30, 60 and 90 min). Subsequently, samples were cooled on ice, evaporated and washed with 500 μL of ethanol twice. The washed samples were dried overnight at 40 °C in a vacuum oven, 500 μL of DMSO were added, and samples were shaken overnight at room temperature. The dissolved samples, as well as untreated fructose and levan were analyzed by high performance size exclusion chromatography coupled to ultraviolet and refractive index detection (HPSEC-UV-RI). An Agilent 1100 Series HPLC with variable wavelength detector (G1316A) and refractive index detector (1260 Infinity II) equipped with an DMSO-Phil-P-150 column (300 × 8 mm, 10 μm, AppliChrom, Oranienburg, Germany) was used. DMSO was used as eluent at a flow rate of 0.2 mL/min and column temperature was 75 °C. The RI detector was operated at 35 °C and UV detection was carried out at 420 nm.

3. Results and discussion

As described above, we assumed that ultrasonication and hydrolysis conditions can influence the results obtained from methylation analysis of bacterial HoEPS. To prove this hypothesis, we used three model HoEPS: A water insoluble α-glucan (produced by a recombinant glucansucrase from *Leuconostoc citreum*), a water soluble dextran (produced by a recombinant glucansucrase from *Limosilactobacillus reuteri*), and a water soluble levan (produced by fermentation with *Gluconobacter albidus*). These polysaccharides were chosen because they are representative for the types of HoEPS formed by lactic and acetic acid bacteria. During methylation analysis, ultrasonication could result in a reduction of molecular weight which would lead to higher portions of terminal units. Thus, we investigated whether an elimination of different ultrasonic treatments results in lower portions of terminal units. Furthermore, appropriate hydrolysis conditions are crucial for correct results: They should result in a complete hydrolysis of the polysaccharides with as little concomitant degradation of liberated monosaccharide units as possible. Therefore, we applied harsh conditions (2 M TFA, 121 °C for 60 and 90 min) which are usually used for all kinds of polysaccharides as well as 1 M TFA at 121 °C for 90 min and mild conditions (1 M TFA, 70 °C for 30 min) which are commonly used for fructans to elucidate how individual conditions influence reproducibility, portions of glycosidic linkages, and the ratio between terminal and branched units. The latter is usually expected to be around 1 and higher portions of terminal than branched units indicate an overestimation (Lindberg et al., 1973; Sims et al., 2018). However, higher portions of terminal units can also result from low molecular weight compounds in the polysaccharide samples or from variations in PMAA stability.

3.1. Ultrasonication

To evaluate the influence of sonication, we applied different combinations of the three ultrasonic treatments shown in Fig. 1 in methylation analysis of the three model HoEPS. The results are shown in Fig. 2 and will be discussed in the following sections.

3.1.1. Water insoluble glucan and dextran

Application of the standard protocol (three ultrasonic treatments) demonstrated that the water insoluble glucan used is composed of 60 % 1,6- and 24 % 1,3-linked Glc*p*. Terminal units account for 13 % and branched units (1,3,6-linked) for 3 %. This high portion of terminal units is most likely due to residual sucrose and/or low molecular weight glucans (Münkel et al., 2019). When different ultrasonication treatments were applied, the portions of the glycosidic linkages clearly varied. Notably, all samples which were derivatized without the second sonication treatment, show higher portions of terminal glucose units. Because the portions of branched units remained unaltered, these results indicated an overestimation of terminal units. The higher portion of terminal units may result from the fact that the low molecular weight compounds present in the sample are easily solubilized without sonication whereas the insoluble glucan is not. Therefore, application of the second ultrasonic treatment seems to be necessary for reliable results. Furthermore, the results suggest that the last ultrasonic treatment which

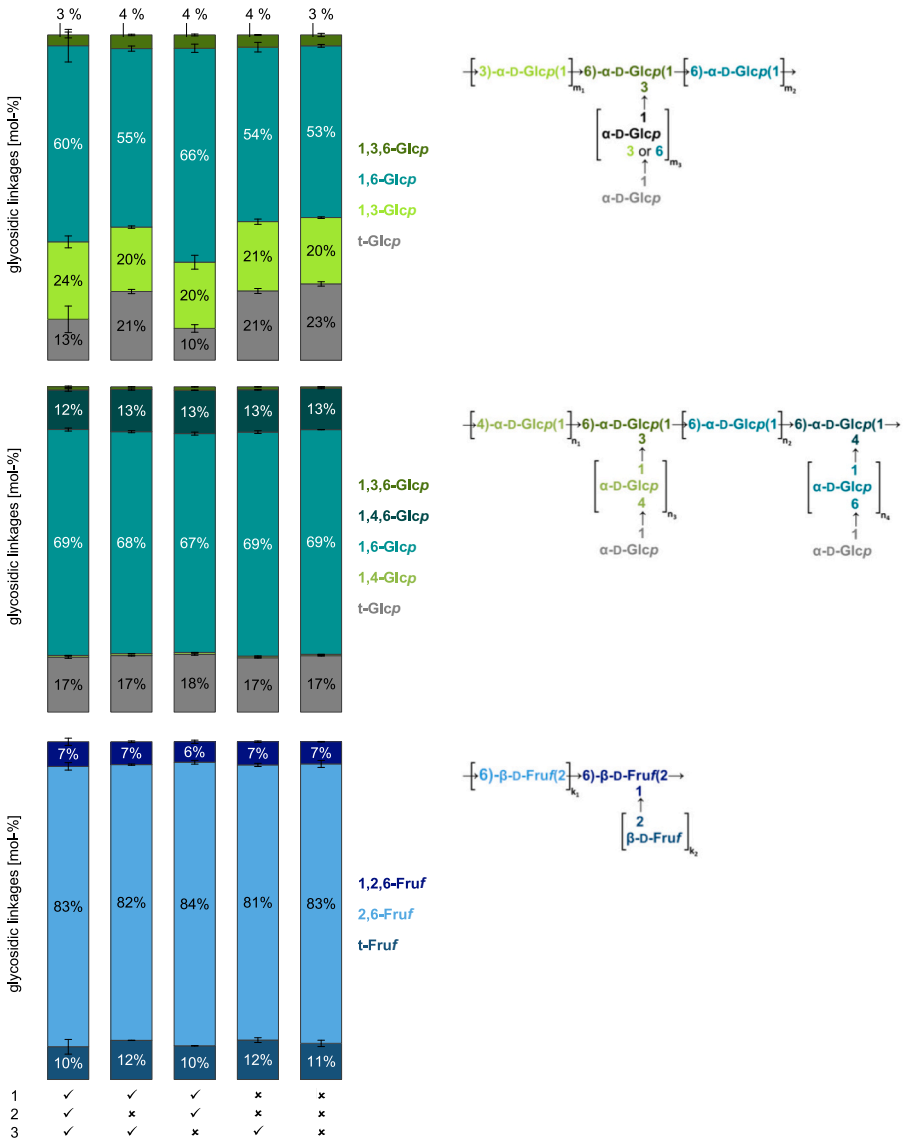

Fig. 2. Glycosidic linkages (mol-%) of levan (bottom), dextran (center) and water insoluble glucan (top) determined by methylation analysis. Ticks and crosses indicate the application of ultrasonic treatments 1, 2 and 3 (Fig. 1). All analyses were performed in duplicate and mean values with half range uncertainties are shown. Furthermore, schematic structures of the analyzed polysaccharides are shown. t: terminal, Glc*p*: glucopyranose, Fru*f*: fructofuranose, Numbers indicate the position of substitution in the monosaccharide unit.

is applied after addition of methyl iodide can be left out to increase reproducibility (compared to the standard protocol). Thus, dissolving and swelling of the polysaccharides seems to be of greater importance than mixing the suspension during the reaction with methyl iodide.

The results obtained for dextran by applying the standard protocol show that this polysaccharide is mainly composed of 1,6-linkages. Furthermore, 12 % of the monosaccharide units are 1,4,6-linked and a slightly higher portion of terminal units (17 %) were detected besides minor portions (≤1 %) of 1,4- and 1,3,6-linked units. Applying different combinations of the three ultrasonic treatments does not seem to have an influence on the results. Neither portions of the PMAAs nor reproducibility change. Thus, chain disruption of polysaccharides, which would result in higher portions of terminal units, does not occur to a significant extent.

3.1.2. Levan

As expected, the standard protocol demonstrated that levan is mainly 2,6-linked and has about 10 % terminal and 7 % 1,2,6-linked units. The application of different ultrasonic treatments does not seem to have significant influence on the results of methylation analysis. Notably, a slightly better reproducibility is obtained by leaving out at least one of the three ultrasonic treatments. However, our results suggest that ultrasonication is not a critical step for polysaccharides which are soluble in water and DMSO.

3.2. Hydrolysis

To completely cleave (permethylated) polysaccharides to monomers, appropriate hydrolysis conditions are crucial. Therefore, we used different hydrolysis conditions during methylation analysis of water insoluble glucan, dextran, and levan in the course of methylation analysis. The resulting portions of the glycosidic linkages are shown in Fig. 3.

3.2.1. Water insoluble glucan and dextran

The results clearly demonstrate that 2 M TFA is necessary to sufficiently hydrolyze water insoluble glucan and dextran. Here, high portions of terminal units were found after hydrolysis in 1 M TFA for 90 min at 121 °C, whereas no PMAAs were detected after mild hydrolysis with 1 M TFA for 30 min at 70 °C. Thus, it can be hypothesized that terminal units are most susceptible to hydrolysis. A kinetic study on the acid hydrolysis of dextran also showed that small molecules are preferably released, because glycosidic bonds near the ends of the polysaccharides have a higher reactivity than those in the center (Basedow et al., 1978). However, the increased portions of terminal units at mild hydrolysis

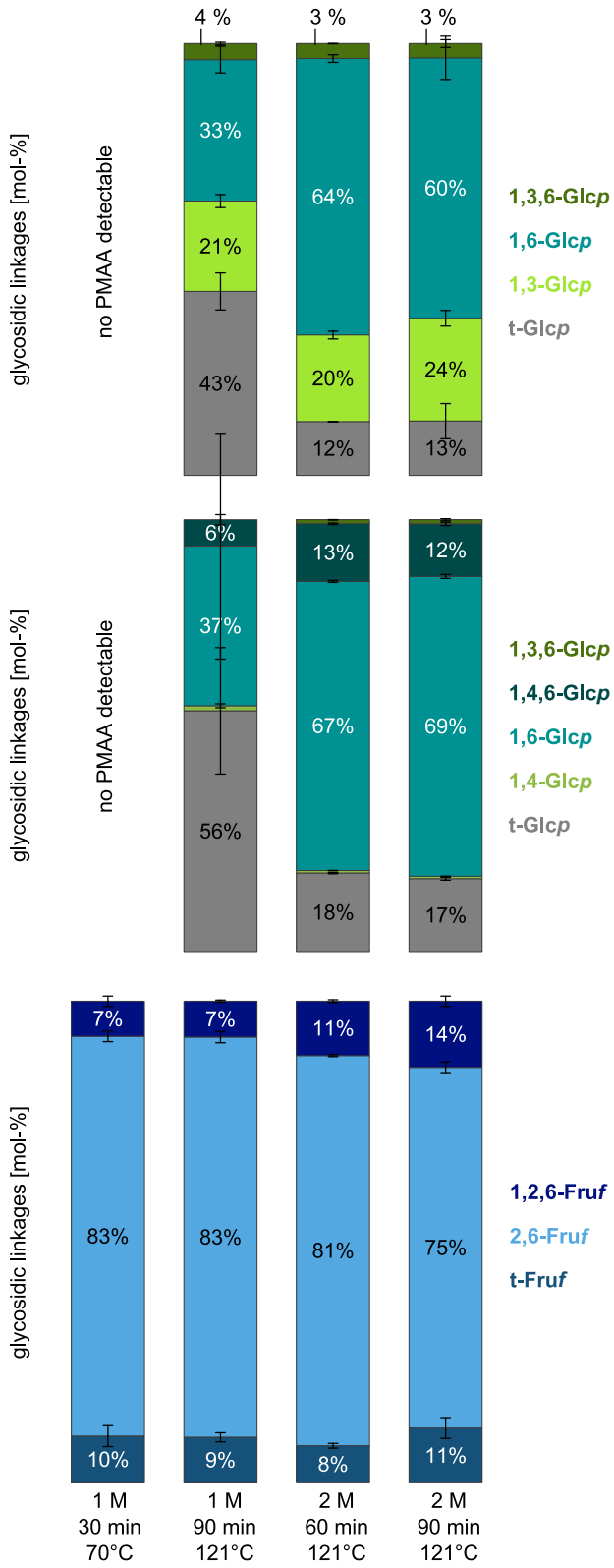

Fig. 3. Glycosidic linkages (mol-%) of levan (bottom), dextran (center) and water insoluble glucan (top) determined by methylation analysis. The application of different TFA concentrations (M), durations (min), and temperatures (°C) during hydrolysis is indicated below the bars. All analyses were performed in duplicate and mean values with half range uncertainties are shown. t: terminal, Glc*p*: glucopyranose, Fru*f*: fructofuranose, Numbers indicate the position of substitution in the monosaccharide unit.

conditions may also result from the hydrolysis of low molecular weight compounds.

When 2 M TFA was used, a higher portion of 1,3-linkages is observed in water insoluble glucan after 90 min instead of 60 min. This could be explained by a higher resistance of 1,3-linkages compared to 1,6-linkages, but this remains speculation at this point. For dextran, the application of 2 M TFA yields almost the same portions of glycosidic linkages for both durations of hydrolysis which indicates that degradation of the liberated partially methylated alditol acetates does not influence the results.

3.2.2. Levan

Because their glycosidic bonds are less stable, fructans are usually hydrolyzed by using milder conditions to avoid destruction of liberated fructose units (Biermann, 1988; Carpita et al., 1991). However, in expectation of glucose residues in inulin or levan, harsher conditions, e.g. 2 M TFA for 2 h at 110 °C, are sometimes used (Zhang et al., 2013). In our study, portions of the glycosidic linkages and reproducibility remained unchanged after application of 1 M TFA for 30 min at 70 °C or 90 min at 121 °C. However, increasing the TFA concentration to 2 M caused increased portions of branched units which were even higher than the portions of terminal fructose units. This ratio cannot result from the polysaccharide structure (every ramification needs to end with a terminal unit), thus, our results show that the PMAAs representing terminal fructose units are lost when harsh hydrolysis conditions are applied. This could result from a preferred hydrolysis (also indicated by our results for glucans) and a subsequent degradation of the liberated partially methylated monosaccharides. This assumption is supported by the detection of additional peaks in the GC chromatogram after hydrolysis with 2 M TFA for 90 min at 121 °C (and by the results reported in the following section). However, to obtain information about the polysaccharide structure, the ratio between 2,6-Fru*f* and 1,2,6-Fru*f* is more important than the portion of terminal units. Because it is unclear whether the levan backbone is completely hydrolyzed with 1 M or 2 M TFA, we analyzed methylated and hydrolyzed levan by HPSEC-UV-RI.

3.2.2.1. HPSEC-UV-RI of methylated and hydrolyzed levan. To analyze the degree of hydrolysis and degradation reactions, levan was methylated and hydrolyzed by using 1 M TFA at 70 °C (for 10 min, 20 min and 30 min) and 2 M TFA at 121 °C (for 30 min, 60 min and 90 min). Furthermore, fructose was methylated and treated with 1 M TFA at 70 °C for 30 min to gain information about the elution pattern derived from degradation of methylated fructose. Untreated fructose and levan were analyzed as elution time standards.

The samples were optically different when dissolved in DMSO for HPSEC analysis: Solutions were colorless after mild hydrolysis with 1 M TFA at 70 °C, whereas beige to brown solutions were obtained when TFA concentration, duration, and temperature increased. Therefore, UV detection was used to specifically detect chromophoric reaction products besides the universal RI detection. It was expected that incomplete hydrolysis leads to residual early-eluting high molecular weight compounds whereas complete hydrolysis leads to later-eluting low molecular weight compounds.

The results of the HPSEC-UV-RI analysis are shown in Fig. 4. Methylated, acid-treated fructose yielded a rather broad RI elution pattern at a similar retention volume than untreated fructose. As expected for hydrolyzed polysaccharides, all samples contained compounds eluting at this volume while early eluting compounds were absent or of low abundance. Although slight variations can be seen in the intensity of the earlier-eluting peaks, these results indicate that the high molecular weight part of levan is hydrolyzed in all samples.

However, although it was expected that increases in TFA concentration, duration, and temperature lead to reduced/increased intensities for the polysaccharide/monosaccharide peaks in the RI elugrams, harsher hydrolysis conditions (2 M TFA and 121 °C) led to a higher

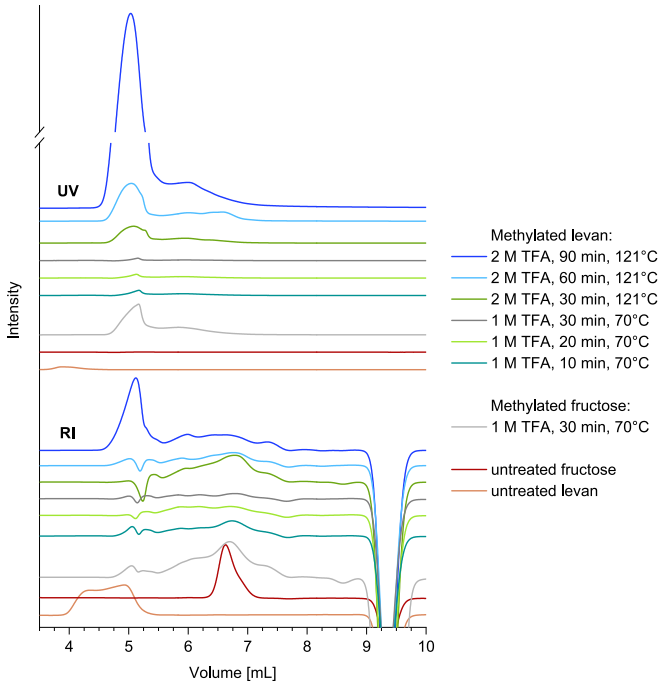

Fig. 4. HPSEC-UV-RI elugrams of levan and fructose (not methylated and hydrolyzed) and of methylated fructose and methylated levan after different hydrolysis procedures (TFA concentration, duration and temperature). UV detection was carried out at 420 nm. RI: refractive index, UV: ultraviolet, TFA: trifluoroacetic acid.

portion of polymeric material. This suggests the occurrence of a polymerization reaction. The UV elugrams at 420 nm confirmed that its products are responsible for the brown color observed when dissolving the samples. Furthermore, significant portions of these chromophoric compounds can be detected in all levan samples hydrolyzed with 2 M TFA at 121 °C, although the peak intensity increases with the incubation time. In contrast, levan samples hydrolyzed at mild conditions contain clearly less of these UV-absorbing products than those hydrolyzed at harsh conditions. Notably, some high molecular weight products are also observed after methylation of fructose and subsequent treatment with 1 M TFA for 30 min at 70 °C (RI and UV signal). This demonstrates that the formation of these compounds is not specific for harsh conditions, but for the exposure of (partially) methylated monosaccharides to acid and high temperatures. The formation of UV-absorbing, polymeric compounds is most likely the result of condensation and dehydration (Kent, 1953; Krol, 1978; Silberman, 1961). However, reaction products of methylated fructans/methylated fructose in acidic environments have not been described in the literature yet. Therefore, the polymerization reaction occurring during TFA hydrolysis of methylated levan could be subject of future research.

Our results confirm that levan is effectively hydrolyzed by the acid treatments used and that partially methylated fructose which is liberated during hydrolysis contributes to the formation of high molecular weight products. Therefore, these compounds cannot be avoided during acid hydrolysis. Hydrolysis with 1 M TFA at 70 °C for 20 or 30 min is well suited for levan analysis, because only low amounts of polymeric material were detected by RI and UV. An approach to avoid decomposition of liberated partially methylated monosaccharides could be to hydrolyze under reductive conditions. This approach is supposed to reduce liberated partially methylated monosaccharides to their corresponding alditols which are more stable in the acidic environment. To evaluate if this approach yields improved results for methylated levan, we used the reductive hydrolysis procedure described by Stevenson and Furneaux (1991).

3.2.2.2. Reductive hydrolysis in methylation analysis of levan. The formation of polymeric products could be hindered by immediate reduction of the anomeric carbon of liberated permethylated fructose which results in a stabilization. Therefore, this approach could also reduce overestimation of branched units after hydrolysis with 2 M TFA. Reduction during acid hydrolysis in situ can be achieved by MMB (Garegg et al., 1988; Stevenson & Furneaux, 1991). Based on the previously described protocol, different conditions were selected for reductive hydrolysis of levan (0.5 M final TFA concentration at 70 °C for 30 and 60 min and at 121 °C for 90 min as well as 1 M final TFA concentration at 70 °C for 30 and 60 min). However, initial experiments showed that the MMB treatments applied did not result in a complete reduction of partially methylated monosaccharides. Therefore, the hydrolyzed samples were subsequently treated with $NaBD_4$ to achieve complete reduction. By using this approach, deuteration can be used to differentiate between PMAAs resulting from MMB reduction and those reduced by $NaBD_4$. The results obtained from the application of reductive hydrolysis with subsequent $NaBD_4$ reduction are shown in Fig. 5.

After hydrolysis with 0.5 M TFA for 30 min at 70 °C, a clearly elevated portion of terminal units is observed which is most likely the result of incomplete hydrolysis. Hydrolysis of levan with 0.5 M TFA for 60 min at 70 °C resulted in portions of glycosidic linkages similar to those obtained with 1 M TFA for 30 min at 70 °C without using MMB (Fig. 3). In contrast, samples hydrolyzed with 1 M TFA for 30 or 60 min at 70 °C and with 0.5 M TFA for 90 min at 121 °C show higher portions for branched than for terminal units. These results suggest that reductive hydrolysis does not stabilize liberated terminal units sufficiently when harsher conditions are used. This observation is supported by the high portion of monosaccharides which are reduced by deuteride and not by MMB (Fig. S1). The low degree of MMB reduction could be the result of degradation of MMB in aqueous acid and/or of the reduction potential of MMB being too weak for partially methylated fructose (Stevenson & Furneaux, 1991). However, Garegg et al. (1988) reported that reduction of fructose was complete during hydrolysis of sucrose in 0.5 M TFA with

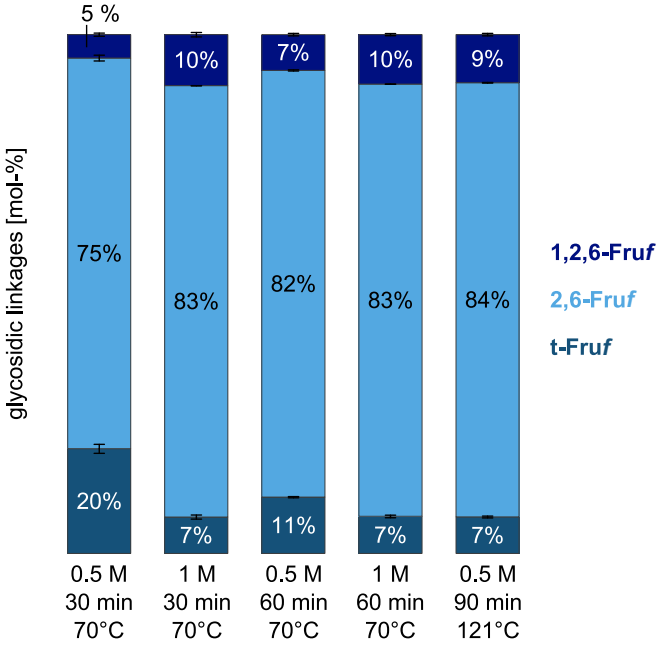

Fig. 5. Glycosidic linkages (mol-%) of levan determined by methylation analysis. The application of different TFA concentrations (M), durations (min), and temperatures (°C) during reductive hydrolysis with MMB (4-methylmorpholine-borane) is indicated below the bars. All analyses were performed in duplicate and mean values with half range uncertainties are shown. t: terminal, Fruf: fructofuranose, Numbers indicate the position of substitution in the monosaccharide unit.

MMB for 15 h at room temperature whereas glucose reduction was incomplete. Besides factors such as monosaccharide identity, temperature, and acid concentration (Garegg et al., 1988), permethylation is likely to influence reductions with MMB. Overall, application of reductive hydrolysis is not improving results in methylation analysis of levan under the conditions used.

3.2.3. Methylation analysis of a mixture of levan and dextran

Mixtures of dextrans and fructans may occur in exopolysaccharide isolates from some lactic acid bacteria. This complicates structural analysis because the polysaccharides require different conditions during acid hydrolysis, as seen in Sections 3.2.1 and 3.2.2. Separation of dextran and fructan would be an option, however, this step is quite work-intensive. Therefore, we analyzed a 50:50 (w/w)-mixture of levan and dextran by using methylation analysis with different hydrolysis conditions. With this approach we aimed to evaluate if the same results are obtained in the presence of another polysaccharide and if the glycosidic linkage portions resemble the overall monosaccharide composition of the mixture. Methylation analysis was performed by using 1 M TFA for 30 min at 70 °C and for 90 min at 121 °C as well as 2 M TFA at 121 °C for 60 and 90 min and 3 M TFA at 121 °C for 90 min. The comparably mild hydrolysis with 1 M TFA proved to be suitable for levan analysis (Section 3.2.2) whereas the application of 2 M TFA at 121 °C for 60/90 min was suitable for dextran (Section 3.2.1). The harsh hydrolysis conditions with 3 M TFA were applied to elucidate if glucan can be determined without detection of levan PMAAs.

Fig. 6 shows the portions of PMAAs derived from dextran and levan after methylation analysis of the mixture with different hydrolysis conditions. As expected from our previous results, only PMAAs derived from levan can be detected when mild hydrolysis conditions (1 M TFA, 30 min, 70 °C) are used. With increasing TFA concentration, duration, and temperature, increasing amounts of dextran and decreasing amounts of levan are found. At harsh conditions (2 and 3 M TFA, 90 min, 121 °C), dextrans are clearly predominant. However, low amounts of levan PMAAs can still be detected, although harsh conditions also led to the formation of detectable amounts of levan-derived degradation products.

Consequently, even harsh hydrolysis conditions can be used to qualitatively assess fructans by methylation analysis which is in good agreement with the literature. For example, van Geel-Schutten et al. examined the exopolysaccharides of *Limosilactobacillus reuteri* LB121 by using 2 M TFA for 2 h at 120 °C in methylation analysis. In combination with monosaccharide analysis, they were able to identify levan and a branched glucan simultaneously. However, the relative amounts of glucan linkages were assessed semiquantitatively, whereas fructan was only assessed qualitatively (van Geel-Schutten et al., 1999). Ravenscroft et al. (2009) analyzed a fructan oligosaccharide with single glucosyl units in the main chain by methylation analysis with varying hydrolysis temperatures. The methylated oligosaccharides were hydrolyzed in 2 M TFA for 40 min at 60, 100 and 125 °C prior to derivatization to alditol acetates and GC–MS analysis. An increasing amount of glucose derived PMAAs was detected with increasing temperature which is in good agreement with our results.

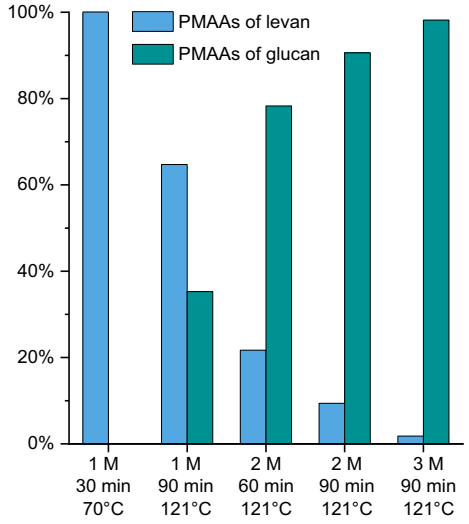

Fig. 6. Portions of the sum of partially methylated alditol acetates derived from dextran (terminal, 1,4-, 1,6- and 1,4,6- linked glucopyranose) and levan (terminal and 2,6-linked fructofuranose) after methylation analysis of a 50:50 (w/w) mixture of the two polysaccharides. Portions of 1,2,6-fructofuranose and 1,3,6-glucospyranose were not incorporated due to ambiguous assignment because of partial peak overlap. The application of different TFA concentrations (M), durations (min) and temperatures (°C) is indicated below the bars.

To evaluate if the different hydrolysis procedures can be used for semiquantitative analysis, the portions of the individual glycosidic linkages were calculated for levan and dextran (Fig. 7). The results were compared to the individually analyzed polysaccharides (Fig. 3).

Levan glycosidic linkage portions determined after analysis of the mixture and hydrolysis with 1 M TFA at 70 °C for 30 min and at 121 °C for 90 min, as well as with 2 M TFA for 60 min at 121 °C were comparable to those obtained for pure levan. However, an increased portion of terminal units was observed, whereas 1,2,6-linked units were of lower abundance. Thus, the presence of dextran may slightly impede fructan hydrolysis. Nevertheless, results after hydrolysis with mild conditions still yield valuable information on levan composition. Harsher conditions (2 M TFA and 3 M TFA at 121 °C for 90 min) led to incorrect results for levan especially due to a partial coelution of PMAAs derived from 1,2,6-Fru*f* and 1,3,6-Glc*p*. Nevertheless, levan can be unambiguously identified after harsher hydrolysis conditions.

For dextran, comparable portions of glycosidic linkages are obtained for the mixture and for the pure polysaccharide when 2 M TFA at 121 °C is used for hydrolysis. Slight variations are observed when samples were hydrolyzed for 60 min, whereas the application of 90 min yielded almost identical portions of glycosidic linkages. Hydrolysis with 3 M TFA does only have a minor influence on the portions of dextran PMAAs. After milder hydrolysis conditions (1 M TFA, 121 °C for 90 min) terminal, 1,4-linked and 1,4,6-linked units are overestimated and after 1 M TFA at 70 °C for 30 min no glucan derived PMAA was detectable. This is in good agreement with the results described in Section 3.2.1. Thus, the presence of fructan only has a minor influence on the hydrolysis of dextrans.

Overall, PMAAs derived from levan can be detected together with PMAAs derived from glucan because levan is stable enough to yield some PMAAs at high TFA concentrations, durations, and temperatures. Although monosaccharide analysis after complete acid hydrolysis also yields information about the polysaccharides in the sample, methylation analysis additionally provides information about the linkage types of the monosaccharides. Thus, this method also allows for conclusions on the type of glucan or fructan. Nevertheless, monosaccharide analysis after complete hydrolysis should be performed complementary to unambiguously identify glucose and fructose and to assess their portions. Semiquantitative analysis of linkage types in each polysaccharide can be carried out by using appropriate hydrolysis conditions (2 M TFA, 60 min at 121 °C). Alternatively, glycosidic linkages can be analyzed after separation of the polysaccharides. This can for example be achieved by selective enzymatic degradation with *endo*-dextranase or *endo*-levanase, followed by dialysis of the sample.

4. Conclusion

Our results clearly confirm our hypothesis and demonstrate that sonication and hydrolysis are critical steps in the derivatization procedure of methylation analysis of bacterial HoEPS. While dextran and levan proved to be unaffected by ultrasonic treatments, sonication was crucial for water insoluble glucans to swell and deprotonate the

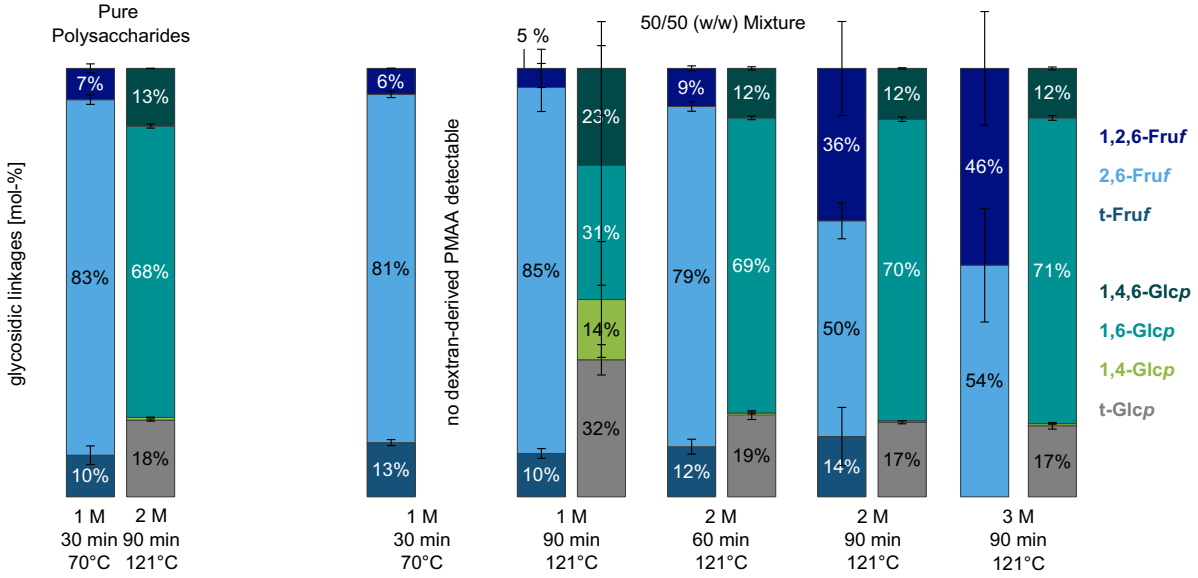

Fig. 7. Glycosidic linkages (mol-%) of levan and dextran determined by methylation analysis of the two pure polysaccharides (left two columns) or the 50:50 (w/w)-mixture of both. Hydrolysis conditions TFA concentration (M), duration (min), and temperature (°C) are shown beneath the columns. The glycosidic linkage portions of the pure polysaccharides obtained after the application of other hydrolysis conditions are shown in Fig. 3. Due to partial peak overlap of PMAAs derived from 1,2,6-Fru*f* and 1,3,6-Glc*p* the letter was not included, because its portion is only 1 % in pure dextran.

polysaccharides prior to methylation. However, avoiding the sonication step after the addition of methyl iodide resulted in better reproducibility. Hydrolysis of glucans required 2 M TFA at 121 °C for complete hydrolysis. As expected, methylated levan can be hydrolyzed at milder conditions (1 M TFA, 70 °C, 30 min). Nevertheless, levan derived PMAAs were also detected when 2 M TFA was used in combination with longer heating times and higher temperature (121 °C), although branched units (1,2,6-Fru*f*) were overestimated and harsh conditions led to the formation of polymeric condensation products. The identity of these compounds remains unknown and should be investigated further. Reductive hydrolysis conditions with MMB can also be used, but no advantages arose from this procedure. When mixtures of levan and dextran are analyzed, 2 M TFA, 60 min, and 121 °C can be used for simultaneous identification of both polysaccharides in unknown HoEPS samples. However, semiquantitative estimation of glycosidic linkages should preferably be carried out by analyzing each polysaccharide separately.

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CRediT authorship contribution statement

Luise Ernst: Conceptualization, Investigation, Methodology, Formal analysis, Validation, Visualization, Writing – original draft. **Annemarie Werner:** Investigation, Methodology, Formal analysis. **Daniel Wefers:** Conceptualization, Resources, Supervision, Writing – review & editing.

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.

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