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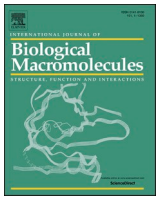

Isolation and characterization of exopolysaccharides from kombucha samples of different origins

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ABSTRACT

Kombucha is prepared by fermenting sugared green or black tea with a symbiotic culture of bacteria and yeast (SCOBY). Some of the bacteria within the SCOBY are known to form exopolysaccharides (EPS) from sucrose. However, it is yet unknown whether water-soluble EPS are formed in kombucha, and if so, which specific EPS are present. Therefore, different kombucha samples were prepared by fermentation of green and black tea with SCOBYs from different manufacturers. Subsequently, the EPS were isolated and characterized by using various chromatographic methods, partial enzymatic hydrolyses and NMR spectroscopy. It was demonstrated that levans with a varying degree of branching at position *O*1 (4.3–7.9 %) are present, while only trace amounts of glucans were detected. Furthermore, levans isolated from kombucha had a comparably low molecular weight and the content of levan within the kombucha samples varied from 33 to 562 mg levan/L kombucha. Therefore, our study demonstrated that levans are the main EPS type in kombucha and that levan amounts and structures varied when different starter cultures and ingredients were used. Furthermore, we provide a comprehensive data set on the structural variability of levans from kombucha.

1. Introduction

Kombucha is a beverage which is traditionally produced by fermenting sugared black or green tea. Fermentation is initiated by adding a starter culture, the symbiotic culture of bacteria and yeasts (SCOBY) [1–3]. The most abundant organism in kombucha is *Komagataeibacter* (*K.*) *xylinus* [4,5] which is responsible for the formation of the cellulose scaffold of the SCOBY. However, other acetic acid bacteria, lactic acid bacteria, and yeasts are also present [4,6] and the microbial composition varies significantly [3,4]. Concerning yeasts, genera such as *Zygosaccharomyces* (e.g. *Zygosaccharomyces lentus*), *Dekkera* (e.g. *Dekkera bruxellensis*), *Candida* (e.g. *Candida stellimalicola*), and *Lanchancea* (*Lanchancea fermentati*) are commonly found. Within acetic acid bacteria, the most prevalent genera in kombucha besides *Komagataeibacter* are *Gluconobacter* and *Acetobacter*. Lactic acid bacteria, specifically bacteria from the genera *Lactobacillus*, *Lactococcus* and *Leuconostoc*, are present in smaller quantities [4–7].

Due to its ingredients and the fermentation, kombucha contains sugars (glucose, fructose, and sucrose), a variety of organic acids (e.g. acetic acid, gluconic acid, glucuronic acid, and lactic acid), ethanol, carbon dioxide, vitamins, proteins, phenols, and tea polyphenols [2,8–11]. Water-soluble bacterial exopolysaccharides (EPS) may also be present because acetic and lactic acid bacteria are known for their ability to produce β-fructans and α-glucans from sucrose [12–14]. The microbial synthesis of these EPS occurs extracellularly and is catalyzed by fructansucrases and glucansucrases. In the initial step, these enzymes cleave the glycosidic linkage of sucrose, releasing one monomer. The other monomer is bound to the active center from which it is transferred onto an acceptor molecule. This reaction may result in hydrolysis (acceptor: water) or oligo- and polysaccharides (acceptor: carbohydrates) [15].

A wide variety of glucans with diverse structures can be formed by glucansucrases. Glucans are often classified based on the predominant linkages of the EPS backbone. Dextrans, for instance, are composed of an α-1,6-linked glucopyranose (Glc*p*) backbone which can be branched at position *O*2, *O*3, or *O*4. Less common α-glucans are mutans (predominantly 1,3-linkages), alternans (alternating α-1,6- and α-1,3-linkages), and reuterans (1,4- and 1,6-linkages) [15].

Fructans are mainly divided into inulin-type fructans and levan-type fructans. Levans are composed of a β-2,6-linked backbone which can be branched at position *O*1, while inulin is made up of a β-2,1-linked backbone [13,14,16]. Inulin is often found in plants [16], whereas bacterial inulin is only produced by a few strains of the genera

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Abbreviations

SCOBY	symbiotic culture of bacteria and yeast
EPS	exopolysaccharides
TFA	trifluoroacetic acid
HPAEC-PAD	high performance anion exchange chromatography with pulsed amperometric detection
PMAA	partially methylated alditol acetate
HPSEC	high performance size exclusion chromatography
RI	refractive index
Fru*f*	fructofuranose
Glc*p*	glucopyranose

Lactobacillus, *Leuconostoc*, and *Streptococcus* [14]. Notably, plant inulin is linear, while bacterial inulin can be branched at position *O*6 [17,18]. Microbial synthesis of levans is widespread and has been observed in various gram-positive and gram-negative bacteria, as well as fungi of the genera *Aspergillus* and *Rhodotorula* [12,19]. Besides cellulose, *K. xylinus*, as well as other acetic acid bacteria, is also able to produce levan and the heteropolysaccharide acetan [4,5,20].

Altogether, kombucha contains multiple EPS-forming organisms as well as the substrates needed for EPS formation. Thus, different water-soluble EPS could be present, either individually or as a mixture. However, the type, structural composition, and amount of water-soluble EPS in kombucha has not been analyzed to date. Thus, information on the variation of EPS type, content and structure in kombucha samples obtained by using different starter cultures and ingredients are also not available. We hypothesized that substantial amounts of EPS with a high structural diversity are formed in kombucha. Therefore, the aim of this study was to gain detailed insights into the structure of EPS produced in kombucha samples which were prepared with different starter cultures as well as black and green tea.

2. Experimental

2.1. Materials

The kombucha starter cultures were provided by 4 different German kombucha manufacturers. Sucrose as well as black and green tea were purchased in a local store. *Endo*-dextranase (EC 3.2.1.11, from *Chaetomium* sp., 490 U/mg) and *endo*-levanase (EC 3.2.1.65, from *Bacteroides thetaiotaomicron*, 210 U/mg) were purchased from Megazyme (Bray, Ireland). If not stated otherwise, all other 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. Kombucha

For kombucha production, green and black tea (10 g/L) was prepared with tap water and sucrose was added to obtain a final concentration of 80 g/L. After cooling to room temperature, 9 volumes of this solution were mixed with the solid SCOBY and 1 volume of batch solution. Subsequently, the kombucha was fermented for 10 days at room temperature. After fermentation, the liquid was filtered through filter paper to remove large particles and freeze dried. The obtained crude extract was used for EPS isolation.

2.3. Exopolysaccharide isolation

To isolate EPS from the dried kombucha extract, the lyophilized kombucha samples were first suspended in 1 M sodium hydroxide solution to remove proteins and microbial cells. The use of alkaline conditions to remove impurities derived from proteins or cells was already successfully applied to isolate high molecular weight dextrans and levans from culture media [21]. The insoluble fraction (proteins/cell debris) was removed by centrifugation (9000 rpm, 15 min, 4 °C) and the supernatant was neutralized with hydrochloric acid. The obtained solution was dialyzed (molecular weight cut-off (MWCO): 14 kDa) against ultrapure water for 96 h and subsequently freeze-dried. Additionally, the polymeric fraction was isolated from untreated green and black tea by using the same procedure. The isolates derived from the SCOBYs of different manufacturers were named K1-4. To specify the type of tea used, bt or gt were added as a prefix for black and green tea, respectively.

2.4. Monosaccharide analysis

The monosaccharide composition of the isolated EPS fractions was determined after hydrolysis with trifluoroacetic acid (TFA) [22]. For this procedure, the isolates from kombucha were dissolved in ultrapure water and an aliquot (20 μg) was dried in a 1.5 mL vial. Subsequently, 500 μL of 2 M TFA were added and the polysaccharides were hydrolyzed for 1 h at 121 °C. Because fructose cannot be determined by using these harsh conditions, a second hydrolysis with 1 M TFA (500 μL) for 30 min at 70 °C was carried out [23]. After hydrolysis, the acid was removed by evaporation and co-evaporation with ethanol. For the analysis of monosaccharides, the samples were redissolved in ultrapure water. Monosaccharides were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS-6000 system (Thermo Scientific Dionex, Sunnyvale, CA) equipped with a CarboPac PA20 column (150 mm × 3 mm i.d., 6.5 μm particle size, Thermo Scientific Dionex). The column temperature was 30 °C and the detector compartment temperature was 25 °C. The following gradient composed of (A) ultrapure water, (B) 0.01 M sodium hydroxide, (C) 0.2 M sodium hydroxide, (D) 0.2 M sodium hydroxide +0.2 M sodium acetate at a flow rate of 0.4 mL/min was used: Before the run, the column was equilibrated for 20 min with 100 % B. After the injection, the following gradient was applied: 0–8 min isocratic 100 % B, 8–10 min linear from 100 % B to 50 % A and 50 % C, 10–20 min linear from 50 % A and 50 % C to 50 % A, 35 % C and 15 % D, 20–35 min isocratic 100 %, 35–50 min isocratic 100 % C.

Because TFA hydrolysis is not suited to hydrolyze crystalline cellulose, sulfuric acid hydrolysis was carried out [24]. The polymeric fraction (3 mg) was swollen in 12 M sulfuric acid (50 μL) for 30 min on ice and incubated at room temperature for 2 h. After adding 325 μL of ultrapure water, the isolates were hydrolyzed for 3 h at 100 °C. To analyze the content of amorphous cellulose (and other glucans) for comparison, the isolates were directly hydrolyzed in diluted sulfuric acid (1.6 M) for 3 h at 100 °C. Subsequently, samples were centrifuged, neutralized with sodium hydroxide, diluted with ultrapure water, and analyzed by HPAEC-PAD equipped with a CarboPac PA20 column by using the conditions described above, but with a slightly modified gradient: Before the run, the column was equilibrated for 20 min with 100 % B. After the injection, the following gradient was applied: 0–15 min isocratic 100 % B, 15–30 min isocratic 100 % D, 30–40 min isocratic 100 % C.

All hydrolyses were performed in duplicate. The monosaccharides were quantitated by using an external calibration with standard compounds.

2.5. Methylation analysis

Methylation analysis was performed as described previously by Ernst et al. [25]. Conditions which are suitable for the analysis of fructans (also in a mixture of fructan and glucan) were used. Briefly, one mg of isolate was dissolved in 2 mL of dimethyl sulfoxide, incubated overnight at room temperature, and sonicated for 30 min. Finely ground NaOH was added and the polysaccharides were incubated for 90 min in an

ultrasonic bath and for 90 min at room temperature. After the addition of methyl iodide, the sample was incubated at room temperature for 1 h. Methylated polysaccharides were extracted into dichloromethane and the organic phase was washed once with 0.1 M sodium thiosulfate and twice with water. After evaporation and drying overnight, methylation was repeated. The methylated fructans were hydrolyzed by adding 1 M TFA and by incubating at 70 °C for 30 min. After evaporation, methylated monosaccharides were reduced with sodium borodeuteride and the reaction was stopped by adding glacial acetic acid. After acetylation with acetic anhydride and 1-methylimidazole, the samples were extracted into dichloromethane and the organic phase was washed with water. The residual water was frozen out overnight at −18 °C and the partially methylated alditol acetates (PMAAs) were analyzed by GC–MS (HP6890 + 5973, Agilent, Santa Clara, CA, USA) and GC-FID (HP5850, Agilent). Mass spectrometry was used for identification and flame ionization detection was used for semiquantitative estimation of the PMAA portions by using the molar response factors described by Sweet et al. [26]. Details on the conditions applied were described by Ernst et al. [25].

2.6. NMR spectroscopy

For NMR measurements, the samples were dissolved in deuterium oxide (final concentration: 10 mg/mL). Acetone was added to calibrate the spectra (^{1}H 2.22 ppm, ^{13}C 30.89 ppm according to Gottlieb et al. [27]). NMR experiments were carried out on an Agilent Technologies 500 MHz DD2 spectrometer (Agilent Technologies, Santa Clara, CA). The ^{1}H NMR spectra were recorded with 128 scans, whereas 8 scans were used for the COSY experiment and 32 scans were used for the HSQC experiment. Levan from *Gluconobacter albidus* TMW 2.1191 [30] as well as sucrose were used as standard compounds.

2.7. Enzymatic hydrolysis

Hydrolysis with *endo*-dextranase was performed as described previously by Münkel and Wefers [28]. The polymeric fractions were dissolved in water (1 mg/mL) and 5 U *endo*-dextranase/mg isolate were added. Subsequently, the samples were incubated for 24 h at 40 °C under continuous agitation. After digestion, the enzyme was inactivated at 95 °C for 10 min. For the hydrolysis of levans, *endo*-levanase (5 U enzyme/mg isolate) was added to a 1 mg/mL solution of the polymeric fractions and the mixture was incubated for 24 h at 40 °C. Both the enzymatically hydrolyzed and the native samples were centrifuged, and the supernatants were used for HPAEC-PAD analysis (see next section).

2.8. Oligosaccharides analysis

Enzymatic hydrolysates were analyzed on the HPAEC-PAD system described in 2.4 equipped with a CarboPac PA200 column (250 × 3 mm i.d., 5.5 μm particle size, Thermo Scientific Dionex). The following gradient composed of (A) ultrapure water, (B) 0.01 M sodium hydroxide, (C) 0.2 M sodium hydroxide, (D) 0.2 M sodium hydroxide +0.5 M sodium acetate at a flow rate of 0.4 mL/min and a column temperature of 30 °C was used: Before the run, the column was equilibrated for 15 min with 100 % B. After the injection, the following gradient was applied: 0–10 min isocratic 100 % B, 10–20 min linear from 100 % B to 50 % B and 50 % C, 20–45 min linear from 50 % B and 50 % C to 50 % B, 30 % C and 20 % D, 45–55 min linear from 50 % B, 30 % C and 20 % D to 50 % B and 50 % D, 55–65 min linear from 50 % B and 50 % D to 100 % D, 65–80 min isocratic 100 % D, 80–95 min isocratic 100 % C. Isomaltose and several previously characterized isomalto-oligosaccharides were used as standard compounds.

2.9. Size exclusion chromatography

To analyze the molecular weight of the EPS samples before and after enzymatic hydrolysis, high performance size exclusion chromatography coupled with a refractive index detector (HPSEC-RI, Agilent 1100 Series, Agilent 1260 Infinity II, Agilent Technologies, Santa Clara, CA) was used. The untreated isolates were dissolved in ultrapure water and enzymatic hydrolysates were used after dilution with eluent to a final concentration of 0.5 mg/mL. A TSKgel G5000 PW_{XL} column (30 cm × 7.8 mm i.d., 7 μm particle size, 20 nm pore size, Tosoh Bioscience GmbH, Germany) was used for separation and sodium azide in ultrapure water (0.05 %) was used as eluent at a flow rate of 0.4 mL/min. The column temperature was 40 °C and the RI detector was operated at 35 °C.

To estimate the molecular weight of the polymeric fractions of the kombucha isolates, dextrans with a weight average molecular mass (M_w) of 12, 64.3, 270 and 670 kDa were used as standard compounds.

3. Results and discussion

As described in the introduction, kombucha contains several microorganisms which are able to form different types of water-soluble EPS. To analyze the type and the structure of the EPS or EPS mixtures present, we prepared different kombucha samples, developed an isolation procedure, and characterized the isolates in detail. Isolates from non-fermented tea samples were used as controls.

The fermentation of the green and black tea led to visible growth of the SCOBYs as well as an acidification of the medium. The pH was monitored during the fermentation of the kombucha samples (see Fig. S1) and decreased from pH 4.0–3.2 to pH 3.6–2.7 after 10 days. By using the isolation procedure described in Section 2.3, significant amounts of a beige to brown powder were obtained from the different kombucha samples as well as from the untreated teas. The yields ranged from 318 mg/L to 944 mg/L and only gtK1 gave a lower yield than the controls from the unfermented tea samples (Table S1). Thus, the procedure which was developed for the isolation of the polysaccharide fraction proved to be suitable for this purpose. However, the obtained fractions were not completely water-soluble. Together with the observed beige to brown color, this indicates that the isolated polymeric fractions were not completely composed of pure, water-soluble glucans and/or fructans. It is possible that plant cell wall polysaccharides from the tea as well as non-carbohydrate compounds such as polyphenols and proteins were present in the isolated fractions. To obtain information about the polysaccharides present in the isolates, monosaccharides were analyzed after acid hydrolysis.

3.1. Monosaccharide composition of the polymeric fractions

To analyze the monosaccharide composition of the kombucha isolates, TFA hydrolysis with harsh as well as comparably mild conditions was used to analyze the contents of glucose, galactose etc. as well as the content of the comparably acid-labile fructose. The results are shown in Table 1.

Significant amounts of glucose and fructose were detected in all isolates, including the unfermented teas. However, the glucose content only showed slight increases for gtK1 and btK1–4 compared to the isolates from green and black tea, while gtK2–4 even showed decreased glucose contents. In contrast, fructose contents clearly increased in all isolates (up to 59 g/100 g in gtK4) compared to the small amounts of fructose in the unfermented teas. However, clear differences can be observed for the samples derived from the fermentation of green and black tea as well as the SCOBYs from different manufacturers. These results indicate that fructans are the predominantly produced EPS in green as well as black tea kombuchas. Assuming that *K. xylinus* is the predominant bacterium in kombucha [4], these data are consistent with the literature. Besides cellulose and acetane, it has been shown that *K. xylinus* is able to synthesize fructans [20].

Aside from glucose and fructose, mannose, galactose, rhamnose and arabinose were detected. However, these monosaccharides, which are

Table 1
Contents of glucose and fructose in the polymeric fraction of the kombucha samples and unfermented teas. Glucose was determined by HPAEC-PAD after hydrolysis with 2 M trifluoroacetic acid (TFA) at 121 °C as well as after different hydrolysis procedures with sulfuric acid (see Section 2.4). Fructose was determined by HPAEC-PAD after hydrolysis with 1 M TFA hydrolysis at 70 °C. All hydrolyses were performed in duplicate. gt: green tea, bt: black tea, K1–4: isolates from kombuchas of manufacturer 1–4.

	TFA hydrolysis		Sulfuric acid hydrolysis	
Sample	Fructose [g/100 g sample]	Glucose [g/100 g sample]	Glucose (including crystalline cellulose) [g/100 g sample]	Glucose (without crystalline cellulose) [g/100 g sample]
black tea	0.05 ± 0.00	2.47 ± 0.01	2.87 ± 0.09	2.93 ± 0.45
btK1	39.26 ± 0.64	4.80 ± 0.12	3.27 ± 0.41	4.03 ± 0.35
btK2	40.50 ± 0.45	3.64 ± 0.15	3.19 ± 0.45	3.34 ± 0.20
btK3	6.77 ± 0.56	4.30 ± 0.28	3.40 ± 0.00	3.87 ± 0.31
btK4	18.81 ± 0.27	4.64 ± 0.18	3.58 ± 0.13	3.90 ± 0.07
green tea	0.13 ± 0.03	9.43 ± 1.03	16.94 ± 2.14	18.32 ± 2.72
gtK1	16.36 ± 0.01	13.45 ± 0.56	11.42 ± 0.12	13.94 ± 0.26
gtK2	46.53 ± 0.71	4.93 ± 0.13	4.75 ± 0.31	4.96 ± 0.61
gtK3	15.09 ± 0.14	8.66 ± 0.08	10.05 ± 0.69	9.43 ± 0.15
gtK4	59.53 ± 0.63	6.02 ± 0.03	4.34 ± 0.03	4.67 ± 0.22

most likely derived from the plant cell wall polysaccharides of the tea leaves [29], only occurred in small amounts (0.5 g/100 g to 3.5 g/100 g). In addition, their content did not change during the fermentation.

Because *K. xylinus* is able to produce cellulose [20] and the isolates were not completely soluble, it is possible that water-insoluble, amorphous and crystalline cellulose is present in the fractions. Because TFA hydrolysis is not well suited for the analysis of insoluble polysaccharides, Saeman hydrolysis [24] was performed (with and without pretreatment with 12 M H_2SO_4, which is responsible for hydrolysis of crystalline cellulose). For almost all isolates, the glucose contents were similar after TFA hydrolysis and the two sulfuric acid hydrolyses. Only the unfermented green tea showed a clearly higher glucose content after sulfuric acid hydrolysis. Thus, this sample may contain some cellulose. However, the results suggest that cellulose is not present in significant amounts in the polymeric fraction of all kombucha samples.

3.2. Structural characterization

3.2.1. Methylation analysis

To obtain information about the structure of the fructans, glycosidic linkages were analyzed by methylation analysis. All samples contained three fructan-derived linkage types: 2,6-linked fructofuranose (Fru*f*), 1,2,6-linked Fru*f*, and terminal Fru*f* units. Thus, branched levans were present in all kombucha isolates. Especially the portions of terminal Fru*f* units showed broad variation, which is most likely caused by varying portions of low molecular weight compounds. To allow for a better comparison of the structural composition of the levans, the degree of branching was calculated from the ratio between the branched backbone units (1,2,6- Fru*f*) and all backbone units (2,6- and 1,2,6-Fru*f*). Terminal units were not used for the calculation because they may also be derived from residual sucrose and the non-reducing end of levans. In addition, their portion may in some cases be overestimated [25]. The degree of branching of the levans from the kombucha samples is shown in Fig. 1. It varied between 4.3 and 7.9 % and clear differences were observed for green and black tea kombuchas as well as the different

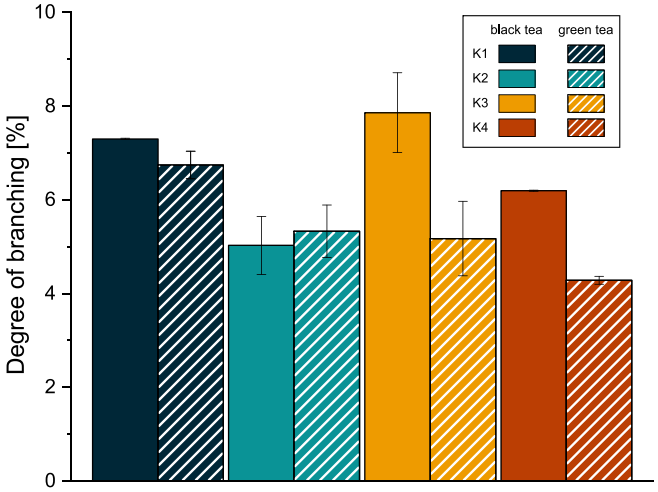

Fig. 1. Degree of branching of the levans isolated from different green and black tea kombucha samples (K1–4). The degree of branching was calculated from the ratio between the branched backbone units (1,2,6-fructofuranose) and all backbone units (2,6- and 1,2,6-fructofuranose). All analyses were performed in duplicate and mean values with half range uncertainties are shown.

manufacturers. Levans from btK3 showed the highest degree of branching, whereas gtK3 contained levans with a clearly lower degree of branching. The same trend was observed for the K4 samples, while the K1 and K2 isolates yielded levans with comparable degrees of branching. Overall, levans from the kombucha samples showed a similar degree of branching to levans in the literature [25,30]. The obtained results suggest that levan structures are clearly influenced by the starter culture as well as the type of tea used. The differences observed may be derived from the activity of different microorganisms or from variations in the activity or product of the fructansucrase(s) involved.

3.2.2. NMR spectroscopy

NMR spectroscopy was applied to confirm the presence of levans in the polymeric fractions of the isolates, to gain additional information on levan structures and to detect other soluble components. ^{1}H, COSY, and HSQC experiments were performed, and the spectra were compared with levan from *Gluconobacter albidus* TMW 2.1191 [30]. The ^{1}H NMR spectra of all kombucha isolates are shown in Fig. 2. Levan-derived signals (H1: 3.66 and 3.77 ppm, H3: 4.18 ppm, H4: 4.09 ppm, H5: 3.94 ppm, H6: 3.89 and 3.54 ppm) were assigned by using previously published data [12,23,31] as well as the COSY and HSQC spectra (Figs. S6 & S7). The very similar spectra demonstrate that levans are the main component in all kombucha isolates. This is in good agreement with the results from monosaccharide analysis. The intensity of the signals also correlates with the amount of fructose detected after TFA hydrolysis which is exemplified by the K3 samples. Besides the levan signals, a characteristic signal at 5.40 ppm as well as other smaller signals were detected in all samples. By using a standard compound, these signals were assigned to residual sucrose. Other signals were not present in the ^{1}H NMR spectrum, thus, water-soluble levans are indeed the main polymeric components of the water-soluble, polymeric fractions.

3.2.3. Analysis of native and enzymatically treated kombucha isolates by HPSEC-RI

The molecular weight of the EPS in the kombucha isolates was estimated by comparing their HPSEC retention time with dextran size standards. However, it must be emphasized that this approach is only an estimation of the molecular weight due to the lack of levan size standards. An exact determination using multi-angle laser light scattering was not possible because the isolates contained insoluble impurities.

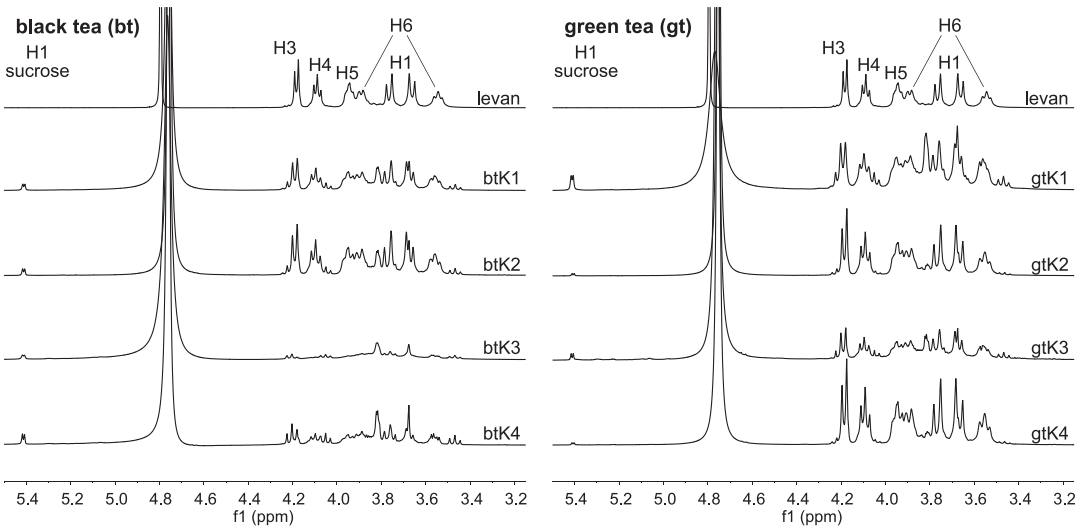

Fig. 2. ^{1}H NMR spectra of the polymeric fraction of black tea (bt) and green tea (gt) kombucha samples (K1–4) which were prepared by using starter cultures from different manufacturers. The spectrum of *Gluconobacter albidus* TMW 2.1191 levan is shown as a reference. The spectra were referenced on acetone (2.22 ppm).

Therefore, it was not possible to determine the exact injected concentration which would be necessary for a reliable determination of the molecular weight. Thus, HPSEC-RI with dextran size standard was considered the best approach. The samples were measured unmodified and after enzymatic hydrolysis with *endo*-dextranase and *endo*-levanase. Both enzymes specifically cleave the linear segments of the EPS backbones (dextrans: α-1,6-linked Glc*p*, levans: β-2,6-linked Fru*f*) which results in the release of the corresponding monosaccharides, small linear oligosaccharides as well as branched oligosaccharides. The individual enzyme solutions were also analyzed as controls. The elugrams before and after *endo*-levanase hydrolysis are shown in Fig. 3.

All untreated samples showed a broad peak, but some variations in the peak shape were observed between the individual isolates. However, most components were eluted after the 64 kDa dextran standard and also contained later eluting compounds (elution after 12 kDa dextran). Nevertheless, all samples, especially gtK2 and gtK4, contained small amounts of polymers which eluted between the 64 kDa dextran standard and the 670 kDa dextran standard. However, our results clearly demonstrate that most polymeric levans from kombucha have a comparably low molecular weight compared to levans described in the literature (from 10^5 to 10^9 Da) [12,30,32,33].

In the elugrams of the *endo*-levanase treated kombucha isolates, the broad peak which represents high molecular weight compounds showed a lower intensity or disappeared completely (Fig. 3). Unfortunately, the *endo*-levanase preparation yielded two very intense peaks (11.0 mL and 11.9 mL) which partially coeluted with the EPS. Therefore, detailed

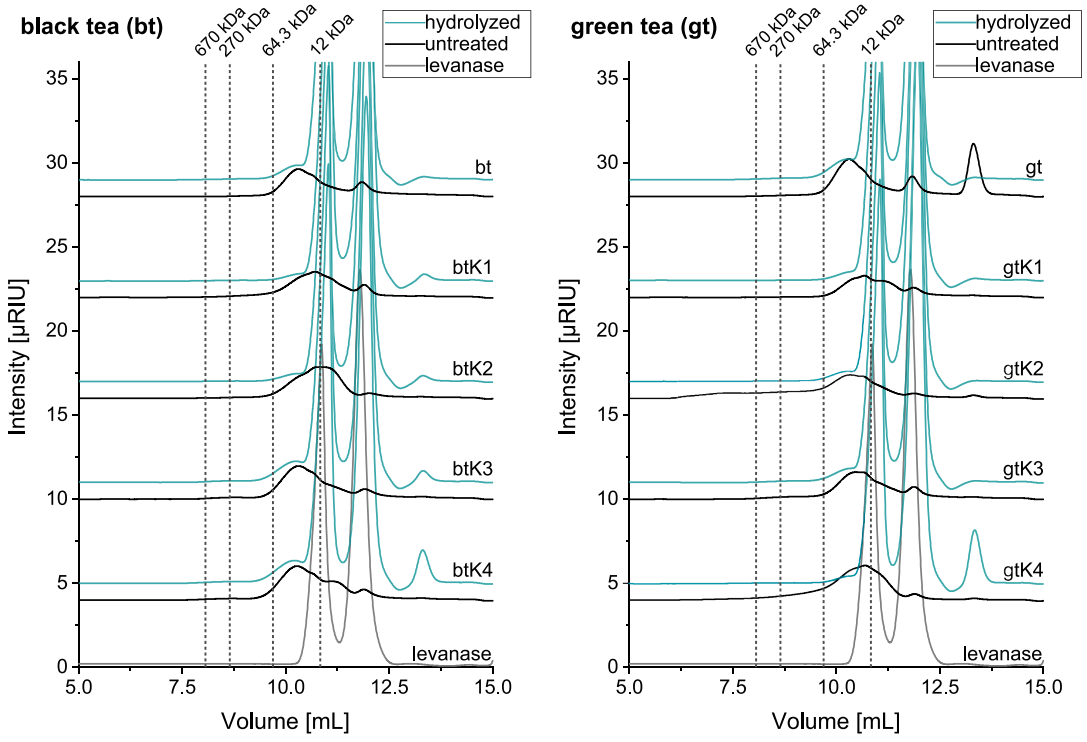

Fig. 3. HPSEC-RI elugrams of the polymeric fraction of black (bt) and green (gt) tea kombucha samples (K1–4, prepared by using starter cultures from different manufacturers) before and after *endo*-levanase hydrolysis. The elution volumes of dextran size standards are provided as reference lines.

information on the elution pattern cannot be obtained from the elugrams. Nevertheless, it can be concluded that the broad peaks originate from polymeric levans which can be partially degraded by *endo*-levanase.

The elugrams of the kombucha isolates after *endo*-dextranase hydrolysis are shown in Fig. S8. An altered portion of the polymeric, early eluting compounds was not observed. Minor changes in the peak shapes can be attributed to the enzyme which causes peaks at 10.5 mL and 12.1 mL.

3.2.4. Analysis of enzymatically liberated oligosaccharides by HPAEC-PAD

In order to gain more detailed insights into the enzymatic hydrolysates of the kombucha isolates, they were analyzed by HPAEC-PAD. This technique allows for the detection of low molecular weight compounds which result from the enzymatic hydrolysis. The chromatograms of the hydrolysates as well as the untreated controls are shown in Fig. 4.

All untreated kombucha isolates showed humps in the range between 60 and 70 min, indicating the presence of polysaccharides. In contrast, these humps were not detected in the chromatograms of the black and green tea isolates. After *endo*-dextranase hydrolysis, trace amounts of isomaltose were present in all isolates (Fig. S9) while gtK1, gtK3, and btK4 also showed low intensity peaks in the range of 30–40 min. Since isomaltose and isomalto-oligosaccharides were not detected after *endo*-dextranase hydrolysis of the green tea and black tea isolates, these results suggest that small amounts of dextrans are present in the kombucha samples. However, the humps derived from polysaccharides between 60 and 70 min were not impacted by *endo*-dextranase hydrolysis. After *endo*-levanase hydrolysis, the polysaccharide hump disappeared completely which suggests that the detected polymeric compounds are levans. In addition, several peaks were detected in the hydrolysates which can be assigned to fructo-oligosaccharides. The same peaks were obtained for all hydrolysates, however, some variations in the relative intensities were observed. This may be a result of the different degrees of branching observed after methylation analysis or a varying fine structure. Overall, these results confirm that levans from the kombucha samples have a complex molecular structure.

3.3. Estimation of the levan content in kombucha

Based on the yield obtained from the isolation procedure as well as the fructose content in the isolate, it was possible to estimate the minimum amount of levan in the different kombucha samples. The estimated levan contents are shown in Fig. 5. The overall highest amount of

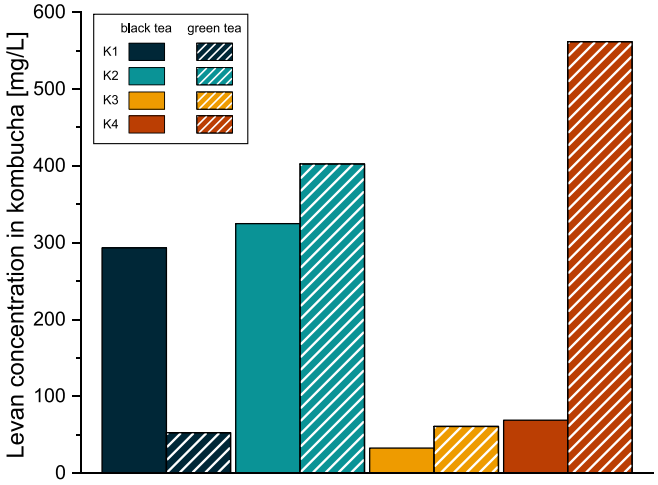

Fig. 5. Estimated levan concentration in the black and green tea kombucha samples (K1–4). The levan concentration was calculated based on the amount of the isolated polymeric fraction and the fructose content in this fraction determined by HPAEC-PAD after trifluoroacetic acid hydrolysis.

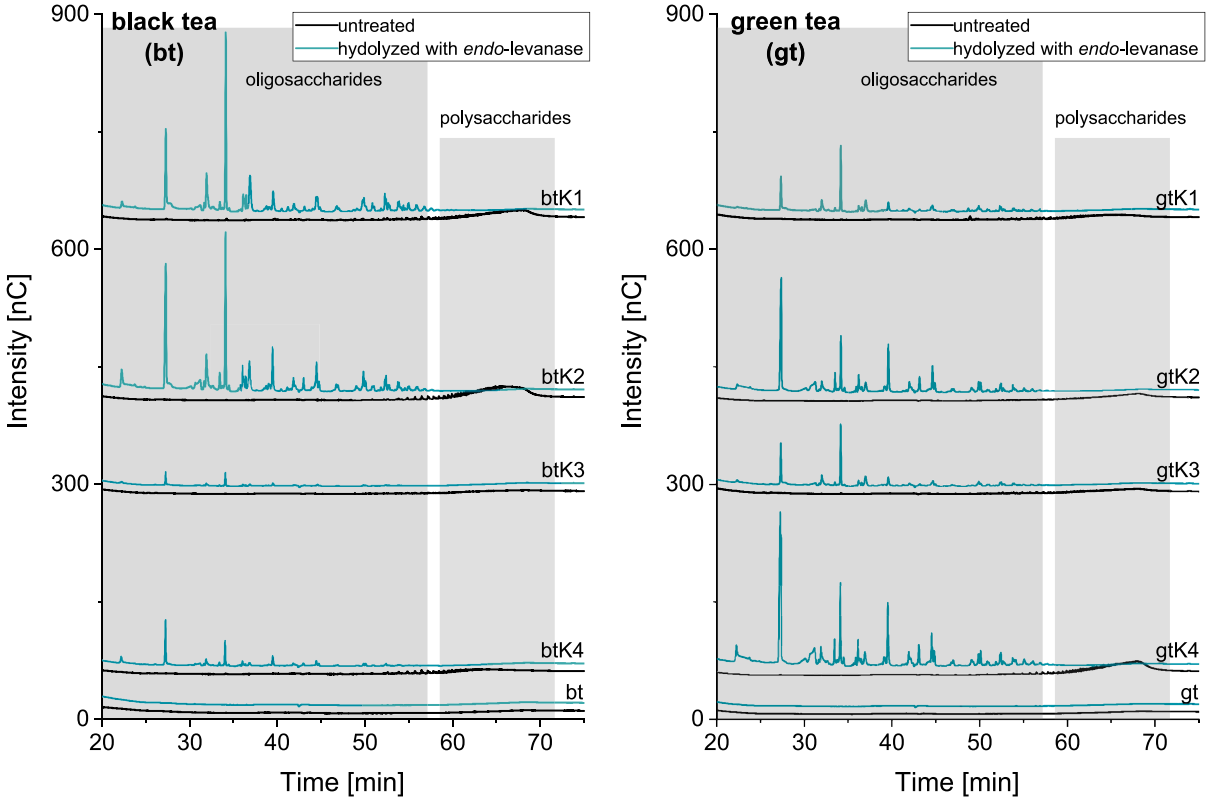

Fig. 4. HPAEC-PAD chromatograms of isolates from black (bt) and green tea (gt) and isolates from black and green tea kombucha (K1–4), before and after hydrolysis with *endo*-levanase.

levan was observed in the K4 green tea kombucha with 562 mg levan/L kombucha, the lowest amount was detected in the K3 black tea kombucha (33 mg/L). The other samples showed levan contents which are in the same order of magnitude than these two samples. Notably, clear variations were observed for the levan contents of K1 and K4 samples from black and green tea, whereas K2 and K3 samples contained comparable amounts of levan. In addition, the levan amount is also clearly dependent on the type of starter culture. It should also be noted that the estimated amounts are the minimum levels of levan in the kombucha samples, because low molecular weight compounds are removed by dialysis. Furthermore, some fructose from levans is most likely lost during TFA hydrolysis. However, the levan contents found in kombucha are quite low, so it is unlikely that they modify the texture or mouthfeel of the beverage. In addition, the levan amounts are probably too low to exhibit a beneficial effect, e.g. due to fermentation in the large intestine. Furthermore, it must be emphasized that although the kombucha samples produced in this study show significant variation, the application of other SCOBYs may lead to even higher (or lower) levan contents. A more detailed investigation of the levan formation could be in the focus of future studies.

4. Conclusion

Overall, we were able to establish a method to isolate EPS from kombucha and demonstrated that substantial amounts of EPS are present herein. From the chemical analysis, we can conclude that levans with a complex molecular structure are the predominant type of water-soluble EPS in kombucha. In addition, we showed that the application of different starter cultures and tea types influences the levan amount and the degree of branching. Our analyses clearly show that levans from kombucha have a high structural diversity. Future work could focus on the optimization of the ingredients used to obtain kombuchas with higher contents of EPS or the analysis of levans produced by individual bacteria isolated from kombucha.

CRediT authorship contribution statement

Verena Hassler: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Nele Brand:** Formal analysis, Investigation, Methodology. **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.

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Appendix A. Supplementary data

pH values observed during kombucha fermentation, yields of the kombucha isolates, TIC chromatogram of the methylation analysis, MS spectra of all fructose PMAAs, COSY and HSQC spectra of a kombucha isolate, HPSEC-RI elugrams of all kombucha and tea isolates before and after *endo*-dextranase hydrolysis, HPAEC-PAD chromatograms of the kombucha isolates after *endo*-dextranase hydrolysis. Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.131377.

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