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Structural characterization of the exopolysaccharides from water kefir

Lea Fels[a], Frank Jakob[b], Rudi F. Vogel[b], Daniel Wefers[a,*]

[a] Department of Food Chemistry and Phytochemistry, Institute of Applied Biosciences, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, 76131 Karlsruhe, Germany

[b] Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising, Germany

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ABSTRACT

Water kefir is a beverage which is produced by initiating fermentation of a fruit extract/sucrose solution with insoluble kefir grains. Exopolysaccharides that are formed from sucrose play a major role in the kefir grain formation, but the exopolysaccharides in the kefir beverage and the detailed structural composition of the whole kefir grains have not been studied yet. Therefore, kefir grains and the corresponding kefir beverage were analyzed for exopolysaccharides by multiple chromatographic approaches and two-dimensional NMR spectroscopy. Furthermore, different fractionation techniques were applied to obtain further information about the exopolysaccharides. The exopolysaccharide-fraction of the investigated kefir beverage was predominantly composed of *O*3- and *O*2-branched dextrans as well as lower amounts of levans. The insoluble dextrans from the kefir grains were mostly *O*3-branched and contained an elevated portion of 1,3-linked glucose units compared to the soluble dextrans. The structurally different exopolysaccharides in water kefir suggest the involvement of multiple bacteria.

1. Introduction

Water kefir is a fermented beverage which is produced by adding kefir grains and fruits or fruit extracts to an aqueous sucrose solution. The irregularly shaped, gel-like kefir grains contain a consortium of various microorganisms which quickly metabolize the sugar (Gulitz, Stadie, Wenning, Ehrmann, & Vogel, 2011; Gulitz, Stadie, Ehrmann, Ludwig, & Vogel, 2013; Laureys & De Vuyst, 2014; Pidoux, 1989; Reiss, 1990). The fermented yellowish beverage contains carbon dioxide, ethanol, acetic acid, lactic acid, and various other fermentation products including volatile aroma compounds (Laureys & De Vuyst, 2014; Reiss, 1990). The kefir grain microbiota contains various yeast and bacterial species. Among the bacterial species, lactic acid bacteria predominate besides acetic acid bacteria and bifidobacteria (Gulitz et al., 2011, 2013; Laureys & De Vuyst, 2014). Lactic acid bacteria are well known to be capable of producing different α-glucans and fructans from sucrose (Torino, de Valdez, & Mozzi, 2015; Zannini, Waters, Coffey, & Arendt, 2016). The formation of these exopolysaccharides is catalyzed by dextransucrases or fructansucrases. These enzymes cleave the glucose-fructose linkage of sucrose, release one of the monosaccharides, and transfer the other monosaccharide to an acceptor molecule, e.g. the growing polysaccharide chain (Leemhuis et al., 2013; van Hijum, Kralj, Ozimek, Dijkhuizen, & van Geel-Schutten, 2006). Lactic acid bacteria derived α-glucans have a high structural heterogeneity and are usually divided into groups based on the linkage type of the backbone. The most frequently described α-glucans are dextrans, which are composed of a backbone of α-1,6-linked glucose units. The linear dextran backbone may be branched to varying extents at positions *O*2, *O*3, and *O*4. Other, less abundant α-glucans are mutan (1,3- and 1,6-linkages), alternan (alternating 1,3- and 1,6-linkages), and reuteran (1,4- and 1,6-linkages). Fructans are usually divided into levan-type fructans (β-2,6-linkages) and inulin type fructans (β-2,1-linkages) (Torino et al., 2015; Zannini et al., 2016).

It has already been demonstrated that different lactic acid bacteria which were isolated from water kefir grains are capable of producing partially branched α-1,6-linked dextrans (Pidoux, Brillouet, & Quemener, 1988; Pidoux, Marshall, Zanoni, & Brooker, 1990; Waldherr, Doll, Meissner, & Vogel, 2010). In addition, high portions of 1,6-linked glucose units and minor portions of 1,3,6- and 1,3-linked glucose units were detected in polysaccharides isolated from kefir grains, suggesting that kefir grains are mainly composed of *O*3-branched dextrans (Horisberger, 1969; Pidoux et al., 1988). However, the whole untreated kefir grains and the soluble kefir exopolysaccharides have not been investigated yet. Consequently, no information about the

Abbreviations: COSY, H,H-correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; HSQC, heteronuclear single quantum coherence; PMAA, partially methylated alditol acetate; RI, refractive index; TFA, trifluoroacetic acid

* Corresponding author.
E-mail address: daniel.wefers@kit.edu (D. Wefers).

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detailed structural composition or about the structural differences between the soluble and insoluble polysaccharides are available.

Therefore, the aim of this study was to structurally characterize the exopolysaccharides present in water kefir grains and the corresponding beverage. In addition, different fractionation techniques were applied to obtain information about different polysaccharide fractions present in the water kefir grains or the beverage.

2. Experimental

2.1. Water kefir

Because water kefir is an unstandardized beverage, water kefir grains from the stock of the Chair of Technical Microbiology at the Technical University of Munich were used. The water kefir beverage was prepared by first dissolving 100 g of sucrose in 1 L of tap water. For preparation of the water kefir, ~80 g of kefir grains were suspended in 1 L of the sucrose solution in a glass bowl (1 L capacity) and two slices of figs and lemon were added as nitrogen sources. The glass bowl was finally covered with a cotton cloth. The water kefir was incubated at 21 °C without shaking for 72 h. Subsequently, kefir grains were harvested by filtration, washed with water, and both the kefir grains and the kefir beverage were freeze-dried. The kefir grains were milled two times (30 s at 25000 rpm) in a tube mill (IKA Tube-Mill Control) prior to analysis.

2.2. Monosaccharide analysis

The monosaccharide composition of the partially insoluble kefir grains was determined after sulfuric acid hydrolysis (Saeman, Bubl, & Harris, 1945). The milled kefir grains (10 mg) were swollen in 12 M sulfuric acid (150 µL) for 30 min on ice and for 2 h at room temperature. Subsequently, 975 µL of water were added and the samples were incubated at 100 °C for 3 h. After filtration and dilution, the hydrolysate was analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS-5000 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). A gradient composed of A) bidistilled water, B) 0.1 M sodium hydroxide, C) 0.1 M sodium hydroxide + 0.2 M sodium acetate and a flow rate of 0.4 mL/min were used at 25 °C: Before every run, the column was rinsed with 100% B for 10 min and equilibrated for 20 min with 90% A and 10% B. After injection, the following gradient was applied: 0–1.5 min, linear to 96% A and 4% B; 1.5–22 min, isocratic, 96% A and 4% B; 22–32 min, linear to 100% B; 32–42 min, isocratic, 100% C.

The monosaccharide composition of the freeze-dried kefir beverage was determined after methanolysis and trifluoroacetic acid (TFA) hydrolysis (De Ruiter, Schols, Voragen, & Rombouts, 1992). Methanolic HCl (500 µL, 1.25 M) was added to the sample (20 µg), and hydrolysis was performed for 16 h at 80 °C. After evaporation, the samples were hydrolyzed with 500 µL of 2 M TFA at 121 °C for 1 h. After evaporation of the acid and co-evaporation with ethanol, the sample was redissolved in water and analyzed by HPAEC-PAD as described above.

To confirm the presence of polymeric fructans, the polysaccharide samples were hydrolyzed with 1 M TFA (500 µL) at 70 °C for 30 min (Carpita, Housley, & Hendrix, 1991). After evaporation and ethanol co-evaporation, the sample was analyzed by HPAEC-PAD as described above.

2.3. Methylation analysis

Methylation analysis was performed as described previously (Nunes, Reis, Silva, Domingues, & Coimbra, 2008; Wefers & Bunzel, 2015) and only minor modifications were applied. The samples (5 mg) were dissolved in 2 mL of dimethyl sulfoxide by sonication (30 min) and an overnight incubation at room temperature. Freshly ground sodium hydroxide (100 mg) was added, and the mixture was incubated for 90 min in an ultrasonic bath and 90 min at room temperature. After addition of methyl iodide (1 mL), the sample was sonicated for 30 min and incubated for 30 min at room temperature. The methylated polysaccharides were extracted into dichloromethane (3 mL), and the organic layer was washed with 0.1 M sodium thiosulfate (5 mL) and twice with water (5 mL). The solvent was evaporated, and samples were dried overnight in a vacuum oven at 40 °C. Subsequently, the methylated polysaccharides were hydrolyzed by adding 2 mL of 2 M TFA and incubation at 121 °C for 90 min. The TFA was removed by evaporation, and $NaBD_4$ (20 mg) in aqueous NH_3 solution (2 M) was added. After 1 h at room temperature, the reaction was terminated by adding glacial acetic acid. For acetylation, 450 µL of 1-methylimidazole and 3 mL of acetic anhydride were added under ice cooling, and the solution was incubated for 30 min at room temperature. After the addition of water (3 mL), the solution was extracted with 5 mL of dichloromethane. The organic layer was washed three times with water, and residual water was removed by freezing overnight at −18 °C. The partially methylated alditol acetates (PMAAs) were analyzed by GC–MS (GC-2010 Plus and GCMS-QP2010 SE, Shimadzu) on a DB5-MS column (30 m × 0.25 mm i.d., 0.25 µm film thickness, Agilent Technologies, CA) using the following conditions: Initial column temperature 140 °C, held for 2 min; ramped at 1 °C/min to 180 °C, held for 5 min; ramped at 10 °C/min to 300 °C, held for 5 min. Helium was used as carrier gas at 40 cm/sec. Split injection with a split ratio of 30:1 was used and the injection temperature was 250 °C. The transfer line was held at 275 °C, and electron impact mass spectra were recorded at 70 eV. The relative quantification of the partially methylated alditol acetates was carried out on a GC-FID system (GC-2010 Plus, Shimadzu) by using the same conditions as described for the GC–MS analysis except for a reduced split ratio of 10:1. Nitrogen was used as makeup gas, and the FID temperature was 240 °C. All analyses were performed in duplicate and the molar response factors described by Sweet, Shapiro, and Albersheim (1975) were used for semiquantitative relative quantification.

2.4. NMR spectroscopy

NMR spectroscopy was carried out on an Ascend 500 MHz spectrometer (Bruker, Rheinstetten, Germany) equipped with a Prodigy cryoprobe. The samples were dissolved in 500 µL of D_2O and acetone was added for spectrum calibration (^1H 2.22 ppm, ^{13}C: 30.89 ppm according to Gottlieb, Kotlyar, and Nudelman (1997)). Standard Bruker pulse sequences and parameter sets were used to acquire the ^1H, H,H-Correlated Spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC) spectra of the water kefir exopolysaccharides and standard compounds such as isomaltotriose.

2.5. Precipitation of exopolysaccharides from the water kefir beverage

To isolate the polymeric fraction from the water kefir beverage, the freeze-dried beverage was dissolved in water (10 mg/mL) and ethanol was added to a final concentration of 80% (v/v). The precipitate was isolated by centrifugation, washed with 80% ethanol (v/v), and freeze-dried. The stepwise precipitation of the exopolysaccharides from the water kefir beverage was performed by adding ethanol (final concentration 40% (v/v)) to a 10 mg/mL solution of the freeze-dried kefir beverage. After precipitate formation and centrifugation, the supernatant was removed, the precipitate was washed with 40% ethanol (v/v), and freeze-dried. Subsequently, ethanol was added to the supernatant to a final concentration of 50% (v/v). The centrifugation and precipitation steps were repeated to reach final ethanol concentrations of 60%, 70%, and 80%. The ethanol portion of the wash solution was adjusted to the ethanol portion used for precipitation. The supernatant from the graded precipitation was concentrated by rotary evaporation