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Fractionation of sugar beet pulp by introducing ion-exchange groups

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ABSTRACT

Sugar beet pulp (SBP) was chemically modified with the goal to utilize this method for the preparation of water-soluble polysaccharides. Yields of the trimethylammoniumhydroxypropylated (TMAHP) polysaccharide fractions prepared under vacuum in absence of NaOH or KOH, as well as their molar masses, were higher than those obtained when the samples were only extracted with hydroxide at ambient pressure. The hydroxypropylsulfonated (HPS) fractions extracted at ambient pressure with NaOH had higher yields and molar mass than the TMAHP fractions extracted under vacuum with KOH. Introduction of both ion-exchange groups in one step under vacuum with NaOH gave better yields than with KOH, but smaller than for the solely sulfonated sample. The molar masses of chemically modified fractions were smaller than those extracted only with alkali or water. According to the monosaccharide composition all the fractions are mixtures of arabinogalactans and rhamnogalacturonoans.

1. Introduction

Sugar beet (*Betula vulgaris* L.) pulp (SBP) represents an important agricultural by-product, which is a potential source of polysaccharides. Until now it was used for preparation of L-arabinose (Tebble, Keech, McDonnell, & Punter, 2005), composites (Liu, Fishman, Hicks, & Liu, 2005), or for emulsion production (Siew & Williams, 2008). It has the advantages of being low in lignin and protein content (Šimkovic & Csomorová, 2006). By quaternization of wood lignocellulose materials, polysaccharide derivatives were prepared which are suitable as paper additives (Antal, Ebringerová, & Micko, 1991). This method proved to be effective also for extraction of agricultural residues (Šimkovic, Mlynár, Alfoldi, & Micko, 1990). Since the monosaccharide composition of the pectin fractions is dependent upon both the source of the plant material and the fractionation method used, there are still questions about pectin fine structure and many hypothetical models have been proposed (Ishii & Matsunoga, 2001; Ralet, Bonnin, & Thibault, 2002; Sakamoto & Sakai, 1995; Zykwinska, Rondeau-Moro, Garnier, Thibault, & Ralet, 2006). Our goal was to isolate the polysaccharide fractions from sugar beet pulp with the highest possible yield, selectivity and molar mass, and compare the effectiveness of alkaline extraction and chemical treatment-procedures using cationic and anionic ion-exchange agents. The procedure utilizes either vacuum evaporation or ambient pressure treatment, since the alkylating agent is hydrolyzed by water, which lowers the conversion efficiency. Solubilized fractions were dialyzed and membrane filtered to separate the smaller molecular mass fractions from polysaccharides. Membrane filtration of sugar beet extracts proved to be more efficient than alcohol precipitation (Yapo, Wathelet, & Paquat, 2007). Consequently, we used dialysis and membrane filtration to separate the extracts into two fractions: 1–10 kDa and above 10 kDa. The fractions retained by the membrane were analyzed by SEC-MALS technique and NMR spectroscopy to ascertain the molar mass distribution, structures and chemical connectivities of the components. In addition, MALDI-TOF-MS was applied for analysis of the 1–10 kDa fractions to obtain more information about the carbohydrate structure and linkage of the substituents. Similar procedures were previously run on corn fiber (CF; Šimkovic, Yadav, Zalibera, & Hicks, 2009). Part of the research program is to compare different sources of polysaccharides for possible applications.

2. Experimental

2.1. Materials and procedures used

SBP (P, 1.07%; N, 1.38%; C, 40.94%; H, 6.08%) pellets obtained from Willamette Valley, Idaho (Milling & Grain), were Wiley milled to 4 mm screen size. Glycidyltrimethylammonium chloride (GTMAC, Fluka), 3-chloro-2-hydroxypropylsulfonic acid, sodium salt (CHPS, Aldrich) and all other chemicals used were of

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commercial grade and were not further purified. All the chemical modification experiments were run in a rotary vacuum evaporator at either ambient or at 3.3–4 kPa pressure, by mixing SBP (0.5 g) with water (5 ml), alkylating agent (0.5 g), NaOH or KOH (0.5 g) for 3 h at 60 °C in a 500 ml round-bottom flask. The mixture was neutralized to pH 7 with HCl and filtered through a fritted glass funnel (G4, Koch-Light) to separate the residue from the soluble fraction. All the soluble fractions were retained by 1 kDa MWCO dialysis tubing and subsequently passed through an ultrafiltration membrane (10 kDa MWCO, Amicon) under nitrogen pressure (3.5 kg/cm^2). Elemental analyses were performed on a Fisons EA-1108 instrument.

2.2. Sugar analysis

Sugars were analyzed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using methanolysis, combined with TFA hydrolysis (Yadav, Johnston, & Hicks, 2007). The samples were first dissolved in de-ionized water (1 mg/ml). An aliquot (100 μL) of this solution along with 100 nmoles myo-inositol (internal standard) were dried in a Teflon-lined screw cap glass vial by blowing with filtered nitrogen followed by drying in a vacuum oven at 50 °C overnight. These samples were methanolyzed with 1.5 M methanolic HCl in the presence of 20% (v/v) methyl acetate for 16 h, cooled to room temperature and dried by blowing with filtered N_2 after adding five drops of *t*-butanol. The methanolyzed samples were hydrolyzed with 0.5 ml 2 M TFA at 121 °C for 1 h, evaporated by blowing with filtered N_2 at 50 °C and the residue was washed by sequential addition and evaporation of three aliquots (0.5 ml) of methanol. In three separate glass vials were placed 200, 300 and 500 nmoles of a mixture of standard sugars containing fucose, arabinose, rhamnose, galactose, glucose, xylose, glucuronic acid and galacturonic acid. Then, 100 nmoles of myo-inositol (internal standard) was added to each vial, evaporated and dried as above. These standard samples were also methanolyzed and hydrolyzed as described above and used for quantification. Hydrolyzates were analyzed for neutral and acidic sugars by HPAEC-PAD using a Dionex ICS-2500 system that included a CarboPac PA10 column and guard column, a GP 50 gradient pump, an ED50 electrochemical detector utilizing the quadruple potential waveform (gold working electrode and pH reference electrode), an AS50 autosampler with a thermal compartment (30EC column-heater), and a PC10 pneumatic controller post column addition system. The mobile phase consisted of isocratic 25 mM KOH eluant for 30 min followed by 100 mM KOH and 20 mM CH_3COOK for 10 min at a flow rate of 0.5 mL/min at ambient temperature. A 5-min column wash with 500 mM KOH followed by 15-min equilibration with 25 mM KOH at a flow rate of 1 mL/min at ambient temperature was required to yield highly reproducible retention times for the monosaccharides. The total run time was ca. 60 min. In order to minimize baseline distortion due to change in pH of the eluant during monosaccharides detection by PAD, 730 mM KOH was added to the post-column effluent *via* a mixing tee. The hydrolysates were also analyzed by HPAEC-PAD using a CarboPac PA20 column and pre-column operated at 0.5 mL/min (Zhao et al. 2008). The mobile phase consisted of 14 mM NaOH isocratic for 13 min, then a 0–120 mM CH_3COONa gradient in 100 mM NaOH by 30 min. The mobile phase was 14 mM NaOH for 40 min prior to the next injection. The results from both HPAEC-PAD analysis methods were differing up to 5% and average values from both methods were reported.

2.3. SEC-MALLS analysis

Dry samples (2–5 mg/ml) were dissolved in 50 mM $NaNO_3$ mobile phase over night, centrifuged at 50,000*g* for 10 min and filtered through a 0.22 μm Millex HV filter (Millipore Corp., Bedford, MA). The flow rate was set at 0.7 mL/min, the solvent delivery system, model 1100 series pump, degasser and auto sampler and pump (Agilent Corp.) The injection volume was 200 μL. Samples were run in triplicate. The column set consisted of two PL Aquagel OH-60 and one OH-40 size exclusion columns (8 μm particle size, Polymer Laboratories, Amherst, MA) in series. The columns were kept in a water bath set at 35 °C. Column effluent was detected with a Dawn DSP multi-angle laser light scattering photometer (MALLS) (Wyatt Technology, Santa Barbara, CA), in series with a model H502 C differential pressure viscometer (DPV) (Viscotek Corp., Houston TX) and an Optilab DSP interferometer (RI) (Wyatt Technology). Electronic outputs from the 90° light scattering angle, DPV and RI were sent to one directory of a personal computer for processing with TRISEC software (Viscotek Corp.). Electronic outputs from all the scattering angles measured by the MALLS, DVP and RI were sent to a second directory for processing with ASTRA^{TM} software (Wyatt Technology). Some characterizations (data listed in Table 1 in brackets) were performed using an additional on-line model H502 C differential viscometer (DV) detector from Viscotek Corp. (Houston, TX, USA). The SEC-MALLS system consisted of an Alliance 2690 separation module, a 2414 differential refractometer (DRI) from Waters (Milford, MA, USA), and a MALLS Dawn DSP-F photometer from Wyatt (Santa Barbara, CA, USA). The wavelength of the MALLS laser was 632.8 nm. The light scattering signal was detected simultaneously at fifteen scattering angles ranging from 14.5° to 151.3°. The calibration constant was calculated using toluene as standard assuming a Rayleigh factor of 1.406×10^{-5} cm^{-1}. The angular normalization was performed by measuring the scattering intensity of a BSA globular protein in the mobile phase assumed to act as an isotropic scatterer. The RI increment, dn/dc of polymers with respect to the solvent was measured by a KMX-16 differential refractometer from LCD Milton Roy (Riviera Beach, FL, USA). The running SEC conditions were the following: carbonate buffer pH 10 as SEC mobile phase, temperature 35 °C, flow rate 0.8 mL/min, injection volume 100 μL. Two aqueous TSKgel PW columns (G4000 and G3000) from Tosoh Bioscience (Stuttgart, Germany) were used.

2.4. Mass spectrometry analysis

Matrix-Assisted Laser Desorption/Ionization mass spectrometry with automated tandem time of flight fragmentation of selected ions (MALDI-TOF/TOF) were acquired with a 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Framingham, MA) in the positive reflectron mode with a 200 Hz Nd-YAG 355 nm laser. Spectra were obtained by averaging 1000 acquired spectra in the

Table 1
Molecular characterization of SBF fractions.

Sample	Recovered [%]	M_w/M_n	M_w [kg/mol]	R_g [nm]	$[\eta]$ [dL/g]
VE_1	66	3.4	804	30.9	1.09
E_1	74	2.5 (6.6)	120 (64)	22.3	0.67
VE_2	51	7.0	413	33.0	1.09
VE_3	94	1.7	73	22.7	1.62
E_3	41	5.7	681	33.0	2.80
VE_4	91	2.0 (2.3)	14 (34)	–	0.16
VE_5	–	–(1.6)	–(27)	–	–
VE_7	82	2.6 (10.4)	143 (112)	18.4	0.32
E_9	46	5.0	501	34.2	1.77
VE_{11}	71	6.0	370	33.3	1.31

Key. Recovered [%] = recovered sample mass [%] calculated from refractometer area; M_w/M_n = polydispersity index; M_w = weight-average molar mass; M_n = numeric-average molar mass; R_g = radius of gyration from on-line MALLS detector; $[\eta]$ = intrinsic viscosity from on-line DV detector; The numbers in brackets were measured by the second system used (see Section 2).

MS mode and 3000 in the MS/MS mode. Conversion of time of flight to mass (Da) for the monoisotopic ions was based on calibration of the instrument with a peptide standard calibration kit (Applied Biosystems). An aliquot of 1 μL of sample in a concentration of approximately 1 mg/ml in water was mixed with 10 μL of 2,5-dihydroxybenzoic acid matrix solution (10 mg/ml dissolved in acetonitrile–water- 50:50 v/v) and 0.7 μl of the solution was spotted on the MALDI plate for analysis.

2.5. NMR analysis

The spectra were recorded at 9.4 Tesla with a Varian Inova spectrometer, and a 5 mm inverse detect, Z-PFG probe in D_2O solution at 40 °C. The ^1H spectra, at 400 MHz, and the ^{13}C spectra, at 100 MHz, were referenced to internal TSP. The DEPT experiment was used to determine the number of attached protons, while gradient versions of COSY, TOCSY, HMQC, and HMBC were used to determine the assignments and connectivities of ^1H and ^{13}C chemical shifts. The gHMBC was performed with three different J_{nCH} values (5, 8 and 16 Hz) to capture long, intermediate and short-range, through-bond couplings. The 2D gHMQC were acquired in either phase-sensitive or phase-insensitive mode, although both were processed in absolute value mode. The TOCSY used a 40 μs mixing time. All 2D experiments were performed with ^1H spectral widths of 5000 Hz, and ^{13}C spectral widths of 25000 Hz. The numbers of acquired transients in the directly detected dimension were either 16 or 32 for homonuclear experiments and were between 64 and 200 for heteronuclear experiments. The number of indirectly detected transients for homonuclear experiments was 400 or 512 and for heteronuclear experiments they were 96 or 200. The F1 dimension of all heteronuclear spectra were forward linear predicted up to 2 times the number of data points, using either half or the full data set. Spectra were apodized with a sine-squared function and a shift of 70°.

3. Results and discussion

3.1. Fractionation

The yields of individual fractions obtained by the extraction procedure, with or without applied vacuum, are listed in Fig. 1. The values listed in brackets are yields of materials which were retained by 1 kDa dialysis tubing and passed through a 10 kDa membrane (see Section 2). As can be seen, the extraction with water gave the smallest yield of eluent at ambient pressure (E_1). Under vacuum (VE_1) the yield of the fraction retained by the 10 kDa – membrane increased to 2%, while the amount of the 1–10 kDa part was the same as at ambient pressure. The yields of the soluble fractions obtained with NaOH and KOH were dramatically higher than those obtained following the water treatment. More material was extracted with NaOH than with KOH at ambient pressure due to higher molar concentration used at equal weight ratios. Under vacuum KOH solubilized more material than NaOH. These results are similar like those obtained by variation of temperature and NaOH concentration (Zykwinska et al., 2006). Much higher yields of soluble polysaccharide could be obtained from corn fiber (CF) than from SBP (Šimkovic et al., 2009). It might be due to the fact that CF contains different types of polysaccharides than SBP.

When SBP was quaternized under vacuum with GTMAC and without the use of alkali, the yield of the extract (13% of fraction above 10 kDa or 5% yield of fraction between 1 and 10 kDa) was higher than extraction only with NaOH (4/5%). When the residue after water extraction was quaternized under vacuum in presence of NaOH or KOH, the yields were smaller than those when SBP was directly extracted with alkali or with GTMAC without alkali. The yields were greater with NaOH at ambient pressure (12/6%). It indicates that although HO^- ions in water cause hydrolysis of GTMAC their presence also supports the nucleophilic attack at the ionized polysaccharide hydroxyl groups. The higher nitrogen contents of samples were obtained at ambient pressure (4.27% for E_6 in comparison to 2.41% for VE_6). It indicates a higher conversion of GTMAC at ambient pressure.

The direct chemical modification scheme of SBP when NaOH or KOH at ambient pressure or vacuum were applied is in Fig. 2. The yields of the solubilized fractions were larger when sulphonated at ambient pressure in the presence of NaOH compared to KOH. Values obtained under vacuum were higher for KOH. The introduction of this group under ambient pressure shows better yield than with NaOH alone. More nitrogen could be introduced into the material with GTMAC than sulfur using CHPS under identical conditions (N, 4.27% for E_9 in comparison to S, 1.56% for E_8). The yields were similar to those observed for the sugar beet pectin obtained under acidic conditions and higher when the 10 kDa permeates and retentates were compared (Yapo et al., 2007). In comparison to CF treatment the yields were lower but the trend with higher yields of sulfonated samples in presence of KOH was retained (Šimkovic et al., 2009).

When the SBP was quaternized and sulphonated in one step the yields (13/20%) in the presence of NaOH under vacuum were bigger than with KOH (Fig. 3). Also the fraction with molar mass above 10 kDa obtained under vacuum resulted in higher yield than at ambient pressure. Yields of fractions run in presence of KOH and under vacuum were similar to data obtained at ambient pressure. Nitrogen contents (4.72/3.37% for fraction >10 kDa; E_{12}/VE_{12}) were higher than the values for sulfur (0.60/0.75%) due to higher conversion of the quaternizing agent at ambient pressure.

3.2. SEC-MALLS analysis

The water-extracted under vacuum (VE_1) sample had the highest weight-average molar mass (M_w) of 804 kg/mol, R_g of 30.9 nm, weight-average intrinsic viscosity ($[\eta_w]$) of 1.1 dL/g and

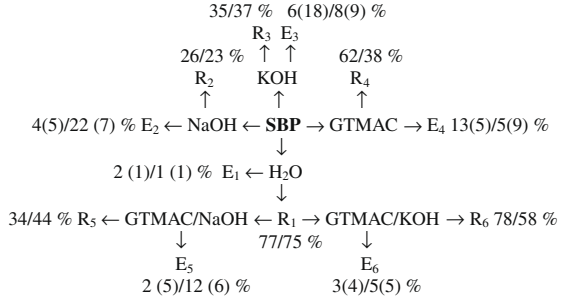

Fig. 1. Fractionation yields of SBP under vacuum/at ambient pressure (the values in brackets are yields of 1–10 kDa fractions).

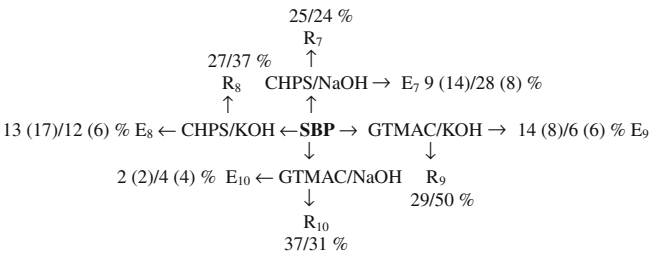

Fig. 2. Chemical modification results under vacuum/at ambient pressure (the values in brackets are yields of 1–10 kDa fractions).

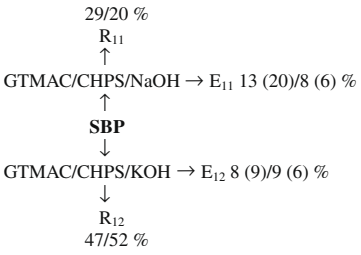

Fig. 3. Chemical modification results with both agents under vacuum/ambient pressure (the values in brackets are yields of 1–10 kDa fractions).

polydispersity (M_w/M_n) of 3.4 (Table 1). At ambient pressure (E_1), the water-extracted M_w was about 7 times lower and the E_1 weight-average intrinsic viscosity was half that of VE_1. As seen from the data obtained on the other SEC-MALLS system (in brackets), the polydispersity is decreasing due to degradation which resulted in lower M_w. The NaOH extract (VE_2) had about half of M_w of VE_1, twice the polydispersity while R_g and $[\eta_w]$ remained the same. The KOH extract (VE_3) had more than ten times lower M_w, half the polydispersity and seven times lower $[\eta_w]$ than VE_1. This M_w was also smaller than when NaOH was used. At the ambient pressure (E_3), the M_w value increased when compared to VE_3. One-step quaternization/extraction of SBP in water (VE_4), led to the lowest M_w observed (Table 1). The quaternization in presence of NaOH (VE_5) resulted in smaller M_w than extraction with NaOH under vacuum (VE_2). Hydroxypropylsulfonation under alkaline conditions (VE_7) produced higher M_w compared to quaternization in water (VE_4). On this sample the M_w determined with both systems resulted in similar value but different D. The bigger value means that the mixture could be more separated by the second system used. The direct quaternization of SBP in presence of KOH (E_9) resulted in bigger M_w than hydroxypropylsulfonation with NaOH under vacuum (VE_7), but slightly smaller than KOH extraction (E_3). The treatment with both alkylating agents gave polysaccharide (VE_{11}) with higher value of M_w than just quaternization under identical conditions (VE_7). In comparison to sugar beet pectins isolated under ambient acidic conditions the molar mass of quaternized sample (VE_4) was smaller while the sulphonated fraction (VE_7), quaternized fraction (E_9) or fraction treated with both alkylating agents in presence of alkali (VE_{11}) had higher M_w (Yapo et al., 2007). When compared to microwave-assisted extraction of SBP from a different source, the molar masses and size were similar to that determined in VE_1. The conventional alkali extracted or chemically modified fractions gave smaller molar masses (Fishman, Chau, Cooke, & Hotchkiss, 2007). Also extraction of sugar beet fiber with acids resulted in higher molar mass values (Levigne, Ralet, & Thibault, 2002).

3.3. Monosaccharide composition

The sugar composition of isolated fractions is listed in Table 2. The water extract obtained from SBP treated under vacuum has identical arabinose (Ara) content as the sample run at ambient pressure. After Ara the second most abundant sugar observed is galacturonic acid (GalA) and galactose (Gal) is third. The content of Ara was much smaller when SBP was treated under vacuum with NaOH in comparison to KOH. At ambient pressure with KOH the content of Ara and GalA was similar and smaller amount of GalA was degraded. When SBP was quaternized in water under vacuum and without additional base the amounts of Ara, Gal, and GalA were similar. When run at ambient pressure the content of Ara and GalA decreased (compare E_4 with VE_4). The quaternization of residue after water extraction (VE_5, E_5, and E_6) resulted in fractions with lower Ara and GalA content than was present in VE_1 and E_1 fractions. When SBP was hydroxypropylsulfonated in the presence of NaOH the contents of Ara and Gal increased, but GalA was more degraded (VE_7). The content of Ara was almost doubled when the experiment was run at ambient pressure (E_7). Lower contents of Ara were observed when hydroxypropylsulfonation was run in the presence of KOH (VE_8 and E_8) than when NaOH was used (VE_7 and E_7). The highest Ara and smallest GalA content was achieved when SBP was quaternized under vacuum in the presence of KOH (VE_9). This indicates that arabinogalactan is not linked to polygalacturonic acid or GalA was dramatically degraded. At ambient pressure much less Ara and more GalA was present in the fraction (E_9). The amount of Ara decreased when NaOH was used (VE_{10} and E_{10}). According to the analysis of fractions VE_{11}, E_{11}, VE_{12}, and E_{12} the most complicated mixture of polysaccharides was isolated when both alkylating agents were used. The results indicate that SBP cell wall is a quite heterogeneous material with predominant amounts of arabinogalactans in most of the extracts. It remains unclear if there are independent arabinan or galactan polysaccharides and how are they linked to pectin component. In comparison to pectin fractions extracted from SBP under acidic conditions, our fractions had a greater Ara to GalA ratio (Yapo et al., 2007). This indicates that our extraction conditions preferentially cleaved the rhamnogalacturonan backbone in pectin but left glycosidic linkages between arabinose- and galactose-rich side-chains and rhamnose intact. When we compare the yields from SB, with fractions from CF, we could consider the difference in extractability between SB and CF material to be related to absence of pectin in CF (Šimkovic et al., 2009).

3.4. Mass spectrometry analysis

The fractions for MALDI-TOF-MS analysis were dialyzed through 1 kDa using de-ionized water prior to 10 kDa membrane filtration and the 1–10 kDa fractions used for analysis. For the water extracts (E_1 and VE_1) analyzed in positive mode above *m/z* 700 the base peak containing hexose (Hex), deoxyhexose (Dhex) and hexuronic acid (HexA) was at 817.4 $[HexDhex_3HexA+Na]^+$, (817.3). The rest of ions were consistent with structures containing pentose (Pen) oligomers. The most intense from them was ion, *m/z*: 701.3 $[Pen_5 + Na]^+$, (701.2), followed with ions, *m/z*: 833.3 $[Pen_6 + Na]^+$, (833.3); 965.4 $[Pen_7 + Na]^+$, (965.3); 1097.4 $[Pen_8 + Na]^+$, (1097.3); 1229.4 $[Pen_9 + Na]^+$, (1229.4); 1361.5 $[Pen_{10} + Na]^+$, (1361.4); 1493.4 $[Pen_{11} + Na]^+$, (1493.45); 1625.4 $[Pen_{12} + Na]^+$, (1625.5); 1757.5 $[Pen_{13} + Na]^+$, (1757.55); and 1889.5 $[Pen_{14} + Na]^+$, (1889.6) in decreasing intensity. By KOH extraction under identical conditions the sample VE_3 gave spectrum in positive mode with ion at *m/z* 817.4 $[Dhex_4HexA + K]^+$, (817.4).

The positive mode MALDI-TOF-MS spectrum of the quaternized sample (E_4 or VE_4) presented ions at *m/z* 910.5 consistent with structures: $[HexADhex_3Hex\text{-}CH_2CH(OH)CH_2N(CH_3)_3]^+$, (910.4) and a less intense ion, *m/z* 908.5 to $[HexA\text{-}en\text{-}PenDhex_2HexA\text{-}CH_2CH(OH)CH_2N(CH_3)_3]^+$, (908.4). This indicates a formation of double bound on hexuronic acid on nonreducing end by beta-elimination (Šimkovic, Alföldi, & Matulová, 1986). In fraction of quaternized residue after water extraction (E_5; Fig. 1), ion, *m/z* 1296.7 $[Hex_5HexA_2CH_2CH(OH)CH_2N(CH_3)_3]^+$, (1296.5) was observed as a most intense peak above 600 Da. The hydroxypropylsulfonated sample in presence of KOH (E_8) analyzed in positive mode at *m/z* over 600 contained non-substituted ion at *m/z*: 817.4 $[Dhex_4HexA + K]^+$, (817.4). Also fraction modified with both types of ion-exchanging groups in presence of KOH (E_{12}) contained the same structure. The presence of ion at *m/z* 908.5 is consistent with structures $[NaO_3SCH_2CHOHCH_2Dhex_3HexA\text{-}CH_2CH(OH)CH_2N(CH_3)_3]^+$, (908.3) suggesting the presence of both substituents. As seen the

Table 2
Monosaccharide composition (mol%) of obtained fractions.[a]

Fraction	Ara	Gal	GalA	Rha	GlcA	Xyl	Fuc	Glc
Vacuum/water eluent (VE_1)	52	12	22	3	2	2	0	7
Water soluble part (E_1)	52	8	31	5	1	0	0	3
Vacuum/NaOH (VE_2)	15	22	13	9	3	21	3	14
Vacuum/KOH (VE_3)	56	17	4	6	2	7	2	7
Ambient pressure (AP)/KOH (E_3)	24	14	21	9	1	15	4	11
Vacuum/GTMAC (VE_4)	23	22	26	16	2	4	2	5
AP/GTMAC (E_4)	16	28	14	16	3	7	5	21
R_1/Vacuum/GTMAC/NaOH (VE_5)	38	31	5	13	3	2	1	3
R_1/AP/GTMAC/NaOH (E_5)	22	23	22	12	2	9	2	8
R_1/AP/GTMAC/KOH (E_6)	12	23	11	10	2	16	8	19
Vacuum/CHPS/NaOH (VE_7)	30	21	6	8	2	16	3	14
AP/CHPS/NaOH (E_7)	58	19	11	8	1	1	0	2
Vacuum/CHPS/KOH (VE_8)	33	20	8	10	1	13	5	9
AP/CHPS/KOH (E_8)	36	20	16	9	2	8	2	7
Vacuum/ GTMAC/KOH (VE_9)	66	19	3	6	1	2	1	2
AP/GTMAC/KOH (E_9)	19	18	20	9	1	16	4	7
Vacuum/GTMAC/NaOH (VE_{10})	25	28	6	9	2	12	4	14
AP/GTMAC/NaOH (E_{10})	33	22	14	11	2	8	2	7
Vacuum/GTMAC/CHPS/NaOH (VE_{11})	17	20	16	9	2	19	4	17
AP/GTMAC/CHPS/NaOH (E_{11})	18	17	15	10	1	22	4	14
Vacuum/GTMAC/CHPS/KOH (VE_{12})	27	20	14	9	2	11	4	12
AP/GTMAC/CHPS/KOH (E_{12})	23	15	33	8	2	7	2	10

[a] The numbers in abbreviations correspond to fractions as shown in Figs. 1–3.

$Dhex_3HexA$ and $Dhex_4HexA$ were the most frequent structures observed.

3.5. NMR analysis

According to the DEPT experiment of the unmodified water-soluble fraction (E_1, data not shown), the CH_2 groups of primary hydroxyls are at 69.80, 69.40, 69.10, 68.70, 64.00 and 63.00 ppm. Some of them (63.70 and 69.40 ppm) could be also observed in the HMQC spectrum (Fig. 4A). Analysis of the cross-peak correlation patterns in the COSY spectrum (not shown) yielded the following resonance assignments: H-1 at 5.12 ppm, H-2 at 4.30 ppm, and H-3 at 4.10 ppm. The corresponding pentose signals in the HMQC spectrum are at 84.30 (C-2) and 85.00 (C-3). The signals at 84.00 (C-4) and 69.40 (C-5) ppm were assigned on the basis of known data on similar structures (Cardoso, Ferreira, Mafra, Silva, & Coimbra, 2007). The anomeric signal at 110.39/5.12 ppm in the HMQC spectrum correlates with signals at 110.39/4.10 (C/H-3) ppm in the HMBC spectrum (Fig. 4B). Analogically, the signal at 84.00/4.16 (C/H-4) in the HMQC spectrum correlates with peak at 84.00/5.12 ppm in the HMBC spectrum. Because the predominant monosaccharide unit of this polysaccharide fraction is arabinose we assume that the identified polysaccharide NMR data belongs to arabinan component. The additional signals at 63.70/3.86 and 3.70 ppm identified as CH_2 signals we also ascribe to arabinan signals. In addition, this fraction contains GalA and Gal as the second and third most abundant monosaccharide unit, which might be assigned to remaining anomeric signals at 102.50/5.04 and 103.0/4.96 ppm in an enlarged HMQC spectrum (not shown). It seams that this fraction is a mixture of arabinogalactan and polygalacturonic acid.

In the HMQC spectrum of the quaternized fraction (VE_4; Fig. 5A), there are, in addition to signals observed in E_1, new signals at 56.20/3.26 (CH_3), 70.30/3.50, 3.62 and 3.75 (CHOH of the

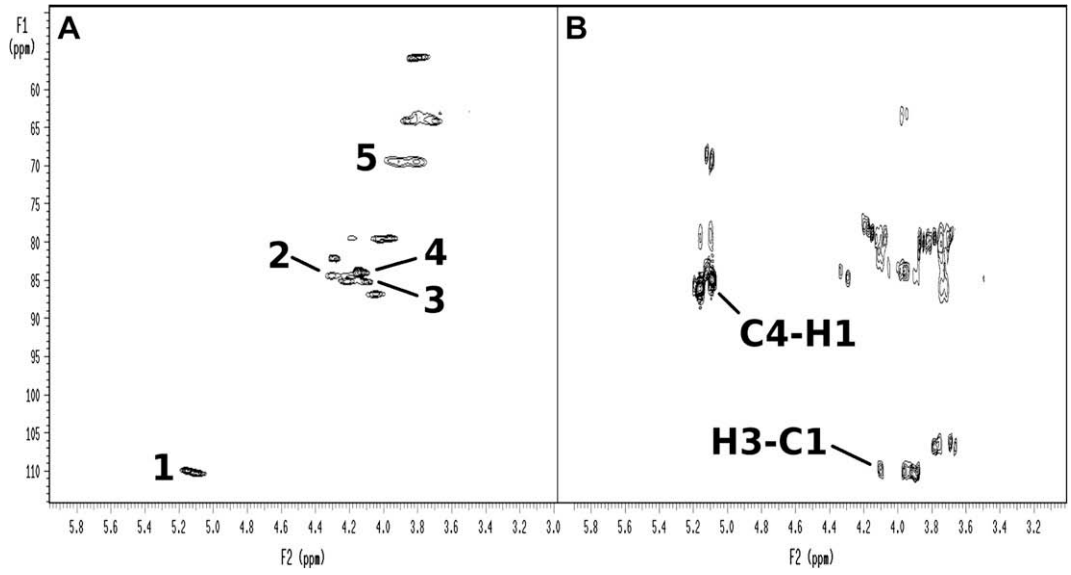

Fig. 4. gHMQC (A) and gHMBC (B; 8 Hz *n*C–H coupling constant) spectra of E_1 (the numbers in HMQC window indicate Ara residue positions and in HMBC window are assigned the respective H–C correlations).

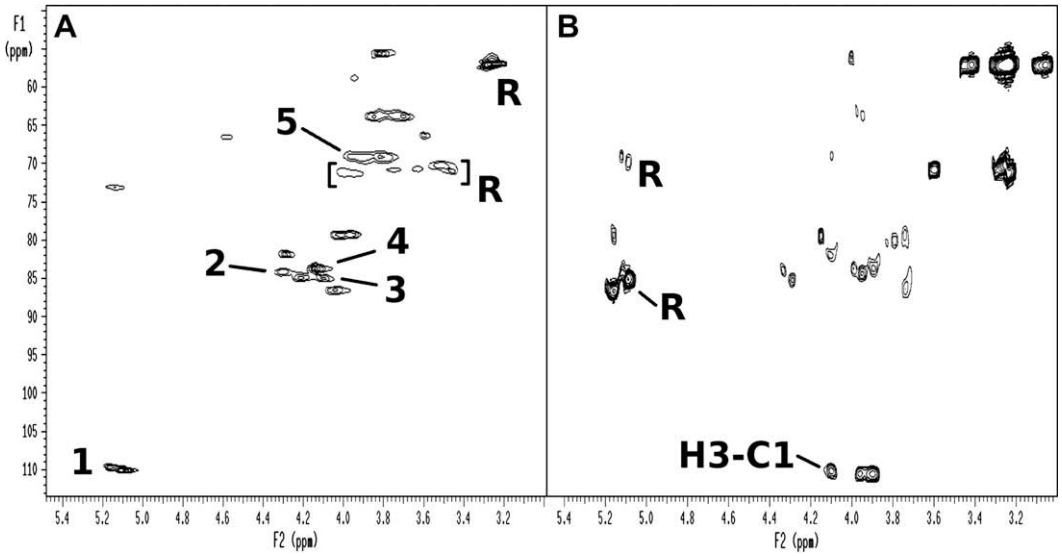

Fig. 5. gHMQC (A) and gHMBC (B; 16 Hz *n*C–H coupling constant) spectra of VE_4 (the numbers in HMQC window are same as for Fig. 4 and R is due to substituent; in the HMBC window R means the correlation with HMQC signals due to substituent).

substituent) and 71.2/4.00 and 3.94 ppm (CH_2 groups according to the DEPT experiment). The CH_2 signals correlate with resonances in the HMBC (Fig. 5B) experiment at 5.09 and 5.16 ppm, which are vertically in-line with multiplets at 85.10 and 85.70 ppm. By comparison with the COSY experiment, these signals were assigned in the HMQC spectrum to the C/H-2 and C/H-3 of the arabinose unit. This indicates that some of the C-2 and C-3 atoms of Ara units might be substituted with the TMAHP-group. It also confirms that the SBP arabinan is a (1 → 5)-glycosidically linked polysaccharide (Keenan, Belton, Matthew, & Howson, 1985). In addition, there are several anomeric signals at 110.47/5.12, 110.47/5.10, 110.01/5.16 and 109.40/5.25 ppm, which are typical for arabinan (Cardoso et al., 2007; Dourado, Cardoso, Silva, Gama, & Coimbra, 2006), and the peaks at 106.80/4.63, 102.80/4.97 and 102.20/5.14 ppm were assigned to Gal*p* and GalA units (Mukhiddinov, Khalikov, Abdusamiev, & Avloev, 2000).

The HMQC (Fig. 6A) spectrum of the 2-hydroxypropylsulfonated fraction (VE_7, Fig. 2) is also similar to the E_1 sample, but also new signals are also observed at 56.60/3.20, 3.15 and 3.04 ppm (CH_2 groups according to the DEPT experiment). These were absent in the spectra of the VE_4 (Fig. 6A). The other new signals are at 69.20/4.27 (CH_2 group according to DEPT), 71.60/4.40 and 3.86, as well as 70.70/3.80 and 3.76 ppm. In the HMBC spectrum (Fig. 6B), there are peaks at 64.10/3.66 and 64.20/3.55 ppm, as well as a multiplet at 69.20/3.10–3.07 ppm, which were not present in the spectrum of the unmodified sample (Fig. 4B). As the signal of the substituent in HMQC spectrum (69.20/4.27 ppm) correlates with the primary alcoholic groups of Ara at 69.20 ppm, we assume that the substituents are linked to primary CH_2OH groups of arabinofuranose at the nonreducing ends. It is also possible that a small percentage may be linked to the primary hydroxyls of the Gal units, which is the second most abundant monosaccharide unit

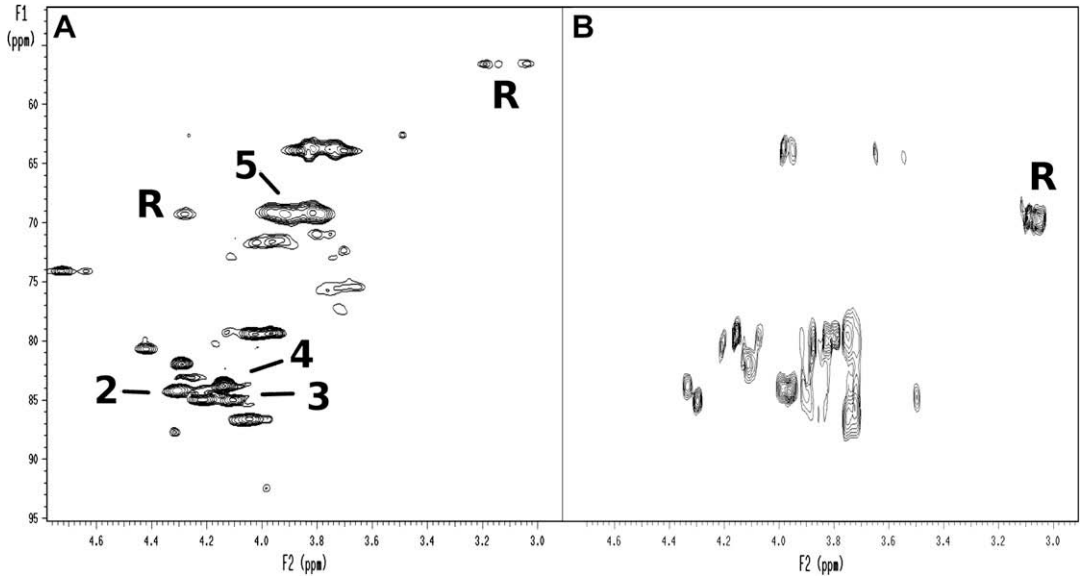

Fig. 6. gHMQC (A) and gHMBC (B; 8 Hz *n*C–H coupling constant) spectra of E_7 (the symbols are the same as in Fig. 5).

(Tab. 2). These might be related to the less intense peaks at 64.10/3.66 and 64.20/3.55 ppm of the HMBC spectrum.

4. Conclusions

SBP could be advantageously extracted and simultaneously modified with trimethylammonium-2-hydroxypropyl- or 2-hydroxypropylsulfonate- groups. The solubilized polysaccharides were obtained in higher yields than by classical alkaline extraction. The molar masses of isolated fractions were smaller than those obtained by sole alkaline extraction. The monosaccharide composition shows a predominance of arabinose for all the used methods. The GalA content was equal to Ara when SBP was quaternized in water in absence of NaOH or KOH. The NMR spectroscopic data provides evidence that the linkages of substituents were mainly at the C-2 or C-3 positions of Ara units in the case of TMAHP-group, and at the C-5 position of Ara or at the C-6 position of Gal, when the HPS was attached. The structures identified by MALDI-TOF-MS confirmed the presence of up to 14 pentose units and the possible linkages of the ion-exchanging groups to Hex, Dhex, or GalA unit.

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