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Isolation and characterization of water-soluble hemicelluloses from flax shive

Anna Jacobs,[a,*] Magnus Palm,[b] Guido Zacchi,[b] Olof Dahlman[a]

[a] *STFI, Swedish Pulp and Paper Research Institute, P.O. Box 5604, SE-114 86 Stockholm, Sweden*
[b] *Department of Chemical Engineering 1, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden*

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Abstract

Partially depolymerized, water-soluble hemicelluloses were solubilized from flax shive employing hydrothermal microwave treatment and thereafter subjected to ion-exchange chromatography, enzymatic purification and/or size-exclusion chromatography (SEC). The oligo- and polysaccharide fractions thus obtained were characterized with respect to molar mass, molar mass distribution, degree of polymerization (DP) and degree of substitution with acetyl moieties (DS_{Ac}) by employing SEC in combination with MALDI-TOF mass spectrometry. The major portion of the water-soluble flax hemicellulose consisted of an *O*-acetyl-4-*O*-methylglucuronoxylan exhibiting a DP_p value (i.e., peak-average DP) of 28. When the DS_{Ac} for this *O*-acetyl-4-*O*-methylglucuronoxylan was calculated on the basis of the MALDI-MS spectra obtained without and following deacetylation, a value of 0.7 was obtained. In addition, an *O*-acetyl-glucomannan ($DP_p = 9$, $DS = 0.4$) and minor quantities of small neutral *O*-acetyl-xylooligosaccharides were also isolated from the mixture of water-soluble hemicelluloses released from the flax shive by microwave treatment.

Keywords: Flax shive; Hemicellulose; Xylan; Glucomannan; O-Acetylation; Size-exclusion Chromatography; Microwave treatment; MALDI-TOF-MS

1. Introduction

During the processing of flax (*Linum usitatissimum*), an important source of natural fibers, bast fibers are separated from the woody core of the straws,[1] referred to as the flax shive. This shive accounts for a considerable portion of the flax straw, and is potentially an important renewable source of novel semi-synthetic biomaterials. One example along these lines is the utilization of flax shive to prepare adsorbent materials.[2,3]

In recent years, a growing interest in using hemicelluloses as raw material for various novel technological applications, e.g., the synthesis of cationic polymers,[4] hydrogels,[5,6] long-chain ester derivatives[7,8] and thermoplastic xylan derivatives,[9] has developed. To this end, hemicelluloses have been isolated and characterized from the residues remaining from production of different agricultural materials e.g., wheat straw[10] and brewery's spent grain.[11] Hemicelluloses present in other agricultural materials, such as jute and luffa fruit fibers,[12] rice straw[13] and red gram husks[14] have also been characterized.

Several studies on the composition of pectic substances present in flax[15,16] have been performed. In contrast, although flax is known to contain a significant quantity of hemicelluloses, few studies have focused on these polymers. In early studies Geerdes and Smith[17,18] employed aqueous alkaline extraction to isolate 4-*O*-methylglucuronoxylan from flax straw. More recently, McDougall[19] obtained several different hemicelluloses from flax fibers, including xylans, xyloglucans and mannans, by sequential alkaline extractions. Furthermore, van Hazendonk and co-workers[20] isolated O-acetylated xylan and glucomannan from flax fibers and characterized the structures of these polymers employ-

* Corresponding author. Tel.: +46-8-6767152; fax: +46-8-108340.
E-mail address: anna.jacobs@stfi.se (A. Jacobs).

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ing NMR spectrometry. In addition, Morrison and co-workers[21] analyzed the chemical composition, including carbohydrates, of retted flax fibers. However, neither of these latter two studies looked for the presence of 4-*O*-methylglucuronic acid residues in the flax xylans examined.

Gorshkova and co-workers[22] have demonstrated that the carbohydrate compositions of the hemicelluloses present in the bast fibers and woody core of flax differ. Moreover, Akin and co-workers[23] reported that the flax core contains a larger proportion of 4-*O*-methylglucuronoxylan and less glucomannan than do the fibers. However, we have been unable to find any articles concerning the precise structure and molecular properties of the hemicelluloses in flax shive.

We have previously described the isolation of partially depolymerized, water-soluble hemicelluloses from aspen and spruce wood employing microwave treatment, along with characterization of these polymers by NMR spectroscopy[24,25] and matrix-assisted laser desorption/ionization (MALDI) Time-of Flight (TOF) mass spectrometry (MS).[26,27] MALDI-MS is a particularly powerful tool for analyzing the molar masses of oligo- and polysaccharides[28,29] (for extensive reviews see the articles by Harvey[30,31]). We have previously achieved detailed characterization of complex mixtures of hemicelluloses employing MALDI-MS in combination with size-exclusion chromatography.[32–35]

As described previously,[26,36] acidic xylooligosaccharides can be effectively separated from neutral oligo- and polysaccharides by size-exclusion chromatography (SEC) employing elution with de-ionized water. Similar ionic exclusion of certain acidic arabinogalactans in connection with aqueous SEC has also been observed.[37,38] In the present study, such SEC was utilized both to monitor the progress of the purification procedure and as the final step in isolation of the glucomannan.

The present study describes the isolation of a partially depolymerized, water-soluble hemicellulose fraction from flax shive following microwave treatment. The hemicellulose mixture thus obtained was further fractionated by SEC and the fractions subjected to MALDI-MS investigation in order to determine molar masses, molar mass distributions, degrees of polymerization and degrees of substitution with acetyl moieties.

2. Results and discussion

2.1. Fractionation of hemicelluloses

The fractionation of flax shive hemicelluloses employed here is depicted schematically in Fig. 1. The starting material, dry flax shive (designated as fraction F0), was impregnated with water and thereafter subjected to hydrothermal treatment in a microwave oven in a manner similar to that described previously.[25,27] Following this treatment, approx 50% of the 4-*O*-methylglucuronoxylan and almost all of the glucomannan originally present in the flax shive were recovered in the aqueous phase. This mixture of water-soluble hemicelluloses, fraction F1, was then fractionated according to the scheme presented in Fig. 1. The carbohydrate compositions (analyzed employing a procedure recently developed in our laboratory and involving enzymatic hydrolysis and subsequent capillary zone electrophoresis[39]) of the raw flax shive and hemicellulose fractions are documented in Table 1, while the chromatograms obtained upon SEC of the hemicellulose fractions are shown in Fig. 2.

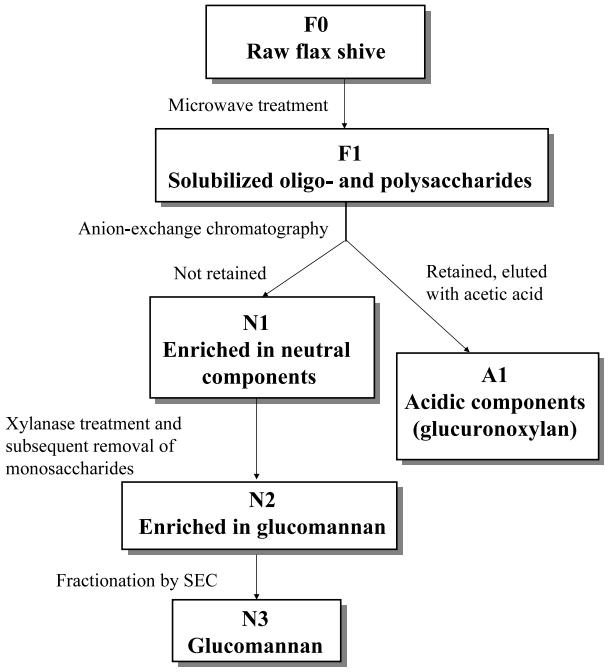

Fig. 1. Schematic representation of the procedure employed to separate water-soluble acidic and neutral oligo- and polysaccharides obtained by microwave treatment of water-impregnated flax shive.

F1 was treated with a cation-exchange resin (in the H^+-form) in order to protonate all of the uronic acid residues present in the xylan. Thereafter, this solution was passed slowly through a column containing an anion-exchange resin, which adsorbed most of the acidic saccharides. All of the neutral saccharides (i.e., a mixture of glucomannan and neutral xylooligosaccharides) were recovered in the flow-trough, fraction N1, which also contained a certain amount of acidic saccharides, not retained by the anion-exchange column.

In order to obtain a pure glucomannan fraction, the xylan and glucuronoxylan polysaccharides in fraction N1 were hydrolyzed with xylanase. The small xylosaccharides thus obtained were removed by fractionation

Table 1
Carbohydrate compositions of raw flax shive and of the hemicellulose fractions isolated from this material

Fraction [a]	Component (mass%)							
	Xyl	Glc	Man	Ara	Gal	4*O*Me-GlcA	GlcA	GalA
F0	33.3	58.3	2.6	0.6	1.3	3.8	n.d.	n.d.
F1	63.3	6.9	11.1	3.0	5.0	9.0	0.8	0.9
A1	83.2	2.8	0.6	1.0	3.4	9.0	n.d.	n.d.
N1	66.8	11.4	6.6	4.3	5.7	5.2	n.d.	n.d.
N2	53.6	15.9	10.2	4.6	6.5	9.1	n.d.	n.d.
N3	7.4	42.2	37.8	3.9	8.8	n.d.	n.d.	n.d.

n.d., not detectable, i.e., $<0.05\%$.
[a] The designations are explained in Fig. 1.

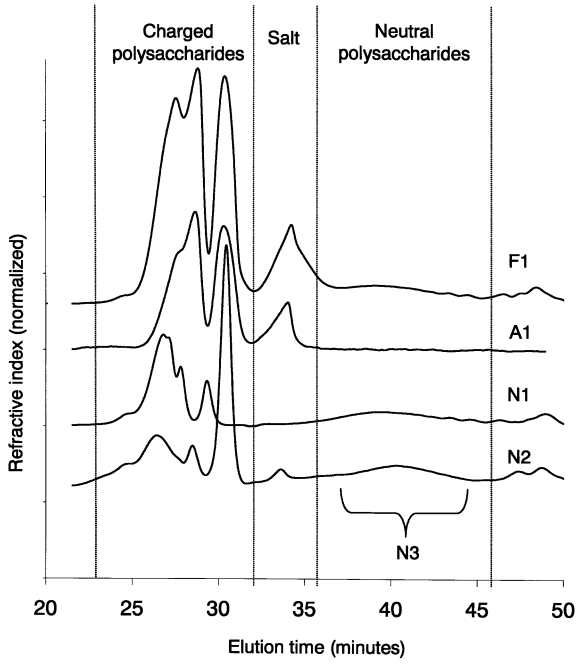

Fig. 2. Size-exclusion chromatography of the fractions containing charged and neutral oligo- and polysaccharides obtained from hemicelluloses solubilized from flax shive by microwave treatment. The designations F1, A1, N1, N2 and N3 are explained in Fig. 1. Charged polysaccharides containing 4-*O*-methylglucuronic acid residues eluted first with this SEC procedure.

using a short gel filtration column to yield fraction N2, which was considerably enriched in neutral saccharides (Fig. 2). However, xylanase acts primarily on unsubstituted regions of β-(1→4)-linked xylosaccharide chains[40] and thus does not hydrolyze *O*-acetyl-glucuronoxylans completely. Consequently, fraction N2 still contained acidic 4-*O*-methylglucuronoxylan saccharides that were large enough to be excluded by the gel filtration column. Therefore, a final step employing SEC was utilized to obtain a pure glucomannan fraction on a semi-preparative scale. This glucomannan fraction (N3) was collected from the outlet of the refractometer during the time interval required for elution of the neutral polysaccharides in fraction N2 (see Fig. 2). Finally, acidic glucuronoxylan polysaccharides retained on the anion-exchange column were eluted with aqueous acetic acid, yielding an almost pure (~90%) 4-*O*-methylglucuronoxylan fraction, A1.

2.2. Characterization of the *O*-acetyl-4-*O*-methylglucuronoxylan isolated from flax shive

As can be seen from Fig. 2, SEC of fraction A1 yielded no signals with an elution time corresponding to that of neutral saccharides (i.e., 36–46 min). Thus, it was concluded that this fraction contained only acidic oligo- and polysaccharides. Carbohydrate analysis of A1 (Table 1) confirmed the presence of *O*-acetyl-4-*O*-methylglucuronoxylan with a purity of >90% and exhibiting a 4-*O*-methylglucuronic acid–xylose ratio of 1:13. This *O*-acetyl-4-*O*-methylglucuronoxylan fraction was subsequently characterized with respect to its molecular properties, i.e., molar mass, molar mass distribution, degree of polymerization (DP) and degree of substitution with acetyl moieties (DS_{Ac}) (Table 2).

Prior to determination of the DP and DS_{Ac} values for A1, the *O*-acetyl substituents were removed. An earlier study in our laboratory on water-soluble hemicelluloses isolated from spruce and aspen demonstrated that the DS_{Ac} values obtained in this manner are in good agreement with the corresponding values obtained by

Table 2
Molecular properties of the water-soluble hemicelluloses isolated from flax shive

Fraction [a]	Property					
	M_p	M_n	M_w	M_w/M_n	DP_p	DS
A1	4900	4000	4500	1.14	28	~0.7
N3	1700	1600	1700	1.07	9	~0.4

[a] The designations are explained in Fig. 1.

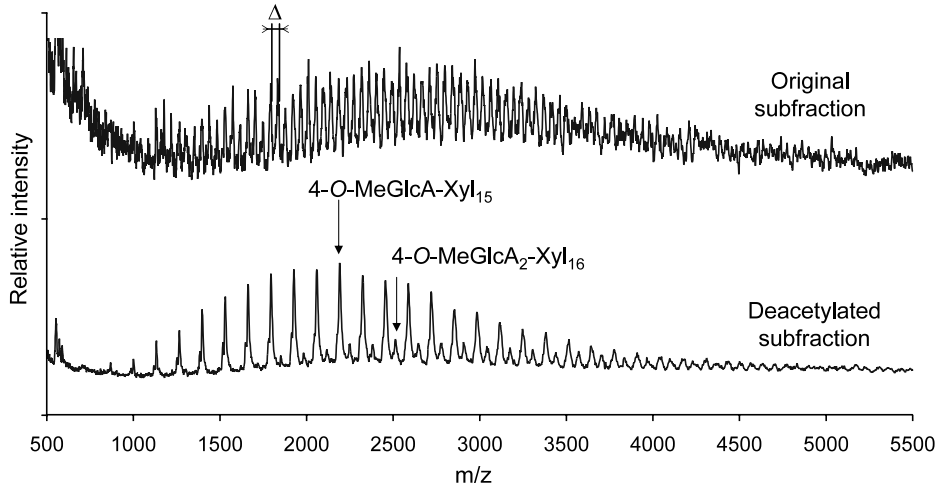

Fig. 3. MALDI-MS spectra of the acidic oligosaccharides present in a subfraction of A1. A1 contained water-soluble, acidic oligosaccharides solubilized by microwave treatment of water-impregnated flax shive (Fig. 1) and the subfraction in question was collected after 27 min of SEC (Fig. 2) using de-ionized water as the mobile phase. Following deacetylation (lower spectrum), this subfraction can be seen to consist of acidic xylooligosaccharides containing one or two 4-*O*-methylglucuronic residues. Without such deacetylation (upper spectrum), a series of weak signals (Δ) separated from one another by 42 mass units (i.e., the mass of an acetyl group) is observed.

NMR.[26] In Fig. 3, the MALDI spectra of the SEC fraction eluting after 27 min (see Fig. 2) with (upper spectrum) and without (lower spectrum) removal of the *O*-acetyl moieties are depicted.

In the case of saccharides derived from the *O*-acetyl-4-*O*-methylglucuronoxylan, the differences in their molar masses were less than the limit of resolution of the MALDI-MS instrument employed here, which explains the poor resolution obtained in the upper spectrum of Fig. 3. Nonetheless, this spectrum can be seen to contain a series of poorly resolved signals separated from one another by 42 mass units (designated as Δ) and deriving from *O*-acetyl-4-*O*-methylglucuronoxylan oligosaccharides with different DP values and numbers of *O*-acetyl substituents.

Following deacetylation, the MALDI-MS spectrum of this same subfraction (the lower spectrum in Fig. 3) revealed the presence of acidic xylooligosaccharides containing one or two 4-*O*-methylglucuronic acid residues (with DP values of 5–30). Similar MALDI-MS analyses, with and without deacetylation, were also performed on five additional subfractions of A1 collected by SEC (Fig. 2). Altogether, these six determinations yielded an average DS_{Ac} value of 0.7.

As pointed out earlier, SEC using a mobile phase of low ionic strength separates acidic saccharides on the basis of both their charges and their size. Consequently, this approach (Fig. 2) could not be utilized to determine the average molar masses of the acidic polysaccharides present in fraction A1. Instead, this determination was achieved by SEC employing a buffer solution containing 50 mM ammonium acetate as the eluent and Fig. 4 depicts the chromatogram thus obtained.

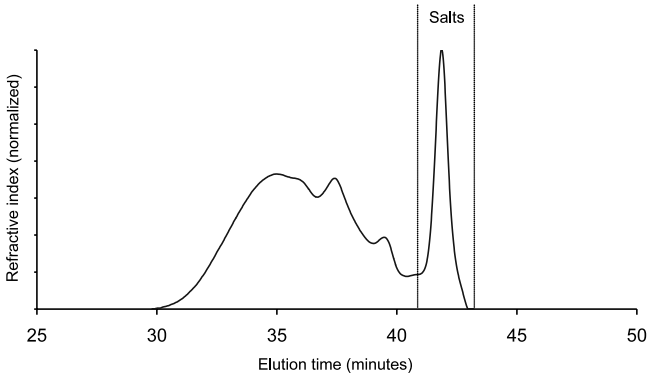

Fig. 4. Separation of the *O*-acetyl-4-*O*-metylglucuronoxylans present in fraction A1 (Fig. 1) by SEC employing 50 mM ammonium acetate as the mobile phase. With this procedure, charged polysaccharides containing 4-*O*-methylglucuronic acid moieties are separated on the basis of their molecular size.

The peak-average molar mass (M_p) values for the SEC fractions collected at the outlet of the refractometer were determined employing MALDI-MS and subsequently used to establish a calibration curve for this SEC system. This curve was then used to determine the number- and weight-average molar masses (M_n and M_w) of the entire distribution of saccharides present in fraction A1 (Table 2).

In summary, the *O*-acetyl-4-*O*-methylglucuronoxylan saccharides present in fraction A1 had an overall 4-*O*-methylglucuronic acid–xylose ratio of 1:13. The number- and weight-average molar masses were 4000 and 4500, respectively; while the peak-average DP value was 28 and the average degree of acetylation estimated to be 0.7 (Table 2). Van Hazendonk and co-workers[20] re-

ported a similar DS_{Ac} (0.5) for *O*-acetylated xylan in hemicelluloses extracted from flax fibers with DMSO. However, these investigators did not document the presence of any 4-*O*-methylglucuronic acid in their xylan, and furthermore, the DP value they obtained, determined by SEC employing calibration with dextran standards, was very large (DP 450–1500). This molar mass value is probably an overestimate resulting from the differences in the behavior of the acidic glucuronoxylans and the neutral dextran utilized for calibration in connection with SEC. The relatively low average molar mass of the *O*-acetyl-glucuronoxylans examined in the present study can be explained by partial depolymerization during microwave treatment.

2.3. Characterization of neutral water-soluble hemicelluloses isolated from flax shive

SEC/MALDI-MS analysis revealed that the neutral components (eluted in the time interval 36–46 min in Fig. 2) of N1 consisted of a mixture of glucomannan and neutral xylooligosaccharides. The presence of neutral xylooligosaccharides in fraction N1 was also demonstrated by its lower relative content of 4-*O*-methylglucuronic acid in comparison to fraction F1 (Table 1).

In contrast, corresponding SEC/MALDI analysis of fraction N3 (i.e., the neutral components of N2) detected only oligosaccharides composed of hexose residues (upper spectrum, Fig. 5). Carbohydrate analysis of this fraction confirmed that the saccharides present in largest amounts were glucose and mannose. However, the presence of xylose indicated that a certain amount of xyloglucan might also have been present in N3. Thus, the glucose–mannose ratio of the flax shive glucomannan can only be estimated to be roughly 1:1.

The number of hexose residues and *O*-acetyl groups contained in the oligosaccharides present in the original subfraction could be calculated by comparing the mass numbers obtained by MALDI-MS mass analysis without (Fig. 5, upper spectrum) and following (Fig. 5, lower spectrum) deacetylation. In these spectra, peaks corresponding to $Hexose_9$ and $Hexose_8$-Ac_2 can be observed. Since the former glucomannan saccharide had a DS_{Ac} value of zero, whereas the corresponding value for the latter oligosaccharide was 0.25, it can be concluded that the acetyl substituents are distributed irregularly along the glucomannan backbone.

The molecular properties of the neutral hemicellulose fraction N3 obtained here from flax shive, as determined by SEC/MALDI-MS, are summarized in Table 2. In contrast to their acidic counterparts, the average molar masses of these neutral hemicelluloses could be determined directly by SEC/MALDI-MS, since the latter are separated on the basis of their molecular size alone by SEC using de-ionized water as the mobile phase.[26,36]

In summary, the water-soluble *O*-acetyl-glucomannan obtained by microwave treatment of flax shive (i.e., fraction N3) has an approximate glucose–mannose ratio of 1:1, a peak-average DP value of 9 and an average $DS_{Ac} = 0.4$. Van Hazendonk and co-workers[20] detected an *O*-acetyl-glucomannan in hemicelluloses extracted from flax fibers by DMSO that exhibited a similar DS_{Ac} value (0.5) and glucose–mannose ratio (1:1–1:1.5). However, the molar mass of this glucomannan, as determined by SEC employing calibration with dextran standards, was found to be considerably larger (1000–60,000) than that of the glucomannan examined here. The low molar mass of the glucomannan in the present case can be explained by partial depolymerization during microwave treatment and is thus not directly relevant to the native *O*-acetyl-glucomannan present in flax shive.

3. Experimental

3.1. Materials

All reagents employed were of analytical grade and obtained from commercial suppliers. The water used to prepare reagent and buffer solutions was first purified utilizing a Millipore Milli-Q Plus apparatus (Millipore, Milliford, USA).

3.2. Hydrothermal treatment

Dry flax shive (11 g) was impregnated with 110 mL water to obtain a final flax shive concentration of 9% (w/w). This preparation was then subjected to hydrothermal treatment for 2 min at 200 °C in a Mega Microwave MLS-120 microwave oven (Milestone, USA). Subsequently, the liquid aqueous phase, containing solubilized hemicelluloses, was collected by filtration, leaving approx 85% of the original flax shive behind as a solid residue on the filter.

3.3. Preparative ion-exchange chromatography

The aqueous phase thus obtained was lyophilized and 1 g of the resulting solid material re-dissolved in 100 mL water. This solution, designated as fraction F1, was then passed through a 6-mL column containing the cation-exchange resin Amberlite IR-120, H^+ form (BDH Laboratory Supplies, UK), in order to protonate all of the uronic acid residues present. Thereafter, the eluent obtained was placed onto a 45-mL column containing the anion-exchange resin Amberlite IRA-93, carbonate form (Fluka, Switzerland). Upon washing this column with 150 mL water, neutral saccharides (fraction N1) were recovered in the void volume. Acidic saccharides (fraction A1) were subsequently removed from this

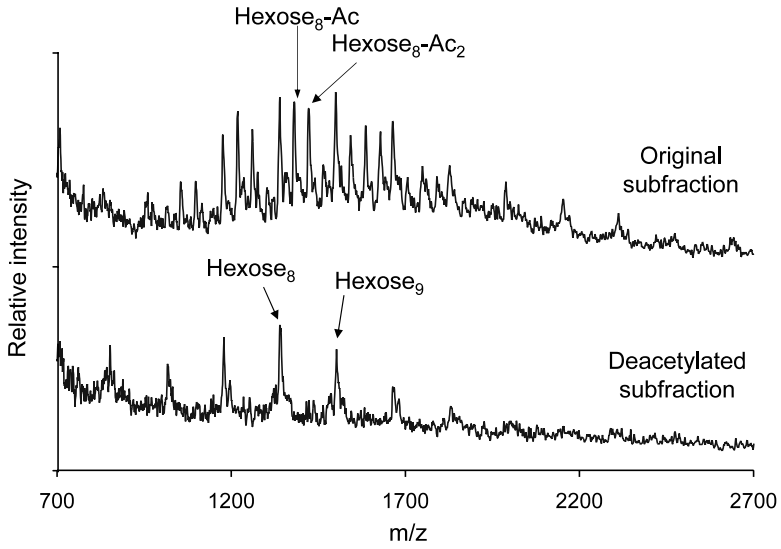

Fig. 5. MALDI-MS spectra of the neutral oligosaccharides present in a subfraction of N3 (see Fig. 1) and obtained by SEC employing de-ionized water as the mobile phase (Fig. 2). In the spectrum of this untreated subfraction (upper spectrum), the distance between adjacent peaks in each oligomer cluster corresponds to 42 mass units (i.e., the mass of an acetyl group). Spectral analysis of the same subfraction following alkaline hydrolysis (lower spectrum) revealed peaks originating from glucomannan oligomers with DP values ranging from 6 to 11.

column by elution with 100 mL 50% (v/v) aqueous acetic acid.

Thereafter, fraction N1 was further purified by enzymatic hydrolysis involving incubation with a combination of *Trichoderma reesei* xylanase II, β-xylosidase and acetyl xylan esterase in 50 mM ammonium acetate buffer, pH 5 for 24 h at 40 °C.[41] Fraction N2 was obtained by removing monosaccharides and low-molecular-mass oligosaccharides from this hydrolyzate by gel filtration on a PD-10 column (Pharmacia Biotech, Sweden). N2 was thereafter subjected to SEC and the final glucomannan fraction N3 collected at the outlet of the refractometer during the time period required for elution of neutral saccharides.

3.4. Analysis of the carbohydrate compositions of hemicellulose fractions

Carbohydrate analysis of isolated hemicellulose fractions was performed employing enzymatic hydrolysis and capillary zone electrophoresis (CZE), as described previously,[39] with the modification that the enzyme mixture utilized contained equal volumes of Novozym 188, Celluclast 1.5 L (Novozymes, Denmark) and Econase 400-TX (Röhm, Finland). In brief, this mixture of purified enzymes (0.5 mL) and sodium acetate buffer (0.5 mL, pH 4.0) were stirred together with the sample (10 mg) for 30 h at 40 °C in order to achieve complete enzymatic hydrolysis. The hydrolyzate thus obtained was supplemented with 0.040 mL of a ribose solution (10 mg/mL) (used as an internal standard for quantitation) and an aliquot (0.2 mL) of this mixture removed for derivatization with *p*-aminobenzoic acid ethyl ester and subsequent analysis by CZE.

3.5. Analysis of the carbohydrate compositions of raw flax shive

Raw flax shive was pretreated by swelling and delignification with peracetic acid and thereafter subjected to enzymatic hydrolysis and subsequent determination of neutral and acidic monosaccharides by CZE in the same manner as described above for isolated hemicellulose fractions. The pretreatment step, which was necessary to render the flax shive susceptible to enzymatic hydrolysis, involved treating pulverized flax shive (10 mg) with 1 mL of a peracetic acid solution (150 mg/mL water) at 70 °C for 16 h, as described in detail previously.[42]

3.6. Analysis of the carbohydrate composition of fraction N3 employing TFA/CZE

Approximately 0.2 mg N3 was dissolved in 50 μL 2 M trifluoroacetic acid (TFA) and this solution maintained thereafter at 80 °C for 4 h. Subsequently, the solvent was evaporated under a flow of nitrogen gas and the residue dissolved in sodium acetate buffer (50 mM, pH 4.0) and derivatized for CZE analysis according to the procedure described earlier.[39]

3.7. Size-exclusion chromatography (SEC)

The SEC system employed here consisted of three columns containing Ultrahydrogel 120, 250 and 500

(Waters Assoc. USA), respectively, linked in series to each other and to a refractometer (Waters Assoc. USA). Two different eluent systems were utilized, i.e., deionized water and 50 mM ammonium acetate pH 7. Approximately 1 mg hemicellulose was injected into this SEC system. The signal from the refractometer was processed on a standard PC using the PL Caliber SEC software and interface (Polymer Laboratories Ltd., UK). At regular intervals during the elution from the SEC system, 100-μL fractions were collected at the outlet of the refractometer.

3.8. Molecular mass calibration of the SEC system

The peak-average molar mass (M_p) values of fractions collected at the outlet of the refractometer were determined by MALDI-MS analysis. The logarithm of the M_p value thus obtained for each fraction was plotted as a function of elution time. In this manner, linear relationships between log M_p and elution time were established for each of the three hemicellulose preparations A1, N1 and N2 and subsequently employed to determine the number- and weight-average molar masses (M_n and M_w) of the entire hemicellulose distribution.[35]

3.9. Removal of *O*-acetyl substituents

O-Acetyl moieties on oligo- and polysaccharides were removed by alkaline hydrolysis in the presence of ammonium hydroxide. For this purpose, approx 50 μL 25% aq ammonium hydroxide was added to each SEC fraction and this mixture maintained thereafter at 80 °C for approx 30 min.

3.10. Determination of DS_{Ac} by SEC/MALDI-MS

The DS_{Ac} values for the isolated glucomannan were calculated from the M_p values of each fraction without and following deacetylation, as determined from the MALDI spectra. The values given are means for six separate SEC fractions in the case of each hemicellulose sample.

3.11. MALDI mass spectrometry

The MALDI analyses were performed using a Hewlett-Packard G2025 A MALDI-TOF mass spectrometer equipped with a linear detector, employing 1–5 μJ energy pulses of the UV (337 nm) laser beam. The spectra depicted routinely represent the sums of 20–50 laser shots. Both positive- and negative-ion spectra were recorded.

Twenty-five microlitres saturated, aqueous solution of 2,5-dihydroxybenzoic acid (DHB) (the matrix) was added to each fraction (approx 100 μL) collected from the SEC system, and approx 0.5 μL of this mixture then applied to the MALDI probe. The molar mass of the peak of maximal intensity in the MALDI-MS spectrum was taken to be the peak-average molar mass (M_p) of each fraction. The weight- and number-average molar masses (M_w and M_n) for each fraction were calculated from the MALDI-MS spectra using the Hewlett-Packard G2025 MALDI-TOF software.

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