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Isolation and partial characterisation of the non-cellulosic polysaccharides of flax fibre

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ABSTRACT

The non-cellulosic polysaccharides of flax fibre cells were isolated using chemical extraction methods. Extraction of mature retted flax fibre with the calcium chelating agent *trans*-1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetic acid (CDTA) removed polysaccharides enriched in galacturonic acid. These pectic polysaccharides were fractionated by their solubility in water into two fractions that differed in their neutral sugar content. Extraction of the fibre with aqueous 24% (w/v) potassium hydroxide produced the first hemicellulosic fraction (Hc1), which was enriched in xylose and, to a lesser extent, glucose- and galactose-containing polysaccharides. Digestion of aliquots of this fraction using the fungal carbohydrase mixture, Driselase, yielded xylobiose and isoprimeverose, which are characteristic limit digestion products of xylans and xyloglucans, respectively. Digestion of Hc1 with a crude cellulase preparation from *Aspergillus niger* yielded oligosaccharide products, one of which was similar to a characteristic nonasaccharide subunit of xyloglucan. Further extraction of the fibre residue with aqueous 18% (w/v) potassium hydroxide supplemented with 4% (w/v) of boric acid removed a second hemicellulosic fraction (Hc2), which was enriched in mannose-containing polysaccharides. Digestion of this fraction with a highly purified endo-(1 → 4)-β-D-mannanase converted 67% of the total polysaccharide into oligosaccharides within one hour. The β-D-mannanase-derived oligosaccharides had a monosaccharide composition of mannose–glucose of 1.6:1.0. Analysis of the oligosaccharides by TLC and high-performance anion exchange chromatography revealed products characteristic of a glucomannan similar to those found in hardwoods. The presence of β-linked D-glucose residues was confirmed by the removal of glucose from the β-D-mannanase-derived oligosaccharides by a purified β-D-glucosidase. A final extraction of the fibre with aqueous 90% Me_2SO containing 4% (w/v) of boric acid removed a further pectic fraction (Hc3) enriched in neutral sugars. Preliminary analyses suggest that this fraction may contain a polysaccharide akin to rhamnogalacturonan I.

INTRODUCTION

Flax (*Linum usitatissimum* L.) is a commercially important fibre crop in the European Community[1]. Flax fibres are long, thin, and lustrous, and produce textiles and linen cloth of high quality[2]. Flax fibres are bast fibres originating from procambial cells of the protophloem within the young stem[3]. They are initially

thin-walled, but they start to develop secondary walls early in the life cycle of the plant[4].

The mature wall of fibre cells consists of cellulose (70–75%, w/w), hemicellulose (~ 15%, w/w), and pectic material (10–15%, w/w)[5,6]. The pectic material is particularly associated with the middle lamellae and its partial removal by a semi-controlled rotting (retting) process allows the fibre bundles to separate from the surrounding cells of the stem[7]. As a result of this commercial application, the pectic components of flax fibre have been extensively studied[6,8,9].

The role of the hemicellulose components in fibre structure is less clear. They do not appear to be absolutely required for the formation of a well-defined secondary wall, as they are absent in cotton fibres[10]. However, bast fibres probably have a different structural role from the seed fibres of cotton. Retting removes little hemicellulose from flax fibre, but the remaining hemicellulose is more easily solubilised[9]. Complete removal of hemicellulose, after depectination, by boiling in aqueous 6% (w/v) sodium hydroxide drastically reduces the strength of the fibre and results in the disintegration of fibre bundles into elementary fibres[8]. This suggests that hemicelluloses are present near the surfaces of the fibre.

Previous work has shed little light on the composition of the hemicelluloses of flax fibre. The presence of mannose-containing polysaccharides was suggested by the identification of this sugar in acid hydrolysates of flax fibre[11], and the presence of xylan-like polysaccharides was suggested by the success of enzyme mixtures which contained xylanase in digesting flax fibre[12]. Also, polysaccharides enriched in galactose and glucose were obtained as a hemicellulose fraction after boiling in aqueous 6% (w/v) sodium hydroxide[6]. However, polysaccharides extracted in this manner would be subject to extensive alkaline degradation[13]. This paper initiates research into the composition and function of flax fibre hemicelluloses.

RESULTS

Extraction of dewaxed fibre with the calcium-chelating agent, CDTA, at 85°C removed two fractions, Pa and Pb (Table I), enriched in uronic acids. These fractions differed in their solubility and their neutral sugar content. Fraction Pa contained only 6.8% (w/w) of neutral sugars, whereas fraction Pb contained 21.6% (w/w) of neutral sugars, of which half (11%) was galactose. Galacturonic acid was the only uronic acid identified in these extracts by TLC.

The first hemicellulosic fraction (Hc1) was enriched in xylose and, to a lesser extent, glucose- and galactose-containing polysaccharides (Table I). Digestion of this fraction with the fungal carbohydrase mixture, Driselase, produced two disaccharide products which co-chromatographed with xylobiose and isoprimeverose (Fig. 1). Xylobiose and isoprimerverose are characteristic limit digestion products of xylan and xyloglucan polysaccharides, respectively[14].

Digestion of Hc1 with a crude cellulase preparation produced a number of oligosaccharides which were soluble in aqueous 55% (v/v) acetonitrile. These

TABLE I

Monosaccharide composition of extracts from flax fibre

Fraction	% Recovery (g/100 g of fibre)	Monosaccharide composition (% of polysaccharide [a])							Uronic acid content [b]
		Fuc	Rha	Ara	Gal	Glc	Xyl	Man	
Pa	2.52	N.D. [c]	0.8	2.0	1.0	3.0	0.6	N.D.	91.6
Pb	2.30	N.D.	3.1	5.2	11.2	1.5	0.6	N.D.	69.2
Hc1	1.65	1.8	4.0	3.2	17.1	21.4	40.8	7.0	11.5
Hc2	2.39	N.D.	1.6	1.6	8.4	33.8	5.0	46.0	4.4
Hc3	0.48	N.D.	14.6	15.3	38.9	8.3	N.D.	N.D.	20.8

[a] Monosaccharide composition expressed as μg of monosaccharide obtained per 100 μg of polysaccharide hydrolysed. [b] Uronic acid content expressed as μg of GalA equivalents per 100 μg of polysaccharide. [c] N.D., Not detected.

oligosaccharides were separated by HPLC on an amino-silica column (Fig. 2). Oligosaccharide 5 has a similar monosaccharide composition (i.e., Glc–Xyl–Gal–Fuc–Man, 4.1 : 2.7 : 1.0 : 0.8 : 0.1) to the nonasaccharide, XG9, which is a characteristic subunit of xyloglucans[18].

The second hemicellulosic fraction (Hc2) was enriched in mannose- and glucose-containing polysaccharides (Table I). Digestion of this fraction with a highly purified endo-(1 → 4)-β-D-mannanase converted 67% (by hexose content) of the polysaccharide into oligosaccharides soluble in aqueous 50% (v/v) acetonitrile in 1 h. These β-D-mannanase-generated oligosaccharides had a monosaccharide com-

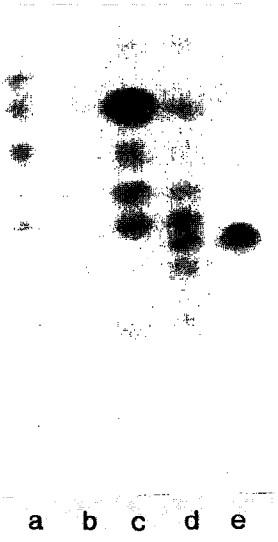

Fig. 1. Driselase and acid hydrolysis products of fraction Hcl. Lane a, mixture of standard monosaccharides, least mobile first: GalA, Gal, Glc (Man, Ara not resolved), Xyl (Fuc not resolved), Rib, Rha; b, isoprimeverose standard; c, acid hydrolysate of Hcl; d, driselase digest of Hcl; e, xylobiose standard.

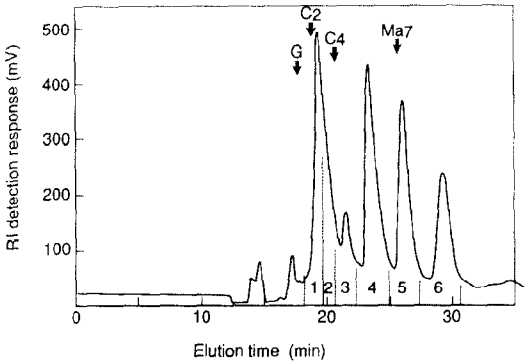

Fig. 2. Separation of oligosaccharides generated by cellulase digestion of fraction Hc1, by HPLC on amino-silica.

position of mannose–glucose–galactose of 1.6 : 1.0 : 0.02. In gel permeation chromatography on Bio-Gel P2, the majority of the oligosaccharides eluted around Kav 0.75–0.90 with the remainder eluting between Kav 0.5 and 0.75 (Fig. 3a). This suggests that the products range in size from disaccharide to > heptasaccharide. The material eluting between Kav 0.40 and 1.00 was pooled and freeze-dried, and aliquots were analysed by high-performance anion exchange chromatography (HPAEC) on the CarboPAC Pa1 column (Fig. 3b). Eight distinct peaks were noted (A–H). From the general chromatographic properties of the digest, it is likely that peak A is a disaccharide and peak H probably a hepta- or octa-saccharide. Digestion of the β-D-mannanase-generated oligosaccharides with a partially purified β-D-glucosidase altered the products obtained (Fig. 3c). Monosaccharides (mainly glucose and some mannose) were generated and identified by TLC. Certain oligosaccharides were removed completedly or reduced (i.e., A and B removed; D, E, F, G, and H reduced), whilst oligosaccharide C was relatively unaffected. The β-D-glucosidase did not remove either glucose or mannose residues from the intact polysaccharide in Hc2.

The final extraction with Me_2SO–boric acid removed a pectic fraction enriched in neutral sugars (Table I). The ratio of galacturonic acid to rhamnose content is ~ 1.4 to 1.0, which is approaching the 2 : 1 ratio found in rhamnogalacturonan I[20] (Rg1). Total acid hydrolysis yielded a compound which had a lower mobility in TLC than either galacturonic or glucuronic acid (Fig. 4, lanes d–f). This compound stained orange–red with aniline phthalate, which is the characteristic colour reaction of uronic acids[14]. As uronic acid residues in polysaccharides are known to be resistant to acid hydrolysis[21], this compound may be an aldobiouronic acid derivative similar to the galacturonsyl-rhamnose disaccharide isolated from rhamnogalacturonan I by acid hydrolysis[22]. On the other hand, 75% of the arabinose and 17% of the galactose content of this fraction, measured by HPAEC, can be released by mild acid hydrolysis (Fig. 4, lane c). The acid lability of the arabinose residues suggests that they are present in the furanose form[14].

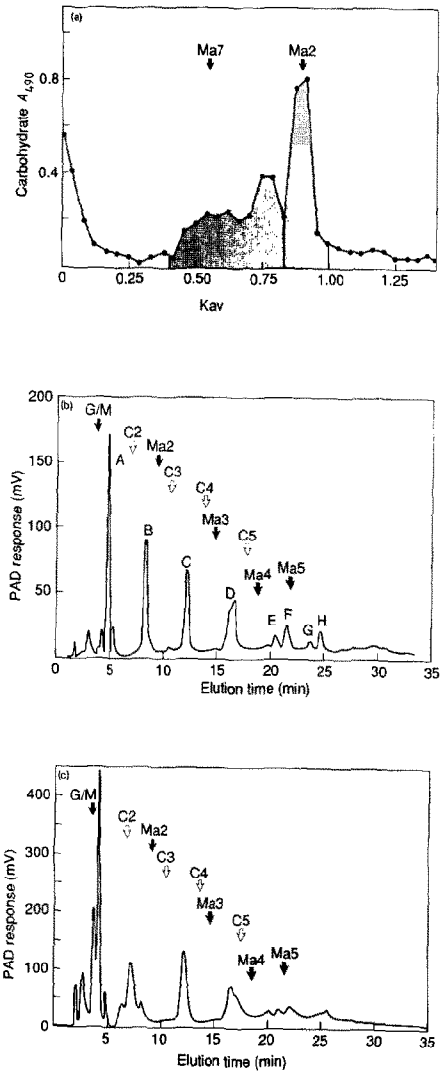

Fig. 3. (a) Separation of β-D-mannanase-generated oligosaccharides by gel permeation on Bio-Gel P2. (b) Separation of β-D-mannanase-generated oligosaccharides by HPAEC. (c) Separation of oligosaccharides generated by β-D-glucosidase digestion of β-D-mannanase-derived oligosaccharides by HPAEC. Abbreviations: G/M, glucose/mannose; Ma_2, maltose; Ma_3, maltotriose, etc.; C_2, cellobiose; C_3, cellotriose, etc.

DISCUSSION

It has been shown that retting of flax fibre preferentially removes pectins of high galacturonic acid and low neutral sugar contents[6]. However, extraction of the retted fibre in this study with hot CDTA solution removed a fraction enriched in these types of polymers (i.e., average galacturonic acid content > 80%). The fibre

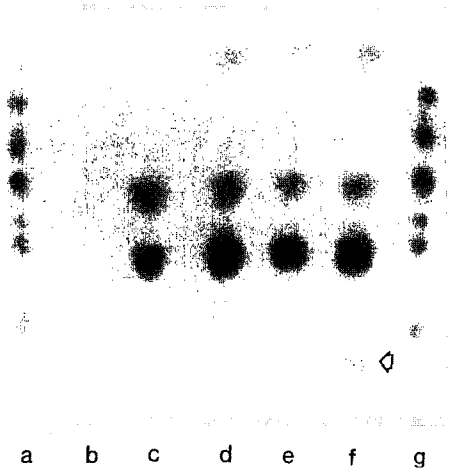

Fig. 4. Products generated from fraction Hc3 by different acid hydrolysis treatments: each lane b–f contains an equivalent amount of hydrolysate. Lanes a and g, mixture of standard monosaccharides: see Legend Fig. 1; b, no CF_3CO_2H, 100°C, 1 h; c, 0.1 M CF_3CO_2H, 100°C, 1 h; d, 0.5 M CF_3CO_2H, 100°C, 1 h; e, 1.0 M CF_3CO_2H, 100°C, 1 h; f, 2.0 M CF_3CO_2H, 100°C, 1 h. Unknown compound noted by arrow.

used in this study appears to be considerably under-retted, as a network of pectic material (presumably the remnants of middle lamellae of surrounding cells) can be observed on the surface of the fibre, using staining with ruthenium red (results not shown).

Rhamnogalacturonan I (RgI) has been suggested to be preferentially removed from flax fibres by extraction with hot water or EDTA solution[6]. In this case, a fraction that appears to contain an RgI-like polysaccharide was obtained by Me_2SO–boric acid treatment of the fibre residue after previous depectination in CDTA and alkaline extraction. Since RgI appears to be specifically localised in the middle lamella[23], this suggests that Me_2SO can swell the fibre bundles sufficiently to extract material from between the elementary fibres.

The first alkaline extract contained xylan and xyloglucan-like polysaccharides. Xyloglucan is known to be a component of cotton fibres, but it is thought to be restricted to the primary wall[24].

Extraction of plant material with alkaline boric acid is known to favour the removal of mannose-containing polysaccharides[25]. Two-thirds of the polysaccharides in this extract (Hc2) could be digested by endo-(1 → 4)-β-D-mannanase within one hour. The monosaccharide composition of these β-D-mannanase-derived oligosaccharides (mannose–glucose 1.6 : 1.0) suggests the presence of a glucomannan similar to those extracted from hardwoods (angiosperms)[26]. These oligosaccharides contain β-linked D-glucose that can be removed by β-D-glucosidase treatment. However, the polysaccharide itself is resistant to β-D-glucosidase digestion. This suggests that the glucose residues are inaccessible in the polysaccharide, perhaps in the backbone of the polysaccharide.

The endo-(1 → 4)-β-D-mannanase from *A. niger* has a restricted action pattern on glucomannans[27]. For example, the active site binding requirements are such that it will not form oligosaccharides with a glucose residue at the reducing terminal. Other oligosaccharides (e.g., mannosyl–glucosyl–mannose and mannosyl–glucosyl–mannobiose, MGM and MGMM) cannot be formed as a result of other binding restraints. These constraint on the enzyme action may be of use for the identification of individual oligosaccharides.

The glucomannan content of flax fibre, assuming that the weight lost during the two alkaline extractions is distributed between Hc1 and Hc2 in the ratio of the recoveries noted in Table I, is ~ 2.5%. This is similar to the figure for hardwood glucomannans. The role of glucomannan is unknown, but it is likely to be closely associated with cellulose microfibrils in the secondary wall. Future work will involve using sera raised against the glucomannan oligosaccharides obtained here to immunolocalise this polysaccharide in the secondary wall of flax fibres.

EXPERIMENTAL

General.—Thin-layer chromatography (TLC) was performed on aluminium-backed cellulose plates in *A*, butan-1-ol–acetic acid–water, 3:1:1; followed by *B*, EtOAc–pyridine–water, 10:4:3; and on silica gel, double-developed in *C*, propan-1-ol–EtOH–water, 7:1:2. Monosaccharides were stained using aniline phthalate[14] and oligosaccharides using vanillin–H_2SO_4–EtOH. Total acid hydrolysis was carried out in 2 M CF_3CO_2H at 120°C for 1 h, and mild acid hydrolysis in 0.1 M CF_3CO_2H at 100°C for 1 h. Samples were dried in vacuo and analysed by TLC on cellulose in irrigants *A* then *B*. Galacturonic and glucuronic acids were separated by TLC on cellulose in butanol-1-ol–pyridine–water; 4:3:4. Quantitative analysis of sugars was carried out by high-performance anion exchange chromatography (HPAEC) on a CarboPAC PA1 column (250 × 4 mm), using electrochemical detection with a pulsed amperometric system[15] (Dionex Ltd, UK). Samples were quantified against standard mixtures of monosaccharides run under identical conditions. The uronic acid content of polysaccharides was determined by the method of Blumenkrantz and Asboe-Hansen[16], and total carbohydrate by the phenol–H_2SO_4 method[17].

Extraction of polysaccharides.—Mature, retted, scutched, and hackled Belgian flax fibre was obtained as a gift from Craigview Mills, Inverbervie, UK. Fibre was washed using aq 0.5% (v/v) non-ionic detergent at 50°C, rinsed with cold water, and dried at 60°C. The fibre was cut into ~ 2-cm lengths by hand. The only milling procedure effective on fibre, retch-milling, produced excessive heat and damaged the fibre.

Fibre was dewaxed in toluene–EtOH (1:1) for 24 h at 20°C. All extractions were carried out in at least a ten-fold excess of extractant to fibre. The dewaxed fibre was washed with EtOH then water, and dried at 60°C. Dewaxed fibre was extracted with 50 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid

(CDTA) pH 7.5 for 1 h at 85°C. The warm extract was clarified by filtration through glass fibre and then cooled to 5°C overnight. A precipitate (fraction Pa) was collected by centrifugation (2500*g*, 30 min, 5°C). The supernatant solution (fraction Pb) was dialysed against distilled water and then freeze-dried.

Alkaline extractions.—The CDTA-extracted fibre was extracted with aq 24% (w/v) KOH containing 1% (w/v) of $NaBH_4$ for 24 h at 20°C. The alkaline extract was separated from the residue by filtration and clarified by filtration through glass fibre, and the filtrate neutralised at 5°C using glacial acetic acid. Insoluble material was removed by centrifugation (2500*g*, 30 min, 5°C) and the supernatant solution (Hc1) dialysed against water and freeze-dried. The fibre residue was washed extensively with water until the eluant was neutral, dried, and then extracted using aq 18% (w/v) KOH and 1% (w/v) $NaBH_4$ supplemented with 4% (w/v) of boric acid for 24 h at 20°C. After clarification, neutralisation, centrifugation, and dialysis as above, the extract (Hc2) was freeze-dried.

The residue was finally extracted with aq 90% (v/v) Me_2SO containing 4% (w/v) of boric acid for 24 h at 20°C. The extract was clarified as above, concentrated ten-fold by rotary evaporation, and diluted with four parts of water, and polysaccharides were precipitated by the addition of ice-cold EtOH to 50% (v/v). The precipitated polysaccharides (Hc3) were resuspended in water, dialysed against water, and then freeze-dried. The fibre residue after these extractions accounted for 81% of the original weight.

Borate associated with Hc2 and Hc3 could be partially removed by resuspending the polysaccharides in MeOH–glacial acetic acid–water (5:1:4) and repeatedly drying the material from MeOH–water (2:3) on a rotary evaporator.

Enzymic digestions.—Polysaccharide (100 mg) was dissolved in 1 mL of aq 1% (v/v) pyridinium acetate buffer (pH 4.0) containing 10 units of a highly purified endo-(1 → 4)-β-D-mannanase from *Aspergillus niger* (Megazyme Ltd, New South Wales, Australia) and incubated with stirring at 100 rpm at 37°C for 1 h. The reaction was terminated by either adding 1 mL of HPLC grade acetonitrile or by heating at 100°C for 10 min.

Samples were treated with a partially purified β-D-glucosidase (Sigma Chemical Co. Ltd, product G4511) at 10 units/mL at 37°C in aq 1% (v/v) pyridinium acetate (pH 4.5) for up to 18 h, and the reaction was terminated by the addition of an equal volume of acetonitrile. The β-D-glucosidase preparation is an exoenzyme and lacked activity against *p*-nitrophenyl α- and β-D-mannopyranosides, α- and β-D-galactopyranosides, and α-D-glucopyranoside. Hemicellulosic fraction Hcl (170 mg) was hydrolysed using a crude cellulase preparation from *A. niger* (Sigma, product C2415) at 4 mg/mL in aq 1% (v/v) pyridinium acetate buffer (pH 4.5) at 37°C for 20 min. The reaction was terminated by the addition of acetonitrile to a final concentration of 50% (v/v). Hemicellulose fraction Hcl (100 mg) was hydrolysed using Driselase at 5 mg/mL in aq 1% (v/v) pyridinium acetate buffer (pH 4.5) at 37°C for 4 h. The reaction was terminated by the addition of acetonitrile to 50% (v/v). Xylobiose was purchased from Sigma Chemical Co. Ltd, and iso-

primerverose was obtained as the major product of Driselase digestion of Nasturtium xyloglucan carried out under the same conditions outlined above.

Chromatographic procedures.—Gel-permeation chromatography was carried out on a column (200-cm^3 bed volume) of Bio-Gel P2 preequilibrated in aq 1% (v/v) pyridinium acetate (pH 4.7). Blue dextran (2 mg/mL) and cobalt chloride (5 mg/mL) were added as markers of the void and included volumes, respectively. Fractions were collected and assayed for carbohydrate. HPLC of oligosaccharides was carried out on semipreparative column of amino-bonded silica (Spherisorb-NH_2, 5 μM, 250 × 10 mm, Merck, Poole, UK) using acetonitrile–water mixtures as eluents. Peaks were detected by refractive index (Gilson model 131), collected by hand, and dried in vacuo. Oligosaccharides were separated by HPAEC on the CarboPAC Pa1 column, using a linear gradient of 25 mM NaOAc in 100 mM NaOH to 100 mM NaOAc in 100 mM NaOH over 20 min at 1 mL/min. Sodium hydroxide (500 mM) was added at 1 mL/min post column, but predetector, to maximise the sensitivity of the pulsed amperometric detection.

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