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Structural features of galactans from flax fibres

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Successive extraction of mechanically-isolated flax fibres with ethylene-diamine-tetraacetic acid partially neutralized with NAOH EDTA–Na_2 and NaOH solubilized about 25% of the mass of fibres. The cation exchange capacity (CEC) of the fibres decreased from 0.100 ± 0.025 meq g^{-1} to 0.035 ± 0.015 meq g^{-1} after EDTA–Na_2 extraction and then was reduced to virtually nil by extraction with NaOH. This final reduction in CEC by alkali was accompanied by the extraction of small charge molecules, notably phenolic acids. These may influence the binding selectivity of the fibre towards monovalent cations. Polymers solubilized by this extraction procedure included two main types of galactans; the first type consisted of β-1→4 galactans attached to a rhamnogalacturonan I-like polymer and the second type was β-1→3, β-1→6 linked galactans attached to proteins in some unknown manner. In addition, some β-1→3 glucan, β-1→4 glucan and glucomannan-like polymers were detected.

INTRODUCTION

The differentiation of secondary cell walls is one of the major events in the development of flax fibres (*Linum usitatissimum*). The formation of fibres consists of a initial rapid elongation step occurring for most cells during the flowering period and a progressive centripetal differentiation phase that mainly takes place during capsulation and seed maturation (Morvan *et al.*, 1989*b*). However, the exact mechanism involved in fibre differentiation is still poorly understood. Light has been demonstrated to be an important factor (Milthorpe, 1945) mainly with respect to the biosynthesis of cellulose precursors (Goubet *et al.*, 1993). Ripoll *et al.* (1993) have provided evidence that the differentiation of fibres in young plants of flax is correlated with a dramatic increase of the sodium to calcium concentration ratio in the cell walls. In mature fibres, the main cations which neutralize the negative charges in cell walls of fibre bundles are calcium ions but magnesium, sodium and potassium also account for about one-tenth of the total cation exchange capacity (Morvan *et al.*, 1989*a*).

Negative charges in cell walls are mainly borne by pectins, such as polygalacturonic and rhamnogalacturonic acids, proteins and phenolic acids (McNeil *et al.*, 1984) and the extent of the involvement of each of these components depends on the tissue, the state of plant development and the species (Jarvis *et al.*, 1988). In flax stems, the main part of the cation exchange capacity of cell walls is borne by polygalacturonic acids in the epidermis (Morvan *et al.*, 1991). Besides, using successive extractions from either green flax (Morvan *et al.*, 1988) or under-retted flax (Morvan *et al.*, 1989*a*, 1990; Davis *et al.*, 1990) or flax hypocotyls (Jauneau *et al.*, 1994; Rihouey, 1994; Rihouey *et al.*, 1995), we concluded that rhamnogalacturonans of type I rather than polygalacturonans were present in the primary cell walls and cell junctions of the fibre bundles of flax. On the other hand, polysaccharides enriched with galactose and glucose (Morvan *et al.*, 1989*a*), mannose (Derminot & Tasdhomme, 1977; McDougall, 1993) or xylose (Sharma, 1987) are also present and can be extracted in a hemicellulosic fraction.

In the present work, we have taken advantage of the availability of elementary fibres from mechanically processed flax, and have extracted, on the one hand, the constituents of their primary cell walls (with the ethylene-diamine-tetraacetic acid (EDTA) calcium chelator) and, on the other, the compounds associated with the secondary deposits of cellulose (using NaOH). We have analysed these fractions for their sugar composition and linkages.

MATERIALS AND METHODS

Plant material

Elementary flax fibres were obtained from mechanical treatment (carding) of bundles extracted from scutched

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stems that had been field retted. These were a gift of Rubalin S.A. (Bacqueville en Caux, France). They were abundantly washed with chloroform–methanol in order to solubilize the remaining cell components.

Preparation of polymers

Chloroform–methanol washed fibres were first treated with the calcium chelator EDTA–Na_2 (0.5% w/v, 100°C, 2 × 1 h) and then with NaOH (6% w/v, 100°C, 2 h under N_2 flux). Polymers solubilized with EDTA (P_{EDTA}) and NaOH (P_{NAOH}), respectively, were obtained after ultrafiltration through Pellicon membrane (10 kDa, Millipore) and then freeze dried.

Ion exchange chromatography

P_{EDTA} (50 mg in 10 ml H_2O) was submitted to ion exchange chromatography on a DEAE Sepharose CL 6B (Pharmacia) column (17 × 1.8 cm) equilibrated in H_2O pH 5 (carbonated water). After sample loading, the column was washed with 100 ml of the same eluent, giving the fraction DEAE-1. Bound polysaccharides were then eluted with a NaCl gradient (400 ml, 0–1 M). The eluate was monitored for absorbance at 214 and 280 nm and appropriate acid fractions were pooled (DEAE-2–DEAE-5), ultrafiltered and concentrated as required for further analysis.

Size exclusion chromatography

Size exclusion chromatography was carried out on a column of Sephacryl S200 (Pharmacia, 60 by 2.5 cm 100 ml h^{-1}), using 1 M NaCl. Polysaccharides (50 mg in 10 ml 1 M NaCl) were loaded onto the column and subsequently detected by absorbance at 214 nm. The excluded and void volumes were estimated with dextran blue and galacturonic acid, respectively.

Sugar composition

To estimate the total sugar composition, including uronic acids, samples (1 mg) were methanolysed (24 h at 80°C) and methylsialated (4°C, overnight) in 1% trimethylchlorosilane in N,O bis (trimethylsilyl-fluoroacetamide) (Quemener & Thibault, 1990) and analysed by gas liquid chromatography on a DB 225 capillary column (J.W. Instruments) as previously described (Goubet *et al.*, 1993).

Sugar linkages

Samples (1 mg) were solubilized in 1 ml of DMSO, frozen and methylated by incubation with 200 mg of NaOH and 1 ml of methyl iodide for 45 min at 4°C. The reaction was terminated by addition of 2 ml of $Na_2S_2O_3$ (Harris *et al.*, 1984). Methylated polymers were extracted four times with chloroform (0.5 ml). The organic phase was washed ten times with water, dried and then solubilized in methanol and chloroform (1.1 v/v). Contaminating ions (Na^+, $S_2O_3^{2-}$) were eliminated by size exclusion chromatography (LH-20 Sephadex; Pharmacia, France). Methylated polymers were hydrolysed at 100°C in 2 M trifluoroacetic acid for 2 h and acetylated by the method of Albersheim *et al.* (1967). The partially methylated alditol acetates were solubilized in dichloromethane and placed on a Ross injector. The material was fractionated on a DB225 capillary column (J.W. Instruments). The temperature was raised from 120°C to 230°C at 2°C min^{-1} and was held at 230°C for 15 min. Methylation was performed with duplicate samples and the derivatives were identified by reference to Jansson *et al.* (1976).

Cation exchange capacity of fibres

The cation exchange capacity (CEC) of chloroform–methanol fibres was measured as previously described (Morvan *et al.*, 1985; Goldberg *et al.*, 1986). The walls were put into the H-form and then titration was performed to pH 7 in the presence of 0.1 M $CaCl_2$. The cations which neutralized the negative charges of fibres were eluted by 0.1 M HCl and estimated by spectrophotometry.

Miscellaneous

Total sugar was estimated by the phenol–H_2SO_4 method (Dubois *et al.*, 1956). Proteins were estimated using the micro-Bradford method (Bradford, 1976).

RESULTS

Cation exchange capacity (CEC) of elementary fibres

The CEC of 20 batches of mechanical elementary fibres of different varieties or degrees of retting was estimated as 0.100 ± 0.025 meq g^{-1} dry mass. The CEC of EDTA-treated fibres decreased to 0.035 ± 0.015 meq g^{-1} dry mass and was effectively zero after NaOH treatment. In both cases, the standard deviation was high due to the difference in varieties or degrees of retting. Within one batch the deviation did not exceed 10%.

Composition of elementary fibres of flax

Flax fibres obtained after mechanical treatments (carding) contained mainly elementary fibre cells and few cells still attached through their cell junctions. Extractions of the chloroform–methanol cleaned fibres with the calcium chelator EDTA and then with NaOH removed about one-quarter of the dry matter. The

second extraction removed 22 ± 2% of the dry fibre mass, a percentage which was much larger than that of the EDTA–Na_2 extraction (1.5 ± 0.5%). Therefore, flax fibres contain appreciable amounts of other constituents associated with cellulose which might explain why they have a much higher mechanical resistance (Bossuyt, 1941) than cotton fibres which are about 95% cellulose.

After ultrafiltration, the polymeric fraction P_{EDTA} represented between 40 and 70% of the extracted material, depending on the experiment. Such variation originated from the variability in the molecular mass distribution, which might be due to some differences in the enzymatic degradation during the retting of stems and the mechanical treatments (scutching and carding).

Approximately 90% of the NaOH-solubilized components was lost during ultrafiltration. This indicates that the NaOH extract was mainly composed of molecules < 10 kDa. This is probably the result of the breakdown during alkaline extraction. Assuming a molecular mass of 180 per sugar, the maximum degree of polymerization of the components is < 60. Therefore, the majority of NaOH extracted compounds were oligomers or small polymers. In some cases, these low molecular weight compounds were recovered using a proton exchange resin to eliminate NaOH but most of the polymers linked to proteins bound to the exchanger via electrostatic interactions with the exchanger. Therefore, either a polymeric P_{NAOH} fraction or low molecular mass compounds could be collected after NaOH extraction.

Low molecular mass fraction contained sugars (mainly galactose) but also phenolics, notably hydroxybenzoic acids as deduced from their UV behaviour (Fry, 1988). Also, unpublished results suggest that flax stem walls contain bound hydroxycinnamates (McDougall, pers. commun.). Chrysanthemin or cyanidin 3-glucoside could be candidates too, as inferred from solid ^{13}C NMR data (Love *et al.*, 1993). It is worth noting that the phenolic-enriched oligomers showed an affinity towards sodium ions which might explain the sodium to calcium ratio found in the cell walls of fibres (Ripoll *et al.*, 1993). These components were not further analysed and the following study mainly deals with the polymeric fractions.

Composition of P_{EDTA} fraction

P_{EDTA} contained mainly sugars as indicated by the sulfuric acid–phenol reaction (Table 1). Also, this fraction reacted with Bradford reagent (1976) and had appreciable absorbance at 280 nm. This suggests it contained some proteins or/and phenolics; most of these components were lately eluted from S200-gel, probably retained through non-specific interactions (Hourdet & Muller, 1987).

P_{EDTA} and fractions recovered after fractionation on anion exchange and S200 size exclusion chromatographies were methanolysed and their sugar composition estimated by gas–liquid chromatography (GLC) analysis. As shown in Table 1, P_{EDTA} mainly comprised neutral sugars, including arabinose, galactose, mannose and glucose. The rhamnose to galacturonic acid ratio of the main fractions S200-2 to S200-3 (which together accounted for 56% of the mass of P_{EDTA}) and DEAE-3 (which represented about half of the mass of P_{EDTA}) was around 0.8–1 which is suggestive of a rhamnogalacturonan I (RGI)-type polysaccharide. This structure

Table 1. Composition of the polymeric fractions obtained by treatment of fibres with EDTA

Fraction	P_{EDTA}	S200-1	S200-2	S200-3	S200-4	S200-5	DEAE-1	DEAE-2	DEAE-3	DEAE-4	DEAE-5
Yield[a]	–	2	20	36	23	19	10	9	49	24	8
Proteins[b]	1.9	0.2	0.3	1.1	2.6	3.3	1.6	1.2	0.7	3.5	11.3
DO 280 nm[c]	0.336	0.169	0.556	0.937	2.780	0.800	0.041	0.202	–	0.496	1.266
Sugar amount[d]	0.5	0.7	0.6	0.4	0.4	–	0.7	1.1[e]	0.8	0.7	0.5
Molar % sugar composition[f]											
Rham	18.4	18.6	20.8	16.4	6.6	3.0	6.6	4.5	24.6	18.8	18.8
Man	8.0	4.0	3.3	9.2	18.7	21.8	27.0	10.5	3.4	3.7	4.3
Ara	6.2	2.7	5.4	6.6	6.5	2.7	7.5	13.6	4.8	4.7	5.5
Xyl	0.5	0.3	0.9	4.4	2.7	1.6	3.6	8.6	2.2	2.2	2.4
Glc	8.9	21.2	5.7	10.1	18.4	37.9	24	11.3	5.6	7.2	9.6
Gal	33.6	47.9	42.0	32.8	22.5	31.2	31.2	50.0	33.0	30.7	32.8
GalU	24.4	5.3	21.9	20.5	24.6	1.8	0.1	1.0	26.4	31.2	25.5

P_{EDTA} was obtained as descxribed in Materials and Methods and was ultrafiltrated. Some P_{EDTA} was loaded either on DEAE or on S200 equilibrated with NaCl or H_2O (see Materials and Methods). S200-1 to S200-5 (fractions obtained after size exclusion chromatography of P_{EDTA} onto S200) and DEAE-1 to DEAE-5 (fractions obtained after ionic exclusion chromatography of P_{EDTA} onto DEAE) were desalted and freeze dried.

[a]The yield of each fraction was the percentage of the mass recovered after fractionation.

[b]Proteins were estimated using the Bradford reaction, in mg per g of lyophilised polymer.

[c]DO was the absorbance measured at 280 nm for 1 mg of lyophilised polymer per 1 ml H_2O.

[d]The sugar amount in g per g of lyophilised polymer was estimated using the sulfuric acid–phenol reaction (Dubois *et al.*, 1956).

[e]In some cases, the sulfuric acid–phenol reactive was unexpectedly larger than 1.

[f]Sugar composition was expressed as a molar percentage, 100% being the sum of acid and neutral sugars.

was confirmed using nuclear magnetic resonance analysis (NMR) (data not shown, Davis *et al.*, 1990). Therefore, P_{EDTA} contains an acidic RGI-like polymer of intermediate molecular mass (25,000–50,000). The high galactose content in fractions S200-2, S200-3 and DEAE-3 may arise from β-linked galactose side-chains of RGI, as previously inferred from NMR analysis of polymers extracted with EDTA from scutched fibres (Davis *et al.*, 1990). In addition, low molecular mass material that eluted in S200-4 had a galacturonic acid to rhamnose ratio around 4 and probably represents polygalacturonans. These polymers may have side-chains of galactose and arabinose in a 1:0.3 ratio.

The non-retained (DEAE-1) fraction contained substantial amounts of glucose and mannose in a ratio close to 1 which suggests the presence of a glucomannan-like polysaccharide. This result agrees with that of McDougall (1993) who identified such a structure in flax scutched fibres. However, fractions S200-4 and S200-5 were also enriched in glucose and mannose (ratios 1 and 1.7, respectively) and this suggests that the polysaccharides might have been depolymerized during extraction. In these fractions, galactose might arise from co-eluted galactans or be attached to these glucomannans. Lastly, the minor and high molecular mass fraction (S200-1) contained around 50% glucose, without much mannose or xylose, which may arise from glucan-type polysaccharides.

Preliminary methylation analysis (Table 2) gave a pattern of glycosidic linkages in P_{EDTA} fractions consistent with the presence of long 1→4 chains of galactose (S200-1 and DEAE-1 and 2). The high yields of 2,3,4,6 tetra-*O*-methyl and 2,3,6 tri-*O*-methyl galactose from the main fractions S200-2, S200-3 and DEAE-3 indicate that 1-4 β-linked galactosyl and terminal galactosyl residues were common as side-chains, as found in RGI-like polysaccharides. However, some 1-3 linked galactosyl residues also appear to be present. On the other hand, the abundance of 2,3,6 tri-*O*-methyl derivatives suggest that 1→4 linked glucosyl residues were present in fractions S200-4, DEAE-1 and DEAE-2, DEAE-4 and DEAE-5. Fractions S200-2 to S200-4 and DEAE-5 yielded 2,4,6 tri-*O*-methyl derivatives of glucose, indicating the presence of β-1→3 linked glucose (i.e. callose).

Table 2. Methylation analysis of P_{EDTA} fractions

Methyl ether	P_{EDTA}	S200-1	S200-2	S200-3	S200-4	S200-5	DEAE-1	DEAE-2	DEAE-3	DEAE-4	DEAE-5	Mode of linkage
2,4,6-Me_3-glc	–	–	10	12	4	–	–	–	–	–	23	→3)-glc*p*-(1→
2,3,6-Me_3-glc	12	–	–	–	58	–	12	16	–	19	36	→4)-glc*p*-(1→
2,3,4,6-Me_4-gal	–	0	34	27	–	–	–	9	30	–	–	gal*p*-(1→
2,4,6-Me_3-gal	15	–	4	16	–	–	42	13	5	–	18	→3)-gal*p*-(1→
2,3,6-Me_3-gal	46	82	52	37	–	–	35	62	45	24	–	→4)-gal*p*-(1→
2,3,4-Me_3-gal	–	–	–	6	17	–	–	–	–	–	10	→6)-gal*p*-(1→
2,3-Me_2-gal	15	7	–	–	21	–	–	–	–	40	12	→4,6)-gal*p*-(1→

P_{EDTA} was obtained as described in Materials and Methods and was ultrafiltrated. S200-1 to S200-5 were the fractions obtained after size exclusion chromatography of P_{EDTA} onto S200. DEAE-1 and DEAE-5 were the fractions obtained after ionic exclusion chromatography of P_{EDTA} onto DEAE.

Composition of P_{NAOH}

Methanolysis of P_{NAOH} gave very high yields of galactose (Table 3). The appreciable yields of glucose without significant amounts of xylose or mannose suggest the presence of hydrosoluble glucans. After size exclusion chromatography of P_{NAOH}, the glucans were either found in high molecular mass polymers (S200-1) or detected in fractions S200-4 to 6 (i.e. in rather low molecular mass polymers (S200-1) or detected in fractions S200-4 to 6 (i.e. in rather low molecular mass

Table 3. Composition of the polymeric fractions obtained by treatment of fibres with NaOH

Fraction	P_{NAOH}	S200-1	S200-2	S200-3	S200-4	S200-5
Yield[a]	–	14	40	36	5	5
Proteins[b]	4.3	1.3	0.5	0.7	1.9	0.4
DO 280 nm[c]	0.408	0.315	0.535	0.446	0.222	0.182
Sugars[d]	0.9	1.0	0.8	0.9	0.5	0.5
Molar % sugar composition[e]						
Rham	3.1	2.4	4.2	5.6	3.8	2.7
Man	1.3	2.9	4.7	2.4	2.9	17.0
Ara	4.1	2.3	2.9	9.4	5.2	2.3
Xyl	0	0	1.2	1.9	0	0
Glc	13.5	23.7	5.0	4.6	8.3	20.0
Gal	78.1	68.7	82.0	76.1	79.8	58.0
GalU	0	0	0	0	0	0

P_{NAOH} was obtained as described in Materials and Methods. S200-1 to S200-5 (fractions obtained after size exclusion chromatography of P_{NAOH} onto S200) were desalted and freeze dried.
[a]The yield of each fraction was the percentage of the mass recovered after fractionation.
[b]Proteins were estimated using the Bradford reaction, in mg per g of lyophilised polymer.
[c]DO was the absorbance measured at 280 nm for 1 mg of lyophilised polymer per 1 ml H_2O.
[d]The sugar amount in g per g of lyophilised polymer was estimated using the sulfuric acid–phenol reaction (Dubois *et al.*, 1956).
[e]Sugar composition was expressed as a molar percentage, 100% being the sum of acid and neutral sugars.

compounds). The latter materials (S200-5) were also enriched in mannose and the glucose:mannose ratios around 0.8 suggest a glucomannan-like polysaccharide similar to those extracted with EDTA.

Fractions S200-2 to S200-4 which represented more than 75% of the dry mass of P_{NAOH} contained very high levels of galactose. This indicates that intermediate molecular mass galactans constituted the bulk of P_{NAOH}.

Methylation experiments for galactosyl residues are presented in Table 4. They gave a pattern of glycosidic linkages in P_{NAOH} fractions consistent with the presence of relatively long 1→3-galactans since 2,4,6 tri-*O*-methyl derivatives of galactose represented most of the galactosyl residue of P_{NAOH}. The absence of 2,3,4,6 tetra-*O*-methyl derivative might also suggest that galactose was one site of linkage with other compounds. These polymers were mainly intermediate molecular mass ones (S200-2 and S200-3 of P_{NAOH}). In the last fraction, 2,3,6 tri-*O*-methyl galactosyl derivatives indicate some 1,4-linked galactose. In addition, in the minor low molecular mass fractions, S200-4 and S200-5, 2,4 di-*O*-methyl galactosyl derivatives suggested 3,6 galactans.

DISCUSSION

Cell walls normally have net negative charges, owing to a preponderance of uronic acid residues. These uronic acids are the galacturonic acid of pectins in primary walls and glucuronic acid of xylans in secondary walls (McNeil *et al.*, 1984). The results reported in this paper show that, in flax elementary fibres, part of the cation exchange capacity of cell walls was borne by rhamnogalacturonic acids which were extracted in EDTA. As they were easily solubilized, they most probably originated from the external part of the fibres, namely, the primary cell walls. However, these polyelectrolytes would have higher electroselectivity towards the divalent cations and hence would not be responsible for the sodium signal that has been reported in flax fibres (Ripoll *et al.*, 1993). The lower CEC remaining after the

Table 4. Methylation analyses of P_{NAOH} fractions

Methyl ether	P_{NAOH}	S200-1	S200-2	S200-3	S200-4	S200-5	Mode of linkage
2,4,6-Me_3-gal	92	94	93	57	–	–	→3)-gal*p*-(1→
2,3,6-Me_3-gal	2	–	7	30	–	30	→4)-gal*p*-(1→
2,3,4-Me_3-gal	6	4	–	–	–	6	→6)-gal*p*-(1→
2,4-Me_2-gal	–	–	–	–	100	64	→3,6)-gal*p*-(1→
2,3-Me_2-gal	–	2	–	13	–	–	→4,6)-gal*p*-(1→

P_{NAOH} was obtained as described in Materials and Methods. S200-1 to S200-5 were the fractions obtained after size exclusion chromatography of P_{NAOH} onto S200.

EDTA treatment must be due to the presence of phenolic acids and/or to some acidic proteins rather than to uronic acids as these were rarely detected. It is interesting to note that only phenolic enriched compounds presented an affinity towards sodium ions, which might explain the presence of these cations in flax fibres.

Also, polysaccharides enriched in galactose were solubilized from flax elementary fibres in both EDTA and NaOH solutions. Galactose is an ubiquitous constituent of cell wall polysaccharides found in all higher plants. Most galactose is present in *O*-linked galactoprotein or arabinogalactans and their derivatives. The arabino-4-galactans generally belong to pectic compounds (Selvendran, 1985). In contrast, the 3,6-galactans are complex hemicellulosic polysaccharides and the proportion of 1,3-β- and 1,6-β linkages differs between plant sources, as does the arabinose:galactose ratio (Aspinall, 1980).

Our results suggest that the first type, i.e. 4-galactans, easily extracted by EDTA–Na_2 may be localized in the primary cell walls of fibres. They were either long neutral polymers, as has been previously described in the cell walls of mung bean hypocotyl (Hervé du Penhoat *et al.*, 1987) or short side-chains branched onto a RGI as has been previously described for scutched under-retted flax (Davis *et al.*, 1990). These polymers, which were very pure and represented the main compounds of EDTA–Na_2, have been previously located in cellular junctions of fibres of flax hypocotyls in early stages of development (Jauneau *et al.*, 1994).

On the other hand, NaOH treatment which disrupts the cellulosic network of secondary deposits in fibres (Sotton & Monrocq, 1977) solubilized polysaccharides greatly enriched in 3-galactans and to a lesser extent in 3,6 galactans. Cell walls of suspension-cultured cells of flax also contain both 1,4-galactans and 1,3,6-galactans (Goubet & Morvan, 1994). To our knowledge, that is the first report on the presence of 3,6-galactans in flax fibres. Naturally, we are aware that these polymers only represented a small part of the components associated with cellulose. Further studies are needed to complete this preliminary work. In particular, the ways in which the galactans are linked into the secondary cell wall and their interactions with proteins, phenolics and cellulose call for further investigations.

Finally, as previously reported, some forms of glucose (Morvan *et al.*, 1989*a*) were also obtained as hemicellulosic components of flax fibres. McDougall (1993) identified glucomannans in a hemicellulosic fraction. Glucomannans form the major hemicellulose of the secondary cell walls of gymnosperms as well as being a minor component of angiosperm secondary walls (Brett & Waldron, 1990). Our results indicate that polysaccharides akin to glucomannan are not only found in NaOH but also in EDTA extracts, which might suggest a localization of glucomannans at the interface of primary and secondary walls of flax fibres. Further investigations, such as immunolocalization are now needed to test this hypothesis.

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