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# Polysaccharides, tightly bound to cellulose in cell wall of flax bast fibre: Isolation and identification

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**Abstract**

Part of matrix polymers of flax bast fibre cell wall is tightly bound to cellulose and can not be extracted by conventional methods. To analyze these polymers, the residue, remaining after cell wall treatment with chelators and alkali, was dissolved in solution of lithium chloride in *N*,*N*-dimethylacetamide. Cellulose was precipitated by water and completely degraded by cellulase, giving the possibility to separate matrix polysaccharides, which remained in polymeric form. The obtained polymers were fractionated by gel permeation chromatography and characterized by monosaccharide analysis, staining with LM5 antibody and Yariv reagent, $^{1}H$ and $^{13}C$ NMR. The total yield of the polysaccharides that are tightly bound to cellulose in flax fibre, was 4.6%. The major fractions (molecular mass 100–400 kDa) were composed of galactose, accompanied by two other significant monomers, GalA and Rha, with the ratio 1.1–1.4. Composition and structure of the cellulose bound galactan permit to consider it as fragment of the high-molecular mass (2000 kDa) galactan, synthesized by the developing fibres, while forming the secondary cell wall of gelatinous type.


*Keywords:* *Linum usitatissimum* L.; Flax; Fibre; Galactan; Cellulose; Secondary cell wall; Gelatinous fibre; $^{1}H$ and $^{13}C$ NMR

## 1. Introduction

Plant cell walls are complex, multi-component, supramolecular structures, with numerous functions. Key components of these structures are polysaccharides – cellulose microfibrils, cross-linking glycans, and pectic substances. The biochemical analysis of plant cell wall composition and isolation of certain components are usually performed by fractionation, using chelators and alkali. Some polysaccharides remain unextracted even after treatment with concentrated alkali (Hayashi, 1989; Mooney, Stolle-Smits, Schols, & de Jong, 2001; Pauly, Albersheim, Darvill, & York, 1999; Rose, Hadfield, Labavitch, & Bennett, 1998). These polymers are characterized, as a rule, by acid hydrolysis, which permits to determine monosaccharide composition only. Therefore, not much is known about molecule masses and structures of these polysaccharides in native form. However, polysaccharides that are tightly bound to cellulose, may play an important role in the formation of cell wall supramolecular structure and the determination of its properties. In order to identify the polymers and to understand the types of interactions between them, firstly a method has to be developed to isolate them in polymeric form for further characterization.

This problem is especially pronounced for gelatinous type cell walls, in which almost the half of all non-cellulosic polysaccharides could not be extracted by chelators and alkali, as was indicated by monosaccharide analysis (Mooney et al., 2001). Gelatinous cell wall is a special type of secondary cell wall, which is present in fibres of many bast fibre crops and of tension wood. The major polysaccharides of gelatinous cell walls are galactose-containing ones, namely galactan and arabinogalactan proteins, which were intensively characterized in flax fibres (Gorshkova &

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Morvan, 2006). The β-(1 → 4)-galactan, formed in developing flax fibres is the version of rhamnogalacturonan I with very high degree of substitution (>90%), and proportion of side chains galactose accounting for up to 85%. Side chains are divergent in structure and length, reaching several tens galactose residues (Gorshkova et al., 1996; Gur'janov, Gorshkova, Kabel, Schols, & van Dam 2007; Gur'yanov, Gorshkova, Kabel, Schols, & van Dam, 2006). The polymer has molecular mass around 2000 kDa before incorporation into the cell wall. Polysaccharides of similar structure were found in fractions extracted from flax fibre cell wall by ammonium oxalate, DMSO and hot water (Davis, Derouet, Hervé du Penhoat, & Morvan, 1990; McDougall, 1993; Morvan et al., 2003; Van Hazendonk, Reinerink, de Waard, & Van Dam, 1996) but they had significantly lower molecular mass (100–300 kDa) (Gorshkova & Morvan, 2006). However, the comparison of galactose content in various fractions and in total cell wall shows, that the major portion of galactose (over 70%) is not extracted by concentrated alkali and remains bound to cellulose (Mooney et al., 2001). The application of cellulase to isolate these polymers gave only partial yields: enzymatic hydrolysis of fibre cell wall cellulose is hindered by its high crystallinity and degrades one third of the microfibrils at most (Girault, His, Andeme-Onzighi, Driouich, & Morvan, 2000). The purpose of our work was to develop method for effective isolation of polysaccharides that are tightly bound to the cellulose in flax fibre cell wall, and to characterize these polymers.

## 2. Materials and methods

### 2.1. Plant material

Flax plants (*Linum usitatissimum* L., cv Novotorzhski) were grown in soil to yellow ripeness. Dried, non-retted flax stems were used for manual isolation of bast fibre bundles. The isolated fibre bundles (18.9 g) were gently grinded (with pestle) in a mortar with an excess of water (1 l), which removed soft tissues and short fibres; the treatment was repeated three times. The remaining fibre bundles were washed in acetone and dried in air; the yield was 9.3 g. Cleaned fibres were twice boiled in a mixture of chloroform and ethanol (1:2 v/v; 300 ml; 5 min) and dried in air. Then the fibres were heated in 1% ammonium oxalate, pH 5.0 on boiling water bath (300 ml; 1 h), grinded gently in mortar and rinsed with pure water. The extraction was twice repeated with shorter time of heating (10–15 min). The fibres cleaned with ammonium oxalate were treated with 4 N KOH + 3% $H_3BO_3$ (300 ml; 2 h), grinded gently in mortar and rinsed several times with water. The alkaline treatment was twice repeated for shorter time (5–10 min). Fibre bundles were washed with water to neutral pH and dried at 50 °C; the yield was 6.4 g. The treatments with water, ammonium oxalate and alkaline extracted pectin and hemicellulose polysaccharides and helped to separate the residues of parenchyma cells from bast fibres.

The cleaned fibre bundles were powdered in liquid nitrogen and again subjected to extractions with 1% ammonium oxalate, pH 5.0 (100 ml, boiling water bath, 30 min), and 4 N KOH + 3% $H_3BO_3$ (100 ml; 30 min). The cell wall residue was washed with water to neutral pH and dried in air. The yield of undissolved cell wall residue was 6.0 g.

### 2.2. Extraction of polysaccharides from cell wall residue by hot water and dimethylsulphoxid (DMSO)

The cell wall residue (5.9 g) was suspended in 50 ml water and heated on a boiling water bath (30 min). The solution was filtered through a Buchner funnel with a nylon filter and concentrated by evaporation under reduced pressure at 40 °C; the extract was named W1. The insoluble residue was dried in air and subjected to extraction by DMSO (60 ml; 1 day); the treatment was repeated three times. The DMSO solutions were combined, filtered through a Buchner funnel with a nylon filter and mixed with water (2:1 v/v). The DMSO was removed on Sephadex G-25 column, eluted with 0.02% $NaN_3$ and the solution concentrated by evaporation under reduced pressure at 40 °C; the extract was designated as DMSO. The residing pellet was again suspended in 50 ml water (boiling water bath, 30 min) and the mixture was filtrated through a Buchner funnel with a nylon filter. After removing DMSO, the water solution was concentrated by evaporation under reduced pressure at 40 °C; this extract was named W2. The remaining cell wall residue was dried in air.

### 2.3. Preparation of solution of LiCl in N,N-dimethylacetamide (DMA) and cellulose activation

DMA was dried over molecule sieves (4 Å) for several days. The lithium chloride (8 g) was dried in conical flask at 180 °C during 4–5 h, cooled to room temperature and dried DMA (100 ml) was added to it. The flask was closed by glass stopper and mixed on shaker till LiCl was dissoluted.

Cellulose of cell wall residue was activated by solvent change from water to acetone and then to dried DMA as follows: cell wall residue (1 g) was suspended in water (10 ml; 40–60 min), and the pellet was separated on a Buchner funnel with nylon filter, rinsed with acetone and suspended in acetone (10 ml; 40–60 min). Then in a similar manner the pellet was washed by *N*,*N*-dimethylacetamide (10 ml × 2; 40–60 min) and kept in fresh portion of DMA overnight (Dupont, 2003; Fernandez, 2003).

### 2.4. Isolation of polysaccharides from cell wall residue after cellulose dissolution

Cell wall residue was separated from DMA by filtration and suspended in solution of LiCl in *N*,*N*-dimethylacetamide (100 ml) to dissolve cellulose (Dupont, 2003; Fernandez, 2003). The flask was closed with a glass stopper and mixed on a shaker at room temperature till cellulose dissolution (1–2 days). The viscous solution was diluted by DMA

(100 ml) and mixed to a homogeneous liquid. To precipitate cellulose the mixture was added drop by drop to water (200 ml) at stirring and kept overnight. The cellulose pellet was removed on Buchner funnel with a nylon filter and washed by water (2–3 times). The filtrate and water washes were combined; LiCl and DMA were removed on Sephadex G-25. The solution was concentrated by evaporation under reduced pressure at 40 °C; the extract was named DMA1.

To digest the precipitated cellulose, the pellet was collected on a filter, suspended in 0.01 M NaAc, pH 5.2 + 0.02% $NaN_3$ solution (50 ml), containing 0.1 ml cellulase (Cellusoft-L; Novo Nordisk Bioindustrrie S.A., Paris, France; 750 EGU/G) and the mixture was kept at 33 °C overnight. The solution was filtered and the precipitate was resuspended in fresh cellulase solution (2–3 days). Then solutions were filtered, combined, heated on boiling water bath (5–10 min), desalted on Sephadex G-25 and concentrated by evaporation under reduced pressure at 40 °C. This extract was designated as DMA2. Cellusoft-L has no glycosidase activities that could cleave galactans, rhamnogalacturonans or AGPs (Girault et al., 2000).

### 2.5. Size-exclusion chromatography

The concentrated extracts were applied to a column (0.9 × 90 cm) of Sepharose CL-4B (Pharmacia, Sweden) and eluted with 0.1 M NaCl + 0.05% $NaN_3$ solution (flow rate 0.25 ml/min, 1.8 ml fractions). Calibration was performed using pullulan standards with molecule weights 1600, 400, 200, and 100 kDa (P-1600, P-400, P-200, and P-100; Waters). Sugar content in each fraction was measured by phenol-sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Fractions were combined according to the peaks and concentrated by evaporation under reduced pressure. The fractions were desalted on Sephadex G-25 eluted with volatile buffer (0.01 M pyridine/acetic acid solution, pH 4.5), dried and used for monosaccharide analysis, NMR spectrometry and immunochemistry.

### 2.6. Isolation of high-molecular mass galactan from developing fibres

High-molecular mass galactan was isolated from developing fibres as was described earlier (Gorshkova et al., 1996; Gur'janov et al., 2007). Briefly, the fibre-rich phloem strips of flax plant were peeled from the xylem part in 10 cm stem portions below snap point. Phloem strips were homogenized in liquid nitrogen and the buffer-soluble material was extracted by 50 mM K-phosphate buffer, pH 6. After centrifugation of the homogenate (7000*g*, 5 min) the supernatant was brought to 80% (v/v) ethanol to precipitate the buffer-soluble polymers (overnight, 4 °C). The resulting pellet was washed three times with 80% ethanol and dissolved in 10 mM NaAc + 0.02% $NaN_3$, pH 4.5. After centrifugation (12,000*g*, 5 min), the supernatant was fractionated on Sepharose CL-4B as described above.

### 2.7. Monosaccharide analysis

The samples were hydrolyzed in 2 M TFA at 120 °C, 1 h, dried under air stream at 30–40° C. The sugar composition was carried out using a high-performance anion-exchange chromatography (HPAEC, Dionex) on CarboPac PA-1 column (4× 250 mm, Dionex) using pulsed amperometric detection (PAD, Dionex). Elution buffers were: A, 0.015 M NaOH; B, 1 M NaAc and 0.1 M NaOH. Column was equilibrated with A buffer and eluted with following linear gradient: 0–20 min A 100%; 20–21 min A 90% and B 10%; 21–31 min A 70% and B 30%; 31–32 min B 100%; 32–42 min B 100%; 42–43 min A 100%; 43–73 min A 100%; flow rate 1 ml/min at 30 °C. Monosaccharide standards were treated with 2 M TFA at 120 °C, 1 h before they were used for calibration. Mannitol was used as internal standard.

### 2.8. Immunodot binding assays (IDA) with LM5 antibody; staining with the Yariv reagent

Fractions of extracts separated on Sepharose CL-4B column were analyzed by dot binding assay, using the Yariv reagent and LM5 antibody. The Yariv reagent was synthesized according to the method of Yariv, Rapport, and Graf (1962). Two microlitre of each fraction were put on nitrocellulose membrane, dried for 10 min at room temperature, incubated for 10 min with the Yariv reagent and washed with water.

For immunodot binding assay 2 μl aliquots were applied to nitrocellulose in a twofold dilution series starting with <1 μg per dot. Membranes were allowed to air dry for 30 min and washed for 5 min in PBST (phosphate-buffered saline with 0.05% Triton X-100). PBS containing 5% BSA (PBS/BSA) was used to block the nitrocellulose sheets for 1 h. The primary antibody LM5 (PlantProbes), which recognizes an epitope of 1,4-β-D-galactan (Jones, Seymour, & Knox, 1997), was applied as a 50-fold dilution in PBST for 1 h. Immunostaining was performed using the Elite ABC Kit (Vector Laboratories) Membranes were subsequently washed three times for 15 min with PBST and incubated with secondary biotinylated antibodies for 30 min. The membranes were washed again three times in PBST for 15 min and then incubated for 30 min in ABC reagent (avidin-biotinylated horseradish peroxidase complex). The membranes were then washed again three times in PBST for 15 min and one time in water. 1,4-Galactan was visualized by addition of solution containing hydrogen peroxide and nickel-enhanced DAB (diaminobenzidine) (Vector Laboratories).

After staining, membranes were air dried and scanned. Controls for specificity included: samples incubated with diluted gum arabic (Sigma) and arabinogalactan from larch wood (Sigma) and nitrocellulose sheets processed without incubation in primary antibody, secondary antibody, or ABC complex solutions.

### 2.9. NMR spectrum analysis

The structure of galactan was determined by $^{1}H$ and $^{13}C$ NMR spectroscopy. Sample was dissolved in $D_2O$ (99.96 at % D). $^{1}H$ and $^{13}C$ spectra were recorded on Bruker Avance 400 NMR spectrometer at room temperature. Chemical shifts are expressed in ppm relative to external acetone-$d_6$ ($^{1}H$, $\delta$ 2.05; $^{13}C$, $\delta$ 30.5 ppm; Brucker).

## 3. Results

### 3.1. Isolation and fractionation of polymers, tightly bound to cellulose

To analyze the polymers that are tightly bound to cellulose in flax fibre cell walls, especial precautions were taken to clean the fibres from the surrounding tissues. The cleaned fibre bundles were completely devoid of the surrounding tissues as was checked by light microscopy (data not shown). The obtained fibres were thoroughly treated by ammonium oxalate and 4 M KOH and the remaining cell wall residue subjected to further analysis (Fig. 1).

Part of matrix polymers from cell wall residue could be extracted by hot water and dimethylsulfoxide treatments (extracts W1, DMSO, and W2) (Fig. 1), applied in previous studies (McDougall, 1993; Van Hazendonk et al., 1996). Gel-chromatography of these extracts was performed on Sepharose CL-4B column, and the fractions were combined according to the peaks (Fig. 2), and subjected to monosaccharide analysis (Table 1). The yield of all these fractions together was 0.28% from the dry mass of cell wall residue.

The approach that we applied to isolate matrix polymers from the pellet after the above treatments was to dissolve

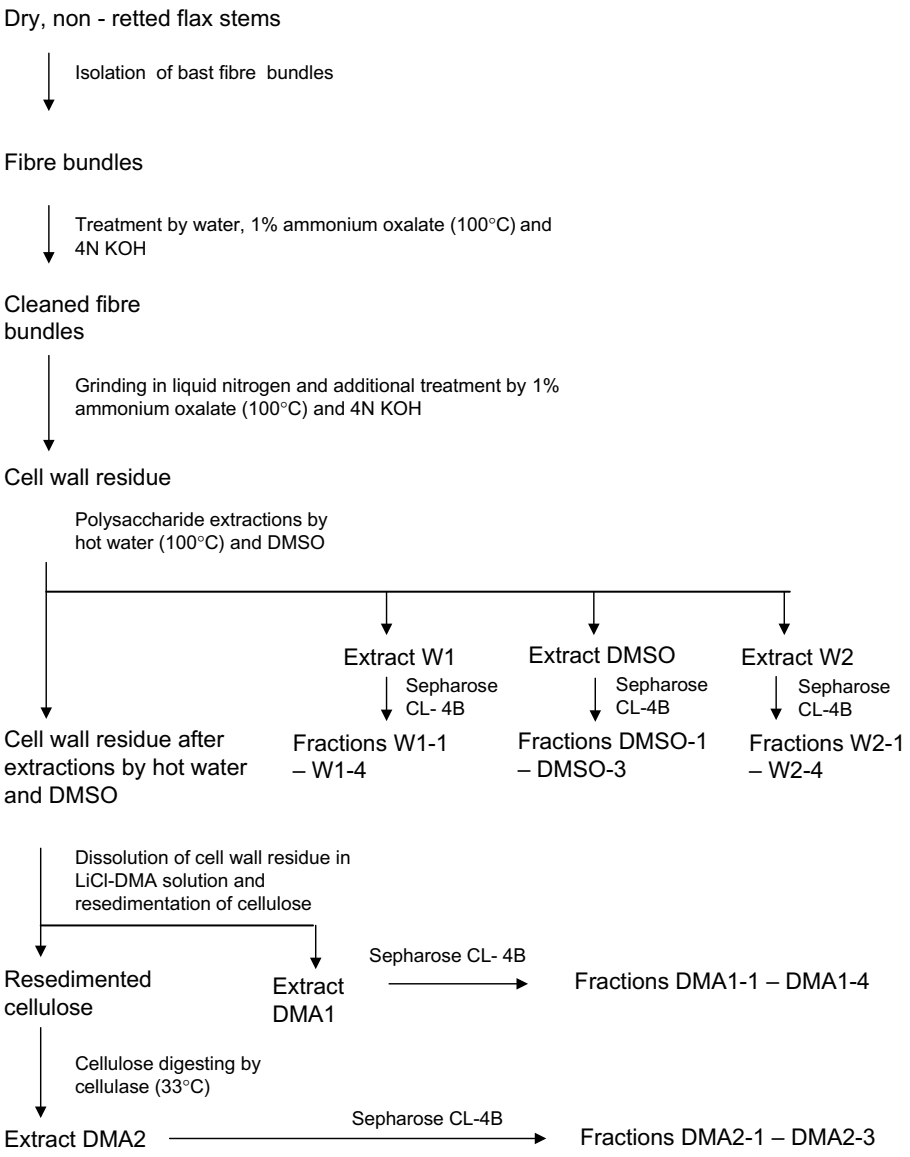


Fig. 1. Isolation scheme of the extracts from alkali-insoluble cell wall residue of flax bast fibre.

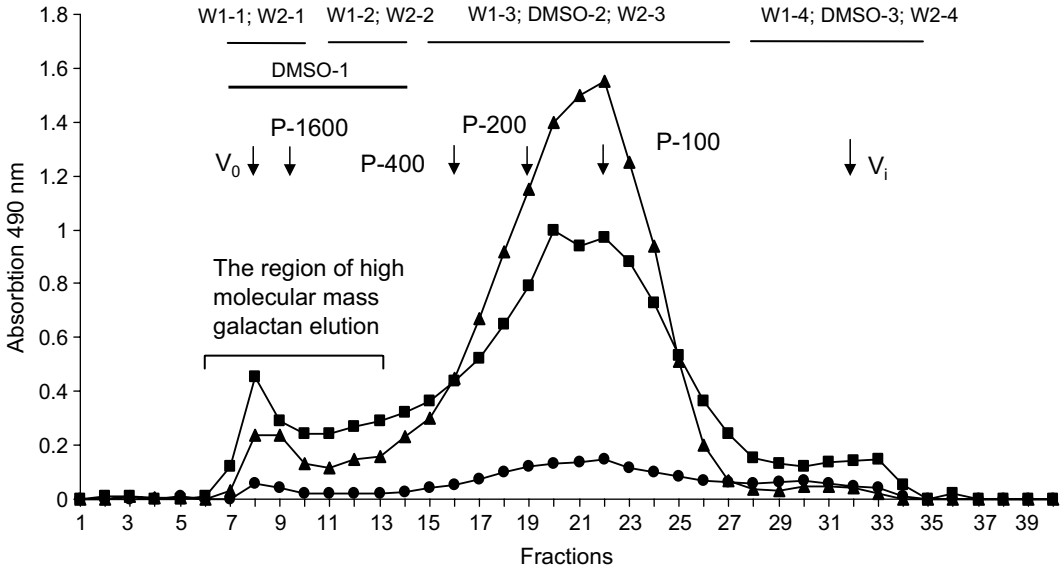


Fig. 2. Fractionation of the extracts (W1, DMSO, and W2) on Sepharose CL-4B, elution with 0.1 M NaCl, containing 0.05% $NaN_3$. ■—■, W1 extract; ●—●, DMSO extract; and ▲—▲, W2 extract. Calibration was performed using pullulan standards with molecular masses 1600, 400, 200, and 100 kDa. The extracts were obtained from 5.9 g of cell wall residue.

Table 1
Sugar composition (mol %) of fractions and yield of galactan from dry cell wall residue

| *Extract*/fraction | Yield of extract[a],% from cell wall residue dry mass | Fraction proportion in extract (%) | Monosaccharide composition (mol %) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Rha | Ara | Gal | Glu | Xyl | Man | GalA | GluA |
| *Extract W1* | 0.11 | | | | | | | | | |
| Fraction W1-1 | | 0.6 | 10.5 | 9.5 | 46.6 | 26.7 | 0.0 | 0.0 | 6.6 | 0.0 |
| Fraction W1-2 | | 9.4 | 16.7 | 5.1 | 60.4 | 0.3 | 0.0 | 0.0 | 17.5 | 0.0 |
| Fraction W1-3 | | 86.0 | 18.7 | 5.9 | 48.4 | 0.0 | 0.0 | 0.0 | 27.0 | 0.0 |
| Fraction W1-4 | | 4.0 | 12.2 | 22.0 | 43.0 | 12.0 | 0.0 | 0.0 | 10.3 | 0.4 |
| *Extract DMSO* | 0.02 | | | | | | | | | |
| Fraction DMSO-1 | | 1.8 | 4.9 | 24.2 | 49.6 | 11.8 | 0.0 | 0.0 | 8.4 | 1.2 |
| Fraction DMSO-2 | | 74.8 | 12.9 | 7.7 | 65.2 | 0.0 | 0.0 | 0.0 | 14.2 | 0.0 |
| Fraction DMSO-3 | | 23.4 | 9.8 | 24.2 | 52.7 | 0.6 | 0.0 | 0.0 | 12.3 | 0.5 |
| *Extract W2* | 0.15 | | | | | | | | | |
| Fraction W2-1 | | 1.9 | 7.1 | 8.3 | 80.0 | 2.2 | 0.0 | 0.0 | 2.3 | 0.0 |
| Fraction W2-2 | | 4.9 | 14.8 | 3.0 | 65.4 | 0.0 | 0.0 | 0.0 | 16.9 | 0.0 |
| Fraction W2-3 | | 92.2 | 18.7 | 4.4 | 53.3 | 0.0 | 0.0 | 0.0 | 23.6 | 0.0 |
| Fraction W2-4 | | 0.9 | 5.3 | 24.5 | 64.3 | 0.9 | 0.0 | 0.0 | 4.9 | 0.0 |
| *Extract DMA1* | 0.80 | | | | | | | | | |
| Fraction DMA1-1 | | 1.7 | 4.9 | 1.4 | 44.7 | 44.1 | 0.0 | 0.0 | 2.8 | 2.0 |
| Fraction DMA1-2 | | 73.2 | 8.6 | 4.6 | 75.7 | 0.0 | 0.0 | 0.0 | 11.2 | 0.0 |
| Fraction DMA1-3 | | 12.3 | 8.9 | 8.6 | 68.4 | 0.0 | 0.0 | 0.0 | 13.8 | 0.4 |
| Fraction DMA1-4 | | 12.7 | 7.4 | 7.8 | 51.1 | 22.5 | 0.0 | 0.0 | 10.8 | 0.3 |
| *Extract DMA2* | 3.51 | | | | | | | | | |
| Fraction DMA2-1 | | 66.7 | 11.5 | 1.3 | 73.1 | 0.0 | 0.0 | 0.0 | 14.2 | 0.0 |
| Fraction DMA2-2 | | 12.6 | 13.0 | 1.5 | 40.2 | 0.0 | 0.0 | 25.6[b] | 19.1 | 0.7 |
| Fraction DMA2-3 | | 20.7 | 3.1 | 2.5 | 12.4 | 38.9 | 3.9[b] | 12.6[b] | 26.2 | 0.2 |

[a] Yield was estimated as sum of all sugars (determined by HPAEC) in extract after fraction separation by Sepharose CL-4B.
[b] Part of mannose and xylose of fractions DMA2-2 and DMA2-3 was from enzyme.

cell wall residue in solution of lithium chloride in *N*,*N*-dimethylacetamide and to precipitate cellulose by water addition. Such treatment transfers the native plant cellulose I into cellulose II, which is formed in vitro (Atalla & Vanderhart, 1984). The precipitated cellulose was susceptible to complete degradation by cellulase, giving the possibility to separate matrix polysaccharides, which remained in polymeric form. Without such treatment flax cellulose was not effectively degraded by the enzyme (data not shown), as also was reported previously (Girault et al.,

2000). Dissolution of cellulose in the mixture of LiCl and DMA was chosen, among other methods to dissolve cellulose, because the treatment is performed at room temperature and these chemicals are neutral towards carbohydrates (Dupont, 2003; Fernandez, 2003).

Flax fibre cell wall residue was completely dissolved in 8% LiCl in DMA and the dissolved cellulose was precipitated by addition of water. Matrix polymers, remaining in the supernatant (extract DMA1), were chromatographed (after concentration) on Sepharose CL-4B and combined, according to the peaks, into fractions DMA1-1 to DMA1-4 (Fig. 3).

Part of matrix polymers that precipitated together with cellulose, were released after cellulose degradation by enzymatic treatment (Cellusoft-L). These polymers (extract DMA2) were chromatographed similarly to DMA1, and subdivided, according to the peaks, into fractions DMA2-1 to DMA2-3 (Fig. 3).

The major extract was DMA2 – the one, obtained after cellulase treatment of precipitated cellulose; it comprised 76% of the weight of all extracts (Table 1). The total yield of the polysaccharides, tightly bound to cellulose, was around 4.6%, being in good agreement with galactose content (3%) in the flax fibre cell wall residue, remaining after alkali extraction (Mooney et al., 2001).

The extracts differed mainly in their yield; the elution profile of all showed the broad major peak (W1-3; DMSO-2; W2-3; DMA1-2; and DMA2-1) in the region between 100 and 400 kDa. Some of the extracts contained peaks (W1-1; W1-2; DMSO-1; W2-1; and DMA1-1), which eluted close to the void volume of the column and resembled the high-molecular mass galactan that was reported to be abundant in the buffer-soluble fraction of developing fibres (Fig. 4) (Gorshkova et al., 2004, 1996; Gur'janov et al., 2007; Gur'yanov et al., 2006). The yield of these fractions was quite low.

### 3.2. Monosaccharide analysis of the fractions

The major fractions (DMA2-1 and other ones with molecular mass around 100–400 kDa) were mainly composed of galactose accompanied by two other significant monomers, GalA and Rha, with ratio 1.1–1.4 (Table 1). Arabinose was present as minor monosaccharide, with a lower than rhamnose content. The monosaccharide composition of these fractions was similar to that of high-molecular mass galactan from developing fibres (Gur'janov et al., 2007; Gur'yanov et al., 2006), but the molecular mass of these polymers was roughly 10 times lower (Figs. 2–4). Neither alkali, nor cellulase treatments, used in our experiments degraded the high-molecular mass galactan noticeably (data not shown).

The low-molecular mass fractions of DMA2 contained a significant proportion of mannose (Table 1), which probably came from glucomannans known to be present in flax fibre cell wall (McDougall, 1993; Morvan et al., 2003; Van Hazendonk et al., 1996). However, the cellulase used to obtain the extract does contain some mannose- and xylose-containing polymers of the similar molecular mass (data not shown). The proportion between these sugars, coming from fibre cell wall, and those from the enzyme, remains to be elucidated.

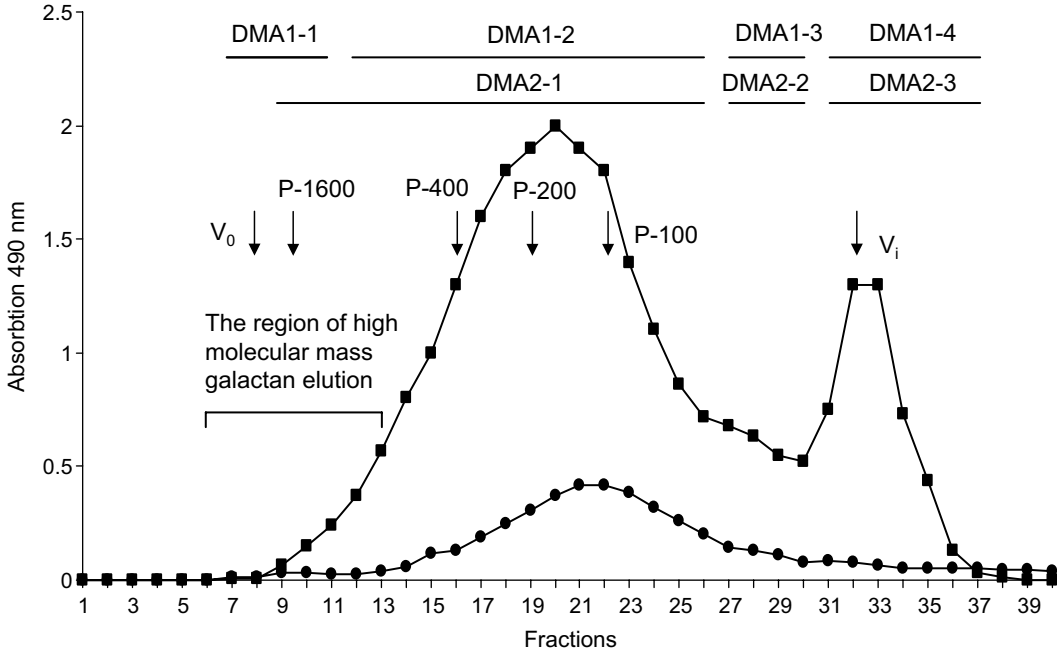


Fig. 3. Fractionation of the extracts (DMA1 and DMA2) on Sepharose CL-4B, elution with 0.1 M NaCl, containing 0.05% $NaN_3$. ●—●, DMA1 extract and ■—■, DMA2 extract. Calibration was the same as in Fig. 2. The extracts were obtained from 1.0 g of cell wall residue.

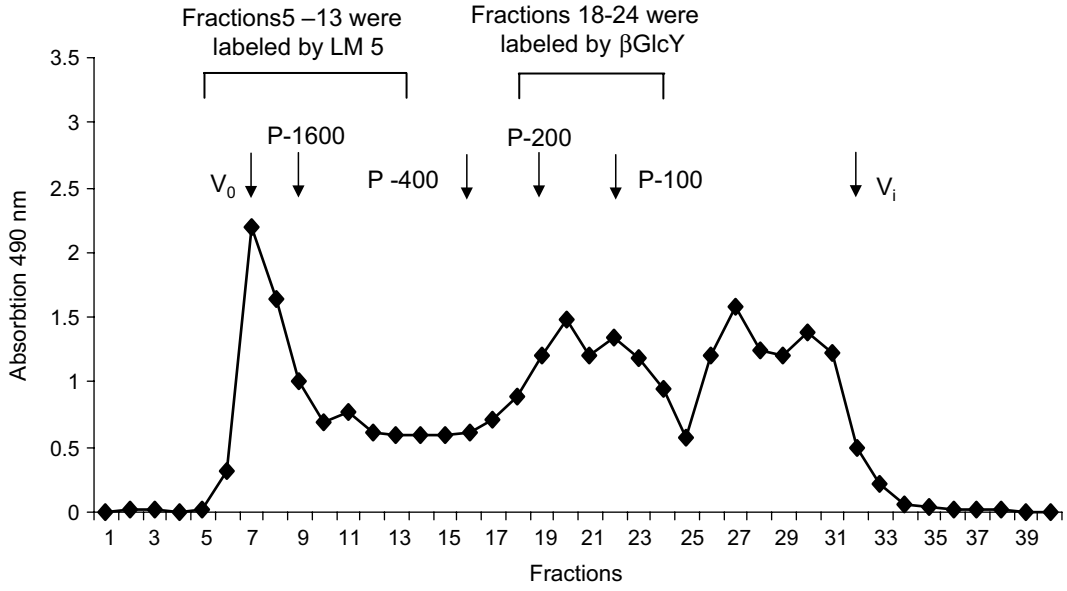


Fig. 4. Fractionation of buffer-soluble polymers from fibre-rich phloem strips of flax plant on Sepharose CL-4B, elution with 10 mM Na-acetate, containing 0.05% $NaN_3$, pH 4.5. Only high molecular mass fractions 5–13 were labeled by antibody LM5; only fractions 18–24 were stained with Yariv reagent (βGlcY).

The low-molecular mass fractions of DMA2 also contained a significant proportion of GalA (Table 1), indicating the presence of polygalacturonans, which were suggested to be part of polymers, that are tightly bound to cellulose, on the basis of monosaccharide composition of the fraction (Mooney et al., 2001). Some glucose in the fractions DMA1-4 and DMA2-3 was probably coming from fibre cellulose fragments but mostly glucose of DMA2-3 fraction was from enzyme.

Thus, the analysis of monosaccharides indicates that the major cell wall polymers, that are tightly bound to cellulose, have the same composition as high-molecular mass galactan and may originate from the tissue- and stage-specific polymer of flax fibre. To get further support for this statement we analyzed the polymers by means of binding assays and NMR.

### 3.3. Immunodot binding assays (IDA) with LM5 antibody and staining with Yariv reagent

Flax fibre contains galactose-containing polymers: tissue-specific galactan of RG I type, and arabinogalactan, which is not tissue-specific (Andème-Onzighi, Girault, His, Morvan, & Driouich, 2000; Gorshkova et al., 1996). Two galactans of flax fibre cell wall can be distinguished by staining with LM5 antibody and Yariv reagent (βGlcY), which are specific, respectively, for β-(1 → 4)-linked tetragalactoside (Jones et al., 1997) and arabinogalactan proteins (Yariv et al., 1962). In agreement with biochemical data (Gorshkova et al., 1996), LM5 labels only the high-molecular mass (2000–700 kDa) part of the elution profile of buffer-soluble polymers, while Yariv reagent stains only fractions from the middle part of the profile (100–200 kDa) (Fig. 4). However, cell wall fractions of similar molecular mass (DMA2-1 and DMA1-2) were not stained by Yariv reagent, but were heavily labeled with LM5 (Fig. 5).

### 3.4. NMR data analysis

The $^{1}H$ and $^{13}C$ NMR spectra of the major fractions (Fig. 6) were similar to those of flax buffer-soluble high-molecular mass galactan (Gur'janov et al., 2007; Gur'yanov et al., 2006) and of isolated cell wall galactans (Davis et al. 1990; Girault et al., 1997). The intensive peaks in the regions 3.80–3.68 ppm and 72–75 ppm (H2, H3, H5, H6 and C2, C3, C5 of galactose residue, correspondingly), and the peaks at 4.17 and 78.16 ppm (H4 and C4 of (1 → 4)-linked galactose residue) (Colquhoun, de Ruiter, Schols, & Voragen, 1990; Davis et al., 1990; Hannuksela & Herve du Penhoat, 2004; York, van Halbeek, Darvill, & Albersheim, 1990) indicate that (1 → 4)-linked galactose predominates. The signals at 1.31 and 1.25 ppm of rhamnose methyl group, at 98.32 and 17.21 of C1 and C6 rhamnose residue, and at 99.40 and 173.25 ppm of C1 and C6 galacturonic acid residue suggest the presence of rhamnogalacturonan I backbone. The signals at 3.53 and 3.36 ppm, which could originate from H2 of terminal galactose and H4 of linear 2-linked rhamnose were also well pronounced. The signals at 4.64 and 104.86 ppm confirmed that galactose was present in the form of β-anomer.

The intensive signal at 61.23 ppm originates from C6 of galactose that does not contain glycosidic bond at C6 position (Willför, Sjöholm, Laine, & Holmbom, 2002). There is no pronounced signal in the region 82–84 ppm and therefore (1 → 3)-linkage (Davis et al. 1990; Willför et al., 2002) is not present in the galactan of DMA2-1 fraction.

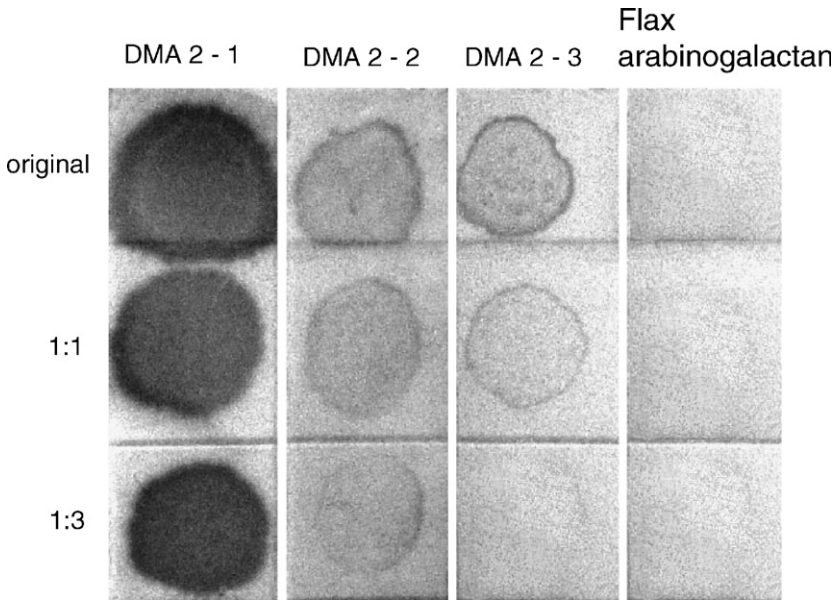


Fig. 5. Immunodot binding assays (IDA) of fractions of DMA2-1, DMA2-2, DMA2-3, and flax arabinogalactan, using LM5 antibody. Flax arabinogalactan was obtained from fractions 18–24 (Fig. 4).

Thus, the arabinogalactan (protein), which in flax fibre has $(1 \rightarrow 3)$ and $(1 \rightarrow 6)$-linkages of galactose (Girault et al., 1997; Gorshkova et al., 1996), was absent in the analyzed fractions. The signal at 2.73 ppm of DMA1-2 fraction was not identified.

## 4. Discussion

### *4.1. The developed method permits to isolate the polysaccharides, that are tightly bound to cellulose*

The residue remaining after treatment of plant cell wall with strong alkali contains cellulose and a small amount of matrix polysaccharides, which are thought to be entrapped by cellulose microfibrils during their crystallization and to play an important role in the formation of cell wall supramolecular structure (Cosgrove, 1997). Matrix polysaccharides, tightly bound to cellulose, are present in cell walls of various types and were found as monosaccharide fragments after acid hydrolysis (Mooney et al., 2001; Rose et al., 1998). From primary cell wall such polymers can be released by cellulase treatment (Chambat, Barnoud, & Joseleau, 1984; Hayashi, 1989; Pauly et al., 1999).

Cellulose microfibrils in fibres of flax, hemp, ramie, etc., which have gelationous cell wall, are characterized by larger size of crystalline unit and lower surface/volume ratio than in most other plant sources (Šturcová, His, Apperley, Sugiyama, & Jarvis, 2004; Viëtor, Newman, Ha, Apperley, & Jarvis, 2002). Cellulose molecules within crystal unit are inaccessible to the surrounding solvent and are resistant towards hydrolysis (Wickholm, Larsson, & Iversen, 1998). For instance, only one third of flax fibre cell wall was degraded by cellulase (Girault et al., 2000). To isolate matrix polysaccharides, that are tightly boud to cellulose, from cell wall of such type cellulose was dissolved in organic solvent and then precipitated it with water. Such treatment turned cellulose I into cellulose II and made it digestible with cellulase. During precipitation of dissolved cellulose less than 20% of matrix polysaccharides remained in solution, while the bulk of them precipitated together with cellulose and was released only after digestion of cellulose with the enzyme. The yield of matrix polysaccharides obtained by the applied procedure was 4.6% and was in good agreement with galactose content in cell wall residue, as determined in previous monosaccharide analysis (Mooney et al., 2001).

Extraction of cell wall residues with agents, such as DMSO, that cause swelling of cellulose microfibrils (Fernandez, 2003; McDougall, 1993), released only a minor portion of non-cellulosic polymers (Table 1). However, their elution profile on Sepharose CL-4B column and composition of fractions were similar to those of the remaining in the cell wall residue (Figs. 2 and 3).

### *4.2. The major flax fibre polysaccharide, that is tightly cellulose bound, has similar structure with tissue-specific high-molecular mass galactan*

Developing flax fibres, while making secondary cell wall, synthesize tissue-specific high-molecular mass (2000–700 kDa) galactan, that can be extracted in homogenization buffer, when not yet fixed within cell wall (Gorshkova et al., 2004, 1996). This galactan is built as RG-I type polymer and is characterized by high galactose content and low arabinose (Gur'janov et al., 2007; Gur'yanov et al., 2006). Small amounts of such polymer were shown to be present in hot water extracts of mature flax fibre and to elute close to the void volume (Fig. 2). Similar monosaccharide com-

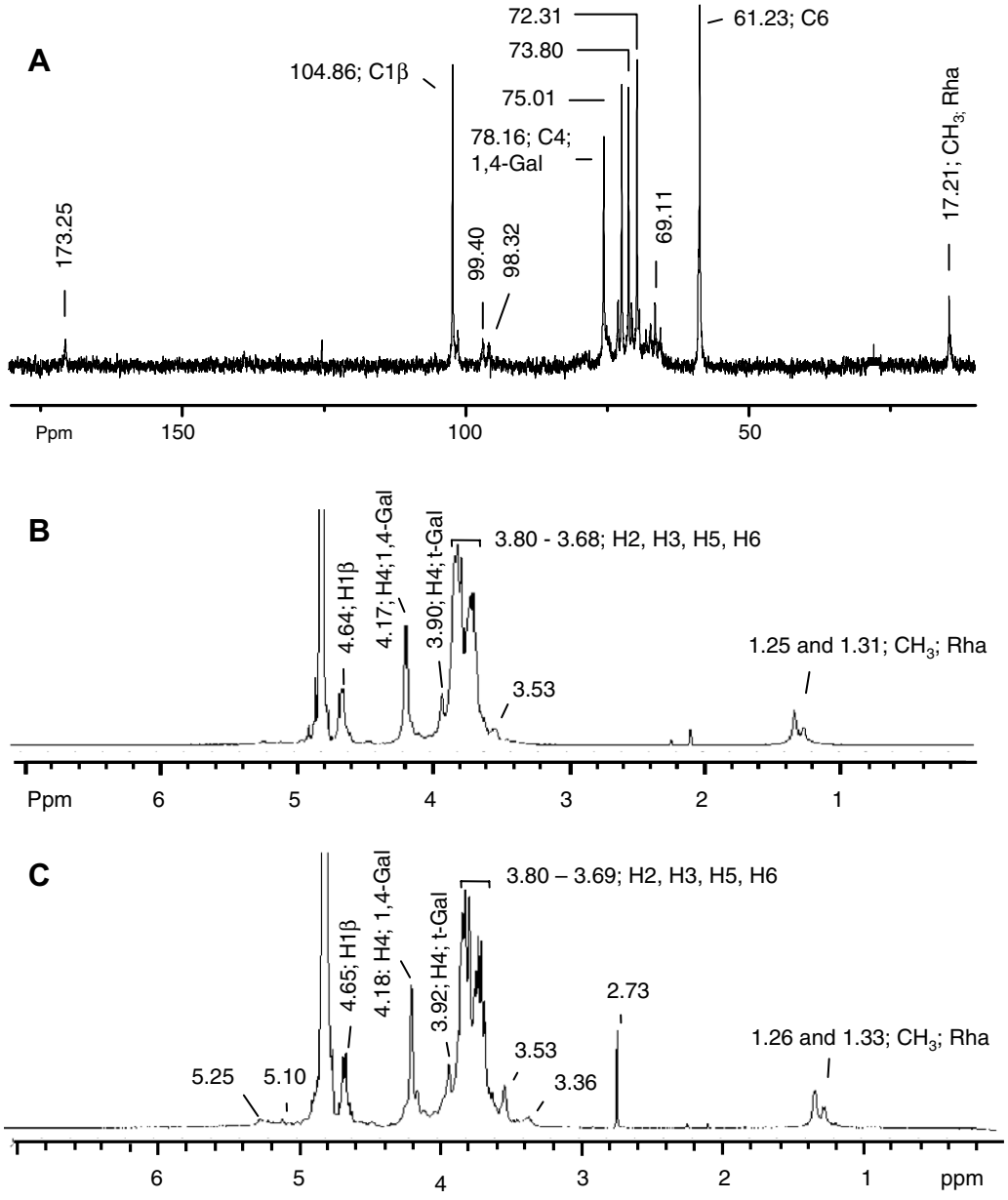


Fig. 6. $^{13}C$ NMR and $^{1}H$ NMR spectra of the galactans that are tightly bound to cellulose: (A) $^{13}C$ NMR spectrum of DMA2-1 fraction, (B) $^{1}H$ NMR spectrum of DMA2-1 fraction, and (C) $^{1}H$ NMR spectrum of DMA1-2 fraction.

position was found for the major fractions of polymers that were more tightly bound to cellulose (Table 1). However, the molecular mass of the bulk of cell wall galactan was in the range 100-400 kDa, which is characteristic for another galactose-containing polymer of developing flax fibre – arabinogalactan of type II (Gorshkova et al., 1996; Morvan et al., 2003; Gorshkova et al., 2006).

Flax fibre arabinogalactan has β-(1 → 3)- and β-(1 → 6)-types of galactose linkages (Gorshkova et al., 1996) and can be distinguished from the galactan of RG-I type by antibody LM5, specific for β-(1 → 4)-linked galactose, and by Yariv reagent, which stains arabinogalactan proteins. Staining of fractions from the elution profile of buffer-soluble polymers from the developing flax stem (Fig. 4) showed, in accordance with previous chemical characterization (Gorshkova et al., 1996), that the high-molecular part of the profile contained β-(1 → 4)-galactan, while 100–200 kDa part contained arabinogalactan protein (Fig. 4).

The major fractions of polymers from flax cell wall residue were heavily labeled with LM5 antibody and were not stained by Yariv reagent. Both $^{1}H$ and $^{13}C$ NMR spectra of DMA2-1 fraction also confirmed that it is composed of β-(1 → 4)-galactan. Partial hydrolysis of flax fibre cell wall with cellulase released, probably, part of the same galactan, as can be suggested from the monosaccharide analysis of the fraction (Girault et al., 2000). This polymer was also partly extracted by hot alkali, but the molecular mass of

it was reduced to 10 kDa (Girault et al., 1997). Polygalacturonic acid and mannan can be among the minor components of matrix polymers, tightly bound to cellulose.

The galactan, tightly bound to cellulose, could be the fragment of high-molecular mass tissue-specific galactan of developing fibres. Both have similar monosaccharide composition, and types of linkages; the only reliably revealed difference is in molecular mass. The fragmentation most probably occurs in the RG backbone of the polysaccharide, since the monosaccharide compositions of 2000 kDa and 200 kDa polymers are similar. It should be noted that neither alkali, nor cellulase in the concentration and time of application, used to prepare fractions, had any noticeable effect on the isolated high-molecular mass galactan from the soluble fraction of developing fibres (data not shown).

Thus, in flax fibre cell wall the major polysaccharide fraction, strongly attached to cellulose, is identified as the galactan, which accounts for over 80% of these polymers. Small amount of similar polysaccharide can be extracted by chelators, hot water or DMSO (Davis et al., 1990; McDougall, 1993; Van Hazendonk et al., 1996). It remains to be elucidated whether the nuances in structure, differences in function or time of deposition distinguish these galactan subfractions.

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