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Characterization of mucilage polysaccharides, arabinogalactanproteins and cell-wall hemicellulosic polysaccharides isolated from flax seed meal: A wealth of structural moieties

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ABSTRACT

The present study aimed at analyzing the structural features of seed mucilage and cell-wall polysaccharides which accounted for 41% of the mass of flax meal (FM). A combination of high molar-mass mucilage-like polysaccharides (rhamnogalacturonan and arabinoxylan) was released from FM in water, together with arabinogalactan proteins and glucans. About half of FM homogalacturonans was extracted using a calcium chelator and boiling water. Hemicellulosic xyloglucans and xylans were further extracted with 1 M KOH, in ~13% FM-sugars yield. Structural characterization of the xyloglucan using specific enzyme hydrolysis, ion exchange chromatography (HPAEC) and matrix assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectroscopy showed the presence of XXXG type xyloglucan, but also that of XXGG-structure, possibly characteristic of flax seeds. Hydrolysis of xylans with *endo*-(1 → 4)-β-D-xylanase, and analysis of the neutral and acidic oligosaccharides by MALDI-TOF-MS showed that xylan consisted of β-(1 → 4)-linked-D-xylopyranose backbone with some zones (DP 5–7) substituted with 4-*O*-MeGlcA\GlcA\Glc residues.

1. Introduction

Flax (*Linum usitatissimum* L., family Linaceae) is one of the most ancient of cultivated crops in India. Here, the agronomic value of the plant lies in the seeds, whereas in Europe, flax is grown chiefly for production of fibers. Linseed contains approximately 38–40% fat, 23–25% proteins and 15–20% carbohydrates of which one quarter to one-third consisted of mucilage (Susheelamma, 1989). Most of the seeds produced in India are utilized for production of oil, with seed cake as by-product, its production being in the range of 0.1–0.3 million tons per year. The residual cake might contain up to 35–45% sugars, but it is primarily used as a protein-rich livestock feed (e.g. Bell & Keith, 1993). Growth depression and reduced feed efficiency were reported for chickens (Trevino, Rodriuez, Ortiz, Rebolé, & Alzueta 2000), which might be due to limited degradation of the mucilages and cell-wall (CW) polysaccharides in the digestive tract of monogastric animals. In that context, understanding the composition and structural features of mucilage and CW polysaccharides is essential for the better use of this agricultural by-product.

The structure of mucilage extracted from seeds has been largely investigated (e.g. Anderson, 1933; BeMiller, 1973; Cui, Mazza, & Biliaderis, 1994; Muralikrishna, Salimath, & Tharanathan, 1987; Naran, Chen, & Carpita, 2008; Wannerberger, Nylander, & Nyman, 1991; Warrand et al., 2005a). They mainly consisted of two moieties, rhamnogalacturonan-I (RG-I) and an arabinoxylan (AX). The α-D-galactosyluronic acid-(1 → 2)-L-rhamnose aldobiouronic acid consists of the repeating unit of the RG-I-backbone with a single terminal L-Gal (Anderson, 1933) and L-Fuc (Naran et al., 2008) residues attached to the Rha. On the other hand, the (1 → 4)-β-D-xylan backbone of AX is substituted by terminal L-Araf residues, generally doubly branched on the *O*-2 and *O*-3 of a xylose residue (Muralikrishna et al., 1987). Terminal GlcA (Naran et al., 2008) and D-Gal residues (Warrand et al., 2005a) were also found to be attached to the xylan chain. Importantly, the composition and

Abbreviations: AEC, anion exchange chromatography; AGP, arabinogalactan protein; AX, arabinoxylan; CT, fraction extracted with chelating agent; CW, cell-wall; FM, defatted flax meal; HG, homogalacturonan; HPAEC, high performance anion exchange chromatography; HWE, fraction extracted with hot water; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of-flight mass spectrometry; RG-I, rhamnogalacturonan I; SEC, size exclusion chromatography; WE, fraction extracted with room temperature water.

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properties of mucilages vary according to their solubility (Naran et al., 2008), and extraction processing (Fedeniuk & Biliaderis, 1994; Mazza & Biliaderis, 1989).

Basically, the flax-meal water-soluble polysaccharides were of similar structure although high level of glucose, lignans and proteins were additionally detected in the mixtures (e.g. Bhatty & Cherdkiatgumchai, 1990; Ho, Cacace, & Mazza, 2007; Warrand et al., 2005b). Oomah, Kenaschuk, Cui, and Mazza (1995) attributed the high level of glucose (21–41%) to the use of buffer (at 85 °C) for the extraction of carbohydrates from flax seeds. They concluded that it is due to the preferential extraction of another glucose-containing component. In flax, glucose was mainly reported to originate from starch (Alix, Marais, Morvan, & Lebrun, 2008), lignans were released from the broken inner part of the seed coat (Attoumbré et al., 2011) and proteins were solubilized from the endosperm. Due to polymer/polymer interactions, such mixtures might exhibit other physicochemical behavior than soluble mucilages which potentially could contribute to other uses than food stabilizers (for examples: in pharmaceuticals, as additives in cosmetics or as lubricant in chemical industries).

On the other hand, less attention has been paid to the structural features of polysaccharides originating from the cell-walls of Indian samples of flax seed. Especially, structural information on hemicellulosic polysaccharides is missing for flax seed. Generally, hemicelluloses in seed cell walls were identified to be mannans, xyloglucans, xylans and β-glucans being stored in either endosperm or cotyledons. Two general types of xyloglucan, poly-XXXG and poly-XXGG, predominate, although poly-XXXX may also exist, differing in the degree of backbone substitution with Xyl (Vincken, York, Beldman, & Voragen, 1997). Other alkali-soluble polysaccharides (e.g. isolated from the seeds of date) were shown to consist of β-linked D-xylopranosyl residues having branches of D-xylopyranosyl and 4-*O*-methyl-α-D-glucopyranosyluronic acid (Ishurd et al., 2003). In the seeds of some grapes, another xylan was identified substituted by L-arabinofuranosyl, α-D-xylopyranosyl and 4-*O*-methyl-α-D-glucopyranuronosyl residues (Igartubury, Pando, Rodriguez-Luis, & Gil-Serrano, 1998).

In order to provide profiles on the CW composition and structure of flax meal polysaccharides for green applications, and in the context of the importance of *L. usitatissimum* as a model plant system to study a number of processes where cell walls play a key role (Roach & Deyholos, 2007), we have performed a thorough analysis of the pectic and hemicellulosic polymers present in flax meal. To generate these reference data profiles, we have used a combination of techniques including monosaccharide compositional analysis, spectroscopy, comprehensive methylation analysis and oligosaccharide mass finger printing as well as more in-depth approaches using chemical and chromatographic fractionation methods. We propose that this combination of tools provide a ready-to-use approach to profile the different wall polymers present in the seeds of *L. usitatissimum* plant populations.

2. Experimental

2.1. Plant material

Flax-seed cakes were obtained from the local market of Burdwan (West Bengal, India) and were treated sequentially with hexane (48 h) and acetone (24 h) in a Soxhlet apparatus. The defatted flax meal (FM) was then air dried and ground.

2.2. Isolation of polysaccharides from FM

FM (10 g) was extracted sequentially with: (i) water (pH 6, 3 × 500 ml) at 30–35 °C for 6, 12 and 6 h (fraction named WE); (ii) 1,2-cyclohexanediaminetetraacetic acid (100 mM, 2 × 500 ml) at 30–35 °C for 12 and 6 h (fraction named CT) and (iii) water (pH 6, 3 × 500 ml, 3 × 1 h) at 100 °C (fraction named HWE). Solubilized material was separated from the insoluble residue by centrifugation (10,000 × *g*, 15 min), and the supernatant filtered through glass filter (G 3). The hot water extract was concentrated, dialyzed against water. The CT extract was dialyzed first against 1 M NaCl and then against distilled water. The hot water extract was submitted to graded precipitation with ethanol. All the dialyzed extracts were concentrated and freeze dried.

The residue was then extracted twice with 700 ml of 1 M KOH solutions containing 20 mM $NaBH_4$ for 14 h at 4–8 °C. The extract was acidified with cold 4 M AcOH (ice bath) to pH 6 and dialyzed extensively against water. The precipitate was removed by centrifugation to yield A (250 mg) fraction. The soluble fraction from the 1 M KOH extract has been designated as B (750 mg).

2.3. Isolation of arabinogalactan protein (AGP) with β-glucosyl Yariv reagent

AGP was isolated according to Yariv, Rapport, and Graf (1962). Briefly, to a solution of WE in 1% NaCl (w/w) was added an equal volume of Yariv reagent dissolved in 1% NaCl. The mixture was kept at 4–8 °C for 18 h and then centrifuged. The pellet was washed with 1% NaCl followed by pure methanol (three times each). The pellet was then dried and treated with sodium metabisulfite (10%). The resulting solution was then dialyzed and freeze dried to yield the arabinogalactan protein (AGP).

2.4. Preparation of oligosaccharides

The hemicellulosic fraction BN (15 mg) was dissolved in 6 ml of 50 mM NaOAc (pH 5.0) and the mixtures incubated with 30 units of *endo*-(1 → 4)-β-D-glucanase (Megazyme International, Ireland) for 24 h at 35–40 °C. The enzyme resistant polymer was then precipitated in 80% ethanol (v/v) and removed by centrifugation. The soluble fraction containing the xyloglucan oligosaccharides was concentrated under reduced pressure, desalted on a column of Sephadex G-25 and finally lyophilized to yield the xyloglucan oligosaccharides (XGose).

Hydrolysis of 10 mg of the xylan-rich fraction (A) in 4 ml of 50 mM NaOAc pH 5.5 was performed using 40 units of *endo*-(1 → 4)-β-D-xylanase (Megazyme International Ireland, xylanase M6) at 35–40 °C for 24 h. One unit of enzyme solution liberates 1 nmol of reducing sugar per minute. To remove polymeric material, hydrolyzate was precipitated with 4 volumes of cold ethanol. After centrifugation, the oligomers present in the supernatant were concentrated yielding a fraction named as Xose.

2.5. Sugar analysis

Total sugars (TS) were estimated as anhydroglucose by the phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Depending on sugar composition, the evaluation was corrected afterwards. Corrections were also made for interference from galacturonic acid. Total uronic acids were assayed as anhydrogalacturonic acid using m-phenyl phenol color reagent (Blumenkrantz & Asboe-Hansen, 1973). All fractions were hydrolyzed in 1 M sulfuric acid (3 h, 100 °C) for measurement of individual neutral sugar. Sugars were reduced, acetylated and analyzed as their alditol acetate by GLC (Blakeney, Harris, Henry, & Stone, 1983) on columns of DB-225 (JW) and by GLC/MS (Shimadzu QP 5050A). Myo-inositol was used as internal standard. Alternatively, trimethylsilyl (TMS) derivatives of methyl glycosides were

analyzed by gas chromatography (York, Darvill, O'Neill, Stevenson, & Albersheim, 1985).

2.6. Amino acid analysis

Protein content was measured in the insoluble residue by estimating total nitrogen (Kjeldhal method) and multiplying the value with 6.25. In the soluble material the Bradford method was assayed using bovine serum albumin as standard. Amino acids were released by hydrolysis with 6 M HCl at 110 °C for 22 h in a sealed tube. Protections were done for cysteine, methionine and tyrosine using proper protecting reagents. The liberated amino acids were analyzed by Pharmacia LKB ALPHA PLUS amino acid analyzer.

2.7. Glycosidic linkage analysis

Glycosidic linkage position of constituent sugars of the xyloglucan rich fraction (BN) was determined by the methylation analysis (Blakeney & Stone, 1985). The polysaccharide was methylated (twice), hydrolyzed with 2.5 M trifloroacetic acid at 120 °C for 75 min, reduced with 1 M $NaBD_4$ in 2 M NH_4OH for 3 h at room temperature and acetylated using perchloric acid as a catalyst. The partially methylated alditol acetates (PMAA) were analyzed by GLC and GLC/MS using DB-225 (JW) column. The temperature program was 170 °C for 15 min, 170–210 °C at 5 °C min^{-1} and 210 °C for 15 min. The mass spectra were recorded with Shimadzu QP 5050A GLC/MS instrument at 70 eV.

2.8. Size exclusion chromatography (SEC)

SEC is an important technique for purification of polysaccharides and also for the determination of molecular mass. In system A, solutions (~5 ml) of different extracts in 500 mM sodium acetate buffer (pH 6.0) were loaded to a column of (90 cm × 2.6 cm) Sephacryl™ S-1000 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with the same buffer. The column was eluted ascendingly with the same buffer at 20 ml h^{-1}, and the temperature was 30–35 °C. Elution of polysaccharide was expressed as a function of the partition coefficient K_{av} [$K_{av} = (V_e - V_0)/(V_t - V_0)$ with V_t and V_0 being the total and void volume of the column determined as the elution volume of glucose and blue dextran, respectively, and V_e is the elution volume of the sample]. The column was calibrated with standard dextrans within a Mw range of 10,000–10,00,000. Fractions (7.5 ml) were collected and analyzed for TS content using glucose as standard.

In system B, solutions were injected on a HiPrep™ 26/10 Desalting column (Amersham Bioscience). The desalted materials were concentrated and then lyophilized.

2.9. Anion exchange chromatography (AEC)

AEC was used to purify the polysaccharides on the basis of their charge density. A solution of WE or CT polysaccharide was passed down a column of Amberlite resin IR 120 (H^+), titrated to pH 5.0 with 100 mM NaOH solution, and loaded onto a DEAE-Sepharose CL-FF column (25 cm × 1.6 cm) equilibrated with 0.05 M sodium acetate buffer, pH 5.0. The column was washed with the same buffer (150 ml: fraction **I**) and then eluted successively with 150 ml of 0.2 M (fraction **II**) and 1 M NaOAc buffer pH 5.0 (fraction **III**) in a stepwise manner. Residual bound polysaccharides were washed from the column with 150 ml of 0.2 M NaOH (fraction **IV**). The collected fractions (**I**–**IV**) were concentrated, dialyzed against distilled water, concentrated and freeze-dried.

Besides, a solution of polysaccharide (B in water) was passed down a column of Amberlite resin IR 120 (H^+), titrated to pH 5.0 with 100 mM NaOH solution, dialyzed and loaded onto a DEAE-Sepharose CL-FF column (25 cm × 1.6 cm) equilibrated with 0.05 M sodium acetate buffer (pH 5.0). The column was washed with the same buffer (150 ml), the eluted material dialyzed against distilled water, concentrated and finally lyophilized (fraction BN).

2.10. High performance anion exchange (HPAE) chromatography

Enzyme-generated fragments were analyzed by HPAE chromatography equipped with a CarboPac PA-1 column (Dionex) combined with pulse amperometric detection (PAD). The flow rate was 1 ml min^{-1} and the eluate was monitored using a pulse amperometric detector (PAD, Dionex). Samples (30–100 μl) were injected and eluted with the following gradient of NaOAc in 100 mM NaOH: 0–5 min, linear gradient of 0 → 5 mM NaOAc; 5–30 min, linear gradient of 5 → 8 mM NaOAc; 30–35 min, linear gradient of 8 → 13 mM NaOAc; 35–40 min, linear gradient of 13 → 15 mM NaOAc; 40–42 min, linear gradient of 15 → 100 mM NaOAc. The eluents were degassed and stored under helium. After each run, the column was washed with 1 M NaOAC in 100 mM NaOH, and subsequently equilibrated for 15 min with the starting eluent. The 1 M NaOAc gradient in 100 mM NaOH used for the analysis of xylan oligomers (Xose) was as follows: 0 → 5 min, zero gradient; 5 → 20 min, linear gradient of 0–50 mM NaOAc; 20 → 42 min, linear gradient of 50–150 mM NaOAc.

2.11. Matrix-assisted laser desorption ionization-time of-flight mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS analysis of the enzyme-generated oligosaccharides (XGose and Xose) was carried out on a Bruker Daltonics flexAnalysis MALDI-TOF mass spectrometer as described (Chattopadhyay et al., 2008). Briefly, mass spectra were recorded in reflectron mode and in positive ion detection using 2,5-dihydroxybenzoic acid (10 mg/mL) as matrix. Each of the samples (2 μl) was mixed with 2 μl of the matrix solution in 1.75:0.75 trifluoroacetic acid–acetonitrile. A 2 μl portion of this solution was applied on a sample plate and allowed to dry under diminished pressure.

3. Results and discussion

3.1. Sugar composition of defatted flax meal (FM)

Defatted flax meal (FM) obtained from Indian flax seed, contained 41% sugars of which about 7% were uronic acids (Table 1). The major neutral sugars were Xyl, Ara, Glc, Gal and Rha. In addition to polysaccharides, FM contained 29% protein (estimated by Kjeldhal method) and 11% of Klason lignin.

3.2. Water and chelator extract rhamnogalacturonan I, arabinoxylan, glucan and arabinogalactan protein

Sequential treatments with water (WE and HWE fractions) and chelating agent (CT fraction) released 20% of FM of which half consisted of sugars (Table 1).

In water at 30–35 °C (WE), the sugar composition as well as the values of the ratios Gal/Rha (1.1), Fuc/Rha (0.25) and Ara/Xyl (0.36) were close to the values reported in the literature for mucilages (e.g. Muralikrishna et al., 1987; Wannerberger et al., 1991; Warrand et al., 2005a). Compared to mucilages extracted from European flaxseed-cakes (Warrand et al., 2005b), the ratio Rha/Xyl was much higher. These mucilages, loosely linked with cell walls, were made of rhamnogalacturonan I (RG-I accounting for 43% of WE total sugar), arabinoxylan (AX ~ 37%) and homogalacturonan (HG ~ 7%), and represented 4–5% of the mass of FM. Importantly, WE amount

Table 1
Yield and molar sugar composition of *Linum usitatissimum* meal and of fractions[a] isolated there from.

	FM	WE	CT	HWE	A	B	BN
Yield[b]	(100)	10	4	6	2.5	7.5	–
TS[c]	41	51	37	64	51	54	87
UA[c]	7	10	25	13	15	5	Tr
Rha[d]	12	16	5	3	1	3	Nd
Fuc[d]	2	4	1	Tr	1	3	1
Ara[d]	22	12	26	33	12	20	3
Xyl[d]	33	33	36	34	61	27	34
Man[d]	1	2	1	Tr	1	1	Nd
Gal[d]	14	18	15	16	8	16	11
Glc[d]	16	16	15	14	16	30	51

[a] Sequential treatments of defatted flax meal (FM) with water (WE fraction), chelating agent (CT fraction), hot water (HWE fraction) and 1 M KOH extract (A and B fractions: corresponding to the precipitates and soluble polymers, respectively) released 30% of matter.
[b] Weight percentage of 'FM' dry weight.
[c] Percentage weight of fraction dry weight.
[d] Molar percentage of neutral sugars. TS, total sugar; UA, uronic acid; Tr, trace; Nd, not detected; –, not determined.

was not larger than the amount of mucilage released in water from intact flax seeds (e.g. Oomah et al., 1995); this indicated a moderate impact of cake processing. According to Wannerberger et al. (1991), Glc rarely exceeded 5% in mucilage released from living seeds; in the present work, only 13% of Glc was detected in WE, which might partly belong to mucilages and partly originate from starch or other contaminants (Alix et al., 2008; Barbary, Al-Sohaimy, El-Saadani, & Zeitoun, 2009; Mazza & Biliaderis, 1989). Consequently, we considered that WE globally consisted of AX and RG-I mucilages, although, a small part of Gal and Ara (and possibly Xyl, see below Table 3) might originate from arabinogalactanproteins or other components.

On the other hand, the 5% of FM sugars released in CT and HWE would originate from different cell-walls of the seeds, especially the coat and endoderm tissues. In CT, the high ratio of GalA over the total sugar showed that more than 60% consisted of HG. The other part of CT contained mainly Ara and Xyl. The Ara/Xyl ratio was 0.75. The level of Rha was very low, and the ratio Gal/Rha estimated to be around 3 was much larger than the WE one.

The HWE fraction was mainly composed of Ara, Xyl, Glc, Gal and GalA residues. It was poor in Rha, a figure comparable with that of CT. Consequently, most of detected GalA was linked in a HG backbone. Compared to the ones in WE and CT, the ratio Gal/Rha was increased to 5 and the ratio Ara/Xyl was shifted up to 1.

Altogether, about a quarter of the neutral sugars and 40% of GalA were extracted from FM. Fig. 1 shows a clear difference in sugar composition between the 3 extracts. As expected for myxogenic seeds, Rha was mainly found in mucilage-enriched WE. The pectic HG was mainly detected in CT (and to a less extent in HWE). Like in *Arabidopsis*, part of CT-HG might originate from the inner mucilage-layer (Macquet, Ralet, Kronenberger, Marion-Poll, & North, 2007). More globally, CT-HG would be localized in the cell-junctions of all seed tissues. Another significant part of HG was extracted in HWE, indicating some covalent cross-linking and a putative localization in cell-wall rather than in cell-junctions. Some JIM5/JIM7 labeling should be performed with flax seeds to precise the HG localizations. Interestingly, more than about half of HG present in FM was successfully extracted within the series. Besides, the main part of arabinose was detected in HWE; the increasing value of the ratio Ara/Xyl (and Gal/Rha) from WE to HWE underlined the possible presence in CW of polymers such as arabinans or arabinogalactan protein (AGP, see below).

3.3. Fractionation of WE on size exclusion chromatography (SEC)

Using SEC onto Sephacryl™ S-1000, the water extracted carbohydrate polymers (WE) were resolved into two main peaks, designated as WE-F1 and WE-F2, and a minor one named WE-F3 (Fig. 2A). Based on calibration with dextrans, the apparent molecular mass of WE-F1, WE-F2 and WE-F3 would be >2000, 500–1000 and 50–500 kDa, respectively. It should be noted however that polysaccharides in WE-F2 containing large amount of uronic acid (Table 2), might have a different hydrodynamic volume than dextrans and, therefore, their molar mass might be less.

The carbohydrate polymer present in WE-F1 peak contained a large amount of Glc, and to a less extent Xyl and Ara together with smaller amount of Man residues (Table 2). Undoubtedly, in such a proportion, Glc could not appear only from mucilage. Two possible

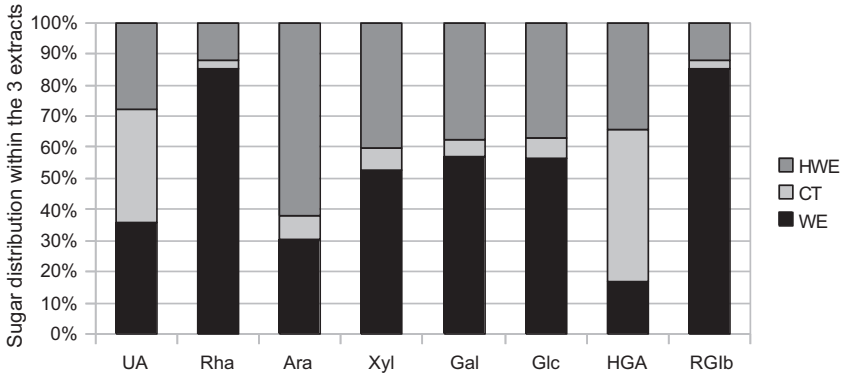

Fig. 1. Distribution of sugars and pectic polysaccharides in water (WE), chelating agent (CT), and hot water (HWE) extracted materials. UA, uronic acid; HGA, homogalacturonan; RGIb, backbone of rhamonogalacturonan I (≈2× Rha).

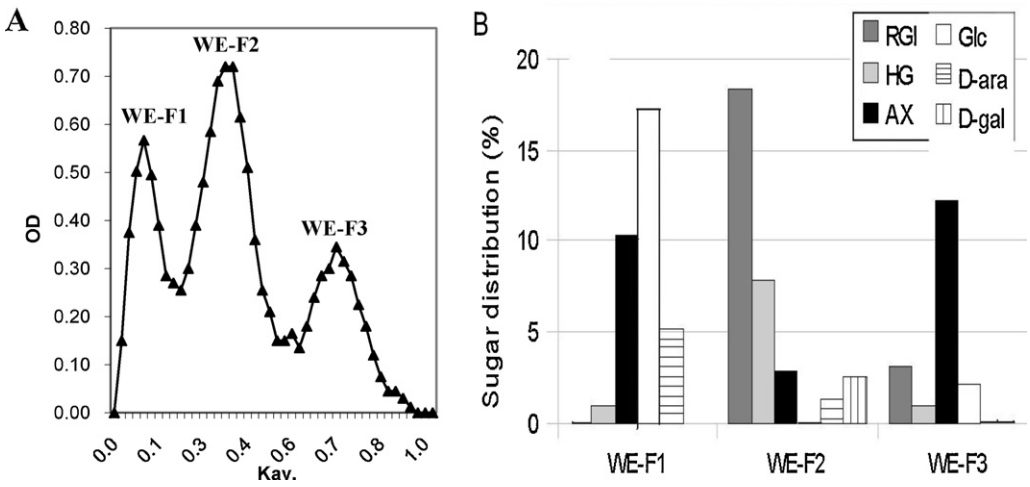

Fig. 2. Size exclusion chromatography of water extracted (WE) materials on Sephacryl™ S-1000 and distribution of the main polysaccharides in the fractions. RG-I and AX were calculated taking into account the Gal/Rha and Ara/Xyl ratios of WE. The excess of Ara and Gal were designated as D-Ara and D-Gal respectively.

polymers, starch and/or cellulose-like might be of high molecular mass. In *arabidopsis* seed, where the epidermis columella was surrounded by a thickened cellulosic secondary cell wall, cellulose was reported to be a component of mucilage (e.g. Windsor, Symonds, Mendenhall, & Lloyd, 2000). Besides, due to the lack of Rha and GalA, no pectic-AX complex did exist in WE-F1. The ratio Ara/Xyl was significantly higher than the one estimated in the total WE fraction and WE-F3, indicating that independent high molar-mass Ara-enriched polysaccharides might co-elute with AX in the void volume. The trace level of Gal was not in favor of AGP moieties. Alternatively the Ara-rich polysaccharide might consist of an arabinan, as those released from seed coat (Arsovski et al., 2009) or endosperm (Dourado, Cardoso, Silva, Gama, & Coimbra, 2006; Habibi, Mahrouz, & Vignon, 2005; Tharanathan, Bhat, Krishna, & Paramahans, 1985). This Ara-enriched moities, if not branched, might be interacting with cellulose-like glucans if some were eluting in WE-F1 fraction (e.g. Zykwinska, Ralet, Garnier, & Thibault, 2005).

The carbohydrate polymer present in major WE-F2 peak was composed mainly of GalA, Gal and Rha residues. The high ratio GalA over TS and the significant percentage of Rha indicated the presence of HG and RG-I. The ratio Gal/Rha = 1.5 was larger than the one measured in WE. Such an excess of Gal together with the few percent of Ara might belong to AGP (see below). Only a low percentage of Xyl was present and no Glc was detected in this fraction.

The minor WE-F3 fraction was mainly composed of Xyl residues. Ara and Glc were the two other significantly represented sugars. The ratio Ara/Xyl (<0.30) resembled to that of AX mucilages. Nevertheless, the presence of a few Rha and GalA eluting in the same fraction might indicate some complex between RG-I and AX. Possibly, this fraction might originate from higher molecular mass moieties due to the presence of enzymes in the mucilages (Paynel et al., 2012; Rasmussen & Meyer, 2010).

Altogether (Fig. 2B), SEC data indicated that the mucilage AX was distributed in about equal amount in WE-F1 and WE-F3. The structure of glucans abundantly present in WE-F1 and their interaction with Ara enriched moieties and AX have to be investigated. Interestingly, it was previously indicated that cellulose could be encapsuled by the high molar mass AX mucilage (Tharanathan, Muralikrishna, Salimath, & Raghavendra, 1987). Besides, RG-I and HG eluted in the same WE-F2 fraction, together a small amount of AX. Anion exchange chromatography (AEC) was hence performed to check the possible presence of a composite AX-RG-I mucilage as described in Naran et al. (2008).

3.4. Anion exchange chromatography (AEC) of WE extract

Using DEAE Sepharose anion exchange chromatography, WE fraction was separated into one neutral (WE-I) and three acidic fractions (WE-II, WE-III and WE-IV). The sugar yields were moderate (≤50%) indicating that some polysaccharides have been lost during the AEC.

The neutral fraction was devoid of GalA and Rha and had a chemical composition similar to that of a mucilage arabinoxylan, with a

Table 2
Monosaccharide composition of fractions obtained either by size exclusion chromatography of water extracted (WE) polysaccharides on Sephacryl™ S-1000 or by anion exchange chromatography on DEAE-Sepharose CL-FF.

	Size exclusion chromatography on Sephacryl S-1000			Anion exchange chromatography onto DEAE Sepharose			
	WE-F1	WE-F2	WE-F3	WE-I	WE-II	WE-III	WE-IV
Total sugar[a]	37	35	17	73	57	55	49
Uronic acid[a]	1	14	2	1	9	15	11
Rha[b]	Nd	29	7	Tr	31	32	28
Fuc[b]	Nd	10	Tr	Nd	8	3	5
Ara[b]	22	10	17	23	3	3	13
Xyl[b]	21	10	60	58	6	7	17
Man[b]	9	Tr	Tr	Tr	5	3	Nd
Gal[b]	Tr	41	2	Tr	37	38	32
Glc[b]	48	Tr	14	19	10	14	5

[a] Percent weight of fraction dry weight.
[b] Percent mole of neutral sugars. Tr, trace; Nd, not detected. The SEC yield of recovered polymers varied from 75% (WE-F2) to 95% (WE-F1 and WE-F3).

Table 3
Sugar and amino acid composition of the arabinogalactan protein (AGP) isolated from the water extracted fraction (WE) of flax meal.

Sugar composition	Mol %	Amino acids	Mol %
Rha	2	Aspartic acid/asparagine	11
Fuc	Tr	Threonine	3
Ara	28	Serine	10
Xyl	16	Glutamic acid/glutamine	26
Man	9	Proline	5
Gal	39	Glycine	16
Glc	6	Alanine	6
		Valine	3
		Isoleucine	2
		Leucine	5
		Tyrosine	1
		Phenyl alanine	3
		Histidine	2
		Lysine	2
		Arginine	5

Tr, trace.

Table 4
Monosaccharide composition of fractions (CT-I to CT-IV) obtained by anion exchange chromatography of 1,2-cyclohexanediaminetetraacetic acid extracted fraction (CT) on DEAE-Sepharose CL-FF.

	CT-I	CT-II	CT-III	CT-IV
Total sugars[a]	25	18	15	13
Uronic acid[a]	3	12	14	7
Rha[b]	Nd	2	3	2
Fuc[b]	Tr	Nd	Nd	Nd
Ara[b]	16	1	5	23
Xyl[b]	47	12	32	25
Man[b]	2	Nd	Nd	Tr
Gal[b]	14	23	12	20
Glc[b]	22	62	47	30

[a] Percent weight of fraction dry weight.
[b] Percent mole of neutral sugars. Nd, not detected. Tr, trace.

ratio Ara/Xyl of 0.39 (Table 2). No neutral Glc or Ara rich moieties could be detected.

Fractions WE-II, WE-III and WE-IV were mainly composed of Gal, Rha and GalA in proportion characteristic of RG-I (Additional Fig. 1). Besides, Ara and Xyl were also present but in very low amount in WE-II and WE-III fractions. In WE-IV, the ratio Xyl to Rha was higher so as the presence of a composite structure made of AX and RG-I might be hypothesized. Conversely, the three later fractions were very poor in HG, in comparison with the significant HG percentage detected in WE-F2 after SEC fractionation. Thus, HG was not efficiently eluted, due to the strong electrostatic interactions with the gel DEAE Sepharose. Incidentally, the data suggest that such an HG may free from the RG-I moieties. Also, the percentage of Glc and the value of the ratio Ara/Xyl were much less in AEC fractions than in WE-F1 SEC fraction. Like HG, part of Glc and Ara enriched moieties were not eluted from AEC, possibly due to their high molar mass and aggregative properties, as hypothesized above for Glc and Ara eluted in WE-F1.

3.5. Flax meal contains arabinogalactan-protein

Considering the significant yield of fraction WE, and its high content of protein (21%), we have tested its reactivity with β-glucosyl Yariv reagent. About 22% (w/w) of WE was precipitable with the Yariv reagent. Sugar compositional analysis of this precipitate (named AGP) showed that Gal and Ara residues were the main monosaccharides (Table 3). The Gal/Ara ratio of this fraction was 1.4. The main amino acids were glutamic acid/glutamine, glycine, aspartic acid/asparagine and serine (Table 3). This AGP displayed an amino acid composition not far from that of the protein bulk of flax seeds (Chung, Lei, & Li-Chan, 2005; Oomah & Mazza, 1993; Udenigwe et al., 2012), although they contained more glycine (16% compared to 4–6%) and serine (10% compared to 4–5%) and less arginine (5% compared with10–12%). Their composition was also close to that found for AGP present in the secondary wall of flax fibers (Girault, His, Andeme-Onzighi, Driouich, & Morvan, 2000). Putatively, this class of plant glycoproteins and/or proteo-glycans might display pharmacological impact on human cartilaginous disease and/or deal with food industry applications (BeMiller, 1973). On the other hand, proteins/peptides enriched in arginine might display some antihypertensive activity (Udenigwe et al., 2012).

Thus, AGP, a class of plant glycoprotein with many pharmacological and food industry applications, was solubilized in water together mucilages. The latters were characterized to be high molar mass AX and RG-I and could be easily fractionated on SEC and AEC chromatographies. The origin of Glc and Ara enriched moieties has to be further investigated.

3.6. AEC of CT extract revealed the presence of a complex network containing AX, long chain of galactan and arabinan, together with HG and cellulose

In order to check whether HG in CT was linked or interacting with Ara, Xyl, Gal and/or Glc enriched moieties (see Table 1), the extract was submitted to AEC onto the gel DEAE Sepharose. As for WE chromatography, the recovery yield was less than 50%. The percentage of polysaccharides in the four eluted fractions accounted for 35%, 25%, 21% and 18% of the total recovered carbohydrates.

The not-retained fraction (CT-I) contained mainly Xyl (Table 4); the ratio Ara/Xyl of 0.34 was similar to that of WE and characteristic of mucilage AX. Other sugars consisted of Glc (22%), Gal (14%) and GalA (12%) residues. Neither Rha nor Fuc was detected in this fraction.

The three other fractions were rich in GalA, especially CT-III that was composed mainly of uronic acid (93%), attesting that this moieties was not linked to the other polysaccharides. In CT-II, Glc was the main neutral sugar suggesting that HG might also interact in some way with Glc enriched moieties. In CT-IV there was significant amount of Ara, Xyl, Gal and Glc. The ratio Gal/Rha was much higher than in the whole CT fraction, suggesting that long galactan chains were present possibly interacting with cellulose, HG and AX.

Altogether, the AEC showed a clear separation of AX and HG (in CT-III) but also the presence of a complex network including AX, long chain of galactan and arabinan, together with HG and possibly cellulose in the other fractions (Additional Fig. 2). Such a complex mixture might be used for drug encapsulation.

3.7. Ten percent of the dry weight of flax meal is extracted by potassium hydroxide

Hemicellulosic polysaccharides were isolated from the depectinated cell-walls of flax meal by extraction with KOH; they accounted ~10% of the FM (Table 1). This extract, which formed precipitates during neutralization, was separated into two fractions: 'A' corresponding to the precipitates and 'B' corresponding to the soluble polymers. Xyl was the major monosaccharide of 'A', indicating the presence of xylan. Sugar analysis of 'B' mainly revealed the presence of Glc, Xyl, Ara and Gal, suggesting that they were composed of xyloglucan/(galacto)xyloglucan and arabinogalactan.

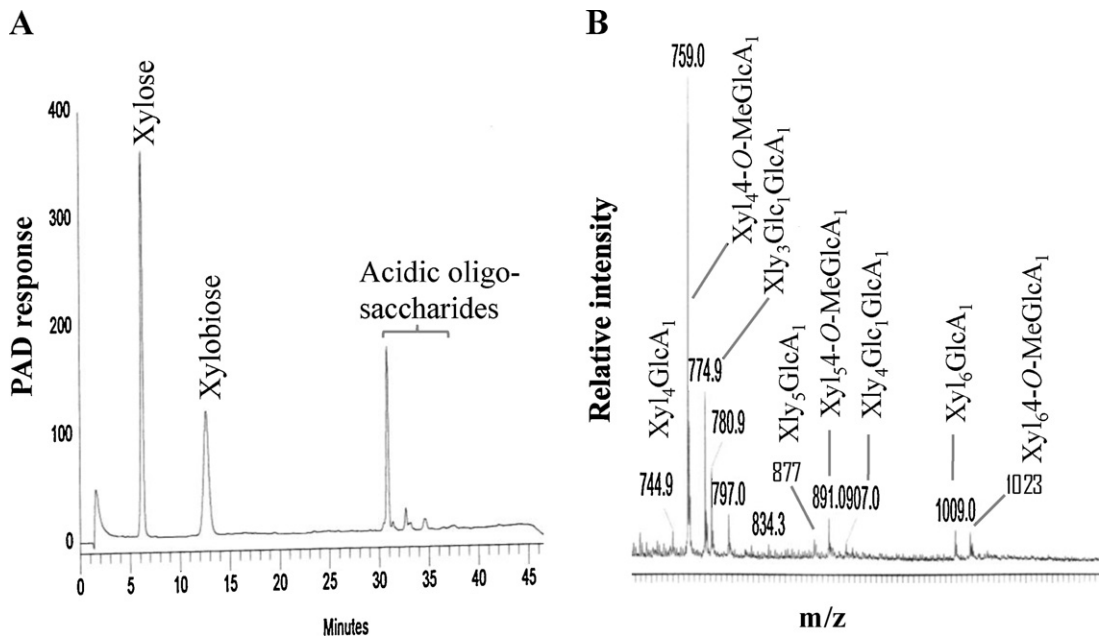

Fig. 3. HPAE-PAD chromatographic and MALDI-TOF-MS analyses of Xose. (A) HPAE-PAD chromatography. The fraction contains soluble xylo-oligosaccharides generated by the action of *endo*-xylanase on fraction A. Peaks were eluted with a gradient of sodium acetate (0.05–1 M) in 0.1 M NaOH. (B) MALDI-TOF-MS. Psuedomolecular ion $[M+Na]^+$ corresponding to acidic oligosaccharides containing Xyl, Glc, GlcA and 4-*O*-MeGlcA residues are visible in this mass spectrum.

3.7.1. β-(1 → 4)-Linked-D-xylopyranose backbone of flax meal xylan contains zones substituted with 4-O-MeGlcA/GlcA/Glc residues

The hemicellulosic A fraction on treatment with *endo*-(1 → 4)-β-D-xylanase generated a water soluble xylo-oligosaccharide rich fraction (designated as Xose) containing Xyl (93%), Glc (6%) and Ara (1%) residues in ~10% yield. HPAE-PAD analysis of this fraction (Xose) indicated the presence of D-Xyl monomer together with β-(1 → 4)-D-xylobiose, as well as peaks arising from xylan-derived acidic oligosaccharides eluted with high concentration of sodium acetate (Fig. 3A).

The xylanase-generated oligomers were further analyzed by MALDI-TOF mass spectrometry (Fig. 3B). The mass spectrum showed the presence of various acidic fragments. The main peak at $m/z = 759$ is in agreement with the $[M+Na]^+$ adduct of a 4-*O*-MeGlcA residue linked to four pentose residues. Considering that Xyl is the unique pentose detected in the fraction, this ion was assigned to Xyl_4-4-*O*-$MeGlcA_1$. In the same way, ions at $m/z = 891$ and 1023 were assigned to $[M+Na]^+$ of Xyl_5-4-*O*-$MeGlcA_1$ and Xyl_6-4-*O*-$MeGlcA_1$. Ions at 745, 877 and 1009 would correspond to Xyl_4-$GlcA_1$, Xyl_5-$GlcA_1$ and Xyl_6-$GlcA_1$, respectively, indicating the presence of another set of fragments with substitution of a 4-*O*-MeGlcA by a GlcA residue. It is remarkable to see a third set of ions at m/z 775 and 907 correspond to Xyl_3-Glc_1-$GlcA_1$ and Xyl_5-Glc_1-$GlcA_1$, respectively, in this spectrum.

Neutral and acidic oligosaccharides were obtained from flax meal by treatment of hemicellulosic A fraction with an *endo*-(1 → 4)-β-D-xylanase. Based on HPAEC-PAD and MALDI-TOF MS analysis, it appeared that flax xylan is composed of a β-(1 → 4)-linked-D-xylopyranose backbone with some zones substituted with GlcA and/or 4-*O*-MeGlcA residues. Surprisingly, the presence of glucose residue is also indicated, but further study is needed to ascertain its location. In xylans from many higher plants the β-(1 → 4)-linked-D-xylopyranose backbone can also be substituted with arabinosyl residue (Ebringerova & Heinze, 2000; Izydorezyk & Biliaderis, 1995; York, Kumar Kolli, Orlando, Albersheim, & Darvill, 1996). Finally, all these oligosaccharides might be useful to substitute chemical additives (Guilloux, Gaillard, Courtois, Courtoix, & Petit, 2009) for food preservations because of either their ability to stimulate the growth and development of gastrointestinal microflora (Ebringerova & Heinze, 2000).

3.7.2. Flax meal xyloglucan is XXXG rich but also contains XXGG structure

Xyloglucan, a key polysaccharide in the regulation of mechanical properties of cell walls during plant development and plant usages, is a neutral macromolecule. The hemicellulosic B fraction of present study was, therefore, purified by AEC using DEAE-Sepharose matrix. The isolated neutral fraction (named as BN) was made up mainly of Xyl and Glc residues with a ratio Xyl/Glc = 0.6 (Table 1). Gal was the other significantly present sugar. Methylation analysis yielded partially methylated alditol acetates corresponding to T-Xylp (terminal xylopyranose, 8 mol%), 1,2-Xylp (2-linked xylopyranose, 16 mol%), T-Gal (2 mol%), 1,4-Glcp (18 mol%), and 1,4,6-Glcp (57 mol%) residues, consistent with the presence of xyloglucan (Additional Fig. 3).

Besides, a combination of chromatographic and mass spectrometric analyses was performed on the digestion products obtained after an *endo*-glucanase treatment of BN. Firstly, sugar composition analysis of oligomeric products, namely, XGose, showed that it contained Xyl (ca. 37 mol%) and Glc (ca. 56 mol%) as the major monosaccharides together with small amount of Gal (ca. 7 mol%) and trace amount of Ara residues. Secondly, methylation analysis of these oligosaccharides yielded partially methylated alditol acetates corresponding to T-Xylp (terminal xylopyranose), T-Galp, 1,2-Xylp, 1,6-Glcp and 1,4,6-Glcp residues, consistent with the presence of galactoxyloglucan fragments. Thirdly, high performance anion exchange-pulse amperometric detection (HPAE-PAD) chromatographic analysis of XGose yielded a complex mixture of oligomeric fragments consisting of at least four major oligosaccharides (Fig. 4A). The retention time of the main fragments were found to be identical to those of XXXG, XXLG, XLXG and XLLG (named according to Fry et al., 1993) isolated from *Brassica*, *Sesamum*, *Argania*, *Benincasa* cell walls and used as standards (Ghosh et al., 2004, 2005; Ray, Loutelier-Bourhis, Condamine, Driouich, & Lerouse, 2004; Mazumder, Lerouge, Loutelier-Bourhis, Driouich,

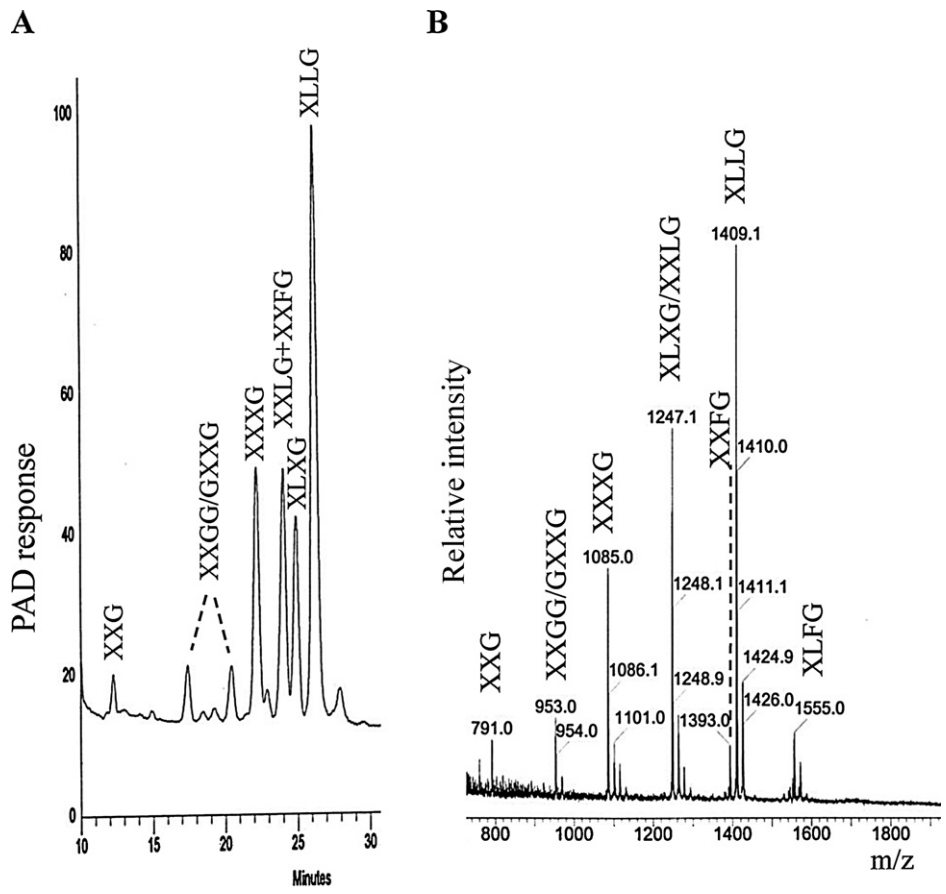

Fig. 4. HPAE-PAD chromatographic and MALDI-TOF-MS analyses of xyloglucan oligosaccharides (XGose). (A) HPAE-PAD chromatography. The peaks were eluted using a gradient of 0–0.1 M NaOAc in 0.1 M NaOH. Xyloglucans are classified as several types with a β-1,4-D-Glc backbone whose Glc (G) are unbranched or substituted with Xyl (X). Xyl can also be substituted by Gal or Gal and Fuc, according to the following nomenclature: F, Fuc 1,2-Gal 1,2-Xyl 1,6-Glc; L, Gal 1,2-Xyl 1,6-Glc; X, Xyl 1,6-Glc. (B) MALDI-TOF-MS. Psuedomolecular ion $[M+Na]^+$ corresponding to pentasaccharide (XXG) to decasaccharide (XLFG) are visible in this mass spectrum.

& Ray, 2005). Xyloglucans, based upon the types of oligosaccharides released after hydrolysis by endo-glucanase, were classified as XXXG and XXGG type (Vincken et al., 1997). A XXXG-type in which side chains is substituted with Xyl-Gal-Fuc residues and a XXGG type whose side chains contain terminal Gal residues such as in tobacco and tomato cell walls. The fragments generated from flax meal xyloglucan are classical fragments of XXXG type xyloglucan characteristics of many dicots (Hayashi, 1989; Vincken et al., 1997). Additional oligomers XXG or XXGG specific to flax were generated from XXGG-type of xyloglucans. These oligosaccharides might have been generated by the *endo*-glucanase enzyme, depending on the presence or absence of *O*-acetyl groups on the unbranched Glc residues. Such a XXGG-type of branching pattern is not common in seeds but has also been previously found in the cell walls of tobacco leaves and tomato suspension cultures, two solanaceous species (Hoffman et al., 2005; Jia, Qin, Darvill, & York, 2003).

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometric analysis provided further detailed structural information on the components present in XGose fraction (Fig. 4B). Taking into consideration the specificity and mode of action of the *endo*-glucanase and information obtained by sugar and linkage analyses, and HPAE-PAD chromatography, pseudomolecular ions in the MALDI-TOF-MS were assigned to xyloglucan oligosaccharide fragments of the XXXG-type from the heptasaccharide XXXG (m/z 1085) to the nonasaccharide XLLG (m/z 1409). The signals at m/z 1393 and 1555 that corresponded to fucosylated fragments were of very low abundance, which was consistent with the low level of Fuc detected in BN. Notably, the dominant side chains subunit is rather XLFG than XXFG as usually found. On the other hand, signals at m/z 791 and 953 confirmed the presence of XXGG type oligosaccharides.

Altogether, enzymatic digestion of the hemicellulosic fractions (A and B) coupled to chromatographic separation and mass spectrometric analysis of generated oligosaccharides, as well as monosaccharide compositional and linkage analysis of the associated fractions, proved a powerful approach to probe for detailed structural information. The *endo*-xylanase was suitable for profiling acidic CW xylans present in flax meal. This needs further quantitative confirmation, particularly as not all of the oligosaccharides released are yet identified. Similarly, the *endo*-glucanase enzyme was found to be suitable for profiling the main XXXG type but also the minor but specific XXGG-type of xyloglucans present in flax meal. Detailed analyses to elucidate the mechanism(s) associated with the XXGG-specific activity of these enzymes are necessary.

4. Conclusions

In conclusion, the findings of this study highlight several novel and important aspects of the Flax meal, FM, derived polysaccharides with regard to their structures: (i) it contained not only mucilages but also cell-wall pectins and hemicelluloses, (ii) the presence of AGP, a class of plant glycoprotein with many pharmacological and food industry applications has been indicated, (iii) the mucilages, which were characterized to be high molar mass AX and RG-I, could be easily fractionated on SEC and AEC

chromatographies, (iv) the presence and successful extraction of homogalacturonan was described, (v) hemicellulosic polysaccharides could be extracted in ten percent yield using 1 M KOH, (vi) the β-(1 → 4)-linked-D-xylopyranose backbone of flax meal xylan contains zones substituted with 4-*O*-MeGlcA/GlcA/Glc residues, (vii) the xyloglucan is XXXG-type but also contained XXGG structure, specific to flax seed and (viii) altogether theses moieties represented about 30% of the FM and might be individually used in various industries, either as polymers or as oligosaccharides.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.12.034.

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