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# Cell wall polysaccharides of *Brassica campestris* seed cake: isolation and structural features

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**Abstract**

*Brassica campestris* seed cake is an important source of water-soluble polysaccharides. The polysaccharides extracted with acid and dilute alkali represented 270 mg/g of defatted meal. These polymers, which have high amount of neutral sugars are arabinan, rhamnogalacturonan I and arabinogalactan proteins. These polymers have been analyzed by size exclusion chromatography. Two overlapping peaks have been found. Structural characterization of 4 M KOH-soluble hemicellulosic polysaccharides using specific enzyme hydrolysis, ion exchange chromatography (HPAEC) and matrix-assisted laser desorption ionization-time of flight mass spectroscopy showed that mustard meal xyloglucan is of XXXG type and contained XXXG, XXFG, XXLG and XLFG (named according to Fry et al. [Physiol. Plant. 89 (1993) 1]) as the major building sub-units. Hydrolysis with endo-β-(1 → 4)-D-xylanase and analysis of the xylan derived oligosaccharides showed the presence of xylose (27%), xylobiose (26%) and acidic xylan fragments containing 4-*O*-methyl-D-glucuronic acid residue (47%).


*Keywords:* *Brassica campestris*; Seed cake; Arabinan; Arabinogalactan protein; Rhamnogalacturonan I; Xyloglucan; Xylan

## 1. Introduction

*Brassica campestris Linn (Brassica rapa Linn)* field mustard is a variable annual or biennial herb cultivated in India as a cool season crop. The agronomic value of the plant lies in the seed. Major portion (95%) of mustard seed produced in India is utilized for production of oil and seed cake as by-product. The average yield of mustard seed is about 500 kg/ha (Wealth of India, 1988). Mustard meal is the residue obtained after the extraction of oil. This important agricultural by-product contains polysaccharides, proteins and is highly lignified. However, due to the limited degradation of these plant cell wall polysaccharides in the digestive track of monogastric animals, mustard seed meal is poorly utilized in animal feed. This is probably caused by the complex structure of the walls, which makes them not easily accessible to enzymatic degradation. Understanding the composition, structure and location of cell wall polysaccharides is therefore essential to the development of a better use for this agricultural by-product.

A number of studies related to the isolation of pectin from *B. campestris* seed meal using CDTA (Eriksson, Andersson, & Aman, 1997), oxalate (Siddiqui & Wood, 1971) as chelating agents has been described. Less attention has been paid, however, to hemicellulosic polysaccharides of this plant. To the best of our knowledge, only one study dealing with the sugar composition and linkage analyses (Siddiqui & Wood, 1977) has been reported, but detailed structural information is missing. Unfortunately, partial structural characterization is not sufficient to be interpreted in terms of functional properties and behavior, and further knowledge is required. Therefore, the objective of the present study is to investigate in details the structural features of the hemicellulosic polysaccharides. The structural analysis was carried out using an enzymatic degradation of the hemicellulosic fractions with specific enzymes and analysis of the resulting fragments by combination of GC, GC-MS, high

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performance anion exchange-pulse amperometric detection (HPAE-PAD) chromatography and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. In addition, as mustard seed meal is an important source of water-soluble polysaccharides, we isolated polysaccharides using a simple mild acidic treatment, a process used for the industrial extraction of pectin from apple pomace and citrus peels (Aravantinos-Zafiris & Oreopoulou, 1992). The yield and chemical composition of the dilute alkali extracted polysaccharides is also presented and discussed.

## 2. Experimental

### 2.1. General analyses

Klason lignin was determined as previously described (Adams, 1965). The analyses were made at least in duplicate and the results presented are their mean values. All evaporations were carried out under reduced pressure at temperature ≤50 °C. The degree of methylation (DM) were estimated by HPLC according to Voragen, Schols, and Pilnik (1986).

### 2.2. Material and preliminary treatments

The seed cake was obtained from the local market and was treated sequentially with hexane (48 h) and acetone (24 h) in a Soxhlet apparatus to remove lipids. The defatted mustard meal (DMM) was then air dried and ground.

### 2.3. Isolation of polysaccharides

#### 2.3.1. Extraction with diluted HCl

Extractions of the defatted seed cake (DMM) with 0.05 M HCl, pH 1.8 (w/v ∷ 1:100) were conducted at 80 °C for 30 min under constant stirring for three times. Separation of the un-extracted residue from the liquid was performed by centrifugation followed by filtration of the centrifugates through glass filter (G-3). The residue was briefly washed with additional distilled water and the wash was collected to maximize polysaccharide recovery. Polysaccharides present in the acidic extract were precipitated with 95% ethanol (3 vol.) and then collected by centrifugation (12,000*g* for 30 min). The pellet was then washed with 80%, 90% ethanol and acetone, and dried in vacuum over $P_2O_5$ at 35 °C overnight to yield (HCl-M).

#### 2.3.2. Delignification

Lignins were removed from the above residue using sodium chlorite in acidic solutions (pH 4.7) at 80 °C for 15 min (twice) and the material recovered was dialyzed and referred to as sodium chlorite-soluble material (SC-M).

#### 2.3.3. Extraction with alkali

Polymers were extracted from the delignified material using the following extraction conditions: (i) 0.05 M KOH + 0.4% $NaBH_4$ for 16 h at 4–6 °C followed by 4 h at 30–35 °C (OH-M), (ii) 1 M KOH + 0.4% $NaBH_4$ for 4 h at 30–35 °C followed by 16 h at 4–6 °C (1OH-M) and (iii) 4 M KOH + 0.4% $NaBH_4$ for 4 h at 30–35 °C followed by 16 h at 4–6 °C (4OH-M) (Fig. 1). All extracts were acidified to pH 5 with acetic acid over ice-bath, concentrated, dialyzed exhaustively and finally lyophilized. The resulting KOH un-extractable residue was washed thoroughly with water containing acetic acid, and then with deionized water, and finally dried by solvent exchange to yield the INS-M residue.

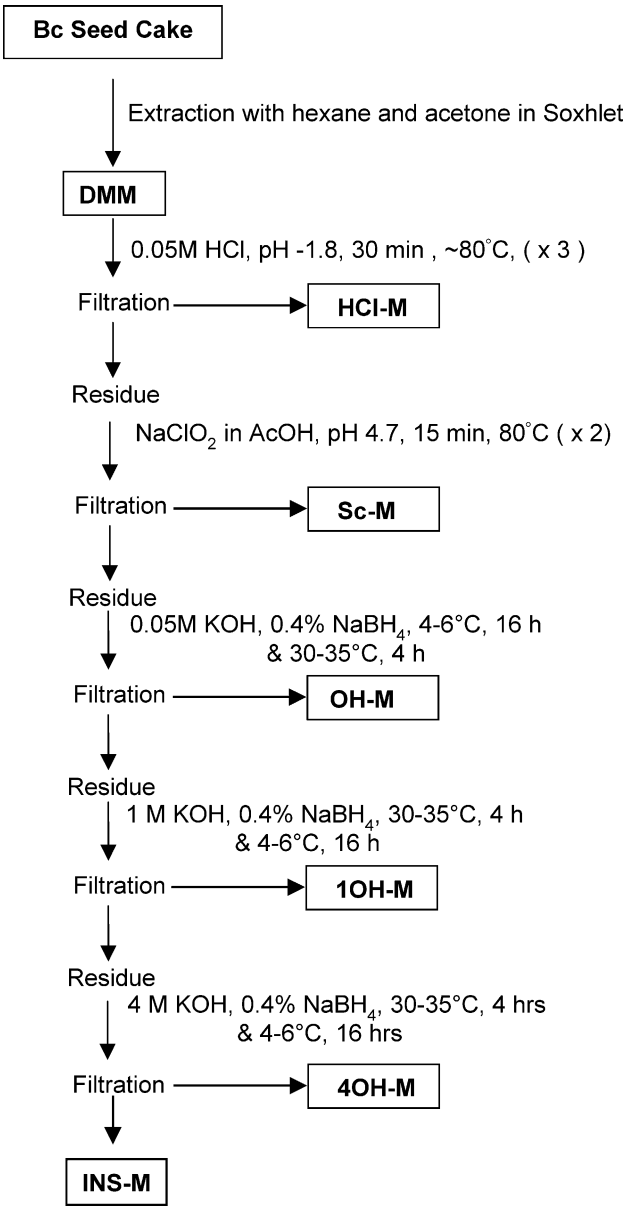


Fig. 1. Flow chart for the extraction of polysaccharides from the defatted seed cake of *Brassica campestris* by sequential extraction with inorganic solvents.

*2.3.4. Isolation of arabinogalactan proteins with β-glucosyl Yariv reagent*

AGPs were isolated according to Schultz, Johnson, Currie, and Bacic (2000). Briefly to a solution of HCl-M in 1% NaCl (w/w) was added an equal volume of Yariv reagent also in 1% NaCl. The mixture was kept at 4 °C for 18 h and then centrifuged. The pellet was washed with 1% NaCl followed by pure methanol (3 times each). The pellet was then dried and treated with sodium metabisulphite (10%). The resulting solution was then dialyzed and freeze dried to yield the mustard arabinogalactan proteins (AGPs).

*2.4. Sugar analysis*

Total sugars were determined by the phenol–sulfuric acid assay using galactose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Total uronic acid of polymers was assayed colorimetrically by the *m*-hydroxydiphenyl assay according to Ahmed and Labavitch (1977). The neutral sugar composition was determined after hydrolysis with sulfuric acid (2 M, 100 °C, 2 h), reduction and acetylation (Blakeney, Harris, Henry, & Bruce, 1983). Alternatively, oligosaccharides and enzyme-resistant materials were hydrolyzed with TFA (2 M, 110 °C, 2 h), converted into a mixture of methyl glycoside by treatment with 1 M methanolic HCl (1 M, 80 °C, 18 h) and the methyl glycosides were separated as their TMS-derivatives (York, Darvill, McNeil, Stevenson, & Albersheim, 1985) by GC. The gas chromatograph was equipped with a flame ionization detector and a WCOT fused silica capillary column (length 25 m, i.d. 0.25 mm and film thickness 0.4 μm) with CP-Sil 5 CP as stationary phase. The oven temperature program was: 2 min at 120 °C, 10 °C/min to 160 °C, and 1.5 °C/min to 220 °C and then 20 °C/min to 280 °C.

*2.5. Protein and amino acid analysis*

Proteins and amino acids were analyzed as previously described (Mazumder et al., 2002).

*2.6. Size exclusion chromatography*

Size exclusion chromatography (SEC) of the acid extracted fraction was done on a Sephacryl S-1000 column ($90 \times 2.6$ cm$^2$, BioRad) calibrated with standard dextrans using 0.01 M borax (pH 9.2) at a flow rate of 30 ml/h.

*2.7. Preparation of xyloglucan oligosaccharides*

The fraction 4OH-M (3 mg) was dissolved in 2 ml of 50 mM NaOAc (pH 5.0) and the mixture was incubated with 10 units of endo-cellulase (Megazyme International, Ireland) for 24 h at 37 °C. The enzyme-resistant polymer (M4G-RF) was then precipitated in 80% ethanol (v/v) and removed by centrifugation. The ethanol-soluble fractions containing the xyloglucan oligosaccharides (M4XGose) were concentrated and finally lyophilized.

*2.8. Preparation of xylan oligosaccharides*

Hydrolysis of 2 mg of xylan rich fraction (4OH-M) in 1 ml of 50 mM NaOAc pH 5.0 was performed using 20 units of endo-(1 → 4)-β-D-xylanase (Megazyme International, Ireland) at 37 °C for 24 h. The enzyme-resistant polymers (M4X-RF) and the 80% ethanol (v/v) soluble xyloglucan oligosaccharides (M4Xose) were isolated as given in this section.

*2.9. Methylation analysis*

The pool of xyloglucan-derived oligosaccharides (M4XGose) was permethylated according to Ciucanu and Kerek (1984). The permethylated product was then hydrolyzed, converted into partially methylated alditol acetates (PMAA) and analyzed by GLC-MS.

*2.10. HPAE-PAD chromatography*

The 80% ethanol soluble oligo- and monosaccharides were analyzed on a Dionex DX 500 system equipped with a GP 50 gradient pump, an eluent degas module, a CarboPac PA-1 column and a pulse amperometric detector (PAD). The gradient was obtained by mixing solutions of 0.1 M NaOH and 1 M NaOAc as described (Lerouxel et al., 2002).

*2.11. MALDI-TOF mass spectrometry*

MALDI-TOF MS in reflectron mode was performed using a Micromass (Manchester, UK) Tof spec E MALDI-TOF mass spectrometer. 2,5-Dihydroxybenzoic acid (10 mg/ml) was used as matrix. Mass spectra were calibrated with a mixture of standard peptides.

*2.12. Nomenclature*

Xyloglucan derived oligosaccharides are named according to Fry et al. (1993).

## 3. Results and discussion

*3.1. Isolation and partial characterization of polysaccharides*

*3.1.1. Composition of defatted mustard meal*

Prior to any extraction and analysis of wall polysaccharides, mustard meal was defatted with hexane and acetone. The yield of the DMM was 800 mg/g of the native meal. It contained arabinose, glucose, galactose and xylose as the major neutral sugars (Table 1). Mannose, rhamnose and fucose were also present but in a very small amount. It also

Table 1
Yields and sugar composition of the fractions isolated from *Brassica campestris* meal and of oligosaccharides derived therefrom by digestion with endo-glucanase and endo-xylanase (see text for identification of fractions)

| | DMM | HCl-M | AGPs | SC-M | OH-M | 1OH-M | 4OH-M | INS-M | M4Xose | M4XGose |
|---|---|---|---|---|---|---|---|---|---|---|
| Yield[a] | 100 | 11 | 9[b] | 10 | 16 | 3 | 3 | 21 | Nd | Nd |
| NS[c] | 22 | 20 | 32 | 22 | 18 | 37 | 57 | 30 | Nd | Nd |
| UA[c] | 6 | 9 | 3.3 | 8 | 3 | 4 | 3 | 5 | Nd | Nd |
| Rha[d] | 1 | 1 | 2 | 3 | 2 | 1 | Tr | 2 | 1 | Tr |
| Fuc[d] | 1 | 1 | Tr | 1 | 2 | 2 | 3 | 3 | – | 6 |
| Ara[d] | 39 | 63 | 22 | 57 | 49 | 24 | 19 | 27 | 7 | 2 |
| Xyl[d] | 10 | 10 | 10 | 18 | 16 | 42 | 42 | 8 | 84 | 32 |
| Man[d] | 4 | 1 | 6 | 1 | 4 | 2 | 5 | 1 | Tr | 8 |
| Gal[d] | 22 | 18 | 47 | 16 | 21 | 14 | 11 | 10 | 2 | 38 |
| Glc[d] | 23 | 6 | 13 | 4 | 6 | 15 | 20 | 49 | 6 | 14 |

Tr, trace; Nd, not determined; NS, neutral sugar; –, not detected; UA, uronic acid. Each value is the mean of at least two determinations and the mean value is given to its nearest whole number. We have calculated the standard deviation for all the analysis performed and found that the standard errors in all cases lie within the range of ±4%.

[a] Percentage weight of the DMM dry weight.
[b] Percentage weight of the HCl-M dry weight.
[c] Percentage weight of fraction dry weight.
[d] Mol percent.

contained 6% uronic acid. In addition to polysaccharides, which represent 28%, DMM contained approximately 18% of Klason lignin.

### 3.1.2. Dilute HCl extracted material (HCl-M)

In order to gain information on the families of polysaccharide present and to solubilize major part of non-cellulosic polysaccharides, DMM was sequentially extracted with different inorganic solvents as shown in Fig. 1. The yields of various fractions obtained therefrom and their sugar composition are given in Table 1. Mild acid treatment has been reported to remove large amount of polymers from citrus, apple or sugar-beet pulp (Aravantinos-Zafiris & Oreopoulou, 1992), probably by cleavage of the cross-links of the matrix polymers, and other linkages such as hydrogen or ionic bonds. Extraction of DMM using dilute HCl yielded 11% of material (HCl-M), containing 29% of carbohydrate. Using 0.05 M CDTA, pH 6.0 as extractant Eriksson et al. (1997) obtained 32.6% yield starting from dehulled, defatted meal. These authors reported that the yield of the hull-free material is 32% of the whole defatted whole seed. Assuming that this value is same in Indian seed as well, then the yield of dilute HCl extracted polymers (HCl-M) increase to 34.5%. Therefore, dilute acid, seems to be an efficient solvent for the extraction of polysaccharides from defatted seed meal. It is much less costly and less toxic than the usually used chelating agents, such as CDTA or EDTA.

The neutral sugar composition of the HCl-M fraction shows the presence of very high amount of arabinose (63-mol%) along with galactose, xylose, glucose and a small amount of rhamnose. The high amount arabinose suggests the presence of arabinan. These data demonstrating the presence of arabinan are consistent with the earlier results as obtained from dehulled seed meal using chelating agents (Eriksson, Andersson, Westerlund, Andersson, & Aman, 1996; Tharanathan, Bhat, Krishna, & Paramahansa, 1985). The presence of rhamnogalacturonan I in this fraction is indicated by the high content of galacturonic acid (9% w/w), the identity of which was confirmed by TLC and by the presence of small amount of rhamnose residue (Table 1). Moreover, the acid extracted material shows absorption bands at 1016 and 1093 $cm^{-1}$ characteristic of polymers containing galacturonic acid (Kacurakova, Capek, Sasinkova, Wellner, & Ebringerova, 2000).

The acid-extracted polymers were further analyzed by gel permeation chromatography. As seen in Fig. 2, the polysaccharides gave two overlapping peaks, within the Kav. ranges from 0.03 to 0.86. The first peak (F1), which accounted for 26% of the total sugar eluted, have Kav. from 0.03 to 0.44, whereas the second peak (F2) elutes between 0.44 and 0.86.

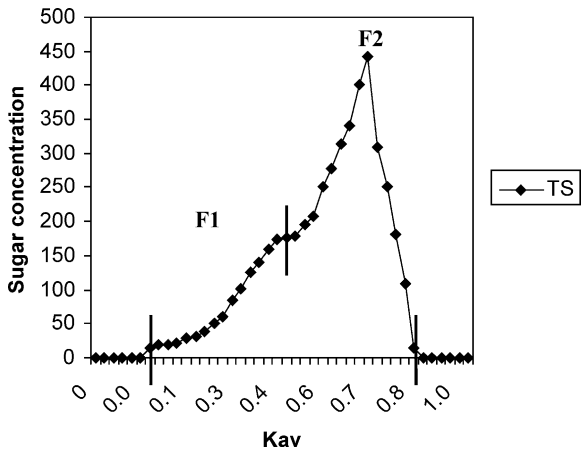


Fig. 2. SEC elution profile of dilute acid soluble polysaccharide fraction (HCl-M) obtained from defatted seed cake of *Brassica campestris*.

*3.1.3. HCl-M contains AGPs*

The total nitrogen value of the HCl-M was 3.5%, which corresponds to a protein content of 21.9% (w/w), indicating the presence of proteins and probably AGPs. Thus, to check for the presence of AGPs, the reactivity of the HCl-M polymers with β-glucosyl Yariv reagent was tested. We found that part of the HCl-M material was precipitable with the Yariv reagent. Sugar composition analysis of this material shows that it is enriched in galactose residues and, to a lesser extent, arabinose confirming the presence of AGP (Table 1). The Gal/Ara ratio of this fraction is 2.1, whereas that of HCl-M fraction is 0.28. About 5% of the arabinose and 35% of the galactose present in HCl-M was precipitated with Yariv reagent. It also contains mannose and xylose residues probably originating from *N*-glycans and polymers containing xylose. The amino acid composition of proteins associated with HCl-M shows that glutamic acid/glutamine, proline and glycine are the major constituents (Table 2).

*3.1.4. Removal of lignin*

To remove the lignin present in the seed meal, the acid un-extractable residue was treated with sodium chlorite. The sugar composition of the material (SC-M) extracted during sodium chlorite treatment was found to be similar to that of the HCl-M fraction (Table 1). This is not surprising, as sodium chlorite treatment is known to solubilize wall polysaccharides as well.

*3.1.5. Dilute alkali extracted material (OH-M)*

Even after solubilization of more polysaccharides during sodium chlorite treatment, some remained cross-linked within the walls and could be extracted with dilute alkali (0.05 M KOH). Indeed, approximately 16% of the DMM was extracted and the uronide content of the dilute alkali extracted material (OH-M) was 3% (Table 1). The neutral sugar composition of this fraction matched that of acid and chlorite extracted polymers, which contained arabinan as the major polysaccharides (Table 1).

Table 2
Amino acid composition of HCl-M fraction isolated from the defatted seed cake of *Brassica campestris* by extraction with diluted HCl

| Amino acids | % mol |
|---|---|
| Aspartic acid/asparagine | 4 |
| Threonine | 4 |
| Serine | 5 |
| Glutamic acid/glutamine | 37 |
| Proline | 9 |
| Glycine | 10 |
| Alanine | 6 |
| Cysteine | 1 |
| Valine | 3 |
| Isoleucine | 2 |
| Leucine | 5 |
| Tyrosine | 1 |
| Phenyl alanine | 2 |
| Histidine | 2 |
| Lysine | 6 |
| Arginine | 3 |

*3.2. Extraction and characterization of hemicellulosic polysaccharides*

The residue was then successively extracted with strong alkali (1 and 4 M KOH) to isolate the hemicellulosic polysaccharides. Monosaccharide compositional analysis of the isolated fractions (1OH-M and 4OH-M) shows that they are enriched with xylose and glucose, and the ratio of xylose to glucose is 2.8 and 2.1, respectively (Table 1). It is, therefore, very likely that these two alkali extracts are mainly composed of xyloglucan and xylan.

*3.2.1. Xylan structure*

To gain information about the structure of xylan present in 4OH-M fraction, it was treated with an endo-xylanase. Sugar compositional analysis of the enzyme-generated oligomers (M4Xose fraction) indicated the presence of xylose residue as the major sugar (Table 1). Small amounts of glucose and trace amount of other sugars including 4-*O*-methyl glucuronic acid (4-*O*-MeGlcA) were also found.

The HPAEC-PAD elution profile (Fig. 3a) of M4Xose fraction indicated the presence of xylose (27%) and xylobiose (26%), as well as peaks eluted with high concentration of sodium acetate arising from xylan-derived acidic oligosaccharides (47%). The xylanase-generated oligomers were analyzed by MALDI-TOF mass spectrometry (Fig. 3b), which showed the presence of various acidic fragments. Considering that xylose is the unique pentose detected in this fraction (Table 1), peaks at *m/z* 759, 891, 1023 and 1155 were assigned to $Xyl_4$-4-*O*-MeGlcA, $Xyl_5$-4-*O*-MeGlcA, $Xyl_6$-4-*O*-MeGlcA and $Xyl_7$-4-*O*-MeGlcA, respectively. Additional ion at *m/z* 1009 arises from a hexapentosyl fragment bearing a GlcA residue, instead of a 4-*O*-MeGlcA.

*3.2.2. Xyloglucan structure*

Hemicellulosic fraction (4OH-M) was treated with endo-(1 → 4)-β-glucanase, a xyloglucan-degrading enzyme and the generated oligosaccharides were isolated from the digest as described in Section 2. Methylation analysis data, which indicates the presence of T-Xyl*p* (terminal xylopyranose), T-Fuc*p*, T-Gal*p*, 1,2-Xyl*p* (2-linked xylopyranose), 1,2-Gal*p*, 1,6-Glc*p* and 1,4,6-Glc*p* residues, and sugar compositional analysis (Table 1) of M4XGose fraction are consistent with the presence of xyloglucan fragments.

HPAE-PAD chromatographic analysis of the endoglucanase-generated fragments indicates the presence of four major oligomers (Fig. 4a). The retention time of the main fragments were found to be identical to those of XXXG, XXFG, XLFG, XXLG and XLLG isolated from *Arabidopsis thaliana* cell walls and used as standards (Lerouxel et al., 2002).

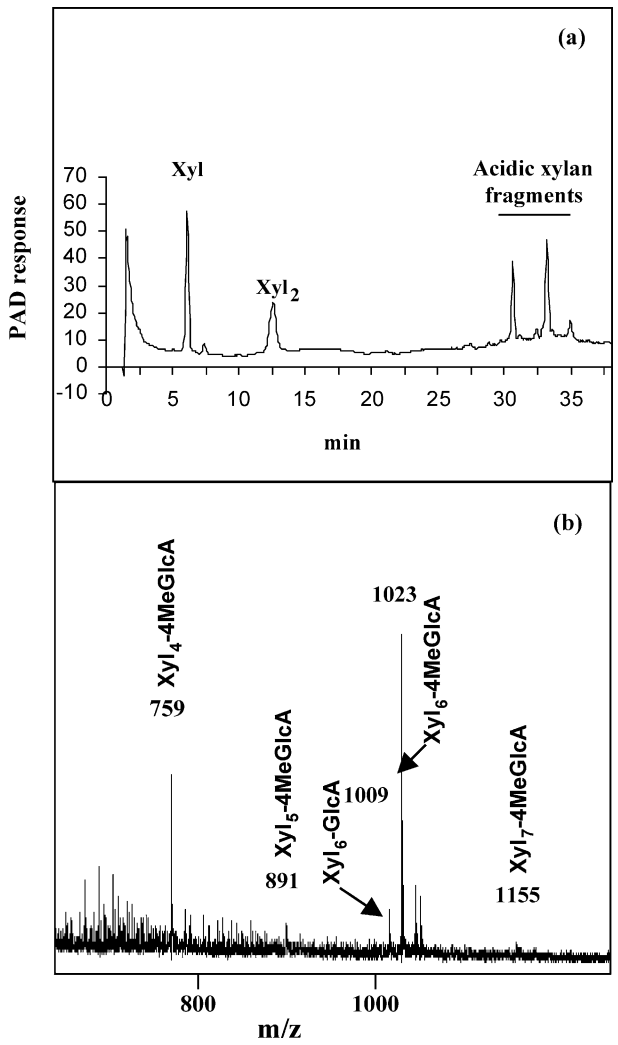


Fig. 3. (a) HPAE-PAD chromatographic elution profile and (b) MALDI-TOF mass spectrum of the M4Xose fraction obtained from 4OH-M fraction of *Brassica campestris* meal after endo-(1 → 4)-β-xylanase degradation. See text for the identification of fractions.

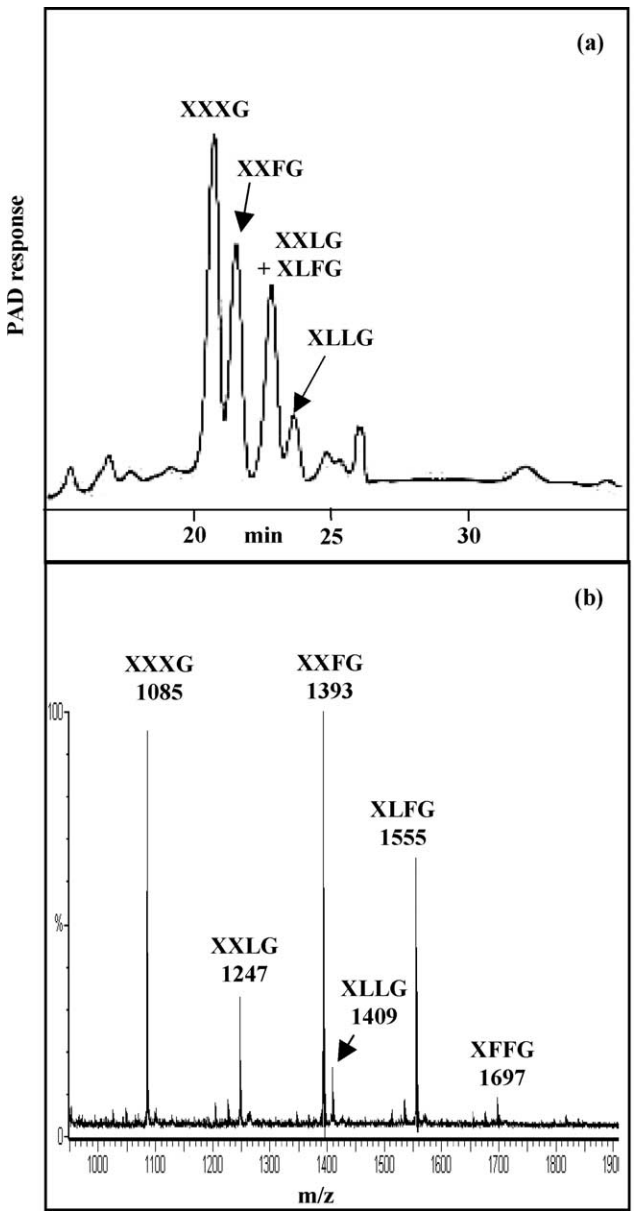


Fig. 4. HPAEC elution pattern (a) and MALDI-TOF mass spectrum (b) of the 4 M KOH-soluble fraction (4OH-M) of *Brassica campestris* seed meal after degradation by endo-(1 → 4)-β-glucanase.

The MALDI-TOF mass spectrum (Fig. 4b) of M4XGose revealed the presence of four main $[M + Na]^+$ ions at *m/z* 1085, 1247, 1393 and 1555 together with two minor $[M + Na]^+$ species at *m/z* 1409 and 1697. Taking into consideration the 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 partially xyloglucan oligosaccharide fragments of the XXXG-type from the heptasaccharide XXXG to the difucosylated XFFG (Fig. 4b).

## 4. Conclusions

In conclusion, this study shows that the major non-cellulosic polysaccharides in the defatted seed cake of *B. campestris* are arabinan, AGPs', rhamnogalacturonan I and hemicellulosic polymers. Over 43% of the seed meal could be isolated by successive treatment with various inorganic solvents. Dilute hydrochloric acid is an efficient as well as a low cost reagent for the isolation of polysaccharides from DMM. Large amount of polysaccharide was extracted during and after delignification. Since arabinan is the major non-hemicellulosic polymer extracted from mustard seed meal, further studies on the physical properties and structure of the extracted polymer will be of interest from scientific as well as industrial purposes. We also show for the first time that DMM contains AGPs, a class of plant proteoglycans with many pharmacological and food-industry applications.

We show that the xyloglucan of *B. campestris* consists mostly of the XXXG, XXFG, XLFG and XXLG sub-units

whereas its xylan is composed of a β-(1 → 4)-linked-D-xylopyranose backbone substituted with 4-*O*-MeGlcA residues, as observed for many other higher plants (Izydorezyk & Biliaderis, 1995; Sims, Munro, Currie, Craik, & Bacic, 1996; Timell & Syracuse, 1967; York, Kolli, Orlando, Albersheim, & Darvill, 1996).

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