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Structural characterisation of hemicellulosic polysaccharides from *Benincasa hispida* using specific enzyme hydrolysis, ion exchange chromatography and MALDI-TOF mass spectroscopy

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Abstract

Benincasa hispida fruit is an important source of water-soluble polysaccharides. Hemicellulosic polysaccharides extracted with 1- and 4-M KOH represented 120 mg/g of alcohol insoluble residues. The intrinsic viscosity of the extracted polymers (named B1OH and B4OH fractions) was 284 and 262 ml/g, respectively. The extracted polymers, which were further characterised by size exclusion chromatography, give one broad peak. The apparent molecular weight of the isolated polysaccharide was estimated to be around 130±40 kDa. Sugar composition analysis of both fractions demonstrates the presence of xyloglucan (XG) and xylan. Structural characterisation of hemicellulosic polysaccharides using specific enzyme hydrolysis, ion exchange chromatography (HPAEC) and Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) mass spectroscopy showed that *B. hispida* fruit XG is of XXXG type and contained XXXG, XXLG, XXFG and XLFG (named according to Fry et al. 1993) in the ratio of 31:28:41 as the major oligomeric subunits. This fucogalactoxyloglucan carries *O*-acetyl substituents exclusively on galactose-containing oligosaccharides subunits. Finally, enzyme hydrolysis together with HPAEC and MALDI-TOF-mass spectrometric analysis of the generated fragments shows that the xylans, also present in both fractions, exhibit a classical structure with a backbone of β-(1→4)-linked xylopyranosyl residues substituted with three 4-*O*-methyl glucuronic acid per 97 xylopyranosyl unit.

Keywords: Benincasa; Hemicellulose; Viscosity; SEC; Enzyme hydrolysis; HPAEC; MALDI-MS

1. Introduction

Benincasa hispida (Thunb) Cogn (Cucurbitaceae), an annual shrub is endemic to India (The Wealth of India, 1962). The mature fruit is the mainly used part of the plant, although stems and leaves are sometimes cut, cooked and eaten. In Indian traditional medicine, it is used for the management of peptic ulcers, epilepsy and other nervous disorders (Chatterjee & Pakrashi, 1997; Sharma, 1984). Fruit juice of *B. hispida* has shown anti-inflammatory and anti-ulcer activities (Grover, Adiga, Vats, & Rathi, 2001; Grover, Rathi, & Vats, 2000; Ramesh, Gayathri, Appa Rao, Prabhakar, & Seshagiri Rao, 1989). Unfortunately, the harvested fruits are utilised and used either as an ingredient

Abbreviations: BAIR and BINS, alcohol- and alkali-insoluble residues of *Benincasa* fruit; B1OH and B4OH, 1- and 4-M KOH extracted polymers from BAIR, respectively; FT-IR, Fourier transform infra red; GLC/MS, gas liquid chromatography/mass spectrometry; Z-GRF, glucanase resistant fractions generated from fraction entitled 'Z'; HPAEC, high performance anion exchange chromatography; MALDI-TOF-MS, matrix assisted laser desorption ionisation-time of flight mass spectrometry; NS, neutral sugar; PAD, pulse amperometric detector; SEC, size exclusion chromatography; TFA, trifluoro acetic acid; TMS, tetra methyl silane; Z-XGose, xyloglucan derived oligosaccharides generated from fraction entitled 'Z'; Z-Xose, xylan derived oligosaccharides generated from fraction entitled 'Z'; Z-XRF, xylanase resistant fractions generated from fraction entitled 'Z'; UA, uronic acid.

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in curries or candied to make petha (a kind of sweet dish), but are often used as cattle feed. Better utilisation of this fruit would contribute to lower the economic burden on communities cultivating it. The isolation of particular high value component, such as water-soluble polysaccharides, could probably stimulate better utilisation of this resource. We have previously characterised the polysaccharides present in *B. hispida* juice (Mazumder, Ray, & Ghosal, 2001) and found them to consist mostly of arabinogalactan. In a subsequent paper, we have shown that the alcohol insoluble residues from the fruit contain high amount of homogalacturonan and β-(1→4)-D-galactan together with a small amount of acidic arabinan (Mazumdar, Morvan, Thakur, & Ray, 2004). To the best of our knowledge, there have been no studies on the characterisation of hemicellulosic polysaccharides present in this fruit, although hemicelluloses have considerable potential for application in foods (Fooks & Gibson, 2002; Garrote, Dominguez, & Pajaro, 2002; Kabel et al., 2002), pharmaceuticals (Burgalassi, Chetoni, & Saettone, 1996; Kato, Uchida, Ito, & Mitsuishi, 2001; Miyazaki, Kawasaki, Kubo, Endo, & Attwood, 2001), and in paper and cotton (Glicksman, 1986; Sims et al., 1998) industries. Since structure and functions are intimately related, knowledge of the chemistry of this material is of crucial importance for its proper utilisation. Therefore, the present study aims at isolating hemicellulosic polysaccharides from the cell walls of *Benincasa* fruit and determining their structural features. Using specific enzyme hydrolysis, GC, GC-MS as well as HPAEC and MALDI-TOF mass spectrometry, we have been able to establish the structure of the xyloglucan and xylan polysaccharides.

2. Experimental

2.1. Materials

Alcohol insoluble residues (BAIR) of *B. hispida* fruit were obtained as described previously (Mazumder et al., 2001).

2.2. Isolation of hemicellulosic polysaccharides by extraction with alkali

Three grams of BAIR were depectinated as described (Majumdar, Morvan, Thakur, & Ray, 2004) and the remaining residue was extracted sequentially with: (i) 250 ml of 1 M KOH + 20 mM $NaBH_4$ at 4 °C for 16 h and then 4 h at 30–35 °C (B1OH) and (ii) 250 ml of 4 M KOH + 20 mM $NaBH_4$ at 30–35 °C for 4 h followed by 16 h at 4 °C (B4OH). After each extraction, the solubilised material was separated from the insoluble residue by centrifugation (10,000 × *g*, 15 min) and filtration of the supernatant through a glass filter (G 3). Extracts were acidified to pH 5 immediately with drop-wise addition of 5-M acetic acid in an ice bath. All the extracts were dialysed, concentrated and kept in the frozen state. To determine the yield, a part of the frozen solution was lyophilised. The final residue was suspended in water, acidified to pH 5.2, dialysed and lyophilised to produce the insoluble residue (BINS).

2.3. General

All experiments in this study were conducted at least in duplicate. Evaporation was carried out under reduced pressure at 50 °C. Dialysis against distilled water was performed with continuous stirring in the presence of toluene to inhibit microbial growth. Moisture was determined by drying ground material in an air-circulated oven at 110 °C for 3-h. IR spectra (KBr disc) were obtained with a JASCO FTIR 420 spectrophotometer. Protein was estimated by the method of Bradford using bovine serum albumin as standard.

2.4. Sugar analysis

For the determination of monosaccharide composition, the polysaccharides in the samples were hydrolysed using trifluoro acetic acid (2 M, 2 h at 110 °C), followed by an 18 h methanolysis at 80 °C with dry 2 M methanolic-HCl. The generated methyl glycosides were converted into their TMS-derivatives (York, Darvill, O'Neill, Stevenson, & Albersheim, 1985) and separated by gas chromatograph (GC) with H_2 as carrier gas. The monosaccharide composition of the insoluble residues was determined after Seaman hydrolysis. The GC was equipped with a flame ionisation 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. Viscosity

To determine the intrinsic viscosity [η] of pectin, the flow time of the pectin solutions at different concentrations were determined at 30 °C using an Ubbelohde type viscometer. The reduced viscosity (η_{sp}/C) was then plotted against the polymer concentration (C in g/mL) and the intrinsic viscosity [η] was derived from extrapolation to $C = 0$.

2.6. Gel-filtration chromatography

Size exclusion chromatography on column (50 × 2.3 cm) of Sephadex G-200 using 500 mM sodium acetate buffer pH 4.0, was done as described (Mondal, Ray, Ghosal, Teleman, & Vuorinen, 2001). Standard dextrans were a gift from Dr Tapani Vuorinen.

2.7. Anion exchange chromatographic method

Fractions (B1OH and B4OH) were submitted to anion exchange chromatography on DEAE-cellulose (acetate form) column of (21 cm×4 cm) equilibrated previously with 0.05-M sodium acetate buffer, pH 5.5. After loading with sample, the column was eluted with the same buffer (500 ml) at a flow rate of 50 ml/h to obtain the non-retained fractions (named B1OH-N and B4OH-N).

2.8. Preparation of xyloglucan-derived oligosaccharides

Samples (2–3 mg) were separately dissolved in 2 ml of 100 mM NaOAc (pH 5.0) and incubated with 10 units of endo-(1→4)-β-D-glucanase (Megazyme International, Ireland) for 24 h at 37 °C with constant shaking. The incubation was stopped by heating (15 min at 100 °C). After cooling, the digests were treated with 4 volumes of cold 95% ethanol, centrifuged and the resulting supernatants were concentrated to a small volume under nitrogen at 40 °C, and finally lyophilised. In this manner, xyloglucan oligosaccharides B1-XGose, B4-XGose and BAIR-XGose were generated from B1OH-N, B4OH-N and BAIR, respectively (Fig. 1). Glucanase-resistant fractions, designated as B1-GRF, B4-GRF and BAIR-GRF, were obtained by lyophilisation of the ethanol insoluble residues (Fig. 1).

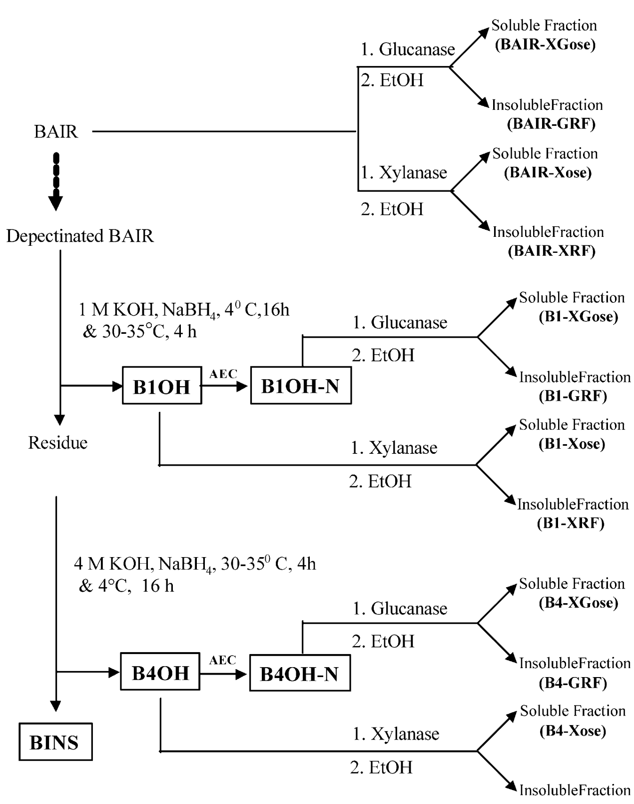

Fig. 1. Flow chart for the isolation of hemicellulosic polysaccharides from *Benincasa hispida* fruit and fragments derived therefrom.

2.9. Preparation of xylan derived oligosaccharides

Hydrolysis of the xylan rich fractions (BAIR and B1OH) 0.1% (w/v) in 2–3 ml of 100 mM NaOAc (pH 5.0) was performed using 22.5 units of endo-(1→4)-β-D-xylanase (Megazyme International, Ireland) at 37 °C for 24 h with constant stirring. Xylanase-derived oligomers and the enzyme-resistant fractions were isolated in the same way as shown in Fig. 1.

2.10. Matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry

Each of the samples (2 μl) were mixed with 2 μl of the matrix solution (2,5-dihydroxybenzoic acid 10 mg/ml in TFA:CH_3CN::1.75:0.75; v/v) and a total of 2 μl of this solution was applied to a stainless steel sample slide and dried under vacuum. MALDI-TOF mass spectra of the generated oligomers were recorded on a Micromass (Manchester, UK) Tof spec E MALDI-TOF mass spectrometer. Spectra were acquired in the reflectron mode.

2.11. High performance anion exchange-pulse amperometric detection (HPAE-PAD) chromatography

Xyloglucan-derived oligosaccharides were also analysed by HPAEC on a Dionex DX 500 system equipped with a GP 50 gradient pump and a CarboPac PA-1 column. The flow rate was 1-ml/min 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 1M-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.

2.12. Methylation analysis

The pool of oligosaccharides (B1-XGose) generated from *B. hispida* fruit xyloglucan was permethylated according to Ciucanu and Kerek (1984). Permethylated material was extracted, dried, hydrolysed, converted into its partially methylated alditol acetates (PMAA) and was separated by GC (HP6890 series) on a column (30 m×250 μm×0.25 μm) with a phase of (Optima 5-MS, Macharey-Nagel) and analysed by MS using a Autospec (Micromass, Manchester, UK) GLC-MS.

3. Results and discussion

3.1. Polysaccharide isolation and sugar composition

The alcohol insoluble residue obtained from *B. hispida* fruit (BAIR) was firstly depectinated to facilitate the subsequent extraction of polymers. Isolation of hemicellulosic polysaccharides was then carried out by sequential extraction with 1 and 4 M KOH containing 20 mM $NaBH_4$ (Fig. 1) leading to two fractions, so-called B1OH and B4OH.

The yields and chemical composition of various fractions obtained are given in Table 1. As shown, 5 and 7% of the BAIR dry weight were recovered from the 1 and 4 M KOH soluble fractions, respectively. Sugar composition analysis shows that xylose alone accounts for $\geq$44% of the total carbohydrate of the B1OH and B4OH fractions indicating the probable presence of xylan. Glucose, fucose and a part of xylose may arise from fucosylated xyloglucan, another important hemicellulosic polysaccharide. The high amount of galactose present in both hemicellulosic fractions probably arises from pectic galactan, a polymer known to be present in *B. hispida* fruit (Majumdar, Ray, Lopes da Silva, & Delgadillo, 2001). A part of the galactose residue may, however, come from xyloglucan.

Interestingly, the final alkali-insoluble residue (BINS) still contains high amount of galactose, suggesting that galactan is very firmly bound to the cellulosic microfibrils. BINS also contains 27 mol% of 2 M TFA hydrolysable glucose. As cellulose is normally resistant to hydrolysis under these conditions, this glucose probably derives from a hemicellulosic glycan, which is also firmly bound to cellulose. Nevertheless, we cannot exclude the possibility that the alkali treatment may have rendered a proportion of the cellulose microfibrills susceptible to acid hydrolysis. Finally, after Seaman hydrolysis, BINS shows a very high glucose content (93-mol%) demonstrating the abundance of cellulose in this fraction.

Table 1
Yields and sugar composition (mol%) of the hemicellulosic fractions and α-cellulose isolated from depectinated cell wall of *Benincasa hispida* by sequential extraction with 1 and 4 M-KOH (see experimental for identification of fractions)

	BAIR[a]	B1OH	B4OH	INS[a]
Yield[b]	100	5	7	16
TS	33(16)	60	63	18(31)
Ara	3(tr)	3	3	1(tr)
Rha	3(tr)	2	tr	3(0)
Fuc	1(tr)	3	2	1(0)
Xyl	11(3)	44	45	5(tr)
GlcA	1(1)	4	1	2(1)
GalA	39(3)	7	2	8(1)
Man	3(4)	1	6	4(5)
Gal	34(tr)	26	23	49(tr)
Glc	5(89)	11	18	27(93)

tr, trace.
[a] Values in the parenthesis were obtained after Seamen hydrolysis.
[b] Percentage weight of the BAIR dry weight.

3.2. Viscosity, IR spectroscopy and molecular weight determination

To further characterise hemicellulosic polysaccharides, B1OH and B4OH were analysed by viscometry and size exclusion chromatography (SEC).

The intrinsic viscosity [η] of B1OH and B4OH fractions was found to be 285 and 262 ml/g, respectively. These values are higher than the values obtained from apple pomace XG (244 ml/g), but much lower than tamarind seed XG (Watt, Brasch, Larsen, & Melton, 1999).

The molecular weight distribution was determined by gel permeation chromatography. The elution profiles of B1OH and B4OH fractions obtained using a Sephadex G-200 column are shown in Fig. 2. As indicated by the Kav. values at the front and tail ends of the chromatograms, the polymers present in both fractions elute within the fractionation range of the column. B1OH fraction is highly polydisperse, with one wide peak between Kav. 0.2 and 0.5, whereas the B4OH fraction shows a peak between 0.3 and 0.6 Kav. values. Based on calibration with standard dextrans, the apparent molecular weight of the polymers present in B1OH and B4OH fraction is 130±40 kDa. B1OH fraction contains large population of polymers having molecular weights higher than that of the average value whereas the distribution of B4OH tails to lower values.

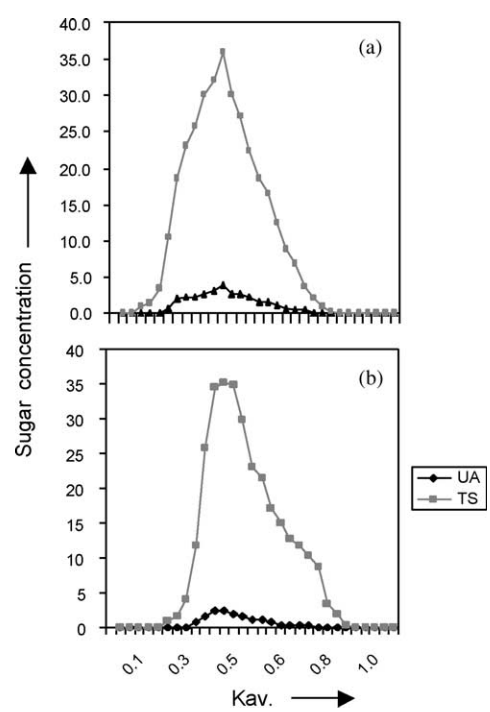

Fig. 2. Elution profile of (a) B1OH, (b) B4OH on a Sephadex G-200 column eluted with 100 mM sodium acetate buffer (pH 4.0) at 30 ml/h (see text for the identification of fractions).

The FT-IR spectra of B1OH and B4OH fractions show presence of bands typical for those of hemicelluloses and indicate the presence of β-linked sugar residues (data not shown).

3.3. Anion exchange chromatography

Attempts had been made to isolate neutral xyloglucan by anion exchange chromatography using DEAE-cellulose column. Two non-retained fractions designated as B1OH-N and B4OH-N were obtained from B1OH and B4OH, respectively. Sugar compositional analysis of these two non-retained fractions shows that they contain high amount of xylose, glucose together with galactose and fucose demonstrating the presence of fucosylated xyloglucan. The B4OH-N fraction, but not the B1OH-N, has a quite high content of mannose (17%) probably reflecting the presence of mannan polymers. In contrast, both fractions are poor in pectins as judged by the very small amounts of galacturonic acid and rhamnose detected.

3.4. Hydrolysis with endoglycosidase

Sugar composition of B1OH, B4OH as well as B1OH-N and B4OH-N fractions indicates that the hemicellulosic fractions contain xyloglucan and xylan polysaccharides. But composition analysis by simple acid hydrolysis may yield ambiguous information (Fry, 1989). Therefore, we further investigated the structure of these hemicellulosic polymers by hydrolysing them with specific endoglycosidase and analysing the resulting fragments by GC, GC-MS, HPAE-PAD chromatography and MALDI-TOF mass spectrometry.

3.5. Structural analysis of the xyloglucan

3.5.1. Hydrolysis with endo-(1→4)-β-D-glucanase

It is known that endo-(1→4)-β-D-glucanase cleaves (1→4)-β-glucosidic linkages of xyloglucan next to an unbranched glucose residue (Fry, 1989) without damaging side chains. The xyloglucan rich fractions were treated with an endo-(1→4)-β-glucanase and oligomers generated from B1OH-N and B4OH-N fractions (Fig. 1) has been designated as B1-XGose and B4-XGose fractions, respectively. To check for the presence of acetyl groups on xyloglucan of *Benincasa*, the alcohol insoluble residue of this fruit (BAIR) was also treated with endo-(1→4)-β-D-glucanase and the oligomers generated designated as BAIR-XGose. Enzyme-resistant polymeric materials B1-GRF, B4-GRF and BAIR-GRF from B1OH-N, B4OH-N and BAIR, respectively, were then removed from the corresponding digests by precipitation with 80% ethanol followed by lyophilisation of the isolated pellet.

3.5.2. Sugar composition of B1-XGose, B4-XGose, BAIR-XGose fractions

As shown in Table 2, xylose and glucose in varying ratios are the major neutral sugars of the three xyloglucan-derived oligomeric fractions (B1-XGose, B4-XGose and BAIR-XGose). Galactose is the other neutral sugars found in those fractions. The sugar composition is thus consistent with the presence of galactoxyloglucan. In addition, the occurrence of fucose indicates that the xyloglucan from *B. hispida* is fucosylated, as is xyloglucan from other dicot plant sources (Fry, 1989; Sims, Munro, Currie, Craik, & Bacic, 1996; York, Kolli, Orlando, Albersheim, & Darvill, 1996). It should be noted that the ratio of xylose to glucose for B1-XGose fraction is 1.3. This reflects that xylan present in B1OH were partially hydrolysed by the commercial preparation of endo-(1→4)-β-D-glucanase, which may contain contaminating xylanase activity. Similarly, the high content of mannose in BAIR-XGose and B4-XGose indicates that mannanase activity may also be present and responsible for mannan hydrolysis. The sugar composition of the glucanase-resistant fractions (B1-GRF and B4-GRF) indicated that they contain pectic galactans. These fractions also contain xylose and glucose, indicating that a part of the xyloglucan is resistant to endo-(1→4)-β-D-glucanase.

Table 2
Sugar composition of polysaccharide fractions and oligosaccharides generated from hemicellulosic polysaccharides of *Benincasa hispida* by digestion with endo-(1→4)-β-glucanase and endo-(1→4)-β-xylanase (see text for identification of fractions)

	Ara	Rha	Fuc	Xyl	GlcA	GalA	Man	Gal	Glc
B1OH-N	2	tr	2	30	1	4	1	37	19
B4OH-N	3	tr	2	28	tr	tr	17	14	39
B1-XGose	0.2	0.1	3.7	42.6	1.7	0.3	2.3	15.3	34.0
B4-XGose	0.2	nd	3.3	25.1	nd	nd	20.6	9.0	41.8
BAIR-XGose	0.9	0.4	7.8	19.8	1.2	1.9	18.0	11.1	39.0
B1G-RF	3.5	tr	nd	13.8	0	5.5	4.8	57.2	15.1
B4G-RF	1.6	tr	nd	32.2	6.1	5.6	1.7	33.4	19.4
B1-Xose	tr	tr	tr	84.5	2.2	4.6	1.7	3.5	2.9
BAIR-Xose	1	tr	tr	70.8	7.9	9.3	tr	9.7	2.2
B1X-RF	tr	nd	tr	12.3	0	12.6	6.8	29.9	38.5

Figures indicate the respective molar composition. tr, trace; nd, not detected.

Table 3
Methylation analysis of B4XGose fraction generated from *Benincasa hispida* fruits (see text for the identification of fraction)

Methylation product	Peak area[a]
2,3,5-Ara[b]	1
2,3,4-Xyl	21
2,3-Xyl	12
3,4-Xyl	6
2,3,4-Fuc	5
2,3,4,6-Man	6
2,3,6-Man	12
2,3,4,6-Gal	7
3,4,6-Gal	4
2,3,4-Glc	3
2,3,6-Glc	10
2,3-Glc	13

[a] Percentage of total area of the identified peaks.
[b] 2,3,5-Ara denotes 1, 4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc.

3.5.3. Glycosyl linkage composition of XG-derived oligomers

The methylation analysis data of the xyloglucan-derived oligosaccharides are shown in Table 3. The presence of (1→4)- and (1→4,6)-linked glucopyranosyl residues, typical for the cellulosic backbone of xyloglucan is revealed. Terminal xylose, fucose and galactose (all) and (1→2)-linked xylose and (1→2)-linked galactose are also present. Together, these results demonstrate the presence of xyloglucan thus confirming sugar composition data. In addition, (1→4)-linked xylose residues, terminal mannose and (1→4)-mannose are present. These data confirms the results of sugar composition analysis where degradation of xylan and mannan by commercial endo-(1→4)-β-D-glucanase preparation has been inferred.

3.5.4. MALDI-TOF-mass spectrometric analysis of XG

MALDI-TOF-mass spectrometry is a convenient tool for the structural analysis of highly branched xyloglucan oligosaccharides, because of its sensitivity and applicability to the analysis of mixtures. We applied this technique to the analysis of the generated oligomers, B1-XGose, B4-XGose and BAIR-XGose. As seen in Fig. 3a, the mass spectrum of B4-XGose fraction indicates the presence of four oligosaccharides having $[M+Na]^+$ at 1085, 1247, 1393 and 1555 of higher abundance together with an oligosaccharide having $[M+Na]^+$ at 1410 of medium abundance and an oligosaccharide having pseudomolecular mass at 1697 of smaller abundance.

Taking into consideration, the specificity and mode of action of the endo-(1→4)-β-D-glucanase, sugar composition and glycosyl linkage data, and molecular masses of the known xyloglucan oligosaccharides, tentative structures for the xyloglucan-derived oligomers are proposed (Fig. 3a). For example, *m/z* value of 1087 corresponds to Hex_4Pent_3 and it is, therefore, assigned as XXXG. Similarly, 1247 as XXLG and/or XLXG, 1393 as XXFG and 1555 as XLFG.

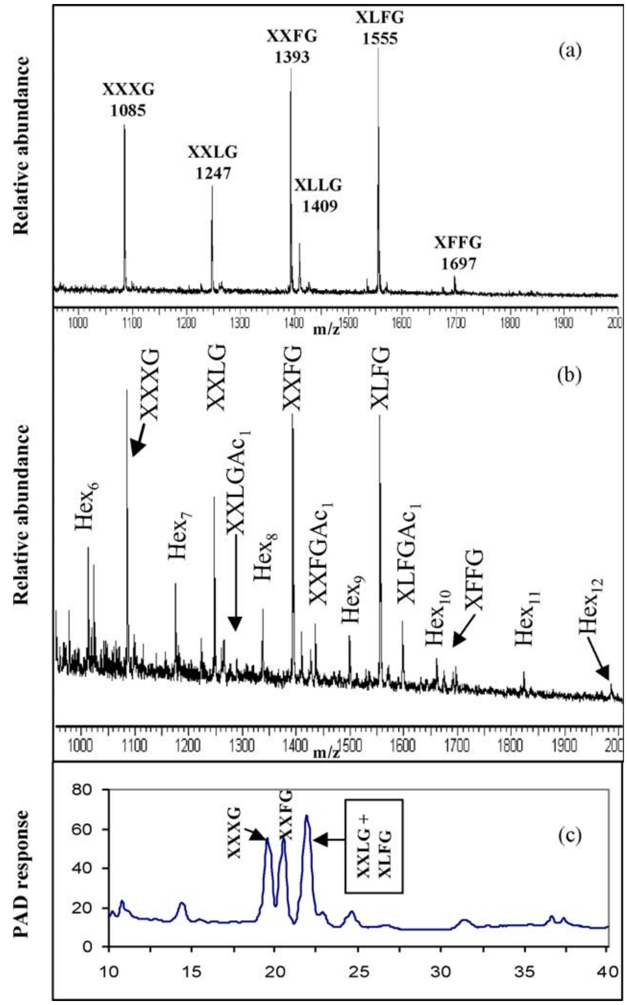

Fig. 3. MALDI-TOF-mass spectra of (a) B4-XGose and (b) BAIR-XGose and (c) HPAE-PAD chromatographic elution profile of B4-XGose fractions generated from *Benincasa hispida* fruit xyloglucan by endo-glucanase degradation (see text for the identification of fractions).

The mass spectrum for the B1-XGose was similar to that of the B4-XGose fraction and therefore, not shown. MALDI mass analysis of the xyloglucan oligosaccharides (BAIR-XGose) generated from BAIR reveals similar structures of XG and indicates that *O*-acetyl substituents are present on XXLG, XXFG and XLFG-type building subunits (Fig. 3b). Interestingly, all oligomers that have *O*-acetyl group also contain galactose residues. Such an occurrence of *O*-acetyl group specifically on galactose unit of xyloglucan oligosaccharide has been previously reported (Fry, 1989; Sims et al., 1996). Besides, a series of ions having a mass difference of 162 Da has also been observed in the mass spectrum with a DP ranging from four to as much as 16. As described above, the sugar analysis of BAIR-XGose fraction shows the presence of considerable amount of mannose residues. Therefore, these pseudomolecular ions, which lack pentosyl units, probably originated from mannan derived oligomers.

3.5.5. HPAE-PAD chromatography of XG oligomers

To further confirm the structure of the xyloglucan-derived oligosaccharides obtained by MALDI spectrometry, these were analysed by HPAE-PAD chromatography. The elution profiles show that the oligosaccharides present in B4-XGose are resolved into three major peaks (Fig. 3c). The retention times of these peaks are similar to those of XXXG, XXFG and XLFG + XXLG, respectively, generated from *Arabidopsis thaliana* (Lerouxel et al., 2002). Qualitatively, the elution profile of B1-XGose fraction is similar to that of B4-XGose fraction, but quantitatively different. The amount of fucosylated building units in the later fraction is higher than that of the former fraction. In contrast, the elution profile of BAIR-XGose fraction shows significant increase in the area of the XXGG subunit compared to those of B1-XGose and B4-XGose. In addition, BAIR-XGose fraction shows a series of peaks of lower abundance at regular intervals probably originating from the mannan oligomers. Based on the HPAE-PAD chromatographic data obtained from B4-XGose and assuming that PAD response of the xyloglucan oligosaccharides are identical, we conclude that XXXG, XXFG and XXLG + XLFG represent 85% of the building units of *B. hispida xyloglucan* in a ratio of 31:28:41.

The branching pattern of the xyloglucan in B1OH and B4OH appears to be similar. SEC data indicates that although the average molecular weight of the 1- and 4-M KOH extracted hemicellulose are the same, the former contains large amount of polymers having molecular weight higher than the average value whereas the distribution for 4-M KOH fraction tails at lower molecular weight. This variation of the molecular weight distribution is, probably, associated with the increase in the hydrodynamic volume of the polymers and hence their viscosity.

3.6. Characterisation of xylan structure

3.6.1. Digestion with endo-(1→4)-β-xylanase and sugar composition

To gain information on the structure of xylan present, selected fractions (B1OH and BAIR) were treated with endo-(1→4)-β-D-xylanase, an enzyme specific for β-D-xylan. Xylan derived oligomers and xylanase-resistant fraction were obtained (Fig. 1) and sugar composition determined (Table 2). The B1-Xose fraction consists mainly of xylose residues (85.5 mol%) together with smaller amount of arabinose, glucose and galactose residues (Table 2) and trace amounts of GlcA and 4-*O*-methyl glucuronic acid (4-*O*-MeGlcA) residues.

3.6.2. MALDI-TOF-MS analysis of XG

MALDI-TOF-mass spectrum of B1-Xose fraction shows one major peak at *m/z* 758.5 which corresponds to a 4-*O*-MeGlcA linked to four xylose residues (Fig. 4a). Peaks at *m/z* 782 and 775 correspond to disodiated and potasiated ions, respectively, of the same oligomer. BAIR-Xose, on the other

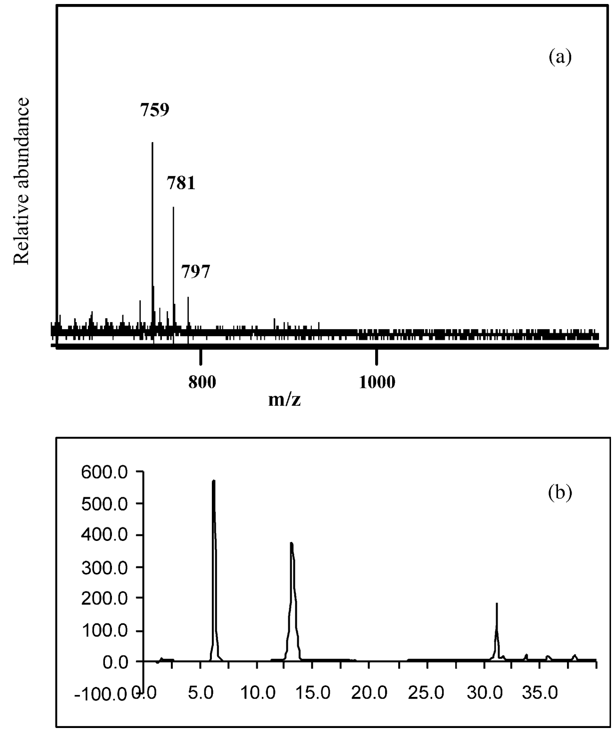

Fig. 4. (a) MALDI-TOF mass spectrum and (b) HPAEC-PAD profile of the xylan derived oligosaccharides (B1-Xose).

hand, gives only one peak at $[M+Na]^+$ at 1022 corresponding to oligomers containing one 4-*O*-MeGlcA and six pentose residues. Although, mass spectrometry cannot distinguish between isomers, but it should be remembered that sugar analysis of B1-Xose reveal the presence of xylose as major pentose unit. The position of the 4-*O*-Me-D-GlcA unit is not known. It has been reported earlier that endo-(1→4)-β-xylanase is not able to remove at least two unsubstituted xylose residues adjacent to a substituted xylose unit towards the reducing end (Kormelink, Gruppen, Vietor, & Voragen, 1993). Although B1-Xose and BAIR-Xose contains very high amount of xylose, [M + Na] + ions of free xylose, and xylobiose, the probable end product of the reaction between endo-xylanase and xylan, were not observed. They were buried under the strong matrix peaks and, therefore, were not characterised.

3.6.3. HPAE-PAD chromatography

As shown on Fig. 4b, the HPAE-PAD chromatography elution profile of B1-Xose indicates the presence of three major peaks. The retention times of the first two peaks are identical with that of xylose and xylobiose. The last peak could originate from the oligomer containing one 4-*O*-MeGlcA linked to four xylose residues. Assuming the response factor for all the oligomers are the same and taking into account only the three major peaks it can be concluded that the B1-Xose contains monomeric xylose (48%), xylobiose (37%) and acidic oligosaccharides (15%) mainly composed of pentose and uronic acid.

4. Conclusions

To summarise, the study presents, for the first time, the characterisation of hemicellulosic polysaccharides from *B. hispida* fruit. We show that (1) the sequential use of 1 and 4 M KOH extracted most of the hemicellulose present in the fruit cell walls, (2) both of the extracted fractions have a sugar composition consistent with the presence of xyloglucan and xylan, (3) that xyloglucan is of XXXG type, and contain XXXG, XXFG, XXLG and XFLG as major oligomeric building sub-units and (4) xylan has a classical structure with a backbone of β-(1 → 4)-linked xylosyl residues substituted with 4-*O*-methyl glucuronic acid. In addition, we show that the intrinsic viscosity of hemicellulosic polysaccharides of *Benincasa* fruit is higher than apple pomace xyloglucan. This study also demonstrates that molecular weight is the major factor influencing the viscosity of two structurally related xyloglucans. Moreover, the enzyme derived oligosaccharides are now available and will be used to characterise their potential biological activity.

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