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An Arabinogalactan from the Seeds of *Limonia acidissima*: Isolation, Purification and Structural Features

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Polysaccharides were extracted from the seeds of *Limonia acidissima* with water. The chemical compositions of the soluble extracts and insoluble materials were determined. Presence of pectic substances, polymers containing xylose and glucose and xylans were indicated. The material isolated by water was fractionated by anion exchange and gel permeation chromatography and the structure of the purified pectic polysaccharides was investigated using acid hydrolysis, methylation analysis, periodic oxidation and spectroscopic experiments. Type II arabinogalactan was the major polysaccharide in the water extract.

INTRODUCTION

Plant polysaccharides are important biopolymers. Various biological activities like antitumoral, antiviral, anticoagulant, etc. have been attributed to polysaccharides[1–4]. Moreover, polysaccharides have immensely large and wide applications in industries. To meet the increasing and varied demands of industries polysaccharides of natural origin are continuously being examined. *Limonia acidissima* W. and A syn *L. crenulata* Roxb. (Fam. Rutaceae), is a tree with green foliage. It grows abundantly in different parts of India. The pharmacological activities of the leaves, fruits and roots of this plant in the treatment of different tropical diseases are well-documented[5–7]. Root-bark of this plant contains coumarins[8, 9] and antifungal compounds[10], whereas its stem-bark contains alkaloids[11, 12] and has insecticidal property[13]. A new tyramine derivative[14] has been obtained from the fruits of *Limonia acidissima*. The leaves of this plant afforded coumarins, triterpenoids and steroids[15]. But less is known about the polysaccharides present in its seed. In order to develop the uses of this biopolymer better knowledge of its chemistry is necessary. The aim of the present research was isolation of different polysaccharides present in the seed, their fractionation and chemical characterization. This work is in continuation to our studies on polysaccharides of *Limonia acidissima* plant[16].

EXPERIMENTAL

General and analytical methods

Chemicals used were analytical grade or best available. All determinations were done at least in duplicate. Polysaccharide samples were dried at 40°C in vacuum over phosphorus pentoxide prior to analysis. GLC[17] of the derived alditol acetates[18] was performed on a glass column packed with 3% SP-2340 on Supelcoport 100–120 meshes. The injector and detector temperatures were 220 and 250°C, respectively, and the oven temperature was 195°C isothermal.

Alternatively, a SGE BP 225 column (25 m × 0.32 mm × 0.25 μm) operating isothermally at 195°C was also used. The temperature program for analyzing partially methylated alditol acetates was 165°C for 15 min, 165–205°C at 5°C/min and 205°C for 10 min.

Protein of SWE fraction was estimated by the methods of Lowry *et al.*[19] Bovine serum albumin was used as standard. Neutral sugars were determined by the phenol-sulfuric acid assay[20] using galactose as standard. Total uronic acids were assayed colorimetrically by the *m*-hydroxydiphenyl assay according to procedure outlined by Ahmed and Labavitch[21] using galacturonic acid as standard. Polysaccharide fractions were hydrolyzed either with 1 M sulfuric acid (3 h at 100°C) for pectic substances or by treatment with 72% (w/w) H_2SO_4 (1 h at 30°C), followed by 1 M—H_2SO_4 (3 h at 100°C) for water insoluble residues. *Myo*-Inositol was used as internal standard. Acetic acid and methanol contents were estimated by HPLC according to Voragen *et al.*[22] The DA and DM were calculated as the molar ratios of acetic acid and methanol to uronic acid.

NMR Spectroscopy: The sample was dissolved in D_2O and ^1H NMR spectrum was acquired on JEOL GSX-400 spectrometer operated at 400 MHz at 60°C.

IR Spectroscopy: Infrared spectra were recorded on a JASCO FTIR 420 spectrophotometer using a KBr disc. Samples were dried at 35–44°C in vacuum over phosphorus pentoxide for 72 h prior to making pellet.

Thin Layer Chromatography: The hydrolysates containing monosaccharides were neutralized with $BaCO_3$, decationised with amberlite IR-120 (H^+ form) and concentrated to a small volume. These concentrated solutions were then applicated on a silica gel G TLC plate impregnated with 0.5 M NaH_2PO_4. The solvents used were ethanol-phenol-pyridine 0.1 M phosphoric acid (10 : 2 : 2 : 4) and/or 2-propanol-methanol-water (16 : 1 : 3) and detections were carried out with saturated aniline phthalate (100°C/30 min). Galacturonic acid and glucuronic acid were used as standard.

Material

The seeds used in this study were obtained from the fruit of *Limonia acidissima* as described elsewhere[16]. The air-dried seed (S) was then ground using a warring blender into powder (LaS).

Extraction of LaS with water: The seed powder (500 mg) was extracted in a thermostated reactor at 35°C with 100 mL of water. The dispersion was stirred continuously using a mechanical stirrer during extraction for 1.5 h period. Next, the dispersion was centrifuged (15 min at 16,000 g) and the pellet re-extracted with 100 mL water. The combined supernatant was dialyzed exhaustively first against running tap water and then deionized water. The retentate after concentration (*ca.* 30 mL) in a rotary evaporator was diluted with 120 mL dehydrated ethanol. The precipitate was recovered by centrifugation (15 min, 16,000 g), washed thoroughly with 70% ethanol, dehydrated ethanol, acetone and diethyl ether and finally dried over silica gel in vacuum (SWE, yield 15 mg). The water unextracted residue was also dried by solvent exchange as described above (WUR; yield 422 mg).

Gel permeation chromatography: 15 mg portion of polysaccharide (SWE)

fraction was dissolved in 0.5 M sodium acetate buffer (pH 4.0), and applied to a Sephadex G-200 column (31 cm × 1.3 cm) equilibrated with the same buffer. The column was eluted ascendingly with the same buffer and the rate of elution was 20 mL/h. Fractions (2 mL) were collected and analyzed for the uronic acid and neutral sugar contents with corrections for mutual interferences. The void volume (V_0) and total volume (V_t) were determined as the elution volumes of amylopectin and glucose respectively. Results are expressed as a function of $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the fraction. Appropriate fractions were pooled, extensively dialyzed against water and the concentrated retentate lyophilized (F).

Carboxyl reduction: Uronic acids in polymer (F) dissolved in 8-M aqueous urea (10 mg/mL) were reduced twice as by the method of Conrad and Taylor[23] as described elsewhere[24] to obtain the carboxyl reduced material (FR).

Methylation analysis: Methylation analysis of the pectic polysaccharide (F) and its carboxyl-reduced derivative (FR) were carried out by the methods of Blakeney *et al.*[25] as described elsewhere[26].

Periodate oxidation and Smith degradation: The method of periodate oxidation and Smith degradation of water-soluble fraction is similar to that described previously[16]. The polysaccharide (480 mg) was dispersed in 250 mL solution of sodium periodate (prepared by dissolving 2.6 g of $NaIO_4$ in 0.25 M formic acid, pH adjusted to 3.7 with 1 M NaOH). Oxidation was allowed to proceed at 4–6°C in the dark with occasional shaking. After 144 h the excess of periodate was removed with 1,2-ethane diol, and the solution was dialyzed and reduced with 0.5 M $NaBH_4$ in 1 M NaOH at 4–6°C overnight. After reduction, the solution was made neutral with *ca.* 70 mL acetic acid and evaporated to dryness. The last traces of boric acid were removed fromthe residue by repeated addition and evaporation of methanol. The residue was hydrolyzed with 0.01 M TFA (pH 2) at 100°C for 10 min. After removing the excess acid with water under vacuum the material once again submitted to periodate acid oxidation as described. But the remaining material was isolated after desalting on a Sephadex G-10 column (38 cm × 2.2 cm). The recovered Smith degraded material (SD) was methylated and the partially methylated alditol acetates derived therefrom were analyzed by GLC.

RESULTS AND DISCUSSION

The yields of *Limonia acidissima* seed (LaS) extractions are given in Table-1. All values are calculated on the basis of defatted starting material and represent the mean of duplicate experiments. Table-1 shows the neutral sugar composition and uronic acid content of the different fractions isolated. The water-soluble fraction (SWE) obtained at an ethanol concentration of 70% (v/v) contained 30.1% neutral sugar and 4.7% uronic acid. Sugar analysis revealed that arabinose, galactose and uronic acid were the main sugars together with smaller quantities of xylose, glucose, mannose and traces of rhamnose residues. The presence of galacturonic acid, confirmed by TLC analysis of the acid hydrolysate of the native polymer (SWE) and GC analysis of alditol acetates derived from carboxyl reduced derivatives of the native polymer, suggests the presence of pectic polysaccharides. In contrast to the water extracted pectin (PWEL) isolated from the fruit of the same

plant where arabinose was the major neutral sugar, galactose was found at a higher concentration than arabinose in the SWE fraction, where it probably originated from the galactose-rich side chain. The degree of acetylation (DA) of water extracted material was 4.3%. The positions of acetyl groups are not known although in a number of cases it is attached to the uronic acid residues. It should be pointed out that other sites of attachment are known to exist. The data in Table-1 also show that the uronic acid in water-soluble fraction, *i.e.*, SWE, in contrast with the highly methylated pectin commonly found in fruits and vegetable tissues, was poorly methyl esterified. The degree of methylation (DM) of the SWE fraction was 9.1%. Water extracted 3.3% of the material present in LaS. This fraction solubilises only 2.8% of the uronic acid present in LaS. The water unextractable residue (WUR) represents more than 60% (w/w) of the defatted seed powder and contains *ca.* 20.5% sugar. Results of acid hydrolysis (with and/or without prehydrolysis with 72% H_2SO_4) of the two water unextractable residues shows that xylose was the predominant sugar component in all the residues comprising 64.7–71.4% of the total sugars present. These results indicated that the water unextractable residues of *Limonia acidissima* seed are mainly composed of xylan. Xylans can potentially be used as a raw material for xylose production. Besides it could be a potential source for the production of xylitol, a sugar substitute of similar calorific values and sweetness as saccharose. The sugar contents determined with or without Seaman hydrolysis were very different for glucose and xylose, but similar for other neutral sugars. This result suggests the probable presence of xyloglucan or xylan and glucan strongly bound to the xylan. Some galacturonic acid containing polymers were also tightly associated to the xylan.

TABLE-1
YIELDS AND CHEMICAL COMPOSITION OF THE DEFATTED POWDER, THE WATER EXTRACT AND WATER UNEXTRACTABLE RESIDUE OF *Limonia acidissima* SEED

	LaS	SWE	OXUR
Yield	100	3	60.2
NS[a]	21.9	30.1	15.8
UA[a]	3.3	4.7	4.75
Rha[b]	1.3 (0.9)[c]	0.9	Tr (1.1)
Fuc[b]	(0.1)	–	–
Ara[b]	8.7 (4.3)	31.9	6.5 (6.1)
Xyl[b]	71.4 (41)	6.5	64.6 (29.6)
Man[b]	Tr	1.8	Tr
Gal[b]	5.1 (2.8)	35.2	4.3 (Tr)
Glc[b]	1.1 (44.7)	4.2	1.4 (34.2)
UA[b]	12.95 (6.3)	20.4	19.2 (30)

[a]Percentage weights of fraction dry weight; [b]Percentage mol; [c]Values in parentheses are results obtained from hydrolysis with 1 M sulfuric acid whereas other values are results obtained from experiments with prehydrolysis. Tr: trace; –: not detected.

TABLE-2
PARTIALLY METHYLATED ALDITOL ACETATES (MOL %) FROM THE F FRACTION, AND ITS SMITH DEGRADED PRODUCT (SD)
[See Experimental for the Identification of Fractions]

	F	SD
2,3,5-Me_3-Ara	20.5	
2,3-Me_2-Ara	14.1	
2-Me-Ara	3.5	
Total	38.1	
2,3-Me_2-Xyl	6.9	
3,4-Me_2-Rha	0.9	
3-Me-Rha	3.2	
Total	4.1	
2,3,4,6-Me_4-Gal	4.1	4
2,4,6-Me_3-Gal	5.7	19.3
2,3,6-Me_3-Gal	4.6	
2,3,4-Me_3-Gal	6.8	3.2
3,6-Me_2-Gal	3.9	
2,4-Me_2-Gal	23.1	5.6
Total	48.2	32.1
2,3,6-Me_3-Glc	1.5	
2,3,6-Me_3-Man	1.4	

[a]2,3,5-Me_3-Ara = 2,3,5-tri-O-methyl-1,4-di-O-acetyl arabinitol etc.

The water-soluble material was fractionated on Sephadex G-200 column in order to assess its purity. Only one fraction having K_{av} between 0 and 0.4 was obtained indicating polydispersity. The varying ratio of uronic acid to neutral sugars found in different fractions indicates the presence of heterogeneity in pectic polymers isolated (F).

The F fraction was de-esterified with cold dilute alkali, and methylated. The native (F) and reduced (FR) fractions were analyzed by GC and GC/MS after conversion into the partially methylated alditol acetates[27, 28]. The methylation analysis of F has revealed some interesting results. The contents of individual monosaccharide constituents (as estimated from GC analysis of derived alditol acetates) are in good agreement with the sugar composition calculated from GC analysis of methylated alditol acetates. The presence of 2,3,5-tri-O-methyl-1,4-di-O-acetyl arabinitol and 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl galactitol indi-

cates that arabinose and galactose are the terminal sugar residues. The rhamnose residues are 1,2-linked with branching through O-4 and about 77% of the total rhamnose units are branched. Appearance of 2,3-di-O-methyl-1,4,5,6-tetra-O-acetyl galacititol, 2,4-di-O-methyl-1,3,5,6-tetra-O-acetyl galactitol and 2-mono-O-methyl-1,3,4,5-tetra-O-acetyl arabinitol suggests that the hairy region of this pectic polymer be extensively branched. The presence of 1,3,6-linked galactose and terminal arabinose residues indicates the presence of arabinogalactan of type II. A major part of the side chain also contains 1,4-linked galactose residues. All the glucose, mannose and xylose residues are 1,4-linked. The presence of increased amount of 2,3,6-tri-O-methyl-1,4,5-tri-O-acetyl galactitol as obtained from the permethylated FR shows that the galacturonic acid residues are 1,4-linked (data not shown).

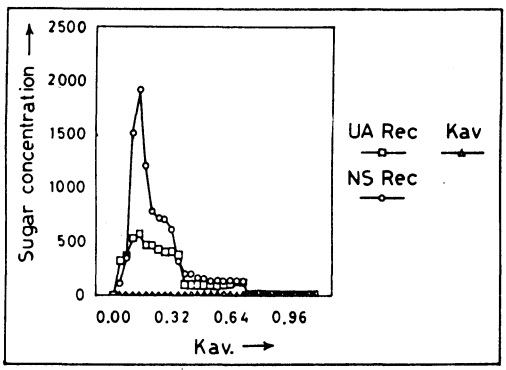

Fig. 1. Size-exclusion chromatography of subfraction F. Elution profile of neutral sugars and uronic acids.

Periodate oxidation studies corroborated the results of methylation analysis. No arabinose was therefore detected after Smith degradation. The 1,5 and terminally linked arabinose units were oxidized by periodic acid. The 1,2-linked arabinose residues were perhaps hydrolyzed during oxidation and lost during dialysis. Methylation analysis data revealed that the arabinose residues are present in the furanose form and are, therefore, susceptible to hydrolysis under mild condition. The proportions of 1,3-linked galactose residues greatly increased after periodate oxidation. This result can be explained if polymer contains 1,3-linked galactan chain with 1,6-linked galactan side chain attached to position 6. But the presence of 1,6-linked galactan chain with 1,3-linked galactan side chain attached via C3 cannot be ruled out.

The ^{1}H NMR spectrum of native polysaccharide at 60°C is very complex (Fig. 2). A partial assignment of resonances by comparison with the spectra of related compounds[29, 30] were possible. The dominating ^{1}H NMR signals that appear in the typical carbohydrate spectral region (3.5 to 5.5 ppm) is from the terminal arabinose residues. The presence of two signals at 5.2 and 5.4 indicates the presence of two α-linked sugar residues in the polymer. Methylation analysis and

Smith degradation studies indicate the presence of terminal and 1,5-linked Ara*f* residues. Therefore, these two signals were tentatively assigned to the α-Ara*f* residues. Based on the values published for similar polysaccharides[29, 30] the signal at 5.4 was assigned to the H-1 of terminal α-Ara*f* residues and the other, therefore, from the α-(1,5)-linked Ara*f* residues. Signal, occurring in the 3.5 to 5.5-ppm region, which are characteristic of carbohydrate, are complex. However, based on the values of coupling constants some assignments could be made. The signals centred at 3.74 is from H-2 of β D-galactose ($J_{2,3} = 9.9$ Hz and $J_{1,2} = 8.1$ Hz). Similarly the sharp singlets at about 3.57 ppm, are from methyl ester groups of the galacturonic acid residues. By comparison with the spectra of similar compounds the signals at about 4.32, 4.22 and 4.02 were assigned to the H-2, H-4 and H-3 of terminal α-Ara*f* residues. Two singlets originating from the protons of acetyl groups of the polysaccharide could be observed at δ 1.97 and 2.26.

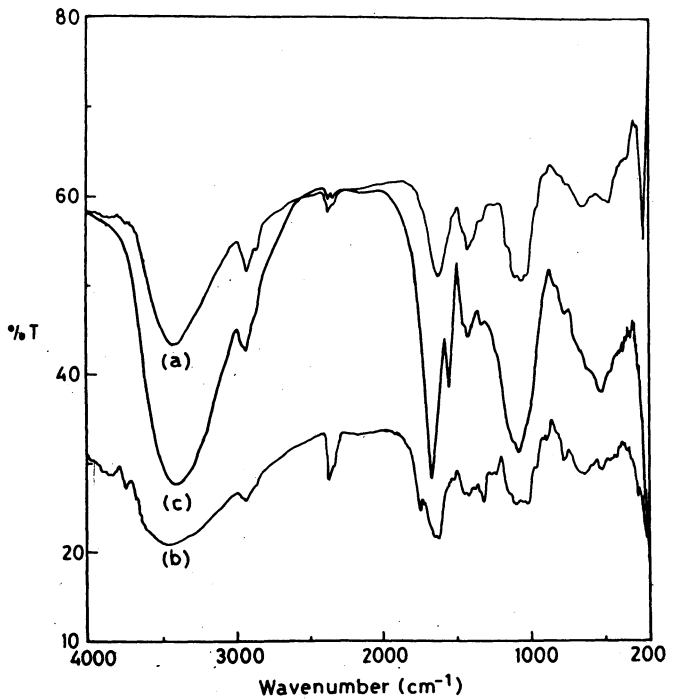

Fig. 2. FT-IR spectra of the defatted seed powder of *Limonia acidissima* (a), the water extracted pectic polysaccharides (b) and the water unextracted residues (c).

The FTIR spectra of the defatted powder, the water extracts and water unextractable residue of *Limonia acidissima* seed are shown in Fig. 3. The spectra show high absorption at wave numbers characteristic of polysaccharides: 3440 cm^{-1} hydroxyl, 1741 cm^{-1} ester carbonyl, 1630 cm^{-1} carboxylate and 1200–850

cm^{-1} carbohydrate[31-35]. The absorption at 1741 cm^{-1} in the spectrum of the water extracts is probably due to the presence of acetyl and ester groups of galacturonic acid (C=O stretching band) residues. The pectic polymers show a band[31-33] in the region 1739 cm^{-1}. Methyl and methylene group vibrations appeared around 2922 cm^{-1} and were present in the spectra of all fractions. The broad band between 3600 and 3000 cm^{-1}, corresponding to vibrations of the hydroxylic groups appeared to be similar in all the spectra. Structural features arising from particular conformations around the glycosidic bond of the pectins are observable in the 1100–990 cm^{-1} region. For example, the bands at wave numbers 1104, 1014 and 945 which are characteristic of pectic polysaccharides[31, 32] appear in the spectrum of SWE fraction (b). The bands around 1260, 1380 and 1420 cm^{-1} represent C—H and O—H bending vibrations[32, 33]

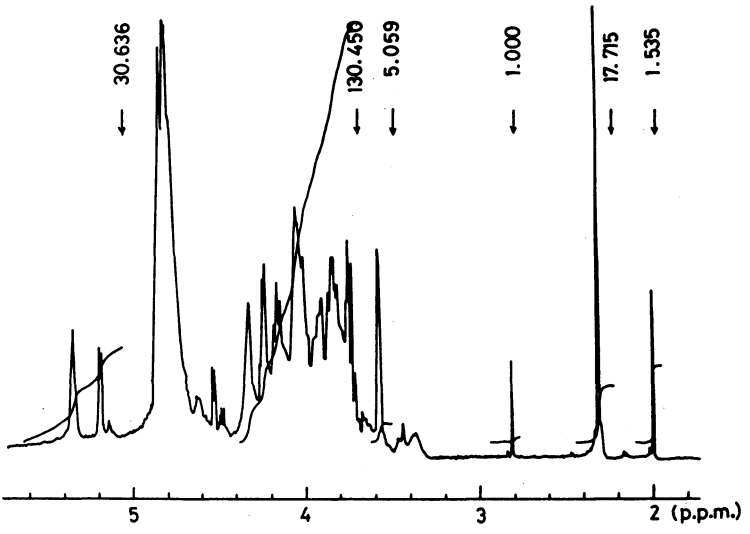

Fig. 3. ^1H NMR Spectrum of water extracted pectic polysaccharides.

Conclusion

The pectic substances, isolated from the seeds of *Limonia acidissima* consist of 30.1% netural sugar and 4.7% galacturonic acid and has a $K_{av.}$ between 0 and 0.4. The carbohydrate part consists of arabinose (31.9 mol %), galactose (35.2 mol %), xylose (6.5 mol %), glucose (4.2 mol %), mannose (1.8 mol %), rhamnose (0.9 mol%), and galacturonic acid (20.4 mol %) residues. The polymer is partly acetylated (DA = 4.3) and has a low degree of methylation (DM = 9.1). The polysaccharides consist of a network of (1,3)-linked galactan chain with (1,6)-linked galactan side chains attached through O-6. The arabinose residues are present either in the terminal positions or (1,5)-linked. The rhamnose residues are

(1,2)- or (1,2,4)-linked whereas the galacturonic acid residues are (1,4)-linked. In addition, the presence of xylan and xyloglucan were also indicated. On the basis of the foregoing discussion it may be concluded that three types of polysaccharides namely arabinogalactan type II, xyloglucan and xylan are present in *Limonia acidissima* seed. The isolation of oligosaccharides for further structural analysis of these polysaccharides from *Limonia acidissima* is in progress.

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