Appendix -I
LIST OF PUBLICATIONS
List of publications

1. Isolation, Purification and some structural features of the mucilaginous exudates from *Musa paradisiaca*.

2. Structural features of a water soluble gum polysaccharide from *Murraya paniculata* fruits.


5. Isolation and characterization of pectic polysaccharides from the fruits of *Naringi crenulata*.
Isolation, purification and some structural features of the mucilaginous exudate from *Musa paradisiaca*

Saroj K. Mondal, Bimalendu Ray*, Swapnadip Thakur, Pradyot K. Ghosal

Department of Chemistry, The University of Burdwan, Burdwan 713 104, W.B., India

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Abstract

The water-soluble polysaccharides isolated from the vascular gel of *Musa paradisiaca*, were fractionated via anion exchange chromatography into four fractions. Fractionated polymers contained arabinose, xylose and galacturonic acid as major sugars, together with traces of galactose, rhamnose, mannose and glucose residues. Methylation analysis revealed the presence of a highly branched arabinoxylan with a significant amount of terminal arabinopyranosyl units and an arabinogalactan type I pectin. Periodate oxidation studies supported the results of methylation analysis. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Musa paradisiaca*; Polysaccharides; Pectin; Acidic arabinoxylan

1. Introduction

*Musa paradisiaca* L. (Musaceae), banana, is a perennial treelike herb widely distributed in moist tropics. Due to enriched food value and versatile medicinal value, banana is one of the most important fruits and vegetable crops of India. The therapeutic effect of the fruit in the tropical treatment of diarrhoea and dysentery,

*Corresponding author. Fax: +91-342-64452.
E-mail address: konica2@dte.vsnl.net.in (B. Ray).
and in the healing of intestinal lesions in colitis is well documented in folk medicine [1]. Processes for the production of paper pulp, biogas, alcoholic beverages, etc., from banana plant have been described [2-4]. This plant produces gel in response to stresses caused by mechanical injury, and/or attack by pathogens. Much work has been done to determine the cause, and mechanism of gel formation [5-8], but less attention has been given to study the chemical structure of the water-soluble material obtained from the gel of this plant. The water-insoluble material obtained from the Fusarium induced gels from M. acuminata is rich in neutral sugars (arabinose, glucose and xylose) and uronic acid that are also found in the cell wall of the host [6]. The aim of the present research was isolation, purification and determination of the structural features of the different polysaccharides present in the mucilaginous exudate from banana plant.

2. Experimental

2.1. Plant material

Colourless and transparent gel was collected during the month of November from the pseudo stem stipules of banana plant grown locally.

2.2. Isolation and purification of the polysaccharides

The mucilaginous exudate (100 g) was extracted with water (2 l). The extract, after concentration in a rotary evaporator, was diluted with EtOH (6 l) to produce a white precipitate. This pelleted material was redissolved in water and to the solution absolute EtOH was added slowly at room temperature, until a concentration of 75% (v/v) was reached. The precipitate was recovered by centrifugation (20 min, 8000 × g), washed thoroughly with 90% EtOH, acetone and Et₂O, and finally dried over silica gel (MWE, yield: 0.8 g).

2.3. Anion exchange chromatography

Solutions (70 ml) of MWE (60 mg) in 10 mM NaOAc buffer pH 6.0 were loaded onto a diethylaminoethyl (DEAE)-Sepharose CL-6B column (1.3 cm × 35 cm) previously equilibrated with the same buffer. The column was then washed with the same buffer (50 ml) and then eluted successively with 0.1 M, 0.3 M, 0.5 M, 0.7 M and 1 M NaOAc buffer pH 6.0 (50 ml each), and finally with 0.2 M NaOH (50 ml). The collected fractions (10 ml) were analysed for neutral sugar and uronic acid content by the phenol-sulfuric acid [9] and m-hydroxydiphenyl (MHDP)-sulfuric acid assay [10], respectively. All DEAE-bound fractions were pooled and then dialysed. The concentrated retentate was diluted with three volumes of absolute ethanol at room temperature. The precipitate was recovered by centrifugation (20 min at 8000 × g); the pellet was dehydrated by solvent exchange as described above and finally dried, under vacuum, over silica gel. Fractions obtained by
elution with 0.1 M NaOAc, 0.3 M NaOAc, 0.5 M NaOAc and 0.2 M NaOH were designated as MF1, MF2, MF3 and MFn respectively. The yields of the fractions are given in Table 1. In a separate experiment MWE was chromatographed on a column of DEAE-Cellulose (OH−).

Table 1
Yield and sugar composition of fractions (MF1 – n) obtained from anion exchange chromatography of the water-soluble part of M. paradisiaca vascular gel (MWE)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MWE</th>
<th>MF1</th>
<th>MF2</th>
<th>MF3</th>
<th>MFn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield UA</td>
<td>(100)</td>
<td>1.8</td>
<td>74.6</td>
<td>6.4</td>
<td>15.7</td>
</tr>
<tr>
<td>Yield NS</td>
<td>(100)</td>
<td>15.9</td>
<td>62.6</td>
<td>8.1</td>
<td>13.4</td>
</tr>
<tr>
<td>UA</td>
<td>17</td>
<td>3.7</td>
<td>18.4</td>
<td>14.7</td>
<td>15.4</td>
</tr>
<tr>
<td>NS</td>
<td>35.5</td>
<td>59.5</td>
<td>32.9</td>
<td>38.2</td>
<td>28.1</td>
</tr>
<tr>
<td>Protein</td>
<td>7.6</td>
<td>8.3</td>
<td>5.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>4.5</td>
<td>–</td>
<td>tr</td>
<td>–</td>
<td>1.9</td>
</tr>
<tr>
<td>Arabinose</td>
<td>27.4</td>
<td>36.1</td>
<td>27.5</td>
<td>31.0</td>
<td>27.0</td>
</tr>
<tr>
<td>Xylose</td>
<td>40.9</td>
<td>59.3</td>
<td>42.3</td>
<td>46.1</td>
<td>38.2</td>
</tr>
<tr>
<td>Mannose</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Galactose</td>
<td>tr</td>
<td>–</td>
<td>tr</td>
<td>–</td>
<td>2.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>tr</td>
<td>tr</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>UA</td>
<td>27.2</td>
<td>4.6</td>
<td>30.2</td>
<td>23.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Ara/Xyl</td>
<td>0.67</td>
<td>0.61</td>
<td>0.65</td>
<td>0.67</td>
<td>0.71</td>
</tr>
</tbody>
</table>

*a* Percentage weight of total uronic acid (UA) recovered.

*b* Percentage weight of total neutral sugar (NS) recovered.

*c* Percentage weight of fraction dry weight.

*d* Percentage mol of anhydrosugars.

--, not detected; tr, trace.

2.4. Gel permeation chromatography

A solution (1 ml) of polysaccharide MF2 in 100 mM sodium acetate buffer pH 4.5, was loaded onto a Sephadex G-200 column (1.3 cm × 30 cm) equilibrated with the same buffer for gel permeation chromatography. The column was eluted ascendingly with the same buffer at 15 ml/h. Fractions (1 ml) were collected and analysed for the total sugar content [9]. The peak forming fractions were pooled and treated as above. The void volume ($V_0$) and the total volume ($V_T$) were determined as the elution volume of amylopectin and glucose, respectively. In a separate experiment, neutral sugar composition of polymeric materials recovered from the peak forming fractions, i.e. at elution volume of 8–10 and 16–18 ml, were determined separately as described below.

2.5. Analytical methods

Chemicals used were of analytical grade. All determinations were done at least in duplicate. Neutral sugars were estimated by the phenol-sulfuric acid assay [9]
using xylose as standard. The uronic acid content was determined by the MHDP assay [10], using galacturonic acid as standard. Correction was made for the interference of neutral sugars in the determination of uronic acid. For the determination of monosaccharide composition, the polysaccharides in the samples were hydrolysed using 4 N sulfuric acid (3 h at 100°C). Neutral sugar composition of the hydrolysates was determined by GLC [11] after conversion of the monosaccharide to alditol acetates. myo-Inositol was used as internal standard, and hydrolytic losses were accounted for by using an external standard. Protein content was estimated according to Lowly et al. [12]. Bovine serum albumin was used as standard. The carboxyl group in the native polymer was reduced by the method of Conrad and Taylor [13].

2.6. Methylation analysis

Methylations of polysaccharides were carried out according to a modified Hakomori method as described by Blakeney et al. [14]. After methylation the reaction mixtures were worked up by dialysis. Next, the methylated samples were hydrolysed with 2 M trifluoroacetic acid (120°C, 75 min) using myo-inositol as internal standard. The partially methylated neutral sugars were then reduced, acetylated and analysed by GLC as previously described [15]. The effective carbon response factors as given by Sweet et al. [16] were used for calculation of molar quantities from peak areas as determined by GLC.

2.7. Periodate oxidation

Periodate oxidation of polysaccharide MF1 was carried out as described by Fry [17].

3. Results and discussion

Cold water extraction of the gel obtained from banana plant afforded a fraction (MWE; 0.8% of the starting gel wet weight) containing 17% uronic acid. MWE was constituted of arabinose, xylose and uronic acid together with traces of rhamnose, galactose, mannose and glucose (Table 1). The sugar composition of this fraction was qualitatively similar to that of the water insoluble part of the gel obtained from banana root [6], but quantitatively there was a difference since the gel from the banana root contained a higher amount of glucose. The present analysis deals only with water-soluble material, which, unlike the water-insoluble part of the gel from banana root, contained no cellulosic material and, therefore, gave traces of glucose. Besides, it was reported [6] that the amount of glucose found in Fusarium-induced gel samples collected at different times was also varying. An increase in the proportion of galactose in the carboxyl reduced material suggests the presence of galacturonic acid (results not shown) in the native polymer. This fraction contained, on fraction dry weight basis, 7.6% protein.
The water-soluble material (MWE) by anion exchange chromatography on DEAE-Sepharose CL-6B (AcO-) column yielded four acidic fractions (Figs. 1 and 2). One fraction was eluted with 0.1 M NaOAc (designated as MF1), one with 0.3 M NaOAc (MF2), and another one with 0.5 M NaOAc (MF3). Some material was strongly retained on the column and could not be removed even by treatment with 1 M NaOAc buffer. One more fraction, designed as MFn, was eluted with 0.2 M NaOH solution. Incidentally, it may be mentioned that MWE also bound to the DEAE-Cellulose (OH-) column. The recovery yield from the anion exchanger was 90.5 and 88%, respectively, of the total uronic acid and neutral sugars injected. The yields and sugar compositions of the fractions are given in Table 1. The neutral sugar composition of fractions obtained from the anion exchange chromatography of MWE showed small differences. Arabinose and xylose were the main neutral monomers in all the cases. Further sugars found were uronic acid together with trace amounts of galactose, rhamnose, glucose and mannose. The uronic acid content varied between 3.7 and 18.4%. MF1, the fraction eluting first from the anion exchanger accounted for 1.8 and 15.9% of the recovered uronic acid and neutral sugar, respectively, and was mainly composed of arabinose and xylose together with smaller quantities of uronic acid. The high arabinose and xylose contents together with a small amount of uronic acid in this fraction indicated that acidic arabinoxylans were present. Further evidence for this was obtained from methylation analysis of the same fraction. The major fraction, MF2, which accounted for 62.6% and 74.6% of the total neutral sugars and uronic acids recovered from the anion exchanger, respectively, had a sugar composition close to that of the water-soluble polymeric material (MWE). Since arabinose and xylose amounted to ~70 mol% of the total sugar, this fraction may contain arabinoxylan type polymer. It also contained high amounts of uronic acid indicative of pectic polymers. The protein content of this fraction is 5.8% on a fraction dry weight basis. Fraction MF3, whose arabinose to xylose ratio is similar to that of the parent fraction, contained 14.7% uronic acid on fraction dry weight basis. In addition to these fractions, one more fraction, MFn, containing the smallest amount of neutral sugar was obtained by elution with 0.2 N NaOH. Polymers with the highest arabinose to xylose ratio were eluted at the highest sodium acetate buffer concentration. Neutral sugars and uronic acid were not distributed evenly in each polysaccharide fraction.

When chromatographed on Sephadex G-200, fraction MF2 revealed two main peaks; the first moving fraction (designated as F), containing 47.9% of the neutral sugar injected, was eluted at the exclusion volume of the column and the slow moving fraction (designated as S), consisting of 43.4% of the neutral sugar injected, was eluted at Kav. 0.4 (Fig. 2). It was found, however, that the neutral sugar compositions of both sub fractions (F and S) were very close to that of the parent fraction.

The linkage compositions of MF1 and MF2 fractions obtained by anion exchange chromatography of MWE were determined by methylation analysis. The neutral sugar composition of the permethylated polymer MF1, calculated from the partially methylated alditol acetates, was in good agreement with the proportion of the
sugars determined before methylation. For materials obtained from the other fraction MF2, the results was less quantitative, probably because of β-elimination of the polymer during methylation and loss of the degradation products during dialysis of the derivatized samples. Following methylation, acid hydrolysis, reduction and acetylation, MF1 yielded a simple pattern of partially methylated alditol acetates (Table 2). The presence of 1,4-di-O-acetyl, 2,3,5-tri-O-methyl arabinitol (32.4 mol%) and 1,4,5-tri-O-acetyl-2,3-di-O-methyl xylitol (38.2 mol%) indicates that the major sugars present in the polymer fraction analysed were terminal
arabinofuranosyl and \((1 \rightarrow 4)\)-linked xylopyranosyl residues. The presence of 1,2,3,4,5-penta-O-acetyl xylitol (8.5 mol%), 1,3/2,4,5-tetra-O-acetyl-2,3-O-methyl xylitol (16.1 mol%) and 1,2,3,4,5-penta-O-acetyl arabinitol (2.6 mol%) suggested that the polysaccharide was highly branched and contained, inter alia, \((1 \rightarrow 2,3,4)\)-linked xylosyl, \((1 \rightarrow 2/3,4)\)-linked xylosyl and \((1 \rightarrow 2,3,5)\)-linked arabinosyl units. Evidence for the presence of \((1 \rightarrow 2)\)-linked arabinofuranosyl and terminal xylopyranosyl units were obtained from the identification 1,2,4-tri-O-acetyl-3,5-di-O-methyl arabinitol (1.2 mol%) and 1,5-di-O-acetyl-2,3,4-tri-O-methyl xylitol (1 mol%), respectively; 38.6% of the xylopyranosyl residues of this arabinoxylan carried a substituent at O-3/O-2 and 13.3% at both O-3 and O-2. The ratio of unbranched to branched xylose for this polymer was 1.59. Permethylated MF2 fraction demonstrated the presence of terminal- (33 mol%), \((1 \rightarrow 5)\)- (1 mol%), and \((1 \rightarrow 2,3,5)\)- (1.2 mol%) linked arabinose; \((1 \rightarrow 4)\)- (34.1 mol%), \((1 \rightarrow 2/3,4)\)- (17.3 mol%), and \((1 \rightarrow 2,3,4)\)- (9.9 mol%) linked xylose, and \((1 \rightarrow 4)\)-linked galactose (3.5 mol%) residues (Table 2). The presence of \(1,4\)-linked xylosyl and terminal arabinosyl residues suggested the presence of arabinoxylan in the MF1 fraction; 38.6% of the xylopyranosyl residues of this arabinoxylan carried substituents at O-3/O-2 and 13.3% at both O-3 and O-2. The ratio of unbranched to branched xylose for this polymer was 1.59. Permethylated MF2 fraction demonstrated the presence of terminal- (33 mol%), \(1,5\)- (1 mol%), and \(1,2,3,5\)- (1.2 mol%) linked arabinose; \(1,4\)- (34.1 mol%), \(1,2/3,4\)- (17.3 mol%), and \(1,2,3,4\)- (9.9 mol%) linked xylose, and \(1,4\)-linked galactose (3.5 mol%) residues (Table 2). The MF2 fraction similarly contained linkages indicative of arabinoxylan type polymer. Since arabinoxylans usually do not contain \((1,4)\)-linked galactose residues (except for birch arabinoxylans), the presence of small but significant amounts of \(1,4\)-linked galactose residues and high amounts of galacturonic acid indicated that the arabinoxylan was probably contaminated with arabinogalactan type I pectin. Anion exchange chromatography showed that the arabinoxylans present in the MWE fraction possessed acidic characteristics and bound to the anion exchanger. This result was in contrast with previous studies on water-soluble arabinoxylans from wheat flour, which could not be fractionated on DEAE-cellulose column [18]. Substantial proportions of all the water unextractable arabinoxylan from wheat flour cell walls, however, bound to the anion exchanger [19]. Fractionation of arabinoxylans on DEAE-cellulose column, in some chromatographic mode, was probably due to adsorption of arabinoxylans to the column matrix rather than to ion exchange mechanisms [20]. Although the mechanism of binding of arabinoxylans to the DEAE column is not clear, it has been suggested that protein may be involved in the binding of the material to DEAE column [19]. The presence of uronic acid residues in various subfractions might explain their binding to the DEAE-Sepharose CL-6B column.

To verify the results of methylation analysis, the polysaccharide MF1 was subjected to periodate oxidation. Sequential periodate oxidation, borohydride reduction and acid hydrolysis of polysaccharide destroyed most of the arabinose residues. This verified earlier finding that these sugar residues occupied terminal positions. Alditol acetates from the periodate oxidised sample included xylitol and
Table 2
Partially methylated alditol acetates (mol%) from MF1 and MF2 fractions of the water-soluble part of *M. paradisiaca* vascular gel

<table>
<thead>
<tr>
<th>Partially methylated alditol acetates</th>
<th>MF1</th>
<th>MF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-Di-0-acetyl-2,3,5-tri-0-methylarabinitol</td>
<td>32.4</td>
<td>33.0</td>
</tr>
<tr>
<td>1,2,4-Tri-0-acetyl-3,5-di-0-methylarabinitol</td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>1,4,5-Tri-0-acetyl-2,3-di-0-methylarabinitol</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>1,2,3,4,5-Penta-0-acetyl-arabinitol</td>
<td>2.6</td>
<td>1.2</td>
</tr>
<tr>
<td>1,5-Di-0-acetyl-2,3,4-tri-0-methyloxytitol</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>1,4,5-Tri-0-acetyl-2,3-di-0-methyloxytitol</td>
<td>38.2</td>
<td>34.1</td>
</tr>
<tr>
<td>1,3,4,5-Tetra-0-acetyl-2-O-methyloxytitol and/or 1,2,4,5-Tetra-0-acetyl-3-O-methyloxytitol</td>
<td>16.1</td>
<td>17.3</td>
</tr>
<tr>
<td>1,2,3,4,5-Penta-0-acetyl-oxytitol</td>
<td>8.5</td>
<td>9.9</td>
</tr>
<tr>
<td>1,4,5-Tri-0-acetyl-2,3,6-tri-0-methylgalactitol</td>
<td>-</td>
<td>3.5</td>
</tr>
</tbody>
</table>

-, not detected.

one tetritol. The occurrence of tetritol indicated the presence of 1,4-linked units without substituents at positions O-2 and O-3. The presence of xylitol confirmed the presence of 1,3,4- and/or 1,2,3,4-linked xylopyranose residues in the polymer.

Thus, the present data indicate that the water-soluble polysaccharides obtained from the vascular gel of *Musa paradisiaca* contain two main types of polysaccharides: arabinoxylan and pectin. Several arabinoxylan fractions of similar composition but of different molecular weights were obtained from the anion exchanger. They are highly branched and acidic in nature.

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References

Structural features of a water soluble gum polysaccharide from *Murraya paniculata* fruits

Saroj K. Mondal a, Bimalendu Ray a,*, Pradyot K. Ghosal a, Anita Teleman b, Tapani Vuorinen c

a Department of Chemistry, The University of Burdwan, Burdwan 713 104, India
b VTT Chemical Technology, PO Box 1401, FIN-02044 VTT, Helsinki, Finland
c Laboratory of Forest Products Chemistry, Helsinki University of Technology, PO Box 6300, FIN-02015 HUT, Helsinki, Finland

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Abstract

A water soluble gum polysaccharide was isolated from *Murraya paniculata* fruits. Hydrolytic experiments, methylation analysis, periodate oxidation studies and NMR data revealed that the polysaccharide was extensively branched and it consisted of 1,3-, and 1,3,6-linked β-D-galactopyranosyl units, terminal β-D-galactopyranosyl units and terminal α-D-glucopyranosyl 1,4-β-D-galactopyranosyl units. Small amounts of 4-O-methylglucuronic acid residues were also present. © 2001 Published by Elsevier Science B.V.

Keywords: *Murraya paniculata*; Rutaceae; Polysaccharide; Galactan; Glucogalactan

1. Introduction

*Murraya paniculata* (Linn.) Jack syn. *Murraya exotica* L. of the family Rutaceae is an evergreen shrub available almost throughout India [1]. Various parts of the shrub have been used in folk medicine [1–4]. For example, the leaves are stimulant and astringent: they are reported to be used for diarrhea and dysentery in the Philippines [1–3]. They also possess antibiotic activity against Micrococcus pyogenes and Escherichia coli [1]. Root bark and leaves are sometimes used against rheumatism, coughs and hysteria [1–3]. Much research has been carried out on the low molecular weight substances such as alkaloids [5,6], terpenoids [7], flavonoids [8,9], etc. present in various parts of this plant. During the last two decades various biological activities like antitumoral, antiviral, anticoagulant, etc. have been attributed to polysaccharides [10–14]. Although the antifertility action of the protein polysaccharide obtained from *M. Paniculata* has been reported [15], little attention has been given to the high molecular weight compounds such as protein, polysaccharide, etc. present in various parts of that plant. One particularly interesting feature of its immature fruits is that on being squeezed they exuded a significant amount of colorless viscous gum. Since structure and functions are intimately related an in-depth study of the structure of the polysaccharides present therein would be of interest. Therefore the aim of this research was to investigate the structural features of the constituent polysaccharide of this gum.

2. Experimental

2.1. Material

Immature fruits of *Murraya* were collected during August 1991 from plants grown at the Golapbag campus of Burdwan University (West Bengal, India), and were identified as *M. Paniculata* from the morphology and collection site. Fruits of uniform size and free from blemishes were selected.
2.2. Isolation and fractionation

On being squeezed by hand the fruits (1 kg) exuded a colorless viscous material. This gummy material was dispersed in deionised water (2.5 l). Next, the dispersion was centrifuged (15 min at 8000 g) and the supernatant obtained therefrom was dialyzed. The retentate after concentration in a rotary evaporator was diluted with 3 vol. of dehydrated ethanol. The precipitate was recovered by centrifugation (15 min, 8000 x g), washed thoroughly with 90% ethanol, acetone and diethyl ether and finally dried over silica gel in vacuo (MeWE, yield 3.1 g).

To an aqueous solution (100 ml) of MeWE (1 g) was added, in drop-wise manner, 15 ml of 7% (w/v) copper (II) acetate solution with continuous stirring. As no precipitate was formed ethanol (55 ml) was added slowly onto the solution. The precipitate (F1) formed was pelleted by centrifugation (20 min at 8000 g). To the supernatant 15 ml of 7% (w/v) copper (II) acetate was added once more but as no precipitates appeared, the solution was once again diluted with 45 ml of dehydrated ethanol whereby a precipitate (F2) formed which was collected by centrifugation. Dilution of the above supernatant with more ethanol (25 ml) afforded one more fraction (F3). The precipitate (F1) was decomposed by 5% ethanolic HCl on an ice bath. Next, the mixture was centrifuged and the centrifugate obtained was diluted with 3 vols. ethanol. After centrifugation, the collected precipitate was washed thoroughly with ethanol, dissolved in water and then dialyzed. Next, the concentrated retentate was diluted with 3 vols. of ethanol. The pellet collected by centrifugation, was dried by solvent exchange as described above (MeWE F1; yield 300 mg). The other two precipitates (F2 and F3) were treated in the same way and the materials thereby recovered were designated respectively as MeWE F2 (yield 150 mg) and MeWE F3 (yield 200 mg).

2.3. Anion exchange chromatography

Fraction MeWE F1 (25 mg) was dissolved in 0.005 M sodium acetate buffer (30 ml), pH 5.5, and applied to a column (1.3 x 30 cm) of DEAE-Sepharose CL-6B, equilibrated with the same buffer. After loading with sample the column was washed with the same buffer (100 ml) and then eluted successively with 0.2, 0.5 and 1 M sodium acetate buffer, pH 5.5 at 45 ml/h. Fractions (5 ml) were assayed by phenol-sulfuric acid method for total sugar contents, using galactose as standard.

2.4. Size exclusion chromatography

Aqueous solutions (1 ml) of polysaccharide (1 mg) were loaded onto a Superose 12 HR column (1 x 30 cm) equilibrated previously with dilute sodium sulfate (0.05 M). The sample was injected to the column through an injection loop (200 µl). A dilute solution of sodium sulfate (0.05 M) was used as the eluent and the rate of elution was 30 ml/h. The amount of carbohydrates in the eluent was determined by following how much the eluent consumed periodate [16]. The void volume (V0) and total volume (V) of the column were determined as the elution volume of dextran (molecular weight 2000000) and Methyl β-D-glucopyranoside respectively. The periodate test was performed on-line with an Alpkem segmented flow analyzer. In separate experiments, solutions (1–2 ml) of polysaccharides (~3 mg) in 400 mM sodium acetate buffer pH 4.0, was loaded onto a Sephadex G-200 column (2.3 x 50 cm) equilibrated with the same buffer. Elution was also performed by the same solvent at a flow rate of 20 ml h⁻¹. Fractions (4 ml) were collected and analyzed for the total sugar, uronic acid and protein content (by the absorbance at 280 nm). The column was calibrated with standard dextrins with a molecular-weight range of 10000 to 500000. The total (V) and void (V0) volume of the column was determined as the elution volume of glucose and dextran (molecular weight 2000000), respectively.

2.5. Sugar analysis

The monosaccharide compositions of the polymers were determined by methanolation and the methyl glycosides were separated as their TMS-derivatives [17] by GLC. The gas chromatograph was equipped with a flame ionization detector and a fused silica capillary column (i.d. 0.32 mm, 25 m) coated with methylpolysiloxane (NB-30). The oven temperature program was: 2 min at 100 °C, 4 °C/min to 200 °C, and 2 min at 200 °C.

Neutral sugars of the carboxyl reduced polymer were determined after hydrolysis with 2 M H2SO4 for 2 h at 100 °C. Reduction and acetylation were carried out as described elsewhere [18] and the alditol acetates were analyzed by GLC. Total sugars were estimated by the phenol-sulfuric acid assay [19] using galactose as standard.

2.6. Protein analysis

Protein was estimated by the methods of Lowry et al. [20] using bovine serum albumin as standard.

2.7. Carboxyl reduction

Uronic acids in polymer (100 mg) dissolved in 8 M aqueous urea (10 mg/ml) were reduced twice as described [21] (yield 60 mg).
2.8. Periodate oxidation

Periodate oxidation of polysaccharide was carried out as described by S. C. Fry [22]. Briefly, a solution of 50 mg of polysaccharide in 25 ml of reagent (50 mM NaIO₄ made up in 0.25 M formic acid, pH adjusted to 3.7 with 1.2 M NaOH) was incubated at 4–6 °C in the dark for 144 h. Next, the excess of periodate was decomposed with 1 ml ethane-1,2-diol, and the solution stirred for a further 1 h period at room temperature. The solution was subsequently cooled on an ice-bath and the pH of the solution was adjusted to ~5 by dropwise addition of glacial acetic acid. The resulting solution was then dialyzed, the retentate concentrated to a small volume, and finally diluted with 5 vols. of ethanol. The precipitate that was collected by centrifugation was then dried by solvent exchange as described above (yield 34.7 mg).

2.9. Methylation analysis

Methylation of the native polysaccharide and its carboxyl-reduced derivative were carried out by the methods of Blakeney et al. [23]. Samples (3–5 mg) were dried overnight over phosphorus pentoxide in vacuo. To each sample, 0.7 ml of dry dimethyl sulphoxide (DMSO) was added and the mixture ultrasonicated. Next, lithium methyl sulphinyl carbanion in DMSO (0.7 ml) prepared as described [23] was added and the solutions were stirred for 1 more hour at room temperature under nitrogen. Cold methyl iodide was then added with external cooling, and the resulting solution stirred for 1 h. The excess of methyl iodide was removed under a stream of air and the methylated samples were extracted with 2:1 (v/v) chloroform-methanol (950 mg of NaBH₄ in 1 M NaOH was added and the mixture was kept at room temperature for 12 h. The solution was subsequently cooled on an ice-bath and the pH of the solution was adjusted to 7.2. The mixture was kept at room temperature for 12 h. The excess of methyl iodide was removed under a stream of air and the methylated samples were hydrolyzed with 2 M trifluoro acetic acid. The resulting solution was then dried by solvent exchange as described above (yield 34.7 mg).

2.10. Mass spectrometry

The mass spectra were recorded with Fisons MD 800 GLC/MS instrument at 70 eV. The partially methylated alditol acetates were identified on the basis of their published mass spectra [27]. The fragmentation of the trimethylsilyl derivatives of the partially methylated methyl glycosides followed the general routes outlined by Petersson [28]: methyl 2,3,4,6-tetra-O-methylpyranosides, m/z 75, 88 (100%), 101; methyl 2,4,6-tri-O-methyl-3-O-trimethylsilylgalactopyranosides, m/z 133, 146 (100%), 159; methyl 2,3,6-tri-O-methyl-4-O-trimethylsilylgalactopyranosides, m/z 75, 88 (100%), 159; methyl 2,3,6-tri-O-methyl-5-O-trimethylsilylgalactofuranosides, m/z 101 (100%); methyl 2,4-di-O-methyl-3,6-di-O-trimethylsilylgalactopyranosides, m/z 133, 146 (100%), 159.

2.11. NMR spectroscopy

Dried polysaccharide samples were dissolved in D₂O and the pH was adjusted to 7.2. The ¹H NMR and ¹³C NMR spectra were obtained at 599.86 and 150.85 MHz, respectively on a Varian UNITY 600 MHz spectrometer. Both one-dimensional and two-dimensional (COSY, RELAY COSY, TOCSY and NOESY) NMR spectra were collected at 70 °C [29].

3. Results and discussion

Cold water extraction of the gum obtained from immature Murraya fruit afforded a water extractable material (designated as MeWE). This fraction (MeWE) amounted to 0.31% of the starting fruit wet weight and contained 85.7% polysaccharide and 2.9% protein. It was composed mostly of galactose and glucose together with smaller amounts of 4-O-methylglucuronic acid and arabinose. Fractionation of MeWE with copper (II) acetate (vide experimental section for identification of fractions) yielded three acidic subfractions (MeWE F1, MeWE F2, and MeWE F3). All the subfractions had practically identical monosaccharide compositions (Table 1). MeWE F1, MeWE F2 and MeWE F3 represented 30, 15 and 20%, respectively, of the initial weight of the crude extract. They also had similar ¹H NMR spectra (Fig. 1). The only difference between these three samples, as judged by gel permeation chromatography, seems to be the

<table>
<thead>
<tr>
<th>Sugar unit</th>
<th>MeWE F1</th>
<th>MeWE F2</th>
<th>MeWE F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>1.5</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Galactose</td>
<td>77.7</td>
<td>76.3</td>
<td>77.2</td>
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<tr>
<td>Glucose</td>
<td>18.5</td>
<td>19.8</td>
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<tr>
<td>4-O-Methylglucuronic acid</td>
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</table>
molecular weight. The fractionation range of the Superoxide 12 HR column was 1000–300 000 for globular protein and for dextrans about the same. Sample MeWE F1 was eluted almost completely at the exclusion limit whereas ~50% of the polymers in sample MeWE F3 had molecular weights lower than the exclusion limit of the column (ie in the range of 80 000–300 000). In separate experiments, all the fractions were rechromatographed on a Sephadex G-200 column calibrated with standard dextran. The elution profiles of the MeWEF1 and MeWE F3 fractions are shown in Figs. 2 and 3 respectively.

Attempts were also made to purify the MeWE F1 fraction using anion exchange chromatography. Based on the total sugar basis the recovery yield from the anion exchanger was 96%. No material was eluted with the starting buffer. 91% of the recovered sugar material was eluted with 0.2-M sodium acetate buffer and the rest portion (9%) of the bound material was eluted from the column by increasing the buffer concentration.

The 1H NMR spectrum of MeWE F1 showed three groups of anomeric resonances with the following chemical shifts and abundances: 4.97 ppm (21.3%), 4.7 ppm (38.3%), and 4.5 ppm (36.0%). In the non-anomeric region a singlet corresponding to a O-methyl group was observed at 3.50 ppm. The intensity of the O-methyl resonances (7.8% of the intensity of the anomeric signals) corresponded to the amount of 4-O-methylglucuronic acid obtained from the methanolysis analysis.

The anomeric resonance at 4.97 ppm originated from a α-D-glucopyranosyl structure as evidenced by its chemical shift and the small $J_{H_1,H_2}$ coupling constant (3.3 Hz). A complete assignment of the non-anomeric protons by COSY, RELAY COSY and TOCSY, and comparison with calculated chemical shifts (CASPER [31] of α-D-Glcp (1→4) D-Gal verified the structure (Table 2).

The similar intensities of the two remaining anomeric resonance groups at 4.5 and 4.7 ppm indicated that the polysaccharide contains at least two types of β-D-galactopyranosyl units. Both the chemical shifts and $J_{H_1,H_2}$ coupling constants (9 Hz at 4.5 ppm, 8 Hz at 4.7 ppm) excluded the possibility of α-D-galactopyranosyl and galactofuranosyl structures. A complete assignment of the non-anomeric proton chemical shifts of the β-D-galactopyranosyl units by two-dimensional NMR tech-

![Fig. 1. 1H NMR spectrum of MeWE F1 (8.2 mg/0.74 ml D₂O).](image1)

![Fig. 2. Elution profile of MeWE F1 on Sephadex G-200 column (2.3 x 50 cm), eluted with 400 mM sodium acetate buffer (pH 4) at 20 ml/h.](image2)

![Fig. 3. Elution profile of MeWE F3 on Sephadex G-200 column (2.3 x 50 cm), eluted with 400 mM sodium acetate buffer (pH 4) at 20 ml/h.](image3)

<table>
<thead>
<tr>
<th>Sugar unit</th>
<th>H-1</th>
<th>H-2</th>
<th>H-3</th>
<th>H-4</th>
<th>H-5</th>
<th>H-6</th>
<th>H-6'</th>
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<tbody>
<tr>
<td>α-D-Glcp</td>
<td>4.97</td>
<td>3.57</td>
<td>3.78</td>
<td>3.47</td>
<td>4.12</td>
<td>3.81</td>
<td>3.79</td>
</tr>
<tr>
<td>β-D-Galp</td>
<td>4.71</td>
<td>3.67</td>
<td>3.76</td>
<td>3.68</td>
<td>4.06</td>
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<td>3.74</td>
<td>4.20</td>
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<tr>
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<td>3.83</td>
<td>4.18</td>
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<tr>
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<td>3.56</td>
<td>3.66</td>
<td>3.95</td>
<td></td>
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</tr>
</tbody>
</table>
niques (COSY, RELAY COSY, TOCSY) was not possible probably because of inefficient coherence transfer via the small J1H,1H of galactopyranosyl residues [32].

A NOESY crosspeak at 4.97/4.06 ppm showed that the α-D-glucopyranosyl unit was attached (1→4) to a β-D-galactopyranosyl unit that had anomeric resonance at 4.71 ppm (cf. Table 2). 13C NMR spectrum of MeWE showed narrow anomeric resonances at 100.9 (α-D-Glcp) and 105.2 ppm (4-O-linked β-D-Galp) together with a broad resonance group at 103.4–105.0 ppm. The presence of 4-O-linked galactopyranosyl unit was further verified by a narrow C-4 resonance at 78.4 ppm [33]. A resonance group at 82.5–83.5 ppm indicated the presence of 1,3-linked galactopyranosyl units [33].

Methylation analysis MeWE F1 and its carboxyl reduced derivative yielded 1,5-di-O-acetyl-2,3,4,6-tetra-0-methyl glucitol, 1,5-di-O-acetyl-2,3,4,6-tetra-0-methyl galactitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl galactitol, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl galactitol and 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl galactitol. These corresponded to terminal gluc- and galactopyranosyl units (both), 1,4-linked galactopyranosyl units, 1,3-linked galactopyranosyl units and 1,3,6-linked galactopyranosyl units.

Analysis of the partially methylated methyl glucosides from permethylated MeWE F2 also showed the presence of terminal glucopyranosyl units (18.4%), terminal galactopyranosyl units (10.9%), 1,3-linked galactopyranosyl units (13.9%), 1,4-linked galactopyranosyl units (20.1%) and 1,3,6-linked galactopyranosyl units (36.7%). No other linkages types were detected (Fig. 4).

The neutral sugar composition of the permethylated MeWE F2 subfractions were in good agreement with the proportion of the sugars determined before methylation. 100% of the substituted residues carried a substituent at O-6. Of the total galactopyranosyl residues 13.3% are present as terminal group. The galactopyranosyl residues that carried substituent at position 2 are (1,3)-linked. Of the total galactopyranosyl residues 62% are (1,3)-linked and 24.6% are (1,4)-linked. All the glucopyranosyl and 4-O-methyl-glucuronopyranosyl residues are present as terminal group.

To verify the results of methylation and NMR analyses, the polysaccharide was subjected to periodate oxidation. Sequential periodate oxidation, borohydride reduction and acid hydrolysis of polysaccharide completely destroyed glucose and 4-O-methyl-glucuronic acid residues. This verified the earlier finding that these sugar residues occupy terminal positions. Alditol acetates from the periodate oxidized sample included galactitol and one tetritol. The occurrence of tetritol indicated the presence of 1,4-linked hexopyranosyl units without substituents at positions O-2 and O-3. The presence of galactitol confirmed the presence of 1,3- and/or 1,3,6-linked galactopyranose residues in the polymer.

4. Conclusion

The results of the present investigation show that the water soluble gum polysaccharide of M. Paniculata fruits is a heterogalactan and is extensively branched. The structure of this gum polysaccharide bears similarity with those of Acacia [34–39] and Aegle mermalos [40]. The latter gums have been shown [34–39] to contain an internal core of β-1,3-linked D-galactopyranosyl units having side chains, joined to it by 1,6-linkages. Gum arabic is believed to be a member of the arabinogalactan protein group of proteoglycan [34–39]. We did not find any absorption at 280 nm during the gel filtration chromatography of the purified fractions of Murraya gum, although the native polysaccharide contained very small amount of protein. Besides, this gum exhibits a simple peak contrary to gum arabic whose elution pattern is discontinuous and broad [39]. Even the individual pooled fractions are made up only of polysaccharide and have very high molecular weight. The constancy in sugar composition and NMR spectra among these fractions can be interpreted by a polymer system having a distribution in molecular weights. Further research will be directed towards a more detailed characterization of the polymer by chemical and enzymatic degradations.

References

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

**SAROJ K. MONDAL, BIMALENDU RAY*, SWAPNADIP THAKUR**

and **PRADYOT K. GHOSAL**

Department of Chemistry, Burdwan University, Burdwan-713 104, India
Fax: +91 342 64452; E-mail: konica2@dte.vsnl.net.in

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. 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. Root-bark of this plant contains coumarins and antifungal compounds, whereas its stem-bark contains alkaloids and has insecticidal property. A new tyramine derivative has been obtained from the fruits of *Limonia acidissima*. The leaves of this plant afforded coumarins, triterpenoids and steroids. 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.

**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 of the derived alditol acetates was performed on a glass column packed with 5% 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 assay20 using galactose as standard. Total uronic acids were assayed colorimetrically by the m-hydroxydiphenyl assay according to procedure outlined by Ahmed and Labavitch21 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) H₂SO₄ (1 h at 30°C), followed by 1 M—H₂SO₄ (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 D₂O 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 EfiR 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 BaCO₃, decactionised with amberlite IR-120 (H⁺ form) and concentrated to a small volume. These concentrated solutions were then applied on a silica gel G TLC plate impregnated with 0.5 M NaH₂PO₄. 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 elsewhere16. 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 \( V_1 \) were determined as the elution volumes of amylopectin and glucose respectively. Results are expressed as a function of \( K_{av} = (V_e - V_0)/(V_1 - V_0) \), 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 \textit{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 \( \text{NaIO}_4 \) in 0.25 M formic acid, pH adjusted to 3.7 with 1 M \( \text{NaOH} \)). 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 dioi, and the solution was dialyzed and reduced with 0.5 M \( \text{NaBH}_4 \) in 1 M \( \text{NaOH} \) at 4-6°C overnight. After reduction, the solution was made neutral with \( \text{ca. 70 mL} \) acetic acid and evaporated to dryness. The last traces of boric acid were removed from the 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 \textit{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 I 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% H₂SO₄) 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 caloric 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 cellulose.

### TABLE I

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<th>LaS</th>
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<tr>
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<td>4.7</td>
<td>4.75</td>
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<td>Rha</td>
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<td>0.9</td>
<td>Tr (1.1)</td>
</tr>
<tr>
<td>Fuc</td>
<td>(0.1)</td>
<td>–</td>
<td>–</td>
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<td>Ara</td>
<td>8.7 (4.3)</td>
<td>31.9</td>
<td>6.5 (6.1)</td>
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<tr>
<td>Xyl</td>
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<td>6.5</td>
<td>64.6 (29.6)</td>
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<td>Tr</td>
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<tr>
<td>Gal</td>
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<td>4.3 (Tr)</td>
</tr>
<tr>
<td>Gla</td>
<td>1.1 (44.7)</td>
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<td>1.4 (34.2)</td>
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<tr>
<td>UA</td>
<td>12.95 (6.3)</td>
<td>20.4</td>
<td>19.2 (30)</td>
</tr>
</tbody>
</table>

*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.
The water-soluble material was fractionated on Sephadex G-200 column in order to assess its purity. Only one fraction having $K_v$ 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. 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 galactitol, 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).

![Size-exclusion chromatography of subfraction F. Elution profile of neutral sugars and uronic acids.](image)

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 $\alpha$-linked sugar residues in the polymer. Methylation analysis and
Smith degradation studies indicate the presence of terminal and 1,5-linked Araf residues. Therefore, these two signals were tentatively assigned to the α-Araf residues. Based on the values published for similar polysaccharides, the signal at 5.4 was assigned to the H-1 of terminal α-Araf residues and the other, therefore, from the α-(1,5)-linked Araf 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 (J2,3 = 9.9 Hz and J1,2 = 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-Araf 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.
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 hydroxyl 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.](image)

**Conclusion**

The pectic substances, isolated from the seeds of Limonia acidissima consist of 30.1% neutral sugar and 4.7% galacturonic acid and has a \(K_a\) 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
An Arabinogalactan from the Seeds of *Limonia acidissima*

(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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Short Communication

Cell-wall polysaccharides from the fruits of Limonia acidissima: isolation, purification and chemical investigation

Saroj K. Mondal, Bimalendu Ray, Jean Francois Thibault, Pradyot K. Ghosal

Department of Chemistry, Burdwan University, Burdwan 713 104, India

INRA, URPOI, BP 1627 Nantes 44316 Cedex 03, France

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Abstract

Polysaccharides were isolated from the fruits of Limonia acidissima by sequential extraction with water, and 1 M and 4 M KOH. The water extract contained pectic polymers substituted with side chains comprising mainly of 1,5-, 1,3,5-linked arabinose together with 1,4-, 1,6-, 1,3,6-linked galactose, and lesser amounts of 1,2,4- and 1,3-linked galactose residues. Galactosyl and arabinofuranosyl groups terminated most of the branched residues. The alkaline extracts contained both pectic and hemicellulosic polymers. The insoluble material consists mainly of cellulose-rich material. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Limonia acidissima; Rutaceae; Fruit; Cell wall; Pectin; Hemicellulose; Cellulose

1. Introduction

Limonia acidissima W. and A syn L.crenulata Roxb. (Fam. Rutaceae), a tree with green foliage, grows abundantly in different parts of India. Different parts of this plant has been found to contain various types of chemical compounds (Bandara, Gunatilake, Sotheeswaran, Wijeratne & Ranasinghe, 1989; Chatterjee, Sarkar & Shoolery, 1980; Chopra, Nayer & Chopra, 1956; Ghosh, Bandyopadhyay, Thakur, Tamura & Matsumoto, 1989; Jain, Srivastava & Srivastava, 1989; Khan, Siddiqui & Zaman, 1975; Macleod, Peter, Patra, Bandora, Gunatilake & Wijeratne, 1989; Patra, Mishra & Choudhury, 1988; Zarga, 1986). However, the polysaccharides present in the fruits of Limonia acidissima have not been investigated before. This communication describes isolation, fractionation and chemical investigation of the polysaccharides present in the fruit.

2. Experimental

Air-dried ripe fruits (1.2 kg) of Limonia acidissima were extracted sequentially with 2.5 l of petroleum ether (60–80°C) and methanol in a Soxhlet apparatus for 33 and 50 h, respectively. The defatted fruit material (yield 590 g) was separated using tweezers into following two groups: (1) the seeds (S), and (2) the pericarp (P). All subseqeuential extraction, purification and analysis were carried out using the pericarp (P) or material derived therefrom.

The grounded pericarps (1 g) were sequentially extracted with (i) water, 150 ml for 4 h (twice); (ii) 1 M KOH containing 0.4% NaBH₄ (50 ml) at 35°C for 1.5 h (twice); and (iii) 4 M KOH containing 0.4% NaBH₄ (50 ml) at 35°C for 1.5 h (twice). All extracts were centrifuged and dialyzed exhaustively; alkaline extracts were acidified to pH 5 with HOAc prior to dialysis. All the dialyzed extracts were concentrated. The water extract was submitted to graded precipitation with ethanol to yield the less soluble fraction PWEL and the more soluble fraction PWEM. The alkane extracts were acidified to pH 5 with HOAc prior to dialysis. All the dialyzed extracts were concentrated. The water extract was submitted to graded precipitation with ethanol to yield the less soluble fraction PWEL and the more soluble fraction PWEM. The alkaline extracts were acidified with ethanol. In all cases the precipitate was recovered by centrifugation, dehydrated by solvent exchange and finally dried under vacuum over P₂O₅.

The resulting KOH unextractable residue was washed thoroughly with water containing acetic acid, and then with deionised water, and finally dried by solvent exchange. Solutions (150 ml) of PWEL (340 mg) in 10 mM NaOAc buffer pH 6.5 was applied to a column (2.2 x 24 cm) of DEAE-Sepharose CL-6B (OAc−) and then eluted successively with 0.01, 0.1, 0.3, 0.4 and 1 M NaOAc buffer pH 6.5 (160 ml each) in a stepwise manner and finally with 0.2 M NaOH (100 ml). The collected fractions (10 ml) were analysed for neutral sugar and uronic acid content and the
Fig. 1. Scheme for extraction and fractionation of polysaccharides obtained from the pericarp of *Limonia acidissima* fruit.

Table 1

<table>
<thead>
<tr>
<th></th>
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</table>

* Percentage weights of the P dry weight.

Percentage weights of fraction dry weight.

* Percentage mol.

The uronic acids present in the major fraction F2 (40 mg) obtained from anion exchanger, were reduced twice by the method of Taylor and Conrad (1972), as described by Ray and Lahaye (1995a) to yield the carboxyl-reduced polysaccharide (F2R, yield 25 mg).

Methylation of F2 fraction and its carboxyl-reduced derivative (F2R) were carried out by the methods of Blakeney and Stone (1985). Conversion to the partially methylated alditol acetate (PMAA) and GC and GC–MS on columns of SGE BP 1 and DB-225 (JW) were done as described (Ray & Lahaye, 1995a).

Protein was estimated by the methods of Lowry, Rosebrough, Lewisfarr and Randall (1951) using bovine serum albumin as standard. Acetic acid and methanol contents were estimated by HPLC according to Voragen, Schols and Pilink (1986). The degree of acetylation (DA) and degree of methylation (DM) were calculated as the molar ratios of acetic acid and methanol to uronic acid. The uronic acid content was determined by the m-hydroxydiphenyl assay as described by Ahmed and Labavitch (1977) using galacturonic acid as standard. Neutral sugars were released by Saeman or 1 M H₂SO₄ and analysed as their alditol acetates (Blakeney, Harris, Henry & Bruce, 1983) by GLC on columns of SGE BP 225 and DB-225 (JW) as...
described (Ray & Lahaye, 1995b). Phenolic acids were determined by HPLC after saponification and extraction as described (Thibault, 1988). The sugars in the acid hydrolysates were also analysed by TLC.

3. Results and discussion

The flow-sheet for the isolation and fractionation are given in Fig. 1. The yield and chemical composition of the *Limonia acidissima* fruit (pericarp) polysaccharides extracted sequentially with various inorganic solvents are given in Table 1. Water alone extracted 7.3% of the material present in the defatted pericarp (P). This extract, which represents 12% of the uronic acid present in P, was then subjected to a graded precipitation with ethanol. The less soluble fraction (PWEL) obtained at an ethanol concentration of 60% (v/v) represents 27.8% of the material present in the water extract. The sugar composition of this fraction indicates the probable presence of pectic polymers. The ratio of acidic to neutral sugars was around 50 galacturonic acid molecules for 50 neutral sugar residues. The degree of acetylation and the degree of methylation of the PWEL fraction were 3.4 and 11.1%, respectively. No phenolic acids were detected. The more soluble fraction (PWEM), obtained at ethanol concentration of 80%, accounted for 5.2% of the P and contained 64.1% neutral sugar and 17.3% uronic acid. The water un-extractable residue (PWUR) represented 60.5% of the defatted fruit powder and contained 41.9% carbohydrate material.

Most of the polysaccharides containing xylose were extracted with alkali. The 1 M KOH soluble fraction (P1OH), which constituted 17.8% of the P, contained substantial amounts of arabinose, xylose, galactose and glucose, with some deoxyhexose and mannose and 4.9% galacturonic acid (Table 1). The sugar composition of this fraction suggested that it contained a mixture of pectic and hemicellulosic polymers. The galacturonic acid to rhamnose ratios of pectic polymers extracted with alkali is small compared with that of water extracted one. The 4 M KOH soluble fraction (P40H) accounted for 7.5% of the P and was rich in arabinose, xylose, galactose, glucose and uronic acid indicating the presence of pectic and hemicellulosic polymers. The dilute alkali treatments (1 M KOH) dissolve ~6.1% of uronic acid originally present in P, whereas ~4% of uronic acids were solubilised by 4 M KOH. The final residue (POHUR) after extraction with 4 M KOH gave a yield of 24.6% of P and contained 68.2 mol% glucose, of which 59.1 mol% is of cellulose origin. It represented 21.4% of the uronic acid originally present in the P. In total, 32.6% of the P could be extracted using sequential extraction with inorganic solvents. The total carbohydrate content of the polymers soluble in various inorganic

---

Table 2

<table>
<thead>
<tr>
<th>Partially methylated alditol acetate</th>
<th>F2</th>
<th>F2R</th>
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<td>2,3,5-Me₂-Ara</td>
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<td>7.2</td>
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<tr>
<td>2,3-Me₂-Ara</td>
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<tr>
<td>2-Me-Ara</td>
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</tr>
<tr>
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<td>3.4</td>
<td>2.6</td>
</tr>
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</tr>
<tr>
<td>3-Me-Rha</td>
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<td>2.4</td>
</tr>
<tr>
<td>2,3,4,6-Me₆-Gal</td>
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<td>3.0</td>
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<td>2,4,6-Me₆-Gal</td>
<td>1.8</td>
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<td>2,3,6-Me₆-Gal</td>
<td>12.9</td>
<td>50.1</td>
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<td>2,3,4-Me₆-Gal</td>
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<td>6.4</td>
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<td>1.4</td>
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<td>2,3,6-Me₆-Glc</td>
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<td>2,3,6-Me₆-Man</td>
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</table>

* 2,3,5-Me₂-Ara = 2,3,5-tri-O-methyl-1,4-di-O-acetyl arabinitol etc.
The polysaccharides present in the PWEL fraction was further resolved by anion exchange chromatography into five (F1, F2, F3, F4 and F5) charged fractions (Fig. 2). The major fraction, F2 accounted for 56.7 and 83.4% of the neutral sugars and uronic acid recovered from the anion exchanger, respectively, and contained galacturonic acid (57.5 mol%), arabinose (20.6 mol%) and galactose (12.8 mol%) as the major sugar together with smaller quantities of rhamnose (2.4 mol%), mannose (3.2 mol%), glucose (1.1 mol%), xylose (1.9 mol%) and trace amount (12.8 mol%) as the major sugar together with smaller amounts of fucose residues.

The results of methylation analysis indicated that galacturonic acid (GalpA) residues are 1,4-linked (Table 2). Rhamnose is 1,2-linked with branching via C-4. Arabinoses are in furanose form and are 1,5-, 1,3,5- and terminally linked. The presence of 1,6- and 1,3,6-linked `galactosyl`, and 1,3- and terminal arabinosyl residues was indicated.

In conclusion, this study has shown that the major polysaccharides in the cell walls of Limonia acidissima fruit are cellulose, hemicellulosic and pectic polymers. The water-soluble material comprises mainly of pectic polysaccharides. These polymers gave viscous solution, were lightly esterified, and contained acetyl groups. Alkaline extracts contained both pectic and hemicellulosic polymers. Glucose and xylose were the predominant sugars in the 1 and 4 M KOH extract, which may indicate the probable presence of xyloglucan in this extract. The isolation of oligosaccharides for further structural analysis of these polysaccharides is in progress.

Acknowledgements

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References


Isolation and characterization of pectic polysaccharides from the fruits of *Naringi crenulata*

Saroj K Mondal, Bimalendu Ray*, Swapnadip Thakur & Pradhyot K Ghosal
Department of Chemistry, Burdwan University, Burdwan, 713 104, India.
Email: konica2@dte.vsnl.net.in; Tel: +91 342 560810.
Fax: +91 342 564452

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Pectic polysaccharides have been isolated from the fruits of *Naringi crenulata* by extraction with water. The water extract contains large amount of protein. The polymers present in the water extract are fractionated by graded precipitation with ethanol, anion exchange chromatography, and size exclusion chromatography. Characterization of the subfractions obtained therefrom by various chemical and physico-chemical methods of analysis reveals that the water extract contain pectic polymers substituted to various degrees with side chains comprising mainly acetyl groups and give viscous solution in water.

*Naringi crenulata* (Roxb.) Nicolson (Fam. Rutaceae), a tree with green foliage, grows abundantly in different parts of India. The therapeutic effect of the fruits, leaves and roots of this plant in the treatment of different tropical diseases is well documented in folk medicine. Different parts of this plant has been investigated for chemical constituents to some extent, and found to contain various types of chemical compounds. It has been known that root-bark of this plant contains coumarins, anti-fungal compounds, whereas its stem bark contains alkaloids and insecticidal property. The leaves of this plant afforded coumarins, triterpenoids and steroids. A new tyramine derivative was obtained from the fruits of *Naringi crenulata*. However, the fruits of *Naringi crenulata* have been less investigated for the polysaccharide contents before. Many polysaccharides from natural origin have been found to have biological activity. Since structure and functions are intimately related knowledge of the structure of isolated compound is a prerequisite for better understanding and therefore modification, if any, of the activity. The aim of the present research was to isolate the different polysaccharides present in this fruit, to fractionate them and to analyze their structures.

**Results and Discussion**

Pectic polysaccharides were isolated from the pericarp of *Naringi crenulata* fruit by extraction with water (pH 5.5) at 35°C. The water extract, which contains 12.5% protein, was subjected to graded precipitation with ethanol. The less soluble fraction (LSF) contained 32.5% neutral sugar and 38.2% uronic acid. The other i.e., the more soluble fraction (MSF) was enriched in neutral sugar (61.4%) and contained lesser amount of uronic acid (17.3%) compared to LSF. Sugar analysis revealed that arabinose (31.8 mol%), galactose (38.5 mol%), and galacturonic acid (19.4 mol%) were the main sugars found in MSF fractions together with smaller quantities of xylose (4.5 mol%), mannose (2.5 mol%), glucose (2.1 mol%), rhamnose (1.1 mol%), and traces of fucose. The presence of high amount of galacturonic acid (confirmed by TLC analysis) in both the fractions suggests the presence of pectic polysaccharides. Neither glucuronic nor any uronic acids other than galacturonic acid were detected by this method. Arabinose was found at a higher concentration than galactose in the LSF fraction, where it probably originated from arabinose rich side chain. GLC analysis and colorimetric estimations also showed that the ratio of acidic to neutral sugars in LSF was around 50 galacturonic acid molecules for 50 neutral sugar residues (two rhamnose inside the chain and 48 arabinose, galactose and other sugars in lateral branches). But MSF fraction contains 19 galacturonic acid molecules for 81 neutral sugar residues (one rhamnose inside the chain and 80 arabinose, galactose and other sugars in lateral branches). These results revealed that the watersoluble material comprises population of polysaccharides differing in neutral sugars (NS)/uronic acid (UA) ratio. MSF fraction was hydrolyzed and the liberated acetic acid and methanol were measured by HPLC. The degrees of acetylation of MSF and LSF were 6.7% and 3.4% respectively. The actual positions of the acetyl groups are not...
known. The acetyl groups are usually assigned to the galacturonic acid residues, though other sites of attachment are known to exist. These data show that less branched pectic materials with low degree of acetylation were precipitated at lower ethanol concentration while the more highly branched pectic polymers had higher acetylation degrees and precipitated at higher ethanol concentration. The degree of methylation (DM) of the LSF fraction was 11.1%. No phenolic acids were detected. Monosaccharide residues in the furanose form are hydrolyzed under mild conditions compared to residues in the pyranose form. Appearance of arabinose as the only detectable sugar in weak acid hydrolysate indicates the presence of Arar residues. If, however, the hydrolysis is continued upto 12 hr in the same condition the hydrolysate contains arabinose as the major sugar along with minor quantities of galactose, xylose, and glucose residues. The water un­
extractable residue (PWUR) contained a significant amount of pectic polysaccharides. Glucose is the most important sugar in this fraction and most of the glucose present here are of cellulosic origin.

DEAE-cellulose chromatography of LSF fraction resolved it into five (PF1, PF2, PF3, PF4 and PFn) acidic fractions. Based on the mass of the composite sugar residues, 96% of the neutral and 91% of the uronic acid injected onto the column was eluted with various inorganic eluants. The yield and sugar compositions of the fractions obtained are shown in Figure 1 and Table I, respectively. The sugar composition of the pectic fractions (PF2, PF3 and PF4) is qualitatively similar, but quantitatively there is a difference. With regard to the neutral sugar composition, arabinose and galactose dominated in varying ratios. Further neutral sugars found were xylose (1.9 – 24.7 mol%), glucose (trace - 1.1 mol%), mannose (trace – 3.2 mol%), rhamnose (1.4 - 2.4 mol%) and fucose (0.3 mol%). Uronic acid is the major sugar found in all fractions except PF1. The sugar composition of various pectic fractions indicates the probable presence of rhamnogalaturonan. The varying ratio of uronic acid to rhamnose found in different fractions indicates the presence of heterogeneity in pectic polymers isolated (assuming no degradation occurred during extraction and fractionation). Lateral chains of glycans consisting mainly of arabinose and galactose residues play a major role in fractionation; the number of rhamnose residue intercalated in the galacturonan chains decreasing slightly from fraction PF2 to fraction PF4. PF2, the major fraction, accounted for 56.7% and 83.4% of the neutral sugars and uronic acid recovered from the anion exchanger, respectively. The fraction obtained with 400 mM-
buffer concentrations had an ara/gal ratio higher than all other fractions.

The major fraction (PF2) was further characterized by its molecular weight distribution as determined by gel permeation chromatography. Figure 2 shows the chromatogram for this fraction obtained using Sephadex G-200 column. As indicated by the Kav. values at the front and tail ends of the chromatograms (Kav. 0 to 0.7), the PF2 fraction elutes well within the fractionation range of the column. Gel permeation chromatography of PF2 yielded two subfractions: PF2A (fraction no 5 to 9) and PF2B (fraction no 16 to 21). Both the subfractions had practically identical monosaccharide compositions (similar to that of PF2). The only difference between these two subfractions, as judged by gel permeation chromatography, seems to be the molecular weight. The fractionation range of this column was 1,000 - 200,000 for dextrans. Subfraction PF2A was eluted almost completely at the exclusion limit whereas the polymers in subfraction PF2B had molecular weights lower than the exclusion limit of the column (i.e. in the range of 40,000 - 70,000 Da based on calibration with standard dextrans).

The FT-IR spectra of various fractions are given in Figure 3. The broad band between 3600 and 3000 cm⁻¹, corresponding to vibrations of the hydroxyl groups appeared to be similar in all the spectra. Methyl and methylene group vibrations appeared around 2927 cm⁻¹ and were present in the spectra of all fractions. The pectic polymers present in the water extracted material (Figure 3a) and the pericarp itself (Figure 3b) show a band in the region 1739 cm⁻¹ related to the >C = O stretching of the ester group. After de-esterification a reduction of the absorbance band at 1739 cm⁻¹ was observed (data not shown). Structural features arising from particular conformations around the glycosidic bond of the pectin are observable in the 850-1200 cm⁻¹ region. The bands at 1014 to 1101 cm⁻¹ are characteristic of the galacturonic acid residues of the pectic polysaccharides (Figures 3a and 3c).

In order to have an idea about the mode of linkages of the polysaccharide present in the LSF, the native polymer (LSF) and its carboxyl-reduced derivative (LSF-R) were subjected to methylation analysis. The results of methylation analysis (Table II) indicated that rhamnose is 1,2-linked with branching via C-4. Reduction of the galacturonic acid residues resulted in increased values for 1,4-linked Galp residues owing to the reduction of galacturonic acid. The slight increase in the 1,2- and 1,2,4-linked Rhap residues was probably due to better de-polymerization of reduced than that of the native polymer. Indeed, the aldobiouronic acid, β-D-GalA-(1,4)-L-Rha, in pectin is resistant to acid hydrolysis but after reduction of the polymer, the reduced galacturonic acid and rhamnose
Table II — The linkage (mol%) of LSF fraction determined by reduction, methylation and GC/MS. LSF-R is the carboxyl reduced derivative of LSF fraction.

<table>
<thead>
<tr>
<th>Partially methylated alditol acetate</th>
<th>LSF</th>
<th>LSF-R</th>
<th>Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-Me₂-Xyl</td>
<td>6.7</td>
<td>4.1</td>
<td>1,4</td>
</tr>
<tr>
<td>2,3,5-Me₂-Ara</td>
<td>13.2</td>
<td>5.4</td>
<td>Terminal</td>
</tr>
<tr>
<td>2,3-Me₂-Ara</td>
<td>15.1</td>
<td>9.3</td>
<td>1,5</td>
</tr>
<tr>
<td>2-Me-Ara</td>
<td>7.2</td>
<td>4.9</td>
<td>1,3,5</td>
</tr>
<tr>
<td>3,4-Me₂-Rha</td>
<td>1</td>
<td>1.2</td>
<td>1,2</td>
</tr>
<tr>
<td>3-Me-Rha</td>
<td>1.5</td>
<td>1.5</td>
<td>1,2,4</td>
</tr>
<tr>
<td>2,3,6-Glc</td>
<td>1.2</td>
<td>1.8</td>
<td>1,4</td>
</tr>
<tr>
<td>2,3,4,6-Me₂-Gal</td>
<td>7.8</td>
<td>4.2</td>
<td>Terminal</td>
</tr>
<tr>
<td>2,4,6-Me₂-Gal</td>
<td>2</td>
<td>2</td>
<td>1,3</td>
</tr>
<tr>
<td>2,3,6-Me₂-Gal</td>
<td>13.8</td>
<td>51.1</td>
<td>1,4</td>
</tr>
<tr>
<td>2,3,4-Me₂-Gal</td>
<td>7.1</td>
<td>7.5</td>
<td>1,6</td>
</tr>
<tr>
<td>3,6-Me₂-Gal</td>
<td>3.2</td>
<td>1.5</td>
<td>1,2,4</td>
</tr>
<tr>
<td>2,4-Me₂-Gal</td>
<td>10.5</td>
<td>2.3</td>
<td>1,3,6</td>
</tr>
<tr>
<td>2,3,6-Me₂-Man</td>
<td>4.7</td>
<td>3.2</td>
<td>1,4</td>
</tr>
</tbody>
</table>

*2,3,5-Me₂-Ara = 2,3,5-tri-O-methyl-1,4-di-O-acetyl arabinitol etc.

linkage is hydrolyzed more easily and thus a better recovery of rhamnose is obtained. Arabinoses are in furanose form (Araf) and are 1,5-, 1,3,5- and terminally linked. About 16.9% of the galactose residues occupy a terminal position, and 29.7% is branched. The presence of 1,6- and 1,3,6-linked galactosyl, and 1,3- and terminal arabinosyl residues suggests the presence of type II arabinogalactan. Besides, nearly 33% of the galactose residues is 1,4-linked, and a small portion (6.4%) is 1,2,4-linked. Galactopyranosyl (Galp) or Araf groups terminated most of the branched residues.

To verify the results of methylation analysis, the polysaccharide (PF2) was subjected to periodate oxidation. Alditol acetates derived from the periodate oxidized sample included arabinitol, galactitol and a tetritol. The occurrence of tetritol indicated the presence of 1,4-linked pyranosyl and/or 1,5-linked furanosyl units without substituents at positions O-2 and O-3. Survival of some arabinose residues established the presence of 1,3,5-linked arabinofuranosyl units. The presence of galactitol confirmed the results of methylation analysis that some of the galactopyranose residues are 1,3- and 1,2,4-linked and therefore resistant to periodic acid oxidation. Although the galacturonic acid residues are 1,4-linked but a significant amount of this residue survived periodate oxidation.

Conclusion

Pectic polysaccharides isolated from the pericarp of Naringi crenulata fruit yield fractions consisting of population of polysaccharides differing in neutral sugars/uronic acid ratio. These pectic substances consisted of a range of structurally related polymers that differed widely in their behavior towards anion exchanger. These polymers gave viscous solution, were lightly esterified, and contained acetyl groups.

Experimental Section

Plant material and polysaccharides. Pericarps of the fruits from Naringi crenulata were the same as that used previously. Water-soluble polysaccharides were obtained from the dried powdered pericarp (P, 1 g) by extraction with water pH 5.5 as follows. The fruit powder (1 g) was dispersed in 150 mL of deionized water and stirred continuously using a mechanical stirrer over 4 hr period. The supernatant was removed by centrifugation (15 min at 16,000 g) and the pellet re-extracted with 150 mL water. The combined supernatant dialyzed exhaustively against water and then concentrated to give a viscous solution.

Graded precipitation with ethanol. The concentrated dialysate (~200 mL) was then submitted to graded precipitation with dehydrated ethanol. The concentration of alcohol was increased in steps of 20%. Each mixture was placed in the refrigerator for 3 hr. The precipitate obtained at 60% ethanol concentration was collected by centrifugation, washed thoroughly with 80% ethanol, acetone and diethyl ether, and finally dried in vacuum (100°C over P₂O₅) (LSF; yield 21 mg). The second precipitate obtained at 80% ethanol concentration was isolated therefrom by centrifugation was dried by solvent exchange as described above (MSF; yield 52 mg). The water un-extracted residue was also dried by solvent exchange as described (PWUR; yield 605 mg).

Anion exchange chromatography. Approximately 340 mg of LSF was fractionated on a column (2.2x51 cm) of DEAE-Sepharose CL-6B that was previously equilibrated with 10 mM NaOAc buffer pH 6.5. Elution was carried out successively with 10, 100, 300, 400 and 1,000 mM NaOAc buffer pH 6.5 (350 mL each) in a stepwise manner and finally with 200 mM NaOH (300 mL). The collected fractions
were analyzed for neutral sugar and uronic acid contents and were concentrated, dialyzed, and the concentrated retentate was diluted with 3 vols. of absolute ethanol at room temperature. The precipitate was recovered by centrifugation (20 min at 16,000 g); the pellet was dehydrated by solvent exchange as described above and finally dried, under vacuum, over P₂O₅.

Gel permeation chromatography. The polysaccharide (PF₂, 3 mg) was dissolved in 400 mM sodium acetate buffer pH 4.0 and passed through a Sephadex G-200 column (1.3 × 30 cm) equilibrated with the same buffer at 15 ml/h. Fractions (1 mL) were collected and assayed for the neutral sugar and uronic acid contents. Elution of polysaccharide from GPC is expressed as Kᵅₐ [Kᵅₐ = (Vₑ-V₀)/(Vₜ-V₀) with Vₑ and V₀ being the total and void volume of the column (determined as the elution volume of glucose and amylpectin respectively) and Vₜ is the elution volume of the samples]. The column was calibrated with standard dextrins with a molecular-weight range of 10,000 to 500, 000. Fractions 5 to 9 and 16 to 21 (Figure 2) were pooled separately. Each of them was dialyzed, concentrated and lyophilized to yield PF₂A and PF₂B subfractions. Their sugar compositions were determined.

De-esterification. The pH of the polysaccharide solution (2 mg/mL) was increased to 13 using 0.2 M NaOH. After 1 hr at 35°C the pH of the solution was lowered to 5 by dropwise addition of glacial acetic acid. Neutralization was carried out over an ice bath and the solution stirred continuously using a magnetic stirrer bar. The solution dialyzed, concentrated and then diluted with 4 vols of ethanol. The de-esterified material was recovered from the retentate by centrifugation and dried after solvent exchange.

Carboxyl reduction of the LSF fraction. This was carried out by the methods of Taylor and Conrad as described in refs. 23,24 to yield the carboxyl reduced derivative LSF-R.

Methylation analysis. Methylation by the methods of Blakeney et al. 25, conversion to partially methylated alditol acetates (PMAA) and GLC and or GLC/MS on columns of SGE BP 1 column (25 m × 0.22 mm, 0.25 μm) and DB-225 (JW) column (30 m × 0.32 mm, 0.25 μm) as described 12,13,24.

Periodate oxidation. LSF (500 mg) in 0.25 M formic acid, pH adjusted to 3.7 with 1 M NaOH was treated with of NaIO₄ (2.675 g) as described in ref. 24. The product was isolated after desalting on a Sephadex G-10 column (2.0 cm × 28 cm). The recovered material was hydrolyzed and after usual treatments was analyzed by TLC and GLC.

Analytical methods. Chemicals used were analytical grade or best available. All determinations were done at least in duplicate. Protein was estimated by the methods of Lowry et al. 26 using bovine serum albumin as standard. Acetic acid and methanol contents were estimated by HPLC according to Voragen et al. 27. The degree of acetylation (DA) and degree of methylation (DM) were calculated as the molar ratios of acetic acid and methanol to uronic acid. The uronic acid content was determined by the m-hydroxydiphenyl assay as described by Ahmed and Labavitch 28 using galacturonic acid as standard. Correction was made for the interference of neutral sugars in the determination of uronic acid. Neutral sugars were estimated by the phenol-sulfuric acid assay 29 using galactose as standard. In this method corrections were made for interference from anhydro-galacturonic acid. For the determination of monosaccharide composition, the polysaccharides in the samples were hydrolyzed using sulfuric acid (2 M, 3 hr at 100°C), preceded by a 1 hr prehydrolysis at 30°C with aqueous 72% H₂SO₄ for insoluble residues. Neutral sugar composition of the hydrolysates was determined by GLC using a SGE BP 225 column (25 m × 0.32 mm, 0.25 μm) operating isothermally at 195°C with H₂ as carrier gas, after conversion of the monosaccharides to alditol acetates as described by Blakeney et al. 30. myo-Inositol was used as internal standard, and hydrolytic losses were accounted for by using an external standard 31. Alternatively, the alditol acetates were separated on a DB-225 (JW) column (30 m × 0.32 mm, 0.25 μm). The temperature program was 180°C for 7 min and then 180°C-250°C at 5°C/min. Phenolic acids were determined by HPLC after saponification and extraction as described 32.

Identification of uronic acid and neutral sugars by thin layer chromatography

The sugars in the acid hydrolysates were analyzed by TLC on Silica Gel G plate impregnated with 0.5 M NaH₂PO₄. The plate was eluted using ethanol-phenol-pyridine-0.1 M phosphoric acid (10:2:2:4) and/or 2-propanol – methanol - water (16:1:3) as developing agent. Sugars were detected by spraying with saturated aniline phthalate and drying at 100°C.
IR spectroscopy. Samples were dried at 35°-44°C in vacuum over phosphorus pentoxide for 72 hr prior to making pellet with KBr. Infrared spectra were recorded on a JASCO FTIR 420 spectrophotometer.

Partial acid hydrolysis. Polysaccharide (LSF) sample was hydrolyzed with 20-mM oxalic acid at 100°C. The hydrolysate removed from the reaction vial at 3 hr and 12 hr, neutralized with CaCO₃, decactionised with Amberlite IR-120 (H⁺), filtered and then concentrated. The sugars present in the hydrolysates were analyzed either by TLC as described above or by GLC of the derived alditol acetates.

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