



## Note

## Structural studies of a heteropolysaccharide (PS-I) isolated from hot water extract of fruits of *Psidium guajava* (Guava)

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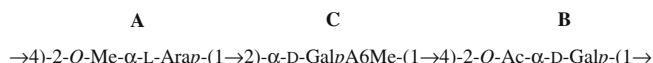
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## ABSTRACT

A water-soluble polysaccharide was isolated from hot aqueous extracts of fruits of *Psidium guajava*. The polysaccharide was found to contain 2-*O*-methyl-*L*-arabinose, 2-*O*-acetyl-*D*-galactose, and *D*-methyl galacturonate in a molar ratio of approximately 1:1:1. On the basis of acid hydrolysis, methylation analysis, periodate oxidation, and NMR studies (<sup>1</sup>H, <sup>13</sup>C, TOCSY, DQF-COSY, NOESY, ROESY, HMQC, and HMBC), the structure of the repeating unit of the polysaccharide was established as



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*Psidium guajava* (Guava) is an important medicinal plant. The fruits show the presence of moisture (77–86%), crude fiber (2.8–5.5%), protein (0.9–1.0%), fat (0.1–0.5%), ash (0.43–0.7%), carbohydrates (9.5–10%), minerals, and vitamins,<sup>1</sup> and exhibit antioxidant<sup>2,3</sup> and hypoglycemic properties.<sup>4</sup> Natural products such as terpenoids<sup>5</sup> from leaves and flavonoids<sup>6</sup> from seeds have been isolated. Extracts of leaves shows antidiabetic,<sup>7</sup> hypotensive,<sup>8</sup> and antimicrobial effects.<sup>9,10</sup> In our laboratory a polysaccharide was isolated from the fruits by hot water extraction followed by acetic acid treatment and gel filtration. No works relating to the polysaccharide of the fruits have been reported. We are reporting herein for the first time the detailed structural studies of the polysaccharide isolated from the guava fruits.

The hot water extract of fruits (1.0 kg) was cooled and then centrifuged. After exhaustive dialysis the filtrate was precipitated in EtOH and residue freeze dried followed by acetic acid treatment and centrifugation to yield 1.5 g crude polysaccharide, which on fractionation through a Sepharose 6B column in aqueous medium yielded two fractions, PS-I and PS-II. We report herein the structural characterization of PS-I of this polysaccharide.

The pure polysaccharide (PS-I) showed a specific rotation of  $[\alpha]_D^{25} +187.70$  (c 0.068, water). The molecular weight<sup>11</sup> of this PS-I was estimated from a calibration curve prepared with standard dextrans as  $\sim 1.63 \times 10^5$  Da. The PS-I was hydrolyzed with 2 M

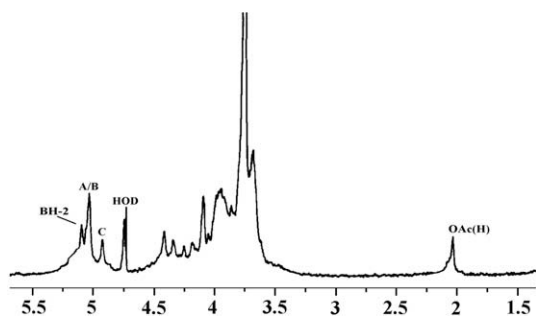
CF<sub>3</sub>COOH for 18 h at 100 °C. Paper chromatographic analysis<sup>12</sup> of the hydrolyzed product showed the presence of galactose, galacturonic acid, and a slow-moving spot nearer to arabinose. The absolute configuration of the monosaccharides was determined by the method of Gerwig et al.<sup>13</sup> taking intact polysaccharide and carboxyl-reduced<sup>14</sup> polysaccharide. Galactose and galacturonic acid had *D* configuration, but arabinose was present in the PS-I with *L* configuration. The GLC analysis of the alditol acetates of the sugars showed the presence of 2-*O*-methyl-arabinose and galactose in a molar ratio of nearly 1:1. But carboxyl-reduced polysaccharide (PS-I) on acid hydrolysis, followed by GLC analysis of the corresponding alditol acetates, showed the presence of 2-*O*-methyl-arabinose and galactose in a molar ratio of nearly 1:2. This result indicated that galacturonic acid was present in the polysaccharide (PS-I). The PS-I was then methylated using the Ciucanu and Kerek method<sup>15</sup> followed by hydrolysis and alditol acetate conversion.<sup>16</sup> The GLC–MS analysis of alditol acetates of the methylated polysaccharide revealed the presence of 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-*L*-arabinitol and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-*D*-galactitol. These results indicated the presence of (1→4)-linked-*L*-arabinopyranosyl or (1→5)-linked-*L*-arabinofuranosyl and (1→2,4)-linked-*D*-galactopyranosyl moieties in a molar ratio of nearly 1:1 in the polysaccharide. The alditol acetate of methylated, carboxyl-reduced<sup>17</sup> PS-I showed the peak corresponding to 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-*D*-galactitol along with the above peaks in a molar ratio of nearly 1:1:1. The appearance of 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-*D*-galactitol indicated the presence of (1→2)-linked *D*-galacturonic acid in the

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polysaccharide (PS-I). The more satisfactory result obtained from the GLC–MS analysis of alditol acetates of carboxyl-reduced methylated PS-I showed the presence of 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-*L*-arabinitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-*D*-galactitol, and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-*D*-galactitol in a molar ratio of nearly 1:1:1. This result further confirmed the presence of (1→2)-linked *D*-GalpA in PS-I and also indicated that galactose was present as (1→4)-linked moiety along with –OAc group at 2-position. The periodate oxidation experiment<sup>18,19</sup> was carried out with PS-I for further linking information of sugar moieties. The periodate-oxidized, reduced material of PS-I upon hydrolysis with trifluoro acetic acid followed by GLC analysis showed the presence of 2-*O*-methyl-arabinose and galactose. A part of periodate-oxidized PS-I on hydrolysis showed the absence of galacturonic acid in the paper chromatographic examination indicating that it had been destroyed during oxidation. GLC–MS analysis of periodate-oxidized, reduced, and methylated PS-I showed the peaks corresponding to 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-*L*-arabinitol and 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methyl-*D*-galactitol. Thus, periodate oxidation experiment confirmed that *L*-arabinose and *D*-galactose were retained while *D*-galacturonic acid had been destroyed.

The 500 MHz <sup>1</sup>H NMR spectrum (Fig. 1, Table 1) of PS-I at 27 °C contained two signals at  $\delta$  5.06 and 4.93 for anomeric protons. The integral value of the signal at  $\delta$  5.06 was almost double that of the other peak. Hence, the signal at  $\delta$  5.06 corresponded to two sugar residues while the signal at  $\delta$  4.93 corresponded to one sugar residue. They were designated as **A**, **B**, and **C** according to their decreasing anomeric proton chemical shifts (Table 1). In <sup>13</sup>C NMR (125 MHz) spectrum (Fig. 2, Table 1) at 27 °C, three anomeric signals appeared at  $\delta$  105.3, 101.0, and 100.4. On the basis of the ano-

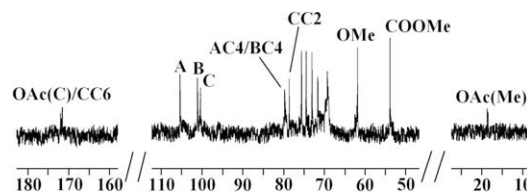


**Figure 1.** <sup>1</sup>H NMR spectrum (500 MHz, D<sub>2</sub>O, 27 °C) of polysaccharide (PS-I), isolated from fruits of *Psidium guajava*.

meric proton assignments, the anomeric carbon signals were readily assigned from the HMQC spectrum. Signals at  $\delta$  105.3, 101.0, and 100.4 were assigned for anomeric carbons of **A**, **B**, and **C** residues, respectively. All the <sup>1</sup>H and <sup>13</sup>C signals were assigned using DQF-COSY, TOCSY, HMQC, and HMBC (Fig. 4, Table 3) experiments. The proton coupling constants were measured from DQF-COSY experiments.

Residue **A** was assigned to *L*-Arap as it showed two H-5 signals at  $\delta$  4.09 and  $\delta$  3.87. The anomeric proton chemical shift for moiety **A** at  $\delta$  5.06 ( $J_{H-1,H-2} \sim 7.7$  Hz) and  $J_{H-1,C-1} \sim 160$  Hz indicated that *L*-arabinopyranosyl residue was  $\alpha$ -linked. The anomeric carbon signal of residue **A** at 105.3 ppm was confirmed by the presence of a cross-peak (**A** C-1, **C** H-2) in the HMBC experiment (Fig. 4, Table 3). The appearance of inter-residual coupling between methoxy proton ( $\delta$  3.74) and C-2 atom ( $\delta$  75.5) of residue **A** [OCH<sub>3</sub> (H), **A** C-2] as well as methoxy carbon ( $\delta$  61.8) and H-2 atom ( $\delta$  3.67) of residue **A** [OCH<sub>3</sub> (C), **A** H-2] in the HMBC spectrum (Fig. 4, Table 3), indicated that one methoxy group was linked at C-2 of residue **A**. The C-4 signal of residue **A** at  $\delta$  79.6 showed a down field shift compared to that of standard methyl glycosides<sup>20,21</sup> due to the effect of glycosylation indicating that it was (1→4)-linked. The carbon signals of residue **A** at  $\delta$  75.5, 72.9, and 68.8 corresponded to C-2, C-3, and C-5, respectively. These results indicated that residue **A** was (1→4)-linked 2-*O*-Me- $\alpha$ -*L*-arabinopyranose moiety.

The anomeric signal of residue **B** at  $\delta$  5.06 ( $J_{H-1,H-2} \sim 3.1$  Hz) and  $J_{H-1,C-1} \sim 170$  Hz indicated that it was an  $\alpha$ -linked residue. A large  $J_{H-2,H-3}$  ( $\sim 8.9$  Hz) and relatively small coupling constant  $J_{H-3,H-4}$  ( $\sim 3.6$  Hz) for residue **B** indicated that it was a  $\alpha$ -*D*-galactosyl residue. The anomeric carbon chemical shift of moiety **B** at  $\delta$  101.0 was confirmed by the presence of a cross-peak (**B** C-1, **A** H-4) in the HMBC experiment (Fig. 4, Table 3). The presence of an acetyl group at C-2 position in residue **B** was confirmed by the appearance of a cross-peak between H-2 proton ( $\delta$  5.12) of residue **B** and acetyl carbonyl carbon ( $\delta$  171.8) [**B** H-2, OAc (C)] in HMBC experiment



**Figure 2.** <sup>13</sup>C NMR (125 MHz, D<sub>2</sub>O, 27 °C) spectrum of polysaccharide (PS-I), isolated from fruits of *Psidium guajava*.

**Table 1**

<sup>1</sup>H NMR<sup>a</sup> and <sup>13</sup>C NMR<sup>b</sup> chemical shifts of the polysaccharide (PS-I) isolated from fruits of *Psidium guajava* recorded in D<sub>2</sub>O at 27 °C

Sugar residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5a, H-5b/C-5	H-6a, H-6b/C-6	2-O-Me	COOMe	2-O-Ac
→4)-2- <i>O</i> -Me- $\alpha$ - <i>L</i> -Arap-(1→ <b>A</b>	5.06 105.3	3.67 75.5	3.77 72.9	4.11 79.6	4.09, 3.87 68.8		3.74 61.8		
→4)-2- <i>O</i> -Ac- $\alpha$ - <i>D</i> -Galp-(1→ <b>B</b>	5.06 101.0	5.12 69.1	3.96 71.5	4.19 79.6	4.35 72.9	3.82, 3.72 61.8			2.04 <sup>c</sup> 18.5 <sup>d</sup> , 171.8 <sup>e</sup>
→2)- $\alpha$ - <i>D</i> -GalpA6Me-(1→ <b>C</b>	4.93 100.4	3.64 78.5	4.06 69.1	4.26 71.5	4.43 74.3	171.8		3.78 53.8	

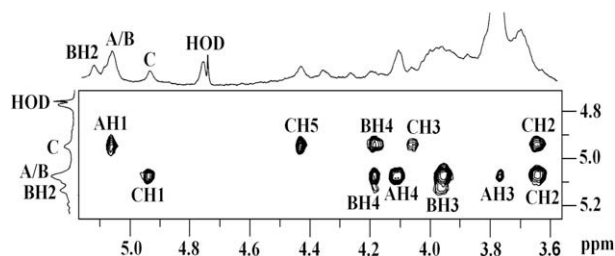
<sup>a</sup> The values of chemical shifts were recorded with respect to the HOD signal fixed at  $\delta$  4.74 ppm at 27 °C.

<sup>b</sup> Values of chemical shifts were recorded with reference to acetone as internal standard and fixed at  $\delta$  31.05 ppm at 27 °C.

<sup>c</sup> Value of the acetyl methyl proton.

<sup>d</sup> Value of the acetyl methyl carbon.

<sup>e</sup> Value of the acetyl carbonyl carbon.



**Figure 3.** The NOESY spectrum of the polysaccharide (PS-I), isolated from fruits of *Psidium guajava*. The NOESY mixing time was 300 ms.

(Fig. 4, Table 3). The down field shift of C-4 at  $\delta$  79.6 indicated that residue **B** was (1 $\rightarrow$ 4)-linked- $\alpha$ -D-galactopyranosyl. The carbon signals of residue **B** observed at  $\delta$  69.1, 71.5, 72.9, and 61.8 corresponded to C-2, C-3, C-5, and C-6, respectively. These indicated that residue **B** was (1 $\rightarrow$ 4)-linked 2-O-Ac- $\alpha$ -D-galactose.

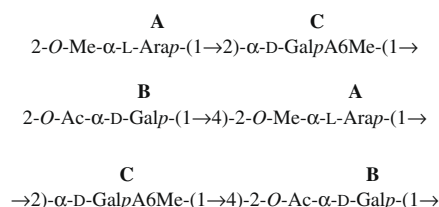
The spin-system of residue **C** consisted of only five protons with a high chemical shift of the H-5 signal ( $\delta$  4.43) and weak coupling between H-3, H-4, and H-5 indicated that residue **C** was D-GalpA. The anomeric signal for moiety **C** at  $\delta$  4.93 ( $J_{H-1,H-2} \sim 3.2$  Hz) and  $J_{H-1,C-1} \sim 171$  Hz indicated that D-galacturonosyl residue was  $\alpha$ -linked. The C-1 signal of residue **C** at  $\delta$  100.4 was confirmed by the appearance of cross-peak (**C** C-1, **B** H-4) in the HMBC experiment (Fig. 4, Table 3). The down field shift of C-2 ( $\delta$  78.5) carbon signal indicated that residue **C** was a 2-linked  $\alpha$ -D-galacturonic acid moiety. The carboxyl group in galacturonic acid was present as methyl ester confirmed by the appearance of inter-residual coupling between ester carbonyl carbon ( $\delta$  171.8) and carboxy methyl proton ( $\delta$  3.78) [COOCH<sub>3</sub> (H), **C** C-6] in HMBC experiment (Fig. 4, Table 3). The carbon signals of residue **C** observed at  $\delta$  69.1, 71.5, 74.3, and 171.8 corresponded to C-3, C-4, C-5, and C-6 (ester carbonyl), respectively. These results indicated that residue **C** was the methyl ester of (1 $\rightarrow$ 2)-linked  $\alpha$ -D-galacturonic acid. The above discussion indicated that acetyl carbonyl carbon and ester carbonyl resonated at the same position.

The sequence of glycosyl residues of the polysaccharide was determined from NOESY (Fig. 3, Table 2) as well as ROESY experiments followed by confirmation by HMBC. The NOESY experiment showed inter-residual contacts from **A** H-1 to **C** H-1 and **C** H-2, **B** H-1 to **A** H-4, and **C** H-1 to **B** H-4 with other intra-residual contacts. Hence, the following sequences were established as

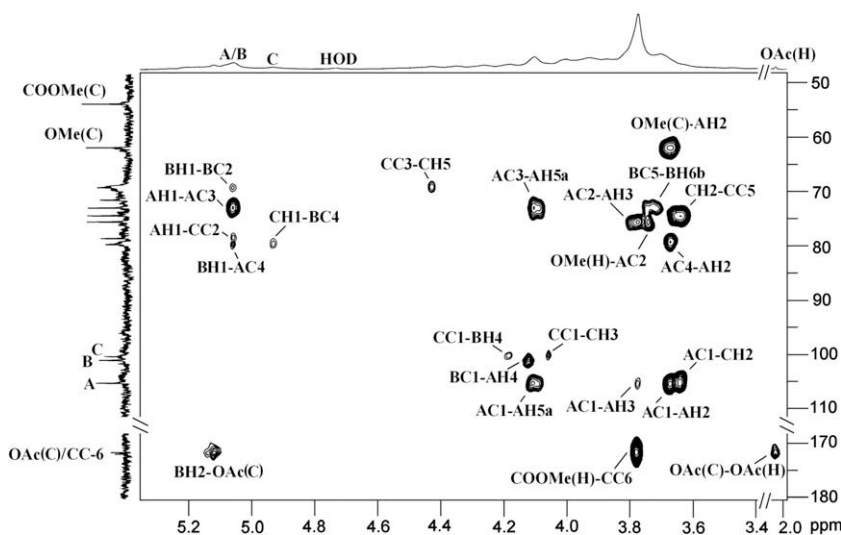
**Table 2**

The NOE data for the polysaccharide (PS-I) isolated from fruits of *Psidium guajava*

Anomeric proton	NOE contact protons		
Glycosyl residue	$\delta_H$	$\delta_H$	Residue, atom
$\rightarrow$ 4)-2-O-Me- $\alpha$ -L-Arap-(1 $\rightarrow$ ) <b>A</b>	5.06	3.77	<b>A</b> H-3
		4.11	<b>A</b> H-4
		4.93	<b>C</b> H-1
		3.64	<b>C</b> H-2
$\rightarrow$ 4)-2-O-Ac- $\alpha$ -D-Galp-(1 $\rightarrow$ ) <b>B</b>	5.06	4.11	<b>A</b> H-4
		3.96	<b>B</b> H-3
		4.19	<b>B</b> H-4
$\rightarrow$ 2)- $\alpha$ -D-GalpA6Me-(1 $\rightarrow$ ) <b>C</b>	4.93	5.06	<b>A</b> H-1
		4.19	<b>B</b> H-4
		3.64	<b>C</b> H-2
		4.06	<b>C</b> H-3
		4.43	<b>C</b> H-5



The sequence of the glycosyl residues in PS-I had been confirmed by the HMBC experiment (Fig. 4, Table 3) which showed cross peaks of both the anomeric proton and carbon of each of the sugar moieties were examined, and both intra- and inter-residual connectivities were observed (Fig. 4, Table 3). Cross-peaks were observed between H-1 ( $\delta$  5.06) of residue **A** and C-2 ( $\delta$  78.5) of residue **C** (**A** H-1, **C** C-2), C-1 of residue **A** ( $\delta$  105.3) and H-2 ( $\delta$  3.64) of residue **C** (**A** C-1, **C** H-2). The cross-peaks between H-1 ( $\delta$  5.06) of residue **B** and C-4 ( $\delta$  79.6) of residue **A** (**B** H-1, **A** C-4), C-1 ( $\delta$  101.0) of residue **B** and H-4 ( $\delta$  4.11) of residue **A** (**B** C-1, **A** H-4) were observed. Similarly, the cross-peaks between H-1 ( $\delta$  4.93) of residue **C** and C-4 ( $\delta$  79.6) of residue **B** (**C** H-1, **B** C-4), C-1 ( $\delta$  100.4) of residue **C** and H-4 ( $\delta$  4.19) of residue **B** (**C** C-1, **B** H-4) were observed. Intra-residue couplings between C-1 of residue **A** with its own H-2 (**A** C-1, **A** H-2), H-3 (**A** C-1, **A** H-3), and H-5a (**A** C-1, **A**



**Figure 4.** HMBC spectrum of polysaccharide (PS-I), isolated from fruits of *Psidium guajava*. The delay time in the HMBC experiment was 80 ms.

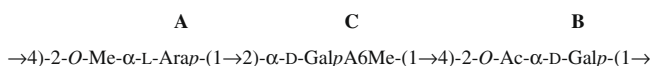
**Table 3**

The significant  $^3J_{\text{H,C}}$  connectivities observed in an HMBC spectrum for the anomeric protons/carbons of the sugar residues of the polysaccharide (PS-I) isolated from fruits of *Psidium guajava*

Residue	Sugar linkage	H-1/C-1 $\delta_{\text{H}}/\delta_{\text{C}}$	Observed connectivities		
			$\delta_{\text{H}}/\delta_{\text{C}}$	Residue	Atom
<b>A</b>	$\rightarrow 4$ )-2-O-Me- $\alpha$ -L-Arap-(1 $\rightarrow$	5.06	78.5	<b>C</b>	C-2
			72.9	<b>A</b>	C-3
			3.64	<b>C</b>	H-2
			3.67	<b>A</b>	H-2
			3.77	<b>A</b>	H-3
<b>B</b>	$\rightarrow 4$ )-2-O-Ac- $\alpha$ -D-Galp-(1 $\rightarrow$	5.06	4.09	<b>A</b>	H-5a
			79.6	<b>A</b>	C-4
			69.1	<b>B</b>	C-2
			4.11	<b>A</b>	H-4
			101.0	<b>A</b>	H-4
<b>C</b>	$\rightarrow 2$ )- $\alpha$ -D-GalpA6Me-(1 $\rightarrow$	4.93	79.6	<b>B</b>	C-4
			100.4	<b>B</b>	H-4
			4.19	<b>B</b>	H-4
			4.06	<b>C</b>	H-3
			2-O-Me $\delta_{\text{H}}/\delta_{\text{C}}$	Observed connectivities	
<b>A</b>	$\rightarrow 4$ )-2-O-Me- $\alpha$ -L-Arap-(1 $\rightarrow$	3.74	75.5	<b>A</b>	C-2
			61.8	<b>A</b>	H-2
			2-O-Ac $\delta_{\text{C}}$	Observed connectivities	
			$\delta_{\text{H}}$	Residue	Atom
			5.12	<b>B</b>	H-2
<b>B</b>	$\rightarrow 4$ )-2-O-Ac- $\alpha$ -D-Galp-(1 $\rightarrow$	171.8	COOMe $\delta_{\text{H}}$	Observed connectivities	
			$\delta_{\text{C}}$	Residue	Atom
			171.8	<b>C</b>	C-6
<b>C</b>	$\rightarrow 2$ )- $\alpha$ -D-GalpA6Me-(1 $\rightarrow$	3.78			

H-5a) were observed. Other intra-residual cross-couplings (**A** H-1, **A** C-3), (**A** H-2, **A** C-4), (**A** C-2, **A** H-3), (**A** C-3, **A** H-5a), (**B** H-1, **B** C-2), (**B** C-5, **B** H-6b), (**C** C-1, **C** H-3), (**C** H-2, **C** C-5), and (**C** C-3, **C** H-5) were observed. Inter-residual couplings between methoxy proton ( $\delta$  3.74) and C-2 ( $\delta$  75.5) of residue **A** [ $\text{OCH}_3$  (H), **A** C-2] as well as methoxy carbon ( $\delta$  61.8) and H-2 ( $\delta$  3.67) of residue **A** [ $\text{OCH}_3$  (C), **A** H-2] were observed. Inter-residual coupling between H-2 proton ( $\delta$  5.12) of residue **B** and acetyl carbonyl carbon ( $\delta$  171.8) [**B** H-2, **OAc** (C)] and also inter-residual coupling between acetyl methyl proton ( $\delta$  2.04) and acetyl carbonyl carbon ( $\delta$  171.8) [**OAc** (H), **OAc** (C)] were found. Similarly, cross-peak between carboxy methyl proton ( $\delta$  3.78) and ester carbonyl carbon ( $\delta$  171.8) of residue **C** [ $\text{COOCH}_3$  (H), **C** C-6] was observed.

Thus, based on all these chemical and spectroscopic evidence, the structure of the repeating unit of the polysaccharide (PS-I) was established as



## 1. Experimental

### 1.1. Isolation and purification of the polysaccharide

The fresh fruits of *Psidium guajava* (Guava) were washed with water and boiled with distilled water for 6 h. The whole mixture was then kept overnight at 4 °C and then filtered through linen cloth. The filtrate was centrifuged at 4 °C. The supernatant was precipitated with 1:5 (v/v) EtOH. The precipitated material was collected through centrifugation and dissolved in water followed by dialysis through a cellulose bag (Sigma–Aldrich, retaining  $> M_w$  12,400) to remove low molecular weight materials. The whole solution was then centrifuged at 8000 rpm (using a Heraeus Bio-fuge stratos centrifuge) at 4 °C. The residue was rejected, and the

filtrate (water-soluble part) was freeze dried, yielding 2.1 g of crude polysaccharide. The crude polysaccharide was then allowed to dissolve in 1% acetic acid and heated in a water bath for 90 min to give two fractions; an acetic acid soluble fraction and an acetic acid insoluble fraction. The acetic acid soluble fraction was separated from the acetic acid insoluble fraction by centrifugation, and the acetic acid soluble fraction was precipitated with 1:5 (v/v) EtOH. The precipitated polysaccharide was collected by centrifugation, dialyzed and freeze-dried through cellulose bag, and freeze dried, yielding 1.5 g of acetic acid soluble fraction. The acetic acid soluble fraction (30 mg) was purified by gel permeation chromatography on a column (90  $\times$  2.1 cm) of Sepharose 6B with water as eluant (0.4 mL min $^{-1}$ ) using a Redifrac fraction collector. 95 Test tubes (2 mL each) were collected, and monitored spectrophotometrically at 490 nm with phenol–sulfuric acid reagent<sup>22</sup> using a Shimadzu UV–vis spectrophotometer, model-1601. Two homogeneous fractions, test tubes number PS-I (27–38) and PS-II (42–52) were obtained. Then PS-I (yield 10 mg) was collected. This purification process was carried out several times.

## 1.2. Monosaccharide analysis

### 1.2.1. Alditol acetate analysis

PS-I (3.0 mg) was hydrolyzed with 2 M  $\text{CF}_3\text{COOH}$  (2 mL) in a round-bottomed flask at 100 °C for 18 h in a boiling water bath. The excess of acid was completely removed by co-distillation with water. Then the hydrolyzed product was divided into two parts. One part was examined by paper chromatography in solvent systems X and Y. Another part was reduced with  $\text{NaBH}_4$  (9 mg), followed by acidification with dilute  $\text{CH}_3\text{COOH}$ , and then co-distilled with pure  $\text{CH}_3\text{OH}$  to remove excess boric acid. The reduced sugars (alditols) were acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h to give the alditol acetates, which were analyzed by GLC using column (A) 3% ECNSS-M on Gas Chrom Q (100–120 mesh) and column (B) 1% OV-225 on Gas Chrom Q (100–120 mesh) at 170 °C. Gas-liquid chromatography–mass spectrometric (GLC–MS) analysis was also performed on Hewlett–Packard 5970A automatic GLC–MS system, using an HP-5 capillary column (25 m  $\times$  25  $\mu\text{m}$ ). The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C min $^{-1}$  up to a final temperature of 200 °C. Quantitation was carried out from the peak area, using response factors from standard monosaccharides.

### 1.2.2. Preparation of carboxyl-reduced polysaccharide<sup>14</sup>

PS-I (1.0 mg) was dissolved in 1 M imidazole–hydrochloric acid buffer, pH 7.0 (200  $\mu\text{L}$ /mg), and cooled on ice.  $\text{NaBH}_4$  (40 mg) was then added and reacted on ice for at least 1 h. The excess  $\text{NaBH}_4$  was decomposed by adding glacial acetic acid (100  $\mu\text{L}$ /40 mg  $\text{NaBH}_4$ ) slowly to the cooled sample. An equal volume of redistilled water was then added, and the reduced polysaccharide was precipitated by adding 3–4 volumes of 95% (v/v) ethanol (2 mL). The sample was reprecipitated two more times with 95% ethanol and freeze dried. The carboxyl-reduced polysaccharide was divided into two portions. One portion was converted to alditol acetates and another portion was methylated using the Ciucanu and Kerek method.<sup>15</sup>

## 1.3. Methylation analysis

PS-I (4.0 mg) was methylated using the Ciucanu and Kerek method<sup>15</sup> using distilled DMSO and finely powdered dry NaOH. The methylated products were isolated by partition between  $\text{CHCl}_3$  and  $\text{H}_2\text{O}$  (5:2, v/v). The organic layer containing products was washed with 3 mL water for three times and dried. The methylated products were then hydrolyzed with 90% formic acid (1 mL) at



100 °C for 1 h, reduced with sodium borohydride, acetylated with (1:1) acetic anhydride–pyridine, and analyzed by GLC (using columns A and B) and GLC–MS (using HP-5 fused silica capillary column) using the same temperature program indicated above. A portion of the methylated polysaccharide (2.0 mg) was dissolved in dry THF (2 mL), refluxed with  $\text{LiAlH}_4$ <sup>17</sup> (40 mg) for 5 h, and kept overnight at room temperature. The excess of the reductant was decomposed by dropwise addition of ethyl acetate and aqueous THF. The inorganic materials were filtered off. The filtrate was evaporated to dryness giving the carboxyl-reduced product. The carboxyl-reduced methylated product was hydrolyzed with formic acid as before, and the alditol acetates of the reduced methylated sugars were prepared in the usual way and analyzed by GLC and GLC–MS experiments. CMC (1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimide metho-*P*-toluenesulfonate)-reduced polysaccharide was also methylated following the same procedure and analyzed.

#### 1.4. Periodate oxidation<sup>18,19</sup>

PS-I (5 mg) was oxidized with 0.1 M sodium metaperiodate (2 mL) at 27 °C in the dark during 48 h. The excess periodate was destroyed by adding 1, 2-ethanediol, and the solution was dialyzed against distilled water. The dialyzed material was reduced with  $\text{NaBH}_4$  for 15 h and neutralized with acetic acid. The resulting material was obtained by co-distillation with methanol. The periodate-oxidized-reduced material was divided into two portions. One portion was hydrolyzed with 2 M  $\text{CF}_3\text{COOH}$  at 100 °C for 18 h, and this hydrolyzate material was used for paper chromatographic examination as well as alditol acetate preparation as usual. Another portion was methylated by the Ciucanu and Kerek method,<sup>15</sup> and alditol acetates were prepared. Alditol acetates were analyzed by GLC using columns A and B.

#### 1.5. Absolute configuration of monosaccharides

The method used was based on Gerwig et al.<sup>13</sup> PS-I (1.0 mg) was hydrolyzed with  $\text{CF}_3\text{COOH}$ , and then the acid was removed. A solution of 250  $\mu\text{L}$  of 0.625 (M) HCl in *R*-(+)-2-butanol was added and heated at 80 °C for 16 h. Then the reactants were evaporated and TMS-derivatives were prepared with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The products were analyzed by GLC using a capillary column SPB-1 (30 m  $\times$  0.26 mm), a temperature program (3 °C/min) from 150 to 210 °C. The 2,3,4,6-tetra-*O*-TMS-(+)-2-butylglycosides obtained were identified by comparison with those prepared from the *D* and *L* enantiomers of different monosaccharides.

#### 1.6. Optical rotation

Optical rotation was measured on a Jasco Polarimeter model P-1020 at 25 °C.

#### 1.7. Colorimetric estimations

Colorimetric estimations were carried out on a Shimadzu UV-vis spectrophotometer, model 1601.

#### 1.8. Paper chromatographic studies

Paper chromatographic studies were performed on Whatmann Nos. 1 and 3 mm sheets. Solvent systems used were (X)  $\text{BuOH}$ – $\text{HOAc}$ – $\text{H}_2\text{O}$  (v/v/v, 4:1:5, upper phase) and (Y)  $\text{EtOAc}$ –pyridine– $\text{H}_2\text{O}$  (v/v/v, 8:2:1). The spray reagent used was alkaline silver nitrate solution.<sup>12</sup>

### 1.9. Determination of molecular weight

The molecular weight of PS-I was determined by a gel-chromatographic technique. Standard dextrans<sup>11</sup> T-200, T-70, and T-40 were passed through a Sepharose 6B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. The elution volume of PS-I was then plotted in the same graph, and molecular weight of polysaccharide was determined.

#### 1.10. GLC experiments

All gas liquid chromatography experiments were performed on a Hewlett–Packard Model 5730 A gas chromatograph having a flame ionization detector and glass columns (1.8 m  $\times$  6 mm) packed with 3% ECNSS-M (A) on Gas Chrom Q (100–120 mesh) and 1% OV-225 (B) on Gas Chrom Q (100–120 mesh). All GLC analyses were performed at 170 °C.

#### 1.11. GLC–MS experiments

All the GLC–MS experiments were carried out in a Hewlett–Packard 5970 MSD instrument using HP-5-fused silica capillary column. The program was isothermal at 150 °C; hold time 2 min, with a temperature gradient of 4 °C  $\text{min}^{-1}$  up to a final temperature of 200 °C.

#### 1.12. NMR studies

PS-I was kept over  $\text{P}_2\text{O}_5$  in vacuum for several days, and then exchanged with deuterium<sup>23</sup> by lyophilizing with  $\text{D}_2\text{O}$  (99.96% atom  $^2\text{H}$ , Aldrich) for four times. With a Bruker Avance DPX-500 spectrometer,  $^1\text{H}$ , TOCSY, DQF-COSY, NOESY, ROESY, and HMBC NMR spectra were recorded in  $\text{D}_2\text{O}$  at 27 °C. The  $^1\text{H}$  and  $^{13}\text{C}$  (both  $^1\text{H}$ -coupled and decoupled) NMR spectra were recorded at 27 °C. The  $^1\text{H}$  NMR spectrum was recorded by suppressing the HOD signal (fixed at  $\delta$  4.74) using the WEFT pulse sequence.<sup>24</sup> The 2D-DQF-COSY experiment was carried out using standard BRUKER software at 27 °C. The TOCSY experiment was recorded at mixing time of 150 ms, and complete assignment required several TOCSY experiments having mixing times ranging from 60 to 300 ms. The NOESY and ROESY mixing delay was 300 ms. The  $^{13}\text{C}$  NMR spectrum of the polysaccharide solution in  $\text{D}_2\text{O}$  was recorded at 27 °C using acetone as internal standard, fixing the methyl carbon signal at  $\delta$  31.05. The delay time in the HMBC experiment was 80 ms.

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