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STRUCTURAL ANALYSIS OF PAPAYA POLYSACCHARIDE II

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ABSTRACT

The structure of papaya polysaccharide II (PP II) isolated by Chandrasekaran *et al.* [*Carbohydr. Res.*, 60 (1978) 105–115] has been investigated by methylation analysis of the carboxyl-reduced polymer and by partial hydrolysis of both the intact (arabinose, 31.0; rhamnose, 13.3; galactose, 42.6; glucuronic acid, 10.3; and 4-*O*-methylglucuronic acid, 2.8%), and carboxyl-reduced polymers. Methylation analysis of carboxyl-reduced PP II indicated a very highly branched structure in which approximately 39% of the galactopyranose units are disubstituted, 24% are monosubstituted, 20% are trisubstituted, and 17% are nonreducing end units. Methylation analysis of products of partial hydrolysis of both intact and carboxyl-reduced polymers indicated that the backbone of the polysaccharide is made up of galactosyl residues substituted at either O-3 or -6, that the principal aldobiouronic acid fragment is 6-*O*-(glucopyranosyluronic acid)galactose, that the rhamnosyl units are substituted at O-3 with either terminal arabinofuranosyl or galactopyranosyl groups, and that the rhamnosyl residues are themselves linked to glucuronic acid residues through O-4. From this information, a possible statistical fragment with six arabinofuranose and two galactopyranose nonreducing end-groups per 19 sugar units [five units in the main chain of (1→3)-linked galactopyranose units] is proposed.

INTRODUCTION

Two acidic polysaccharides were isolated and identified as contaminants in crude papain (Sigma type II) by Chandrasekaran *et al.*[1]. These polysaccharides were labelled PP (papya polysaccharide) I and PP II, a designation of their order of elution by a salt gradient from DEAE-Sephadex columns. Other samples of crude papain contained additional polysaccharides. All these polysaccharides appeared to belong to a family, the individual members of which differed from

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each other primarily in charge density, *i.e.*, in the amount of uronic acid. The monosaccharide compositions of the two polysaccharides were found to be very similar, consisting of the same sugar units, but differing slightly in the relative amounts of each sugar present, especially in the relative amounts of uronic acid.

The papaya polysaccharides were reported to have some interesting biological properties. When applied to normal, human diploid-fibroblast cells, they effected an aggregation and alignment of cells, and a reduction in growth and in nuclear area; but they had no effect on aneuploid, mouse embryo-fibroblast cells which do not exhibit contact inhibition of growth or movement.

Cell surfaces contain glycoproteins and glycolipids. There is considerable indirect evidence that the carbohydrate residues of these molecules that constitute the glycocalyx are involved in intercellular recognition and adhesion. Because PP I and PP II affect aggregation of fibroblast cells, it is possible that an elucidation of their structure might shed some light on the mechanism of this cell–cell recognition and adhesion, particularly on the nature of the carbohydrate residues involved.

Papain is obtained from the latex of green fruit and leaves of *Carica papaya* plants. Although much work has been done on the isolation of papain from papaya and on the sugar content of the fruits during development, little has been done on the polysaccharides of papaya. In the only previous paper on papaya polysaccharides, Biswas and Rao[2] reported the isolation of a galactan from unripe papaya fruits; this galactan contained, in addition to D-galactose, a considerable proportion of D-galacturonic acid, a small proportion of L-arabinose, and traces of L-rhamnose. This study was undertaken to determine the structure of PP II.

RESULTS AND DISCUSSION

PP II was isolated by the procedure described by Chandrasekaran *et al.*[1]. About 1.5 g of a mixture of polysaccharides was obtained from 100 g of crude papain *via* fractionation on a DEAE-cellulose column; about 0.7 g of pure PP II was obtained from the mixture. Although the amount of PP II isolated varied, it was always the most abundant of the polysaccharides present in every lot examined by us. The relative amounts of the other polysaccharides present varied considerably from one lot to another.

The optimum hydrolysis time for PP II in 0.5M sulfuric acid at 100° was found to be 6 h. Chromatography of the neutralized, de-ionized hydrolyzate revealed the presence of five major components, three monosaccharides (rhamnose, arabinose, and galactose) and two uronic acid-containing oligosaccharides. The uronic acid-containing fragments were separated from the neutral sugars by adsorption onto anion-exchange resins and purified by preparative paper chromatography. The monosaccharides were characterized by g.l.c. of their trimethylsilyloxime[3] and peracetylated aldononitrile (PAAN)[4,5] derivatives. The presence of small amounts of glucuronic acid and glucuronolactone was also evident on paper chromatograms of the PP II hydrolyzate.

Two aldobiouronic acids isolated by preparative paper chromatography from a 4-h hydrolyzate of PP II (UA1 and UA2, UA1 being the major component and the component with the lower R_F value) were reduced and a portion of each was hydrolyzed; the monosaccharides obtained were converted into PAAN derivatives. Another portion of each was methylated before hydrolysis; and partially methylated alditol acetate (PMAA) derivatives[6] of the products of hydrolysis were prepared. G.l.c. of both PAAN and PMAA derivatives showed that glucose and galactitol only were present in both reduced aldobiouronic acids and that they were present in equimolar amounts. Thus, the reducing end-unit of both disaccharides is galactose. In addition, both the retention times and the mass-spectral fragmentation patterns of PMAA derivatives of the sugar components of UA1 were identical with those of UA2; the fragmentation pattern for the component that was eluted first is identical with that of 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methylgalactitol and that of the component that was eluted last is identical with the fragmentation pattern of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. Both methylated components were obtained in equimolar amounts from both UA1 and UA2. Thus, it is clear that the uronic acid at the nonreducing terminus of the major aldobiouronic acid is glucuronic acid; that of the minor aldobiouronic acid, which gives the same methylated product, is presumed to be 4-*O*-methylglucuronic acid*.

Hydrolysis of carboxyl-reduced[1] PP II (PP II-R) in 0.5M sulfuric acid at 100° was complete after 4 h. G.l.c. of PAAN derivatives of the components of neutralized hydrolyzates indicated the presence of only monosaccharides, *viz.*, rhamnose, arabinose, galactose, and glucose. The presence of glucuronic acid in the original polysaccharide was indicated by the fact that glucose was absent from PP II hydrolyzates and present in the hydrolyzates of PP II-R. A minor uronic acid component was presumed to be 4-*O*-methylglucuronic acid*.

PP II-R was partially hydrolyzed in 0.05M sulfuric acid at 100° for 2 h. Chromatography of the hydrolyzate indicated the presence of rhamnose, arabinose, and small proportions of galactose, a disaccharide composed of equimolar amounts of galactose and arabinose, and a polymeric material that remained at the origin (PP II-RA). A portion of PP II-RA, isolated by preparative paper chromatography, was hydrolyzed; the hydrolysis products were converted into PAAN derivatives and subjected to g.l.c. (Table I).

Attempted methylation analysis of PP II was unsuccessful because methylation resulted in the formation of the products of base-catalyzed degradation of uronic acid-containing polysaccharides[7]. Therefore, methylation was restricted to PP II-R and fragments obtained from PP II-R by partial hydrolysis. Complete

*Present in all hydrolyzates was a minor uronic acid component that comprised <3% of the parent polysaccharide. Because this component was present in such small proportions, it could not be rigorously identified. However, primarily because 2,3,4,6-tetra-*O*-methylglucitol was formed from it in both PP II and UA2 by reduction, methylation, hydrolysis, and reduction, the component is presumed to be a partially methylated glucuronic acid, probably 4-*O*-methyl-D-glucuronic acid, based on the composition of other plant arabinogalactans. This suggestion is consistent with other data.

TABLE I

MOLAR PROPORTIONS OF MONOSACCHARIDE COMPONENTS CALCULATED FROM INTEGRATION OF THE PAAN DERIVATIVE PEAKS

Polysaccharide fraction	*Moles (%)*				
	Rha	*Ara*	*Gal*	*Glc*	*Unknown*[a]
PP II	17.5	40.1	42.4	0	0
PP II-R	13.3	31.1	42.6	10.3	2.78
PP II-RA	13.3	18.9	55.8	11.9	0

[a]Probably 4-*O*-methylglucuronic acid.

methylation of PP II-R was confirmed by the absence of a hydroxyl group absorption in the i.r. spectrum. G.l.c. of PMAA derivatives formed from the hydrolyzates of methylated PP II-R (MPP II-R) indicated eleven components that were identified by their retention times and mass spectral fragmentation patterns[8,9]

Two oligosaccharides, of the several obtained after treatment of PP II-R with 0.5M sulfuric acid at 100° for 2 h, were isolated by preparative paper chromatography and subjected to methylation analysis. One oligosaccharide (apparently a trisaccharide) contained only components *D* and *H* (Table II) in a mole ratio of 1:2. The composition of the other (MP 1, apparently an octasaccharide) is given in Table II.

Methylated sugars released upon hydrolysis of completely methylated PP II-RA (MPP II-RA) were also reduced and acetylated. G.l.c. of the PMAA derivatives so produced indicated the presence of ten components (Table II).

From methylation analysis, it was evident that all arabinose residues present

TABLE II

PARTIALLY METHYLATED ALDITOL ACETATES (PMAA) DERIVED FROM HYDROLYZATE COMPONENTS[a]

Component[b]	*Parent methylated alditol*	*Methylated fractions*		
		MPP II-R	*MP 1*	*MPP II-RA*
A	2,3,5-Tri-*O*-methylarabinitol	31.7		17.8
B	2,3,4-Tri-*O*-methylarabinitol	1.7		1.1
C	2,4-Di-*O*-methylrhamnitol	10.4		6.8
D	2,3,4,6-Tetra-*O*-methylglucitol	2.7	21.1	7.5
E	2,3,4,6-Tetra-*O*-methylgalactitol	9.0	13.0	6.7
F	2,4,6-Tri-*O*-methylgalactitol	1.7	10.5	2.0
G	2,3,6-Tri-*O*-methylglucitol	10.1		9.0
H	2,3,4-Tri-*O*-methylgalactitol	0.9	38.3	8.1
I	2,6-Di-*O*-methylgalactitol	1.5		
J	2,4-Di-*O*-methylgalactitol	19.6	11.2	28.1
K	2-*O*-Methylgalactitol	10.5		2.9

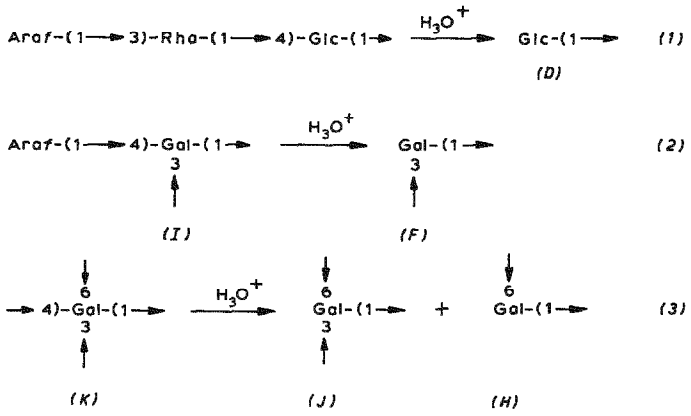
[a]Molar proportions (%) [b]In order of elution from an OV-225 g.l.c. column.

in PP II-R are in the form of nonreducing end-groups and, for the most part, in the furanose ring form (component *A*, Table I); only a few arabinose residues are in the pyranose ring form (component *B*). All rhamnose residues are mid-chain units substituted at O-3 (component *C*). Although most of the D-glucose units (derived from D-glucuronic acid units) are in the form of O-4 substituted chain-units (component *G*), a fraction of the glucose units appears as nonreducing end-groups (component *D*), probably derived from 4-*O*-methylglucuronic acid. Galactose, the most abundant component, is present as nonreducing end-groups (component *E*), mid-chain-units (components *F* and *H*), and branch points units [components *I*, *J*, and *K* (a doubly branched unit)]. The highly branched, compact structure thus indicated is also evidenced by the low viscosity of solutions of this polysaccharide[1].

Because PP II-R has such a large percentage of branch points and terminal arabinofuranose units, it was postulated that mild hydrolysis would remove the easily hydrolyzed furanoside end-groups without depolymerization, thus simplifying the structure. Mild hydrolysis did simplify the structure somewhat, although not as much as had been expected. The methylated product of hydrolysis, MPP II-RA, exhibited a decrease in the number of terminal arabinose units (Table II, components *A* and *B*), the disappearance of all O-3,4 disubstituted galactose units (component *I*, branch point), a decrease in the number of O-3,4,6 trisubstituted galactose units (component *K*, double branch point), and a decrease in the number of O-3 substituted rhamnose units (component *C*). Slight decreases were also apparent in the numbers of terminal galactose units (component *E*) and O-4 substituted glucose units (component *G*). Increases in the number of terminal glucose units (component *D*), O-6 substituted galactose units (component *H*), and O-3,6 disubstituted galactose units (component *J*, branch point) were observed. Based on these changes in the relative amounts of the components in MPP II-RA as compared to MPP II-R, a number of postulates could be made. Because the terminal glucose units increased by about the same amount that the O-3 substituted rhamnose units decreased, it can be hypothesized that these two sugar residues are directly linked; and because rhamnose units are not end-groups, it can be hypothesized that terminal arabinose or galactose units are linked to them because they are the only end-units lost during mild hydrolysis.

The increase in terminal glucose units along with the decrease in O-3 substituted rhamnose units and some terminal arabinose or terminal galactose units may be accounted for by Eq. *1*. Eq. *2* may account for the small increase in O-3 substituted galactose units. Eq. *3* may account for the decrease in doubly branched units (component *K*) and the increases in both singly branched units (component *J*) and the O-6 substituted galactose residues (component *H*). An increase in O-6 substituted galactose residues could also have resulted from hydrolysis of the O-3 bond of some O-3,6 disubstituted branch point units (component *J*) (see Scheme 1).

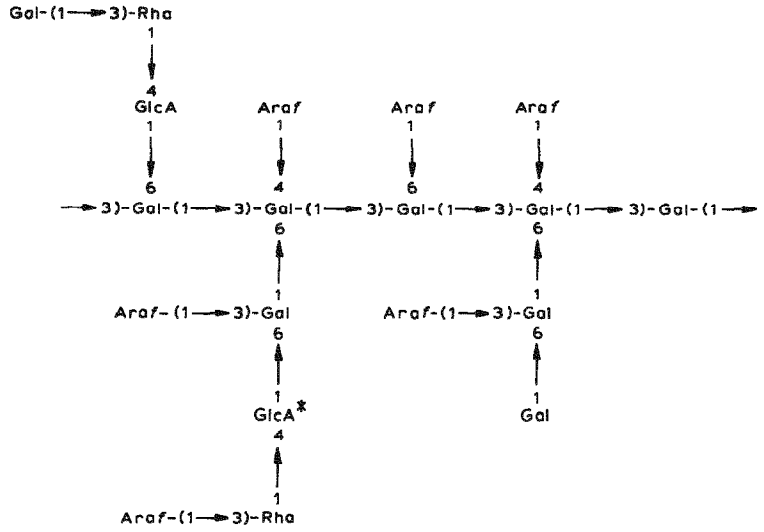
Fragments from more extensive partial hydrolysis provided the information required to deduce the sequence of sugars in PP II-R. Methylation analysis of one fragment indicated that it was a disaccharide consisting of a terminal glucosyl group



Scheme 1.

(component *D*) linked to O-6 of a galactose residue (component *H*), thus confirming that the glucuronic acid residues in PP II are linked to O-6 of the galactosyl residues. Methylation analysis of the isolated and reduced aldobiouronic acid confirmed that it is indeed GlcA-(1→6)-Gal, for only two components were observed, *viz.*, terminal glucose and O-6 substituted galactitol.

From this information, a possible statistical structure consistent with the data could be proposed for PP II, recognizing of course that PP II is undoubtedly both polymolecular and polydisperse as are all known polysaccharides of this type.



Scheme 2. Possible statistical fragment of papaya polysaccharide II. The GlcA unit marked by an asterisk has probably a CH_3O-4 group.

EXPERIMENTAL

Materials. — Papain (Sigma Chemical Co., type II), standard sugars, DEAE-

cellulose, and 3-(3-diethylaminopropyl)-1-ethylcarbodiimide hydrochloride were obtained from Sigma. Dialysis membrane Spectra/por. 6 with a mol. wt. cutoff of 50 000 was obtained from Fisher Scientific Co. All other chemicals used were of reagent grade or the purest available.

PP II was isolated by the procedure of Chandrasekaran *et al.*[1]. Fractions from the DEAE-cellulose column were analyzed for the presence of uronic acids[10]. Plots of fraction number *vs.* absorbance at 520 nm, and fraction number *vs.* conductance were made to determine the fractions that contained PP II, which were pooled, dialyzed exhaustively, concentrated to a small volume, and lyophilized to give pure PP II; yield 0.68 g from 100 g of crude papain.

Reduction of PP II was achieved by the method described by Taylor and Conrad[11]. Uronic acid-containing fragments were reduced as described for PP II, except that excess borohydride was removed by addition of Dowex 50 (H^+) cation-exchange resin. After filtration to remove the resin, the filtrate was concentrated to dryness at 50° under reduced pressure several times with MeOH to remove borate ions as methyl borate.

Hydrolyses. — To determine the optimum hydrolysis time for PP II, PP II (63 mg) was dissolved in 0.5M H_2SO_4 (45 mL). Portions (2.0 mL) were placed in each of 21 centrifuge tubes which were then capped and heated in a boiling-water bath. After 2 h, three tubes were withdrawn; three more tubes were withdrawn every hour thereafter until a total hydrolysis time of 8 h was reached. The contents of each tube were made neutral with solid $BaCO_3$, centrifuged, and filtered. The solid was washed twice with 1.0-mL portions of water; both washings and filtrate were combined and diluted to 5.0 mL with distilled water. Reducing sugar content of the tubes was determined by the Somogyi micro copper reduction method[12].

For complete hydrolysis of PP II and PP II-R, PP II or PP-II-R (75 mg) was dissolved in 0.5M H_2SO_4 (3.0 mL). The solution was heated at 100° for 6 h (determined to be the optimum hydrolysis time). The hot solutions were made neutral with solid $BaCO_3$ (pH 6), filtered, de-ionized with Dowex 50 (H^+) cation-exchange resin, passed through a column (8 × 260 mm) of Amberlite IR-45 (OH^-) anion-exchange resin to remove the uronic acid-containing components, and evaporated to dryness under reduced pressure at 50°.

Partial hydrolysis was achieved by heating PP II-R in 0.5M H_2SO_4 at 100° for 2 h. The mixture was worked up as before, and the components were isolated by preparative descending paper chromatography. Partial hydrolysis with minimal mol. wt. reduction was achieved by heating PP II-R in 0.05M H_2SO_4 at 100° for 2 h.

Isolation of uronic acid residues in PP II. — PP II (500 mg) was heated at 100° in 0.5M H_2SO_4 (25 mL) for 4 h. After neutralization and de-ionization, the uronic acid-containing fragments were isolated by preparative descending paper chromatography on Whatman 3MM paper (24 h); yield of UA1 30 mg, yield of UA2 10 mg.

Methylation. — PP II-R was methylated as described for polysaccharides by Jansson *et al.*[9]. In the case of oligomeric material, the reaction mixture was not

dialyzed but instead diluted with five times its volume of water and extracted with four portions of chloroform. The chloroform extracts were washed with four portions of water, dried ($MgSO_4$) and evaporated to dryness under reduced pressure.

Acid-catalyzed hydrolysis of methylated PP II-R and transformation into partially methylated alditol acetates (PMAA). — Methylated PP II-R (MPP II-R; 50 mg) was treated with 88% formic acid (10 mL) at 100° for 1 h. The acid was removed under reduced pressure, the residue was dissolved in 0.13M H_2SO_4 (15 mL), and heating was continued at 100° overnight. Excess acid was neutralized with solid $BaCO_3$; the solids were removed by filtration and washed twice with 5-mL portions of water. Both washings and filtrate were combined and treated with Dowex 50 (H^+) cation-exchange resin to remove residual Ba^{2+} ions. The resins were removed by filtration, and the filtrate was concentrated to 5.0 mL at 50° under reduced pressure. $NaBH_4$ (500 mg) was carefully added, and the mixture was kept at 50° for 2 h. Excess $NaBH_4$ was removed by addition of Dowex 50 (H^+) cation-exchange resin, which was then removed by filtration. The filtrate was evaporated to dryness three times with 15-mL portions of methanol to remove borate ions as methyl borate.

Acetylation of the partially methylated alditols was achieved by treatment with 1:1 (v/v) pyridine–acetic anhydride (6 mL). After 1 h at 100°, the solvents were removed by evaporation under reduced pressure at 40° with the aid of toluene. The residue was dissolved in dichloromethane (5.0 mL). The solution was washed three times with 3-mL portions of water, dried ($MgSO_4$), concentrated to 1.0 mL, and subjected to g.l.c. on an OV-225 column.

Analytical techniques. — Paper chromatograms were irrigated with 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water in a tank saturated with vapors of the same mixture. Developed chromatograms were dried, sprayed with a 3–4% (w/v) solution of *p*-anisidine hydrochloride in 4:1:1 (v/v) butanol–ethanol–water containing a trace of $SnCl_2$ for stabilization, allowed to dry, and heated until the spots appeared (5 min).

To prepare trimethylsilylated oxime (Me_3Si-oxime) derivatives, neutral sugars (40 mg) of a PP II hydrolyzate were dissolved in a solution of hydroxylamine hydrochloride in pyridine (4.0 mL; 30 mg/mL). The solution was heated in a water bath at 75° for 0.5 h, then allowed to cool to room temperature before addition of hexamethyldisilazane (4.0 mL), followed by addition of trifluoroacetic acid (0.4 mL). After the white precipitate had settled, the clear supernatant was subjected to g.l.c. on a stainless-steel column (0.003 × 9 m) packed with Chromsorb W with a 3% (w/w) coating of SE 52. The column temperature was programmed at 2°/min from 100° to 250°; the injector port temperature was 200° and the detector temperature 290°.

Peracetylated aldononitrile (PAAN) derivatives were prepared by the method described by Lance and Jones[4]. Both PAAN and PMAA derivatives were chromatographed on stainless-steel columns (0.003 × 2.1 m) packed with Gas Chrom Q with a 3% (w/w) coating of OV-225. For PAAN derivatives, the column temperature was increased 2°/min from 190° to 215°, and held at 215° for a total run time of 20 min. For PMAA derivatives, the column temperature was increased 2°/min from 165° to 215°, and held at 215° for a total run time of 30 min. An injection-port temperature of 225° and a detector-block temperature of 240° was used for both derivatives.

Mass spectra were obtained by use of a computer-controlled Finnigan 3300 GC-MS system operated in the electron-impact mode.

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