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STUDIES ON A NEUTRAL POLYSACCHARIDE ISOLATED FROM BAEL (*Aegle marmelos*) FRUIT PULP

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ABSTRACT

The homogeneous, neutral polysaccharide isolated from the crude polysaccharide of the fruit pulp from bael (*Aegle marmelos*) contains arabinose, galactose, and glucose in the molar ratios of 2:3:14. The linkages among the different monosaccharide residues were established through methylation analysis and Smith-degradation studies of the polysaccharide. The anomeric configurations of the different glycosyl groups were determined by study of the chromium trioxide oxidation of the acetylated polysaccharide. Results of these experiments have been discussed in order to assess the structure of the neutral polysaccharide.

INTRODUCTION

The exudate gum[1] from bael (*Aegle marmelos*) trees, and the gummy material[2–4] surrounding the seeds of bael fruit, contain a common, backbone chain of (1→3)-linked, β-D-galactopyranosyl residues, but the side chains of the two gums were found to be different. Homogeneous carbohydrate materials in the fruit pulp have been studied in search of a molecule having a similar backbone chain, in order to examine the proposal[5] that the gums are formed by apposition of additional sugar groups to the outer chains of the polysaccharide already present in the plant. The results of investigations conducted on a homogeneous, neutral polysaccharide isolated from bael-fruit pulp are reported herein.

RESULTS AND DISCUSSION

The crude polysaccharide isolated from an ammonium oxalate extract of the powdered pulp of well-developed, but unripe, bael fruits by precipitation with ethanol had $[\alpha]_D^{23}$ +116° (in water). The light-brown material (polysaccharide A) contained galacturonic acid (53.50), galactose (12.70), glucose (19.37), arabinose (10.75), and rhamnose (2.15%). Under the same conditions, the aqueous extract of the fruit pulp yielded a polysaccharide (polysaccharide B, $[\alpha]_D^{23}$ +101°) containing the same monosaccharide residues. The presence of galacturonic acid in these polysaccharides

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was confirmed by paper chromatography using solvent *C*, and also by preparing carboxyl-reduced polysaccharides, hydrolyzing the products, and then analyzing the contents of the hydrolyzate by g.l.c. Both of these polysaccharide were highly heterogeneous.

In order to obtain a homogeneous polysaccharide, polysaccharide A was dispersed in water and treated with aqueous calcium chloride (5%) at pH 8.5. The calcium pectate precipitated was removed, and the supernatant liquor was treated with cold ethanol, to yield a precipitate of polysaccharide C, which showed $[\alpha]_D^{23}$ +53.5° and contained all of the monosaccharide residues present in polysaccharide A. Polysaccharide C was also heterogeneous (according to high-voltage, paper electrophoresis), and contained 47.2% of uronic acid. To achieve further fractionation, polysaccharide C was resolved on a column of DEAE-cellulose which was eluted successively with (*i*) water, and (*ii*) 1.0M sodium chloride solution, the eluates being monitored by means of 1-naphthol, to yield two polysaccharide fractions, polysaccharide D and polysaccharide E. Polysaccharide E was still heterogeneous, according to paper electrophoresis, and contained all of the monosaccharide residues in A or C. Polysaccharide D was found to be homogeneous by high-voltage electrophoresis in borate buffer (pH 9.28) and phosphate buffer (pH 7.8), and attempted resolution on a column of Sephadex G-100 failed, as the entire material was eluted as a single peak.

This pure and homogeneous polysaccharide D contained only arabinose (10.8), galactose (16.4), and glucose (71.4%); rhamnose and galacturonic acid were absent, as shown by p.c., g.l.c., and use of the carbazole–sulfuric acid reagent. Polysaccharide D was permethylated first by the Hakomori method[6], and then by the Purdie method[7] until the product showed no OH absorption band in its i.r. spectrum. The permethylated polysaccharide D, $[\alpha]_D^{23}$ +56°, was hydrolyzed, first

TABLE I

METHYL ETHERS OF SUGARS FROM THE HYDROLYZATES OF METHYLATED, NEUTRAL POLYSACCHARIDE

Sugars[a]	T[b]	*Approximate mole %*	*Mode of linkages*
2,3,5-Ara	0.43	4.85	Ara*f*-(1→
3,5-Ara	0.78	4.75	→2)-Ara*f*-(1→
2,3,4,6-Glc	1.00	4.89	Glc*p*-(1→
2,3,6-Glc	2.30	54.89	→4)-Glc*p*-(1→
2,3-Glc	4.47	11.91	→4,6)-Glc*p*-(1→
2,3,4,6-Gal	1.17	6.07	Gal*p*-(1→
2,3,6-Gal	2.21	5.17	→4)-Gal*p*-(1→
2,6-Gal	3.17	5.03	→3,4)-Gal*p*-(1→

[a]2,3,5-Ara = 2,3,5-tri-*O*-methylarabinose, *etc.* Traces of 2,5-Ara, 2,4,6-Gal, and 6-Glc were also detected. [b]Retention times of the corresponding alditol acetates, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on a 1% OV-225 column at 173°.

with 85% formic acid for 2 h at 100°, and then, after removal of the formic acid, with 0.5M sulfuric acid for 18 h at 100°. The acid was neutralized with barium carbonate, and the partially methylated sugars were converted into their alditol acetates, and these analyzed by g.l.c. using column (*b*). The results are shown in Table I.

These results gave an indication as to the linkages between the different monosaccharide residues in polysaccharide D. Identification of 2,3,5-tri-*O*-methylarabinose and of 2,3,4,6-tetra-*O*-methyl-glucose and -galactose indicated their presence as the nonreducing end-units in the methylated polysaccharide. In the interior part of the polysaccharide, both glucose and galactose are present as (1→4)-linked residues; of them, the former are preponderant, as a larger amount of 2,3,6-tri-*O*-methylglucose than of 2,3,6-tri-*O*-methylgalactose was found. The presence of 3,5-di-*O*-methylarabinose in the hydrolyzate of the methylated polysaccharide showed that arabinosyl groups are joined through (1→2) linkages. Branch points in the polysaccharide originate from both glucose and galactose. Identification of 2,3-di-*O*-methylglucose and 2,6-di-*O*-methylgalactose indicated that, at branch points, glucose residues are linked through O-1, O-4, and O-6, and galactose residues, through O-1, O-3, and O-4. Traces of 2,5-di-*O*-methylarabinose, 2,4,6-tri-*O*-methylgalactose, and 6-*O*-methylglucose, which were also detected in the hydrolyzate of methylated polysaccharide D, possibly play no significant role in the chemical architecture of the methylated macromolecule.

Smith-degradation studies[8] on polysaccharide D supported the data obtained from methylation analysis of the polysaccharide. The Smith-degraded products were converted into alditol acetates whose analysis by g.l.c. using column (*a*) indicated the presence of alditol acetates of threose, erythrose, arabinose, and galactose in the molar ratios of 1 : 10.8 : 1 : 1, together with glycerol (which could not be correctly estimated). The large amounts of erythritol originated from (1→4)-linked glucosyl residues, which were expected to yield ~13, rather than 10.8, molar proportions of erythritol; this discrepancy could not, however, be explained. The equimolar proportions of threitol, arabinose, and galactose originated from (1→4)-linked galactose, (1→2)-linked arabinose, and the branched galactosyl residues, respectively. In addition, a slight trace of glucose was detected in the Smith-degradation product;

TABLE II

SURVIVAL OF SUGARS[a] IN OXIDATION OF ACETYLATED, NEUTRAL POLYSACCHARIDE WITH CHROMIUM TRIOXIDE

Time (h)	myo-*Inositol*	*Glucose*	*Galactose*	*Arabinose*
0	10	365	49	31
1	10	149	0	29
2	10	147	0	19

[a]The sugars were analyzed, and estimated, by g.l.c. using column *a* at 190°.

this might have originated from the 6-*O*-methylglucose identified in the hydrolyzate of methylated polysaccharide D.

In order to ascertain the anomeric configurations of the different sugar residues, the acetylated polysaccharide D was subjected to oxidation with chromium trioxide[9,10] in acetic acid at 50°, for various intervals of time, using *myo*-inositol as the internal standard. The aliquots at different intervals were diluted with water, and the material extracted into chloroform, and deacetylated; the products were hydrolyzed, and the surviving sugars estimated by g.l.c. (as their alditol acetates). The results, shown in Table II, indicated that the galactosyl groups had the β configuration, whereas the glucosyl groups had both α and β configurations. Because acetylated furanoses are nonspecifically oxidized[11] by chromium trioxide, the anomeric configuration of the arabinosyl groups could not be determined by this experiment. In fact, the rate at which arabinose was hydrolyzed off with 5% formic acid during 2 h at 100° indicated that it possibly had the α configuration.

EXPERIMENTAL

General methods. — All evaporations were conducted at 40° (bath temperature) under diminished pressure. Small volumes of aqueous solutions were lyophilized. Paper partition-chromatography was performed by the descending technique, using Whatman No. 1 chromatographic paper, with the following solvent systems: (*A*) 8:2:1 ethyl acetate–pyridine–water, (*B*) 9:2:2 ethyl acetate–acetic acid–water, and (*C*) 5:5:1:3 ethyl acetate–pyridine–acetic acid–water. The spray reagents used were (*a*) alkaline silver nitrate, and (*b*) 4% pentaerythritol in 0.5M sodium hydroxide solution and silver nitrate solution in acetone.

All specific rotations were recorded with a Perkin–Elmer Model 241 MC spectropolarimeter at 23 ± 1° and 589.6 nm. A Shandon high-voltage electrophoresis apparatus, model L-24, was used for electrophoresis of materials in different buffers. Infrared spectra were recorded with a Beckman IR-20A instrument, and ultraviolet and visible spectra, with a Yanaco-SPI spectrophotometer. The alditol acetates of sugars were prepared as in the case of sugars obtained from exudate gum[1], and chloroform solutions thereof were injected into the g.l.c. apparatus. For gas–liquid chromatography, a Hewlett–Packard 5730A gas chromatograph with flame-ionization detector was used. Resolutions were performed in glass columns (1.83 m × 6 mm) containing (*a*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates of sugars), and (*b*) 1% of OV-225 on Gas Chrom Q (80–100 mesh) at 173° (for alditol acetates of partially methylated sugars).

Extraction of polysaccharide from fruit pulp. — Twenty well-grown, but unripe, bael fruits (average weight, 500 g) were cut up. Pulp was obtained by removing the rinds and seeds, along with the gummy envelope. The pulp was then macerated under ethanol in a blender. The pulpy material was filtered off with a piece of Nylon cloth, and air dried (yield 130 g). The powdered pulp (60 g) was extracted, with stirring, with 0.5% ammonium oxalate solution (1 L) for 4 h at 85°. The extract was

centrifuged at 6,000 r.p.m. for 30 min, and the clear, supernatant liquor was decanted. The same process was repeated once with the residue. The cold, supernatant liquors were combined, acidified with acetic acid (pH 5.40), and the contents precipitated with ethanol (3 vol.). The precipitate was collected by centrifugation, washed three times with ethanol, dissolved in water (600 mL), and reprecipitated with ethanol. The process of dissolution and precipitation was repeated once. The final precipitate was washed three times with ethanol, and dried *in vacuo*. The light-brown material (7.74 g) had $[\alpha]_D^{23}$ +116° (*c* 0.56, water). Another batch of polysaccharide was extracted from the powdered pulp (30 g) with water (750 mL) for 4 h at 85°. Polysaccharide (polysaccharide B) was isolated by following the same procedure as just described; yield 5.5 g; $[\alpha]_D^{23}$ +101° (*c* 0.6, water).

Hydrolysis and sugar analysis of the polysaccharides. — The polysaccharides (10 mg) were hydrolyzed with M sulfuric acid for 20 h on a boiling-water bath. The solutions were cooled, made neutral with $BaCO_3$, and the suspensions centrifuged. Parts of the centrifugates were analyzed on paper using solvents *A*, *B*, and *C*, spots corresponding to rhamnose, arabinose, glucose, galactose, and galacturonic acid, and a faint spot near the base line, being detected. The other parts were converted into alditol acetates, and these analyzed by g.l.c. using column (*a*). When estimation of sugars was desired, a known amount of *myo*-inositol was added to the polysaccharide (known amount) before hydrolysis by the foregoing procedure; in these cases, only g.l.c. analysis (and no p.c.) was conducted.

*Preparation of the carboxyl-reduced polysaccharide with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC)*[12]. — CMC (250 mg) was added to a stirred solution of the polysaccharide (10 mg) in water (10 mL), and the pH of the solution was kept at 4.75 by dropwise addition of 0.01M hydrochloric acid. After 2 h, 2M aqueous sodium borohydride (17 mL) was added dropwise during 45 min, and the pH was kept at 7 by concurrent addition of 4M hydrochloric acid. After being stirred for 1 h, the solution was dialyzed against distilled water for 24 h, and then lyophilized. The whole process was repeated once.

Fractionation of the polysaccharide with calcium chloride. — Polysaccharide *A* (5 g) was dispersed in water (500 mL) by magnetic stirring for 1 h. The suspension was made ammoniacal (pH 8.5) with dilute ammonia, and stirring was continued for 4 h at room temperature, to afford a clear solution. The pectate was precipitated by dropwise addition of 5% calcium chloride solution (65 mL). After addition was complete, the suspension was stirred for 3 h at room temperature, the turbidity gradually increasing. The precipitate was allowed to settle overnight at 10°, the mixture centrifuged, and the supernatant liquor collected. The precipitate was washed several times with water, the washings were combined with the supernatant liquor, and the solution was extensively dialyzed against distilled water and then concentrated to ~300 mL, cooled to 5°, and the contents precipitated by slow addition of ethanol (4 vol.). The precipitate was collected by centrifugation, washed (twice) with absolute alcohol, and the brownish material air-dried. Polysaccharide C (1.156 g) was thus obtained; it had $[\alpha]_D^{23}$ +53.5° (*c* 1.01, water).

Estimation of uronic acid. — The galacturonic acid in polysaccharides A and C was estimated by the carbazole–sulfuric acid method[13] (using D-galacturonic acid as the standard), and found to be 53.5 and 47.1%, respectively.

Resolution of polysaccharide C on a DEAE-cellulose column. — Polysaccharide C (500 mg) was decationized by passing its aqueous solution through a column (12 × 1.5 cm) of Dowex-50W X-8 (H^+) resin. The column was washed, and eluted, with water. The eluate and washings were combined, concentrated, and lyophilized, to yield ~360 mg of material. A solution of the decationized material in water (25 mL) was added to the top of a column (20 × 2.5 cm) of DEAE-cellulose. The column was successively eluted with water and 1.0M sodium chloride solution, elution being monitored with 1-naphthol. The fractions containing carbohydrate materials were pooled. The fraction obtained by water elution was concentrated, and lyophilized, to give polysaccharide D; yield 35.6 mg, $[\alpha]_D^{23}$ +65.9° (*c* 0.5, water). The fraction obtained from the sodium chloride eluate was dialyzed, the salt-free solution concentrated, and the concentrate lyophilized, to give polysaccharide E, yield 91.7 mg, $[\alpha]_D^{23}$ +33° (*c* 0.6, water).

High-voltage electrophoresis of different polysaccharide fractions. — High-voltage, paper electrophoreses were conducted at 70 $V.cm^{-1}$ for 1 h, using (*i*) borate buffer (0.02M, pH 9.28) and (*ii*) phosphate buffer (pH 7.8), and spray reagent *b*. A single spot, at a distance of 1.5 cm towards the anode, was obtained for polysaccharide D, but two spots, or tailings, were detected in other cases.

Column chromatography, on Sephadex G-100, of polysaccharide D. — A solution of polysaccharide D (30 mg) in 0.05M ammonium hydrogencarbonate buffer (5 mL; pH 8.17) was passed through a column (95 × 1.1 cm) of Sephadex G-100, equilibrated, and eluted, with the same buffer, 5-mL fractions being collected, and the elution monitored with a differential refractometer. A single peak appeared on the chromatogram; the pooled fractions (120 mL), after dialysis and lyophilization, yielded 28.6 mg of a polysaccharide having the same specific rotation as polysaccharide D.

Methylation analysis. — Polysaccharide D (5 mg) was dissolved in dry dimethyl sulfoxide (5 mL) in a closed vial, and treated with 2M methylsulfinyl sodium (5 mL) under nitrogen. The solution was stirred overnight, methyl iodide (2 mL) was slowly added, with external cooling, and the mixture was stirred for 2 h. The product was dialyzed (to remove the methylating reagents), lyophilized, and remethylated by the Purdie method (with methyl iodide and silver oxide). Permethylated material (3.9 mg) had $[\alpha]_D^{23}$ +56° (*c* 0.39, chloroform), and showed no OH stretching vibration in the i.r. spectrum. It was hydrolyzed with 85% formic acid for 2 h at 100° and then (after removing the formic acid) with 0.5M sulfuric acid for 18 h at 100°. After the usual treatment, the partially methylated sugars were converted into their alditol acetates, and these were analyzed by g.l.c. using column *b* (see Table I).

Smith degradation[8]. — Polysaccharide D (7 mg) was treated with 0.1M sodium metaperiodate (8 mL) in the dark for 48 h at 10°. The excess of periodate was decomposed with ethylene glycol (2 mL), and the reagents were then dialyzed out. The

product was reduced with sodium borohydride overnight at room temperature, the solution acidified with acetic acid, and then dialyzed. The dialyzate was concentrated to a small volume, and the concentrate lyophilized. The product was hydrolyzed with M sulfuric acid for 20 h on a boiling-water bath, and the hydrolyzate, after the usual treatment, was converted into alditol acetates, and these were analyzed by g.l.c. using column *a*.

Oxidation with chromium trioxide[9,10]. — Polysaccharide D (3.7 mg) was mixed with *myo*-inositol (0.3 mg) as the internal standard, and then dissolved in formamide (0.7 mL). To this solution were added acetic anhydride (0.8 mL) and pyridine (1.2 mL), and the mixture was stirred overnight at room temperature. The acetylation product was isolated by evaporating the mixture to dryness, and then partitioning between water and chloroform. The chloroform layer was evaporated to dryness, and the product was dissolved in glacial acetic acid (4 mL) and treated with chromium trioxide (300 mg), with stirring at 50°. Aliquots were removed at 0, 1, and 2 h, diluted with water immediately after removal, and the solutions extracted with chloroform. The extracts were evaporated to dryness, and the dried materials were deacetylated with sodium methoxide, and the products hydrolyzed. Finally, these were analyzed, as the alditol acetates, by g.l.c. using column *a*.

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