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GRADED-HYDROLYSIS STUDIES ON BAEL (*Aegle marmelos*) GUM*

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ABSTRACT

Graded hydrolysis of purified bael gum afforded three neutral and two acidic oligosaccharides, together with monosaccharides These sugars were identified through periodate oxidation, methylation, reduction with lithium aluminum hydride, co-chromatography. and preparation of crystalline derivatives The neutral oligosaccharides were characterized as 3-*O*-β-D-galactopyranosyl-L-arabinose, 5-*O*-β-D-galactopyranosyl-L-arabinose, and 3-*O*-β-D-galactopyranosyl-D-galactose, and the acidic oligosaccharides as 3-*O*-(β-D-galactopyranosyluronic acid)-D-galactose and 3-*O*-(β-D-galactopyranosyluronic acid)-3-*O*-β-D-galactopyranosyl-D-galactose

INTRODUCTION

The bael plant (*Aegle marmelos*) is found abundantly in India Bael fruit and, particularly, bael gum, known for their antiamebic and antihistaminic actions, are important in Indian Ayurvedic medicine[1] Bael fruit is used against dysentery and diarrhea[2] Marmelosin, present in the fruit, is an exceedingly potent drug and, if taken in doses of 50 mg, acts as a laxative and diuretic, with a slight lowering of respiration and a tendecy towards sleepiness In larger doses, it acts as a strong depressant for the heart[3]. The gum is used to prepare adhesives, water-proofing, and oil-emulsion coatings[4]. Some preliminary investigations on ripe bael gum were made by Parikh *et al* [2], but no detailed work on the elucidation of the structure of the polysaccharides of bael gum seems to have been performed so far In the present paper, we report detailed structural studies on the oligosaccharides obtained by graded hydrolysis of the degraded gum

The bael gum was isolated by first taking out the gummy envelope around each seed of unripe bael fruits. From these envelopes, the pure polysaccharide of the bael gum was obtained by repeated precipitation from aqueous solution with ethanol until its specific rotation remained constant The final precipitate was washed with acetone, and dried over phosphorus pentaoxide *in vacuo* The purified gum had $[\alpha]_D$ $+84°$

*Structural Investigations on Bael (*Aegle marmelos*) Gum, Part I

(c 0 2, water), and contained galactose (71), arabinose (12 5), rhamnose (6 5), and galacturonic acid (7 0%), it was converted into the degraded gum by heating a 1% solution of the gum in 0 05M oxalic acid for 75 min on a boiling-water bath The excess of oxalic acid was removed as calcium oxalate, and the degraded gum was isolated by precipitation from the solution with ethanol The precipitate had $[\alpha]_D$ +44° (in water), and contained galactose (79 5), arabinose (3 5), galacturonic acid (8 6), and rhamnose (4 2%), it was found to be electrophoretically homogeneous, and was used for graded-hydrolysis studies

The graded hydrolysis, guided by the results of pilot experiments for maximal yield of lower oligosaccharides, was conducted by heating a 1% solution of the degraded gum in 25mM sulfuric acid for 16 h on a boiling-water bath The acidic sugars were adsorbed on a column of Dowex-1 X-4 resin Three homogeneous disaccharides (I, II, and III) plus galactose, arabinose, and rhamnose were isolated from the neutral fraction of the hydrolyzate by using Whatman No 3 MM chromatographic paper Disaccharide I contained a galactosyl group and an arabinose residue, the latter being at the reducing end, it proved to be 3-*O*-β-D-galactopyranosyl-L-arabinose, as (*a*) the disaccharide had R_{Glc} value and specific rotation identical with those of an authentic sample, (*b*) its methyl glycoside consumed 1 9 moles of periodate per mole, and (*c*) the permethylated derivative yielded 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,5-di-*O*-methyl-L-arabinose on hydrolysis Disaccharide II also contained a galactosyl group and an arabinose residue, the arabinose being present at the reducing end, and it was characterized as 5-*O*-β-D-galactopyranosyl-L-arabinose as (*a*) the R_{Glc} value and the specific rotation were identical with those of the authentic compound, (*b*) the methyl glycoside consumed 2 9 moles of periodate per mole, and (*c*) on methanolysis and hydrolysis, the permethylated disaccharide yielded 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3-di-*O*-methyl-L-arabinose On hydrolysis, disaccharide III yielded only galactose, and was identified as 3-*O*-β-D-galactopyranosyl-D-galactose, mainly by the results of periodate oxidation and by comparison with an authentic sample

The mixture of acidic sugars was recovered from the column of anion-exchange resin, and galacturonic acid, an aldobiouronic acid, and an aldotriouronic acid were isolated from this fraction The aldobiouronic acid was proved to be 3-*O*-(β-D-galactopyranosyluronic acid)-D-galactose mainly through methylation studies and the results of periodate oxidation of its methyl ester methyl glycoside On hydrolysis, the aldotriouronic acid, $[\alpha]_D$ +40°, afforded galacturonic acid, galactose, and an aldobiouronic acid that was chromatographically indistinguishable from 3-*O*-(β-D-galactopyranosyluronic acid)-D-galactose However, on reduction of the triouronic acid with sodium borohydride, and subsequent hydrolysis, the hydrolyzate showed a spot for the same aldobiouronic acid, together with zones for galactose and galacturonic acid, when the paper was sprayed with aniline oxalate, suggesting that the triouronic acid has the sequence galacturonic acid–galactose–galactose. On periodate oxidation of the methyl ester methyl glycoside of the aldotriouronic acid, 1 9 moles of the oxidant were consumed per mole of the aldotriouronic acid, this

indicated that the linkage between the two galactose residues is (1→3) This conclusion was corroborated by a Smith-degradation study of the aldotriouronic acid, in which only galactose was detected Upon methanolysis and subsequent hydrolysis, the permethylated aldotriouronic acid yielded 2,3,4-tri-*O*-methyl-D-galacturonic acid and 2,4,6-tri-*O*-methyl-D-galactose, showing that the aldotriouronic acid is 3-*O*-(β-D-galactopyranosyluronic acid)-3-*O*-β-D-galactopyranosyl-D-galactose

The results so far obtained give some insight into the structural details of the polysaccharides present in the degraded gum, but are insufficient for proposal of a plausible structure for the gum polysaccharide Characterization of these oligosaccharides indicated that the galactose residues are mostly linked through O-1 and O-3, and that the arabinose residues are linked through O-1 and O-3 and also through O-1 and O-5 The arabinose residues linked through O-1 and O-5 are evidently in the furanose form No disaccharide containing rhamnose could be isolated The low, positive, specific rotation (+44° in water) suggests that both the α and the β type of linkage may be present in the polysaccharide

EXPERIMENTAL

Materials and methods — The solvent systems (v/v) used for paper partition chromatography were (*A*) 18 3 1 4 ethyl acetate–acetic acid–formic acid–water, (*B*) 4 1 5 1-butanol–acetic acid–water, (*C*) 5 5 1 3 ethyl acetate–pyridine–acetic acid–water, (*D*) 8 2 1 ethyl acetate–pyridine–water, and (*E*) 4 1 5 1-butanol–ethanol–water The spray reagents used were (*a*) aniline hydrogen phthalate, (*b*) alkaline silver nitrate, (*c*) aniline hydrogen oxalate, and (*d*) benzidine periodate All specific rotations recorded are equilibrium values Unless otherwise stated, all evaporations were conducted *in vacuo* at 30–40° Whatman No 1 filter paper was used for qualitative, paper chromatography, and quantities up to 200 mg of sugar mixtures were separated on Whatman No 3 MM sheets

Acidic oligosaccharides were converted into their methyl ester methyl glycosides by refluxing with dry, methanolic hydrogen chloride (2 5%), and neutral ones were converted into methyl glycosides by keeping with dry, methanolic hydrogen chloride (2 5%) for 36 h at room temperature Each solution was made neutral with silver carbonate, the suspension filtered, and the filtrate evaporated to a syrup

Periodate oxidation studies were conducted spectrophotometrically[5] The molecular weights of the oligosaccharides were determined by the alkaline hypoiodite method[6]. The monosaccharides from the polysaccharide solutions were estimated by using the L-cysteine–sulfuric acid method[7], taking the dichromatic readings for galactose[7] at 412 and 380 nm, for arabinose[8] at 390 and 420 nm, and for rhamnose[9] at 396 and 482 nm. Uronic acid was determined by the carbazole method[10] at 535 nm *N*-*p*-Nitrophenylglycosylamines of the monosaccharides were prepared[11] by refluxing a methanolic solution of the sugar with a methanolic solution of *p*-nitroaniline containing a trace amount of hydrochloric acid, and crystallizing the derivative from ethanol

Isolation of purified gum — Gum and seeds were collected from 20 green bael-fruits Water (400 ml) was mixed with the gummy material, and the suspension was filtered through nylon cloth To the clear filtrate was added ethanol (5 vol), whereupon a brownish precipitate appeared This was centrifuged off, and successively triturated with ethanol (three times) and acetone, yield 45 g, $[\alpha]_D$ +90° (*c* 0 5, water) The polysaccharide was dissolved in water (400 ml) and precipitated by addition of ethanol (4 vol) The polysaccharide was isolated as already described, and this process of dissolution and reprecipitation was repeated five times, the value of the specific rotation had then become constant at +84° The final polysaccharide was dried over phosphorus pentaoxide *in vacuo*, yield 40.12 g, $[\alpha]_D$ +84° (*c* 0 5, water), moisture 1 93, ash 0 45, uronic acid 7.0, OMe 0 3, OAc 0 0%, and nitrogen negligible

In the hydrolyzate of the polysaccharide, arabinose, galactose, rhamnose, galacturonic acid, and an aldobiouronic acid were detected by paper chromatography using solvents *A*, *B*, and *D*, and spray reagents *b* and *c* The constituent monosaccharides were separated on paper by using solvent *B*, and were identified through preparation of derivatives (see Table I) by methods described earlier. Galactose, arabinose, and rhamnose from polysaccharide solutions were determined as described earlier, found, galactose 71, arabinose 12 5, and rhamnose 6 5%

Preparation of degraded gum — A solution of whole gum (8 g) in 0 05M oxalic acid solution (800 ml) was heated on a boiling-water bath for 75 min (the time previously found for maximal release of the labile arabinose and rhamnose without hydrolysis of galactose residues) The solution was made neutral with 0 05M calcium hydroxide solution, the calcium oxalate was removed by centrifugation, and the supernatant liquor was dialyzed against distilled water and then poured with stirring into ethanol (3 vol) containing 1% of lithium chloride The resulting precipitate was triturated three times with ethanol, and once with acetone, and dried, yield 5 72 g A portion was dissolved in water and the polysaccharide was reprecipitated with ethanol, and isolated as before The specific rotation of these two fractions was the same (+44°) The degraded polysaccharide was electrophoretically homogeneous When subjected to electrophoresis in a Shandon high-voltage, electrophoresis model L24 apparatus, in either borate buffer (pH 9 5) or phosphate buffer (pH 8 0), at a potential gradient of 20 V/cm, it showed a single, clear spot at 1 cm towards the cathode. This homogeneous, degraded polysaccharide contained moisture 0 3, ash 2 6 (as Li), uronic acid 8 6, galactose 79 5, arabinose 3 8, and rhamnose 4 2%, and was used for the detailed, structural studies

Graded hydrolysis of degraded gum — Guided by the results of pilot experiments for the maximal yields of the small oligosaccharides, the degraded gum (3 0 g) was heated with 25mM sulfuric acid (300 ml) for 16 h on a boiling-water bath The solution was cooled, made neutral ($BaCO_3$), and the suspension centrifuged The supernatant liquor was passed through a column of Amberlite IR-120 (H^+) resin and then through a column of Dowex-1 X-4 (HCO_3^-) resin, on which the acidic oligosaccharides were adsorbed (Each column was washed with distilled water until the

TABLE I

CHARACTERIZATION OF MONOSACCHARIDES

Sugars	$[\alpha]_D$ *(in water) (degrees)*	*Lit* $[\alpha]_D$		*Crystalline derivatives*	*M p (°C)*	*Lit m p*		$[\alpha]_D$ *(in pyridine) (degrees)*	*Lit.* $[\alpha]_D$	
		(degrees)	*Ref*			*(°C)*	*Ref.*		*(degrees)*	*Ref*
D-Galactose	+78	+83 3	12(a)	*N p*-nitrophenyl-D-galactosylamine	217	217–218	11	−246	−245	11
L-Arabinose	+110	+108 0	12(b)	*N p*-nitrophenyl-L-arabinosylamine	200–202	202	11	−144	−140 6	11
L-Rhamnose	+10	+9.1	12(c)	*N p* nitrophenyl-L-rhamnosylamine	218–220	220	13	+296	+300	13
D-Galacturonic acid	+52	+53 4	12(d)	galactaric acid	214	212–213	14	—	—	—

final washings gave a negative Molisch test.) The neutral eluate and the washings were combined, and evaporated to a syrup (2 6 g) Paper-chromatographic examination (solvent *B* and *D*) of this neutral fraction indicated the presence of three oligosaccharides (R_{Glc} 0 50, 0 23, and 0 17 in solvent *B*), together with galactose, arabinose, and rhamnose

Examination of neutral oligosaccharides — The mixture of neutral sugars was resolved on Whatman No 3 MM paper, and the zones corresponding to the oligosaccharides were cut out, and eluted with water Before this elution, each of these fractions was tested for homogeneity by paper chromatography

Characterization of 3-O-β-D-galactopyranosyl-L-arabinose — The syrup of I (89 mg) had $[\alpha]_D$ +57° (*c* 0 2, water) (lit [15] $[\alpha]_D$ +64°), R_{Glc} 0 50 (solvent *B*), and mol wt 316 (calc for $C_{11}H_{20}O_{10}$, 312) On hydrolysis, it yielded galactose and arabinose in equal proportions A portion of the sugar was reduced with sodium borohydride and then hydrolyzed After the usual treatment, the product was examined by paper chromatography using aniline hydrogen oxalate spray reagent, when only one spot, corresponding to galactose, was obtained A portion of the disaccharide (10 mg) was converted into the methyl glycosides, and these were oxidized with sodium metaperiodate, 1 9 moles of periodate were consumed per mole of the disaccharide derivatives Part of the mixture was methylated by the Hakomari method[16], and the product methanolyzed and hydrolyzed The resulting components were identified as 2,3,4,6-tetra-*O*-methylgalactose and 2,5-di-*O*-methylarabinose on paper chromatograms The mixture was separated on paper The former had $[\alpha]_D$ +110° (in water) (lit [17] +109°), and the latter, $[\alpha]_D$ +63° (in water) (lit [18] +60°)

Characterization of 5-O-β-D-galactopyranosyl-L-arabinose — The syrup of II (57 mg) had $[\alpha]_D$ −20° (*c* 0 25, water) (lit [19] $[\alpha]_D$ −18°), R_{Glc} 0 23 (solvent *B*), and mol wt 306 (calc for $C_{11}H_{20}O_{10}$, 312) On hydrolysis, it yielded galactose and arabinose in equal proportions Reduction with sodium borohydride, and subsequent hydrolysis, gave galactose as the only reducing sugar The mixture of methyl glycosides was subjected to periodate oxidation, it consumed 2 78 moles per mole A portion of this glycoside mixture was completely methylated by the Hakomori method, and the product hydrolyzed Spots corresponding to 2,3,4,6-tetra-*O*-methylgalactose and 2,3-di-*O*-methyl arabinose were obtained The 2,3-di-*O*-methyl-L-arabinose had $[\alpha]_D$ +100° (in water), lit [20] +104°

Characterization of 3-O-β-D-galactopyranosyl-D-galactose. — The syrup of III (132 mg) had $[\alpha]_D$ +63° (*c* 0 5, water) (lit [21] +60°), R_{Glc} 0 17 (solvent *B*), and mol wt 338 (calc for $C_{12}H_{22}O_{11}$, 342) On hydrolysis, it yielded galactose only One mole of the methyl glycosides of the disaccharide consumed 2 05 moles of periodate The disaccharide, crystallized from methanol–acetone, had m p and mixed m p 163–165° (lit [21] 163–170°)

Examination of the acidic sugars — The washed column of Dowex-1 X-4 resin was eluted with 0 05M sulfuric acid (200 ml) and then with water (500 ml) The solutions were combined, made neutral with barium carbonate, and the suspension centrifuged The supernatant liquor was evaporated to a syrup (300 mg) which, on

paper-chromatographic examination, showed three components; these were separated on paper, and individual components were checked for homogeneity, and then characterized Galacturonic acid was characterized as described earlier (see Table I)

Characterization of 3-O-(β-D-galactopyranosyluronic acid)-D-galactose — This albobiouronic acid (43 6 mg) had $[\alpha]_D^{30}$ +50° (*c* 0 5, water) (lit [22] +56 2°), R_{GalA} 0 5 (solvent *A*), and mol wt 349 (calc for $C_{12}H_{20}O_{12}$, 356) On hydrolysis, it gave spots on a paper chromatogram corresponding to galactose, galacturonic acid, and the original aldobiouronic acid Reduction of the aldobiouronic acid with sodium borohydride, followed by hydrolysis of the reduced product, gave galacturonic acid only (but no galactose) as the reducing sugar On reduction with lithium aluminum hydride, the methyl ester methyl glycoside of the aldobiouronic acid gave galactose, only, on hydrolysis On periodate oxidation, the methyl ester methyl glycoside consumed 1 85 moles of periodate per mole

Identification of 3-O-(β-D-galactopyranosyluronic acid)-3-O-β-D-galactopyranosyl-D-galactose — This aldotriouronic acid (23 6 mg) had $[\alpha]_D^{30}$ +40° (*c* 0 2, water), R_{GalA} 0 34 (solvent *A*), and mol wt 512 (calc for $C_{18}H_{30}O_{17}$, 518) On hydrolysis, it yielded galactose, galacturonic acid (trace), and the same aldobiouronic acid as already characterized The aldotriouronic acid was reduced with sodium borohydride, and the product hydrolyzed On paper chromatography (using aniline hydrogen oxalate spray-reagent), spots for galactose, galacturonic acid, and the aldobiouronic acid were detected The methyl ester methyl glycoside was reduced with lithium aluminum hydride, and the product hydrolyzed, on paper chromatography, the hydrolyzate showed a spot for galactose, only On periodate oxidation, the methyl ester methyl glycoside consumed 1 9 moles of periodate per mole On complete methylation, methanolysis, and hydrolysis of the methylated methyl glycosides, the spots on a paper chromatogram corresponded to those for 2,3,4-tri-*O*-methylgalacturonic acid and 2,4,6-tri-*O*-methylgalactose (and no other spots) These methylated sugars could not, however, be studied in detail, as the amounts of them were too small

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