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STUDIES ON BAEL (*Aegle marmelos*) SEED GLYCOPROTEINS

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ABSTRACT

The carbohydrate material isolated from bael (*Aegle marmelos*) seeds was resolved into four, pure glycoprotein fractions. The carbohydrate moiety of one of the fractions (F-I) contained galactose, glucose, arabinose and rhamnose in the molar ratios of 6 2 8 3 The linkages among these monosaccharide residues and the anomeric configurations of the glycosyl residues were determined The structure at the glycosyl–amino acid junction was also established

INTRODUCTION

The structure of the gummy material surrounding bael seeds has been determined[1–3] The structure of the exudate gum from bael trees was also established[4] Both materials contain (1→3)-linked galactopyranosyl residues as the backbone chains in the macromolecules The carbohydrate materials present in the seeds of bael fruit were 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 polysaccharides already present

RESULTS AND DISCUSSION

The crude, carbohydrate material was obtained from defatted bael seeds by extraction with hot water, and then isolated by precipitating it with ethanol It had $[\alpha]_D^{23}$ −51 3°, and contained threose (0 24%), rhamnose (0 68%), arabinose (2 11%), xylose (0 39%), galactose (1 82%), and glucose (0 93%), as determined by g l c (column *A*, using *myo*-inositol as the internal standard) The result indicated the presence of large proportions of non-carbohydrate materials in the crude product The crude material was purified by passing a solution thereof in ammonium hydrogencarbonate–ammonia buffer (pH 10 0) through a column of Sephadex G-100 and eluting the column with the same buffer, the eluate being monitored with a differential refractometer A considerable proportion of the crude material was insoluble in the buffer, and was not used; this part contained only amino acids and no carbohydrates

Four fractions (F-I–F-IV) were thus obtained (see Fig 1) The sugar compo-

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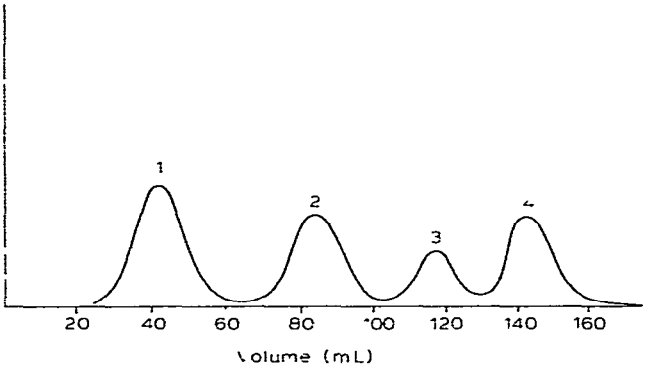


Fig 1 Chromatography of carbohydrate material from bael seed in a column of Sephadex G-100, by elution with ammonium hydrogencarbonate–ammonia buffer (pH 10) and monitoring with a differential refractometer (Assignment of peaks 1, Fraction-I 2 Fraction-II, 3 Fraction-III, and 4 Fraction-IV)

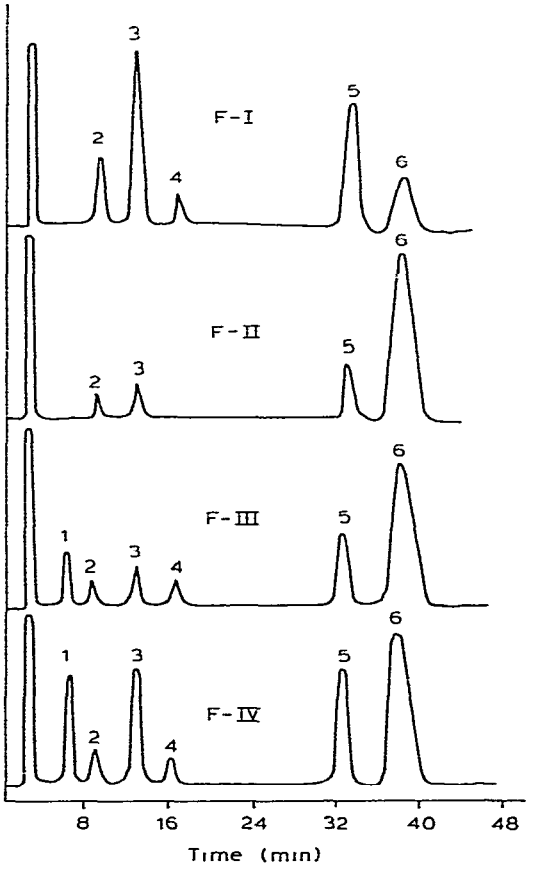


Fig 2 G l c of sugar components of pure glycoprotein fractions (F-I to F-IV) (Assignment of peaks 1, threose, 2, rhamnose, 3, arabinose, 4, xylose, 5, galactose, and 6 glucose)

TABLE I

SUGAR COMPONENTS OF DIFFERENT FRACTIONS FROM BAEL-SEED GLYCOPROTEINS

Fraction	*Yield (mg)*	$[\alpha]_D^{23}$ *(degrees)*	*Sugar (mol%)*[a]					
			Threose	*Rhamnose*	*Arabinose*	*Xylose*	*Galactose*	*Glucose*
F-I	23 6	−11 5	—	14 77	41 97	1 04	31 16	10 72
F-II	19 6	−13 0	—	2 17	4 84	—	6 52	86 95
F-III	2 8	−31 5	4 73	1 30	2 21	1 17	4 93	85 63
F-IV	8 2	−8 3	16 00	3 84	15 12	2 62	21 30	41 08

[a]The sugars were analyzed and estimated by g l c (column *A* at 190°)

TABLE II

METHYL ETHERS OF SUGARS FROM THE HYDROLYZATES OF METHYLATED F-I

Sugars[a]	T[b]	*Approximate mole %*	*Mode of linkage*
2,3,4-Rha	0 35	4 87	Rha*p*-(1→
2,3,5-Ara	0 41	7 68	Ara*f*-(1→
3,5-Ara	0 80	5 16	→2)-Ara*f*-(1→
3,4-Rha	0 87	9 31	→2)-Rha*p*-(1→
2,3,4,6-Glc	**1 00**	5 59	Glc*p*-(1→
2,3-Ara	1 07	31 00	→5)-Ara*f*-(1→
2 4,6-Gal	2 03	5 44	→3)-Gal*p*-(1→
2,4 6-Glc	1 81	4 45	→3)-Glc*p*-(1→
2,3,6-Gal	2 23	10 49	→4)-Gal*p*-(1→
2 4-Gal	5 10	15 19	→3 6)-Gal*p*-(1→

[a]2,3,4-Rha = 2,3,4-tri-*O*-methylrhamnose, etc [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 column (1% of OV-225) at 165°

nents in each fraction were identified and estimated by g l c analyses of the alditol acetates of the sugars in the corresponding hydrolyzates (see Fig 2) The results are summarized in Table I Although a trace of xylose was detected in F-I, neither it nor its derivatives were detected in any chemical investigation of F-I The non-carbohydrate materials present in these fractions were found to contain amino acids, and these fractions were proved to be homogeneous by high-voltage electrophoreses in different buffers, and through poly(acrylamide)–SDS-gel electrophoresis using Tris–glycine buffer (pH 8 3) These results suggested that fractions F-I–F-IV were pure glycoproteins F-I was employed for investigation, as it was obtained in comparatively large proportion

Fraction F-I was permethylated, first by the Hakomori method[6] and then by the Purdie method[7] (twice in succession), until the product showed no OH absorption

band in its i r spectrum The methylated F-I, $[\alpha]_D^{23}$ +4°, was hydrolyzed, and the liberated sugars were converted into their alditol acetates and then analyzed by g l c using column *B*, the results are shown in Table II From the nature of the products of hydrolysis of methylated F-I, it was possible to develop part of the structure of the carbohydrate moiety of glycoprotein F-I

Characterization of 2,3,4-tri-*O*-methylrhamnose, 2,3,5-tri-*O*-methylarabinose, and 2,3,4,6-tetra-*O*-methylglucose (and no tetra-*O*-methylgalactose) in the hydrolyzate of methylated F-I indicated that its (nonreducing) end-groups are rhamnopyranosyl, arabinofuranosyl, and glucopyranosyl groups Identification of both 2,3,6- and 2,4,6-tri-*O*-methylgalactose in the hydrolyzate showed that both (1→3)- and (1→4)-linkages are present between galactosyl residues The glucosyl residues have (1→3)-linkages in the interior part, as only 2,4,6-tri-*O*-methylglucose could be identified Again, characterization of both 2,3- and 3,5-di-*O*-methylarabinose suggested the occurrence of both (1→5)- and (1→2)-linkages for the arabinosyl residues, the rhamnosyl groups are joined by (1→2)-linkages, as only 3,4-di-*O*-methylrhamnose was present in the hydrolyzate Interestingly, the branch points of the molecule are only at O-6 of the galactosyl residues, as no di-*O*-methylhexose other than 2,4-di-*O*-methylgalactose could be identified in the hydrolyzate (there was no mono-*O*-methylpentose)

Fraction F-I was subjected to Smith degradation[8] The products obtained were characterized as threitol arabinitol, glucitol, and galactitol in the molar ratios of ~2 1 1 5 (besides glycerol) Characterization of these products further supported the results of the methylation analysis (see Table II), as (1→4)-linked galactopyranosyl residues would yield threitol by Smith degradation

The anomeric configurations of the sugar residues were ascertained by chromium trioxide oxidation[9,10] of fully acetylated F-I Oxidation was conducted in acetic acid at 50°, using *myo*-inositol as the internal standard Aliquots were removed at intervals of 0 0 5, and 1 5 h, and deacetylated, and the surviving sugars were determined Chromium trioxide oxidizes the sugar residues having the β configuration much more rapidly than those of the α configuration The results (shown in Table III) indicated that only the rhamnosyl residues have the α configuration, and the

TABLE III

SURVIVAL OF SUGARS[a] IN CHROMIUM TRIOXIDE OXIDATION OF ACETYLATED F-I

Time (h)	myo-*Inositol*	*Glucose*	*Galactose*	*Arabinose*	*Rhamnose*
0	10	1 70	4 85	6 41	2 53
0 5	10	0 86	1 17	1 80	2 50
1 5	10	0 34	0 14	0 18	2 05

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

TABLE IV

RESULTS OF AMINO ACID ANALYSIS OF GLYCOPEPTIDE A AND ALKALINE BOROHYDRIDE-DEGRADED GLYCOPEPTIDE B

Amino acids[a]	*A* (mol%)	*B* (mol%)
Aspartic acid	4 92	4 78
Threonine	9 31	6 65
Serine	10 54	10 39
Glutamic acid	11 44	11 75
Glycine	10 48	11 14
Alanine	17 87	17 95
Leucine	8 02	8 83
Tyrosine	27 39	28 47

[a] In addition small quantities of the following amino acids were also detected in both cases isoleucine methionine, phenylalanine proline and valine

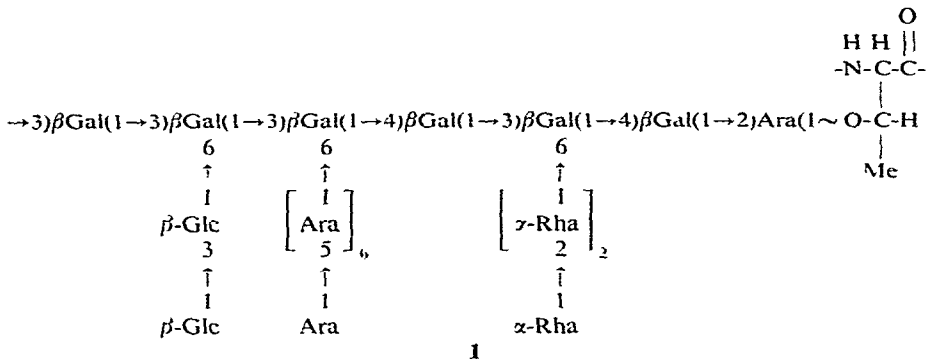
rest have the β configuration but as acetylated furanoses are nonspecifically oxidized[7] by chromium trioxide the anomeric configuration of the arabinosyl groups could not be determined by this experiment In fact, the ease with which the arabinose was hydrolyzed off with mild acid indicated that they might have the α configuration

In order to ascertain the structure at the glycosyl–amino acid junction, the glycoprotein was first degraded with pronase, to afford a glycopeptide that contained only eight amino acids in appreciable proportions (see Table IV) it did not contain any hydroxyproline or hydroxylysine, and was alkali-labile The glycopeptide was degraded with alkaline borohydride and the carbohydrate part and the peptide part were separated The amino acids present in the original glycopeptide and in the degraded peptide were analyzed, after hydrolysis by an amino acid analyzer the results, given in Table IV show a diminution of ~28 5% in the threonine content of the degraded material, indicating its involvement in the glycosyl–amino acid bond The threonine residue is converted into 2-amino-2-butenoic acid and the corresponding amount is lacking from the chromatogram

The hydrolyzate of the carbohydrate part of the degraded material was acetylated, and the product analyzed by g l c Only arabinitol acetate could be identified in the chromatogram by coinjection of individual alditol acetates (the chromatogram contained peaks different from those of the alditol acetates from galactose glucose, and rhamnose) On alkaline borohydride degradation, the sugar residue involved in the glycopeptide bond is converted into the corresponding alditol and on hydrolysis, only this residue would be released as an alditol the remaining sugar residues would be obtained as aldoses The results of this experiment therefore suggest that an arabinosyl residue must have been involved in the glycosyl–amino acid bond Again, on methylation and subsequent hydrolysis, this carbohydrate part yielded all of the partially methylated sugars except 3 5-di-*O*-methylarabinose This result

not only confirmed the involvement of arabinose in the glycosyl–amino acid bond, but also showed that only a (1→2)-linked arabinosyl residue is involved in it, as this residue in the degraded material emerged as *O*-2-linked arabinitol. However, the expected 2,4-di-*O*-acetyl-1,3,5-tri-*O*-methylarabinitol could not be traced in the mixture, as we lacked an authentic sample thereof.

From the experimental results discussed, the partial structure for the carbohydrate moiety of the glycoprotein F-I may be written as shown in **1**. This structure explains all of the results discussed, although there may be other possible sequences of branches that cannot be decided with the data thus far adduced.



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EXPERIMENTAL

General — All evaporations were conducted at ~40° (bath temperature) under diminished pressure. Small volumes of aqueous solution were lyophilized. All specific rotations were recorded with a Perkin–Elmer Model 241 MC spectropolarimeter at 23 ± 1° and 589.6 nm. Paper partition-chromatography was performed by the descending technique, using Whatman No. 1 chromatographic papers with the following solvent systems (*A*) 8:2:1 ethyl acetate–pyridine–water[11] and (*B*) the upper layer of 4:1:5 1-butanol–acetic acid–water[12]. The spray reagents used were (*a*) alkaline silver nitrate[13], (*b*) benzidine periodate[14], and (*c*) ninhydrin[15]. For gas-liquid chromatography, a Hewlett-Packard 5730 A gas chromatograph with flame-ionization detector was used. Resolutions were performed in a glass column (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 165° (for alditol acetates of partially methylated sugars). A Shandon high-voltage electrophoresis apparatus, model L-24, was used for electrophoresing the materials with use of different buffers. Infrared spectra were recorded with a Beckman IR-20A instrument, and ultraviolet and visible spectra with a Yanaco-SPI spectrophotometer.

Extraction of crude, carbohydrate material from the seeds of bael (Aegle marmelos) *fruit* — Forty-five, well-developed, bael fruits were cut horizontally through the middle, and the seeds were collected. They were cleaned, the outer coating was

removed, and the fleshy material was macerated under ethanol in a blender The ethanol was removed by centrifugation, and the white powder (63 g) was defatted by extracting with 1 1 benzene–ethyl ether The defatted material was then suspended in water (1 L), and the suspension heated, with stirring, for 4 h on a water bath at 80° The mixture was cooled, and centrifuged to collect the aqueous extract The residue was re-extracted by repeating the procedure To the combined, aqueous extract (cold) was then added cold ethanol (3 vol , acidified with acetic acid to pH 4 5) The precipitate that separated was centrifuged off, and washed three times with dry ethanol, and then with dry ether The dried material (1 8 g) had $[\alpha]_D^{23}$ −51 3° (*c* 0 4, 0 1M NaOH)

Hydrolysis — (*A*) *For carbohydrate* The carbohydrate material (~5 mg) was hydrolyzed with M sulfuric acid (1 mL) in a sealed tube for 20 h on a boiling-water bath Where estimations were performed, the material was mixed with an appropriate amount of *myo*-inositol, and hydrolyzed The hydrolyzate was made neutral with $BaCO_3$, and the barium salts were removed by centrifugation Part of the supernatant liquor was analyzed by paper chromatography, and the rest was converted into its alditols by reduction with $NaBH_4$, and the product acetylated the alditol acetates were analyzed by g l c

(*B*) *For amino acids* The material (~5 mg) in 6M hydrochloric acid (1 mL) in a sealed tube was heated for 24 h at 100° The acid was removed by repeated evaporation with methanol (to dryness), and part of the residue analyzed by paper chromatography, and part by means of an amino acid analyzer

Fractionation of crude material — The crude material (100 mg) was suspended in 10 mL of ammonium hydrogencarbonate–ammonia buffer (pH 10) The insoluble material was centrifuged off, and the solution was placed on the top of a column (95 × 1 1 cm) of Sephadex G-100 The column was eluted with the same buffer with collection of 5-mL portions in test tubes in an automatic, fraction collector and monitoring with a differential refractometer Four fractions (F-I–F-IV) were obtained (see Table I and Fig 1) Three such batches were made

Test for homogeneity of the glycoprotein fractions — (*A*) High-voltage electrophoreses were conducted at 20 V cm^{-1} for 1 h, using (*i*) pyridine–acetic acid buffer (pH 5 75) and spray reagent *c*, (*ii*) phosphate buffer (pH 7 8) and spray reagent *b* and (*iii*) borate buffer (pH 9 18) and spray reagent *b*

(*B*) Poly(acrylamide)–SDS gel-electrophoreses (8 mA/gel, 2 5 h) were performed, using Tris–glycine buffer (pH 8 3). 7% acetic acid–25% ethanol was used for destaining the gels

Methylation analysis — Fraction F-I (8 mg) was dissolved in dry dimethyl sulfoxide (8 mL) in a closed vial, and then treated with 2M methylsulfinyl sodium (8 mL) under nitrogen The solution was stirred overnight, and then methyl iodide (4 mL) was slowly added, with external cooling The mixture was stirred for 2 h, and the solution of the product was dialyzed (to remove the methylating reagents) and then lyophilized The product was remethylated by Purdie's method with methyl

iodide and silver oxide Permethylated F-I (7 4 mg) had $[\alpha]_D^{23}$ +4° (*c* 0 74, chloroform) and showed no OH stretching-vibration in its i r spectrum

The permethylated sample was hydrolyzed, first 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 processing the partially methylated sugars were converted into their alditol acetates, and these were analyzed by g l c , using column *b* (see Table II)

Smith degradation — Fraction F-I (2 1 mg) was treated with 0 1M sodium metaperiodate (4 mL) in the dark for 45 h at 5° The excess of periodate was decomposed with ethylene glycol (1 mL) and the reagents were then dialyzed out The product was reduced with sodium borohydride overnight at room temperature, and the solution acidified with acetic acid, and dialyzed After concentration, the product was hydrolyzed After the usual treatment the hydrolyzate was converted into the alditol acetates, and these were analyzed by g l c., using column *a*

Oxidation of F-I with chromium trioxide — A mixture of Fraction F-I (2 3 mg) with *myo*-inositol (0 4 mg) as the internal standard was dissolved in formamide (0 5 mL) To this solution were added acetic anhydride (1 mL) and pyridine (1 5 mL), and the mixture was stirred overnight at room temperature The acetylation product was isolated by evaporating the mixture to dryness, and then partitioning the residue between water and chloroform The product (from the latter) was dissolved in glacial acetic acid (4 mL) and treated with chromium trioxide (400 mg) whilst stirring at 50° Aliquots were removed at 0. 0 5, and 1 5 h, diluted with water immediately after removal, and extracted with chloroform 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*

Proteolytic degradation[16] — The crude glycoprotein (1 g) was dissolved in 0 1M Tris–acetate buffer (pH 7 8, 25 mL) containing 5mM calcium chloride Pronase (~4 mg) was added and the mixture was incubated for 72 h at 37°, pronase (1 mg each time) being added after 18 and 47 h The suspension was then heated for 15 min at 65° to deactivate the enzyme The mixture was dialyzed until free from amino acids and peptides (monitored by the ninhydrin test) The solution of the glycopeptide was lyophilized, and the whole process was repeated once The final yield of glycopeptide was 60 mg A similar degradation of F-I (75 mg) was made for the methylation study

Alkaline borohydride degradation[7,8] *of the glycopeptide* — The glycopeptide (20 mg) in 0 05M KOH and M $NaBH_4$ (final volume, 8 mL) was incubated for 15 h at 45° The excess of borohydride was decomposed by careful addition of 4M acetic acid to pH 5 0, and the solution was passed through a column (1 2 × 10 cm) of Dowex 50W X-8 (H^+) ion-exchange resin The column was washed with water, the eluate and the washings were combined and evaporated to dryness, and the boric acid was removed as methyl borate

Identification of the sugar residue involved in the glycosyl–peptide bond — An amount (~5 mg) of the carbohydrate part obtained from the alkaline borohydride degradation was hydrolyzed with M sulfuric acid for 20 h on a boiling-water bath

The hydrolyzate was made neutral ($BaCO_3$), evaporated to dryness under diminished pressure, and the residue acetylated with 1 : 1 pyridine–acetic anhydride The product was analyzed by g l c using column *a* for alditol acetates (by co-injections with authentic alditol acetates)

Methylation of the carbohydrate part obtained by alkaline borohydride degradation of F-I — The carbohydrate part (~5 mg) was methylated twice by the Hakomori method[6], as described earlier The permethylated compound (4 5 mg) had $[\alpha]_D^{23}$ +6° (*c* 0 45, chloroform), and showed no OH stretching vibration in the i r spectrum The permethylated sample was hydrolyzed and processed as usual, and the partially methylated sugars were converted into their alditol acetates, and analyzed by g l c using column *b*

Identification of the amino acid involved in the glycosyl-peptide bond — The Dowex 50W X-8 (H^+) column, which had retained the degraded peptides, was eluted with 30% acetic acid (25 mL) and the eluate was evaporated to dryness The material (~2 mg) was hydrolyzed with 6M hydrochloric acid for 24 h at 100° and the excess of acid was removed by co-distillation with methanol (4–5 times) under diminished pressure The amino acids present in the hydrolyzate were identified and estimated according to the classical method of Spackman *et al*[17], with the aid of an amino acid analyzer (Beckman Multichrom liquid column chromatograph model 4255), the results are given in Table IV

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