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INVESTIGATIONS ON THE PARTIAL STRUCTURE OF A GLYCOPROTEIN FROM BAEL (*Aegle marmelos*) SEED

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ABSTRACT

The crude carbohydrate material isolated from bael (*Aegle marmelos*) seeds was resolved into four fractions. The homogeneous fraction contained 38.5% of carbohydrate and 60.6% of protein, and its carbohydrate moiety consisted of glucose, galactose, rhamnose, and arabinose in the molar ratios of 40:3:1:2. The linkages among various monosaccharide residues were established through methylation analysis and Smith-degradation studies. The anomeric configurations of the glycosyl groups and the structure at the glycosyl–amino acid junction were also determined. From the results of these experiments, a partial structure of the glycoprotein has been proposed.

INTRODUCTION

The structures of the exudate gum[1] and the gummy material surrounding the seeds[2–4] of bael (*Aegle marmelos*) plants have been investigated, and both were found to contain (1→3)-linked D-galactopyranosyl residues in the backbone chains of these macromolecules. The carbohydrate materials present in the seeds of bael fruits were examined for molecules having similar basal chains, to test the proposal[5] that gums are formed by apposition of additional sugar residues to the outer chains of polysaccharides already present.

RESULTS AND DISCUSSION

Crude carbohydrate material was obtained from defatted bael seeds by extraction with hot water, and then isolated by precipitation 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. in column *1*, using *myo*-inositol as the internal standard. The results indicated the presence of a large proportion of non-carbohydrate material in the crude product. The crude material was fractionated on a column of Sephadex G-100 which was eluted with ammonium hydrogencarbonate–ammonia buffer (pH 10.0), a considerable proportion of the

TABLE I

SUGAR COMPONENTS OF DIFFERENT FRACTIONS FROM BAEL SEED GLYCOPROTEINS

Fraction	*Yield*[a] *(mg)*	$[\alpha]_D^{23}$ *(degrees)*	*Sugars (mol %)*[b]					
			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]From 65 mg of buffer-soluble, glycoprotein mixture. [b]The sugars were analyzed by g.l.c. in column *1* at 190°.

TABLE II

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

Sugars	T[a]	*Approximate mol %*	*Mode of linkage*[b]
2,3,4-Tri-*O*-methylrhamnose	0.35	2.21	Rha*p*-(1→
2,3,5-Tri-*O*-methylarabinose	0.41	2.33	Ara*f*-(1→
3,5-Di-*O*-methylarabinose	0.80	1.91	→2)-Ara*f*-(1→
2,3,4,6-Tetra-*O*-methylglucose	1.00	5.20	Glc*p*-(1→
2,3,4,6-Tetra-*O*-methylgalactose	1.18	2.62	Gal*p*-(1→
2,4,6-Tri-*O*-methylgalactose	2.03	3.89	→3)-Gal*p*-(1→
2,3,6-Tri-*O*-methylglucose	2.32	77.37	→4)-Glc*p*-(1→
6-*O*-Methylglucose	5.00	4.42	→2,3,4-Glc*p*-(1→

[a]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 of 1% of OV-225 at 165°. [b]Rha*p* = rhamnopyranose, etc.

crude material remaining insoluble in the buffer; this part contained no carbohydrate. The elution was monitored with a differential refractometer, and four fractions (FI–FIV) were obtained (see Fig. 1 in ref. 6). The sugar residues in each fraction were identified, and estimated, by g.l.c. of the alditol acetates formed from the acid hydrolyzates (see Fig. 2 of ref. 6). The results are summarized in Table I. The non-carbohydrate components in these fractions were found to contain amino acids only. These fractions were proved to be homogeneous by high-voltage electrophoresis in different buffers, and by poly(acrylamide)–SDS gel-electrophoresis using Tris–glycine buffer (pH 8.3). The sugar patterns in these fractions were found to be distinct (see Table I), and to differ from those of the gums[1–4] of this plant. Fraction F-I, which had been shown[6] to be a glycoprotein, contains both (1→3)- and (1→4)-linked galactopyranosyl residues in the basal chain of its carbohydrate moiety. Fraction F-II, which also proved to be a glycoprotein, and which contains mainly glucose in its carbohydrate moiety, was investigated, and the results are reported herein.

Fraction F-II was permethylated, first by the Hakomori method[7], and then twice by the Purdie method[8], until the product showed no OH absorption band in its i.r. spectrum. Methylated F-II, $[\alpha]_D^{23}$ +8.5°, was hydrolyzed, and the sugars liberated were converted into their alditol acetates, and these analyzed by g.l.c. in column *2*; the results are shown in Table II. From the nature of the products of hydrolysis of methylated F-II, it is possible to develop a partial structure for the carbohydrate moiety of glycoprotein F-II.

The carbohydrate part of the glycoprotein F-II is dominated by glucose residues and seems to have a backbone chain of (1→4)-linked glucosyl residues, as a large proportion of 2,3,6-tri-*O*-methylglucose was present in the hydrolyzate of methylated F-II. There is some branching in this molecule, and, interestingly, the branches originate at O-2, O-3, and O-4 of glucosyl residues only; this is evident from the detection of 6-*O*-methylglucose in the hydrolyzate. As 2,3,5-tri- and 3,5-di-*O*-methylarabinose were detected in approximately equimolar proportions, it is probable that an arabinofuranosyl group is present at the nonreducing end, and that another (interior) residue is joined through a (1→2)-linkage. Characterization of about two molar proportions of 2,4,6-tri-*O*-methylgalactose indicated the presence of at least two (1→3)-linked galactosyl groups in the molecule. The nonreducing ends are occupied by all four sugars present in the molecule.

Fraction F-II was subjected to Smith degradation[9]. The products obtained were characterized as galactitol, arabinitol, and glucitol in the approximate molar ratios of 1:1:2 (besides glycerol and a large proportion of erythritol). Characterization of these products further supported the results of methylation analysis, as a large proportion of erythritol would be expected to be released from (1→4)-linked glucosyl residues after Smith degradation.

In order to determine the anomeric configurations of the different sugar residues, the acetylated derivative of F-II was subjected to oxidation with chromium-(VI) trioxide[10,11] in acetic acid at 50° for different intervals of time, using *myo*-inositol as the internal standard. The products were deacetylated, and the surviving sugars were estimated. Chromium trioxide oxidizes sugar residues having the β configuration much more rapidly than those having the α configuration. The results,

TABLE III

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

Time (hours)	myo-*Inositol*	*Glucose*	*Galactose*	*Arabinose*	*Rhamnose*
0	10	7.30	0.51	0.31	0.15
0.5	10	1.00	0.20	0.12	0.13
1.5	10	0.60	0	0.10	0.10

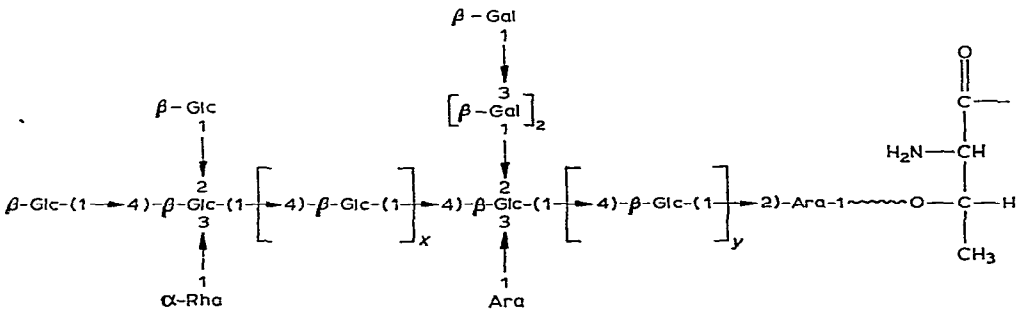
[a]The sugars were analyzed, and estimated, by g.l.c. in column *1* at 190°.

shown in Table III, indicated that only rhamnose has the α configuration, and that the rest have the β configuration, but, as acetylated furanoses are nonspecifically oxidized[12] by chromium trioxide, the problem of the anomeric configuration of the arabinosyl groups could not be settled by this experiment. However, the rate at which arabinose was hydrolyzed by mild acid indicated that it possibly had the α configuration.

In order to characterize the glycosyl–peptide bond present in the glycoprotein of bael seed, the glycoprotein was first degraded with pronase into glycopeptides, which were then degraded with alkaline borohydride, and treated exactly as for F-I (see ref. 6). Only threonine was diminished (by ~30%) in the degraded material, indicating its involvement in the glycosyl–amino acid bond. These threonine residues are converted into 2-amino-2-butenoic acid, and the corresponding amount is absent from the chromatogram.

The material in the hydrolyzate of the carbohydrate part of the degraded material was acetylated, and the products analyzed by g.l.c. Only arabinitol pentaacetate could be identified in the chromatogram by co-injection of individual alditol acetates (the chromatogram contained other peaks, different from those of the alditol acetates from galactose, glucose, and rhamnose). On degradation with alkaline borohydride, the glycosyl–amino acid bond was broken, and the reducing-end unit of the carbohydrate part was converted into its alditol. Methylation analysis (see Table II) of F-II indicated the presence of two arabinosyl residues, one at the nonreducing end, and the other as a (1→2)-linked residue inside the chain. Evidently, this (1→2)-linked arabinosyl residue is the reducing-end unit of the carbohydrate part of degraded F-II. This group is, therefore, involved in the glycosyl–amino acid bond in the glycoprotein.

From the experimental results just discussed, a partial structure for the carbohydrate moiety of glycoprotein F-II may be depicted as in **1**. This structure explains all of the results discussed, except that there may be other possibilities as regards the sequence of branches (which cannot be assigned with the data thus far adduced).



1

where Gal represents a galactopyranosyl group; Glc, a glucopyranosyl group; Rha, a rhamnopyranosyl group; and Ara, an arabinofuranosyl group; and $x + y = 36$.

Consequently, the glycosylamino group of F-II is similar to that[6] of F-I, even though the structure of the carbohydrate part of these two glycoproteins is dissimilar.

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 conducted by the descending technique, using Whatman No. 1 chromatographic paper with the following solvent systems: (*A*) 8:2:1 ethyl acetate–pyridine–water[13], and (*B*) the upper layer of 4:1:5 1-butanol–acetic acid–water[14]. The spray reagents used were (*a*) alkaline silver nitrate[15], (*b*) benzidine periodate[16], and (*c*) ninhydrin[17]. All specific rotations were recorded with a Perkin–Elmer Model 241 MC polarimeter at 23 ±1° and 589.6 nm. A Shandon high-voltage electrophoresis apparatus, model L-24, was used for electrophoresis. Infrared spectra were recorded with a Beckman IR-20A instrument, and ultraviolet and visible spectra, with a YANACO-SPI spectrophotometer. For gas–liquid chromatography, a Hewlett–Packard model 5730 A gas chromatograph with a flame-ionization detector was used. Resolutions were performed in glass columns (1.83 m × 6 mm) containing (*1*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates), and (*2*) 1% of OV-225 on Gas Chrom Q (80–100 mesh) at 165° (for alditol acetates of partially methylated sugars).

Extraction of crude carbohydrate material from the seeds of bael (Aegle marmelos) *fruit.* — Well-developed bael fruits (45) were cut horizontally through the middle, and the seeds were collected. Crude glycoprotein was isolated as described in ref. 6. The dried material (1.8 g) had $[\alpha]_D^{23}$ −51.3° (*c* 0.4, 0.1M NaOH).

Hydrolysis for carbohydrate. — The carbohydrate material (5 mg) was hydrolyzed with M sulfuric acid (1 mL) in a sealed tube on a boiling-water bath for 20 h. Where estimations were performed, the material (accurately weighed) was mixed with the appropriate amount of *myo*-inositol, and hydrolyzed. The hydrolyzate was treated in the usual way. Part of it was analyzed by paper chromatography, and the rest was converted into the alditol acetates, which were analyzed by g.l.c. in column *1*.

Hydrolysis for amino acids. — The material (~5 mg) in 6M hydrochloric acid (1 mL) was heated in a sealed tube for 24 h at 100°. The acid was removed by repeated addition and evaporation of methanol (to dryness), and analyzed partly by paper chromatography and partly by means of an amino acid analyzer.

Fractionation of crude material. — The crude material (100 mg) was suspended in ammonium hydrogencarbonate–ammonia buffer, pH 10 (10 mL). 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 fractions and monitoring with a differential refractometer. Four fractions (FI–FIV) were obtained (see Table I, and Fig. 1 of ref. 6). 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-electrophoresis (8 mL/gel, 2.5 h) was performed, in Tris–glycine buffer (pH 8.3), and 7:25:68 acetic acid–ethanol–water was used for destaining the gels.

Methylation analysis. — A solution of F-II (4.7 mg) in dry dimethyl sulfoxide (5 mL) in a closed vial was 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) and lyophilized. The product was twice re-methylated by the Purdie method (with methyl iodide and silver oxide). Permethylated F-II (3.5 mg) had $[\alpha]_D^{23}$ +8.5° (*c* 0.35, 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 treatment, the partially methylated sugars were converted into their alditol acetates, and these were analyzed by g.l.c. in column *2*.

Smith degradation. — Fraction F-II (2.6 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 was acidified with acetic acid, dialyzed, and concentrated. The product was hydrolyzed, and the hydrolyzate, after the usual treatment, was converted into alditol acetates, and analyzed by g.l.c. in column *1*.

Oxidation of F-II with chromium(VI) trioxide. — Fraction F-II (7 mg) was mixed with *myo*-inositol (1.8 mg) as the internal standard, and then dissolved in formamide (2 mL). To this solution were added acetic anhydride (4 mL) and pyridine (6 mL), and the mixture was kept overnight at room temperature. The acetylation product was isolated by evaporating the mixture to dryness, and then partitioning between water and chloroform. The product was dissolved in glacial acetic acid (8 mL) and treated with chromium(VI) trioxide (800 mg) while being stirred 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, and analyzed as alditol acetates by g.l.c. in column *1*.

Determination of the glycosyl–amino acid bond in F-II. — This involved conversion of the glycoprotein into the glycopeptide, degradation of the glycopeptide with alkaline borohydride, separation of the carbohydrate part from the peptide part with Dowex-50W X-8 (H^+) resin, and identification of the sugar residue and the amino acid residue involved in the glycosyl–amino acid bond. These experiments were performed by following the procedure described in ref. 6.

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