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STRUCTURAL STUDIES OF AN ARABINOGALACTAN–PROTEIN FROM THE GUM EXUDATE OF *Acacia robusta*

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ABSTRACT

The gum exudate from *Acacia robusta* (subspecies *clavigera*) has been found to contain protein (18% w/w), bound to an arabinogalactan having structural features typical of the gum polysaccharides from Acacias of Bentham's Series 4 (Gummiferae), of which *A. robusta* is a member. Three sequential Smith degradations yielded an oligosaccharide of molecular weight ~700, indicating the presence in the galactan core of very small blocks of contiguous (1→3)-linked D-galactopyranosyl residues. Such an oligosaccharide was produced after only one Smith degradation, following removal of the L-arabinosyl units (constituting ~50% of the total carbohydrate) by partial hydrolysis of the arabinogalactan with acid. This partial hydrolysis resulted in the detachment of a small proportion (~25%) of the protein from the polysaccharide, whereas virtually all of it was detached by Smith degradation. These results have implications with regard to the nature of the linkages between the arabinogalactan and the 4-hydroxy-L-proline-rich, protein component of this gum.

INTRODUCTION

Numerous studies in this laboratory have established a general regularity of structure in the galactan cores of the arabinogalactans from the gum exudates of *Acacia* species included in Series 1 (Phyllodineae)[1–5] or Series 2 (Botryocephalae)[1,6,7] of the standard Bentham classification. Subsequent extension of the scope of the investigation to include the polysaccharide occurring in the gum of *Acacia senegal* (Bentham Series 5; Vulgares) has afforded evidence for similar regularity in the molecular structure of this arabinogalactan[8], despite its higher average molecular weight and greater structural complexity. In each of the cases cited, sequential Smith degradations[9] indicated the presence in the galactan core of uniform blocks of (1→3)-linked D-galactopyranosyl residues, the blocks being con-

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nected to one another through sugar residues that are vulnerable to Smith degradation, or become so after removal of protecting branches, by prior Smith degradation or by partial hydrolysis with acid. The number of contiguous, (1→3)-linked, D-galactopyranosyl residues constituting this regularly repeating block in the galactan core varies according to the *Acacia* species involved, ranging from ~12 to >30.

In contrast, structural studies by D. M. W. Anderson *et al.* of the gum polysaccharides from several *Acacia* species that are members of Bentham's Series 4 (Gummiferae)[10–16] indicated that long chains of (1→3)-linked, D-galactopyranosyl residues are not a dominant structural feature of these arabinogalactans. The presence in the galactan core of a high proportion of D-galactopyranosyl residues that are vulnerable to Smith degradation, or can become so, was demonstrated by the very considerable extent of cleavage of the main skeletal chain that was noted when the gum polysaccharides from such species were subjected to two or more successive Smith degradations[10,14,15]. Recently, further evidence for the small size of the blocks of periodate-resistant, (1→3)-linked D-galactopyranosyl residues in the galactan core of the gum polysaccharide from an *Acacia* of Series 4 was obtained in our laboratory by sequential Smith degradations of the arabinogalactan from *A. karroo* gum, which yielded, after three such degradations, an oligosaccharide (having a molecular weight of the order of 800) as the only carbohydrate product[17]. A similar oligosaccharide was obtained after only two successive Smith degradations of a sample of the gum polysaccharide from which protecting L-arabinosyl groups had been removed by partial, acid hydrolysis. These results suggest that the maximum number of contiguous, (1→3)-linked D-galactopyranosyl residues in the galactan core of the *A. karroo* gum polysaccharide is not much greater than four.

Another feature of the gum exudates from Series 4 that have been examined to date is the wide variation in nitrogen content, an indicator of the extent to which proteinaceous material is present in the gum. This is negligible (nitrogen content <0.3%, corresponding to a protein content of <2%, w/w) for the gums of *A. karroo*[17,18] and several other species, such as *A. nilotica*[18–20], *A. nubica*[11,19], *A. seyal*[15,19], and *A. arabica*[10], whereas proportions of nitrogen in the range of 1.0–1.5% (corresponding to protein contents of 6–9%) have been reported for the gums from *A. drepanolobium*[19] and from different subspecies of *A. tortilis*[21], and, more recently, a nitrogen content as high as 9.4% (protein, 59% w/w) has been found in the gum of *A. hebeclada*[22].

Our interest in *A. robusta* gum was prompted largely by the fact that its nitrogen content (2.85%, corresponding to a protein content of 18%) is intermediate between the extremes just noted. The structural study presented here has formed part of a comparative investigation of several gums, differing widely in protein content, from *Acacia* species of Series 4 that is currently in progress in this laboratory[17,23]. In addition to the chemotaxonomic value of such an investigation, gums containing protein in appreciable proportion are of interest *per se*, as the

structures and possible biological functions of arabinogalactan–protein conjugates in plants are the subject of much attention and speculation[24,25].

EXPERIMENTAL

Origin and purification of gum sample. — The specimen of gum was collected at Tshokwane, Kruger National Park, Transvaal, South Africa, from a tree that was labelled as *A. robusta* Burch., subspecies *clavigera*. This was authenticated by courtesy of Dr. E. A. C. L. E. Schelpe and staff of the Bolus Herbarium, University of Cape Town, who also confirmed the classification of this *Acacia* species as a member of Bentham's Series 4 (Gummiferae), subspecies 2 (Medibracteatae).

When a suspension of the crude gum (2.7 g) in water (100 mL) was stirred overnight, most of the gum dissolved, but some gelatinous material remained after removal of insoluble debris (bark, *etc.*) by filtration. This residual gel was solubilized by sonication. Examination of both fractions (water-soluble and solubilized) by chromatography on a Sepharose 4B column showed no difference in molecular-weight distribution, and so the two were combined. Precipitation with ethanol (4 vol.) yielded the arabinogalactan–protein, which was purified by dialysis and then recovered (in a yield of 1.36 g) by freeze-drying.

The product thus isolated had $[\alpha]_D$ +36° (*c* 0.3; solution clarified by sonication) and $\overline{M}_w$ 7.2 × 10^5 (single peak in steric-exclusion chromatography on Sepharose 4B); found, by microanalysis: N, 2.85% (protein content, ~18%).

General experimental methods. — The solvent systems used in paper chromatography were (v/v): *A*, 8:2:1 ethyl acetate–pyridine–water; *B*, 10:4:3 ethyl acetate–pyridine–water; *C*, 4:1:5 (upper phase) 1-butanol–ethanol–water; and *D*, 2:1:1 1-butanol–acetic acid–water. The *p*-anisidine hydrochloride and ammoniacal silver nitrate spray reagents were used to detect sugars and polyols in p.c., and the ninhydrin reagent to detect amino acids.

G.l.c. was performed on column *1* (2 m × 3 mm i.d.), consisting of 3% of OV-225 on Chromosorb W-HP, 80–100 mesh, or 2, a glass capillary column (26 m × 0.35 mm i.d.) coated with Silar 10C, in a Carlo-Erba 4200 gas chromatograph, coupled to a Columbia Supergrator-3A integrator for quantitative analyses. For g.l.c.-m.s., an identical chromatograph was coupled, through a jet separator, to a VG Micromass 16F mass spectrometer. The carrier gas was helium. G.l.c. analysis of mixtures of sugars, as the derived alditol acetates (prepared by the method of Albersheim *et al.*[26]) was performed on column *1*, isothermally at 220°, and that of mixtures of methylated sugars, similarly derivatized, on the same column at 170°. Column *2* was used in the analysis of mixtures of polyols (as their peracetylated derivatives); the temperature program was 170 → 230° at 4°/min.

Molecular weights ($\overline{M}_w$) of the arabinogalactan–protein and its degradation products were estimated by steric-exclusion (gel-permeation) chromatography[27], on Sepharose 4B or Bio-Gel P-10, as appropriate.

The emergence of protein, where present, in the column effluent was moni-

tored by reading the absorbance of each fraction at 220 nm (with a Beckman UV 5260 spectrophotometer), prior to the usual colorimetric assay for carbohydrate (phenol–sulfuric acid method[28]).

Degradation products having molecular weights of <1000 were further examined by l.c. at 5–6 MPa on a cartridge (10 cm × 8 mm) of Dextropak (Waters Associates) which was used under radial compression (Waters Z-Module) with water (at a flow rate of 1 mL/min) as the eluant, as recommended by Cheetham *et al.*[29]. Samples (25–100 μL, ranging in concentration from 100 to 20 mg/mL) were injected through a Waters U6K universal injector. The other components of the modular l.c. system were a Constametric Model II pump (LDC) and a differential refractometer detector (LDC Refracto Monitor).

The proportions of neutral sugars constituting the polysaccharide and products of degradation were determined by g.l.c., following hydrolysis in sealed tubes with 2M trifluoroacetic acid for 8 or 18 h at 100°. The shorter period was considered advisable where L-arabinose was a major constituent. The extent of degradation of this acid-labile pentose under the conditions used for hydrolysis was estimated by analysis (g.l.c. and phenol–sulfuric acid assay) of a standard solution of L-arabinose that had been exposed to the same conditions: heating for 8 and 18 h resulted in losses of the order of 10 and 25%, by weight, respectively. These factors were applied as corrections to all analyses of hydrolyzates containing L-arabinose.

The proportions of uronic acid in the arabinogalactan–protein, before and after partial hydrolysis with acid, were determined by the highly specific, colorimetric method of Blumenkrantz and Asboe-Hansen[30], which is not subject to interference from the protein present, as is the titrimetric procedure for determination of the uronic acid content of polysaccharides.

In methylation analysis of the arabinogalactan–protein, the Hakomori procedure[31] (as modified by Sandford and Conrad[32]) did not result in complete methylation, and three further treatments, by the Purdie method, were required before the i.r. spectrum indicated that methylation was complete. Permethylation of the degraded polysaccharides obtained by partial hydrolysis and by Smith degradation was achieved in a single treatment by the modification of the Hakomori procedure proposed by Phillips and Fraser[33], in which potassium methylsulfinylmethide (dimsyl potassium) is substituted for dimsyl sodium. In all cases, the permethylated polysaccharides were hydrolyzed, in sealed tubes, with 2M trifluoroacetic acid for 18 h at 100°, prior to reduction, acetylation, and g.l.c. analysis as already described. A portion of the methylated arabinogalactan–protein was submitted to base degradation[34,35] with dimsyl potassium, and then remethylated and analyzed as before.

Amino acid analyses. — The presence of amino acids in hydrolyzates of the whole gum, and of fractions isolated from the products of Smith degradation of this and the partially hydrolyzed gum, was demonstrated by paper chromatography (solvent *D*), using ninhydrin as the spray reagent. The proportion of 4-hydroxy-L-proline in the arabinogalactan–protein was determined by the specific, colorimetric method proposed by Leach[37]. For this analysis, a sample (12 mg) was hydrolyzed

under nitrogen, in a sealed tube, with 6M hydrochloric acid for 24 h at 110°. Standard solutions (5–15 μg/mL) of authentic 4-hydroxy-L-proline in 0.4M hydrochloric acid were used for calibration.

G.l.c. analyses of the mixtures of amino acids produced on hydrolysis of the protein component of this proteoglycan were performed, by courtesy of Dr. I. M. Moodie, at the Fishing Industry Research Institute, University of Cape Town. The method used[38] involved derivatization of the amino acids by esterification with 2-butanol containing hydrogen chloride gas (3M), followed by acylation with 2:5 (v/v) heptafluorobutanoic anhydride–ethyl acetate. The derivatized mixture, dissolved in acetic anhydride, was analyzed by g.l.c. on a capillary column coated with OV-101 (temperature program, 80 → 230° at 6°/min). Two hydrolyzates, prepared under different conditions (see footnotes to Table IV), were thus analyzed; norleucine was added as an internal standard in both cases.

Chromatography on hydroxylapatite. — In order to ascertain whether or not all of the protein present in *A. robusta* gum was bound to carbohydrate, a sample of the purified gum was examined by chromatography on hydroxylapatite[39]. The sample (15 mg) was applied to the column (20 × 0.9 cm) in 0.01M phosphate buffer, pH 7.4, and eluted first with this buffer, then with a phosphate gradient 0.1 → 0.4M, and finally with 0.4M phosphate buffer, the pH being maintained at 7.4. The fractions (1 mL) collected were monitored for protein (spectrophotometrically, at 220 nm) and for carbohydrate (phenol–sulfuric acid assay). Protein contents were estimated from the absorbance readings by calibration with a standard solution of the proteoglycan, and carbohydrate by similar calibration of the colorimetric assay.

Partial hydrolysis with acid. — A sample (505 mg) of the arabinogalactan–protein was heated at 100° in 25mM sulfuric acid (100 mL; pH 2.3) for 9 h (under which conditions virtually all of the L-arabinosyl groups were removed from the arabinogalactan of *A. karroo* gum[17]). The specific rotation decreased to +30.0° during the first 5 h of hydrolysis, and reached its equilibrium value of +28.5° at 7 h. The partially hydrolyzed product, A, was recovered, after neutralization ($BaCO_3$) of the acid, by precipitation with ethanol followed by freeze-drying, as previously described[8,17], giving A (216 mg); it had $[\alpha]_D$ −8° (*c* 0.44) and $\overline{M}_w$ 4 × 10^5 (single peak on Sepharose 4B); found: N, 3.8% (~24% of protein). P.c. (solvent *A*) and g.l.c. of a hydrolyzate showed D-galactose to be the only neutral-sugar constituent. The proportion of uronic acid in A was 19% (w/w) of the total, corresponding to 25% w/w (23 mol %) of the carbohydrate portion. P.c. (solvent *D*) of a sample hydrolyzed in 2M trifluoroacetic acid for 6 h at 100° showed the presence of components having the mobility, and color response with the *p*-anisidine spray reagent, of the aldobiouronic acids 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose, and 4-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose.

The fraction remaining in solution in aqueous ethanol after precipitation of A was concentrated to a syrup (205 mg), which was found on p.c. (solvent *A*) to contain L-arabinose as the major component, with some D-galactose and a

TABLE I

DETAILS OF SMITH DEGRADATIONS PERFORMED ON *A. robusta* ARABINOGALACTAN-PROTEIN AND PARTIALLY HYDROLYZED PRODUCT, *A*

	Arabinogalactan–protein			*A*	
Smith degradation	*1*	*2*	*3*	*1*	*2*
Mass of sample (mg)	374	59	10	161	10
Duration of oxidation[a] (h)[b]	144(72)	96(72)	96	72(48)	72
Periodate consumption (mmol.g^{-1})	5.7	3.4	5.8	7.7	2.6
Duration of treatment with acid[c] (h)[d]	96(72)	120(72)	78	90(64)	72
Yields of fractions (mg)					
Insoluble	82[e]	14[f]	[g]	50[h]	[g]
Soluble	80[e]	22[f]	[g]	83[h]	[g]

[a]In 0.12M $NaIO_4$. [b]In parentheses, time after which no further increase in consumption of IO_4^- was observed. [c]M trifluoroacetic acid at room temperature (22°). [d]In parentheses, time after which no further change in molecular-weight distribution was observed. [e]On fractionation in 1:1 (v/v) methanol–acetone. [f]On fractionation in 1:2 (v/v) methanol–acetone. [g]Not isolated. [h]On fractionation in 1:3 (v/v) methanol–acetone.

disaccharide having the mobility of 3-*O*-β-L-arabinopyranosyl-L-arabinose, as well as a trace of L-rhamnose. The syrup also contained some material that did not migrate in solvent *A*: this component, which was strongly fluorescent, gave a yellow coloration with ninhydrin. Addition of water to the syrup caused some precipitation of this apparently proteinaceous material, but it was not possible to isolate it in a quantity sufficient to permit further examination.

Smith degradations. — A series of three sequential, Smith degradations was performed with the intact arabinogalactan–protein as the starting material, and a sample of the partially hydrolyzed product, A, was submitted to two successive Smith degradations. The experimental conditions for these degradations, details of which are summarized in Table I, were, in general, as described previously[1,5,40,41]. Because of the small quantities of material available, the consumption of periodate was monitored in each case by the modification[40,41] of the arsenite method[42] in which graduated syringes are used in the titration of small aliquots (100 μL or less). Reduction with sodium borohydride was terminated by acidification with acetic acid, followed directly by freeze-drying[8]. After the removal of borate by evaporation with methanol, the reduced, oxidized product in the first Smith degradation of the whole arabinogalactan–protein was further purified by dialysis. A portion of the dialyzate was concentrated, and analyzed for carbohydrate (phenol–sulfuric acid assay) and for polyol (chromotropic acid method[43]): no carbohydrate was detected, but polyol (equivalent to ~12 mg in the total sample) was found. This dialysis step was omitted in all other cases. With the exception of the third Smith degradation in the series starting with the whole arabinogalactan–protein, and the second in that starting with A, in which paucity of material necessitated the use of a modified procedure[5,41] involving consumption of the entire reaction-mixture in

steric-exclusion chromatography, the products of the Smith degradations were isolated by fractionation as indicated in Table I. This gave SD1 and SD2 (from the whole arabinogalactan–protein) and SD1A (from A), with soluble syrups. The latter, which were produced in yields constituting a high proportion of the total products in all three cases, were found by p.c. (solvent *C*) to consist mainly of glycerol. The only sugar detected in the soluble fractions (p.c., solvents *A* and *C*) was L-arabinose, which was present in appreciable proportion (~14%, by weight, by the phenol–sulfuric acid assay) in the syrup separated from SD1, but only in traces in that fractionated from SD2; in the latter, p.c. revealed a further component, possibly a glycoside, migrating more slowly than arabinose and galactose.

The syrups separated from SD1 and SD1A also contained, in appreciable proportion, a strongly fluorescent component that did not migrate in p.c. with solvent *A*. This material was precipitated when water was added to the syrups, and it was isolated by centrifugation. A portion of each water-insoluble fraction, dried *in vacuo* at 40°, was submitted to microanalysis: the fractions isolated from SD1 and SD1A had nitrogen contents of 10.7 and 10.5% (w/w), respectively. The proteinaceous nature of these fractions was further demonstrated by the production of amino acids on hydrolysis (p.c., solvent *D*, ninhydrin spray-reagent).

After removal of the water-insoluble fractions, the soluble syrups were further examined by l.c. at 5–6 MPa and (following reduction and acetylation) by g.l.c. (column 2).

Analytical results for SD1, SD2, and SD1A are given in Table II. A sample (4 mg) of SD1A was submitted to 5.24-MPa l.c., which gave a sharp peak at a retention time of ~3 min, and a broad peak having a maximum at ~12 min. Both

TABLE II

ANALYTICAL DATA FOR *A. robusta* ARABINOGALACTAN–PROTEIN, PARTIALLY HYDROLYZED PRODUCT (*A*), AND INSOLUBLE PRODUCTS (TABLE I) OF SMITH DEGRADATION

	Arabino-galactan–protein	*A*	*SD1*	*SD2*	*SD3*	*SD1A*	*SD2A*
$[\alpha]_D$ (degrees)	+36	−8	−33	−9	[a]	−22	[a]
$\bar{M}_W$	720,000[b]	400,000[b]	330,000[b]	3000[c]	700[c]	750[c]	550[c]
Nitrogen (%)	2.8	3.8	~0.3	n.d.[d]		<0.2	
Hence, protein (%)	18	24	<2	n.d.		tr.[e]	
Proportions of constituent residues (mol %)							
Galactose	40	77	60	100		78	
Arabinose	50	—	40	tr.		—	
Rhamnose	1	—	—	—		—	
Uronic acid	9	23	—	—		—	
Glycerol	—	—	—	n.d		22	

[a]Not isolated. [b]Single peak on Sepharose 4B. [c]Single peak on Bio-Gel P-10. [d]n.d = not determined. [e]tr. = trace (<1%).

fractions were collected for further investigation: that eluting early (~15% of the total sample) was found to contain neither carbohydrate nor polyol, but to consist of inorganic salts, as addition of Amberlite IR-120 (H^+) resin caused a solution of this fraction to become strongly acidic, whereas the fraction eluted later contained the carbohydrate. A sample (2 mg) of this material, recovered by freeze-drying, was re-examined by chromatography on Bio-Gel P-10.

A portion (3 mg) of SD1A was hydrolyzed, and the hydrolyzate, after reduction and acetylation, was analyzed by g.l.c. on column *2*, which afforded an estimate of the molar ratio of the residues of D-galactose and glycerol constituting SD1A. The degree of polymerization and homogeneity of SD1A were further verified by p.c. (solvent *B*; ammoniacal silver nitrate as the spray reagent).

RESULTS AND DISCUSSION

The analytical data presented in Table II, and the results of methylation analysis (see Table III), show that the arabinogalactan occurring in the gum exudate of *A. robusta* has structural features that are typical of the gum polysaccharides of Series 4 *Acacia* species. The positive specific rotation and high molecular weight of this gum are characteristic of the Series Gummiferae[10–22], as are the high proportion of L-arabinosyl units in the arabinogalactan and its low proportions of con-

TABLE III

METHYLATION ANALYSES OF *A. robusta* ARABINOGALACTAN-PROTEIN AND DERIVATIVES

Methyl ethers[a]	*Methylated polysaccharide*			
	Arabinogalactan–protein (mol%)[b]	*Base-degraded, remethylated (mol%)*	*A (mol%)*[b]	*SD1 (mol%)*
2,3,4-Rha	1	1	—	—
2,3,5-Ara	11	12	—	19
2,3,4-Ara	8	9	—	—
3,5-Ara	6	9	—	15
2,5-Ara	4	9	—	—
2,3-Ara / 3,4-Ara	11	13	—	—
2,3,4,6-Gal	7	13	16	13
2,4,6-Gal	4	6	17	18
2,3,6-Gal	6	3	3	—
2,3,4-Gal	2	3	16	12
2,6-Gal	—	2	—	—
2,3-Gal	—	—	2	2
2,4-Gal	21	20	18	17
2-Gal	10	—	5	4

[a]As acetylated alditols; identities, indicated by retention times relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, verified by mass spectrometry. [b]Molar proportions adjusted to allow for proportions of uronic acid.

TABLE IV

PROPORTIONS OF AMINO ACIDS IN *A. robusta* ARABINOGALACTAN-PROTEIN

Amino acid[a]	*Proportion (% by weight)*[b]	
	Hydrolyzate 1[c]	*Hydrolyzate 2*[d]
*	—	0.3
Alanine	4.0	3.7
Glycine	3.7	3.4
*	0.4	2.1
Valine	5.0	4.5
Threonine	5.2	5.0
Serine	7.5	7.2
Leucine	7.2	7.5
Isoleucine / Norleucine	3.2	2.9
*	—	1.4
Proline	4.5	5.0
*	0.6	0.5
*	3.0	3.3
Hydroxyproline[e]	18.8	18.1
Methionine	—	0.8
Aspartic acid	9.6	9.4
*	2.3	3.4
Phenylalanine	3.5	3.4
*	1.3	0.5
Glutamic acid	5.9	5.6
Lysine	4.6	3.6
Tyrosine	4.7	4.6
Arginine	2.5	1.6
Histidine	1.2	1.0
*	1.3	1.0

[a]Unknown component represented by asterisk. [b]Expressed as proportion of total amino acids. [c]6M HCl, 24 h, 110°. [d]Constant-boiling HCl, 22 h, 110°. [e]Independent analysis by Leach method gave value of 2.97% (w/w) for proportion of hydroxyproline in total arabinogalactan-protein; this corresponds to 16.7% of protein.

stituent rhamnose and uronic acid. The main structural features that are indicated by the methyl ethers detected on methylation analysis, namely, a branched galactan skeleton, linked (1→3) and (1→6), to which are attached sidechains in which many of the L-arabinosyl units are present not only as terminal groups (L-arabinopyranosyl and L-arabinofuranosyl) but also as chain units, are very typical[10–17]: these chain units include (1→2)- and (1→3)-linked L-arabinofuranosyl residues, as well as (1→5)-linked L-arabinofuranosyl or (1→2)-linked L-arabinopyranosyl units, or both. The changes in the proportions of the methyl ethers constituting the per-*O*-methylated polysaccharide that resulted from base degradation (see Table III) indicate, as in the case of *A. karroo* gum[17], that the uronic acid units are terminal. Their attachment to D-galactopyranosyl residues at O-4 or O-6 is evident from the aldobiouronic acids produced on partial, acid hyd-

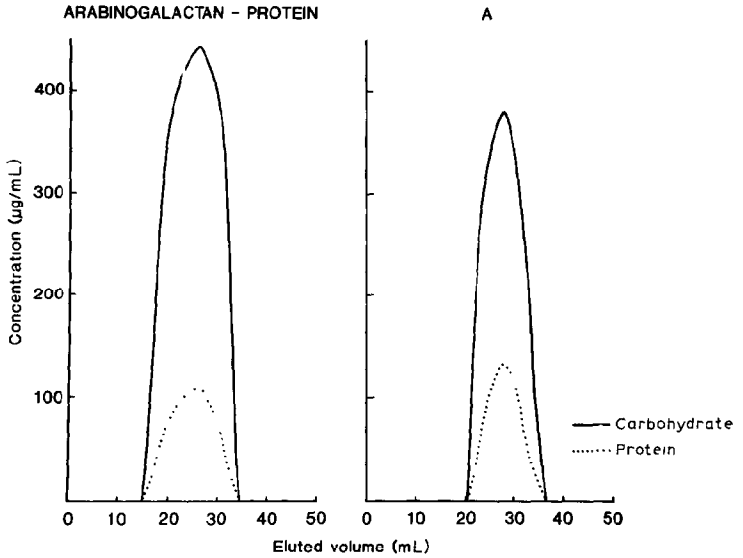


Fig. 1. Chromatography of *A. robusta* arabinogalactan–protein and of partially hydrolyzed product (A) on a column (60 × 0.9 cm) of Sepharose 4B with M sodium chloride as the eluant.

rolysis: these acids, and the arabinobiose 3-*O*-β-L-arabinopyranosyl-L-arabinose, have been reported as products of a number of Series IV gums[10–12,15–17,20].

A major point of difference between the gum of *A. robusta* and the other gums from this Series that have been examined is its protein content, which is higher than those of any for which detailed structural investigations have been reported[10–17]. The amino acid composition of the protein component of the gum is shown in Table IV. The high proportion of 4-hydroxy-L-proline among the constituent amino acids, which was confirmed by the independent, colorimetric assay, is a feature of many arabinogalactan–proteins of plant origin[24,25]. The other amino acids that are present in greater proportions (>7%) than the rest are aspartic acid, serine, and leucine. Of these, serine is generally considered to be another important constituent of plant proteoglycans[24,25], and has been reported as a major component of the proteinaceous fractions of a number of plant gums[44]. High proportions of aspartic acid have been found among the amino acid constituents of the gums from *Acacia campylacantha*, *Azadirachta indica*, and two *Araucaria* species, and leucine is among the preponderant components of the proteinaceous gums from *Lannea* species[44].

When chromatography of the arabinogalactan–protein from *A. robusta* gum on Sepharose 4B was monitored for the emergence of both protein and carbohydrate, it was found that the two were co-eluted (see Fig. 1). There was no evidence for the presence of free protein from this chromatography, or from that on hydroxylapatite. In the latter, neither protein nor carbohydrate was detected in the final fractions, obtained on elution with the 0.4M phosphate buffer, under which

conditions free protein should be desorbed from hydroxylapatite[39] (this was verified by chromatography of a sample of egg albumin). Most (~60%) of the arabinogalactan–protein was eluted from the hydroxylapatite column in a sharp peak (I) near the void volume, but a second fraction (II) emerged during the gradient elution. Samples of these two fractions, isolated by freeze-drying, after dialysis of the appropriate portions of the effluent, were found to differ in protein content (~12% w/w for I, ~26% for II), but their specific rotations (+38° and +32° for I and II, respectively) differed little from the value (+36°) found for the whole proteoglycan, and there was no detectable difference in $\bar{M}_w$ among I, II, and the whole sample. Although these results indicated some heterogeneity in respect of the proportion of protein bound to arabinogalactan, poly(acrylamide)-gel electrophoresis (by courtesy of Mr. D. W. Gammon) gave only a single band.

Interestingly, whereas virtually all of the protein became detached from the polysaccharide as a result of the first Smith degradation of the *A. robusta* arabinogalactan–protein (see Table II), most of it remained bound after partial hydrolysis of the proteoglycan with acid, which removed all of the L-arabinosyl units (see Tables II and III). The bound protein was again co-eluted with the carbohydrate on chromatography of the partially hydrolyzed product, A, on Sepharose 4B (see Fig. 1), and no free protein was detected in this fraction. There was, however, some indication of free protein among those products of the partial hydrolysis that remained in solution in aqueous ethanol, and the discrepancy between the value of 24% that was found for the protein content of A and the value of 32% (calculated from the ratio of the $\bar{M}_w$ values for A and the whole arabinogalactan–protein), predicted if all of the protein initially present were to remain bound in A, suggests that partial hydrolysis, under the conditions described, causes ~25% of the total protein content of this proteoglycan to become detached from the polysaccharide. It may be postulated that this proportion is linked through acid-labile, L-arabinofuranosyl units, and the rest through D-galactopyranosyl residues which are, however, themselves linked to the polysaccharide chain in a manner rendering them vulnerable to cleavage by periodate, and thus, to Smith degradation. The L-arabinofuranosyl units involved in the polysaccharide–protein linkage must also be vulnerable to attack by periodate.

The presence of more than one type of linkage between polysaccharide and protein in an arabinogalactan–protein had been reported previously[24,25]: for example, there is evidence for both *O*-D-galactopyranosyl-serine and 4-glycosyloxy-L-proline linkages in the proteoglycan fractions isolated from *Cannabis sativa* leaves of South African origin[45,46]. The characteristic presence of 4-hydroxy-L-proline in high proportion in many proteoglycans of plant origin strongly suggests the implication of this amino acid in polysaccharide–protein linkages, and glycosylation of 4-hydroxy-L-proline both with D-galactopyranosyl and with L-arabinofuranosyl residues has been detected[24,25]. The nature of the polysaccharide–protein linkage in the *A. robusta* proteoglycan is not yet known, but the occurrence of more than one type is strongly suggested by the results of the present study.

Except for their effects on the binding of the protein component, the degradative processes applied in the investigation of the structure of *A. robusta* gum caused depolymerization to occur in a manner that was similar in most respects to that of the Series IV gums previously examined in detail[10–17]. Partial, acid hydrolysis resulted in an increase in the proportion of uronic acid residues, commensurate with the loss of acid-labile L-arabinosyl and L-rhamnosyl residues, and methylation analysis of the degraded polysaccharide A (see Table III) indicated the presence of (1→6)-linked D-galactopyranosyl residues, unprotected by branching, in appreciable proportion. The decrease in specific rotation that accompanied partial hydrolysis was another point of resemblance between this gum and others of the same Series[10–12,15,17], although the average molecular weight of A, while comparable with that of the corresponding product from *A. karroo* gum[17], was much higher than the values reported for the autohydrolyzed polysaccharides from the gums of *A. arabica*[10], *A. nubica*[11], *A. drepanolobium*[12], and *A. seyal*[15], which were of the order of 5000.

In yielding, after the first Smith degradation, a product (SD1) that retained a comparatively high average molecular weight and a large proportion of the L-arabinosyl units (see Table II), some of which remained as chain units (see methylation analysis, Table III), the *A. robusta* gum polysaccharide again resembles the others from Series IV that have been similarly investigated[10,11,14,15,17]. The marked fall in specific rotation accompanying the Smith degradation is also highly characteristic of Series IV gums[16]. The soluble syrup that was separated from SD1 by fractionation with 1:1 methanol–acetone was found to contain some ethylene glycol and threitol, in addition to glycerol, the major component. The formation of these minor products, which has also been reported in Smith–degradation studies of the gum polysaccharide of *A. nubica*[11], is attributable to cleavage of the L-arabinopyranosyl end-groups and of (1→4)-linked D-galactopyranosyl residues. From the g.l.c. analysis, the molar ratio of glycerol to threitol in the soluble syrup from the first Smith degradation of the *A. robusta* gum arabinogalactan was estimated at 20:1. The presence of free L-arabinose in this syrup (20 mol%, by g.l.c. analysis) was a consequence of the extended period of mild, acid hydrolysis that was required in order to ensure complete cleavage of acetal linkages in the Smith degradation[47,48]. The possible effect of this cleavage of L-arabinofuranosidic linkages on the extent of depolymerization of the galactan chain in subsequent degradations in the sequence, owing to the exposure of interior residues that would not otherwise have been vulnerable, had been noted previously[8]. No components larger than L-arabinose were detected on examination of the water-soluble fraction of the syrup by l.c. and p.c.

The second Smith degradation resulted in considerable depolymerization, to an extent comparable with that observed in similar studies of the gum polysaccharides of *A. arabica*[10], *A. drepanolobium*[14], *A. seyal*[15], and *A. karroo*[17]. It was only after mild, acid hydrolysis of the reduced, oxidized polysaccharide had continued for 72 h that the molecular-weight distribution became constant, with the

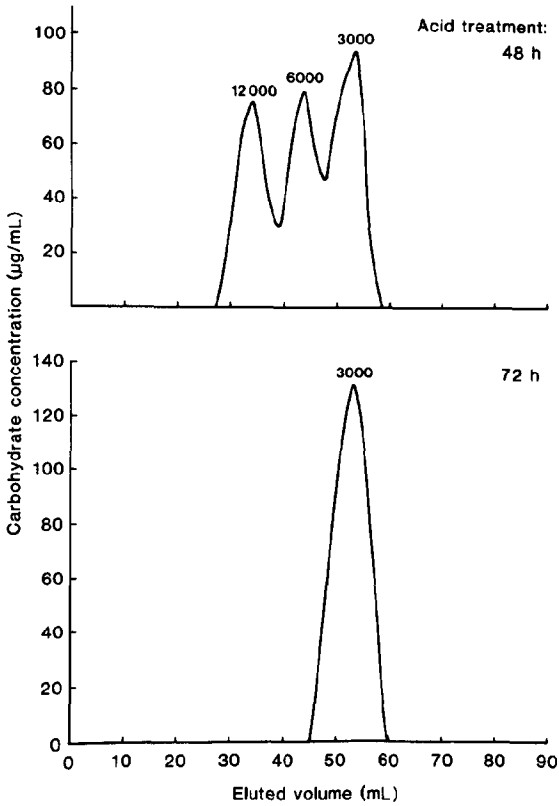


Fig. 2. Chromatography, on a column (52 × 1.5 cm) of Bio-Gel P-10, of samples removed during second Smith degradation in series starting with whole arabinogalactan–protein.

formation of an apparently monodisperse product, SD2, having a molecular weight (~3000) that is close to the value (~2500) found for the analogous product from *A. karroo* gum[17]. The presence of two further peaks, at elution volumes corresponding to molecular weights that are multiples of 3000, in the steric-exclusion chromatogram of a sample removed after mild, acid hydrolysis had proceeded for 48 h (see Fig. 2) suggests the occurrence of uniform, regularly repeating sub-units, separated by sugar residues that are vulnerable to Smith degradation, in the galactan chain of SD1.

The product SD2, like its analog from *A. karroo* gum[17], but in contrast to the corresponding products from the gums of *A. arabica*[10], *A. nubica*[11], *A. drepanolobium*[14], and, to a lesser extent, *A. seyal*[15], was found to consist very largely of D-galactopyranosyl residues, with only a trace of L-arabinose detectable, by p.c. and g.l.c., in the hydrolyzate. The syrup that was separated from SD2 by solvent-fractionation contained free L-arabinose in very low proportion (~3 mol%, by g.l.c., column 2), but a discrepancy between this and the total carbohydrate content of the syrup (9%, w/w) as estimated by phenol–sulfuric acid assay suggests the

presence of further L-arabinose glycosidically linked to glycerol. The occurrence of such a glycoside among the Smith-degradation products of low molecular weight was indicated by p.c., although this component was not resolved in l.c.

The removal, during the second Smith degradation, of virtually all of the L-arabinosyl units present in SD1 suggests that the L-arabinofuranosyl units linked through O-2 that appear, from methylation analysis (see Table III), to be present in appreciable proportion are, although themselves resistant to attack by periodate, in turn linked to periodate-vulnerable residues, possibly to O-6 of D-galactopyranosyl residues, in side-chains. This would result in the appearance of L-arabinosyl units in glycosides of low molecular weight, rather than as constituents of SD2, after the second Smith degradation.

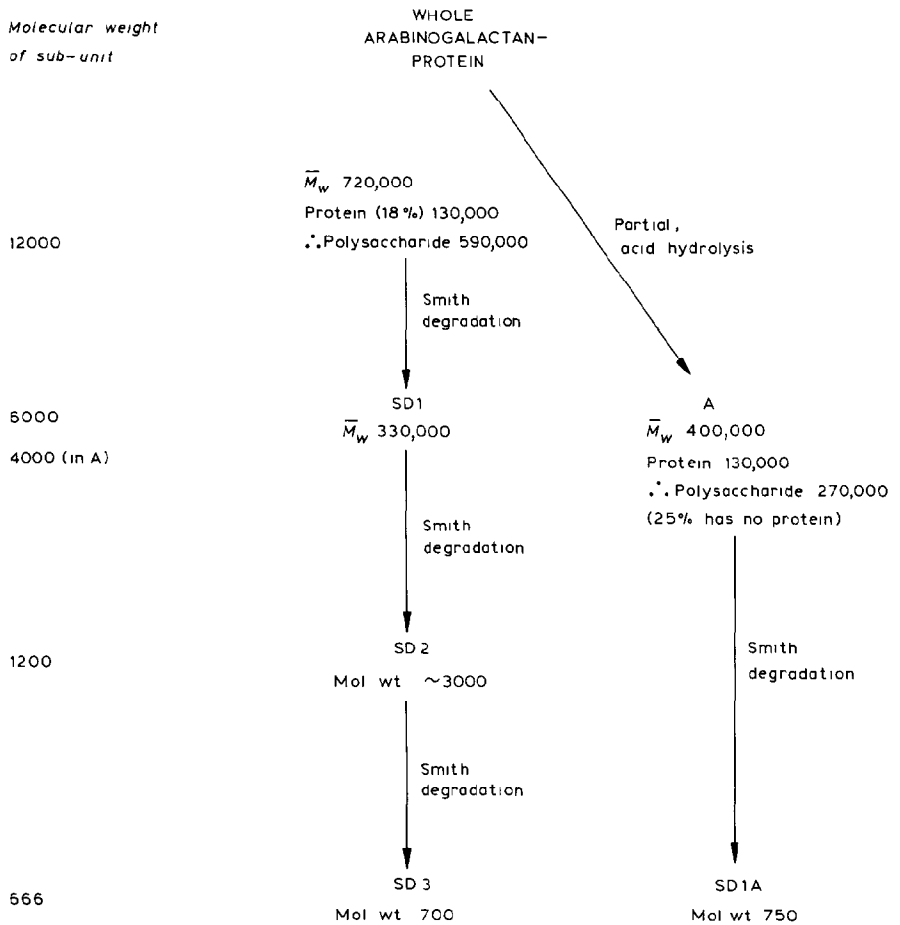
As in the cases of the other Series IV gums similarly investigated[10,14,15,17], further fragmentation of the galactan core resulted from the third Smith degradation of the *A. robusta* gum polysaccharide, which yielded an oligosaccharide (SD3) having a molecular weight of ~700 (see Table II) as the only carbohydrate product. The consumption of periodate by SD2 (5.8 $mmol.g^{-1}$, equivalent to 17 mol per mol) was consistent with the cleavage of ~8 D-galactopyranosyl residues. Although most of these must have been end-groups, the molecular weight of SD3 shows that cleavage also occurred in the middle of the main chain of SD2, with the survival of fragments containing only ~4 D-galactopyranosyl residues. In this respect, there is once again a strong resemblance to the polysaccharide of *A. karroo* gum[17], from which an oligosaccharide having a molecular weight of ~800 was obtained after three successive Smith degradations.

A product similar in size to SD3 was produced after only one Smith degradation of the partially hydrolyzed polysaccharide A. The resulting oligosaccharide, SD1A, had a molecular weight of ~750, which indicated the presence of four hexopyranosyl residues, in addition to a glycerolyl residue expected at the "reducing" end. The molar ratio of the constituent D-galactose and glycerol was estimated, from g.l.c. analysis of the hydrolyzate, at ~4:1 (see Table II), and was thus consistent with this postulated molecular structure. On p.c. in solvent *B*, SD1A moved as a single spot, with R_{Gal} ~0.4, a value lower than that of the trisaccharide (raffinose) used as a marker, whereas, in l.c., the carbohydrate present was eluted at a retention time comparable with that reported[29] for a maltodextrin having a degree of polymerization of ~5. Rechromatography, on Bio-Gel P-10, of a sample of this fraction collected after l.c., gave a chromatogram having a single peak at molecular weight ~750, identical to that given by the total product, SD1A.

Thus, all of the available evidence suggests that SD1A is a monodisperse oligosaccharide, composed of four D-galactopyranosyl residues and a glycerolyl end-group. The consumption of periodate (see Table I) and decrease in molecular weight (to 550; see Table II) on further Smith degradation of a sample of SD1A are consistent with the removal of a single D-galactopyranosyl end-group only, which indicates that the molecule is linear and that the constituent sugar residues are (1→3)-linked.

After removal of the proteinaceous material, the soluble syrup separated from SD1A by solvent fractionation was found, by p.c. and l.c., to contain polyol only. G.l.c. analysis showed the presence of glycerol and threitol, in the molar ratio of ~16:1; the production of threitol may be ascribed to the linkage of some of the uronic acid residues to O-4 of D-galactopyranosyl residues in the parent polysaccharide. This polyol has been found among the products of low molecular weight isolated after Smith degradation of autohydrolyzed *A. nubica* gum[11].

The breakdown pattern of the *A. robusta* gum polysaccharide differs from that observed on similar investigation of *A. karroo* gum[17] in one respect only: whereas the oligosaccharide of low molecular weight is yielded after only one Smith degradation of the partially hydrolyzed *A. robusta* gum, two successive degradations are needed in order to obtain a similar product from autohydrolyzed *A. karroo* gum, which appears to have fewer exposed, periodate-vulnerable residues in



Scheme 1. Chart illustrating breakdown of sub-units on degradation of *A. robusta* arabinogalactan-protein.

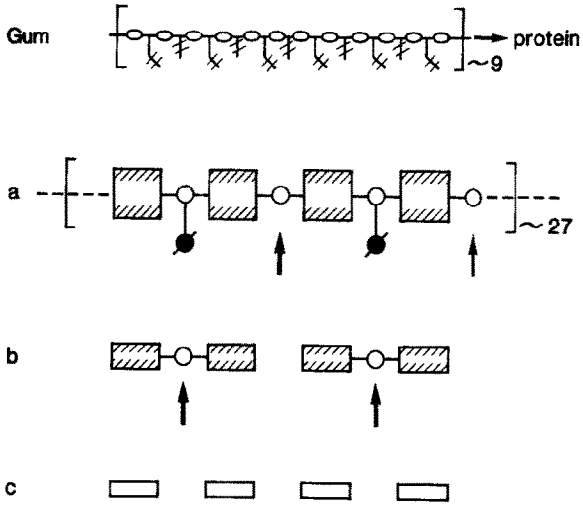


Fig. 3. An impression of sizes of sub-units at various stages of depolymerization of *A. robusta* gum polysaccharide (ellipses represent ~5000 mol.wt. units, these being interspersed by assemblages of ~4 sugar units). Hatched areas denote periodate-vulnerable sugars in side-chains: (a) for SD1, each square is a uniform fragment of mol.wt. ~3000; (b) for SD2, each rectangle is a block of ~7 D-Gal*p* units; (c) for SD3, each strip is a block of four →3)-D-Gal*p*-(1→ units. In a, the symbols -O- indicate linking sugars that are periodate-vulnerable, and -O- are linking sugars protected by side-chains.

the main, skeletal chain. Apart from this, it is evident that the *A. robusta* gum polysaccharide closely resembles that of *A. karroo* in having a galactan core composed of uniform blocks, each containing only about four contiguous, (1→3)-linked D-galactopyranosyl residues, separated by sugars, probably (1→6)-linked D-galactopyranosyl residues, that are vulnerable to attack by periodate, or become so after removal of protecting sugars.

From the size of these blocks, and the other experimental data, conclusions may be drawn regarding the sizes of the corresponding sub-units in the products of Smith degradation, the whole *A. robusta* arabinogalactan–protein, and its partially hydrolyzed derivative. These conclusions, which are illustrated in Scheme 1 and Fig. 3, are summarized as follows.

(i) SD3 probably differs from SD1A only in that half of the fragments have a glycolyl end-group, formed by oxidation of a glycerolyl end-group in SD2, arising, as does that in SD1A and the other half of SD3, from cleavage of unprotected (1→6)-linked D-galactopyranosyl residues in the main chain. Both SD3 and SD1A may be regarded as fundamentally being tetrasaccharides composed of four (1→3)-linked D-galactopyranosyl residues (see Fig. 3c).

(ii) SD2 can be envisaged as containing two of these tetrasaccharide blocks, linked through a periodate-vulnerable, (1→6)-linked D-galactopyranosyl residue, and terminated by a glycerolyl group. The consumption of periodate by SD2 indicates the presence of branching to the extent of at least three D-galactopyranosyl

end-groups in each of these two blocks, so that the size of the sub-unit must be that of a heptasaccharide (each of the uniform rectangles in Fig. 3b represents such a unit).

(iii) The central, (1→6)-linked D-galactopyranosyl residue in SD2 must have been protected by branching in SD1 (see Fig. 3a); as no product analogous to SD2 was afforded by the partially hydrolyzed polysaccharide A, the protecting branch was probably an L-arabinosyl residue. Thus, the galactan core of SD1 may be visualized as being composed of the blocks of four (1→3)-linked D-galactopyranosyl residues, joined through (1→6)-linked D-galactopyranosyl residues, alternately protected and unprotected. The subunit must now be considered to be a chain of nine D-galactosyl residues (that is, two of the tetrasaccharide blocks, joined by the protected residue), bearing six side-chains, each with one D-galactosyl residue surviving into SD2. From the results of methylation analysis of SD1, it may be concluded that these side chains include 2-linked L-arabinofuranosyl and 6-linked D-galactopyranosyl residues, as well as D-galactopyranosyl and L-arabinofuranosyl end-groups. The proportions of these units, all of which are removed on Smith degradation of SD1, total ~60%, from which the molecular weight of each of the blocks represented by squares in Fig. 3a is estimated at ~3000, and that of the sub-unit (two blocks joined through a protected sugar) ~6000. The value of 330,000 found for the $\overline{M}_w$ of SD1 indicates the presence of ~55 such sub-units per molecule: the number of breaks in the main galactan chain accompanying the second Smith degradation of the *A. robusta* arabinogalactan must thus be of this order.

(iv) The proportions of periodate-vulnerable residues in the whole arabinogalactan (calculated from the methylation analysis and the uronic acid content) total ~50%, whence the molecular weight of the sub-unit in the intact polysaccharide is estimated at ~12,000. The observed value of $\overline{M}_w$ includes a contribution from the bound protein (~130,000), so that $\overline{M}_w$ for the arabinogalactan itself must be decreased to ~590,000. The presence of ~55 sub-units per average molecule (calculated in *iii*) would give a polysaccharide having $\overline{M}_w$ ~660,000. The discrepancy may be attributed to the error (~10%) inherent in the method used to determine $\overline{M}_w$, although some polydispersity of both arabinogalactan and protein cannot be excluded as a possible reason for the degree of heterogeneity (with respect to protein content) that was suggested by the fractionation observed on chromatography of the arabinogalactan–protein on hydroxylapatite.

(v) The removal of all L-arabinosyl units from the arabinogalactan by partial, acid hydrolysis results in the exposure of every (1→6)-linked D-galactopyranosyl residue in the galactan core, and the remaining side-chains, which contain residues of D-galactose and uronic acid only, are likewise vulnerable to attack by periodate, so that A is fragmented to the tetrasaccharide blocks after only one Smith degradation. The molecular weight of the repeating sub-unit in A may be calculated by adding, to the fundamental unit of nine D-galactosyl residues in the main chain, the contribution of these periodate-vulnerable residues in side-chains (60%, from methylation analysis and uronic acid content), which gives an estimated value of

~4000. This predicts a value of ~220,000 for the $\overline{M}_w$ of the arabinogalactan in A (~55 repeating units), to which must be added the contribution of the protein (~130,000). The total of ~350,000 is again in fairly good agreement with the experimentally determined value (400,000). In this case, some polydispersity must result from the detachment of protein from arabinogalactan in ~25% of A.

Thus, it may be concluded that the arabinogalactan component of *A. robusta* gum has structural features, of which further details have emerged from the present study, that are considered typical of the gum polysaccharides from *Acacia* species of Bentham's Series IV. The protein component appears to be bound to the arabinogalactan through at least two different types of linkage. The high molecular-weight averages found for SD1, from which virtually all of the protein has been removed, and the partially hydrolyzed product, A, which has lost ~25% of the protein, seem to favor a structure in which there is only one polysaccharide–protein linkage per molecule, rather than the model that has been postulated for some arabinogalactan–proteins, in which the arabinogalactan is envisaged as several "clusters" anchored to a polypeptide core at various points (the "wattle blossom" model[25]). The low viscosity of solutions of the arabinogalactan–protein indicates that the molecule has the overall spheroidal shape that appears to be characteristic of such proteoglycans[24,25]. This is consistent with the highly branched structure indicated for the sub-units constituting the arabinogalactan, which may be linked to one another in a "branch-to-branch" manner, as has been suggested for *A. arabica* gum[10], resulting, as does the flexibility of the protein core[25], in a globular molecule.

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