Carbohydrate Research, 151 (1986) 135–146
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

ARABINOGALACTAN–PROTEIN COMPONENTS OF *Acacia tortilis* GUM*

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(Received August 29th, 1985; accepted for publication, November 25th, 1985)

ABSTRACT

The gum exudate from *Acacia tortilis*, its Smith-degradation products, and the partially hydrolysed gum are associated with protein. The presence of periodate-immune arabinosyl chains which protect otherwise vulnerable galactosyl residues is the cause of only limited breakdown of the gum during Smith degradation. Removal of the arabinosyl groups followed by Smith degradation results in depolymerisation to give short galactose-containing chains, mainly of uniform size. A molecular representation of the core structure, based upon these results and those of methylation analysis, is given.

INTRODUCTION

Studies of the polysaccharide gums from *Acacia* spp. in Bentham Series 4 (Gummiferae), reviewed by Anderson and Dea[1], have demonstrated that the main skeletal chains are composed of D-Gal*p* residues that are decomposed by periodate, a few successive Smith-degradations being sufficient to reduce the size of the resulting blocks of contiguous sugar residues to relatively small fragments. Three such degradations of the acidic arabinogalactan of *A. karroo* yield oligosaccharide material of molecular weight ~800, a similar product being obtained after two degradations if the protecting L-Ara*f* residues were first removed by mild acid hydrolysis[2]. For *A. robusta* gum and its autohydrolysed derivative, three degradations and one, respectively, suffice to yield blocks containing (1→3)-linked D-Gal*p* at the tetrasaccharide level[3].

Apart from polysaccharide, gums from Series 4 may contain up to 9.4% of nitrogen, indicative of various amounts of protein. For *A. robusta* gum, the value of 18% of protein is an intermediate one, and the results of partial acid hydrolysis and Smith degradation point to covalent attachment of carbohydrate to protein through at least two types of linkage[3]. In view of the protein content[4] of the *A. tortilis* product (12%) and of related studies on *A. senegal* gum[5,6], the protein-containing gum polysaccharide from *A. tortilis* has been investigated.

*Dedicated to Roger W. Jeanloz.

0008-6215/86/$ 03.50

EXPERIMENTAL

General methods. — P.c. was performed with *A*, 8:2:1 ethyl acetate–pyridine–water; *B*, 10:4:3 ethyl acetate–pyridine–water; *C*, 2:1:1 1-butanol–acetic acid–water; and *D*, 4:1:5 (upper phase) 1-butanol–ethanol–water; with detection using *p*-anisidine hydrochloride and alkaline silver nitrate (for carbohydrates) and ninhydrin (for amino acids). G.l.c. and g.l.c.–m.s. were performed as described earlier[3], using *1*, a column (2 m × 3 mm i.d.) packed with 3% of OV-225 on Chromosorb W-HP (80–100 mesh); *2*, a glass capillary column (25 m × 0.35 mm i.d.) coated with OV-225; and *3*, a quartz capillary column (30 m × 0.32 mm i.d.) with OV-225 (0.25-μm film thickness) as the bonded phase (Durabond DB-225; J. and W. Scientific).

Determination of molecular-weight distribution, methylation procedures, l.c., and analyses of mixtures of sugars and of methylated sugars in hydrolysates were carried out as previously described[3]. Analyses of mixtures of amino acids present in hydrolysates were performed according to the method of Moore *et al.*[7].

Origin and isolation of gum samples. — The raw material (4.9 g) from an exudate occurring on the bark of *A. tortilis* subsp. *heteracantha* (Forsk.) Hayne (Burch.) Brenan (trees growing near Kimberley, South Africa) was dissolved in water. The product (3.14 g) isolated from this solution by precipitation with ethanol–acetone (1:1, 4 vol.), redissolving the precipitate in water, and freeze-drying was used in the studies reported here.

The analytical results are shown in Table I and the results of chromatography in Figs. 1 and 2. The proportions (mol % of the total) of the amino acids were: Asp (13.4), Thr (5.2), Ser (7.5), Glu (6.5), Gly (7.2), Ala (4.4), Val (8.1), Ile (4.3), Leu (7.0), Tyr (1.3), Phe (7.1), Lys (3.5), His (1.8), Arg (2.1), Hyp (7.5), Pro (8.7), with 4.5% not identified.

Partial acid hydrolysis. — A sample (500 mg) of the *A. tortilis* arabino-galactan–protein (AG-P) was treated with 0.03M H_2SO_4 (50 mL) at 100° for 9 h when $[\alpha]_D$ fell from +75° to +55°. Addition of EtOH (4 vol.) to the neutralised ($BaCO_3$) and filtered solution yielded a precipitate, an aqueous solution of which was dialysed against water. The non-dialysable fraction (*A*, 110 mg) was examined by chromatography (Figs. 1 and 2), analysis for sugar composition (Table I), and methylation analysis (Table II). The dialysable portion contained ~35 mg of carbohydrate, hydrolysis of which gave (p.c.) Gal as the only neutral sugar constituent. The supernatant solution remaining after the fractionation with EtOH was concentrated to a syrup (360 mg) which consisted (p.c.) almost wholly of Ara (230 mg, ~85% of that present in the original gum). The N content (0.65%) was equivalent to ~4% of protein.

Smith degradations. — (*a*) A vacuum-dried sample (51 mg) of *A* was oxidised in 0.12M $NaIO_4$ (50 mL) and the product was reduced ($NaBH_4$) to the polyalcohol (36 mg), which was hydrolysed with acid (see Table III). The product that was insoluble in 1:3 MeOH–Me_2CO was examined by p.c. and chromatographed on

TABLE I

ANALYTICAL DATA FOR *A. tortilis* ARABINOGALACTAN-PROTEIN AND ITS DERIVATIVES

	Arabinogalactan–protein	A	*SD1*	*SD2-I*[a]	*SD2-II*[a]	*SD3-I*	*SD* A
$[\alpha]_D$ (degrees)	+75	−8	+71	+65	+96	+65	−34
$\overline{M}_w$	1.3×10^6	6.3×10^4	5×10^5	1.5×10^5	2200	2300	800
Nitrogen (%)	1.9	2.9	1.3	1.3	0.8	[b]	[b]
Hence, protein (%)	12	18	8	8	5	[b]	[b]
Constituent residues (mol %)							
Ara[c]	66	—	75	80	84	69	—
Gal[c]	23	83	25	20	16	31	100
Uronic acid	8	17	—	—	—	—	—
Other	[d]						

[a]^{1}H-N.m.r. spectra showed signals grouped at δ 4.6, 5.2 and 5.4, indicating β-Gal and the Ara residues; and, at higher field, those of amino acids. [b]Not determined. [c]The L configuration of arabinose (Ara) follows from the high positive rotation of the syrup released from the AG-P on partial hydrolysis; D-galactose (Gal) is found invariably in hydrolysates of *Acacia* gums[1]. [d]Man (3). All other analytical values were in conformity with those reported in ref. 4.

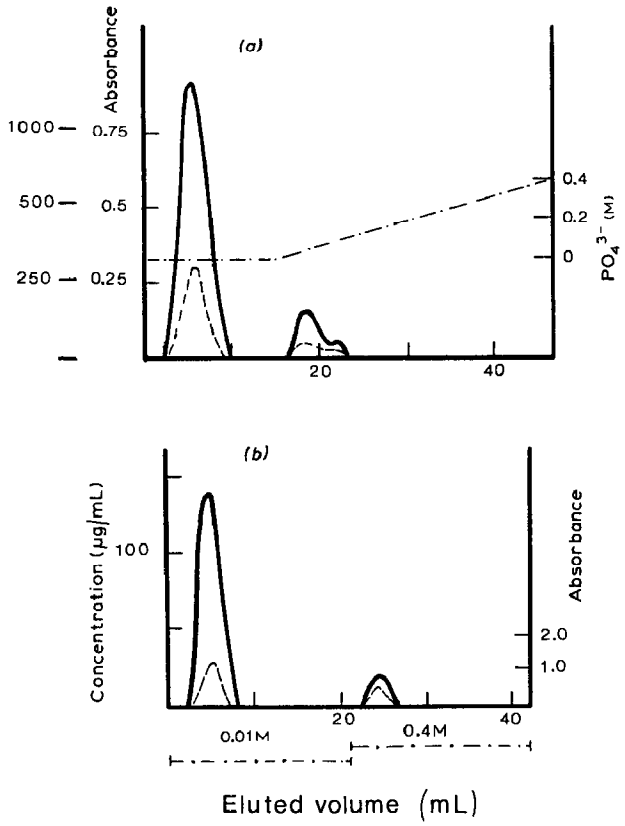


Fig. 1. Chromatography of (*a*) *A. tortilis* AG-P and (*b*) *A* on hydroxyapatite (column, 10 × 0.5 cm); carbohydrate ——, absorbance at 220 nm ----, phosphate buffer (pH 7.4) —·—·—·.

Bio-Gel P-10 (Fig. 4). The soluble fraction contained (p.c.) ethane-1,2-diol (+), glycerol (6+), threitol (+), and traces of sugars and amino acids.

(*b*) Three sequential Smith-degradations were performed on AG-P (1.78 g), using the procedures described previously[3]. Table III outlines the course of the reaction sequences and the intermediate products, and materials soluble or insoluble in mixtures of MeOH and Me_2CO (see Tables I–III and Figs. 3 and 4) were analysed chromatographically.

These degradation experiments, together with others involving partial acid hydrolysis (already described) or treatment with base (see below), are illustrated in Scheme 1, which provides a key to the designation of the products obtained.

The second Smith-degradation yielded two polysaccharide products, separated by fractionation with MeOH–Me_2CO (1:3). The insoluble fraction was purified on Sephadex G-10 and isolated after dialysis to give SD2-I (93 mg). The soluble portion contained, in addition to polyols (see Table III), a polysaccharide (SD2-II) of low mol. wt. which was isolated (95 mg) by using Sephadex G-10.

Smith degradation of SD2-I (Table III) gave SD3-I, which was characterised

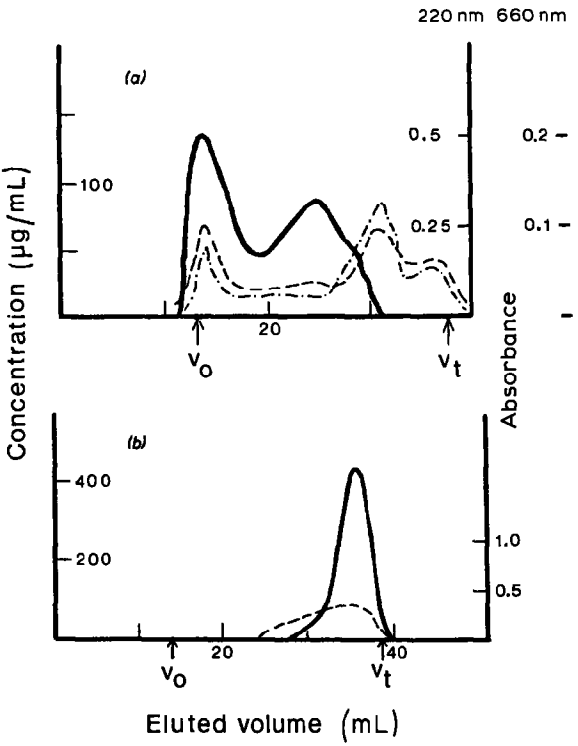


Fig. 2. Chromatography of (*a*) *A. tortilis* AG-P, equilibrated in SDS, and (*b*) *A.* Sepharose 4B column (60 × 0.9 cm), eluted with M NaCl; carbohydrate ——, absorbance at 220 nm ----, absorbance at 660 nm —·—·—·.

TABLE II

METHYLATION ANALYSES OF *A. tortilis* AG-P AND DERIVATIVES

Sugar[a] *linkage*	*Polysaccharide methylated*							
	AG-P	*RMAG-P*[b]	A	*SD1*	*SD2-I*	*SD2-II*	*SD3-I*	*SD3-IIa*
Araf	12	11	—	7	14	15	25	17
→2)-Ara*f*	38	36	—	55	47	49	30	54
→3)-Ara*f*	11	9	—	16	16	15	14	8
Gal*p*	1	2	10	12	2	2	5	2
→3)-Gal*p*	5	3	6	8	7	8	10	7
→4)-Gal*p*	3	3	16	1	—	—	—	—
→6)-Gal*p*	2	1	27	2	6	4	7	6
→3,4)-Gal*p*	1	—	4	tr.	—	—	—	—
→3,6)-Gal*p*	14	20	20	8	8	7	9	7
→3,4,6)-Gal*p*	2	5	tr.	1	—	—	—	—
Uronic acid[c]	8	—	17	—	—	—	—	—
Glc*p*[d]	—	6	—	—	—	—	—	—
Other	[e]	[f]						

[a]From acetylated alditols produced; identities by relative retentions in g.l.c. and by m.s. Quantities as mol %. [b]Carboxyl-reduced methylated AG-P. [c]Assayed colorimetrically in polysaccharide prior to methylation. [d]From terminal Glc*p*A groups. [e]Traces of Man*p*, →2)-Man*p*, and →2,6)-Man*p*. [f]→2)-Man*p* (2).

TABLE III

SMITH-DEGRADATION EXPERIMENTS ON WHOLE GUM AND DERIVED PRODUCTS

Smith degradation	*Arabinogalactan–protein*				A
	First expt.	*Second expt.*	*Third expt.*		
			SD2-I	*SD2-II*	
Sample (mg)	1780	703	46	9	51
Duration of oxidation (days)	5	7	4	3	5
Periodate consumption ($mmol.g^{-1}$)	5.5	2.4	1.5	1.3	5.7
Duration of hydrolysis[a] (days)	4	5	2	2	3
Yields of fractions[b] (mg)					
Insoluble	820[c]	360	16	[g]	5
Soluble	~200[d]	[e]	24[f]		+

[a]M Trifluoroacetic acid (TFA) at 20°. [b]Based on solubility in MeOH–Me_2CO mixtures. [c]Of which 56 mg, containing 9.5% of N, was insoluble in M TFA. [d]Glycerol (9), threitol (1), Ara (tr.), Gal (tr.); $(Ara)_n$-threitol (n ~2) and deuterated 2-Me-erythritol (from Me-erythronolactone) identified after l.c. and further derivatisation. [e]Glycerol, threitol, and residual polysaccharide SD2-II (95 mg); see Table I and Fig. 4. [f]Glycerol, and 5 mg of sugars Ara and Gal in the ratio 5:1. [g]Separated on Trisacryl GF05; SD3-IIa (3 mg) and SD3-IIb (4 mg), both insoluble in MeOH–Me_2CO (1:3 v/v).

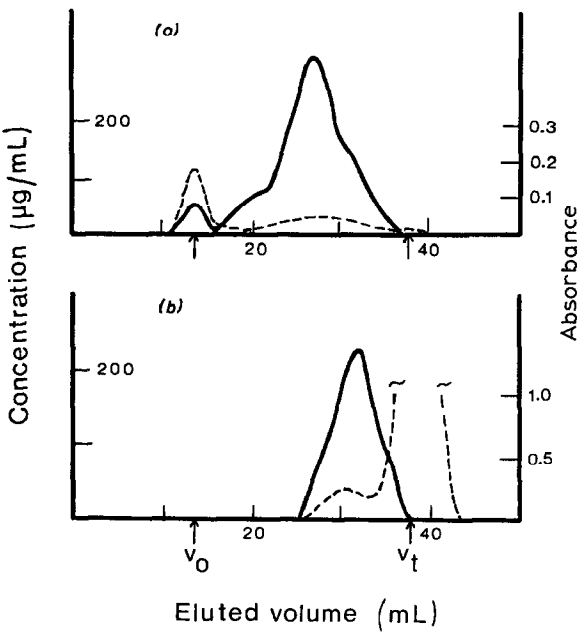


Fig. 3. Products of first and second Smith-degradations of AG-P: (*a*) SD1, (*b*) SD2-I. Chromatography on Sepharose 4B; carbohydrate ——, absorbance at 220 nm ----.

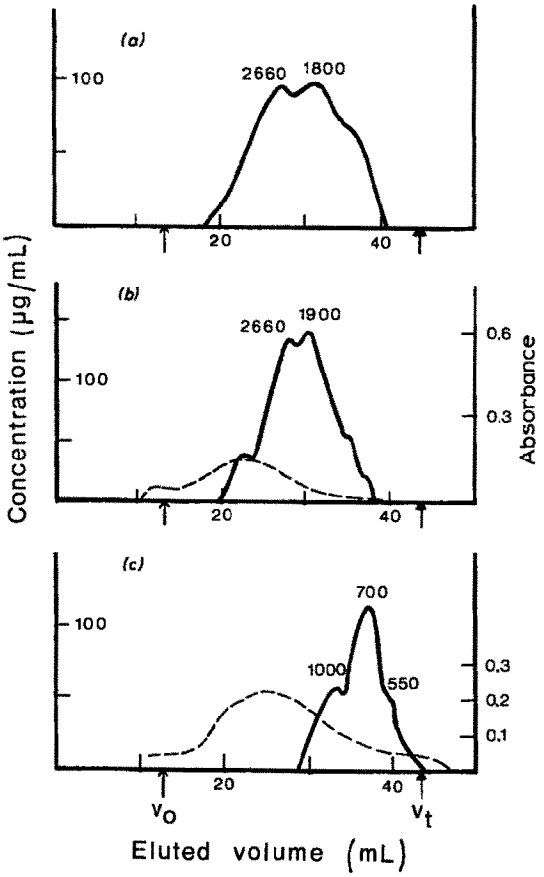
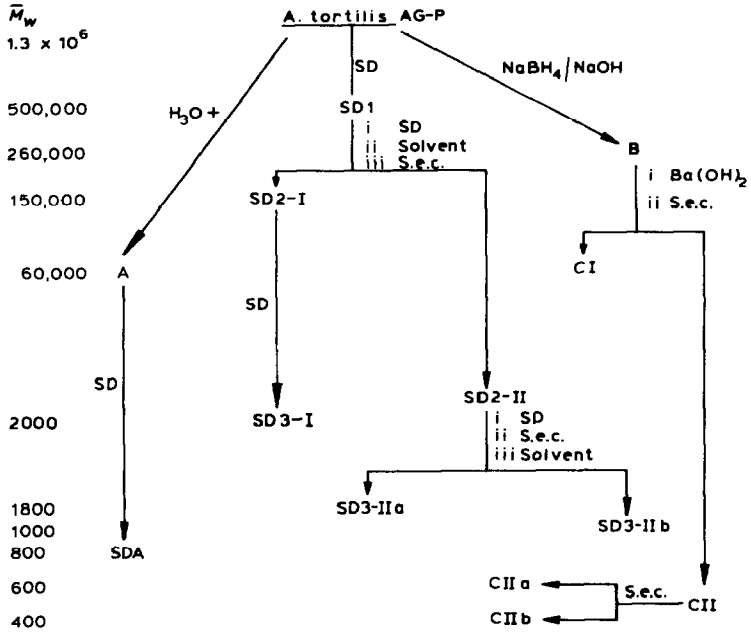


Fig. 4. Products of Smith degradation of AG-P derivatives: (*a*) SD2-II, (*b*) SD3-I, (*c*) SD*A*. Chromatography on Bio-Gel P-10 column (52 × 1.5 cm), eluted with M NaCl; carbohydrate ——, absorbance at 220 nm ----.

as shown in Tables I and II and Fig. 4. Treatment of a portion (8 mg) of SD3-I, in aqueous solution, with $CHCl_3$ and 1-butanol, in order to remove unglycosylated protein (see Fig. 4), yielded a product (7 mg), $[\alpha]_D$ +68°, which still showed strong absorbance at 220 nm.

Smith degradation of SD2-II, followed by elution with water from a column (20 × 1.2 cm) of Trisacryl GFO5, gave SD3-IIa, $[\alpha]_D$ +64°, $\overline{M}_w$ ~2000. Eluted second was SD3-IIb, $[\alpha]_D$ +15°, $\overline{M}_w$ ~1000. Each product was subjected to methylation analysis and the resulting partially methylated alditol acetates were analysed by g.l.c. The linkages present in SD3-IIa are shown in Table II. G.l.c. of the hydrolysate of methylated SD3-IIb indicated the presence of terminal and (1→2)-linked Ara residues (the latter preponderating) with some (1→3)-linked Ara*f*. The e.i.-mass spectrum of methylated SD3-IIb contained prominent ions at *m/z* 175 and 143, 335 and 303, indicating Ara*f* and →2)-Ara*f* in sequence (see Results and Discussion).



Scheme 1. Sequence of degradation experiments; the products are aligned according to the scale of molecular weights shown.

Degradation by alkali. — (*a*) A solution of AG-P (24 mg) in 2M $NaBH_4$ (2.5 mL) was stirred for 4 h at 20°, 0.1M NaOH (2.5 mL) was added, and stirring was continued for a further 24 h. The solution was then dialysed for 48 h against water (5 L) containing Amberlite IR-120 (H^+) resin, and the non-dialysable material (*B*) was recovered by freeze-drying; *B* (25 mg) had $[\alpha]_D$ +65°, contained uronic acid, and was examined further by chromatography on Sepharose 4B.

(*b*) The bulk of *B* was treated with 0.22M $Ba(OH)_2$ (10 mL) at 100° for 6 h, and the solution was neutralised (M H_2SO_4) and freeze-dried to give alkali-degraded *C*. The whole sample was eluted from Trisacryl GF05 with water, and fractions (1 mL) were assayed for carbohydrate and optical rotation (365 nm). CI (6.9 mg), $[\alpha]_D$ +122°, which was eluted near the void volume of the column, and CII (4 mg) were isolated.

Hydrolysis of CI in 2M trifluoroacetic acid at 100° for 18 h released Ara (87) and Gal (10), together with 4-hydroxy-L-proline (Hyp); uronic acid (3) was also present (colorimetric assay)[8]. CII contained traces of free amino acids, and three components having R_{Hyp} 0.30, 0.44, and 0.66 (yellow colour with ninhydrin; p.c., solvent *C*). A sample of CII was methylated and the product was examined by e.i.-m.s. Another portion, on hydrolysis with 2M trifluoroacetic acid at 100° for 8 h, yielded Ara and Hyp. In a separate experiment, a component corresponding to CII was resolved, by elution from Trisacryl GF05 with water, into CIIa and CIIb; the latter contained only the Hyp-glycoside with the highest mobility in p.c. (0.66), and

methylation analysis of this gave derivatives of 2,3,5-Me_3Ara and 3,5-Me_2Ara in equal amounts. Methylation analysis of CII gave 2,3,5-Me_3Ara (1), 3,5-Me_2Ara (3), and a trace of 2,5-Me_2Ara.

RESULTS AND DISCUSSION

Many structural features of the *A. tortilis* arabinogalactan–protein resemble those for *A. robusta* gum[3], including the proportions of constituent sugars and the manner in which they are linked, association with protein, and the molecular sizes of the products formed on Smith degradation (Tables I and II). The amino acid residues present are those found in the *A. robusta* AG-P, although the Hyp content is appreciably less. Apart from the absence of L-Ara*p* residues, either terminal or 2-linked, in *A. tortilis* AG-P, the first notable difference from the *A. robusta* product is seen on partial acid hydrolysis, which cleaves this AG-P into fragments (*A*) which are about one-tenth of the size expected after total removal of Ara*f* residues only. As for the starting material, chromatography of *A* on hydroxyapatite resulted in the co-elution of a major followed by a minor, less-glycosylated component (Fig. 1). Whereas steric-exclusion chromatography (s.e.c.) showed the AG-P to be heterodisperse, and some protein appeared to be detached from the complex, the partially hydrolysed product *A* was eluted as a single peak, again separated from some of the protein (Fig. 2). The fragments *A* contained 18% of protein, which does not, on average, allow the presence of protein exceeding 11,000 in mol. wt.

Smith degradation of the AG-P yielded mainly a uniform product of mean mol. wt. 500,000 (Fig. 3) containing 8% of protein; the diminished size (from ~150,000 to 40,000) of the protein component implied limited proteolysis[9], while the presence of residues of certain of the amino acids accounts for a small part of the periodate consumption (Table III)[10]. Methylation analysis (Table II) showed little change in the modes of linkage of the Ara residues compared with those in the AG-P, apart from a minor increase in →2)-Ara*f* units and a decrease in terminal Ara (suggesting direct attachment of the latter to Gal residues); the expected removal of uronic acid residues was confirmed. The co-elution of protein and carbohydrate during s.e.c. of SD1 (Fig. 3) indicated attachment through a periodate-resistant glycosyl residue. The fraction of the products of Smith degradation that was soluble in organic solvents (see Table III) included arabinosylthreitols, which implied linkage of Ara to O-4 of Gal, and there was evidence for terminal 4-*O*-MeGlcA in the AG-P in the isolation of 2-*O*-methylerythritol.

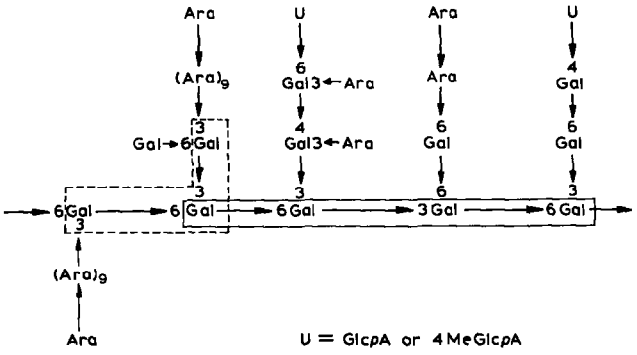
Breakdown of the carbohydrate to products of low mol. wt. after a second Smith-degradation was only partial (Tables I and III, Figs. 3 and 4). The persistence of material of high mol. wt. in SD2-I was attributed to the presence of arabinosyl chains, 5 units in length on the average, protecting otherwise vulnerable Gal*p* residues in the main chains; the β-L-Ara*f* residues contribute to the high positive optical rotation. The fractions SD2-II, $\overline{M}_w$ ~2200 (Table I and Fig. 4), and SD2-I,

$\overline{M}_w$ 150,000 (Table I and Fig. 3), were formed possibly from different populations of molecules in the original AG-P. Again, little change in the proportions of the constituent sugar residues was observed (Table II). Similarly, there was hardly any alteration in composition or size in SD3-IIa, obtained on submitting SD2-II to a third Smith-degradation, but the larger molecules (SD2-I) were considerably reduced in size, the product (SD3-I) having $\overline{M}_w$ ~2300. Because of the survival of Ara residues, a high positive rotation was maintained throughout the three Smith-degradations (Table I). However, relatively more Ara than Gal residues were destroyed in the Smith degradations of SD2-I and SD2-II, and the persistent occurrence of a 6-linked Gal*p* unit (Table II) suggested partial deprotection of a branched unit in the core. Mass spectrometry of methylated SD3-IIb confirmed the presence of 2-linked Ara*f* units, inasmuch as the intensity of the peak at *m/z* 303 relative to that at 335 was high. This finding indicated[11] loss of MeO from C-3 and consequently glycosylation (by Ara) at C-2.

As expected, Smith degradation of *A* gave a product with Gal as the only constituent (see Scheme 2), which averaged only 800 in mol. wt. (Fig. 4c) and had $[\alpha]_D$ −34° (Table I). The yield, representing 25% of that calculated on the basis of the survival of all protected glycosyl residues and the protein in *A*, was too low to permit systematic structural analysis (Table III). The remainder of the intact sugar residues were present as glycosides of low molecular weight, being separated from one another in the parent molecule.

Heating AG-P with alkaline borohydride gave a product (*B*) which contained protein and carbohydrate and had mol. wt. ~260,000; no dialysable carbohydrate was produced. However, similar treatment of *A. karroo* gum polysaccharide, which contains negligible protein, also caused a decrease in $\overline{M}_w$. More vigorous alkaline degradation, designed to cleave much of the polypeptide, yielded a modified product (CI) which contained polysaccharide and Hyp, and had $\overline{M}_w$ ~60,000, together with a series of polypeptides, embodying (1→2)-linked Ara*f* residues and Hyp, which were separated chromatographically (CIIa and CIIb). Of the Ara residues of the AG-P, ~20% were accounted for as arabinosyl-Hyp residues, the remainder of the pentose being incorporated in the polysaccharide as chains or single units attached to Gal residues.

Combining the results of the foregoing experiments leads to the formulation of a model such as that in Scheme 2 which indicates one of the many possible arrangements of the sugar units in the polysaccharide portion of *A. tortilis* AG-P. That there are segments of glycosylated Hyp residues contained in the protein moiety is demonstrated by the isolation of products containing between two and four Ara*f* residues, mutually linked (1→2), attached to Hyp. The series of Smith degradations, in particular, gives credence to the possibility of there being a repetition in the polymer of blocks of sugar residues which are uniformly of the size indicated (see Scheme 1). The core structure, as with *Acacia* gum polysaccharides generally, is a highly branched galactan embodying (1→3) and (1→6) linkages, with associated, mainly terminal, uronic acid groups. These are attached by both



After 3 Smith-degradations, Ara remains attached to a short chain of Gal residues, the framework of which includes the section enclosed within the dotted line.

The solid line encloses residues expected to survive Smith degradation of A.

Scheme 2. One possible structure for *A. tortilis* arabinogalactan (AG-P).

(1→4) and (1→6) linkages, and comprise both D-Glc*p*A and its 4-methyl ether; the ratio of methylated to unmethylated acid is variable, exceeding unity[4].

The close structural relationship between *A. tortilis* AG-P and that from *A. robusta*[3], in the same taxonomic series, is clear. The diversity of types of molecule present and the existence of short arabinosyl chains joined to Hyp is a feature of *A. tortilis* gum, which is in conformity with a growing recognition that these structural features are attributable to plant exudates from different *Acacia* sources.

ACKNOWLEDGMENTS

We thank the C.S.I.R. and the Council of the University of Cape Town for financial support, Miss Bridget Williamson (Mass Spectrometry Unit, University of Cape Town) for technical assistance, Ms. A.-L. Wiid for the preliminary analyses of the AG-P, Profesor W. L. Brandt (Biochemistry) for the analysis for amino acids, and Dr. R. Liversidge (Kimberley) for the raw material and the botanical identification of its source.

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