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The molecular characterisation of the polysaccharide gum from *Acacia senegal*

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ABSTRACT

Gum arabic (*Acacia senegal* gum) was fractionated by hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B and yielded four fractions of similar carbohydrate composition, whilst differing in protein content, amino acid composition, and molecular mass distribution. All four fractions interacted with an array of anti-arabinogalactan–protein (AGP) monoclonal antibodies via anti-carbohydrate epitopes and were precipitated by Yariv's reagent, suggesting that they were all AGPs. Further characterisation using SDS-PAGE and Western blotting showed that all fractions contained proteins of molecular mass 30000–45000 with at least one protein unique to each fraction. The fractions were subjected to enzymatic (with pronase) and alkaline hydrolysis, and the molecular mass distribution of the products was monitored by gel permeation chromatography. The fractions showed various degrees of resistance to hydrolysis, thus illustrating differences in their macromolecular structure.

INTRODUCTION

The gum exudate from *Acacia senegal* is the major source of gum arabic. It is a highly complex polysaccharide consisting of a branched β-(1 → 3)-linked galactose backbone with branches linked through the 1,6-positions, and with arabinose, rhamnose, and glucuronic acid in ramified side chains[1–7].

Although Anderson and Stoddart[3] reported that the gum contained a small proportion of nitrogenous material that could not be readily removed, the structural implications of this were ignored until Akiyama et al.[8] suggested that the gum was a kind of arabinogalactan–protein (AGP). They pointed out that the gum has been previously shown to precipitate with Yariv antigen[9], and found the proteinaceous component (2% w/w of the total) to be rich in hydroxyproline, serine, and

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proline. Their work provided evidence indicating the presence of hydroxyproline–arabinose and serine–carbohydrate linkages.

Vandevelde and Fenyo[10] and Randall et al.[11,12] fractionated the gum, using gel permeation chromatography and hydrophobic affinity chromatography. Three fractions were obtained by hydrophobic affinity chromatography[12], i.e., a high molecular mass fraction (1.45×10^6) which contained 11.8% protein (10.4% w/w of the total gum), referred to as an AGP; a second fraction of molecular mass 2.79×10^5 containing only 0.35% protein (88.4% w/w of the total gum), referred to as an arabinogalactan (AG); and a third fraction of molecular mass 2.5×10^5 containing 47.3% protein ($\sim 1\%$ w/w of the total gum), referred to as a glycoprotein (GP). Further characterisation by NMR and methylation analysis[13] showed that all three fractions had similar branched structures, with each comprised of a galactose core having 3- and 6-linkages with the other sugars occurring peripherally.

Connolly et al.[14,15] carried out enzymatic degradation studies on the whole gum, using pronase, and found that the higher molecular mass components were degraded to a single component of lower molecular mass. They argued that their results were consistent with the "wattle blossom" model for AGPs proposed by Fincher et al.[16], and suggested that the molecules consisted of several polysaccharide blocks of molecular mass ca. 2×10^5 linked to a common protein core.

Recently, Qi et al.[17] fractionated gum arabic into two molecular mass components, using GPC, and referred to them as a glucuronorhamnoarabinogalactan, GAP, which represented 90% of the total, and the remainder as a glycoprotein, GAGP. The GAGP contained ca. 10% of proteinaceous material and on deglycosylation yielded a hydroxyproline-rich polypeptide chain consisting of ~ 400 amino acid residues. The amino acid composition resembled that of the serine-rich extensins since it contained a low proportion of alanine and acidic amino acids. Alkaline hydrolysis of the GAGP and subsequent analysis showed that most of the carbohydrate was attached to the polypeptide backbone as small sub-units of about 30 sugar residues through galactose–hydroxyproline linkages as is commonly found in hydroxyproline-rich AGPs. They concluded that, while the polypeptide backbone of this glycoprotein resembled the extensins, the polysaccharide side chains were characteristic of AGPs. Their data implied a rod-like molecule with numerous small polysaccharide units attached at regular intervals. Direct visualisation on the GAGP molecules, using electron microscopy, demonstrated that these molecules were rod-like in appearance and resembled a "twisted hairy rope".

The present study of gum arabic structure and composition provides novel data on the molecular complexity of this gum. In the light of these findings, the current models of gum arabic structure are discussed.

EXPERIMENTAL

Gum from *A. senegal* originating from the Kordofan region of Sudan was kindly supplied by Agrisales Ltd. (London, UK).

Fractionation using hydrophobic affinity chromatography.—The gum was fractionated by hydrophobic affinity chromatography using a column packed with Phenyl-Sepharose CL-4B gel (Pharmacia) according to the procedure described previously[12]. Separation was achieved by initially passing the gum solution through the column in a mobile phase of 4.2 mol dm^{-3} NaCl and then removing adsorbed material by changing the mobile phase to 2.0 mol dm^{-3} NaCl and finally to water. The eluent was collected and the UV absorbance measured at wavelengths of 214 and 280 nm. The former is sensitive both to the carboxylate groups present in the carbohydrate component of the gum and also to the proteinaceous component, whereas the latter is more sensitive to the protein. All fractions collected were extensively dialysed and then freeze-dried.

Characterisation of gum fractions.—The optical rotation, sugar composition, and the uronic acid, nitrogen, and amino acid contents of the fractions were determined as described previously[11]. The molecular mass distributions were monitored by gel permeation chromatography (GPC) using the Pharmacia FPLC system. Solutions of the gums (0.5 or 1% w/v) were prepared in 0.5 mol dm^{-3} NaCl and, after filtering (0.45 μm Millipore), were injected via a 100-μL loop onto a Superose 6 column and eluted using a mobile phase of 0.5 mol dm^{-3} NaCl at a flow rate of 0.5 cm^3 min^{-1}. The eluent was monitored by UV at a wavelength of 206 nm (LKB 2238 Uvicord SII detector) and by differential refractometry (R401 Waters Millipore differential refractometer). In addition, the eluent was collected in 15-cm^3 fractions that were subsequently pooled and extensively dialysed against distilled water. These were analysed for uronic acid, using the carbazole procedure described previously[11], and were also tested using an immunoassay for gum arabic. Full details of this assay have been given elsewhere[21–23].

SDS-PAGE and Western blotting.—10% Acrylamide slab gels were prepared according to Laemmli[24] and blotted using standard procedures. Gels were stained with silver according to Rabilloud et al.[25]. For immunoblotting, nitrocellulose was blocked with a 3% solution of skimmed milk powder in phosphate-buffered saline pH 7.4 for 1 h and treated for 2 h with a 1/50 dilution of MAC 207 (anti-AGP monoclonal antibody[26]) in the block solution. Bound antibody was detected with an alkaline phosphatase-conjugated goat anti-rat IgG (H + L) antiserum (Sigma Chemicals), using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT) in the developing solution.

Yariv reagent dot blots.—Each sample (1 mg) dissolved in sterile distilled water was dotted onto nitrocellulose that was blocked with a 3% solution of skimmed milk powder in phosphate-buffered saline pH 7.4 (PBS) for 1 h, washed for 5 min in PBS, and subsequently immersed for 15 min in β-glucosyl Yariv reagent, an artificial carbohydrate antigen bound by AGPs[9,27]. The concentration of Yariv reagent was 1.5 mg cm^{-3} in aq 1% NaCl. The nitrocellulose was washed in aq 1% (w/v) NaCl in order to decrease background staining before observations were made.

Immuno-dot blots.—Each sample (1 mg) dissolved in sterile distilled water was

dotted onto nitrocellulose and probed with a series of anti-AGP monoclonal antibodies, which bind to the carbohydrate epitopes of these proteins[28], as described above for Western blots.

Enzymatic hydrolysis.—Solutions (9 cm^3) of whole gum arabic and fractions in 0.5 mol dm^{-3} NaCl (1% w/v) were treated with pronase (Type XXV from *Streptomyces griseus*, pronase E; 1 cm^3 of 1.6 mg cm^{-3}, Sigma). The solutions were adjusted to pH 7.5 and the samples incubated at 37°C in stoppered test-tubes. Aliquots (1 cm^3) of the pronase-treated gum were removed at intervals (24, 48, and 72 h). The effect of degradation on the molecular mass distribution of the gum was monitored at $A_{206\ nm}$ and by RI using the Pharmacia FPLC system and conditions described above.

Alkaline hydrolysis.—The gum was subjected to alkaline hydrolysis using $Ba(OH)_2$ (0.22 mol dm^{-3}) at 100°C for 18 h as described by Qi et al.[17]. Hydrolysis was also performed using a mixture of NaOH (0.2 mol dm^{-3}) and $NaBH_4$ (0.2 mol dm^{-3}) at 50°C for 5 h as described by Akiyama et al.[8].

RESULTS

Fractionation using hydrophobic affinity chromatography.—The UV profiles obtained by monitoring the absorbance of the eluent from the hydrophobic affinity chromatography column at wavelengths of 214 and 280 nm are given in Fig. 1. The profiles show that the gum can be conveniently separated into four fractions. Fractions 1A and 1B were eluted from the column in 4.2 mol dm^{-3} NaCl, whereas fractions 2 and 3 were eluted in 2.0 mol dm^{-3} NaCl and water, respectively. This numbering system was adopted in order to be consistent with our previous work[12].

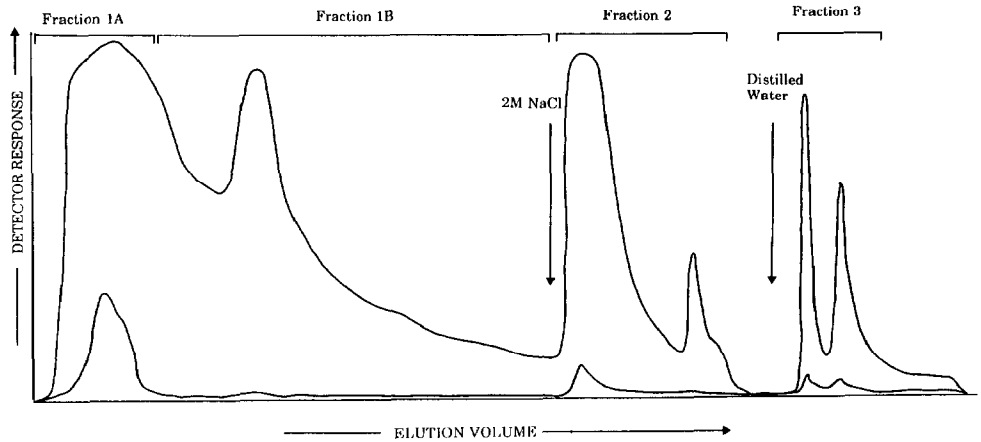

Fig. 1. Elution curve of *A. senegal* gum following fractionation by hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B. Fractions were eluted (step-wise) using 4.2 mol dm^{-3} NaCl, 2.0 mol dm^{-3} NaCl, and distilled water. Upper trace, $A_{214\ nm}$; lower trace, $A_{280\ nm}$.

TABLE I

Fractions recovered following hydrophobic affinity chromotography

Elution medium	Current Study		Previous study[12]		
	Fraction	Yield (%)	Fraction		Yield (%)
NaCl, 4.2 mol dm^{-3}	1A	89.4	1	(AG)	88.4
NaCl, 4.2 mol dm^{-3}	1B	4.7			
NaCl, 2.0 mol dm^{-3}	2	4.4	2	(AGP)	10.4
Water	3	1.5	3A, 3B	(GP)	1.2

In these earlier studies, using a different gum sample, we isolated only one fraction when eluting with 4.2 mol dm^{-3} NaCl. Recent studies (data not shown) of a number of *A. senegal* gums have indicated that the two peaks corresponding to fractions 1A and 1B are a common feature in the hydrophobic affinity chromatograms, although they are not always as well resolved as for this particular sample. The weights recovered for each fraction compared to the total weight recovered are given in Table I together with the yields obtained previously[12]. Since, in the previous study, fractions 1A and 1B were pooled, the proportions of each recovered are very similar for the two fractionation procedures.

Characterisation of gum fractions.—The optical rotation, sugar, uronic acid, and nitrogen analyses of the whole gum and its fractions are given in Table II, and the amino acid analyses in Table III.

The carbohydrate composition of the whole gum is similar to that reported in the literature for gum arabic. Slight variations in carbohydrate composition occur for the fractions as observed previously[12], with fractions 1B and 2 containing more arabinose and fraction 1A having an increased rhamnose content. The full composition of fraction 3 was not determined due to the very small amounts of this component recovered, but it was shown to have a low content of glucuronic acid.

Fraction 1A, which makes up the bulk of the gum, contains only a small

TABLE II

Chemical analysis of the whole gum and fractions

	Whole gum	1A	1B	2	3
$[\alpha]_D^{20}$ (deg)	−29.4	−33.8	n.d. [a]	n.d.	n.d.
Rhamnose (%)	14	18	12	10	n.d.
Arabinose (%)	27	27	31	28.5	n.d.
Galactose (%)	35	32	33	28.5	n.d.
Glucuronic acid (%)	21	23	16	17	3
N (%)	0.31	0.05	n.d.	n.d.	n.d.
Protein [b] (%)	2.4	0.4	n.d.	n.d.	n.d.
Protein [c] (%)	2.4	0.7	7.8	16.2	24.2

[a] n.d., Not determined. [b] From Kjeldahl, using an N conversion factor of 6.60 (ref 29). [c] Determined from amino acid analysis.

TABLE III

Amino acid analysis of the whole gum and fractions

Amino acid	whole gum [a]	1A	1B	2	3
OHpro	256 [b]	593	302	208	112
Asp	91	27	53	90	103
Thr	69	50	83	72	62
Ser	144	113	182	136	125
Glu	36	6	29	51	67
Pro	64	49	62	62	60
Gly	53	25	56	61	85
Ala	28	9	20	31	49
Cys	3	3			
Val	35	6	18	46	54
Met	2	1	2	1	4
Ile	11	10	7	10	20
Leu	69	39	65	78	76
Tyr	13	8	7	21	42
Phe	30	4	18	48	54
His	52	43	57	54	35
Lys	27	9	12	21	37
Arg	14	4	26	8	20

[a] The whole gum and fractions 2 and 3 also contain glucosamine. [b] Values given in residues/1000 residues.

proportion of proteinaceous material while the minor fractions 1B, 2, and 3 contain comparatively high proportions of protein, in agreement with earlier findings. Hydroxyproline, serine and proline are the most abundant amino acids in fractions 1A, 1B, and 2, with hydroxyproline itself accounting for 59, 30, and 21% of the total in each, respectively. In fraction 3, serine, aspartic acid, and hydroxyproline are the most abundant , but together only account for 34% of the total. As can be seen from Table III, there is a much more even distribution of the various amino acids in this fraction as was previously found[12].

SDS-PAGE and Western blotting.—The results obtained for the silver-stained SDS-PAGE gel are shown in Fig. 2 and clearly indicate that fractions 1B, 2, and 3 contain several proteins varying in molecular mass from ~ 30 000 to 200 000. Several of these proteins of molecular mass 30 000–45 000 are present in all three fractions; however, at least one protein present in each fraction appears to be unique (see arrows). Thus, only fraction 1B contains a protein of molecular mass ~ 70 000, only fraction 2 contains a protein of molecular mass ~ 29 000, and only fraction 3 contains a protein of molecular mass ~ 10 000.

The results obtained on immunoblotting of the SDS-PAGE gels as shown in Fig. 2 with anti-AGP monoclonal antibody (MAC 207) are illustrated in Fig. 3. A smeary band with molecular mass > 200 000 was present in all three fractions, but with most reaction observed in fractions 1B and 2. This indicates that all three fractions contain MAC 207-reactive AGP, which is most probably highly glycosylated as suggested by the smeary nature of the bands.

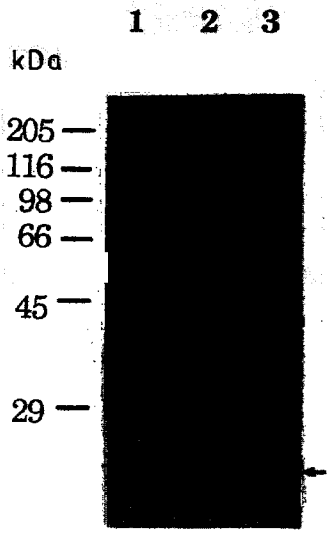

Fig. 2. SDS-PAGE (silver stained) of *A. senegal* gum fractions (each 12 μg). Lane 1, fraction 1B; lane 2, fraction 2; lane 3, fraction 3.

Yariv reagent dot blots.—All four fractions were shown to precipitate with Yariv reagent, as illustrated in Fig. 4, indicating, therefore, that they all contain AGP. The strongest reaction was with fractions 1B and 3.

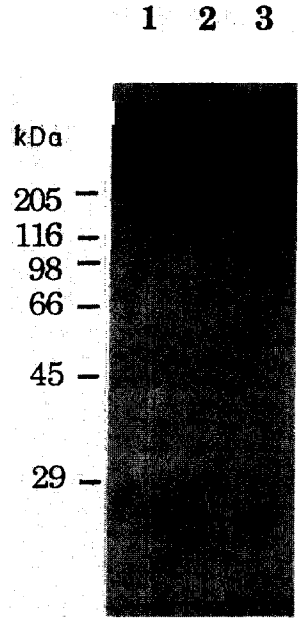

Fig. 3. Western blot of SDS-PAGE gel as shown in Fig. 2, probed with MAC 207. Lanes 1, 2, and 3 as for Fig. 2.

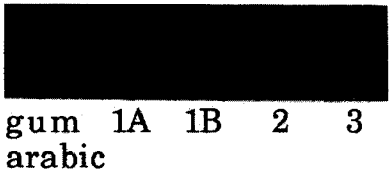

Fig. 4. Yariv reagent dot blots. The samples (each 1 μg) on each blot from left to right are *A. senegal* gum; fraction 1A; fraction 1B; fraction 2; fraction 3.

Immuno-dot blots.—The immunoblots obtained for the whole gum and fractions, using a panel of anti-AGP monoclonal antibodies, are shown in Fig. 5 and suggest that not all of the epitopes recognised by these antibodies are present in equal amounts in each fraction. JIM 13 and JIM 16 showed the strongest reaction, with JIM 16 giving the most pronounced signal for all the samples. JIM 8, JIM 15, and MAC 207 showed a similar yet lower degree of binding, while JIM 4 and JIM 14 showed very little affinity.

Gel-permeation chromatography.—The RI and UV GPC elution profiles of the whole gum and fraction 1A are given in Fig. 6. Since fraction 1A was known to constitute ~89% of the total gum, the concentrations of fraction 1A and whole

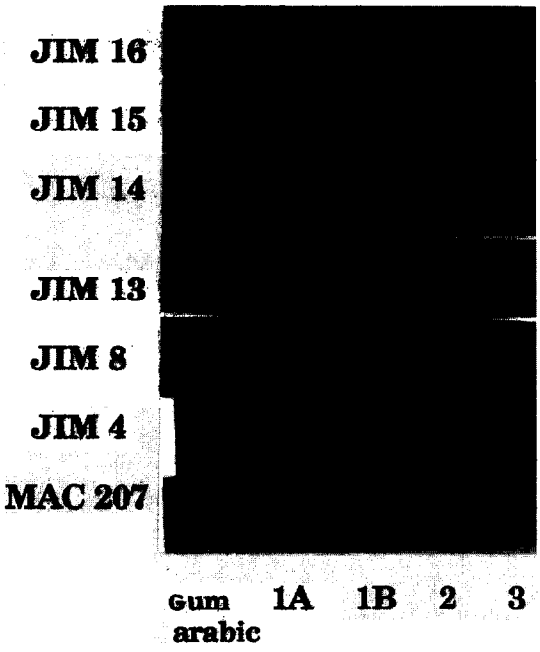

Fig. 5. Immunoblots showing varied reactions of anti-AGP monoclonal antibodies MAC 207, JIM 4, JIM 8, JIM 13, JIM 14, JIM 15, and JIM 16 with *A. senegal* gum and fractions 1A, 1B, 2, and 3. Order of samples and loadings as in Fig. 4.

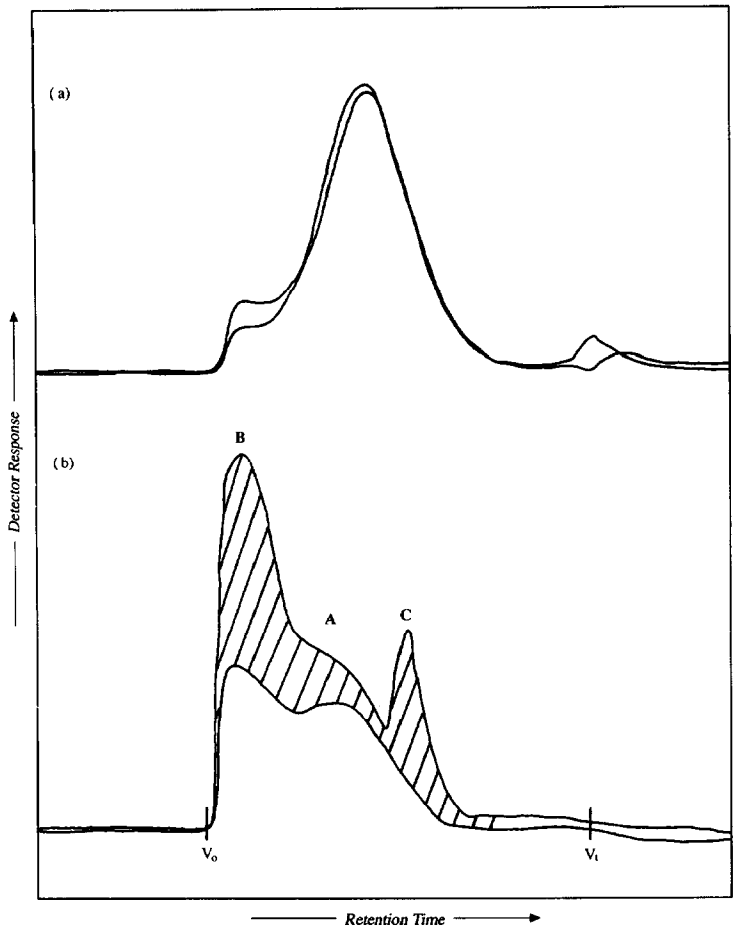

Fig. 6. GPC chromatograms (FPLC) of *A. senegal* gum and fraction 1A on Superose 6. Mobile phase, 0.5 mol dm^{-3} NaCl; flow rate, 0.5 cm^3 min^{-1}; sample, 100 μg dm^{-3} at 1% w/v in 0.5 mol dm^{-3} NaCl. (a) RI detection, (b) UV detection ($A_{206\ nm}$). In both (a) and (b), the upper trace is that of the whole gum.

gum used for the GPC experiments were 0.89 and 1%, respectively, so that the contribution of fraction 1A to the elution curve for the whole gum could be readily observed. As discussed previously[11], the RI curve closely reflects the actual concentration of gum, whereas the UV curve shows enhanced peaks for protein-rich components. For the whole gum, the RI curve shows two peaks, the major one corresponding to a $K_{av} = 0.33$ and a minor one at $K_{av} = 0.04$. The UV curve is very different, consisting of three peaks, two (labelled peaks A and B) corresponding to those in the RI curve and a third (peak C) at $K_{av} = 0.46$. These traces are consistent with previously published data[10,11]. The RI elution profile for fraction 1A is very similar to that for the whole gum, apart from the reduced intensity of the high molecular mass peak at $K_{av} = 0.04$ (peak B). The lower proportion of this molecular mass component in this fraction is more evident in the UV elution

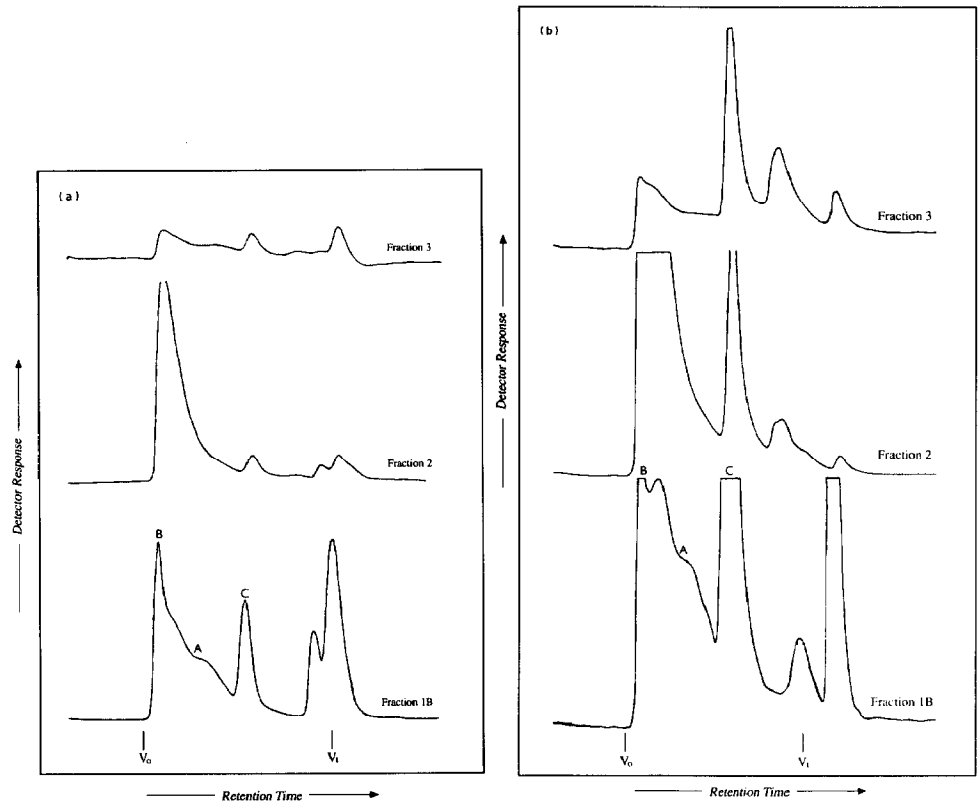

Fig. 7. GPC chromatograms (FPLC) of gum fractions 1B, 2, and 3. Conditions as for Fig. 6. (a) RI detection, (b) UV detection ($A_{206\ nm}$).

curve, which in addition shows that the molecular mass component corresponding to peak C is absent. Fractions 1B, 2, and 3, which together account for only ~ 11% of the total mass, therefore consist of molecular mass components which give rise to the shaded portion of the chromatogram. The RI and UV chromatograms for the isolated fractions are given in Fig. 7. The RI elution profiles show that fractions 1B and 3 consist of material corresponding to both peaks B and C in approximately equal proportions, whereas fraction 2 consists predominantly of material corresponding to peak B. On the other hand, the UV elution curves show that fractions 1B and 3 have a more intense peak C than peak A, suggesting that a significantly greater proportion of proteinaceous material is associated with peak C. The GPC data illustrate that, whereas the molecular mass of the various fractions is generally consistent with previous data, the improved molecular mass separation achieved using the Sepharose 6 GPC column indicates that these fractions are considerably polydisperse with respect to molecular mass. The differences in chemical composition and size, therefore, clearly illustrate the heteropolymolecular nature of the gum as suggested originally by Anderson and Stoddart[3].

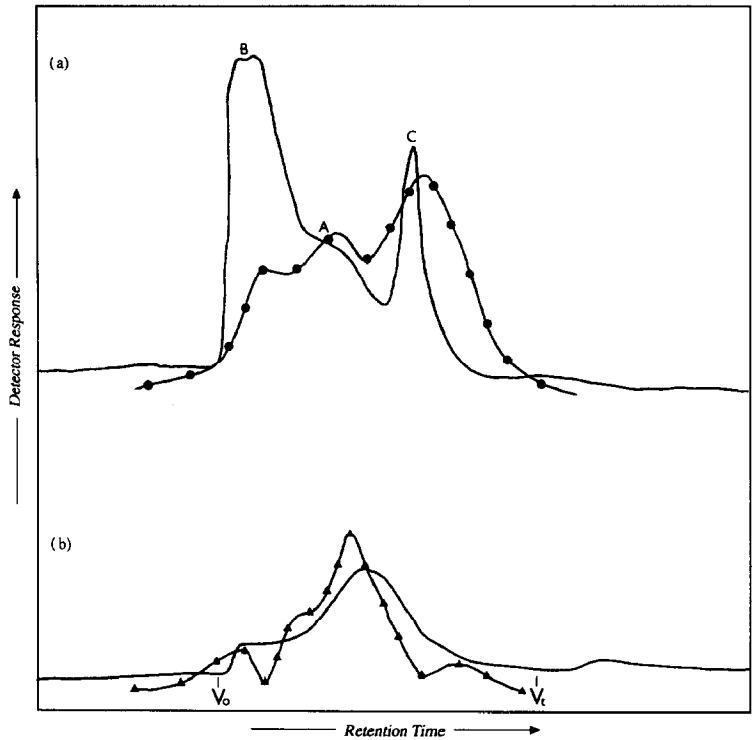

Fig. 8. GPC chromatograms (FPLC) of *A. senegal gum*: (a) detection by UV and immunoassay (ELISA, ●———●), (b) detection by RI and carbazole reaction (▲———▲). Conditions as for Fig. 6.

The results obtained from uronic acid and immunoassay analysis of the eluent following GPC of the whole gum are superimposed on the RI and UV elution profiles in Fig. 8. It is readily seen that the uronic acid profile closely follows the RI curve. Since the latter reflects the actual gum concentration, it is apparent that the uronic acid groups are fairly evenly spread between the various fractions and are not confined to specific molecular mass species, which is in agreement with previous studies[11,12]. The results from the immunoassay show a different trend with the molecular species corresponding to peak C showing enhanced reactivity towards the antibody as has been reported previously[21].

Enzymatic hydrolysis.—The effect of proteolytic enzyme on the molecular mass distribution of the whole gum and the fractions are given in Figs. 9 and 10. For the whole gum (Fig. 9a), the RI elution profiles before and after treatment show that a small proportion of the high molecular mass material corresponding to peak B is removed and that there is a slight shift in the position of the major peak of lower molecular mass (peak A). These changes are more apparent on studying the UV elution profiles (Fig. 10). It is clear that the high molecular mass material giving rise to peak B degrades to material of the same molecular mass as the bulk of fraction 1, giving a slight enhancement in the intensity of peak A and also to other

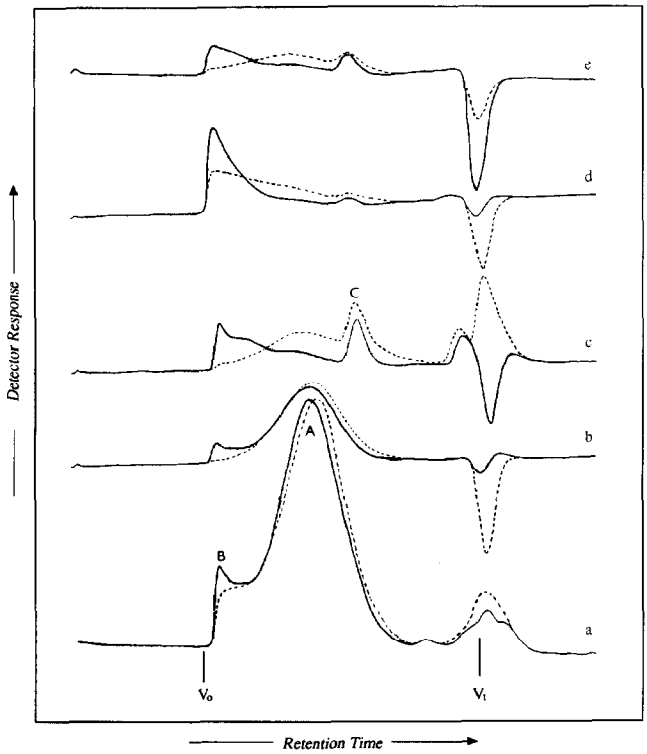

Fig. 9. GPC chromatograms (FPLC) of *A. senegal* gum and fractions before (———) and after (— — —) treatment with pronase and detected by RI: a, whole gum; b, fraction 1A; c, fraction 1B; d, fraction 2; and e, fraction 3.

lower molecular mass material which is eluted at $K_{av} = 0.59$ between peak C and the enzyme peak. It is very interesting that peak C, which is a protein-rich component, remains after enzyme treatment.

The RI and UV elution curves for fraction 1A (Figs. 9b and 10b) show a similar trend to the whole gum, with the high molecular mass component associated with peak B degrading to lower molecular mass material, leading to an enhancement and shift in the intensity and position of peak A.

As reported above, fractions 1B and 3 have very similar RI and UV elution profiles and both are seen to behave similarily on enzyme treatment (Figs. 9c,e and 10c,e). The high molecular mass material associated with peak B observed in both the RI and UV elution profiles is degraded, leading to a reduction in the intensity of this peak and an increase in the intensities of peaks A and C. A small amount of lower molecular mass material which is eluted between peak C and the enzyme peak is also observed. Fraction 2 (Figs. 9d and 10d) is also degraded on enzyme treatment. The intensity of peak B is reduced, as observed in both the RI and UV elution curves, and there is an increase in intensity in peaks A and C. Lower molecular mass material is also eluted between peak C and the enzyme peak.

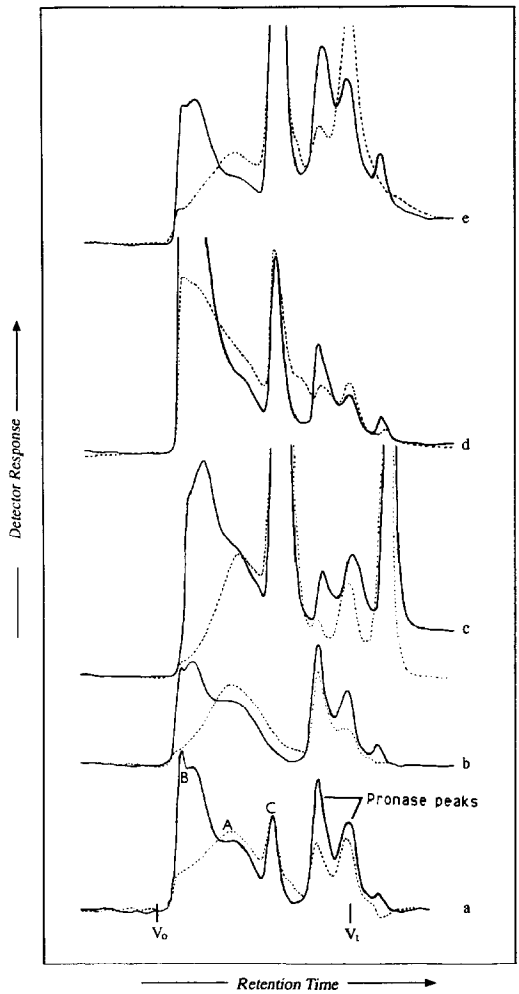

Fig. 10. As for Fig. 9, detected by UV ($A_{206\ nm}$).

Alkaline hydrolysis.—The GPC profiles for the gum before and after $Ba(OH)_2$ and $NaOH–NaBH_4$ hydrolysis are given in Fig. 11. The results show that peak A, which is virtually protein free, is degraded using both alkali systems, indicating that glycosidic bonds have been broken. This is consistent with the results of Young and Sarkanen[30] who found that arabinogalactans were particularly susceptible to alkaline degradation. Interestingly, both hydrolysis procedures give rise to a low molecular mass species at $K_{av} = 0.91$. The structural significance of this, however, can only be appreciated when more is known about the degradation reaction.

DISCUSSION

Gum fractions have been isolated according to their hydrophobic character as defined by their degree of interaction with a Phenyl-Sepharose gel matrix. Hy-

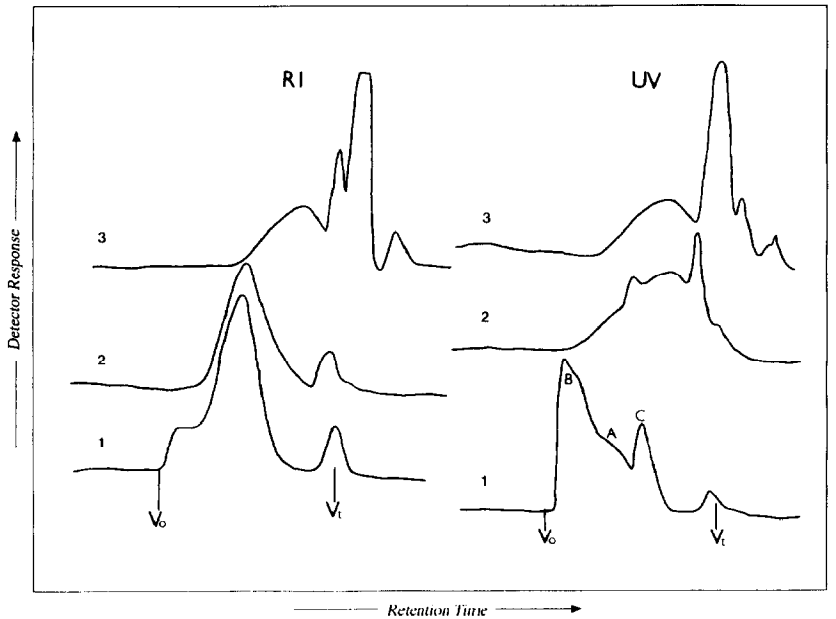

Fig. 11. GPC chromatograms (FPLC) of 1, *A. senegal* gum; 2, 1 after treatment with 0.2 mol dm^{-3} $NaBH_4$ in 0.2 mol dm^{-3} NaOH (50°C, 5 h); and 3, 1 after treatment with 0.2 mol dm^{-3} $Ba(OH)_2$ (100°C, 18 h). Detected by UV ($A_{206\ nm}$) and RI.

drophobicity was found to increase in the order 1A < 1B < 2 < 3. This corresponds to the order in which the protein content increases in the fractions. The data reported in Table II together with results obtained previously[12] indicate that all four fractions have similar galactose : arabinose : rhamnose ratios, while previous NMR and methylation analysis[13] suggests that they also have similar branched structures based on a β-(1 → 3)-linked galactan core. Indeed, the studies reported here using Yariv reagent and anti-AGP antibodies raised against carbohydrate moieties show further similarities, with each fraction containing epitopes previously shown to be associated with AGPs[26,28]. The two fractions isolated by Qi et al.[17] were also found to interact with Yariv reagent. In our studies, the strongest reaction of this reagent was observed with fractions 1B and 2 which are also shown to contain high levels of the three amino acids characteristic of AGPs, namely alanine, serine and hydroxyproline. The differences observed in the relative abundances of the various epitopes may be of functional and/or structural significance in the whole gum.

Characterisation of the isolated fractions by GPC and SDS-PAGE has indicated that each contains a number of molecular species differing in both size and chemical composition. This clearly demonstrates that the gum is composed of many molecular fractions rather than the three previously identified. It is perhaps surprising that, despite the extreme heteropolymolecular nature of the gum, its gross chemical and physico-chemical characteristics appear to remain relatively

constant between different samples. Qi et al.[17] fractionated the gum by preparative GPC and based on our findings it is highly likely that the two fractions isolated were heterogeneous. Their high molecular mass fraction (corresponding to peak B in Fig. 6) was reported to have a molecular mass of 220 000. This was calculated by summation of the molecular mass of the polypeptide backbone (determined by GPC of the deglycosylated fraction as 40 000) and the molecular mass of the attached sugar units (ca. 40 sugar units each of molecular mass ~ 4440 determined by GPC after alkaline hydrolysis of the whole fraction). Their value is very much lower than the value of $\sim 1.5 \times 10^6$ reported previously by us[11,12] and is about half the value accepted as the average molecular mass for the whole gum. The discrepancy may arise from the interpretation of the alkaline hydrolysis experiments. Qi et al. appear to have neglected the possibility that the branched carbohydrate chains could themselves be degraded by alkali, as indeed we found to be the case (Fig. 11), in addition to the cleavage of carbohydrate–polypeptide bonds. Furthermore, they used a specific hydroxyproline assay to monitor the alkaline hydrolysate following chromatography and hence could not detect sugar residues devoid of hydroxyproline. Since the fraction they isolated was almost certainly heterogeneous, it is possible that the rod-like molecules observed by transmission electron microscopy correspond to just one of the components of this fraction.

The results of enzymatic hydrolysis reported in this paper are consistent with earlier findings[11,12,14,15], in as much as the high molecular mass component (peak B) is degraded giving rise to products of molecular mass $2–3 \times 10^5$, and, therefore, are supportive of the "wattle blossom" model described in the Introduction[14–16]. However, as noted by Spiro[31], enzymatic hydrolysis does not always proceed to completion and this is clearly illustrated from our studies since peak C, which corresponds to protein-rich material, is not degraded by the enzyme. Hence, alternative methods which selectively cleave carbohydrate–polypeptide, but not carbohydrate–carbohydrate, bonds need to be established.

In conclusion, therefore, the work presented has shown that the fractions isolated by hydrophobic affinity chromatography have broad similarities in their carbohydrate structure, with each having similar sugar compositions and each showing reactivity to anti-AGP antibodies. This is consistent with earlier findings[13] which also confirmed similar modes of branching in the various fractions. The major difference between the fractions appears to be the molecular size and protein content. The work has also demonstrated the extremely heterogeneous nature of the gum, with each of the fractions containing a number of molecular mass species each of which may also vary in their carbohydrate–amino acid compositions and structural characteristics. The general models put forward by Qi et al. and Fenyo and co-workers, therefore, need to be viewed in the light of this new evidence. It has become abundantly clear that much more extensive fractionation procedures are needed in order to isolate individual molecular species before the work of molecular characterisation can begin.

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