Carbohydrate Polymers **8** (1988) 23–32

Effect of a Proteinase on the Macromolecular Distribution of *Acacia senegal* Gum

Siobhan Connolly, Jean-Claude Fenyo* and Marie-Colette Vandevelde

Laboratoire des Echanges Cellulaires, Scueor, UA CNRS No 203, Faculté des Sciences et Techniques, Université de Rouen, 76130 Mont-Saint-Aignan, France

(Received 12 March 1987; revised version received 6 May 1987; accepted 3 August 1987)

SUMMARY

Molecular size distribution of Acacia senegal *gum on Sephacryl gel S-400 is compared before and after incubation with the proteolytic enzyme Pronase. The profile and elution volumes of chromatograms are greatly modified: a rather broad system of high molecular weight peaks near the exclusion volume is resolved into a single peak at lower molecular weight.*

Physicochemical data from isolated Pronase-treated samples tend to favour, for crude Acacia senegal *gum, a structure where varying numbers of polysaccharide units of MW ca* 2×10^5 *are linked to a protein core. The effect of Pronase on* Acacia mearnsii *and* Combretum nigricans *gums was also investigated.*

Our results are discussed in relation to structural models proposed for these arabinogalactan-protein complexes.

INTRODUCTION

Careful examination of the molecular size distribution of *Acacia senegal* gum (gum arabic) by size-exclusion chromatography (Vandevelde & Fenyo, 1985; Vandevelde, 1986) has confirmed that polydispersity, as previously defined by Gibbons (1972) is largely associated with high molecular weight nitrogen-containing fractions.

Compositional and physicochemical studies on gum arabic samples indicate that their physical, especially viscosity properties, may be attributed to a mixture of charged high molecular weight polysaccharides of fairly constant sugar composition in association with nitrogen-containing

* To whom correspondence should be addressed.

Carbohydrate Polymers 0144-8617/88/$03.50 —

components, that from their analyses, appear to be proteins. Their amino acid composition has been studied in detail (Anderson *et al.*, 1972; Akiyama *et al.*, 1984; Anderson *et al.*, 1985), hydroxyproline and serine being consistently found in high proportions.

Gum arabic is believed to be a member of the arabinogalactan-protein group of proteoglycans (Clarke *et al.*, 1979; Fincher *et al.*, 1983; Akiyama *et al.*, 1984). Akiyama *et al.* (1984) have provided evidence based on treatment with saturated $Ba(OH)_2$ to suggest an arabinosyl–hydroxyproline linkage and have also obtained evidence from alkaline borohydride (β elimination) experiments for linkages to serine.

Various studies have been undertaken with the aim of understanding the relationship between protein and carbohydrate components; however, as discussed by Fincher *et al.*, (1983) and Churms & Stephen, (1984), the nature of the linkage is not yet clear. The different procedures used (chemical, physical, enzymatic) have limitations as concurrent reactions leading to alterations of one or both components may occur.

The striking correlation between high molecular weight fractions and nitrogen content suggested that the consequences of cleavage of protein using a proteolytic enzyme (as opposed to harsher chemical methods) on the molecular size distribution of *Acacia senegal* gum should be investigated.

Pronase, a broad spectrum proteinase, produced by *Streptomyces griseus,* a mixture of endopeptidases and exopeptidases (Narahashi, 1970), was chosen as the proteolytic agent. It has already been used by Akiyama *et al.* (1984) to study the reaction of deglycosylated gum arabic with Yariv antigens but its effect on native gum arabic was not explored.

The results of these experiments are reported in this paper.

EXPERIMENTAL

Materials

Acacia senegal gum samples A and B and *Combretum nigricans* were kindly donated by Iranex SA (Rouen, France). A is a commercial atomized gum, B a crude nodule and the *Combretum nigricans* sample a ground gum. Admixture with other species can be safely disregarded in view of their physicochemical characteristics (Vandevelde & Fenyo, 1985; Anderson, 1978). *Acacia mearnsii* gum was collected and authenticated by Dr J. Vassal of the University Paul Sabatier, Toulouse, France.

All samples were dissolved in water, filtered through Millipore membranes, subjected to ultrafiltration using a Millipore low retention volume system equipped with Pellicon membranes of MW cut off 10^4 daltons and freeze-dried as described earlier (for details see Vandevelde & Fenyo, 1985).

Freeze-dried Pronase for analytical purposes, from *Streptomyces griseus,* was purchased from Boehringer Mannheim, reference 165921 (specific activity *ca* 7×10^3 U g^{-1} on casein as substrate, 40°C, pH 7·5).

Enzymatic treatment

Gums were dissolved in deionized water and the pH of the solution adjusted to 7·5 with NaOH. Pronase was likewise dissolved and added to the gum solution. Usually, 1 ml of Pronase (1·6 mg) was added to 9 ml of gum (1%) and incubated overnight at 37°C.

Polysaccharide hydrolysis is excluded as we have checked that there is no increase in reducing end groups when Pronase is incubated with *Acacia senegal, Acacia mearnsii, Combretum nigricans* gums and arabinogalactan from larchwood (SERVA). There is no decrease in viscosity when commercial galactan from gum arabic (SERVA) is incubated with Pronase.

Methods

Size-exclusion chromatography, light-scattering, viscometry, optical rotation and nitrogen determinations were performed as described by Vandevelde & Fenyo (1985). Elution profiles are measured at 214 nm: at this wavelength, both protein and sugars (especially uronic acids) absorb in proportions relative to their respective extinction coefficients. Analysis of these elution profiles has already been carried out (Vandevelde & Fenyo, 1985).

RESULTS AND DISCUSSION

The two gum samples A and B were chosen as they represent low (15·8 ml g^{-1}) and high (22·0 ml g^{-1}) viscosity gums.

The elution profile of gums A and B on Sephacryl S-400 gel before and after incubation with Pronase are shown in Fig. 1. In each case, a rather broad system of high molecular weight peaks with K_{av} between 0 and *ca* 0·75 found prior to Pronase treatment is resolved into a single

peak of lower molecular weight (K_{av} *ca* 0·7). The peak near V_t ($V_t = 300$ ml) corresponds to the added Pronase.

To study the influence of different reaction parameters, sample A and Pronase were incubated under various conditions. Incubating up to 63 h in the same way as reported in Fig. 1 did not modify the elution profiles.

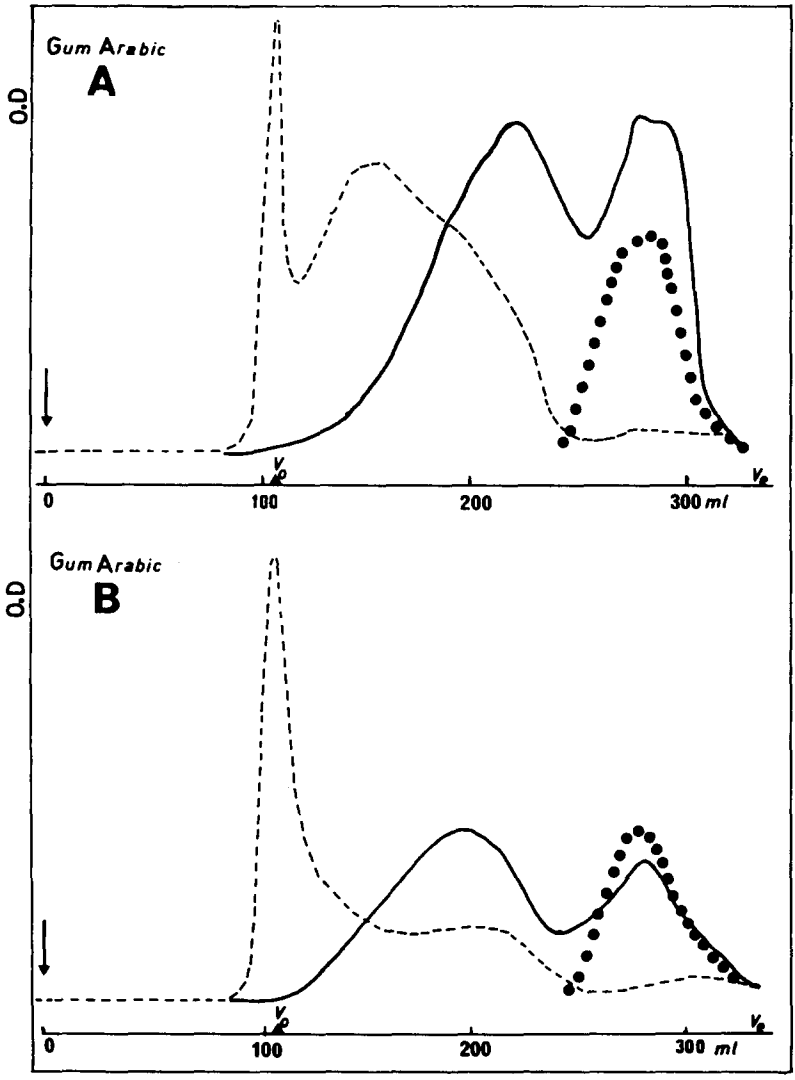

Fig. 1. Size-exclusion chromatography of *Acacia senegal* gum samples A and B, before and after treatment with Pronase. Injection of sample (5 ml, C = 1% in molar sodium chloride) on Sephacryl S-400 gel (column 2·5 × 59 cm), flow rate 128 ml h^{-1}. UV detection at 214 nm, optical path length 0·3 cm. --- gum, un-treated; —— gum 1% + Pronase 1·6 × 10^{-2}% incubated overnight at 37°C; ●●● Pronase alone.

It was also verified that neither the temperature (15°C, 25°C, 50°C) nor gum concentrations (up to 20%) had an effect on the results.

The nitrogen content of gum arabic corresponds to *ca* 2% w/w in protein (Akiyama *et al.*, 1984; Anderson, 1986). In the experimental conditions reported above, the weight ratio protein to Pronase is about 1. It can be seen in Fig. 2 that a decrease in the proportion of the Pronase in the incubation mixture influences the shift of the molecular size distribution of the gum components to lower values.

This effect can be directly monitored by continuously measuring the change in viscosity as shown in Fig. 3. Viscosity falls sharply in the first few minutes and then approaches limiting values of around 11 ml g^{-1} when Pronase is in excess. Thus it can be assumed that overnight incubation at 37°C with a ratio of Pronase to gum of about 1 to 60 w/w is sufficient to complete the change caused by Pronase.

To separate the treated gum from the Pronase after overnight incubation, semi-preparative size-exclusion chromatography was performed. The rechromatography of the fraction collected between K_{av} 0·25 and 0·75 is represented in Fig. 4.

Figure 5 shows results obtained by HPLC using a TSK G-5000 PW column. The observed peak at V_0 (total exclusion volume), and its tail (both due to arabinogalactan-protein complex) disappear after the Pronase treatment, the remaining unique peak being symmetrical. The apparent difference of the elution profile in comparison with Sephacryl

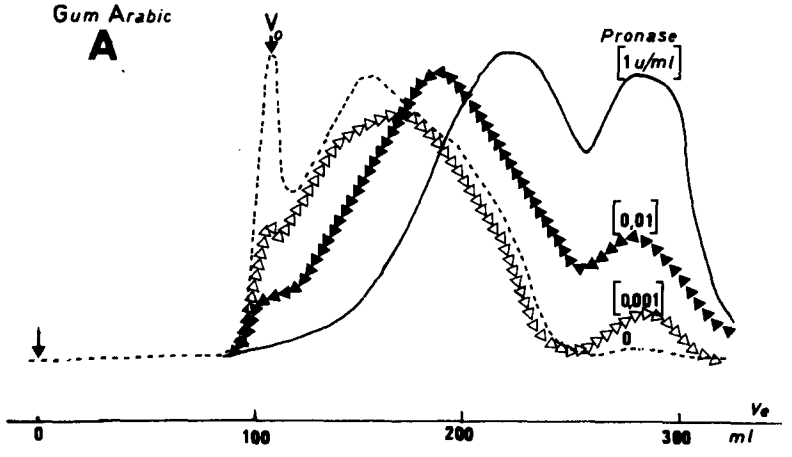

Fig. 2. Size-exclusion chromatography of gum A after incubation with varying concentrations of Pronase. Sephacryl S-400 gel in molar sodium chloride. Same conditions as in Fig. 1. – – – gum 1%, un-treated; ◁ ◁ ◁ gum 1% + Pronase $1{\cdot}6 \times 10^{-5}$% (*ca* 10^{-3} U ml^{-1}); ◀ ◀ ◀ gum 1% + Pronase $1{\cdot}6 \times 10^{-4}$% (*ca* 10^{-2} U ml^{-1}); —— gum 1% + Pronase $1{\cdot}6 \times 10^{-2}$% (*ca* 1 U ml^{-1}). All samples are incubated overnight at 37°C.

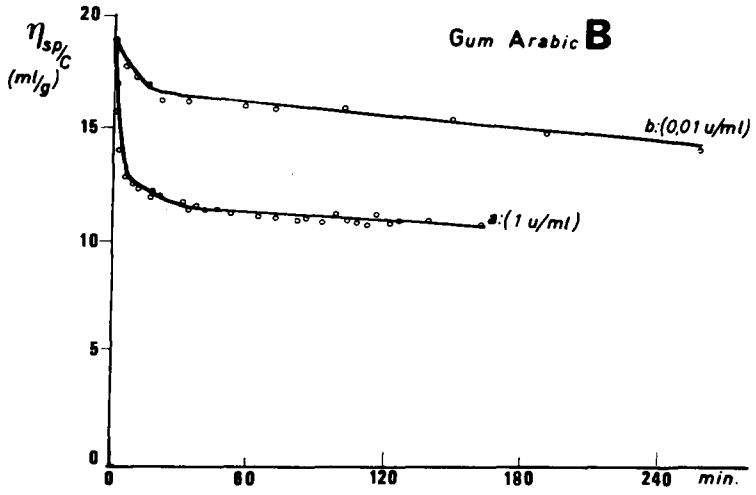

Fig. 3. Viscosity of gum B after addition of Pronase, as a function of time, in molar sodium chloride at 37°C. (a) gum 1%. at $t = 0$, addition of $1{\cdot}6 \times 10^{-2}$% Pronase; (b) gum 1%. at $t = 0$, addition of $1{\cdot}6 \times 10^{-4}$% Pronase.

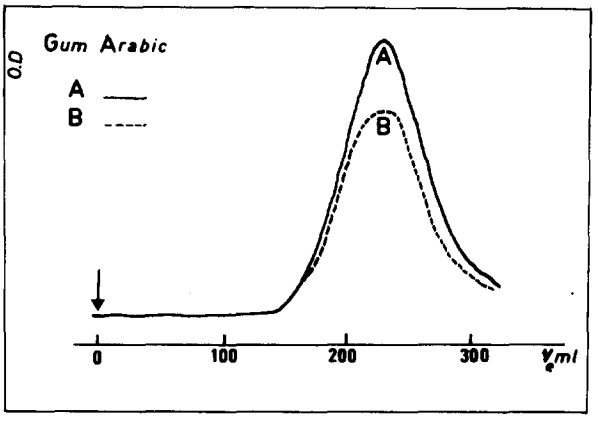

Fig. 4. Rechromatography on Sephacryl S-400 gel of gums A and B after incubation with Pronase. Same conditions as in Fig. 1, except flow rate: 110 ml h^{-1}. —— gum A, 0·35%; - - - gum B, 0·21%.

S-400 gel is due to the type of detection used: elution profiles detected by refractive index are proportional to the concentration of AGP. The HPLC data will be published in more detail in a later paper.

Analytical data for the two gum samples were compared before and after Pronase treatment (see Table 1). Regardless of their initial value, intrinsic viscosity decreases to 11–12 ml g^{-1} and weight average molecular weight to close to 2×10^5.

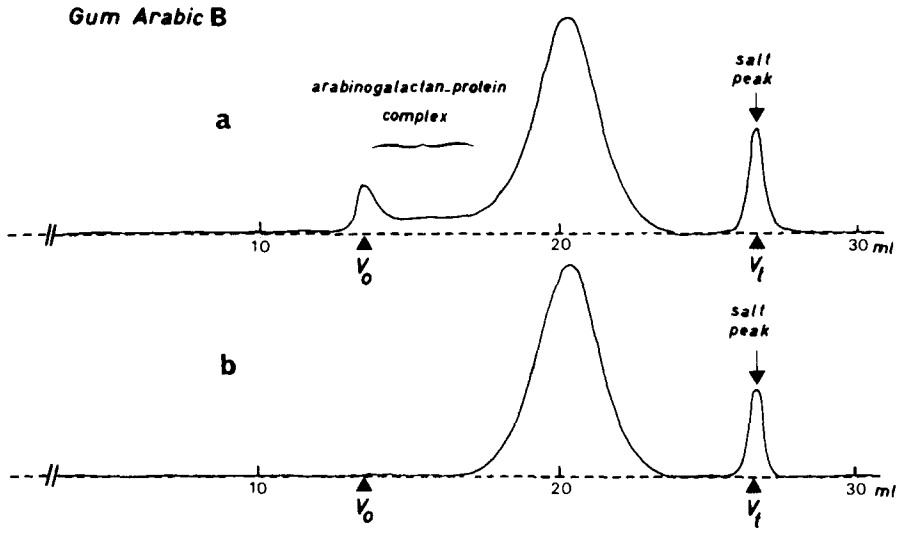

Fig. 5. High performance size-exclusion chromatography of gum B: (a) before; and (b) after incubation with Pronase (same sample as in Fig. 4). HPLC system was comprised of a Waters Assoc. (Milford, Mass., USA) 6000 A pump, a UGK injector, and a differential refractometer. Column: LKB Ultropac TSK G-5000 PW; 17 μm, 7·5 × 600 mm. Eluent: NaCl 1M. Flow rate: 0·7 ml min^{-1}. Pressure: 100 psi. Temperature: ambient. Injected vol.: 25 μl at 2%.

TABLE 1
Analytical Data for Pronase Treated Gum Samples

Sample	$[\eta]$ *(ml* g^{-1}*)*[a]		$\bar{M}_w \times 10^{5}$[b]		$[\alpha]^{25}_{589}$ *(degrees)*[c]	
	(1)	*(2)*	*(1)*	*(2)*	*(1)*	*(2)*
A	15·8	12	4·9	2·0	−29·1	−28·0
B	22·0	11	7·3	1·8	−29·2	−30·2

[a]Intrinsic viscosity in molar sodium chloride.
[b]Weight average molecular weight determined by laser light-scattering photometry.
[c]Specific rotation at 589 nm in molar sodium chloride.
(1) before and (2) after incubation with Pronase, fractionation on Sephacryl S-400 gel, ultrafiltration and freeze-drying.

These limiting values have also been observed by size-exclusion chromatography of crude gum (Vandevelde & Fenyo, 1985) and in earlier studies by Anderson & Stoddart (1966) where viscosities of 10·8 ml g^{-1} are given for the last fraction precipitated by Na_2SO_4.

The data in Table 1 and the symmetry of the peaks in Figs. 4 and 5 are indicative of the homogeneity of the material being released by Pronase

treatment. An indication that the polysaccharide itself is apparently not altered by Pronase treatment is given by the absence of change in the specific rotation values.

Several models for the organization of arabinogalactan–protein molecules have been proposed. For the high nitrogen *Acacia robusta* AGP gum, Churms & Stephen (1984) favour a structure where there is only one polysaccharide-protein linkage per molecule.

Our results for *Acacia senegal* favour the 'wattle blossom' model proposed by Fincher *et al.* (1983). A similar structure has been proposed for the arabinogalactan-peptide from wheat endosperm (McNamara & Stone, 1981; Strahm *et al.*, 1981). For *Acacia senegal* gum, several polysaccharide units of MW *ca* 2×10^5 would be linked to the protein core. Pronase would hydrolyze the protein leading to the liberation of polysaccharide chains which may, however, still bear amino acid residues. It will be of great interest to verify the linkage between protein and polysaccharide.

Published molecular weights of *Acacia senegal* gum exudates vary from several hundred thousand up to 10^6 (Anderson *et al.*, 1968; Vandevelde, 1986), which indicates that crude natural gum may be a mixture of AGP molecules containing one to several polysaccharide chains linked to the protein core.

Street & Anderson (1983), Churms *et al.* (1983), Ullmann (1983) and Defaye & Wong (1986) have recently presented evidence for a more regular structure of the heterogalactan portion of the gum as opposed to the random one considered in earlier work.

It can be calculated from the relative number of sugar residues given by Street and Anderson (1983) for the AG, that the MW of the AGP would be a multiple of *ca* $1{\cdot}5 \times 10^5$, a value fairly close to the one we have observed. On the other hand, Churms *et al.* (1983) have postulated a block organization of the arabinogalactan with sub-units having a molecular weight of *ca* 8×10^3. The intact polysaccharide would contain an average of 64 of these sub-units to give a molecular weight of $5{\cdot}6 \times 10^5$.

The chemical reactions used to establish these structures can denature the protein. This does not of course alter the results relating to structural studies of the polysaccharide alone but may, however, lead to misinterpretations in evaluating molecular weights of the native polysaccharide-protein. Thus in view of our results, considerations relating to the polydispersity of *Acacia senegal* gum by precipitation with organic components such as acetone (Swenson *et al.*, 1968), or alcohol (Alain & McMullen, 1985), which do not take into account the protein core will be misleading.

In order to check if the observed effect of Pronase is specific to *Acacia senegal,* similar experiments were performed on two other gums from *Combretum nigricans* and *Acacia mearnsii,* which are under study in our laboratory.

No significant difference following Pronase treatment was observed by size-exclusion chromatography of *Combretum nigricans* gum (Fig. 6); on the other hand a striking effect is shown in Fig. 6 for *Acacia mearnsii* gum. At first sight it is possible to relate these differences in behaviour to

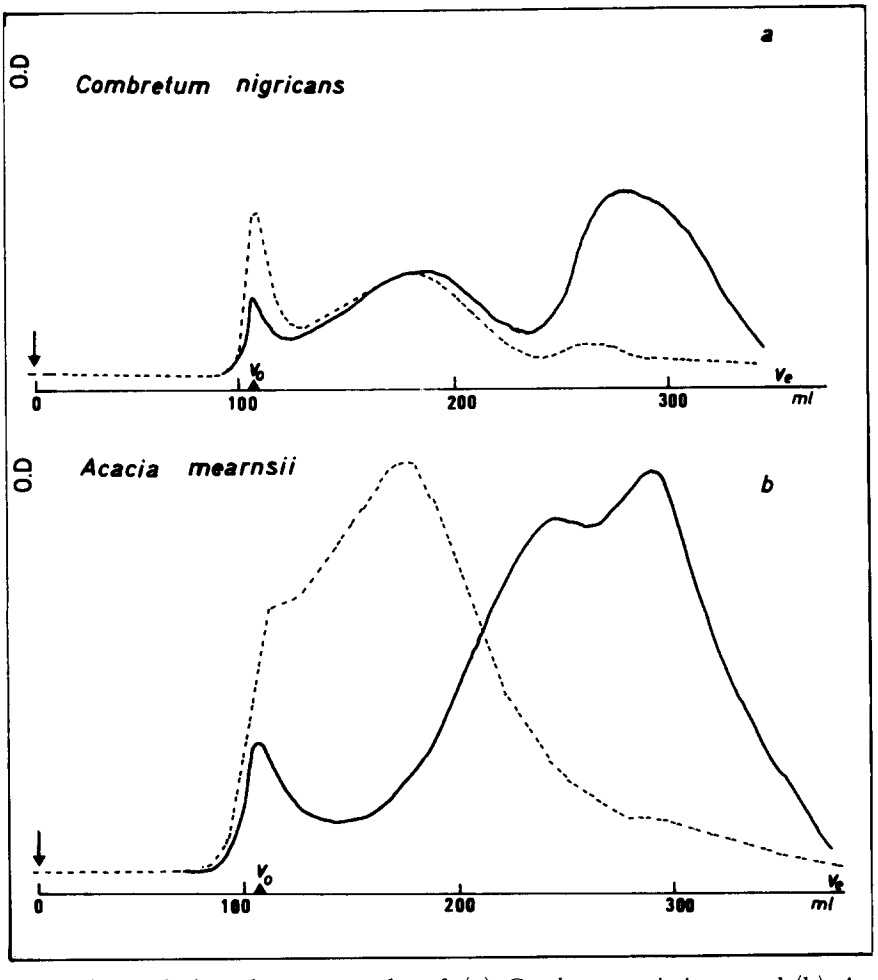

Fig. 6. Size-exclusion chromatography of: (a) *Combretum nigricans* and (b) *Acacia mearnsii* gums before and after incubation with Pronase. Sephacryl S-400 gel in molar sodium chloride. Same conditions as in Fig. 1. --- gum 1%, un-treated; —— gum 1% + Pronase $1{\cdot}6 \times 10^{-2}$% incubated overnight at 37°C. Pronase (not represented) is eluted near V_t (V_t = 300 ml) as in Fig. 1.

the differences in nitrogen content: 0·1% for *Combretum nigricans* and 0·7% for *Acacia mearnsii* gum.

Further investigation on other gums with higher nitrogen contents could be of great interest to demonstrate the generality of our observations.

ACKNOWLEDGEMENTS

The authors express their gratitude to Professors J. P. Joseleau and B. A. Stone for helpful discussions and Dr G. Muller for molecular weight determinations.

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