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HEVAMINE: A CRYSTALLINE BASIC PROTEIN FROM *HEVEA BRASILIENSIS* LATEX*

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Key Word Index—*Hevea brasiliensis*; Euphorbiaceae; latex; basic proteins; isolation; characterisation; hevamine.

Abstract—The isolation and purification of a basic protein from the bottom fraction of *Hevea brasiliensis* latex is described. Two protein fractions were obtained by chromatography on carboxymethyl cellulose which also differed in their electrophoretic mobility in polyacrylamide gel, but the similarity of their other properties precludes their classification as two protein entities at this stage.

INTRODUCTION

Fresh latex, obtained by tapping *Hevea brasiliensis* can be separated into three main fractions by centrifugation [1,2], these are a white upper layer of rubber particles, an aqueous serum and a 'bottom fraction' which contains the lutoid particles first described by Homans and van Gils [3].

Roe and Ewart [4] showed that *H. brasiliensis* latex contains a protein with an isoelectric point of about 9·6, and a similar substance moving towards the cathode on electrophoresis at pH 8·6 was found to be a major constituent (component i) of the latex bottom fraction [5–7]. The importance of these cationic proteins in causing the resealing (plugging) of latex vessels after they had been tapped, was suggested by Southorn and Edwin [8] who showed that bottom fraction contained a powerful latex destabiliser. This paper describes the isolation of two crystalline basic protein fractions from latex bottom fraction, both of which have the same mobility on cellulose acetate and correspond to the two bands separated by Archer *et al.* [7] using polyacrylamide gel electrophoresis. As the amino acid compositions of the two fractions are not sufficiently different to justify the postulation of two separate proteins, it is proposed that the collective name of hevamine should be adopted for them.

RESULTS

Isolation of basic protein from Hevea brasiliensis *latex*

The gel-filtration chromatography of *Hevea* bottom-fraction proteins on Sephadex G-25 has been reported previously [7]. Since hevein is retarded on this column it provides a simple method of removing much of this anionic protein from the preparation, together with low molecular weight impurities. The rechromatography of the unretarded protein from the G-25 column, on Sephadex G-50 is shown in Fig. 1, where two methods of concentrating the protein solution prior to G-50 chromatography are compared. In (a) ammonium sulphate was used to precipitate the protein, whereas in (b) the solution was freeze-dried. Since the proteins of peaks 1 and 2 had very similar electrophoretic mobilities on cellulose acetate, it is very likely that the former was produced by aggregation of the lower molecular weight protein of peak 2 during drying. Disc electrophoresis confirmed the similarity of the proteins of peaks 1 and 2 by showing the presence of two bands of approximately equal intensity, very close together, in both fractions. The protein of peak 1 has a low solubility between pH 5 and 10 whereas the protein of peak 2 is soluble over the entire pH range. It is probable therefore that peak 1 is an artefact and the small amount observed [Fig. 1 (a)] was formed during the freeze-drying stage of the original preparation of the bottom fraction. This conclusion was confirmed by treating a sample of freeze-dried basic protein with 6M urea. Gel-chromatography showed that the untreated material was mainly the high molecular weight protein of peak 1 but urea caused the conversion of 96% of this protein to the protein of peak 2. Peak 3 (Fig. 1) contained a number of protein components of low electrophoretic mobility on cellulose acetate strip at pH 8·6.

Figure 2 shows the elution diagram obtained when the protein of peak 2, from a Sephadex G-50 column, was chromatographed on a carboxymethyl cellulose column. Peaks A and B are both hevamine fractions and each gave a single band on disc electrophoresis at pH 8·5.

Fraction B was slightly more cathodic than A under these conditions. Cellulose acetate strip electrophoresis was not sufficiently sensitive to detect any difference between the two proteins at pH 8·6. Peak X in Fig. 2 contained a mixture of proteins of low electrophoretic

* Part 6 of "The proteins of *Hevea brasiliensis* latex". For part 5 see Tata, S. J. and Moir, G. F. J. (1964) *J. Rubb. Res. Inst. Malaya.* **16**, 155.

mobility at pH 8·6 and appeared to be similar to peak 3 in Fig. 1 These proteins possibly correspond to bands (iii) and (iv) of Karunakaran *et al.* [6].

Properties of the hevamine

The crystals of both hevamine fractions consisted of short hexagonal prisms in contrast to the long needles of hevein which had also been isolated previously from latex bottom fraction [9]. Both hevamine fractions are water soluble over the whole pH range, provided they are not aggregated to high molecular weight compounds. Aggregation occurs readily during freeze-drying of their solutions. The elementary analysis of hevamine fraction A (C, 50·3; H, 6·3; N, 15·5; S, 0·88; P, 0·012; ash, 2·15) is probably not significantly different from that of fraction B (C, 50·5; H, 6·0; N, 15·8; S, 0·79; P, 0·015; ash, 1·62).

The molecular weight of the protein in peak 2 from the Sephadex G-50 column was measured on a Mechrolab osmometer at pH 8·0 in 0·25M Na_2SO_4 at 25°. A linear graph of π/c vs c was obtained, from which M_n was found to be 24800. Sodium sulphate was found to give the most stable solution of the protein over an extended period. Other ions including phosphate, chloride and fluoride caused precipitation of protein within 3 days at 25°.

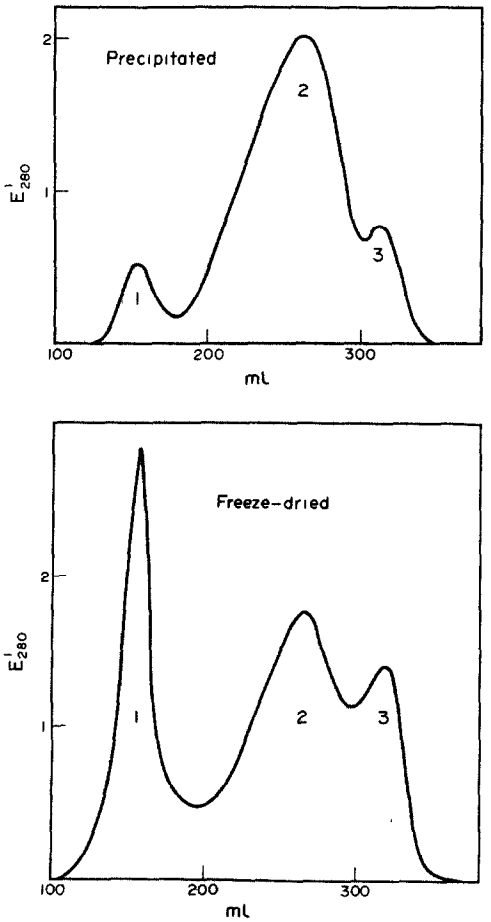

Fig. 1. Chromatography of basic proteins of *Hevea brasiliensis* latex on Sephadex G-50. Protein previously chromatographed on Sephadex G-25 was separated into three components on a Sephadex G-50 column (1000 × 25 mm) at 2° with 0·002M HCl at a flow rate of 1·55 ml min^{-1}. (a) Protein precipitated with $(NH_4)_2SO_4$ and dissolved in 10 ml 0·002M HCl before chromatography. (b) Protein freeze-dried and redissolved in 0·002M HCl before chromatography.

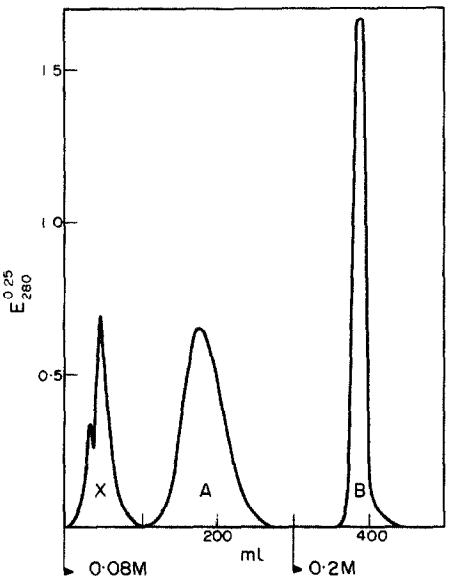

Fig. 2. Ion-exchange chromatography of hevamines. The effluent from the G-50 Sephadex column was applied to a column of Whatman CM-52 (200 × 15 mm) and eluted with 300 ml of 0·08M borate buffer, pH 8·9, at 1·3 ml min^{-1}, followed by 0·2M borate buffer pH 8·9, at the same flow rate. A = Hevamine fraction A, B = fraction B. X = Uncharacterised protein of low electrophoretic mobility.

An attempt was made to measure M_n for the protein eluted in peak 1 from the G-50 column. Owing to the low solubility of this protein anywhere near its isoelectric point, it was necessary to use 0·5M NaCl adjusted to

Table 1. Amino acid analyses of hevamine

Amino acid	Hevamine fraction A		Hevamine fraction B	
	% w/w of residues	Number of residues per mol*	% w/w of residues	Number of residues per mol*
Ala	5·2	18·4	5·1	17·8
Arg	3·4	5·4	4·0	6·4
Asp	13·9	30·3	13·7	29·8
Cys†	2·0	4·9	2·0	4·9
Glu	6·3	12·2	5·9	11·5
Gly	6·5	28·4	6·2	27·2
His	0·7	1·2	0·8	1·4
Ile	6·2	13·6	6·0	13·2
Leu	8·9	19·7	8·4	18·6
Lys	5·4	10·6	5·7	11·2
Met†	1·2	2·2	1·1	2·0
Phe	4·6	7·8	4·5	7·6
Pro	5·5	14·2	5·5	14·2
Ser	7·2	20·8	7·2	20·8
Thr	4·3	10·7	4·4	10·9
Trp‡	3·0	4·0	2·4	3·2
Tyr¶	9·4	14·4	9·0	13·8
Val	3·5	8·7	3·5	8·7
	97·2		95·5	

*Assuming an approximate molecular weight of 25000. † Cys and Met were determined as cysteic acid and methionine sulphone after oxidation of the protein with performic acid. ‡ Trp was measured on a hydrolysate prepared with methane sulphonic acid. ¶ Calculation of the concentration of Tyr from the spectra gave 14·6 and 14·0 residues per mol respectively for fractions A & B.

pH 10·5 with potassium hydroxide, as solvent. A falling osmotic pressure corresponding to an apparent M_n of the order of 10^6 was observed, and precipitated protein was found in the osmometer after the experiment.

DISCUSSION

Little, if any, significant difference between the two hevamine fractions is indicated by their elementary analyses. Similarly the amino acid compositions only differ slightly, (5 Arg and 4 Trp residues in one as compared with 6 Arg and 3 Trp residues in the other). The observed difference in the numbers of residues of Gly, Ile and Leu in the two fractions are probably not significant. The total number of dicarboxylic acid residues (Asp + Glu) in each fraction, considerably exceeds the number of basic residues (Lys + Arg). The basic character of the hevamines (isoelectric point 9·5) suggests that more than half of the Glu and Asp residues are amidated, and small differences, for example in the degrees of amidation, cannot be excluded as the cause of the apparent difference in electrophoretic mobility in agar gel between the two fractions. It is unlikely that either of the two components detectable by gel-electrophoresis, were formed by deamidation during the ion-exchange chromatography stage as they could be detected in the effluent from the gel-chromatography columns, and re-running of either component on the ion-exchange column did not cause any apparent change in their elution volumes or gel-electrophoretic mobilities. It is also unlikely that the initial freeze-drying of the latex bottom fraction gave rise to the second crystalline component as this procedure is known to cause aggregation of the molecules to a product of considerably higher molecular weight (see results). Further work, probably by peptide finger-printing, will be needed before the existence of two different hevamine molecules can be established.

EXPERIMENTAL

Materials. Freeze-dried samples of the bottom fraction of latex, freshly tapped into ice-cooled vessels were used throughout. Collection and centrifugation of the latex, were carried out by the staff of the Rubber Research Institute of Malaysia, as described by Karunakaran *et al.* [6]. After freeze-drying, the samples of bottom fraction were airmailed to England and stored at −20°.

Extraction of proteins. 10g samples of the freeze-dried bottom fraction were homogenised in a Waring blender with 160 ml of 2 mM HCl at 0° under N_2 and centrifuged at 90000 g_{max} to remove most of the suspended matter. Ammonium sulphate was then added at the rate of 470g/l, and the pH adjusted to 7·5 before centrifugation at 5000 g_{max}. The precipitated protein was dissolved in 20 ml of 2 mM HCl and the solution clarified by centrifugation at 90000 g_{max}.

Gel-filtration chromatography. The solution of protein was chromatographed on Sephadex G-25 using 2 mM HCl at 2° for elution. The first peak, shown by monitoring at 280 nm, was collected and the protein recovered from the solution by precipitation with $(NH_4)_2SO_4$ (470g/l), since freeze-drying caused the formation of high molecular weight products. The precipitated protein was dissolved in 2 mM HCl and rechromatographed on a column of Sephadex G-50. Peak 2, which contained the native hevamine, was loaded directly on to the cellulose ion-exchange column.

Ion-exchange chromatography. Columns of carboxymethyl cellulose (Whatman CM52) were equilibrated with 0·04M borate buffer at pH 8·9 (0·006M $Na_2B_4O_7$ + 0·016M H_3BO_3), and loaded directly with the eluate from the G-50 column. Hevamine fraction A was eluted with 0·08M borate buffer and then fraction B with 0·2M borate buffer. Alternatively gradient elution with borate buffer from 0·08 to 0·2M at pH 8·9 could be used.

Crystallisation. Eluates from the ion-exchange column, which contained the two hevamine fractions, were each treated with $(NH_4)_2SO_4$ (470g/l) and the precipitated protein was dissolved in a small vol 0·01M HCl. pH was adjusted to 8·0 with 0·5 M NaOH and any precipitate formed (polymerised hevamine) centrifuged off. $(NH_4)_2SO_4$ was added to give a slight turbidity to the solution which was then left at 0° to crystallise. After two further recrystallisations, each product was stored at 0° under 70% saturated ammonium sulphate.

Elementary analyses. Solutions of the hevamine fractions were exhaustively dialysed against H_2O and then passed through columns of Biodeminrolit resin. After freeze-drying, C, H and N were measured on a Perkin-Elmer 240 analyser and corrected for the water content of the sample, obtained by drying to constant weight at 105°. S was estimated by dry combustion and titration of SO_4^{2-} with $Ba(ClO_4)_2$ using thorin as indicator. Cl was estimated by dry combustion and potentiometric titration with $AgNO_3$. Ash was determined directly on the sample used to determine S, and P was estimated by wet oxidation followed by colorimetry with $(NH_4)_2MoO_4$ and metol.

Amino acid analyses. Protein samples were hydrolysed with 6M HCl under vacuum in sealed tubes at 105° for 24 hr. After removal of HCl, hydrolysates were analysed on a Beckman 120c automatic amino acid analyser. Trp was determined after hydrolysis of the protein with methane sulphonic acid [10]. S-containing amino acids were measured after performic acid oxidation, followed by hydrolysis with HCl in the normal way. No corrections were applied to the values for Thr, Ser, Val or Ile, to allow for their destruction during hydrolysis.

Electrophoresis. This was carried out on 'Oxoid' cellulose acetate strips in a Shandon apparatus for 1 h. The buffer was 0·01M veronal and 0·04M sodium barbitone (pH 8·6), and the potential gradient 20 V cm^{-1}. The protein bands were visualised with a 0·2% solution of Ponceau S in 0·3% trichloracetic acid and excess dye was removed with 5% acetic acid.

Disc electrophoresis. The apparatus of Ornstein and Davis [11] was used. The formulation of the polyacrylamide gel was similar to that of Whitaker [12] for the separation of cationic molecules. The final concentrations in the gel solution (pH 8·5) were: KOH, 0·06M; glycine, 1·90M; acrylamide, 75g/l; *NN'*methylene-bis-acrylamide, 0·75g/l; ammonium persulphate, 0·75g/l; *NNN'N'*-tetramethylethylene-diamine, 5·75 ml/l. The electrode buffer (pH 8·2) contained 0·1 M glycine and 0·05M imidazole. The samples for analysis (100 μg protein in 0·1 ml) were mixed with an equal vol of a buffer soln (pH 10·3), which contained 0·12M KOH, and 0·16M glycine in glycerol–H_2O, 3:1 v/v. No large-pore spacer-gel was used. 250 V was applied for 80 min, with the cathode in the lower compartment of the apparatus. In these laboratories, difficulties have been experienced with the standard methods of staining the basic protein bands after electrophoresis. However the modified staining technique of Chrambach *et al.* [13] has given good results, using 0·06% Coumassie brilliant blue in 12·5% trichloracetic acid for 1 hr followed by washing the gels free of excess dye in the same acid solvent.

MW determination by osmometry. MW of the hevamine, as prepared by chromatography on Sephadex G-25 and G-50, was estimated using the Mechrolab osmometer Type 501 (Hewlett-Packard) with an S & S membrane, type B.19. The protein was dissolved in 0·25M Na_2SO_4, adjusted to pH 8·0 with KOH, and dialysed for 40 hr against three changes of the same solvent, before loading into the osmometer. The protein concentrations in the solutions were measured by UV absorptiometry at 278 nm ($E^{1\%}_{1cm}$ = 20·6).

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