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HEVAINS: SERINE-CENTRED PROTEASES FROM THE LATEX OF *HEVEA BRASILIENSIS*

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Abstract—Hevains *b* and *l*, isolated respectively from the serum and lutoids of freeze-dried latex from *Hevea brasiliensis*, were purified to homogeneity and compared with hevain *a* from commercial, ammonia-treated latex. The M_rs of hevains *a* and *b* are 69 000 and 58 000, respectively, and both exist in several charged forms. The amino acid compositions of the two enzymes differ significantly, but the reactivities to a variety of ester and protein substrates are similar, as are the pH optima. Hevain *l* is a distinct protease of M_r 80 000 and unique amino acid composition. It displays esterolytic activity and will digest insulin B chain, but is not proteolytic to azocollagen, azocasein, bovine serum albumen or haemoglobin. The activities of all three enzymes are dependent on the presence of serine and histidine residues.

INTRODUCTION

In a recently published report [1], we described the isolation, M_r, amino acid composition and some properties of a protease, hevain, isolated from commercially obtained latex of *Hevea brasiliensis* Muell. Arg. The latex had been stored in concentrated ammonia solution, which is a normal practice in the industry to prevent polymerization. It was of interest to extend the investigation to latex not subject to prolonged exposure to high pH, and such material was generously supplied to us by Dr. B. L. Archer of the Palm Oil Research Institute of Malaysia. This has enabled us to isolate and characterize a protease of different composition from *Hevea* latex which had been centrifuged to separate lutoid (lysozomal) material and then freeze-dried, immediately following collection. Another enzyme, displaying only limited proteolytic properties was purified from the lutoid fraction.

To distinguish between the proteolytic/esterolytic enzymes isolated from ammonia-treated [1] and untreated latex sera the former is designated hevain *a*, the latter hevain *b*. The enzyme from the lutoid fraction is hevain *l*.

The enzymes were obtained in homogeneous form from the sources described, their M_rs and amino acid compositions determined, and some properties examined.

RESULTS AND DISCUSSION

All three enzymes were isolated as homogeneous proteins using methods previously described [1], each giving a single band on both anionic and cationic gel electrophoresis. Hevain *a*, from ammonia-treated latex, has an M_r of 69 000 as measured using a calibrated HPLC system and confirmed on Biogel P100 [1]. The enzyme from freeze-dried latex, hevain *b*, has M_rs of 57 000 and 59 000, respectively, using the same two procedures. Hevain *l*, the lutoid esterase, has an M_r of 80 000 estimated by HPLC using a TSK-Spherogel column [1].

When subjected to PAGE in the presence of SDS, all three enzymes are fragmented: hevain *a* into five bands with weights of 54, 49, 41, 25 and 15 000 (compare 1): esterolytic hevain *l* into four bands of weights 45, 39, 27 and 16 000: hevain *b* into three bands of weights 41, 23 and 20 000 with, in addition, proteins of weights 65 and 83 000, which are attributed to aggregations between charged entities unaffected by the presence of SDS.

The amino acid analyses of hevains *a*, *b* and *l* are given in Table 1. To facilitate comparisons we have also included weight per cent compositions of the three enzymes. The data for the first-named enzyme are essentially identical with those previously reported from this laboratory [1]. The results for the lutoid-derived enzyme are incomplete (Cys, Trp and amino-sugars) because of a shortage of material, but the data obtained permit comparisons to be made. The three hevains clearly have different compositions. It is noteworthy that, despite major differences in composition between hevains *a* and *b*, these two proteases have retained similar properties, as will be described below, suggesting that the active centre of hevain *a* is not completely altered by prolonged exposure to concentrated ammonium hydroxide. On comparing the compositions of hevains *a* and *b* large losses of Cys and aromatic amino acid residues are apparent for the former enzyme (Table 1). Hevain *a*, however, has acquired significant amounts of Thr, Glx, Pro, Ala, Val and Lys residues, which may imply covalent coupling of hevain *b* with proteins or peptides from the crude latex during prolonged storage in concentrated base. Reactions of possible relevance to such an hypothesis have been discussed by Fruton [2] and by Ponnamerpuma [3] but definition of the agents directly responsible for the weight enhancement and increase in

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Table 1. Amino acid compositions as residues/mol (weight % in parentheses)

Residue	Hevain *a*	Hevain *b*	Hevain *l*
Cys	2 (0.3)	10 (1.8)	n.m.*
Asx	39 (6.4)	61 (12.0)	105 (15.4)
Thr	73 (10.5)	38 (6.5)	60 (7.7)
Ser	20 (2.5)	32 (4.8)	52 (5.8)
Glx	162 (30.0)	81 (17.7)	71 (11.7)
Pro	97 (13.4)	32 (5.3)	50 (6.2)
Gly	20 (1.6)	38 (3.7)	64 (4.6)
Ala	108 (11.0)	42 (5.0)	36 (3.3)
Val	35 (4.9)	21 (3.5)	39 (4.9)
Met	0 (0)	1 (0.3)	7 (1.2)
Ile	17 (2.7)	28 (5.3)	41 (5.9)
Leu	20 (3.2)	47 (9.1)	62 (8.9)
Tyr	2 (0.5)	13 (3.5)	35 (7.3)
Phe	1 (0.2)	17 (4.3)	30 (5.6)
His	7 (1.4)	7 (1.6)	7 (1.2)
Lys	56 (10.2)	38 (8.3)	32 (5.2)
Arg	6 (1.3)	17 (4.6)	25 (5.0)
Trp	0 (0)	2 (0.7)	n.m.
Glu-N	0 (0)	0	n.m.
M_r	70000	59000	78500

*Not measured.

several amino acid components of the hevain a cannot be made from currently available information. Hevain *l* is a unique molecule with properties that distinguish it from the other two enzymes discussed here (Table 1).

pH optima for the three hevains described were determined, with CGN as the substrate, at 6.6 and 6.3 for hevains *a* and *b* (broad 'bell-shaped' curves), while hevain *l* had two maxima, at pHs of 6.3 and 7.7, thus resembling the euphorbains from *Euphorbia lactea* and *E. lactea cristata* [4].

Hevain *a*, examined by chromatofocusing [1], had a pI of 4.3: this was confirmed in the investigation reported here, using isoelectric focusing, and charged forms with pIs of 4.3, 4.8, 5.2 and 5.7 were then observed. Both hevains *b* and *l* displayed a number of charged proteins: the former at pIs of 4.8, 4.9, 5.1 and 5.3 and the latter at 4.9, 5.0, 5.4, 6.2, 6.6 and 6.9.

In Table 2 are summarized results from tests with a number of ester and protein substrates which demonstrate the differences between hevains *a*, *b* and *l*. While the first two enzymes have both esterolytic and proteolytic abilities, including carboxylesterase properties, hevain *l* is not active to such protease substrates as azocoll, azocasein, bovine serum albumin or haemoglobin. It shares with hevains *a* and *b* reactivity to CGN and an inability to catalyse the hydrolysis of *p*-nitrophenyl butyrate, but, unlike those two enzymes, hevain *l* cannot hydrolyse *p*-nitrophenyl caproate.

When insulin B chain was digested by hevains *a*, *b* and *l*, then subjected to thin layer mapping [5], the results reported in Fig. 1 were obtained. It is clear that hevains *a* and *b* are notably similar in proteolytic ability: with allowance for experimental variation from one map to another, about 14 peptides were judged to be identical, of the 16 found in cleavage with hevain *a*, and 18 with hevain *b*. Hevain *l* produced an entirely different collection of products, showing again that it is a unique entity.

The qualitative similarities between the reactivities of hevains *a* and *b* are apparent in the data reported, and in inhibitor studies described below. In measurements of the Michaelis–Menten parameters of hevains *a* and *b* using several mesyl glycinates [$MeSO_2NHCH_2CO \cdot O \cdot C_6H_4(R)$] as esterolysis substrates [6] a consistent pattern of behaviour of the two enzymes is apparent. The data are summarized in Table 3

Table 2. Summary of reactivities of hevains *a*, *b* and *l* with a variety of esterolytic and proteolytic substrates

Substrate	Hevain *a*	Hevain *b*	Hevain *l*	Method
CGN*	+	+	+	Ref. 1
Azocoll	−	−	−	Ref. 1
Azocasein	+	+	−	Ref. 1
Bovine serum albumin	n.m.	+	−	As azocasein
Haemoglobin	+	n.m.	−	Ref. 4
Mesyl glycinates†	+	+	+	Ref. 6
pNP acetate‡	+	+	+	As CGN
pNP propionate	+	+	+	As CGN
pNP butyrate	−	−	−	As CGN
pNP valerate	+	+	+	As CGN
pNP caproate	+	+	−	As CGN
pNP caprylate	+	+	+	As CGN
pNP caprate	+	+	+	As CGN
pNP laurate	+	+	+	As CGN
pNP myristate	+	+	+	As CGN
pNP palmitate	+	+	+	As CGN

*n.m., Not measured.

†Carbobenzoxy glycine *p*-nitrophenyl ester. *p*-Chloro, *m*-chloro, *m*-NO_2phenyl derivatives.

‡pNP, *p*-nitrophenyl.

A + indicates sensitivity, a − lack of hydrolysis under identical conditions. Measurements in 100 mM Tris–HCl, pH 7.0, at 21°, except that protein substrates reacted at 37°.

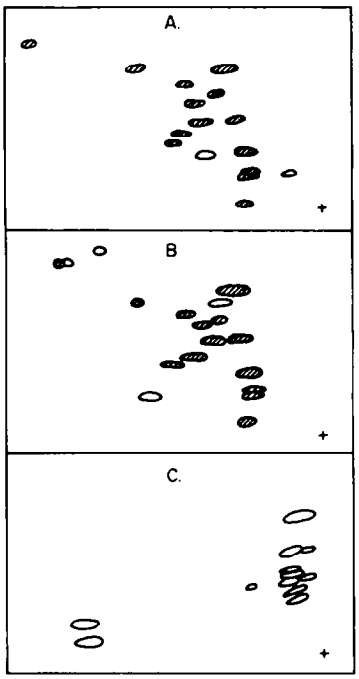

Fig. 1. Thin layer maps of digestions of insulin B chain by hevains *a* (A), *b* (B) and *l* (C) under conditions described in ref. [5].

where it is seen that $K_m(b)/K_m(a)$ is, within experimental error, the same as $V_{max}(b)/V_{max}(a)$ which shows that while the hevain from the freeze-dried latex (*b*) binds the substrate less effectively than that from the base-treated source (hevain *a*), the former protease catalyses the hydrolysis of the glycinates more efficiently. Thus the ratios of V_{max}/K_m for the two enzymes are equivalent for the three substrates reported in Table 3. Structural modifications, which the data of Table 1 show to have occurred on prolonged storage of the *Hevea brasiliensis* latex in concentrated ammonia, have effects on the binding and catalytic properties of hevain *a* which consistently neutralize each other, when comparison is made with the protease from freeze-dried latex.

Results of inhibition studies with the serine-specific reagents di-isopropyl fluorophosphate (DFP) [7] and phenyl methane sulphonyl fluoride (PMSF) [8], as well as the histidine specific reagent diethyl pyrocarbonate (DEP) [9], are collected in Table 4. The three hevains are inhibited at both serine and histidine centres and so resemble several of the euphorbains isolated from other members of the Euphorbiaceae [4, 5, 10]. Hevain *l* is distinguished from hevains *a* and *b*, which are unaffected, by being inhibited to the extent of 30% with serine-directed chymostatin [11] and both carboxyl-directed pepstatin [12] and diazo-DL-norleucine methyl ester [13] when these reagents are in large (10^5) molar excesses.

Hevain *a* was reported [1] to be unaffected by the presence of mercaptoethanol and *p*-chloromercuribenzoate (*p*-CMB). Crude proteases isolated during this work from both latex sera whilst unaffected by mercaptoethanol were, however, activated by *p*-CMB, iodoacetic acid (IAA) and Hg^{2+} in 10^3-fold molar excesses. The extents of activation of hevains *a* and *b* varied from one preparation to another and the phenomenon was not observed with the purified proteases. Apparently an inhibitor was co-purified with the enzyme until the final steps of producing homogeneous enzymes. As large excesses of *p*-CMB, IAA and Hg^{2+} were required before activation was observed, interaction with the assumed inhibitor may not be specifically at thiol groups, and it is possible that the compound affecting the purifications was the inhibitor reported by Archer [14]. The lutoid hevain was again different in that no activation by the three reagents described was observed during its purification.

EXPERIMENTAL

Reagents and procedures used in this work have been described [1].

Hevain *a* was prepared as reported elsewhere [1]. Freeze-dried latex serum and lutoids, obtained by ultracentrifugation of fresh

Table 3. Michaelis–Menten parameters ($K_m \times 10^4$ M; $V_{max} \times 10^7$ M sec^{-1}) for esterolysis of substituted phenyl mesyl glycinates by hevains *a* and *b*

R	*p*-Cl	*m*-Cl	*m*-NO_2
$K_m(b)/K_m(a)$	16.9/3.8 = 4.4	9.06/2.32 = 3.9	10.5/1.76 = 6.0
$V_{max}(b)/V_{max}(a)$	6.5/1.79 = 3.6	9.69/2.39 = 4.1	9.01/1.78 = 5.1

Table 4. Inhibition of hevains *a*, *b* and *l* with serine- and histidine-specific reagents

Inhibitor	Molar excess over enzyme	% Inhibition	Hevain
DFP	2	100	*a*
	20	80	*b*
	400	86	*l*
PMSF	5×10^4	77	*a*
DEP	10^3	100	*a*
	4×10^3	77	*b*
	4×10^4	86	*l*

latex which was collected in cold flasks, were the sources of hevains *b* and *l*, respectively. These two enzymes were prepared by the same method as used for hevain *a* [1]. Peptide mapping was done as reported [5]. Michaelis–Menten measurements were made in 100 mM Tris–HCl buffer, pH 7.0, at 21° using substrates and methods described elsewhere [6].

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