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PROTEASES OF EUPHORBIACEAE

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Key Word Index—*Euphorbia* species; Euphorbiaceae; latices; proteases; properties.

Abstract—From lactices of eight species of the family Euphorbiaceae a total of 17 proteases have been isolated. The major characterstics of these enzymes are collected here for comparisons which demonstrate that the proteases are all serine-centred and carry essential histidine residues. They have, however, different physical properties such as M_rs, amino acid compositions and charged forms, and different reactivities to ester and protein substrates, and to protease inhibitors.

INTRODUCTION

The presence of a proteolytic enzyme, euphorbain, in the latex of *Euphorbia* species was first reported by Ellis and Lennox [1] working with *E. lathyris*. Following the description of euphorbain c (from *E. cerifera* latex [2]) the product from *E. lathyris* was named euphorbain l [3]. The latter enzyme was described as not resembling papain [3] and so not a cysteinyl centred protease, while euphorbain c was reported to be of that type, being activated by reducing agents [2, 4].

We have recently isolated, in homogeneous forms, 13 proteases from latices of six *Euphorbia*. These are: euphorbains l (from *E. lathyris* [5]), p (from, *E. pulcherrima* [6]), y_{1-3} (from *E. cyparissias* [7]), t_{1-4} (from *E. tirucalli* [8]), la_{1-3} (from *E. lactea* [9]) and lc (from *E. lactea cristata* [9]). From latex of *Elaeophorbia drupifera* of the same family, two more proteases were purified: euphorbains $d_{1,2}$ [10] and from *Hevea brasiliensis*, also of the Euphorbiaceae, latex sera yielded hevain b while from the lutoid fraction of the latex was isolated hevain l [11]. These enzymes are all of the class having an essential serine in the active site, and dependence on the presence of a histidine residue, i.e. a group of plant proteases not extensively examined [12].

Because of that we have summarized here the data collected in refs [5–11] and describe results from kinetic measurements with the enzymes, using synthetic esterase substrates. The results obtained show each enzyme family (e.g. euphorbain t_{1-4}) to have unique esterolytic ability. In addition, data are reported from digestion experiments which help define the sites of proteolysis of the euphorbains and hevains discussed. These experiments also demonstrate that the enzymes do not have high specificities, and that each acts uniquely in protein digestion. To extend the comparisons of the proteases which were made with the data from the amino acid compositions obtained during this work, we have also subjected a number of the euphorbains and the hevains to tryptic digestion and prepared 2D maps of the resulting peptides.

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RESULTS AND DISCUSSION

The M_rs of the euphorbains and hevains isolated range from 33 000 to 117 000 with the majority having weights between 60 000 and 80 000 (Table 1). Both unique and multiple charged species are found: in the former category are the euphorbains l, p, y_{1-3}, la_1 and la_3, while the others listed in Table 1 occur in three to five charged forms. The pH optima observed with the proteases changed with the substrates employed (euphorbains y_{1-3}, t_{1-3} in Table 1) and ranged from 5.6 to 7.5 with azocasein, 6–8.3 with azocollagen and 6.3–8.0 with carbobenzoxy glycine *p*-nitrophenyl ester. Of the enzymes examined for amino sugars, hevain b alone was shown not to be a glycoprotein, the others all carrying glucosamine [5, 7–11]. This observation was confirmed in a separate study of the crude latices of the plants which were sources of the proteases discussed here [13]. Using 2D electrophoresis, and staining for both proteins and glycoproteins, it was found that the majority of the proteins in the lattices were glycoproteins.

A selection of the enzymes discussed here was examined for heat stability in 10 mM aqueous solutions with carbobenzoxy glycine *p*-nitrophenyl ester as substrate. Euphorbains $t_{1,2}$, $la_{1,2,3}$ and l, as well as hevain b, were all stable for 60 min at 60°, but rapidly lost activity at higher temperatures, being inactivated in 10 min at 90°.

For facility of comparison, percent weight compositions of 15 euphorbains and of hevains b and l are collected in Table 2, the original data being available elsewhere [5–11]. It is apparent that the euphorbains $y_{1,2,3}$ are similar, as are those from *E. tirucalli* (t_{1-4}). Relationships between the proteases obtained from the various species cannot be clearly discerned in this data, and application of such mathematical tests as those reported by Cornish-Bowden [14] for comparisons of the euphorbains and hevains is precluded by the different M_rs of the proteases (Table 2). As has been observed [5], the amino acid composition of euphorbain 1 is similar to that of cocoonase, another serine-centred protease, but of insect origin.

To examine further the relationship between the euphorbains and hevains, tryptic digestions of several of

Table 1. Summary of some properties of euphorbains and hevains

Enzymes		$M_r \times 10^{-3}$	pI	pH opt.	Glycoprotein
Euphorbains	l	43	4.9	7–7.5†	yes
	p	74	4.7	7.0†	yes
	y_1	67	5.2	6.5–7.0† 5.2(b)	yes
	y_2	33	5.2	6.5–7.0† 5.5‡	yes
	y_3	67	6.3	inact.† 7.0‡	yes
	t_1	74	5.0–5.5(4)*	7.5‡ 7.5§	yes
	t_2	74	4.7–5.2(4)	7–7.5‡ 7.5§	—
	t_3	74	—	7.5–8.0§	—
	t_4	74	4.0–5.0(4)	6–6.5‡	—
	d_1	117	5.8–7.5(5)	6.3 and 7.8‡	—
	d_2	65	5.2–9.1(5)	6.5 and 7.8‡	yes
	la_1	66	7.0	7.5(b)	—
	la_2	44	5–6.4(3)	—	—
	la_3	33	4.5	—	—
	lc	70	5.0–8.0(5)	8.3‡	yes
Hevains	b	58	4.8–5.3(4)	6.3§	no
	l	80	4.9–6.2(3)	6.3 and 7.7‡	—

*Number of isozymes in parenthesis; —, no measurement.
†Assayed with azocasein.
‡With azocollagen
§With carbobenzoxy glycine *p*-nitophenylester.

Table 2. Percent weight compositions of euphorbains and hevains from published data [5–11]

	l	p	y_1	y_2	y_3	t_1	t_2	t_3	t_4	d_1	d_2	l_{a-1}	l_{a-2}	l_{a-3}	lc	hev b	hev l
Cys	1.6	1.3	1.4	1.6	1.5	2.5	—	—	—	—	2.1	—	—	—	2.9	1.8	—
Asx	11.7	11.7	16.6	14.2	16.3	14.9	15.1	15.5	15.0	14.5	13.7	13.3	14.9	15.4	14.0	12.0	15.4
Thr	7.8	6.8	5.2	4.4	4.7	5.6	5.7	5.6	5.9	6.7	6.5	8.6	7.4	6.6	6.6	6.5	7.7
Ser	10.0	8.6	9.7	11.7	10.1	7.9	7.9	7.6	7.7	8.6	7.5	7.6	6.4	6.3	5.9	4.8	5.8
Glx	6.5	8.2	8.8	8.4	8.4	6.3	7.8	7.7	7.8	7.8	5.9	7.4	8.5	8.1	6.6	17.7	11.7
Pro	4.2	4.8	4.6	4.5	3.9	5.5	5.6	5.5	5.9	5.6	5.5	6.3	5.7	6.7	5.4	5.3	6.2
Gly	6.5	6.1	5.8	6.5	5.7	6.2	6.8	6.4	6.5	5.7	5.0	4.5	4.1	4.8	4.5	3.7	4.6
Ala	6.3	5.4	4.8	5.0	5.2	6.5	7.1	6.6	6.8	5.5	5.0	4.8	3.8	5.3	4.3	5.0	3.3
Val	7.2	4.9	6.0	5.5	5.9	7.1	7.0	6.7	6.8	5.9	6.6	5.1	4.9	5.3	5.9	3.5	4.9
Met	0	0.2	0	0	0	1.2	0.7	1.3	1.1	0.8	0.7	1.0	0.6	0.8	0.2	0.3	1.2
Ile	7.5	5.9	4.7	5.6	4.9	6.0	6.6	6.6	6.9	6.3	6.7	5.1	4.5	4.2	6.0	5.3	5.9
Leu	6.4	7.8	5.9	5.2	5.9	7.3	7.2	7.1	7.2	9.2	8.5	8.7	10.1	9.5	7.6	9.1	8.9
Tyr	3.7	4.3	2.4	3.5	3.4	6.4	5.4	6.6	6.8	5.9	5.8	10.6	9.6	12.2	6.2	3.5	7.3
Phe	5.0	5.9	5.2	5.0	5.2	4.0	4.7	4.7	4.9	5.9	5.8	5.9	7.3	7.3	4.9	4.3	5.6
His	2.5	2.6	3.2	4.7	2.6	2.0	2.7	2.7	2.3	2.2	2.1	2.1	2.3	0.9	2.2	1.6	1.2
Lys	5.0	5.5	6.7	7.1	7.6	4.8	5.7	5.5	5.0	3.4	2.6	3.4	4.8	3.6	4.3	8.3	5.2
Arg	3.9	4.3	5.3	3.9	5.3	4.0	4.1	4.1	3.7	6.1	5.3	5.5	5.1	2.9	5.2	4.6	5.0
Trp	3.0	0.5	2.4	1.7	1.9	1.0	—	—	—	—	1.0	—	—	—	3.3	0.7	—
Glc-N	1.1	5.3	1.0	1.5	1.4	0.7	—	—	—	—	3.8	—	—	—	4.0	0	—

these proteases were mapped on 2D-TLC (cellulose). Typical results are shown in Fig. 1 which contains maps of euphorbains y_1 and y_2. It is evident that the enzymes are of similar but not identical sequence, as is suggested by the differences in their (similar) amino acid compositions (Table 2). There is, also, a general resemblance between the map of euphorbain l and those of Fig. 1. Digests of the euphorbains $t_{2,3,4}$ were again similar when mapped, though earlier results [8] suggested some differences, possibly because of incomplete tryptic digestion. They, however, differ from the other maps described here (Fig. 1) as did those of euphorbains p, and la_1, and those of hevains b and l. Thus, in conformity with the amino acid compositions of Table 2, the euphorbains from varying

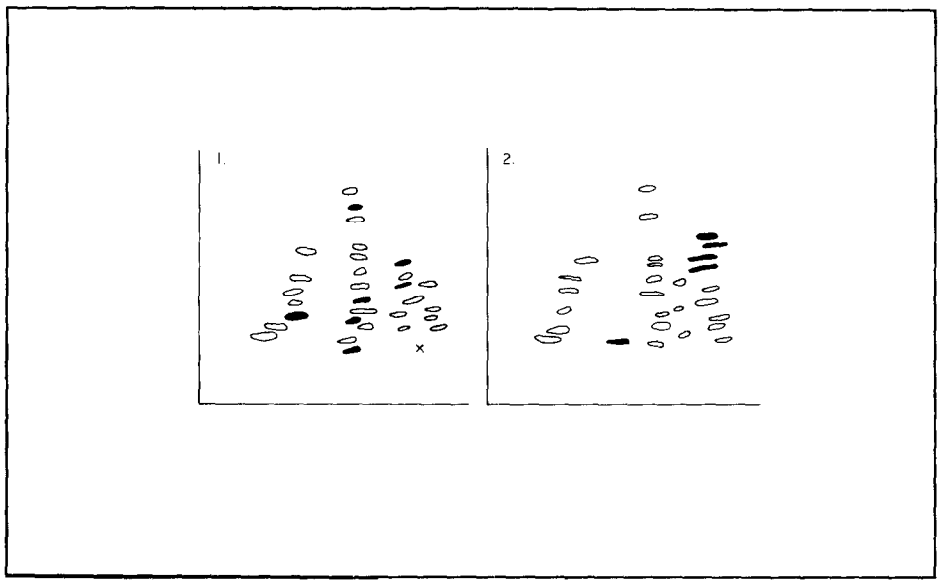

Fig. 1. Two dimensional maps of tryptic digests of euphorbains y_1 (1) and y_2 (2): horizontal separation by electrophoresis, vertical by chromatography. Peptides not common to the digests are shaded.

sources, and the hevains which were mapped displayed no clear relationships of structure.

To compare the esterase activities of the euphorbains and hevains, a series of substituted aromatic esters of *N*-mesylglycine were employed: $MeSO_2NHCH_2CO.O.C_6H_4X$ where X was *p*-methyl, *p*-fluoro, *p*-chloro or *m*-nitro. Michaelis–Menten parameters were determined with each of these substrates, as were the pseudo-first order rate constants, k_ψ at sub-saturating concentrations of substrate. These data were then compared using the Hammet relationship [15]. To this end, plots were prepared, for each enzyme, of V_{max}, K_m and k_ψ *versus* the Hammet σ function, and from the slopes of the straight lines so obtained, ρ values estimated [16]. As $k_\psi = V_{max}/K_m$, $\rho k_\psi = \rho V_{max} - \rho K_m$, so providing a check of the results obtained [16]. These are summarized in Table 3. It is evident that each family of euphorbains and hevains behaves uniquely with the ester substrates employed. Further, the proteases from a single source, e.g. the three from *Euphorbia tirucalli* (euphorbains t_{1-3}) gave the same ρ values. It may be noted that the families of enzymes such as the euphorbains t_1 and t_2 digest insulin B chain in somewhat different ways, as will be discussed below. A wide range of reactivities is evident in Table 3: while euphorbain p and hevain 1 are markedly affected by changes in the X group of the substrate, euphorbains d_1 and d_2 are completely unaffected, and a variety of intermediate responses is apparent.

Digests of oxidized insulin B chain with a number of euphorbains and with hevain b were separated into component peptides by HPLC and those peptides analysed by amino acid analysis. It was thus possible to determine the sites of cleavage affected by the several proteases. A typical set of data, from digestion with euphorbain y_1 is shown in Table 4. A summary, from 10 euphorbains and hevain b, is collected in Table 5. Comparision of those two Tables shows that the latter is composed of data for only the major cleavage points in the digestions. It is evident that there is no consistent exopeptidase activity in the proteases considered, and that those enzymes reacted with no specificity as endopeptidases. While -ser-his-, -val-glu-, -tyr-leu- and -cys(SO_3H)-gly- are favoured sites for scission, none of them is attacked by all of the proteases examined. Again we must conclude that the euphorbains and hevains are each unique in proteolytic reactivity, a conclusion supported by peptide maps made, and reported elsewhere (e.g. [11]).

Table 3. ρ values from Michaelis–Menten parameters and initial rates at sub-saturating conditions for euphorbains and hevains reacting with substituted aromatic esters of mesyl glycine

Enzyme		$\rho V_m - \rho K_m = \rho K_4$	ρk_ψ
Euphorbains:	1	0.66–0.70 = −0.04	0
	ρ	−3.0–(−3.1) = 0.1	0.07
	y_{1-3}	0.37–0 = 0.37	0.37
	t_{1-3}	0.15–(−0.04) = 0.11	0.1
	$d_{1,2}$	0–0 = 0	0
	1c	−0.14–(−0.50) = 0.36	0.24
Hevains	1	−2.5–(−2.6) = 0.1	0.4
	b	0–(−0.8) = 0.8	0.8

Data from studies with various inhibitors of proteases are summarized in Table 6. For that purpose we have indicated only whether the reagents employed were inhibitory (*i*) or not (*n*) and whether they were partly inhibitory (*pi*) or partly activating (*pa*). Details of the work are available elsewhere [5–11].

All the enzymes tested were inhibited by diisopropylfluorophosphate (DEP) or phenyl methyl sulphonyl fluroide (PMSF) which define them as possessing essential serine residues at the active site [17, 18]. Other inhibitors of such serine-centred enzymes as trypsin, namely anti-trypsin, potato I, elastatinal and chymostatin

Table 4. Cleavage points in the oxidized insulin B chain

Time (Hr)	Phe 1	Val 2	Asn 3	Glu 4	His 5	Leu 6	Cys 7	Gly 8	Ser 9	His 10	Leu 11	Val 12	Glu 13	Ala 14	Leu 15
0.5							○				○				
3							◐				○	◐			
48							○	○			○	○			◐
75							○	○			○	◐		○	○	○

Relative frequencies of scission are indicated by ○, one; ◐, two and ●, three or more cleavages.

Table 5. Major sites of reaction: points of scission occurring in short digestions and of continuing importance during longer periods

Enzyme	Bonds split																			
	3/4	4/5	5/6	6/7	7/8	9/10	10/11	11/12	12/13	14/15	15/16	16/17	18/19	19/20	25/26	26/27	29/30
1				×		×								×			
y_1				×			×	×				×		×			
y_2		×		×		×		×	×			×		×			
p		×			×		×										
d									×								
t_1	×					×	×			×	×			×	×		×
t_2	×						×	×		×		×		×		×	
la_1						×			×			×	×				
la_2				×		×			×			×					
la									×		×		×				
hevain b				×		×				×		×					

Table 6. Summary of the effects of various

Enzymes	DFP*	PMSF†	Antitrypsin	Potato I	Elastatinal
Euphorbains					
l	i	n	—	—	—
p	i	i	—	—	—
y_1	i	—	i	i	i
y_2	—	—	i	i	i
y_3	—	—	i	i	i
t_1	—	i	n	n	n
t_2	—	i	n	n	n
t_3	—	—	n	n	n
t_4	—	i	n	n	—
d_1	—	i	—	n	—
d_2	—	i	—	n	—
la_1	i	—	pi	pi	n
la_2	i	—	—	—	—
la_3	—	—	—	—	—
lc	i	pi	—	n	pi
Hevains					
b	i	—	—	—	—
l	i	—		—	

*Di-isopropyl fluorophosphate.
†Phenyl methyl sulphonyl fluride.
‡Diethyl pyrocarbonate.
§*p*-Bromophenycyl bromide.
‖*p*-Chloromercuribenzoate.
¶Iodoacetic acid.
*Re-examination of euphorbain l shows it is inhibited by DFPC: the

after digestions for various times with euphorbain y_1

Tyr 16	Leu 17	Val 18	Cyx 19	Cly 20	Glu 21	Arg 22	Gly 23	Phe 24	Phe 25	Tyr 26	Thr 27	Pro 28	Lys 29	Ala 30
○			○											
○			◐											
○			●	○								○		
○			◐	○	○									

were all inhibitory for the three proteases of *E. cyparissias* (euphorbains y_{1-3}) and some partial inhibitions were observed with other enzymes, chymostatin having the most extensive range. However other conventional reagents such as bovine pancreatic, ovomucoid, soybean and lima bean trypsin inhibitors, and hirudin did not act with any of the proteases discussed here.

Two histidine specific reagents, diethyl pyrocarbonate (DEPC [19] and *p*-bromophenacyl bromide (*p*-BPB [20]) were inhibitory for all of the proteases examined. Thus, as in trypsin and related enzymes, the presence of a histidine as well as a serine residue is apparently essential.

No protease tested in this work was activated by mercaptoethanol, suggesting that they are not cysteine-centred enzymes resembling papain, bromelain and ficin which are also derived from plant latices. Similarly, *p*-chloromercuribenzoate, a reagent specific for free sulphydryl groups, was without effect on any of the proteases listed in Table 6 except euphorbains $d_{1,2}$ which were partly activated, as discussed elsewhere [10]. Those two enzymes were also partly inhibited by iodoacetic acid, another reagent with specificity for sulphydryl groups, which was without effect on any of the other euphorbains of Table 6.

Of the metals tested (Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Hg^{2+} and Zn^{2+}) only the latter two had effects on the euphorbains and hevains, and those were widely variable (Table 6). Antipain, a bacterially derived short-chain substituted peptidal inhibitor of papain [21] reacted with euphorbains y_{1-3} while leupeptin, an inhibitor of some mammalian cysteinyl proteases [22], had no effect on the enzymes discussed here.

No evidence was found in this work to suggest that the euphorbains are metallo-proteins: neither EDTA nor *O*-phenanthroline affected the reactivities of the enzymes. Diazo-DL-norleucine methyl ester, a carboxyl-specific reagent [23] similarly was without effect on the proteases, which, then, do not have aspartic or glutamic acid residues in vital positions. However, pepstatin, which is an inhibitor of pepsin [24], had some effect on euphorbains t_{2-3}.

inhibitors and metal ions on euphorbins and hevains

Chymostatin	DEPC†	*p*BPB§	*p*CMB‖	IAA¶	Hg^{2+}	Zn^{2+}	Antipain
—	i*	—	—	—	n	—	—
—	—	—	—	—	—	—	—
i	i	i	n	pi	i	i	i
i	—	—	n	—	i	i	—
i	—	—	n	—	i	i	—
pi	i	pi	n	n	pi	n	n
pi	i	—	n	n	pi	n	n
pi	i	—	n	—	—	—	n
—	i	pi	—	n	pi	n	n
pi	i	—	pa	—	pa	n	n
pi	i	—	pa	—	pa	n	n
n	i	—	n	—	i	i	n
—	i	—	n	—	i	i	—
—	—	—	—	—	—	—	n
pi	i	—	n	—	i	i	n
n	i	—	n	n	n	—	—
pi	i	—	n	n	n	—	—

original failure to observe this [5] must be ascribed to use of aged reagent.

EXPERIMENTAL

The sources of latices, the reagents and the methods for purifying and characterizing the enzymes have been reported [5–11] except as described below.

Kinetic measurements were made spectrophotometrically, at 21°, and Michaelis–Menten parameters estimated conventionally. Aryl esters of mesyl glycine were prepared, where the substituents were *p*-Me, *p*-Cl, *p*-F and *m*-NO_2. Mps and elemental analyses for these compounds were as reported [15]. Mesyl glycine [25] was converted to the acid chloride with PCl_5 and reacted with the appropriate phenols in CH_2Cl_2 containing equimolar amounts of C_5H_5N with 10 mmol mesyl glycine chloride. After refluxing for 2 hr the reaction mixture was extracted with 100 mM HCl, 2% $NaHCO_3$, H_2O in that order, the extract dried, and the product crystallized.

Protease digestions: Oxidized insulin B chain (Sigma Chem. Co.) was purified by HPLC on a Bio-Rad RP304 column (250 × 4.6 mm) using a gradient of MeCN–H_2O (0.1% in trifluoroacetic acid). Digestions, made with 1:100 ratios of enzyme to substrate, were in NH_4HCO_3 (100 mM) at 21°. Aliquots were subjected to HPLC in the system described above and peaks collected. These were hydrolysed, under vacuum, in 6 M HCl for 22 h at 110°, and the mixtures dried and subjected to amino acid analyses on an automatic analyser. From the results and the known sequence of the substrate, scission points were determined. Tryptic digests of the proteases were prepared and mapped as described elsewhere [26].

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