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ISOLATION AND CHARACTERIZATION OF PROTEASES FROM *EUPHORBIA LACTEA* AND *EUPHORBIA LACTEA CRISTATA*

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Key Word Index—*Euphorbia lactea*; *cristata*; Euphorbiaceae; serine protease; latex; amino acid composition.

Abstract—Latex from *E. lactea* yielded three homogeneous proteases, euphorbains, $1a_1$, $1a_2$ and $1a_3$ with M_r of 66 k, 44 k and 33 k respectively. Euphorbains $1a_1$ and $1a_3$ had unique pIs of, in order, 7.0 and 4.5, while $1a_2$ comprised three charged forms with pIs ranging from 5.0 to 6.4. From the latex of *E. lactea cristata* a single proteolytic euphorbain 1c was isolated which had an M_r of 70 000 and five pIs between 5.0 and 8.0. Euphorbains $1a_1$ and 1c have similar substrate specificities which are different from those of $1a_2$ and $1a_3$. Euphorbains $1a_1$, $1a_2$ and 1c are serine-centred enzymes with vital histidine residues, and the latter protease is activated by Ca^{2+}, Mg^{2+} and Mn^{2+}.

INTRODUCTION

We have previously reported the isolation and characterization of proteases from the latex of several species of the family Euphorbiaceae. Unique proteases were separated from the latices of *Euphorbia lathyris* [1], *E. pulcherrima* [2], and *Hevea brasiliensis* [3] which were named respectively, euphorbains 1 and p, and hevain. Other members of the family, namely *E. cyparissias* [4], *E. tirucalli* [5], and *Elaeophorbia drupifera* [6] each yielded several proteases, respectively euphorbains y_{1-3}, t_{1-4} and d_{1-2} on purification of their latices. All of these enzymes were inhibited by di-isopropylfluorophosphate, and so were considered to be members of the serine-centered group of enzymes, which includes trypsin.

As an extension of those studies we have lately examined latex from two other succulent euphorbs, *Euphorbia lactea* ('candelabra plant'), and *E. lactea cristata* ('brain cactus'), a crested 'monstrosa' variety which bears little physical resemblance to *E. lactea*. Three proteases were isolated from *E. lactea* latex, named euphorbain $1a_{1-3}$, and a single protease from *E. lactea cristata* latex, euphorbain 1c. The purification procedure and some characteristics of these enzymes are reported below.

RESULTS AND DISCUSSION

The three isoenzymes isolated from *E. lactea* (Fig. 1) were recycled on a TSK G3000SW column, each eluting in a single symmetrical peak. The column was calibrated for M_r determination with lysozyme, myoglobin, ovalbumin, bovine serum albumin and human immunoglobulin G. The M_rs of euphorbains $1a_{1-3}$ so obtained were 66 k, 44 k and 33 k respectively. Confirmation of the homogeneity of euphorbains $1a_1$ and $1a_3$ is evident in the finding of single charged forms for each of them, at pIs of 7.0 and 4.5 respectively, using isoelectric focusing. The $1a_2$ enzyme has pIs of 5.0, 5.4 and 6.4, while the single proteolytic enzyme isolated from *E. lactea cristata*, euphorbain 1c, has isoelectric points of pH 5.0, 5.3, 6.8, 7.6 and 8.0. The latter two enzymes thus resemble other proteases isolated from other members of the Euphorbiaceae; the euphorbain y family [5] and also those from *Elaeophorbia drupifera* [6]. Multiple charged forms of proteases of plant origin have been reported for enzymes from latices of *Asclepias syriaca* [7, 8], *Ficus glabrata* [9], *Ficus carica* [10] and *Ananas comosus* [11]. The origins of these multiple charged forms is not yet known. There is no evidence that the majority of them arise from post-translational modifications [8, 10] but attributions to unique genes have not been made.

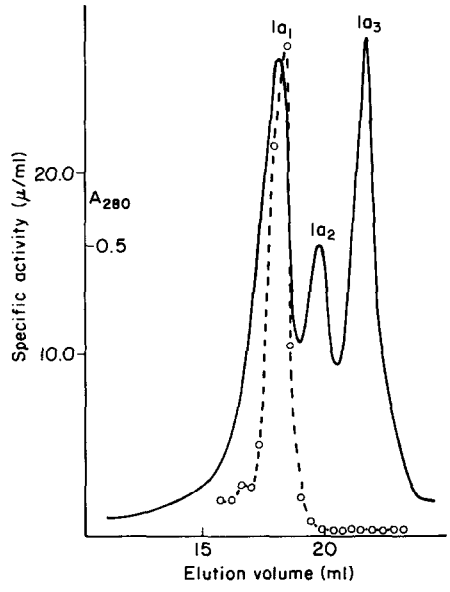

Fig. 1. Separation of euphorbains $1a_1$, $1a_2$ and $1a_3$ on HPLC: conditions as described in text. A_{280} – – –; specific activity (units CBZ-gly-*p*-NP/ml) O——O.

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On SDS gel electrophoresis euphorbain $1a_1$ gave two protein bands of M_rs 66 k and 46 k and $1a_2$ five bands of weights 44 k, 34 k, 29 k, 19 k and 14 k. Euphorbain $1a_3$, with the same technique, gave a predominant band at M_r 34 k, corresponding to the applied material, with minor bands at 32 k, 26 k, 24 k and 18 k. A very faint component of this gel with apparent M_r of 49 k was also observed. As this protein does not correspond to either $1a_1$ or $1a_2$ in weight, and $1a_3$ was recycled on the HPLC column to homogeneity, the high M_r band described is probably not an impurity, but may represent an aggregate of strongly charged peptides resistant to the effects of the detergent.

The protease isolated from *E. lactea cristata*, euphorbain 1c, was homogeneous in gel exclusion HPLC, and by chromatography on a Bio-Gel P150 column calibrated following the method of Andrews [12]. The M_r so obtained was 70 k. SDS polyacrylamide gel electrophoresis confirmed that M_r: three bands of M_rs 68 k, 41 k and 32 k were found.

Amino acid compositions for the four euphorbains discussed here are presented in Table 1 which also contains percent weights of the enzymes to facilitate comparison. Visual inspection suggests that the proteases are related, and this was confirmed using the method of Cornish-Bowden [13]. When the residue composition of $1a_3$ was doubled to enable calculations to be made, $1a_1$ and $1a_3$ were both shown to be related to 1c by the rigorous test described: the methodology does not permit inclusion of $1a_2$ in these calculations [13].

Euphorbain $1a_1$ gave, with azocollagen, a typical 'bell-shaped' pH profile with maximum proteolytic activity at pH 7.5. Euphorbain 1c has an optimum at about pH 8.3. Lack of material prevented comparable measurements with the $1a_2$ and $1a_3$ proteases. A summary of substrate specificities is presented in Table 2. The similarities of euphorbains $1a_1$ and 1c are then apparent, as are the notable characteristics of the proteases $1a_2$ and $1a_3$.

The effects of protease inhibitors on euphorbain $1a_3$ could not be examined because of a lack of purified enzyme. Like other members of this family [1–6] euphorbains $1a_1$, $1a_2$ and 1c were all inhibited by diisopropylfluorophosphate (DFP [14]) as is seen in Table 3. In addition, inhibition with phenylmethanesulphonyl fluoride (PMSF) another serine-specific reagent [15] occurred (Table 3). That table also contains results of reactions with the histidine-specific reagent diethylpyrocarbonate (DEPC [16]) which inhibits some euphorbains [4–6] but not others [1–3]. Clearly the proteases from both *E. lactea* and *E. lactea cristata* are members of the group having essential serine/histidine residues.

Cysteinyl-blocking reagents *p*-chloromercuribenzoate, leupeptin [17] and antipain [18] did not affect the activity of euphorbains $1a_1$, $1a_2$ and 1c (compare refs [1, 2, 4]). Similarly, the inhibitors of carboxyl-centred enzymes pepstatin [19] and diazo-DL-norleucine methyl ester [20] were without effect on those enzymes.

While the activities of euphorbains $1a_1$, $1a_2$ and 1c were unchanged in the presence of the chelating reagents EDTA and *o*-phenanthroline, suggesting that these enzymes are not metallo-proteins, the latter protease was activated by $MgCl_2$ (by 73%), $MnCl_2$ (by 28%) and by $CaCl_2$ (by 39%); results which are unique for this family of proteases [1–6].

The data presented here show that the proteases from both *E. lactea* and *E. lactea cristata* are distinct from those isolated from other members of the genus *Euphorbia*. The euphorbains from these two plants are related to each other in amino acid composition, where comparison can be made, even though they display different physical and biochemical properties.

EXPERIMENTAL

Materials. Euphorbia lactea and *E. lactea cristata* plants were purchased from commercial sources in Ottawa. Latex was

Table 1. The amino acid composition of euphorbains $1a_{1-3}$ and 1c

	Residues/mole (% weight composition)							
Residue	$1a_1$		$1a_2$		$1a_3$		1c	
Cys	—	—	—	—	—	—	19	(2.9)
Asx	75	(13.3)	55	(14.9)	43	(15.4)	83	(14.0)
Thr	55	(8.6)	31	(7.4)	21	(6.6)	45	(6.6)
Ser	56	(7.6)	31	(6.4)	23	(6.3)	46	(5.9)
Glx	37	(7.4)	28	(8.5)	20	(8.0)	35	(6.6)
Pro	42	(6.3)	25	(5.7)	22	(6.7)	38	(5.4)
Gly	51	(4.5)	31	(4.2)	27	(4.8)	54	(4.5)
Ala	44	(4.8)	25	(3.8)	24	(5.3)	41	(4.2)
Val	34	(5.2)	21	(4.9)	17	(5.2)	41	(5.9)
Met	5	(1.0)	2	(0.6)	2	(0.8)	1	(0.2)
Ile	29	(5.1)	17	(4.5)	12	(4.2)	36	(6.0)
Leu	50	(8.7)	38	(10.1)	27	(9.5)	46	(7.6)
Tyr	42	(10.6)	25	(9.6)	24	(12.2)	26	(6.2)
Phe	26	(5.9)	21	(7.2)	16	(7.3)	23	(4.9)
His	10	(2.1)	7	(2.2)	2	(0.8)	11	(2.2)
Lys	17	(3.4)	16	(4.8)	9	(3.6)	23	(4.3)
Arg	23	(5.5)	14	(5.1)	6	(2.9)	23	(5.2)
Trp	—	—	—	—	—	—	12	(3.3)
Glu–NH_2	—	—	—	—	—	—	17	(4.0)
M_r	64 736		42 530		32 025		68 368	

Table 2. Substrate specificities of euphorbains $1a_{1-3}$ and 1c

Substrate	Specificity activity (u/mg enzyme) at pH 7			
	$1a_1$	$1a_2$	$1a_3$	1c
CGN	12.8	0.31	0	21.5
Azocasein	0.044	0.015	0.05	0.065
Azocollagen	0.142	0.127	0.057	0.23
Hemoglobin	0.31	0.32	3.02	—

Table 3. Inhibition of euphorbains $1a_1$, $1a_2$ and 1c with reagents specific for serine and histidine residues

Inhibition	Molar excess over enzyme	% Inhibition	Euphorbain
DFP	$10^2\times$	96	$1a_1$
	$10^2\times$	57	$1a_2$
	$10^2\times$	96	1c
PMSF	$10^3\times$	40	1c
DEPC	$10^4\times$	64	$1a_1$
		73	$1a_2$
		80	1c

collected from incisions and centrifuged at 20 000 *g* for 1 hr. The aq. layer was separated and stored at −20° as the crude enzyme source.

Assays. Esterolytic assays with CBZ-gly-*p*-Np were performed as previously reported [1]. Proteolysis with azocollagen and with azocasein was measured as described [1, 2]. Activity to hemoglobin was assayed by mixing enzyme with 1 ml of 2% hemoglobin in 100 mM Tris–HCl pH 7. The soln was incubated for 45 min at 37° and then 1 ml 5% TCA added. The mixture was centrifuged, and A_{280} nm of the supernatant measured. One unit of activity produced a change of 1 AU/min.

Protein assay. Protein concn was expressed as A_{280} or determined using the BioRad protein reagent.

Polyacrylamide gel electrophoresis. SDS gels were prepared following ref. [21]. Isoelectric focusing using Biolyte ampholytes employed the method of ref. [22]: the gels were fixed in 11.5% sulphosalicylic acid for 1 hr and then stained in 0.09% Coomassie blue in 8% HOAc–50% MeOH.

M_r *determination by HPLC.* A column (600 × 7.5 mm) of TSK Spherogel G3000SW (Altex Scientific Co.), equipped with a 10 cm precolumn, was used with a Beckman Model 110A solvent metering pump and a Varian Model 2050 variable wavelength detector. The buffer used was 200 mM KPi pH 7, with a flow rate of 0.5 ml/min.

Amino acid analyses. Hydrolysates were prepared either in 6 M HCl or, for tryptophan determination, 4 M methanesulphonic acid [23] under vacuum at 110° for 22 hr. Cysteine content was measured after oxidation and hydrolysis [24]. Amino sugars were determined following hydrolysis under vacuum in 4 M HCl for 6 hr. An automatic amino acid analyser was used throughout this work.

Purification of enzymes. All purification procedures were performed at 4° using a 10 mM NaOAc buffer pH 6, except that the Affigel-10-β-D-lactoside chromatography required NaPi buffered saline and the HPLC fractionation was made at 21° with 200 mM KPi pH 7.

Euphorbain 1*a.* Clear *E. lactea* latex serum was applied to a Sephadex G25 column (2.5 × 22.5 cm). The 'break-through' peak of unretained material, which contained all of the proteolytic activity, was fractionated on a DEAE Sepharose CL-6B column (2 × 30 cm) with a linear gradient of 0–1 M NaCl in the acetate buffer. A single active peak, eluting at *ca* 390 mM NaCl, was collected, dialysed against H_2O, lyophilized and redissolved in a small vol. of 200 mM KPi pH 7 for application to the HPLC-TSK gel exclusion column. Three symmetrical peaks were then resolved and denoted euphorbains $1a_1$, $1a_2$ and $1a_3$ in their order of elution (Fig. 1). The first of them contained about 99% of the CBZ-gly-*p*Np hydrolysing activity, and had a specific activity of 12 800 u/g as compared with 190 u/g in the crude latex serum. Euphorbain $1a_2$ contained 1% of this activity with a specific activity of about 310 u/g.

Euphorbain 1c. The clear latex serum of *E. lactea cristata* was applied to a column of Affigel-10-β-D-lactoside (1.5 × 15 cm) equilibrated in 10 mM NaPi containing 150 mM NaCl (pH 7.3), as a preliminary step in the isolation of the lectins from this material [25]. The non-binding protein fraction was concentrated on a PM-10 Diaflo membrane and fractionated on a Sephadex G25 column (2 × 32 cm). The unretained active peak was then applied to DEAE Sepharose CL-6B (1.5 × 40 cm), which was washed with a linear gradient of 0–500 mM NaCl in the 10 mM pH 6.0 NaOAc buffer. Euphorbain 1c was eluted at about 120 mM NaCl, concd on a Diaflo membrane, and applied to a column of Bio-Gel P150 which was calibrated for M_r determination [12]. The post P150 fraction was then concentrated on a Diaflo membrane and subjected to gel exclusion HPLC, whereupon the enzyme eluted as a single protein peak which was coincident with the CBZ-gly-*p*Np activity. The pure enzyme had a specific activity to CBZ-gly-*p*Np of 21 500 u/g; the activity of the crude serum was 2200 u/g.

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