Phytochemistry, Vol. 24, No. 12, pp. 2843–2845, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

TWO PROTEASES FROM THE LATEX OF *ELAEOPHORBIA DRUPIFERA**

K. R. LYNN and N. A. CLEVETTE-RADFORD

Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A OR6

(*Revised received* 7 *June* 1985)

Key Word Index—*Elaeophorbia drupifera*; Euphorbiaceae; euphorbains; serine protease.

Abstract—Two serine-centred proteolytic enzymes containing catalytically essential histidine residues have been purified to homogeneity from the latex of *Elaeophorbia drupifera*. The high (117 K) M_r form, euphorbain d_1, and the low M_r (65 K) form, euphorbain d_2, are each composed of subunits of weight 30 000. The subunits differ slightly, as is seen by tryptic mapping and in the amino acid compositions reported for the proteases. Both enzymes have five isoelectric forms, and both display two pH maxima for proteolytic activity. Large molar excesses of sulphydryl-blocking reagents produce some activation of euphorbains d_1 and d_2.

INTRODUCTION

A number of serine-centred proteases have been isolated in this laboratory from latex-sera taken from *Euphorbia lathyris* [1], *E. pulcherrima* [2], *E. cyparissias* [3] and *E. tirucalli* [4]. They have M_rs ranging from 33 K to 74 K, and although they have some general similarities in amino acid compositions, they are clearly separate entities with differing reactivities [1–4].

Related to the genus of Euphorbia, but taxonomically separate from it [5] is that of Elaeophorbia, which contains the West African succulent tree *Elaeophorbia drupifera* Stapf. As an extension of the investigation of the proteases of plant latices, we have isolated, purified to weight homogeneity and determined the properties of two proteolytically active enzymes from the latex of *E. drupifera*. In conformity with the previously adopted terminology [1] these enzymes have been given the trivial names of euphorbains d_1 and d_2.

RESULTS AND DISCUSSION

The M_rs of the euphorbains from *E. drupifera* were determined from gel exclusion HPLC on TSK-G3000SW as 117 K (d_1) and 65 K (d_2). When the two enzymes were subjected to SDS-PAGE essentially complete dissociation into unit weights of 30 K were observed for both euphorbain d_1 and d_2. Euphorbain d_1 can also be partially separated into esterolytically active units of weight about 60 K by HPLC (Fig. 1).

To examine whether the two enzymes share a common unit, as the M_r measurements discussed above suggest, thin-layer two-dimensional maps were prepared from tryptic digests of both euphorbains described here (Fig. 2). It is apparent that the two maps are very similar but not identical, and so the euphorbains d_1 and d_2 are separate entities. This was confirmed when the amino acid compositions of euphorbains d_1 and d_2 were determined. The results are collected in Table 1. The d_2 molecule

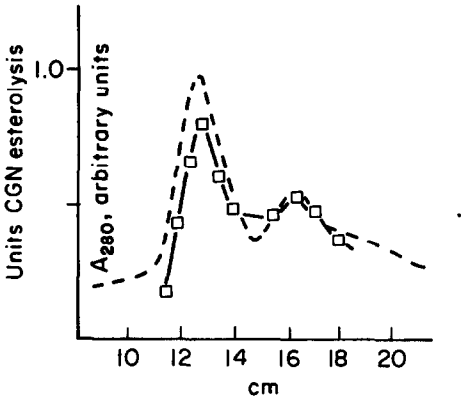

Fig. 1. Partial conversion of euphorbain d_1 (M_r 117 K), left-hand peak, to a unit of weight 61 K shown by HPLC on Spherogel TSK-G3000SW in 200 mM, KP_i, pH 7.0, 10 mM in mercaptoethanol, at 23°. Esterolysis measured with CGN.

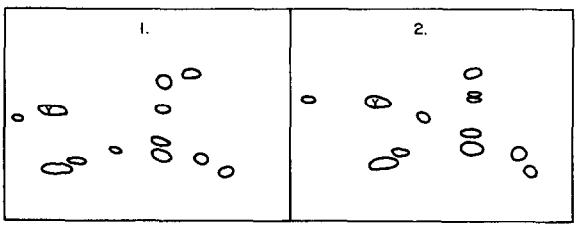

Fig. 2. Thin-layer peptide maps from tryptic digests of euphorbains 1, d_1; 2, d_2.

contains glucosamine but not galactosamine, and, by analogy with all other euphorbains isolated [1–4], d_1 probably also contains glucosamine. For comparison of the two euphorbains discussed here, their percent weight compositions are included in Table 1. It is apparent that the two proteases are of somewhat differing amino acid

*N.R.C.C. No. 24899.

Table 1. Amino acid compositions of euphorbains d_1 and d_2

Amino acid	Residues/mol		Composition (%)	
	d_1	d_2	d_1	d_2
Cys	—	12	—	2.1
Asx	147	70	14.5	13.7
Thr	77	38	6.7	6.5
Ser	114	50	8.6	7.5
Glx	71	27	7.8	5.9
Pro	67	33	5.6	5.5
Gly	117	51	5.7	5.0
Ala	90	41	5.5	5.0
Val	69	39	5.9	6.6
Met	7	3	0.8	0.7
Ile	65	35	6.3	6.7
Leu	95	44	9.2	8.5
Tyr	42	21	5.9	5.8
Phe	47	23	5.9	5.8
His	19	9	2.2	2.1
Lys	31	12	3.4	2.6
Arg	46	20	6.1	5.3
Trp	—	3	—	1.0
GlcN	—	14	—	3.8

compositions, as would be expected from the tryptic maps (Fig. 2). The data of Table 1 also facilitate comparison of euphorbains d_1 and d_2 with proteases isolated previously [1–4]. No clear compositional relationship is evident.

Euphorbains d_1 and d_2 differ in comparatively few residues (Table 1), and both have unit weights of 30 K. This suggests that the two proteases were differentiated recently in evolutionary time from a common ancestral proteolytic enzyme.

Both euphorbains from the latex of *E. drupifera* were subjected to isoelectric focussing: from euphorbain d_1 pIs of 5.8, 6.1, 6.5, 6.7 and 7.5 were observed, and from d_2, pIs of 5.2, 6.8, 7.5, 8.9 and 9.1. These results are comparable with those reported for the proteases of the succulent *E. tirucalli* [4], and differ from those of other euphorbains isolated [1–3].

Proteolysis by euphorbain d_1 of azocoll has two clearly defined pHs of maximal activity, at 6.3 and 7.8 (Fig. 3). Euphorbain d_2 is similar in behaviour, although the maximum pH 6.5 is not as well defined. This form of pH dependence is not common, but one explanation is that a change in the position of the rate-limiting step of the proteolysis occurs which is dependent on pH. Thus if, for example, the enzyme–substrate complex must undergo an ionization step before discharging the product, as described in [6], k_{+1} may be rate-determining at one pH and k_{+2} at another.

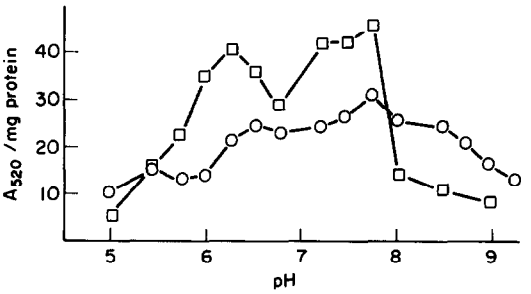

Fig. 3. pH plotted *versus* proteolytic activity to azocoll for euphorbains d_1 (□) and d_2 (○); pH 5–6.3 with 100 mM acetate; pH 6.6–9.3 with 100 mM Tris–HCl; 37°.

$$E^n \xrightarrow{K_e} E^{n-1} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E^{n-1}S \xrightarrow{k_{+2}} E^n + P \qquad (1)$$

Previous work has shown the euphorbains to be serine/histidine centred enzymes [1–4]. This is also demonstrated with the proteases d_1 and d_2 (Table 2) where the latter enzyme is more sensitive to histidine-specific inhibitors than the former. Neither euphorbain was affected by soybean, ovomucoid or potato I inhibitors (compare refs [1–4]).

Euphorbains d_1 and d_2 are not affected by EDTA or *o*-phenanthroline, and so are probably not metallo-proteins. Nor are they carboxyl-centred, being unaffected by pepstatin [9] or diazo-DL-norleucine methyl ester [10].

Unlike other euphorbains isolated [1–4] the two described here were activated by the sulphydryl binding reagent *p*-chloromercuribenzoate: a 15 × molar excess of that reagent increased the activity of euphorbain d_2 by 35%, while d_1 required a 300 × excess to achieve the same activation. With both enzymes a plateau of activation was found with increasing amounts of *p*-CMB (Fig. 4).

Stimulation of enzymatic activity by sulphydryl reagents has been reported for fructose 1,6-diphosphatase [11] and ascribed to conformational changes in the enzyme caused by blocking of cysteinyl groups [12; compare also 13]. The requirement for a large molar excess of *p*-CMB to activate the protease (Fig. 4) suggests that the activation reported here results from non-specific effects of that reagent, a conclusion supported by the 30% activation of euphorbain d_2 by a 10^3 × excess of Hg^{2+}.

Table 2. Effects of serine- and histidine-specific reagents on euphorbains d_1 and d_2

Reagent	Molar excess over enzyme	Inhibition (%)	Euphorbain
Phenyl methine sulphonyl fluoride	10×	70	d_2
	20×	60	d_1
Diethyl pyrocarbonate [7]	10×	95	d_2
	20×	95	d_1
Chymostatin [8]	100×	50	d_2
	200×	30	d_1

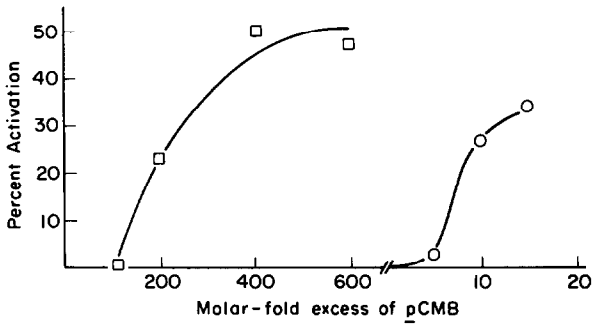

Fig. 4. Percent activation of euphorbains d_1 (□) and d_2 (○), measured in 100 mM Tris–HCl, pH 7.0 at 23°, as esterolytic activity with CGN.

This activation phenomenon is being further examined in a protease isolated from *Hevea brasiliensis* [14].

EXPERIMENTAL

Materials. Elaeophorbia drupifera cuttings, a gift from Dr. G. L. Webster, Dept. of Botany, University of California at Davis, Davis, CA, were grown in the greenhouses, Carleton University, Ottawa by Mr. H. Datema using a 50% sand/soil mixture, 18 hr daylight cycle, at 23°. Latex was collected regularly from incisions, clarified by centrifugation at 20000 *g* for 1 hr and the aq. layer separated and stored at −10° until used.

Assays. Esterolytic assays with CBZ-glycine-*p*-nitrophenyl ester (CGN), and assays with azocollagen (azocoll) were performed as previously described [1]. Protein concns were measured with BioRad protein reagent or as A_{280}.

SDS-PAGE. Gels were prepared and run following Weber *et al.* [15].

Isoelectric focusing. The procedure of Righetti and Drysdale [16] was used with Biolyte ampholytes. Protein staining was with 0.05% Coomassie Brilliant Blue/0.5% $CuSO_4$ in 10% HOAc, 27% EtOH.

M_r *determination by HPLC.* A column of TSK Spherogel G3000SW (600 × 7.5 mm) was used and KPi, 200 mM, pH 7.0, was the solvent employed. A standard line of M_r versus elution time was prepared with immunoglobulin G, bovine serum albumin, ovalbumin, myoglobin and lysozyme.

Amino acid analyses. Hydrolysates were prepared in 6 M HCl or, for tryptophan determination, 4 M methane sulphonic acid [17] under vacuum at 110° for 22 hr. Cysteine content was measured after oxidation and hydrolysis [18] and amino sugars after hydrolysis in 4 M HCl for 6 hr under vacuum at 110°. Amino acid and amino sugar determinations were made with an automatic analyser.

Tryptic digestions and 2D maps. The euphorbains were digested with trypsin in 100 mM NH_4HCO_3 for 20 hr with a ratio of trypsin to substrate of 1:100. 2D-thin layer maps were prepared on Macherey-Nagel polygram Cel-400 sheets as described elsewhere [19].

Purification of euphorbains d_1 and d_2. Latex serum of *E. drupifera* was applied to a column (2 × 33 cm) of Sephadex G-25 equilibrated with 10 mM NaOAc, pH 4.8, at 4°. The esterolytically active eluate was then introduced on to a column of CM-Sepharose CL-6B (1.5 × 44 cm) maintained at 4°, and the column washed with the 10 mM Ac buffer used above, before elution with a linear gradient composed of 300 ml each 0–0.6 M NaCl in that buffer. A single active peak was obtained with a 72% yield of activity and a 2.5-fold purification.

The enzyme from this elution was concentrated on an Amicon UM-10 membrane and subjected to gel exclusion chromatography on Bio-Gel P150 (1.5 × 96 cm) equilibrated with 50 mM NaOAc, pH 6.0 at 4°. Two enzymatically active components were then partly separated and these were further purified to homogeneity on Spherogel TSK-G3000SW using HPLC at room temp. The yield of activity of euphorbain d_1 (the heavier component) was 11%, that of d_2, 7% while the respective purification factors observed were 4.1 and 1.4, calculated from the specific activity of the crude latex serum.

Acknowledgements—The authors wish to express their gratitude to Dr. G. L. Webster, University of California, Davis, CA, for the gift of the *Elaeophorbia drupifera* and to Mr. H. Datema, Carleton University, Ottawa, for growing the trees. They are also indebted to Dr. M. Yaguchi for the amino acid analyses, and to Mr. J. Giroux for technical assistance.

REFERENCES

1. Lynn, K. R. and Clevette-Radford, N. A. (1983) *Biochim. Biophys. Acta* **746**, 154.
2. Lynn, K. R. and Clevette-Radford, N. A. (1984) *Phytochemistry* **23**, 682.
3. Lynn, K. R. and Clevette-Radford, N. A. (1985) *Phytochemistry* **24**, 925.
4. Lynn, K. R. and Clevette-Radford, N. A. (1985) *Phytochemistry* (submitted).
5. Evans, F. J. and Kinghorn, A. D. (1975) *Lloydia* **38**, 363.
6. Dixon, M. and Webb, E. C. (1979) *Enzymes*, 3rd edn, p. 162. Academic Press, New York.
7. Kapoor, M. and MacLean, S. (1976) *Int. J. Biochem.* **7**, 49.
8. Umezawa, H., Aoyagi, T., Morishima, H., Kunimoto, S., Matsuzaki, M., Hamada, M. and Takeuchi, T. (1970) *J. Antibiot.* **23**, 425.
9. Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaki, M., Hamada, M. and Takeuchi, T. (1970) *J. Antibiot.* **23**, 259.
10. Rajagopalan, T. G., Stein, W. H. and Moore, S. (1966) *J. Biol. Chem.* **241**, 4295.
11. Pontremoli, S., Traniello, S., Enser, M., Shapiro, S. and Horecker, B. L. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 286.
12. Little, C., Sanner, T. and Pihl, A. (1969) *Eur. J. Biochem.* **8**, 229.
13. Motion, R. L., Blackwell, L. F. and Buckley, P. D. (1984) *Biochemistry* **23**, 6851.
14. Lynn, K. R. and Clevette-Radford, N. A. (1984) *Phytochemistry* **23**, 963.
15. Weber, K., Pringle, J. R. and Osborn, M. (1971) *Methods Enzymol.* **26**, 3.
16. Righetti, P. L. and Drysdale, J. W. (1974) *J. Chromatogr.* **98**, 271.
17. Simpson, R. J., Neuberger, M. R. and Lui, T. Y. (1976) *J. Biol. Chem.* **251**, 1936.
18. Hirs, C. H. W. (1967) *Methods Enzymol.* **19**, 197.
19. Lynn, K. R. (1979) *Biochim. Biophys. Acta* **569**, 193.