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THREE SERINE PROTEASES FROM THE LATEX OF *EUPHORBIA CYPARISSIAS**

K R LYNN and N A CLEVETTE-RADFORD

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

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Key Word Index—*Euphorbia cyparissias*, Euphorbiaceae, latex, plant protease, serine, amino acid composition

Abstract—Three serine-centred proteolytic enzymes, euphorbains y-1, -2 and -3, were isolated from the latex of *Euphorbia cyparissias* These proteases have different specific activities to azocoll and CBZ glycine *p*-nitrophenyl ester The pIs and M_rs of y-1, -2 and -3 are 5 2 and 67 000, 5 2 and 33 000, and 6 3 and 67 000, respectively The enzymes, which are glycoproteins, are immunologically distinct from euphorbain l, but clearly related to that enzyme in amino acid composition

INTRODUCTION

While cysteine-centered proteases have been found in a number of plants (see ref [1] and refs cited therein) and some of these have been extensively examined both structurally and mechanistically [2], proteolytic enzymes of plant origin carrying an essential serine residue have not been widely reported [3]

From the latex of *Euphorbia lathyris* (caper spurge) and that of *E pulcherrima* (poinsettia), we have recently purified separate proteases which were of differing M_rs and substrate specificities, but similar per cent residue weight compositions [4, 5] Both enzymes, which were given the trivial names euphorbains l and p respectively, were inhibited by diisopropyl fluorophosphate (DFP), suggesting that they each have an essential serine residue at the active site Neither of them was sensitive to the presence of sulphydryl reagents such as mercaptoethanol or *p*-chloromercuribenzoate, and so they are not cysteine-centred proteases Similarly, the presence of metal complexing reagents, and of inhibitors effective against proteolytic enzymes with essential acid residues were without effect on the two enzymes discussed here, supporting their identification as serine proteases This was further confirmed by the close resemblance, in per cent weight compositions, between euphorbain l and cocoonase, which is related to trypsin [4]

In extending the work on the latex of members of the family Euphorbiaceae to *E cyparissias* (cypress spurge), we isolated three proteolytic enzymes These have been named euphorbains y-1, -2 and -3 to distinguish them from a protease reportedly present in *E cerifera* [6] which was named euphorbain c [7] While euphorbains y-1, -2 and -3 are clearly related by composition to the proteolytic enzymes obtained from *E lathyris* and *E pulcherrima*, they display individual properties, which will be described here

RESULTS AND DISCUSSION

Figures 1 and 2 show that euphorbain y-1 is the major component of the mixture of enzymes separated It was recovered as 26% of the original activity in the crude dried latex (10% of the original protein) Euphorbains y-2 and -3 were each recovered as 4% of the original activity and 10 and 13% of the original protein, respectively

The homogeneities of the three enzymes isolated from *E cyparissias* were demonstrated by anionic gel electrophoresis Each gave a single band y-1 and y-2 had R_f values of 0 4, and for y-3 the R_f was 0 3 Isoelectric focusing experiments also showed that the enzymes were homogeneous, with single pIs of 5 2, 5 2 and 6 3 for y-1, -2 and -3, respectively The M_rs reported above were confirmed by SDS gel electrophoresis of the purified enzymes 66 000 and 68 000 being the weights then estimated for y-1 and y-3, and y-2 was found to have an M_r of 31 000 The observation of proteins with M_rs between 30 000 and

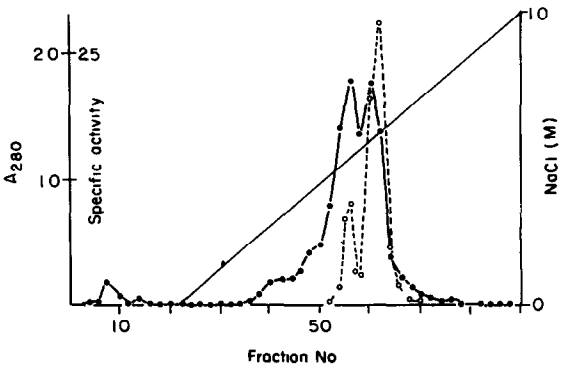

Fig 1 Elution of two euphorbain y proteases from DEAE-Sepharose CL-6B (1 5 × 20 cm) with a linear gradient (0–600 mM) of sodium chloride in 10 mM sodium acetate, pH 6 0, at 4° (●——●) A_{280}, (○——○) specific activity (units CGN/ml)

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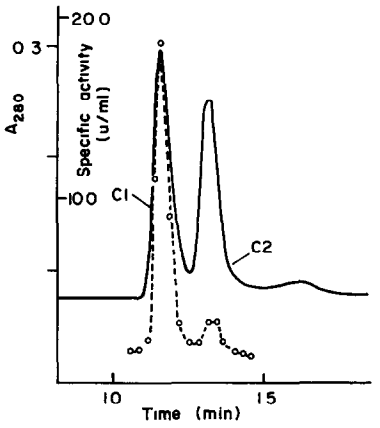

Fig 2 Separation of euphorbains y-1 and y-2 on HPLC (conditions as described in the text) (——) A_{280}, (O——O) specific activity (units CGN/ml)

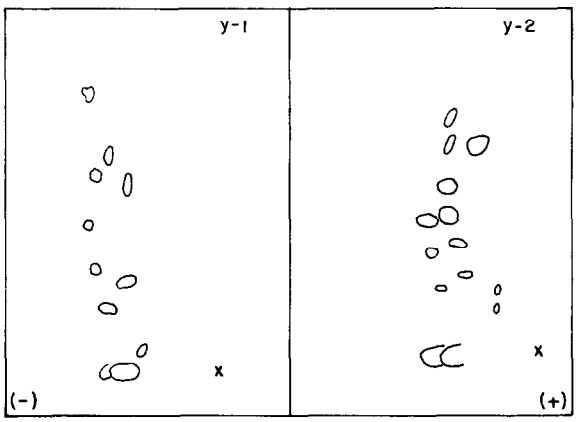

Fig 3 2-D TLC maps of digests of insulin B chain in 100 mM ammonium acetate, pH 7 0, 37° for 18 hr Horizontal dimension—electrophoresis, vertical—chromatography

60 000 after SDS gel electrophoresis suggests the presence of more than one chain in the larger molecules of these euphorbains (y-1 and y-3)

The three euphorbains purified in this work are not only physically distinct, as described above, but also have different activities to both esterolytic and proteolytic substrates Thus the specific activities (units/mg protein) for euphorbains y-1, -2 and -3 were, in that order, for CBZ-gly-pNp, 17 8, 3 0 and 2 1, for azocoll, 0 13, 0 18 and 0 29, and for azocasein, 0 07, 0 07 and 0 06 A comparison can be made of the specific activities reported here with those from purified, homogeneous preparations of euphorbains 1 [4] and p [5] The former of these had a specific activity of 7 3 with CBZ-gly-pNp and 3 3 with azocasein while the comparable figures for euphorbain p were 1 0 and 0 7 The relatively high reactivity of euphorbain y-1 with CBZ-gly-pNp is a noteable aspect of these results

That the euphorbains from *E cyparissias* react in individual ways with protein substrates is further demonstrated from 2D-TLC maps of digests of insulin B chain Such maps are shown in Fig 3, and it can be seen that for the two enzymes examined (y-1, y-2), significant differences were found in the products from prolonged digestion

Mixtures of the three euphorbain y enzymes discussed here were unaffected by the peptide inhibitors of trypsin obtained from bovine pancreas, soybean and ovomucoid (cf ref [4]), nor was hirudin inhibitory However, α-1-antitrypsin and potato inhibitor I were inhibitory when in 1000-fold molar excesses, as were the microbially derived serine protease inhibitors elastatinal and chymostatin Clearly, none of the commonly employed peptide and protein inhibitors of serine proteases are notably efficient against the *E cyparissias* euphorbains Homogeneous euphorbain y-1 was inactivated by 55% after 1 hr in the presence of a 1000-fold molar excess of DFP, and so apparently, like the other proteases isolated from the latices of euphorbs [4, 5], it has a vital serine group at its active site Euphorbain 1 was shown to be related by composition to cocoonase, a serine protease of the trypsin family in which there are vital serine and histidine residues [4] However, histidine-specific reagents such as diethyl pyrocarbonate [8] and *p*-bromophenacyl bromide [9] had no effect on the activity of euphorbain 1 or p [4, 5] These two reagents were, though, immediately inhibitory, in 1000-fold molar excesses, for euphorbain y-1 As will be shown below, euphorbains 1 and y-1 are of markedly similar amino acid compositions, so the results described for the histidine-specific reagents, as well as the incomplete inhibition by DFP reported above (cf ref [4]), suggest that some difference in three-dimensional structures must exist between the proteinases of *E lathyris* and *E cyparissias*

The activities of the euphorbain y enzymes were unaffected by reducing reagents such as mercaptoethanol, or by sulphydryl-blocking reagents such as *p*-chloromercuribenzoate or the peptide inhibitor leupeptin However, another peptide inhibitor which affects cysteinyl-centred enzymes, antipain, did inactivate euphorbain y-1 when in 1000-fold molar excess, and a 2×10^5 molar excess of iodoacetic acid caused 40% inhibition in 1 hr These results suggest the possibility that a cysteinyl residue has a functional role in this enzyme

Such a hypothesis would explain the finding that the proteases of *E cyparissias* were totally inhibited by 10 mM Hg^{2+} and Zn^{2+} Ca^{2+}, Mg^{2+} and Mn^{2+} were without effect on the enzymes The metal complexing reagents EDTA and *o*-phenanthroline did not inhibit the euphorbains described here, which suggests that the enzymes are not metallo-proteins

Reagents which are inhibitors of such carboxylic acid-centred proteolytic enzymes as pepsin, namely pepstatin and diazo-DL-norleucine methyl ester [10], had no effect on euphorbain y-1 when in 2×10^5 molar excess The enzyme is not, apparently, related mechanistically to pepsin

The three proteases isolated from *E cyparissias* displayed somewhat different pH maxima with azocoll as substrate, these being at pHs of 5 2, 5 5 and 7 0 (y-1, -2 and -3, respectively) in flattened, 'bell-shaped' curves of activity vs pH With azocasein as substrate, the euphorbains y-1 and y-2 had maxima at pH 6 5–7 0, and the extent of proteolysis with y-3 increased with increasing pH, to pH 9, the highest value measured The pH maxima for euphorbains y-1 and y-2 are significantly lower than those

Table 1 Amino acid compositions of euphorbains y-1, -2 and -3 (euphorbian 1 data from ref [4])

Amino acid	Residues/mol			Wt composition (%)			
	y-1	y-2	y-3	y-1	y-2	y-3	1
Cys	9	5	10	1 4	1 6	1 5	1 6
Asp	97	40	95	16 6	14 2	16 3	11 8
Thr	35	14	31	5 2	4 4	4 7	7 8
Ser	74	43	77	9 7	11 7	10 1	10 0
Glu	46	21	44	8 8	8 4	8 4	6 5
Pro	32	15	27	4 6	4 5	3 9	4 2
Gly	68	37	67	5 8	6 5	5 7	6 5
Ala	45	23	49	4 8	5 0	5 2	6 3
Val	41	18	40	6 0	5 5	5 9	7 2
Met	0	0	0	0	0	0	0
Ile	28	16	29	4 7	5 6	4 9	7 2
Leu	35	15	35	5 9	5 2	5 9	6 4
Tyr	10	7	14	2 4	3 5	3 4	3 7
Phe	24	11	24	5 2	5 0	5 2	5 0
His	16	11	13	3 2	4 7	2 6	2 8
Lys	35	18	40	6 7	7 1	7 6	5 0
Arg	23	8	23	5 3	3 9	5 3	3 9
Trp	9	3	7	2 4	1 7	1 9	3 0
Glc-NH_2	4	3	6	1 0	1 5	1 4	1 1
M_r	67 152	32 362	67 214				

found with other euphorbains [4, 5], and with other serine proteases such as trypsin, suggesting that these two enzymes may be mechanistically distinct

The amino acid compositions of the three proteases isolated from *E cyparissias* and that of euphorbain 1 are compared in Table 1 All of the enzymes are glycoproteins containing glucosamine but not galactosamine, as does euphorbain p [5] The data further show that the three newly isolated enzymes are of remarkably similar amino acid compositions, euphorbains y-1 and y-3 carrying different charges (see pI values above), and y-2 is probably a monomer unit of one of these two molecules, possibly y-1, from comparison of the pI values measured The relationship between the proteases from *E cyparissias* and the comparable enzyme from *E lathyris* [4] is also evident in Table 1 However, differences in composition (Thr, Ala, Val, Ile, Lys and Trp) are apparent, and the proteases of *E cyparissias* are clearly distinct entities

That euphorbains y-1, -2 and -3 differed from euphorbain 1 was confirmed by immunoelectrophoresis experiments when the proteases from *E cyparissias* showed no precipitation with antibodies raised in rabbits to euphorbain 1

While the latex of *E lathyris*, like that of *E pulcherrima*, was found to contain only a single proteolytic enzyme, the latex of *E cyparissias* has three related proteases Work currently in progress has demonstrated that from the latex of *E tirucalli*, also, several proteolytic enzymes can be obtained, and these are being isolated

EXPERIMENTAL

Materials Euphorbia cyparissias L latex was collected from plants growing wild near Arnprior, Ontario The latex was immediately clarified by centrifugation at 20000 *g* for 1 hr and the aq layer separated and stored at −20° until required

All reagents employed were of analytical grade except as noted Pharmacia (Canada) Ltd supplied the DEAE-Sepharose CL-B, Sephadex G-25, Polybuffer 74 and Polybuffer exchanger 94 Reagents for disc gel electrophoresis, and BioLyte ampholytes were obtained from BioRad Chemicals, Richmond, CA Sigma Chemical Co , St Louis, MO supplied mercaptoethanol, diazo-DL-norleucine methyl ester, diethylpyrocarbonate, *p*-chloromercuribenzoate, CBZ-glycine-*p*-nitrophenyl ester (CBZ-gly-pNp), azocollagen, azocasein, insulin B chain and diisopropyl fluorophosphate (DFP) Bovine pancreatic, soybean and ovomucoid trypsin inhibitors were from the same company, as were hirudin, leupeptin, pepstatin A and α-1-antitrypsin Antipain, elastatinal and chymostatin were purchased from the Protein Research Foundation, Osaka, Japan Pierce Chemical Co, Rockford, IL supplied the methane sulphonic acid, and Eastman Chemical Co Rochester, NY, the *p*-bromophenacyl bromide The potato inhibitor I was prepared as described in ref [11]

Esterolytic assays with CBZ-gly-pNp were performed as described in ref [4] Proteolysis was measured, using reported methods, with azocoll [4] and azocasein [5] Protein contents were measured as A_{280} or with the BioRad protein reagent

PAGE Anionic and cationic gels were used as described in ref [12], SDS gels were employed following ref [13]

Isoelectric focusing BioLyte ampholytes were used in the procedure of Righetti and Drysdale [14] protein staining was with 0 05% Coomassie brilliant blue–0 5% $CuSO_4$ in 10% HOAc–27% EtOH

Chromatofocusing Enzyme in 25 mM imidazole–HCl buffer, pH 7 4, was applied to a column of Pharmacia Polybuffer exchanger 94 (1 × 25 cm) which had been equilibrated with that buffer Elution was effected by a 1 8 dilution of Polybuffer 74 at pH 4 0 Fractions were assayed for pH, protein content and enzymatic activity

M_r *determination by HPLC* A column (600 × 7 5 mm) of TSK Spherogel G3000SW, equipped with a 10 mm precolumn (Altex Scientific Co), was used with a Beckman Model 110A solvent

metering pump and model 153 analytical UV detector The buffer employed was 200 mM KPi, pH 7 0, at 21° and a flow rate of 0 5 ml/min A standard line was prepared, of M_r vs elution times for immunoglobulin G, bovine serum albumin, ovalbumin, myoglobin and lysozyme

Amino acid analyses Hydrolysates were prepared either in 6 M HCl or, for tryptophan determination, 4 M methane sulphonic acid [15] under vacuum at 110° for 22 hr Cysteine content was measured after oxidation and hydrolysis [16] 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, and all hydrolyses were performed in duplicate

Immunoelectrophoresis Rabbit anti-euphorbain 1 serum was produced conventionally after injections of homogeneous enzyme [4] into the animals The immunoelectrophoresis procedure used was essentially that of Weeke [17] Plates 1 5 mm thick of 0 86% agar in 90 mM barbital buffer containing 0 02% NaN_3 were prepared Parallel wells of 3 mm diameter, 6 mm apart, were loaded with, on one side, anti-euphorbain 1 serum and on the other, varying dilutions of the euphorbain y samples The plate was chilled on a water-cooled flat bed electrophoresis apparatus and subjected to 4 V/cm using the barbital buffer in the electrode troughs and saturated paper bridges to the agar plate After 45 min, the plate was stored at room temp in a humid atmosphere and interaction between antigen and antibody was examined visually

2D maps of digests of insulin B chain Digestions of insulin B chain were made with solns of 250 μg/ml in 100 mM NH_4OAc, pH 7 0, at 37° for 18 hr The enzyme/substrate ratio was 1 100 by wt Peptide maps were prepared on thin layer cellulose sheets (Macherey-Nagel Polygram Cel 400) and developed, as described elsewhere [18]

Purification of euphorbain y enzymes All procedures were conducted at 4° except for the final fractionation on HPLC, which was performed at 20°

Clear serum from the latex was subjected to gel chromatography on Sephadex G-25 (2 × 25 cm) in 10 mM NaOAc buffer, pH 6 0 The active material eluted without retention, separating the enzyme from pigments and other low-M_r components of the serum The enzymatically active pool was applied to a column of DEAE-Sepharose CL-6B (1 5 × 20 cm) equilibrated with the acetate buffer used above, and elution was with a linear gradient of 0–600 mM NaCl in the same buffer Two esterolytically active peaks were separated (Fig 1) at *ca* 290 mM NaCl and 340 mM NaCl, respectively On subjecting these to HPLC as described for the determination of M_rs, after concn on a Diaflo membrane, the latter peak was separated into two components of M_rs 67 000 (euphorbain y-1) and 33 000 (euphorbain y-2) (Fig 2) Each of these enzymes had activity and protein peaks which were coincident, showing them to be homogeneous The enzyme eluted first from the ion exchanger (Fig 1), on application to the HPLC following concn, was also shown to be a homogeneous protein, of M_r 67 000 (euphorbain y-3), and again the protein and activity profiles were coincident

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REFERENCES

1 Lynn, K R (1983) *Phytochemistry* **22**, 2485
2 Glazer, A N and Smith, E L (1971) in *The Enzymes* (Boyer, P D, ed), Vol 3, p 501 Academic Press, New York
3 Ryan, C A and Walker-Simmons, M (1981) in *The Biochemistry of Plants* (Marcus, A, ed), Vol 6, p 321 Academic Press, New York
4 Lynn, K R and Clevette-Radford, N A (1983) *Biochim Biophys Acta* **746**, 154
5 Lynn, K R and Clevette-Radford, N A (1984) *Phytochemistry* **23**, 682
6 Castanêda, M, Gavarron, F F and Balcazar, M R (1942) *Science* **96**, 365
7 Lennox, F G and Ellis, W J (1945) *Biochem J* **39**, 465
8 Kapoor, M and MacLean, S (1976) *Int J Biochem* **7**, 49
9 Roberts, M F, Deems, R A, Mincey, T C and Dennis, E A (1977) *J Biol Chem* **252**, 2405
10 Rajagopalan, T G, Stein, W H and Moore, S (1966) *J Biol Chem* **241**, 4295
11 Ryan, C A and Kassell, B (1970) in *Methods in Enzymology* (Perlman, G E and Lorand, L, eds), Vol 19, p 883 Academic Press, New York
12 Davis, B J (1964) *Ann N Y Acad Sci* **121**, 404
13 Weber, K, Pringle, J R and Osborn, M (1971) in *Methods in Enzymology* (Hirs, C H W and Timasheff, S N, eds), Vol 26, p 3 Academic Press, New York
14 Righetti, P L and Drysdale, J W (1974) *J Chromatogr* **98**, 271
15 Simpson, R J, Neuberger, M R and Lui, T Y (1976) *J Biol Chem* **251**, 1936
16 Hirs, C H W (1967 in *Methods in Enzymology* (Perlman, G E and Lorand, L, eds), Vol 19, p 197 Academic Press, New York
17 Weeke, B (1973) *Scand J Immunol* **2** (Suppl 1), 15
18 Lynn, K R (1979) *Biochim Biophys Acta* **569**, 193