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# EUPHORBAIN P, A SERINE PROTEASE FROM *EUPHORBIA PULCHERRIMA**

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**Key Word Index**—*Euphorbia pulcherrima*; Euphorbiaceae; pointsettia; euphorbain; serine protease; HPLC; electrophoresis.

**Abstract**—A serine protease named euphorbain p has been isolated in a homogeneous state from the latex of *Euphorbia pulcherrima*. This multi-chain enzyme, MW 74000, is similar in composition to one in *E. lathyris*, but is larger in size and has a more restricted activity. It has a pI of 4.7, and displays maximum activity at pH 7.0.

## INTRODUCTION

In a continuing study of the proteases of plant latices [1 and references therein], we have recently isolated and characterized a serine protease, euphorbain 1, from the latex of *Euphorbia lathyris* [2]. Euphorbain 1 is not only different from the commonly known proteases of plant origin such as papain and ficin in being a serine rather than a sulphydryl enzyme, but it also displays somewhat more specificity than members of the cysteinyl protease families, and has a significantly larger MW (43 000) [2]. We now report the isolation and characterization of another serine protease (euphorbain p) from the latex of *Euphorbia pulcherrima* L.

## RESULTS AND DISCUSSION

On anionic disc gel electrophoresis the *E. pulcherrima* enzyme gave a single band when stained for protein. A single band was also obtained when the position of proteolytic activity was determined [3] on a gel. Further evidence of the homogeneity of the euphorbain was provided by HPLC when a single symmetrical peak corresponding to a MW of 74 000 was obtained on a column previously calibrated with a mixture of human immunoglobulin G, bovine serum albumin, myoglobin and lysozyme. SDS gel electrophoresis confirmed this MW and also indicated that the enzyme was multi-chain, as a major protein band was detected with a MW of about 35 000, as well as minor bands of MW 20 000 and 15 000. (The gels were calibrated with lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase B.) Similar fragmentation of euphorbain 1 has been observed in this laboratory [2] and the phenomenon is currently being examined further.

While euphorbain p has a MW of 74 000, that of euphorbain 1 is 43 000 [2]. However, the two enzymes are of notably similar amino acid compositions (Table 1). As a close compositional relationship between euphorbain 1 and such proteases as cocoonase [4] has been adduced elsewhere [2] this must also apply to euphorbain p. Like euphorbain 1, euphorbain p contains glucosamine but not galactosamine. Other carbohydrate residues have not been identified.

Euphorbain p not only strongly resembles euphorbain 1 and cocoonase in amino acid composition, suggesting that it, too, is a serine protease, it is effectively inhibited by the classic inhibitor of such enzymes, di-isopropyl fluorophosphate (DFP) [5] and also by a second serine specific inhibitor [6], phenylmethyl sulphonyl fluoride (PMSF). Thus the former reagent at 100 $\mu$M immediately inhibited 71% of the enzyme activity to azocasein, the latter at 14 mM inhibited 50% of the activity. By contrast, conventional reducing agents employed with sulphydryl proteinases were without effect on the activity of euphorbain

Table 1. The amino acid composition of euphorbains p and 1

| Residue | No. of residues | % Residue compositions | |
|---|---|---|---|
| | | Euphorbain p | Euphorbain 1 [2] |
| cys | 9 | 1.3 | 1.7 |
| asp | 74 | 10.9 | 10.6 |
| thr | 49 | 7.2 | 8.0 |
| ser | 71 | 10.4 | 11.8 |
| glx | 46 | 6.7 | 5.2 |
| pro | 36 | 5.3 | 4.5 |
| gly | 78 | 11.4 | 11.8 |
| ala | 55 | 8.1 | 9.2 |
| val | 36 | 5.3 | 7.6 |
| met | 1 | 0.1 | 0 |
| ile | 38 | 5.6 | 6.9 |
| leu | 50 | 7.3 | 6.0 |
| tyr | 19 | 2.8 | 2.4 |
| phe | 29 | 4.3 | 3.6 |
| his | 14 | 2.1 | 1.9 |
| lys | 31 | 4.5 | 4.0 |
| arg | 20 | 2.9 | 2.6 |
| trp | 2 | 0.3 | 1.7 |
| gluNH$_2$ | 24 | 3.5 | 0.7 |
| MW | 72 578 | | |

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p. EDTA had no effect on the activity of that enzyme suggesting that, as for euphorbain 1, metal ions are not critical to the catalytic activity [2].

Euphorbain p had a pI of 4.7 (determined by chromatofocusing) and displayed a typical bell shaped pH profile with a maximum activity at pH 7.0 using azocasein as substrate. While both azocasein and azocoll were satisfactory substrates for the enzyme, its esterase activity as determined with carbobenzoxy glycine *p*-nitrophenylester (CGN) was low, and with the tryptophan analogue of that substrate, zero. Similarly, euphorbain p showed no amidase activity with either benzoylarginine- or leucine-*p*-nitroanilides. With azocasein as substrate the ratio of specific activities of euphorbains p to 1 was 0.2; when CGN was the substrate the ratio was 0.1.

## EXPERIMENTAL

*Materials. E. pulcherrima* (pointsettia), purchased from a commercial source, was grown in a greenhouse which was maintained at 20°, and was illuminated only by the sun. Latex was collected from incisions in the plant stems, frozen overnight and, after thawing slowly, the aq. layer was separated from the rubber and freeze dried. The powder so obtained was stored at 4° and used as a source for the procedure described below. Unless otherwise stated, all reagents employed were of analytical grade.

*Assays.* Azocasein was employed as the protein substrate. To 1 ml 0.2% azocasein in 100 mM Tris–HCl buffer, pH 7.0, at 37° was added a known vol. of enzyme soln. After incubation at 37° for 30 min, 1 ml 5% TCA was added to the reaction mixture which was then centrifuged and the $A_{410\,nm}$ of the supernatant measured. One unit of activity produced a change of 1.0 absorption units (versus a blank)/min.

The esterolytic assay employed CBZ glycine *p*-nitrophenylester. To 2.9 ml Tris–HCl buffer was added 0.1 ml 3 mM ester in acetonitrile soln. On addition of a known vol. of the enzyme soln the rate of hydrolysis was followed at $A_{410\,nm}$ in a Beckman Model 26 recording spectrophotometer. One unit of activity caused an increase of 1.0 absorption units/min.

*Amino acid analysis.* Hydrolysates were prepared under vacuum at 105° for 22 hr in 6 M HCl or, for determination of tryptophan content, 4 M methane sulphonic acid [7]. Cysteine content was measured using the method of Hirs [8]. Amino sugars were determined on an amino acid analyser after hydrolysis for 6 hr in 4 M HCl.

*Disc gel electrophoresis.* Gels were prepared and run as described by Davis [9] and by Weber *et al.* [10].

*MW determination by HPLC.* Samples were applied to a column (600 × 7.5 mm) of TSK Spherogel G300SW equipped with a precolumn (10 mm, Altex Scientific Co.). A Beckman Model 110A solvent metering pump and Model 153 analytical UV detector were used. The buffer was 200 mM KPi, pH 7.0, used at 20° with a flow rate of 0.5 ml/min.

*Chromatofocusing.* To a column (1 × 23 cm) of Pharmacia PBE 94 equilibrated with 25 mM imidazole–HCl buffer, pH 7.4, was added 500 μg euphorbain p in the same buffer. Elution was with a 1:8 dilution of Pharmacia Polybuffer 74 adjusted to pH 4.0 and de-aerated. Fractions were assayed for activity, protein and pH.

*Purification of euphorbain p.* Following the isolation of the crude, dried latex powder described above, all purification steps were conducted at 4°. The latex powder was dissolved in a minimum vol of 10 mM acetate buffer, pH 6.0, which was centrifuged (10 000 g; 10 min) to clarify the soln. This was applied to a column (2.0 × 35 cm) of DEAE-Sephadex CL-6B equilibrated with 10 mM NaOAc, pH 6.0. After washing the column with 200 ml buffer, a linear gradient consisting of 500 ml each of the buffer containing 0 and 1 M NaCl was applied. The enzyme eluted at about 300 mM NaCl with no loss of activity. This material was dialysed against $H_2O$, freeze-dried and passed through Sephadex G-100 and Sephacryl S-300 (each column 1.5 × 84 cm), yielding a single active peak which was electrophoretically homogeneous.

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