Phytochemistry, Vol. 26, No. 3, pp. 655–657, 1987.
Printed in Great Britain.

0031 9422/87 $3.00 + 0.00

ACID PHOSPHATASES FROM LATICES OF EUPHORBIACEAE*

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

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

(*Revised received* 30 *July* 1986)

Key Word Index Euphorbiaceae; latex; acid phosphatase.

Abstract—Five phosphatases were isolated from the latices of three members of the Euphorbiaceae. From *Euphorbia lathyris* were obtained phosphatases l_1 and l_2; from *E. trigona* phosphatase t and from *Elaeophorbia drupifera* the enzymes d_1 and d_2. Phosphatases l_1, l_2 and t were purified to homogeneity. Amino acid compositions are reported and other properties of the enzymes are described. The two enzymes described from *E. lathyris* both have two pH maxima (l_1 at 5.0 and 6.8, l_2 at 5.8 and 7.5) while t, d_1 and d_2 respectively have maxima at pHs of 5.6, 5.6 and 5.0. On the basis of their responses to several residue-specific inhibitors the five phosphatases apparently comprise three groups: l_2 and d_1, t and d_2, and l_1.

INTRODUCTION

Acid phosphatases (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) are widely distributed in animals, plants and bacteria. As part of a continuing investigation of the enzymes in the latices of Euphorbiaceae [1–7] we have recently purified two distinct phosphatases, designated l_1 and l_2, from the latex of *E. lathyris*, a 'leafy' member of that genus. Acid phosphatases were also isolated from the succulents *E. trigona* and *Elaeophorbia drupifera*. The former of these yielded a single, and the latter two phosphatases, respectively phosphatases t, and d_{1-2}.

As phosphatases from latex sources have not previously been described, we here report the purification procedures employed and some properties, including amino acid compositions of these enzymes.

RESULTS AND DISCUSSION

The two enzymes from *E. lathyris* and that from *E. trigona* were homogeneous on gel exclusion HPLC using a TSK G3000SW column, each eluting as a single symmetrical peak. Two phosphatases were also separated from the latex of *Elaeophorbia drupifera* with the procedures used for the other enzymes discussed here, and M_rs were determined on Bio-Rad P150 gel-filtration. On HPLC, however, these phosphatases were found to be not completely homogeneous, and only limited data for them are reported as a shortage of latex prevented attainment of that degree of purification. Some of the properties of the five enzymes discussed here are summarized in Table 1: M_rs were determined by the procedure of Andrews [8] using a P150 column calibrated with bovine serum albumin, ovalbumin, carbonic anhydrase, ribonuclease and lysozyme. All of the enzymes have M_rs between 60 and 72 000: other phosphatases reported from a variety of sources display a wide range of M_rs. However their subunit structures, demonstrated by SDS polyacrylamide gel electrophoresis, vary (Table 1): phosphatases $l_{1\ 2}$ are probably each composed of two identical subunits whereas phosphatase t comprises two non-identical subunits of M_rs 30 and 35 000. The isoelectric points of phosphatases l_1, t and d_{1-2}, determined by isoelectric focusing, are all between 3.7 and 3.9. Phosphatase l_2 is markedly different, having p*I*s of 6.7, 6.9 and 7.0.

In Table 2 are collected the amino acid compositions for phosphatases l_1 and l_2 as well as the percentage weight compositions which are included to facilitate comparisons. It is evident that the enzymes are different from each other: they also show no notable similarities in primary compositions with other acid phosphatases. The amino acid analytical data for phosphatase l_1 showed the presence of ornithine with the homogeneous protein hydrolysed. This was probably from firm attachment of that amino acid to the enzyme throuhout the purification, and is not a component of the phosphatase: ornithine has not been found in any other enzyme isolated. Only with phosphatase l_2 was sufficient protein obtained to allow determination of cysteine and of amino sugar contents. The latter figure (5.9% by weight—for glucosamine) suggests that the enzyme is a glycoprotein, as are the proteases isolated from the latices discussed here [1–7]. All five phosphatases have pH maxima between 5.0 and 5.8, but the two enzymes from *E. lathyris* each have a second pH maximum: for phosphatase l_1 this is at pH 6.8 and is the position of higher hydrolysing ability (the second maximum being at pH 5.0), while for phosphatase l_2 the maximum at pH 7.5 is of secondary importance, the primary one occurring at pH 5.8. The occurrence of the two maxima may be interpreted as suggesting that there is a change in the position of the rate limiting step of phosphatolysis which is dependent on pH. As described by Dixon and Webb [9] if the enzyme–substrate complex must undergo an ionization step before discharging the product, the rate determining step at one pH may be different from that at another. It is notable that this phenomenon of two pH maxima occurs only in phosphatases from *E. lathyris* which is the sole 'leafy' member of the family examined: the phosphatases from the two 'succulent' members of the Euphorbiaceae studied,

*N.R.C.C. No. 26695

Table 1. Some properties of phosphatases from latices of *E. lathyris*, *E. trigona* and *Elaeophorbia drupifera*

	l_1	l_2	t	d_1	d_2
M_r (gel exclusion)	72000	65000	66000	72000	60000
M_r (SDS gels)	38000	34,000	30,35000		
pI	3.7	6.7, 6.9, 7.0	3.7, 3.8	3.7, 3.9	3.9
pH optima	5.0, 6.8	5.8, 7.5	5.6	5.6	5.0

Table 2. Amino acid compositions of phosphatases l_1 and l_2 as number of residues/mole and, in parentheses, % weight composition

	l_1	l_2
Cys		19 (3.1)
Asp	77 (12.6)	79 (14.3)
Thr	52 (7.5)	59 (9.4)
Ser	72 (9.0)	43 (6.0)
Glu	51 (9.4)	46 (9.3)
Pro	36 (5.0)	24 (3.7)
Gly	95 (7.7)	41 (3.7)
Ala	42 (4.3)	22 (2.5)
Val	35 (4.9)	25 (3.9)
Met	2 (0.4)	0 (0)
Ile	40 (6.5)	37 (6.6)
Leu	49 (7.9)	51 (9.1)
Tyr	19 (4.4)	17 (4.4)
Phe	24 (5.0)	12 (2.8)
His	14 (2.8)	7 (1.5)
Lys	27 (4.9)	15 (3.0)
Arg	26 (5.8)	29 (7.1)
Trp		20 (5.9)
Glu-NH_2		15 (3.8)

E. trigona and *Elaeophorbia drupifera*, have single pH maxima: at 5.6, 5.6 and 5.0 for the t, d_1 and d_2 phosphatases, respectively.

The effects of three site-specific inhibitors, namely *p*-CMB [10], PMSF [11] and DEPC [12] are reported in Table 3. None of the inhibitors is notably efficient, minimally 10^3 molar excess being required for complete inhibition. The data suggest that the enzymes fall into three groups: phosphatases l_2 and d_1 where all three reagents have significant inhibitory effects, so implicating cysteine, serine and histidine residues in the active site; phosphatases t and d_2 where only the *p*-CMB is strongly inhibitory, suggesting a vital role for cysteine residues, but PMSF is without effect and the histidine specific reagent DEPC has only minor inhibitory ability. Phosphatase l_1 is unique in this categorization, being inhibited by all three reagents, but without high efficiency.

The enzymes described here react with a range of synthetic phenyl phosphates [13] and preliminary data show them to be analogous in mechanism with other acid phosphatases studied ([13] and references cited therein). Of the physiological substrates examined, while AMP, glucose-1-phosphate, glucose-6-phosphate, ribose-5-phosphate and creatine phosphate were unaffected, ADP and fructose 1,6-diphosphate were hydrolysed, the Michaelis-Menten parameters for them being, respectively, 2.4 and 0.52×10^{-4} M for K_m and 2.3 and 0.14×10^{-8} M sec for V_{max}.

EXPERIMENTAL

Materials. *Euphorbia lathyris* L. was grown from seeds generously supplied by the Botanic Gardens, Lyon, France. *E. trigona* Haw. was a commercially obtained specimen, and *Elaeophorbia drupifera* Thonn. ex Schum., was from the Department of Botany, University of California, Davis. All plants were grown in 50% sand/soil at 23° and a daylight cycle of 18 hr. Latex was collected from incisions in the stems of mature plants, centrifuged at 20000 *g* for 1 hr and the aq. layer separated and stored at −20° for use as required.

Unless otherwise stated, reagents were of analytical grade. All aryl phosphates were synthesized as described elsewhere [13] except *p*-nitrophenyl phosphate, which was obtained from Sigma. That company also supplied the *bis*-*p*-nitrophenyl phosphate, *p*-chloromercuribenzoate (*p*-CMB) and phenylmethyl sulphonyl fluoride (PMSF). Diethyl pyrocarbonate (DEPC) was from Aldrich, Sepharose CM-CL6B from Pharmacia, and Biolyte ampholytes from BioRad, which also supplied the BioGel P150.

Assays. Acid phosphatase activity was routinely determined by incubating 10 μl of enzyme soln with 0.4 ml of 3 mM *p*-nitrophenyl phosphate (*p*-NPP) and 0.2 ml 100 mM NaOAc buffer, pH 5.8 at 37°. After 10 min, 1 ml of 2 M NaOH was added to stop the reaction, and the *p*-nitrophenol released measured

Table 3. Effects of three inhibitors on the phosphatases $l_{1,2}$, t and $d_{1,2}$; bracketed numbers are the molar excesses at which the reported percentage inhibitions were observed

	Phosphatases				
Inhibitor	l_1	l_2	t	d_1	d_2
p-CMB	47 (10^6 ×)	93 (10^3 ×)	100 (10^3 ×)	100 (10^4 ×)	100 (10^3 ×)
PMSF	20 (10^6 ×)	80 (10^4 ×)	0 (10^3 ×)	65 (10^4 ×)	0 (10^3 ×)
DEPC	20 (10^6 ×)	80 (10^4 ×)	22 (10^3 ×)	56 (10^4 ×)	14 (10^3 ×)

at 410 nm. Units of enzyme activity were expressed as μmol *p*-nitrophenol released per min.

Kinetic assays were made by following the rate of product formation spectrophotometrically on a Bausch and Lomb Model 2000 instrument [13]. All measurements were in 100 mM NaOAc buffer, pH 5.8, at 25°.

Protein concn was expressed as A_{280} units or determined using the modified Lowry procedure [14].

The method used for the determination of phosphate was that of ref. [15] using glassware washed with low-phosphate detergent and extensively rinsed with double-distd H_2O. In the inhibition experiments reagents of known concn were mixed with the enzyme in 100 mM NaOAc buffer, pH 5.8 at 21° and residual activity measured as described above.

Polyacrylamide gel electrophoresis. The method of ref. [16] was used in preparing SDS gels; that of ref. [17] with Biolyte ampholytes for isoelectric focusing. Staining for proteins was performed as described in ref. [4].

HPLC fractionation. A 300 × 7.5 mm column of TSK Spherogel G3000SW (BioRad), equipped with a 10 cm precolumn, was used with a Beckman model 110A pump and a Varian model 2050 variable wavelength detector. The column flow rate was 0.5 ml/min using 200 mM KPi buffer, pH 7 at 20°.

Amino acid analyses. Hydrolysates were prepared either in 6 M HCl or 4 M methanesulphonic acid (for tryptophan determination [18]) under vacuum at 110° for 22 hr. Cysteine content was measured after oxidation and hydrolysis [19]. Amino sugars were determined following hydrolysis, *in vacuo*, in 4 M HCl for 6 hr at 110°. A Durrum D-500 automatic amino acid analyser was used in this work.

Purification of enzymes. The enzymes described here were all prepared using the same method. Only the purification of phosphatases I_1 and I_2 is described in detail. All procedures (except HPLC fractionation) were performed at 4° using a 10 mM NaOAc buffer, pH 4.8.

Clear *E. lathyris* latex serum was applied to a Sephadex G-25 column (2 × 28 cm). The unretained peak, which contained all of the phosphatase activity, was subjected to ion-exchange chromatography in CM-Sepharose-CL6B (1.5 × 40 cm) and eluted with a linear gradient of 0–2 M NaCl in the NaOAc buffer. Two active peaks were collected, at 0.5 M and at 0.9 M NaCl and denoted phosphatases I_1 and I_2. The enzyme solns were concd on a Diaflo membrane, and subjected to gel exclusion chromatography, either using HPLC or a Bio-Gel P150 column (1.5 × 90 cm). Phosphatase I_1 so obtained had a sp. act. to *p*-NPP of 0.68 U/mg, I_2 a sp. act. of 2.4 U/mg: that of the crude serum was 0.3 U/mg.

Acknowledgements—Dr. M. Yaguchi performed the amino acid analyses. The *Euphorbia lathyris* was grown by Mr. H. Datema of the Greenhouse, Carleton University, Ottawa. Dr. G. L. Webster, Department of Botany, University of California, Davis, generously provided cuttings of *Elaeophorbia drupifera*.

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. (1984) *Phytochemistry* **23**, 963.
4. Lynn, K. R. and Clevette-Radford, N. A. (1985) *Phytochemistry* **24**, 925.
5. Lynn, K. R. and Clevette-Radford, N. A. (1985) *Can. J. Biochem. Cell Biol.* **63**, 1093.
6. Lynn, K. R. and Clevette-Radford, N. A. (1985) *Phytochemistry* **24**, 2843.
7. Lynn, K. R. and Clevette-Radford, N. A. (1986) *Phytochemistry* **25**, 807.
8. Andrews, P. (1970) *Methods Biochem. Anal.* **18**, 1.
9. Dixon, M. and Webb, E. C. (1979) *Enzymes*, 3rd. edn., p. 162. Academic Press, New York.
10. Boyer, P. D. (1954) *J. Am. Chem. Soc.* **76**, 4331.
11. Fahrney, D. E. and Gold, A. M. (1963) *J. Am. Chem. Soc.* **85**, 997.
12. Kapoor, M. and MacLean, S. (1976) *Int. J. Biochem.* **7**, 49.
13. Lynn, K. R., Clevette-Radford, N. A. and Chuaqui, C. A. (1981) *Bioorganic Chem.* **10**, 90.
14. Lowry, O. H., Roseborough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
15. Lowry, O. H. and Lopez, J. A. (1946) *J. Biol. Chem.* **162**, 421.
16. Weber, K., Pringle, J. R. and Osborn, M. (1971) *Methods Enzymol.* **26**, 3.
17. Righetti, P. L. and Drysdale, J. W. (1974) *J. Chromatogr.* **98**, 271.
18. Simpson, R. J., Neuberger, M. R. and Lui, T. Y. (1976) *J. Biol. Chem.* **251**, 1936.
19. Hirs, G. H. W. (1967) *Methods Enzymol.* **19**, 197.