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INDUCTION OF ACID PHOSPHATASE IN COTTON SEEDLINGS: ENZYME PURIFICATION, SUBUNIT STRUCTURE AND KINETIC PROPERTIES

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Key Word Index—Acid phosphatase; purification; enzyme kinetics; *de novo* synthesis.

Abstract—About a 16-fold rise in acid phosphatase (EC 3.1.3.2) activity was observed during the early stages of germination of cotton embryos. Administration of cycloheximide to the germinating embryos significantly blocked the enhancement of acid phosphatase activity. This indicated that translational activity was essential for the induction of enzyme activity. Conclusive proof for the *de novo* synthesis of the enzyme was obtained by showing the incorporation of ^{35}S from $^{35}SO_4^{2-}$ into the cysteine residues of the purified acid phosphatase. The enzyme was purified (1046-fold) to electrophoretic homogeneity by ammonium sulphate fractionation, CM-Sephadex C-50 and affinity chromatography on concanavalin A-Agarose. PAGE gave two isozyme bands. The M_r of the phosphatase was 200 k as determined by molecular sieving on Sephadex G-200. SDS-PAGE of acid phosphatase revealed a single band of M_r 55 k. Thus the native enzyme is a tetramer of four identical subunits. The K_m of the enzyme with *p*-nitrophenyl phosphate was 0.5 mM. Optimal enzyme activity was observed at pH 5.0, using *p*-nitrophenyl phosphate as substrate. The enzyme activity remained linear for 105 min at 37° and was proportional to the concentration of protein within the range 0.6–2.4 μg.

INTRODUCTION

There are several reports on the purification of acid phosphatase from plant tissues such as lupin seedlings [1], amylaceous tubers of *Dioscorea* [2], maize scutellum [3], aleurone particles in rice grains [4], wheat germ [5], sunflower seed [6] and cultured tobacco cells [7]. These phosphatases exhibit a broad specificity towards natural or synthetic phosphoric esters [8–10]. In tubers of *Dioscorea*, the membrane-bound acid phosphatases preferentially hydrolyse phosphorylated sugars, adenosine 5'-monophosphate and adenosine 5'-diphosphate [2, 11]. In tobacco, the acid phosphatase hydrolyses 3'-AMP most rapidly, and has very little activity towards 2'- and 5'-AMP. Periodic acid Schiff staining of the enzyme and its affinity towards Con A-Sepharose revealed the glycoprotein nature of the acid phosphatase in tobacco and rice cultured cells [7, 12]. The acid phosphatases from *Dioscorea* [2] and sweet potato [8] have been shown to be metalloproteins. At present, very little is known about the regulation of acid phosphatase in plant cells. In germinating excised wheat embryos, Bansal and Sachar [13] reported that the stimulation of acid phosphatase activity was dependent on fresh transcriptional and translational activities. We reported a 2- to 4-fold stimulation of acid phosphatase activity by the exogenous application of chloramphenicol to germinating cotton embryos. The stimulatory effect of the drug on acid phosphatase activity was a dose–response phenomenon. Gel fractionation studies revealed that chloramphenicol induced both a qualitative and a quantitative change in acid phosphatase activity. The effect of the drug does not seem to be absolutely specific for acid phosphatases [14].

In the present investigation, we provide evidence to show that the stimulation of acid phosphatase activity in germinating cotton embryos is achieved by *de novo* synthesis of the enzyme. The enzyme was purified to electrophoretic homogeneity and its kinetic properties and subunit structure were determined.

RESULTS AND DISCUSSION

A time course study revealed that acid phosphatase activity increased during the early stages of germination of cotton embryos. However, although the activity showed a 16-fold increase after 72 hr, the incorporation of [^{3}H]leucine into the protein fraction over the same time period increased only by about 2.3-fold (Table 1).

Isozymes and the molecular form of acid phosphatase

Ion exchange chromatography of the ammonium sulphate precipitated fraction (0–65% saturation) on CM-Sephadex C-50 yielded one major and two minor peaks of acid phosphatase with a linear gradient of sodium chloride (0–400 mM) (Fig. 1), indicating the presence of three distinct isozymes of acid phosphatase in the early phases of germination. Fractionation of the enzyme preparation by gel chromatography on Sephadex G-200 revealed a single molecular form with a M_r of 200 k (Fig. 2).

Purification of acid phosphatase

Acid phosphatase was purified to electrophoretic homogeneity from 72 hr germinated cotton embryos

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Abbreviations: CHI, cycloheximide; PVP, polyvinyl polypyrrolidone; SDS, sodium dodecyl sulphate; Con A-Agarose, concanavalin A-Agarose.

Table 1. Stimulation of acid phosphatase activity and [^{3}H]leucine incorporation into the protein fraction during the early stages of germination of cotton embryos

Period of germination (hr)	Acid phosphatase activity (nmol of *p*-nitrophenol/ mg protein)	Relative activity	[^{3}H]Leucine incorporation into the acid-precipitable protein fraction (10^{-3} dpm/mg protein)	Relative radio-activity
Dry embryo	395	1.0	—	—
24	677	1.7	5.1	1.0
48	2053	5.2	8.7	1.7
72	6417	16.2	11.6	2.3

Embryos were germinated in the dark at 25 ± 2° on 0.4% agar medium for various time intervals. Acid phosphatase activity was assayed in the dialysed ammonium sulphate fraction precipitate (0–65% saturation). Another set of embryos was germinated in the presence of [^{3}H]leucine (25 μCi/set). The incorporation of labelled leucine into the protein fraction was determined as described in the text.

(Table 2). After ammonium sulphate (0–65% saturation) fractionation, the per cent recovery of acid phosphatase was 694. This indicated the presence of some inhibitory factor of acid phosphatase which is eliminated during ammonium sulphate fractionation. On CM-Sephadex C-50, the enzyme fraction was eluted batchwise with sodium chloride (250 mM) in acetate buffer and further chromatographed on a Con A-Agarose affinity column. This gave *ca* 15% yield of enzyme with 1050-fold purification.

The M_r of purified acid phosphatase was determined by molecular sieving on Sephadex G-200. A single molecular form was observed which had a M_r of 200 k.

PAGE

The purified peak fraction was electrophoresed on acrylamide gels (12.5%) to ascertain the homogeneity of the enzyme preparation. The gels were then stained for enzyme activity with Fast Garnet GBC salt and α-

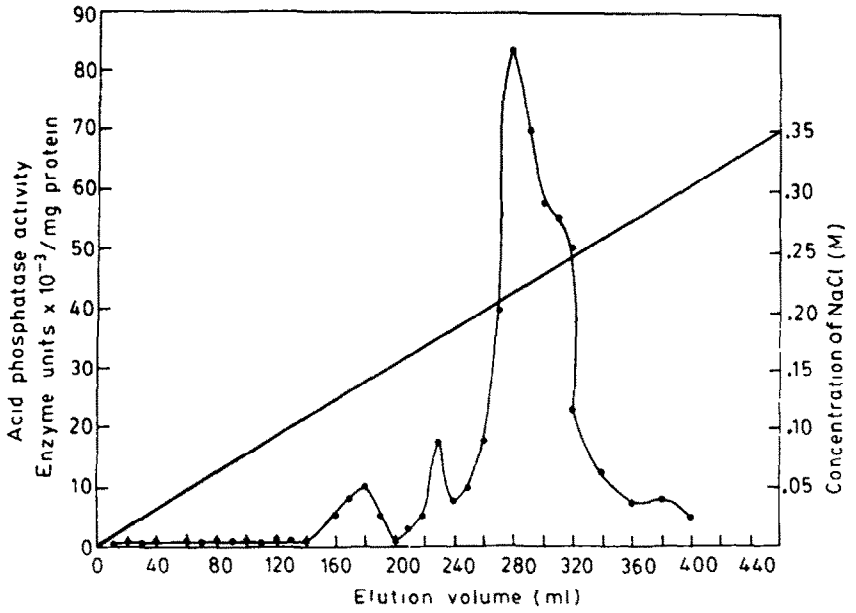

Fig. 1. Chromatography on a CM-Sephadex C-50 column (2.8 x 100 cm) of acid phosphatase from cotton embryos. The fraction volume was 10 ml. ●——●, Enzyme activity; ——, NaCl concentration (M).

Table 2. Stages of purification of acid phosphatase from germinating cotton embryos and the incorporation of ^{35}S into the acid-precipitable protein fraction at various steps of purification

Stages of purification	Specific activity with *p*-nitrophenol (μ mol/mg protein)	Total protein (mg)	Purification (fold)	Yield (%)	^{35}S incorporation into the acid-precipitable protein fraction (10^{-4} dpm/mg) protein	Relative radio-activity
Dialysed crude extract	1.38	1736	1.0	100	2.6	1.0
Ammonium sulphate (65% saturation)	18.5	900	13.4	694	2.2	0.85
CM-Sephadex C-50	38.0	60	27.0	94	6.8	3.1
Con A-Agarose	1444.0	0.24	1046.0	14.5	100.0	38.5

Embryos germinated for 72 hr were used for enzyme purification. Acid phosphatase activity was assayed at different stages of purification. Labelled $^{35}SO_4^{2-}$ was added at the start of germination of embryos and its incorporation into the acid-precipitable protein fraction was determined at various stages of purification of acid phosphatase.

naphthyl phosphate, and for protein with Coomassie Brilliant Blue R-250. Two major activity bands (isozymes) were observed on acrylamide gels which co-electrophoresed with the two protein bands (Fig. 3). No other protein bands could be detected on the gels, indicating the homogeneity of the enzyme preparation. SDS gel electrophoresis yielded only one subunit with a M_r of 55 k. These results clearly suggested that acid phosphatase is a tetramer, made up of four identical subunits.

De novo *synthesis of acid phosphatase*

The enhancement of acid phosphatase activity during the early stages of germination of cotton embryos is dependent on *de novo* protein synthesis, since the presence of cycloheximide (50 μg/ml) in the germination medium strongly inhibited (84.1%) the enzyme activity recovered in the dialysed ammonium sulphate (0–65%) precipitate. More direct proof for the *de novo* synthesis of acid phosphatase in cotton embryos was shown by the presence of radiolabel in the purified enzyme preparation isolated from embryos germinated in the presence of

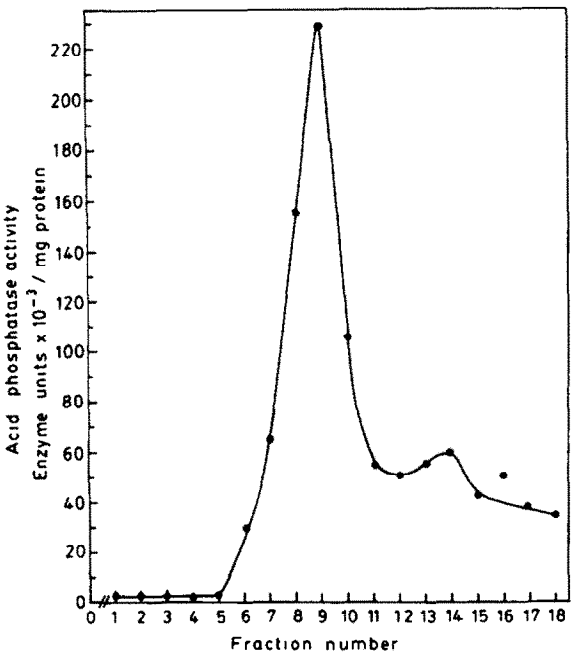

Fig. 2. Chromatography on a Sephadex G-200 column (1.8 × 70 cm) of the peak fraction collected from CM-Sephadex C-50. The fraction volume was 2 ml.

a b

Fig. 3. PAGE of the purified enzyme preparation obtained from Sephadex G-200. Protein (8 μg) was fractionated on the gels. One gel (a) was stained with Coomassie Brilliant Blue R-250 for detecting the position of protein bands, while the other gel (b) was incubated in a solution containing α-naphthyl acid phosphate disodium salt and Fast Garnet GBC salt for developing the activity bands of acid phosphatase.

$^{35}SO_4^{2-}$ (Table 2). Paper chromatography of the hydrolysate of the purified ^{35}S-labelled enzyme followed by autoradiography showed the label to be in the cysteine residues. No label could be detected in the zone of methionine. Thus, the detection of ^{35}S in the cysteine residues of the purified enzyme conclusively proved the *de novo* synthesis of acid phosphatase during the early stages of germination of cotton embryos. A similar approach was adopted in previous studies to show the *de novo* synthesis of isocitric lyase, protease and carboxypeptidase in germinating cotton embryos [15].

Properties of acid phosphatase

The purified enzyme was stable at 4° for 4 weeks, while freezing denatured the enzyme. The pH optimum of enzyme activity was 5.0 (data not presented). A similar pH optimum was reported for acid phosphatase isolated from wheat germ [16], spinach leaf cytoplasm [17], citrus juice [18], white lupin seedlings [1] and *Dioscorea cayenensis* [2]. The optimum temperature for acid phosphatase activity at pH 5.0 was 37° whereas the crude enzyme preparation exhibited optimal activity at 45° (data not presented). Purified cytoplasmic acid phosphatase of *D. cayensis* exhibited a temperature optimum at 50° [2]. The rate of the reaction remained linear for 105 min. The enzyme-catalysed reaction remained linear when the concentration of the purified enzyme protein was varied over the range 0.6–2.4 μg (data not presented).

The K_m of acid phosphatase for *p*-nitrophenyl phosphate at pH 5.0 was 0.5 mM (Lineweaver–Burke plot). The K_m value of acid phosphatase isolated from cultured tobacco cells was 0.56 mM [7] and from lupin seedling, 0.32 mM [1]. A relatively high K_m was observed for this enzyme in *Dioscorea* (K_m 2.8 mM) [2].

EXPERIMENTAL

Material. Marker proteins used for the calibration of Sephadex G-200, SDS marker proteins, concanavalin A-Agarose and Sephadex G-200 were purchased from Sigma Chemical Company (St. Louis, U.S.A.). [^{3}H]Leucine and $^{35}SO_4^{2-}$ were obtained from Bhabha Atomic Research Centre (Bombay, India). CM-Sephadex C-50 was a product of Vallabhai Patel Chest Institute (Delhi, India).

Cotton (*Gossypium hirsutum* L., var. Bikaneri Nerma) seeds were obtained from the Indian Agricultural Research Institute, New Delhi, India.

Germination of embryos. Cotton seeds were soaked in distilled H_2O for 30 min at 25°. The seed coat was removed manually. The excised embryos were surface-sterilized with aq. $HgCl_2$ soln (0.02%) for 4 min and rinsed several times with sterile distilled H_2O. The embryos were germinated for various time intervals in the dark at 25° under aseptic conditions on agar medium (0.4%). The embryos were also germinated (48 hr) in the presence of cycloheximide (50 μg/ml). [^{3}H]Leucine was added to the germinating emrbyos at 24, 48 and 72 hr.

In vivo labelling of embryos with $^{35}SO_4^{2-}$. The seedlings (48 hr) were transferred to sterile H_2O containing $^{35}SO_4^{2-}$ (10 mCi/100 ml). The seedlings contained in Erhlenmeyer flasks were shaken on a reciprocating shaker in the dark for 24 hr at 25°. After incubation, the tissue was frozen in liquid N_2 and stored at −20° prior to enzyme extraction.

Preparation of enzyme extract. The frozen tissue of 72 hr germinated cotton embryos was thawed, mixed with chilled acetate buffer (3 vols, 50 mM, pH 5.0) and PVP (2%, w/v) and homogenized in a blender (at 4°) for 2 min. The homogenate was filtered through four layers of muslin and centrifuged at 27 000 *g* for 15 min at 4°. The supernatant was filtered through a layer of absorbent cotton to remove the fatty layer and was used for the assay of enzyme activity and also for the purification of acid phosphatase.

Assay of acid phosphatase activity. The enzyme activity was determined by the modified procedure described in our earlier report [14].

[^{3}H]*Leucine incorporation into the acid-precipitable protein fraction.* The incorporation of [^{3}H]leucine (5 μCi/ml; 12 000 mCi/mmol) into the acid-precipitable protein fraction and the free pool of [^{3}H]leucine was measured in germinating cotton embryos according to the method of Taneja and Sachar [19]. [^{3}H]Leucine was added to the cotton embryos at the start of germination.

Protein determination. Protein was determined as described in ref. [20].

Enzyme purification. To the crude extract, $(NH_4)_2SO_4$ was added to 65% saturation at 0° with slow stirring. The suspension was chilled and centrifuged for 20 min at 20 000 *g*. The ppt. was suspended in acetate buffer (30 ml, 50 mM, pH 5.0) and dialysed for 48 hr against the same buffer. After dialysis, the denatured proteins were removed by centrifugation and the supernatant chromatographed on a CM-Sephadex C-50 column (2.8 cm × 100 cm) equilibrated with acetate buffer (150 mM NaCl in 50 mM buffer, pH 5.0). The column was washed with two bed vols. of the same buffer containing NaCl (150 mM) and then the acid phosphatase was eluted with 2 bed vols. of acetate buffer containing NaCl (250 mM). The proteins in the 250 mM NaCl fraction were precipitated with 0–80% $(NH_4)_2SO_4$, dissolved in a minimal vol. of Tris–HCl (50 mM, pH 7.5) and dialysed for 12 hr against Tris–HCl buffer (5 mM, pH 7.5). After dialysis, the denatured proteins were removed by centrifugation. An aliquot (10.0 ml) was loaded on to a Con A-Agarose column (1.8 cm × 12 cm) equilibrated with Tris–HCl buffer (10 mM, pH 7.5) containing NaCl (150 mM), $CaCl_2$ (100 mM) and $MnCl_2$ (100 mM). After binding the enzyme fraction to the affinity column, it was washed with 2 bed vols. of the equilibration buffer. The enzyme was eluted with two bed vols. of the same buffer containing α-D-methylmannoside (500 mM). Fractions (5 ml) were dialysed and tested for acid phosphatase activity.

PAGE. Electrophoresis of purified acid phosphatase was performed according to the procedure of Gabriel [21] on acrylamide gels (12.5%). Proteins were stained with Coomassie Blue R-250 and acid phosphatase activity bands were localized using Fast Garnet GBC salt and α-naphthyl phosphate disodium salt as substrate [22]. The procedure of Laemmli [23] was adopted for SDS-PAGE using marker proteins (albumin, ovalbumin, pepsin, trypsinogen, β-lactoglobulin, lysozyme).

Determination of isozymes and the molecular form of acid phosphatase in (72 hr) cotton embryos. The $(NH_4)_2SO_4$ fraction ppt. (0–65% saturation) was chromatographed on a CM-Sephadex C-50 column (2.8 × 100 cm) equilibrated with acetate buffer (50 mM, pH 5.0). The column was washed with two bed vols of the same buffer after binding the enzyme fraction and was eluted with a linear gradient of NaCl (0–400 mM NaCl prepared in acetate buffer, 50 mM, pH 5.0). Fractions containing acid phosphatase activity were concentrated with purified $(NH_4)_2SO_4$ (0–80% saturation). The ppt. was collected on Millipore filter discs at 4°, suspended in a minimal vol. of acetate buffer (50 mM, pH 5.0) and loaded onto a Sephadex G-200 column (1.8 cm × 70 cm) equilibrated with the same buffer. The void vol. (V_0 = 54.0 ml) of the gel column was determined experimentally from the elution profile of Blue Dextran. The specific activity of

acid phosphatase was plotted against the fraction number. The M_r of the purified enzyme was estimated from its distribution coefficient (K_{av}). Marker proteins (ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin and aldolase) were used for the calibration of the Sephadex G-200 column.

Characterization of ^{35}S-*labelled amino acids in the purified enzyme fraction.* The highly purified enzyme fraction (240 μg protein), after affinity chromatography on Con A-Agarose, was transferred into an ampoule containing 6 M HCl. The ampoule was sealed under N_2 and hydrolysis of the protein carried out for 48 hr at 110°. The protein hydrolysate was reduced to a small vol. and the ^{35}S-labelled hydrolysed product subjected to ascending chromatography on Whatman No. 1 filter paper (30 × 20 cm) for 18 hr using a mixture of *n*-BuOH–HOAc–H_2O (12:3:5) as solvent system. Unlabelled methionine and cysteine were run as markers on the same chromatogram. The positions of the standard amino acids were located by spraying ninhydrin. The chromatogram was scanned for the distribution of radioactivity in the sulphur-containing amino acids. The dried chromatogram was exposed to Sakura X-ray films for 7 days at −20°.

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