

ACID PHOSPHATASE IN RICE SEEDLINGS

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Abstract: Acid phosphatase (EC 3. 1. 3. 2) extracted from rice seedlings consisted of ten kinds of isozymes based on separation by polyacryamide gel electrophoresis. One isozyme was purified through ammonium sulfate precipitation, Ultrogel gel filtration, DEAE-trisacryl chromatography and chromatofocusing. This isozyme had a high affinity to p-nitrophenyl phosphate. The molecular weight of this isozyme was around 130 kD examined with FPLC and it consisted of heterodimer with molecular weight of 70 kD and 62 kD. The purified isozyme had a pH optimum at 5.0 and its K_m (p-nitrophenyl phosphate) was 0.69 mM. Mg^{++} and Mn^{++} enhanced the activity, but heavy metal ions were inhibitory. NaF and Na_2MoO_4 also had an inhibitory effect on the isozyme.

INTRODUCTION

The functions of acid phosphatase in plant tissue may include: 1) autolysis of cytoplasmic organelles (Bowen and Bryant, 1978; Browning et al., 1980); 2) the formation of secondary wall (Charvat and Esau, 1975; Cronshaw and Bentwood, 1977); 3) cell secretion (Schulz and Jensen, 1981); 4) the formation of abscission layer (Hall and Sexton, 1974; Moore and Walker, 1981); and 5) sugar transport (Hebant, 1973; Leigh and Walker, 1980).

The isozymes of acid phosphatase may vary dependently on plant species, tissues, and developmental stages (Scandalios, 1974; Teno et al., 1987; Huang and Chen, 1991). Treatments with external factors, e. g., hormones, minerals and water deficiency may affect isozyme activity (De Leo and Sacher, 1970; Ueki and Sato, 1977; Barrett-Lennard and Greenway, 1982). Because of the diversity and complexity of isozymes, purification and characterization of acid phosphatase were difficult in early studies (Joyce and Grisolia, 1960; Mizuta and Suda, 1980). Recent studies showed that some acid phosphatase isozymes are monomeric in maize (Rossi et al., 1981) and soybean (Ullah and Gibson, 1988), dimeric in sunflower (Park and Van Etten, 1986) and tomato (Paul and Williamson, 1987), and tetrameric in cotton (Bhargava and Sachar, 1987).

In an attempt to better understand physiological role of acid phosphatase, efforts were made to purify and characterize an isozyme from rice seedlings.

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MATERIALS AND METHODS

Plant Materials

Seeds of *Oryza sativa* Tainung 67 were surfacedly sterilized in 2% sodium hypochlorite for 20 min. After being thoroughly washed, seeds were soaked in water at 37°C for one day, then transferred to hydroponic culture containing half strength of Kimura medium (Kao, 1980). Seedlings were grown under 14 h photo period at 30°C and 10 h dark period at 25°C. Ten-day-old seedlings were collected for enzyme preparation.

Enzyme Preparation

Rice leaves were homogenized in a glass homogenizer with chilled 50 mM tris-maleate buffer (pH 7.0). Crude extracts were filtered through four layers of cheesecloth before centrifuged at 500 xg for 5 min at 4°C. The supernatant was centrifuged again at 18,000 xg for 20 min. After centrifugation, the supernatant was collected, and used for acid phosphatase assay (Hooley, 1984). Reaction mixture (1 ml) containing 0.1 M sodium acetate (pH 5.0), 0.05 M disodium p-nitrophenyl phosphate, and 50 μ l enzyme preparation was incubated at 37°C for 20 min. Reaction was terminated by adding 4 ml of 0.6 M Na₂CO₃, and absorbance was measured at 400 nm by a spectrophotometer. One unit activity was expressed as the amount of enzyme that liberated 1 μ M p-nitrophenol per min. Protein content was determined by Bradford's method (1976).

Electrophoretic Analysis

Acid phosphatase isozymes were separated by discontinuous polyacrylamide gel according to Blackshear (1984). Approximately 50 μ g of enzyme preparation was loaded into one of the sample slot of a slab gel. After electrophoresis, the gel was removed and put into a stainer containing 0.2 M sodium acetate buffer (pH 5.0), 0.1% fast red TR salt, 0.1% alpha-naphthyl phosphate and 5 mM MgCl₂ for 3 h, and finally destained with distilled water and dried.

Enzyme Purification and Characterization

Leaves of 3-week-old rice seedlings were collected, immediately frozen in liquid nitrogen, and ground with a mill grinder. About 1,000 g of ground sample was homogenized with a Polytron in 2,000 ml of 0.05 M tris-HCl (pH 7.0) containing 0.007 % β -mercaptoethanol (buffer A). The homogenate was centrifuged at 18,000 xg for 5 min, filtered through two layers of cheesecloth, and centrifuged at 18,000 xg for 10 min. Proteins were precipitated from the supernatant with 30-50% saturation of ammonium sulfate and resuspended in 20 ml buffer A. Protein solution (20 ml) was applied to an IBF Ultrogel AcA 34 column (3 \times 60 cm), and with 1,000 ml buffer A at a flow rate of 80 ml/h. The fractions eluted which contained acid phosphatase activity were pooled and applied to a DEAE-Trisacryl M column (2 \times 100 cm), and eluted with 800 ml of 0-0.5 M NaCl linear gradient at a flow rate of 70 ml/h. Activity fractions were pooled and purified further by

using chromatofocusing column (0.9 × 30 cm) containing Polybuffer Exchanger 94 (Pharmacia) at a flow rate of 20 ml/h. Fractions with high acid phosphatase activity were pooled again and vacuum concentrated with Speedvex. The concentrated preparation was then electrophoretically analyzed with 7.5% polyacrylamide gel as described above. The major acid phosphatase was cut from gels and eluted, and molecular weight of subunits was determined by SDS-PAGE on 10% gel at 110 V for 8 h. Molecular weight of the native enzyme was determined by Waters FPLC 300 SW column (0.8 × 30 cm).

Assay for Purified Isozyme.

Purified acid phosphatase isozyme was assayed as described above (Hooley, 1984). Six phosphoric esters (all in 0.05 mM) were examined to judge the substrate specificity of the purified isozyme. Eleven different ions (all in 0.5 mM) affected on the activity of this isozyme were also tested.

RESULTS

Enzyme Purification

The yields of acid phosphatase purified from rice seedlings were relatively low (Table 1). This may be attributable to the eliminations of certain isozymes during steps of purification. However, chromatofocusing did show a 24-fold increment in specific activity of acid phosphatase.

Table 1. Purification of acid phosphatase from rice seedlings.

	Protein (mg)	Activity (μ M/min)	Specific activity	Recovery (%)
Crude extract	9640	537,000	55.7	100
(NH ₄) ₂ SO ₄ (30-50%) precipitation	5910	367,000	62.1	68.3
Gel filtration	1125	109,760	97.6	20.4
DEAE-Trisacryl M	184.4	9,625	52.2	1.8
Chromatofocusing	0.66	871	1319.7	0.16

Proteins obtained from ammonium sulfate precipitation contained ten acid phosphatase isozymes based on separation by polyacrylamide gel electrophoresis (Fig. 1). The isoelectric point of these isozymes varied between pH 4.5 and 6.5 (Fig. 2). Nine isozymes were recovered with additional step of purification by gel filtration. When the enzyme fractions were pooled and eluted through ion exchange column with buffer A, a loosely bound group contained eight isozymes was obtained (Fig. 3A). Continuing elution with 0-0.5 M NaCl gradient released the bound group which contained three isozymes (Fig. 3B). When the bound

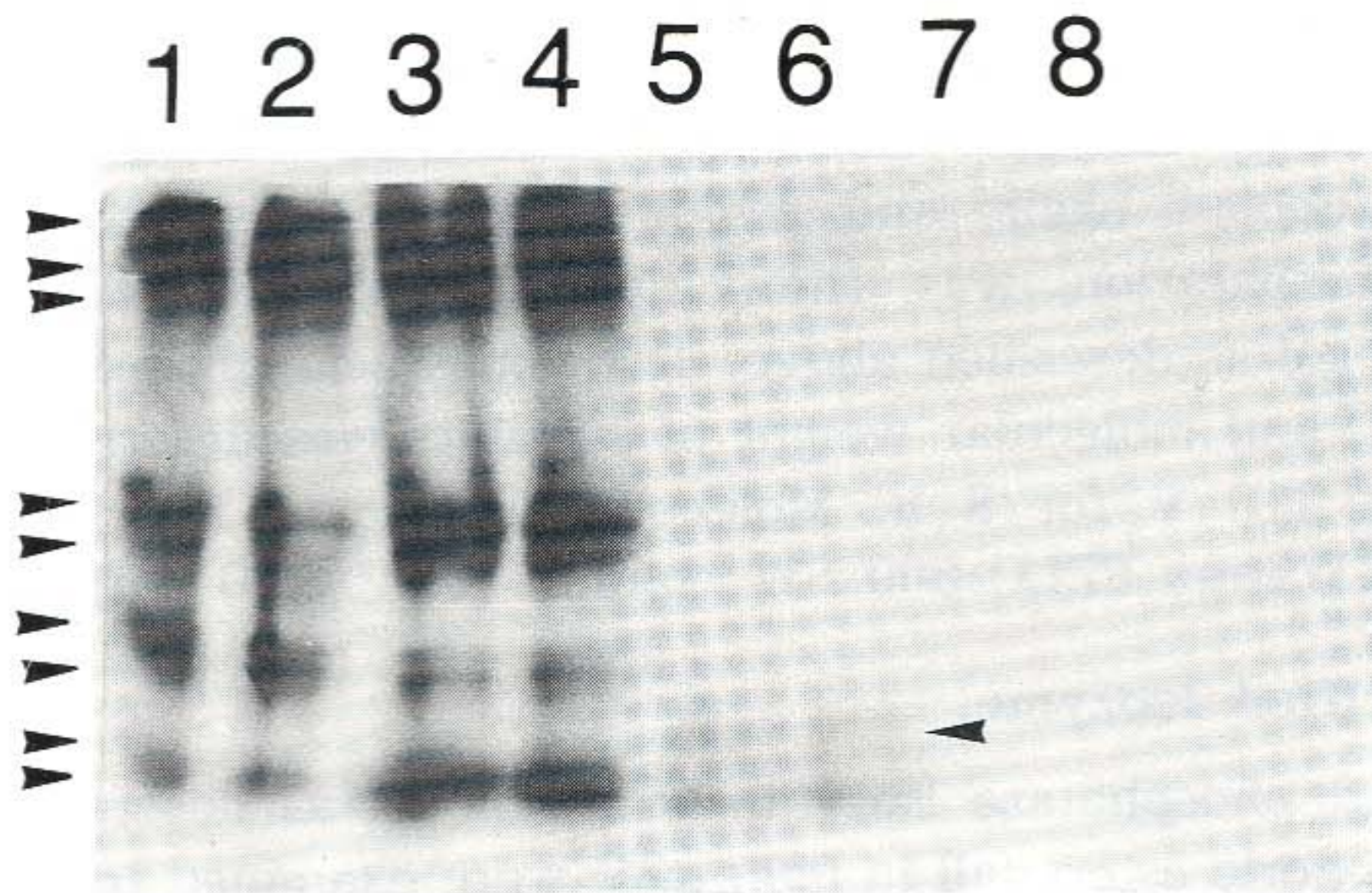


Fig. 1. PAGE-zymogram of acid phosphatase salted out by different concentrations of ammonium sulfate. Different cuts were indicated by the range of salt saturations: 0-30%, lanes 1 and 2; 30-50%, lanes 3 and 4; 50-70%, lanes 5 and 6; and >70%, lanes 7 and 8. Arrows indicated the different isozymes of acid phosphatase.

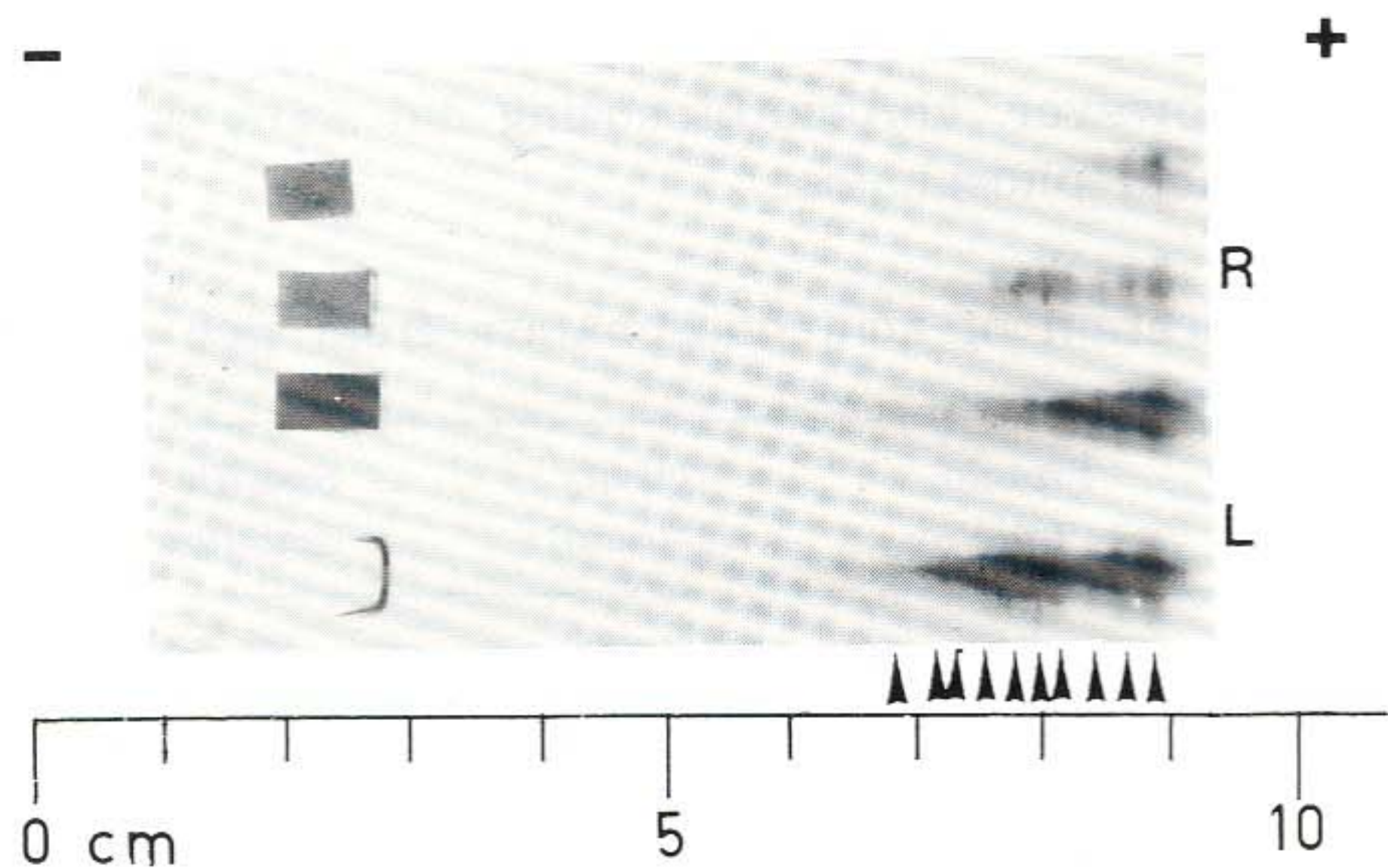


Fig. 2. Separation of acid phosphatases by IEF. R, soluble extract of root (upper lane, lx; lower lane, 3x, concentrated extract). L, soluble extract of shoot (upper lane, lx; lower lane, 3x, concentrated extract)

fractions were combined and eluted through chromatofocusing column, and a major peak of enzyme activity was separated from other four minor peaks. The isozyme obtained from the major peak contained two subunits based on separation by SDS-PAGE, and their molecular weights were 70 kD and 62 kD, respectively (Fig. 4). Molecular weight of the native isozyme determined with FPLC was 130 kD.

Enzyme Kinetics

The purified acid phosphatase isozyme had an optimal pH around 5.0 (Fig. 5) and it had a different substrate-specificity when compared with the total acid

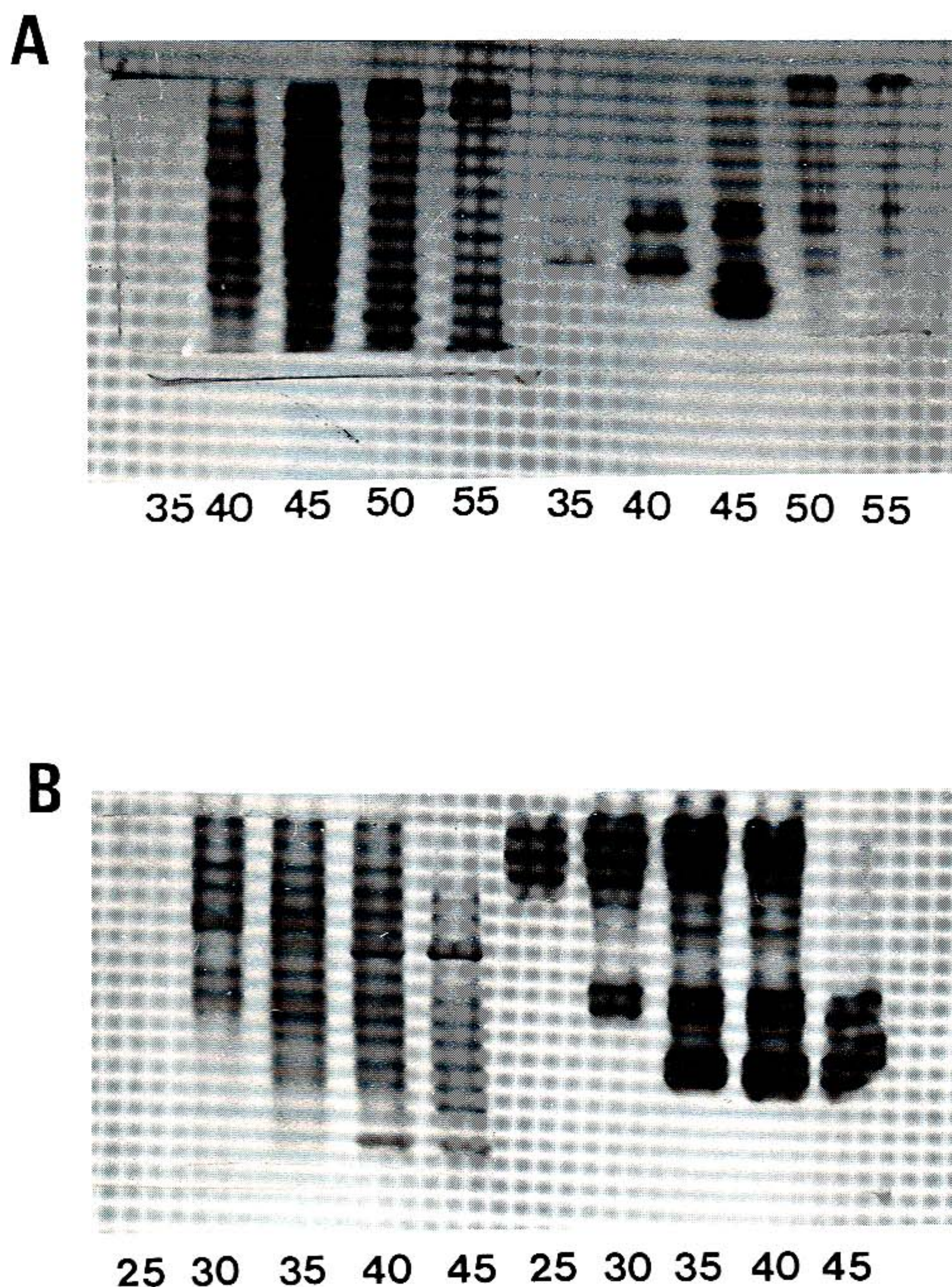


Fig. 3. A, PAGE-zymogram of loosely bound acid phosphatase eluted from DEAE-Trisacryl M column. The left five lanes were stained with Coomassie blue and the right five lanes were assayed for native enzyme. B, PAGE-zymogram of bound acid phosphatase eluted from DEAE-Trisacryl M column with 0-0.5M NaCl gradient. The left five lanes stained with Coomassie blue and the right five lanes assayed for native enzyme.

phosphatases obtained from $(\text{NH}_4)_2\text{SO}_4$ cut. The former preferred p-nitrophenyl phosphate among seven potential substrates tested; while the latter was in favor of fructose-1,6-diphosphate (Table 2). β -glycerophosphate, a popular reagent for cytochemical localization of acid phosphatase, was relatively inactive as a substrate for both purified isozyme and crude enzymes. K_m (p-nitrophenyl phosphate) for the purified isozyme was 0.69 mM. The purified enzyme was more heat labile than that of crude enzyme extract (Fig. 6); purified enzyme

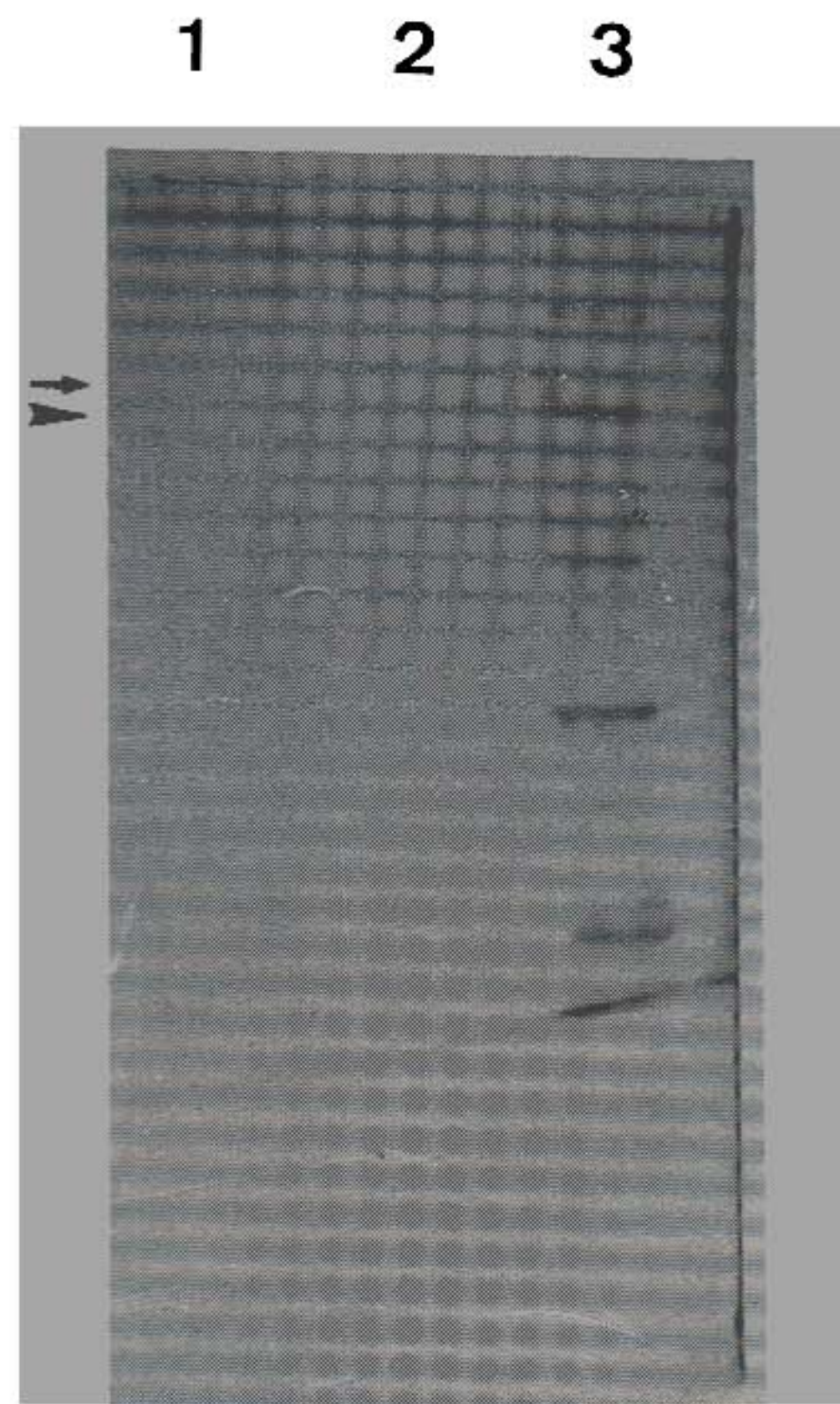


Fig. 4. Estimation of molecular weight of purified acid phosphatase isozyme by SDS-PAGE. Columns 1 and 2 were purified enzyme. Column 3 was protein makers (Phosphorylase b, 97,000; Bovine serum albumin, 66,200; Ovalbumin, 45,000; Carbonic anhydrase, 31,000; Soybean trypsin inhibitor, 21,000; Lysozyme, 14,400). Arrow indicates a subunit at 70,000 and the arrowhead indicates another subunit at 62,000.

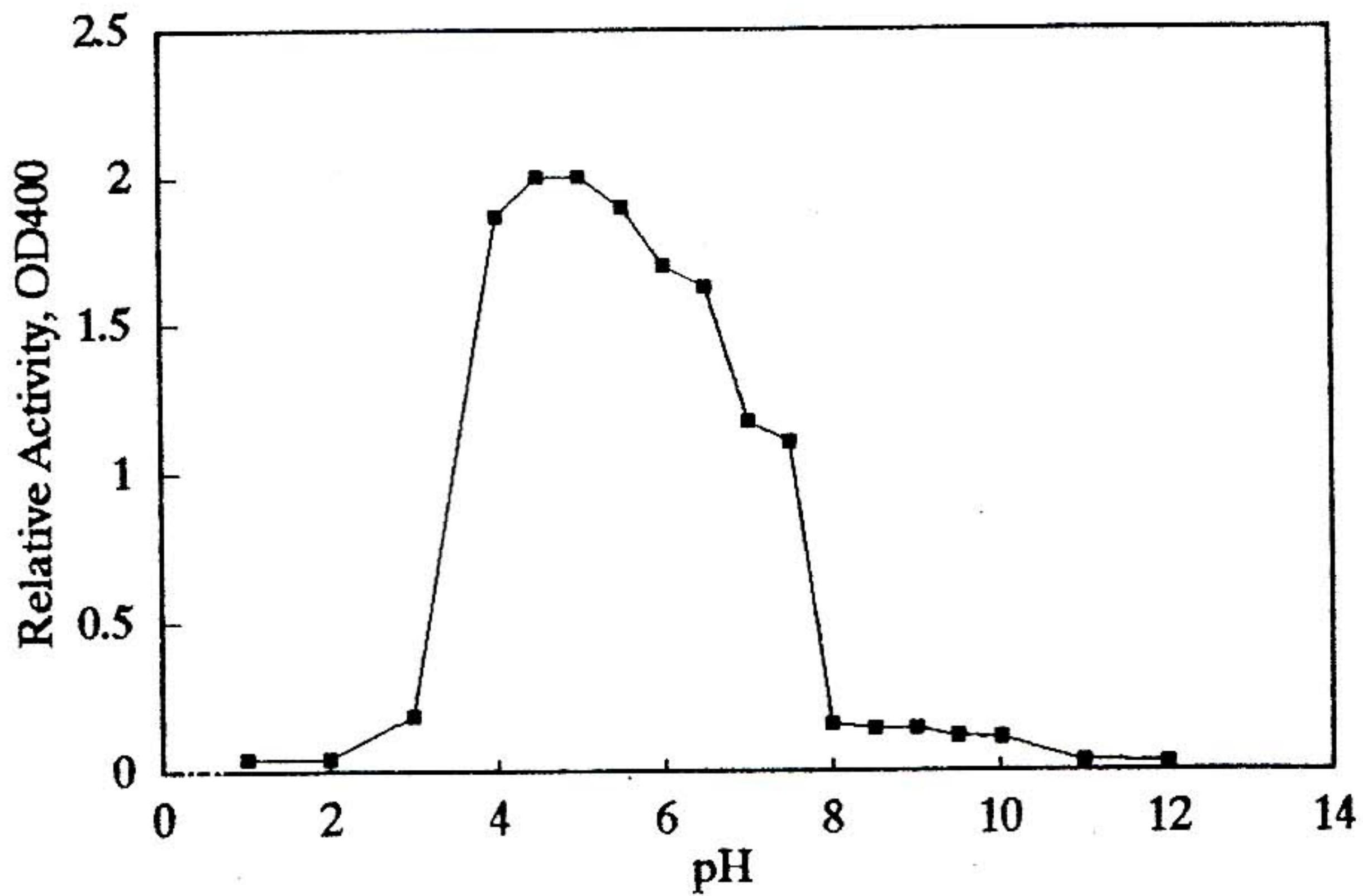


Fig. 5. The effect on pH on the activity of acid phosphatase. Overlapped pH points were used to standardize the value.

Table 2. Substrate specificity between crude and purified acid phosphatase.

Substrate (0.05mM)	Relative activity (%)	
	Crude	Purified
p-Nitrophenyl phosphate	100.0 ± 5.7	100.0 ± 2.3
Fructose-1,6-diphosphate	128.1 ± 4.6	83.1 ± 1.3
Fructose-6-phosphate	17.9 ± 0.9	12.5 ± 0.5
Glucose-1-phosphate	15.2 ± 1.0	3.5 ± 0.4
Glucose-6-phosphate	6.6 ± 0.5	4.5 ± 0.4
β-Glycerophosphate	4.7 ± 0.2	6.4 ± 0.5

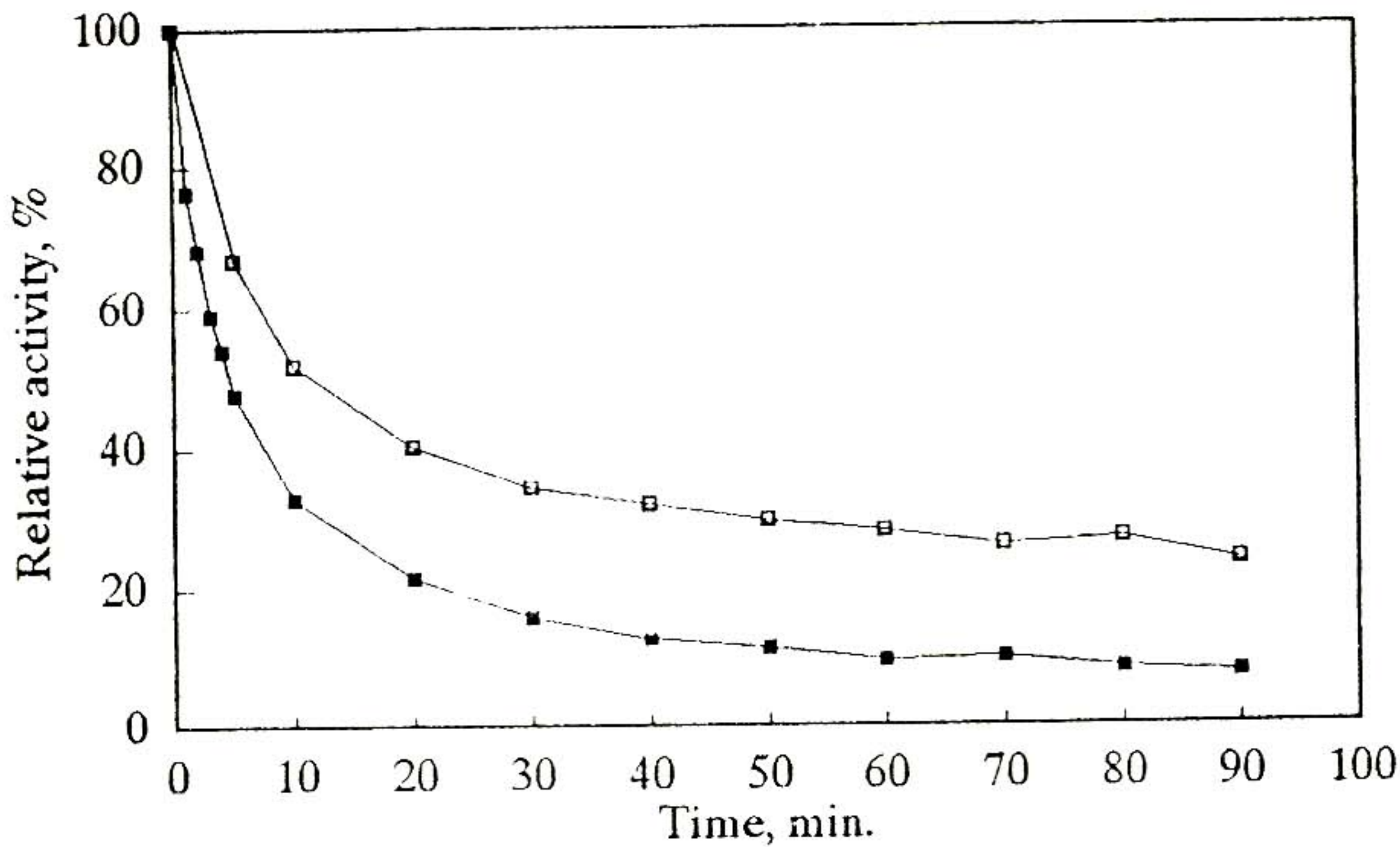


Fig. 6. The comparison of thermal stability between crude and purified acid phosphatase at 60°C. Symbols —■— and —□— denoted the response of purified isozyme and total isozymes, respectively.

preincubated at 60°C for 90 min lost more than 90% of its activity while the crude enzyme extract retained about 22% activity.

Effect of divalent cations on the purified enzyme varied (Table 3). The activity was stimulated by Mn²⁺, Ca²⁺, and Mg²⁺, but inhibited by Cu²⁺, Hg²⁺, and Zn²⁺. Co²⁺ had no effect on the activity. Monovalent cations such as K⁺ and NH₄⁺ had little effect on the purified enzyme. But purified enzyme was sensitive to inhibition by NaF and Na₂MoO₄ (Table 3).

Table 3. Effect of different ions on the activity of acid phosphatase isozyme.

Additives (0.5 mM)	Relative activity (%)		Additives (0.5 mM)	Relative activity (%)	
	Crude	Purified		Crude	Purified
Control	100.0 ± 5.1	100.0 ± 4.3	NaF	54.0 ± 0.4	40.6 ± 3.2
CaCl ₂	100.5 ± 3.5	126.0 ± 13.2	Na ₂ MoO ₄	23.4 ± 1.0	2.0 ± 0.6
MgCl ₂	119.3 ± 2.6	101.8 ± 5.9	NH ₄ NO ₃	80.7 ± 16.1	81.4 ± 7.3
CuSO ₄	42.7 ± 3.1	23.5 ± 0.8	CoCl ₂	107.3 ± 17.0	97.3 ± 6.8
HgCl ₂	11.8 ± 0.3	2.0 ± 0.1	ZnSO ₄	71.7 ± 1.9	53.2 ± 7.2
KH ₂ PO ₄	97.2 ± 4.0	88.7 ± 2.0	MnSO ₄	151.9 ± 13.5	114.4 ± 6.8

DISCUSSION

Acid phosphatase is commonly present in organisms as multiple forms, and usually rather nonspecific, quantitatively small, and unstable in dilute solution (Park and Van Etten, 1986; Paul and Williamson, 1987). The activity and property of isozymes may also vary due to differences in developmental stage and plant species. In addition, the nomenclature of isozymes is inconsistent. Therefore, only limited information is available regarding specificity, localization and physiological function (Baker and Tadakazu, 1973; Charvat and Esau, 1975; Mizuta and Suda, 1980; Ching et al., 1984). However, recent development of chromatographic methods has been helpful in purifying individual isozyme of acid phosphatase for studying physiological functions and genetic regulation (Bhargava and Sachar, 1987; Ullah and Gibson, 1988).

Twelve isozymes of acid phosphatase were purified from bean hypocotyl by using combined methods of DEAE-cellulose chromatography and cell fractionation (Mizuta and Suda, 1980). Soluble acid phosphatases from wheat leaves were separated into various fractions by CM-cellulose chromatography (Barrett-Lennard and Greenway, 1982). Purification and characterization of individual acid phosphatase isozyme by the flow-chart process of DEAE-cellulose Con A-sepharose chromatography were also reported in nematode-resistant tomato cultivar (Paul and Williamson, 1987) and sunflower seed (Park and Van Etten, 1986). In this study, a 24-fold increase in specific activity of the 130 kD isozyme was purified from rice leaves by chromatofocusing method. The poor recovery of acid phosphatase activity at the step of chromatography was, in part, attributable to the elimination of loosely bound phosphatases.

The purified isozyme from rice (130 kD determined by FPLC) was heterodimer consisted of 70 kD and 62 kD subunits when analyzed by SDS-PAGE. Polymorphisms of acid phosphatase are reflected in the expression of allelic or non-allelic genes (Tanksley and Orton, 1983). In tomato, gene *Aps1* coding for the

acid phosphatase-1 was located in chromosome 6 and gene *Aps2* for acid phosphatase-2 was mapped to chromosome 8 (Dupont *et al.*, 1985). The native enzyme of acid phosphatase-1 variant was shown to consist of homodimer of 31 kD polypeptide estimated by SDS-PAGE; however, acid phosphatase-2 contained two different polypeptides, 48 and 56 kD (Paul and Williamson, 1987).

The pH optimum of the purified isozyme was around 5, and it was similar to most acid phosphatase (Bentwood and Cronshaw, 1976; Huang and Chen, 1991; Pan *et al.*, 1987), although acid phosphatase-1 of tomato had a broader optimal pH between 3.5-4.5 (Paul and Williamson, 1987).

Purified acid phosphatases of *Dioscorea cayenesis* (Kamenan and Diopoh, 1983) and *Sorghum vulgare* (Saeed *et al.*, 1987) were stable at 50°C and 55°C, respectively. The purified isozyme, in this study, was more thermal labile than that of crude extract of acid phosphatase. Similar results were observed with cotton isozyme (Bhargava and Sachar, 1987).

Substrate specificities of crude and purified acid phosphatases were slightly different (Table 2), although the K_m of crude extract (8 mM) was much higher than that of the purified acid phosphatase (0.69 mM). The K_m value of purified rice acid phosphatase is slightly higher than that of sunflower, 0.13 mM (Park and Van Etten, 1986) and sorghum, 0.34 mM (Saeed *et al.*, 1987). Nevertheless, it is relatively lower in comparison with K_m of *Dioscorea* acid phosphatase, 2.8 mM (Kamenan and Diopoh, 1983).

The activity of purified rice acid phosphatase was stimulated by $MnSO_4$. This is consistent with a previous suggestion that Mn^{2+} functions as a cofactor for acid phosphatase (Fujimoto *et al.*, 1977). $HgCl_2$ had an inhibitory effect on acid phosphatase. The observation that purified rice acid phosphatase was inhibited by $CuSO_4$, $ZnSO_4$, Na_2MoO_4 and NaF is similar in a manner to that of the pea and tobacco enzymes (Mizuta and Suda, 1980; Pan *et al.*, 1987). It is interesting to note that extranuclear acid phosphatase in rice tissue was inhibited by NaF , whereas nuclear acid phosphatase was not based on a cytochemical study (Chen *et al.*, 1991), indicating an extranuclear location of the purified isozyme.

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水 稻 酸 性 磷 酸 酶

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摘 要

水稻組織的粗抽出液在電泳分析圖譜上呈現十帶的同功酶。此粗抽出液的循序純化經硫酸銨沉澱法、Ultron膠體過濾法、DEAE-trisacryl 色層法及對焦色層法等分析後，取得一與 p-nitrophenyl phosphate (pNPP) 親和性很高的同功酶。其經 FPLC 法所定的分子量為 130 kD，以 SDS-PAGE 法定出次單體的分子則分別為 70 kD 及 62 kD。該同功酸性磷酸酶的最適 pH 為 5.0，對 pNPP 的 K_m 為 0.69 mM，屬熱不穩定性酵素，鎂離子及錳離子的存在可加強酵素活性，汞離子屬強抑制劑，氟化鈉則只具部分抑制性。