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Partial Purification and Kinetic Characterization of Acid Phosphatase from Garlic Seedling

Begüm Yenigün* **and Yüksel Güvenilir**

Istanbul Technical University, Department of Chemical Engineering, 80626 Maslak, Istanbul, Turkey,
E-mail: yenigun@itu.edu.tr or avcibasi@itu.edu.tr

Abstract

The objective of this study was to obtain purer acid phosphatases than produced by prior art by operating under conditions that improve the final product. The study features are the use of a mild nonionic detergent, 40–80% saturation with $(NH_4)_2SO_4$, maintained at low temperature to remove impurity, and the use of chromatografic columns to concentrate the acid phosphatase and remove non–acid phosphatase proteins with lower or higher molecular weights. Acid phosphatase was isolated and purified from garlic seedlings by a streamline method without the use of proteolytic and lipolytic enzymes, butanol, or other organic solvents. Grown garlic seedlings of 10–15 cm height were homogenized with 0.1 *M* acetate buffer containing 0.1 *M* NaCl and 0.1% Triton X-100. After homogenization, the supernatant was filtered with paper filters. Filtrated supernatant was cooled to 4°C, followed by a threestep fractionation of the proteins with ammonium sulfate. The crude enzyme was isolated as a green precipitate that was dissolved in a small amount of 0.1 *M* acetate buffer containing 0.1 *M* NaCl and 0.1% Triton X-100. Garlic seedling acid phosphatase was purified with ion-exchange chromatography (DEAE cellulose). The column was equilibrated with 0.1 *M* acetate buffer. Acid phosphatase was purified 40-fold from the starting material. The specific activity of the pure enzyme was 168 U/mg. A variety of stability and activity profiles were determined for the purified garlic seedling acid phosphatase: optimum pH, optimum temperature, pH stability, temperature stability, thermal inactivation, substrate specificity, effect of enzyme concentration, effect of substrate concentration, activation energy, and effect of inhibitor and activator. The molecular mass of acid phosphatase was estimated to be 58 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The optimum pH was 5.7 and the optimum temperature was 50°C. The enzyme was stable at pH 4.0–10.0 and 40–60°C. Activation energy was between 10 and 20 kcal, and as Michaelis Menten coefficients, V_m values

*Author to whom all correspondence and reprint requests should be addressed.

were 100 and 20 mM/s and K_m values were 21.27 and 8.33 mM for paranitrophenylphosphate and paranitrophenyl, respectively. Studies of the effect of metal ions on enzyme activity showed both an activating and a deactivating effect. While Cu, Mo, and Mn showed strong inhibitory effects, Na, Ca, and K were the significant activators of acid phosphatase.

Index Entries: Enzyme; acid phosphatase; enzyme purification; garlic seedlings.

Introduction

Phosphate esters are widely distributed in any organism. While many metabolic intermediates are activated through the transfer of phosphate groups, it is equally important that phosphate esters can also be rapidly broken down. The hydrolytic removal of phosphate groups from phosphoesters is catalyzed by phosphatases. Depending on the pH at which such phosphatases have optimal activity, one can distinguish between acidic phosphatases (also called acid phosphatases) and alkaline phosphatases. The latter enzymes require divalent metal ions as cofactors and are common in animal tissues and bacteria *(1)*.

Acid phosphatases are enzymes that hydrolyze the terminal phosphate of phosphomonoesters, thus releasing inorganic phosphate. The optimum pH for hydrolysis is in the range of 4.0–6.0. These enzymes are ubiquitous in bacteria, fungi, animals, and plants *(2)*. Studies of acid phosphatases from various plant sources suggested their roles during the solubilization of macromolecular organic phosphates in soils and the mobilization of phosphorous reserves during germination *(3)*. Most studies of acid phosphatase, particularly those from plant sources, are largely of a descriptive nature. With a few exceptions (notably human prostatic acid phosphatase), the acid phosphatases occur in very small quantities, are unstable in dilute solution, and are subject to surface denaturation when purified. These factors, together with a tendency to occur in multiple forms or as isoenzymes, make the isolation of highly purified acid phosphatase difficult *(4)*.

Materials and Methods

Garlic seedlings were grown from Kastamonu Seedlings, Turkey. Chemicals (paranitrophenyl (*p*-NP), paranitrophenylphosphate (*p*-NPP), 4-nitrophenylphosphate) were obtained from Sigma, England. All other chemicals (ammonium sulfate, NaOH, ammonium, sodium acetate, acetic acid, citric acid, NaCl, ammonium hydrogen phosphate, sodium hydrogen phosphate, potassium dihydrogen phosphate, sodium pyrophosphate decahydrate, sodium pyrophosphate, D-glucose-6-phosphate, pyridoxal-5-phosphate) were obtained from Merck, Germany. DEAE-cellulose anion exchanger (Sigma) was used for column chromatography.

Specific Activity Assay

For substrate *p*-NPP, hydrolysis was determined by measuring inorganic phosphate released by the acid phosphatase reaction. Phosphatase

activity was assayed in 0.1 *M* of sodium acetate buffer (pH 5.5) containing 0.1 *M* NaCl and 0.1% Triton X-100, and 50 m*M* substrate at pH 5.5. Enzyme 0.25 mL was added to 2.25 mL of substrate solution. Buffer solution (0.25 mL) instead of enzyme was used for blank sample. The reaction mixtures were incubated at 37°C for 30 min. Reactions were quenched by sequential addition of 2.5 mL of 20 m*M* NaOH solution. The absorbance was read at 405 nm. A standard curve of *p*-NPP was constructed. One unit of phosphatase activity was defined as the amount of enzyme required to produce 1 (mol of free Pi/min from 5 m*M* of *p*-NPP at pH 5.5 and 37°C. Protein concentration was determined with an ultraviolet spectrophotometer at 280 nm.

Purification of Enzyme

Garlic seedlings were grown in 3-, 6-, and 9-wk periods. Crude extract was obtained and ammonium sulfate saturation was applied for each period. It was observed that 3- and 6-wk-old seedlings were quite unstable and lost their activities in a short time. On the other hand, 9-wk-old seedlings did not show activity loss during almost 10–12 wk. As a result, the purification procedure was carried out with 9-wk-old seedlings. The steps for purification were homogenization, centrifugation, saturation, and ion-exchange chromatography.

Garlic seedlings (9 wk old) were first washed free from soil and weighed. They were homogenized in 0.1 L of 0.1 *M* sodium acetate buffer, pH 5.5 (0.1 *M* NaCl, 0.1% Triton X-100), with a Waring blender at low speed for 20 s and then high speed for 30 s. The crude extract was filtered through black filter paper and white filter paper, respectively. The crude extract after filtration was centrifuged for 5 min at 20,000 rpm to get a clear extract. The supernatant and precipitate were obtained. The precipitate was then resuspended in a minimum amount of buffer solution. Solid ammonium sulfate was added to the supernatant to change the solubility of proteins and to precipitate the proteins. First 40% was brought to saturation with ammonium sulfate (25 g/100 mL). Stirring at about 4°C was continued for 2 h, and the mixture was then centrifuged for 30 min at 20,000 rpm. The supernatant and precipitate were separated. The supernatant was retained and ammonium sulfate concentration was increased first to 60% (39 g/100 mL) then to 80% (52.3 g/100 mL). After stirring and centrifuging as just described, the second supernatant and precipitate were obtained, which were then resuspended in a minimum amount of buffer.

A 20-mL sample of supernatant obtained after ammonium sulfate saturation and centrifugation was loaded onto a DEAE cellulose ion-exchange chromatography column (2 × 60 cm) that had been preequilibrated with 0.1 *M* sodium acetate buffer, pH 5.5. The protein was eluted with the same buffer using a fraction size of 5 mL at the rate of 1/7 mL/min. Fractions containing the majority of the acid phosphatase activity were pooled for activity assay. The activity of acid phosphatase at the end of each of the preparative steps was measured by spectrophotometric method.

Stability of Acid Phosphatase and Activity Profiles

Optimum pH, optimum temperature, pH stability and temperature stability, thermal inactivation, substrate specificity, effect of enzyme concentration, effect of substrate concentration, effect of cations, kinetic constants, and activation energy were determined for the partially purified acid phosphatase of tomato seedlings. *p*-NPP was used as substrate.

Optimum pH

Acid phosphatase activity was tested in a pH range of 3.0–10.0 by changing the ionic strength of the substrate. Ammonium and citric acid were used to regulate pH. Sample without enzyme but buffer was used as blank solution. Activity was determined at 37°C for 30 min.

Optimum Temperature

Optimum temperature was determined by measuring the activity in a temperature range (0, 20, 30, 37, 50, 60, and 70°C) using the activity assay procedure at related temperature.

pH Stability

Buffer solutions at different ionic strengths in the range of 2.5–10.0 were incubated at 30°C for 24 h after enzyme solution was added. Activity was determined at 37°C with activity assay procedure. Distilled water without enzyme was used as blank sample.

Temperature Stability

Diluted enzyme fractions of 15 mL were incubated for 15 min at different temperatures (0, 30, 40, 50, 60, 70, and 80°C). After cooling for 5 min, their activities were determined at 37°C according to activity assay.

Thermal Inactivation

Thermal inactivation studies were carried out at 65, 67, and 69°C. Samples of 2 mL of diluted enzyme solution were incubated 0, 10, 20, 40, and 60 min at each temperature. Activity assay at 37°C was applied immediately after incubations.

Substrate Specificity

Acid phosphatase was assayed for activity toward 5 m*M* diammonium phosphate, disodium phosphate, potassium phosphate, sodium pyrophosphate, sodiumpyrophosphate decahydrate, D-glucose-6-phosphate, pyridoxal-5-phosphate, and *o*-phosphorylethanolamine. Each substrate had its own blank sample consisting of buffer solution instead of enzyme. The same activity procedure was applied.

Effect of Enzyme Concentration

Enzyme was first brought to a 17 U/mg concentration and then 8.5, 4.25, and 2.125 U/mg concentrations by diluting with buffer solution. Activities of samples were determined at 37°C.

Effect of Cations on Enzyme Activity

Studies of the effect of metal ions on enzyme activity showed both an activating and a deactivating effect. While Cu, Mo, and Mn showed strong inhibitory effects, Na, Ca, and K were the significant activators of acid phosphatase. Experiments were carried out with different amounts of inhibitors over which 2.25 mL of *p*-NPP substrate was added. After 5 min at 37°C, 0.25 mL of enzyme was added to the 30-min reaction.

Kinetic Constants

Substrate concentration is an important factor in determining the rate of enzyme reaction. When an initial velocity is plotted against substrate concentration while enzyme concentration is kept fixed, a saturation curve (Michaelis-Menten curve) can be obtained. The specificity of garlic seedling acid phosphatase was established by determining the Michaelis constants for two different substrates. *p*-NPP and *p*-NP were preferred substrates. Substrate solutions were prepared by diluting of concentrated solutions with the acetate buffer. Apparent K_m and V_{max} values were estimated using substrate concentrations ranging from 2.5 to 20 m*M*. K_m values were calculated using a Lineweaver-Burk plot.

Activation Energy

Activation energy, *A*, of acid phosphatase is calculated by Arrhenius equation

$$k = A \cdot e^{-E_a RT}$$

by substituting the initial rates, *V*, instead of *k*. Two different substrates (*p*-NPP and *p*-NP) with different concentrations were used.

Results and Discussion

This will be the first report on purification and characterization of acid phosphatase, which was isolated from garlic seedlings, as shown in Table 1. Acid phosphatase of garlic seedlings was purified from crude homogenate by the following steps: centrifugation, treatment with ammonium sulfate, and DEAE cellulose ion-exchange chromatography.

The specific activity of the crude homogenate was found to be 4.33 U/mg of protein, which is a quite good result when compared with previous results from different plant sources in the literature *(2,4–7)*. The specific activities at each of the isolation and purification steps are as follows: 74 U/mg of protein after saturation to 40–80% with ammonium sulfate, and 168 U/mg of protein after DEAE Sepharose column. After a 40-fold purification, the enzyme was obtained in an 18% yield when assayed with *p*-NPP.

After DEAE cellulose column, activity was quite high although the protein was low. This resulted in the enzyme being almost brought to homogeneity. However, most of the acid phosphatase literature is concerned with the seedling and leaf source of the enzyme. Purifications of 100-fold are typical, and although some 700- to 1000-fold purifications

Table 1
Isolation and Purificaiton Steps of Garlic Seedling Acid Phosphatase

Step	Total protein (mg)	Specific activity (U/mg protein)	Total activity (U)	Yield (%)	Purification (fold)
Crude extract	1392	4.33	6029	100	1
Saturation to 40–80% with $(NH_4)_2SO_4$ DEAE cellulose ion-exchange column	6.5	168	1090	18	39

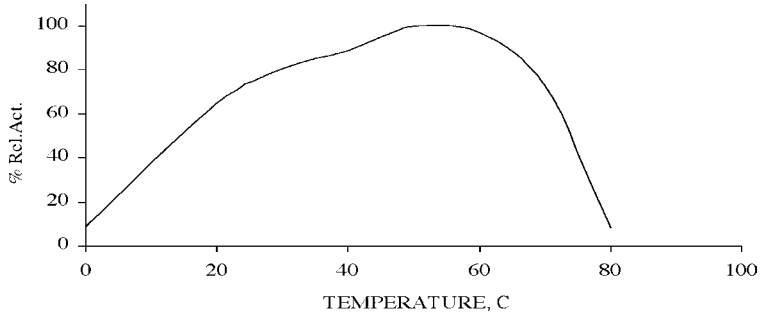

Fig. 1. Optimum temperature.

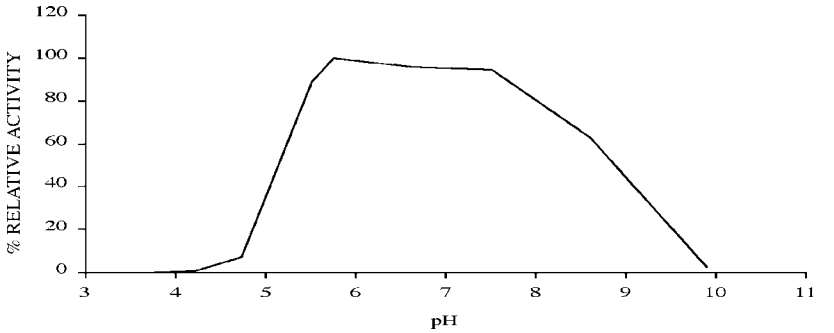

Fig. 2. Optimum pH.

have been described, few present any evidence for the catalytic purity of the preparations *(8)*.

To determine the effect of some parameters on partially purified acid phosphatase enzyme and its stability, pH and temperature assays were done. Activity of garlic acid phosphatase toward *p*-NPP was investigated since some enzymatic activity toward this substrate is present in many plant-sourced acid phosphatases. *p*-NPP could be obtained in an optically active form, with the chirality at phosphorus.

Enzyme activity was stable between pH 5.5 and 7.5 and 40–60°C. Maximum activity was obtained at pH 5.7, and garlic seedling acid phosphatase presented high activity at 50°C, which is higher than described for some other plant phosphatases, such as barley roots (30–35°C) and cotton seed (37°C), when *p*-NPP was used as substrate *(9)* as shown in Figs. 1–4.

The activity of acid phosphatase toward various substrates is shown in Table 2. We used some inorganic and organic phosphates that were not commonly used in the literature, to examine whether the isolated enzyme could be used in remediation as phosphate decomposer. Garlic acid phosphatase did not show activity with most of these substrates, a few of which were utilized successfully with other acid phosphatases reported in the literature *(7)*.

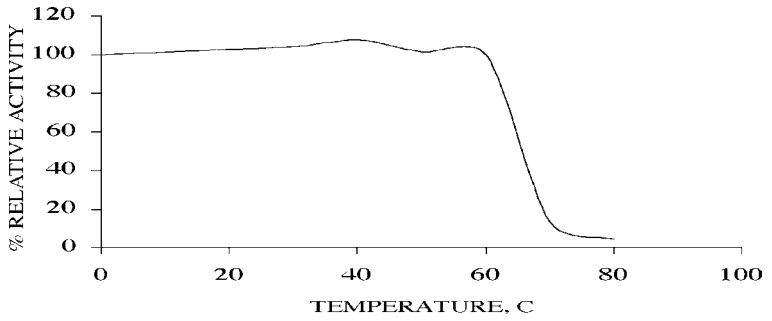

Fig. 3. Thermal stability.

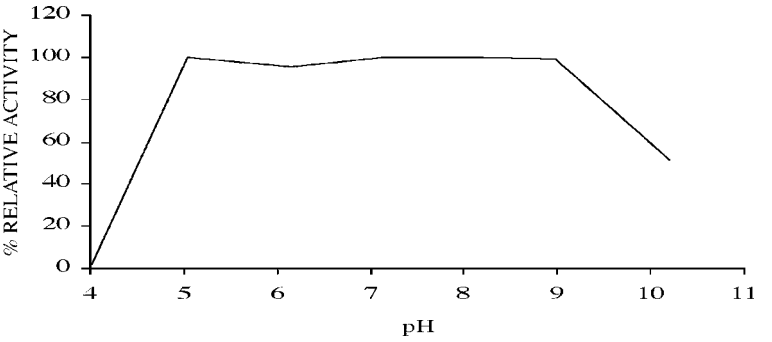

Fig. 4. PH stability.

Substrate concentration is an important factor in determining the degree of enzyme reaction. The results of our study are given in Table 3.

Generally, 5 m*M* *p*-NPP is used as substrate for acid phosphatase studies reported in the literature. In our study, 2.5 and 5 m*M* *p*-NPP showed the same characteristics in the first 10 min, but, on the other hand, 1.25 m*M* *p*-NPP showed quite higher activity. Activities came closer to each other after 30 min, and after 50 min all substrate concentrations showed the same characteristics. This led us to believe that a 1 m*M* *p*-NPP concentration could be used in further studies.

Garlic seedling acid phosphatase lost its activity after 60°C. It can be seen in Fig. 5 how fast this inactivation was, especially for 65°C. Inactivation occurred nonlinearly and fast at 62, 65, and 67°C. It can be concluded that temperature control could be effectively possible up to 65°C. Proteins in enzyme structure will be denatured after 65°C.

The results of changes in enzyme concentration are shown in Fig. 6. Enzyme concentrations of 17, 8.5, and 4.25 U/mg gave linear results but a concentration of 2.125 U/mg showed diversions from linearity. It was thought that there was a relationship between free water molecules coming from dilution and active sites. The same situation had been reported for

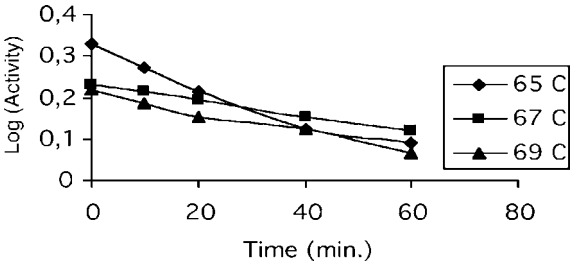

Fig. 5. Thermal inactivation.

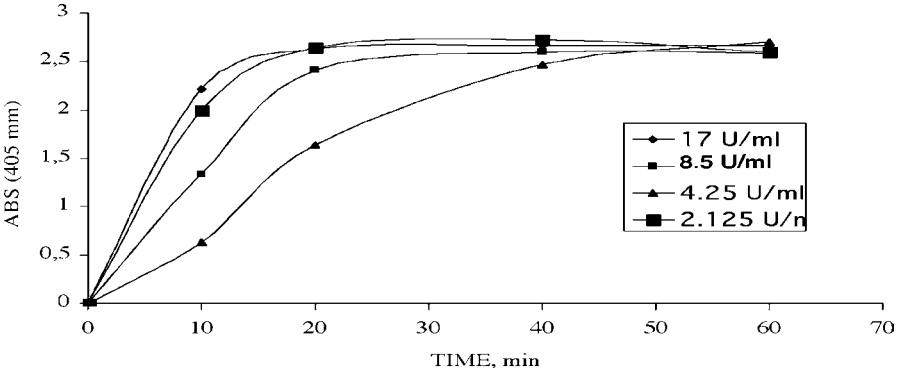

Fig. 6. Enzyme concentration.

Table 2
Substrate Specificity

Substrate name	Specific activity (U/mg)
Di.-ammonium phosphate	0
Di.-sodium phosphate	0
Potassiium phosphate	0
Sodium pyrophyosphate	0
Tetrasodium pyrophosphate decahydrate	0
D-Glucose-6-phosphate	0
Pyridoxal-5-phosphate	1.5
O-Phosphorylethanolamine	0

Table 3
Substrate Concentration

Time (min)	1.25 m*M p*-NPP	2.5 m*M*p-*NPP*	*5 m*M p-*NPP*
10	0.625	0.517	0.577
30	1.388	1.464	1.528
50	1.579	1.552	1.661

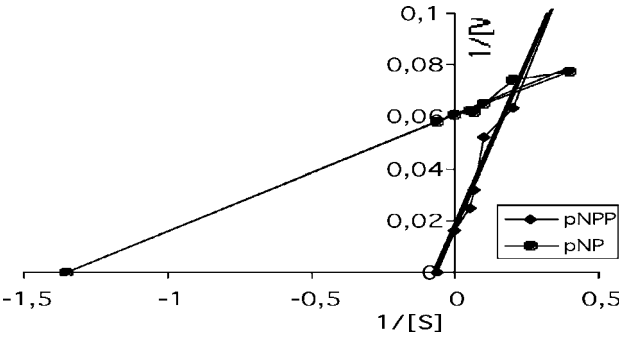

Fig. 7. Lineweaver-Burk plot of acid phosphatase activity with *p*-NPP and *p*-NP as substrate at pH 5.5.

Table 4
Effect of Metal Ions on Enzyme Activites

Metal ions (1 m*M*)	Relative activity (%)[a]
None	100
Copper	66
Molybdate	40
Manganese	66
Dodium	163
Calcium	165
Potassium	215

[a]Relative activity is expressed as percentage of the activity of no addition.

lipases. For further information about this situation, amino acid composition of this enzyme should be investigated.

Only two substrates, *p*-NPP and *p*-NP, were used for Michaelis kinetic constants. Figure 7 shows that V_m for *p*-NPP was 100 m*M*/s and K_m was 21.27 m*M*, while V_m for *p*-NP was 20 m*M*/s and K_m is 8.33 m*M*. It is obvious that the reaction was faster with *p*-NPP. Nonlinear plots may be owing to diesterase contaminants. *p*-NPP could be a substrate for some possible diesterase contaminants since it has been proposed as a specific substrate for certain phosphodiesterases *(5)*.

The effects of several substances on enzyme activity with *p*-NPP as substrate were measured (Table 4). Inhibition of acid phosphatases by copper, manganese, and molybdate has been observed in sweet potato, cultured tobacco cells, rice bran, gladiolus bulbs *(7)*, Japanese radish *(7)*, lentil seeds *(10)* and wheat germ *(3)*. On the other hand, the activators sodium, calcium, and potassium, are also listed Table 4. Calcium has been observed in wheat germ *(3)*, sweet Spanish *(2)*, and rice bran *(11)*.

The activation energies were between 10 and 20 kcal. Substrate concentrations ranged between 1.25 and 20 m*M* for *p*-NPP and *p*-NP, a result quite parallel with findings in the literature. Activation energy was slightly higher with *p*-NP.

Initial velocity rates were determined with different substrate concentrations of 5, 10, 15, and 20 m*M*. Activities after 5 min were found to be 2.8×10^{-3}, 4×10^{-3}, 6.4×10^{-3}, and 6.9×10^{-3}, respectively.

Considering the specific activities despite the little weight of the seedlings and small volume of the crude extract, it can be concluded that the seedlings consist of a significant amount of acid phosphatase enzyme. Further studies using a more appropriate purification procedure may lead to the use of garlic seedlings as an economic source of acid phosphatase enzyme.

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