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Purification and Characterization of Acid Phosphatase from Hypericium (*Hypericum perforatum* L.)

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ABSTRACT

In this research, acid phosphatase was purified and characterized from hypericium (*Hypericum Perforatum* L.) using anion exchange chromatography. Acid phosphatase was eluted separately with a concentration gradient from a DEAE-selulose column. The activity was determined by using pNPP as substrate. Additionally, protein was determined by the Bradford method. The optimum pH and optimum temperature values for acid phosphatase enzyme were determined, and than Km and Vmax values for the same substrate were obtained by mean of Lineweaver-Burk graphics. The optimum pH of acid phosphatase was 5. The optimum temperature was 10°C. The molecular weight of acid phosphatase was found to be 18 kDa and 57 kDa by gel filtration chromatography. Km and Vmax values at optimum pH and 15°C were 0.03 mM, 9.3 μmol/L min for p-nitrophenyl phosphate respectively. The purification degree for acid phospahatase was determined by SDS-PAGE and two distinct bands appeared. On the other hand, MoO_4^{2-} inhibited, but Cu^{2+} increased activity of acid phosphatase. The I_{50} value for MoO_4^{2-} was 0.03 mM.

Key Words: Hypericium; Acid phosphatase; Cupper; Molybdate; Purification-characterization

INTRODUCTION

Acid phosphatases (EC 3.1.3.2) catalyze the nonspecific hydrolysis of phosphate monoesters and oxygen exchange from water into inorganic phosphate in an acidic environment (Hollander, 1971). These enzymes are widely distributed in mammalian serum (Nakanishi *et al*., 2000), plants (Yam *et al*., 1971) and in microorganisms (Nakanishi, 2000).

Plant acid phosphatases have been characterized from roots, tubers, bulbs, seeds, aleurone layer, leaves, maize scutellum, and suspensions cells. These phosphatases, usually presented in multiple forms, display different biochemical properties and exhibit a broad specificity at pH optima below 6.0. Many roles have been ascribed to acid phosphatases in plants, including participation on signal transduction, regulation of metabolism by protein dephosphorylation and the release of inorganic phosphatase from organic phosphate in the environment. Phosphorus not only plays a vital role in energy transfer and in metabolic regulation, but it also is a constituent of phospholipids and nucleic acids. The growth and development of plants is particularly dependent on the availability of phosphate (Ferreira *et al*., 1998).

The purpose of this study was to isolate acid phosphatase from *Hypericum perforatum.* Perforatum is used for "perforated" in Latin. The leaves of *H. perforatum,* when held to the light, reveal translucent dots, giving the impression that the leaf is perforated. The dots are not holes in the leaf, but a layer of colorless essential oils and resin. The flowers are bright yellow-orange. The petals are peppered with black dots. When the black dots are rubbed between the fingers, the fingers become red. Many herbalists say the translucent as "perforations" and the black-red spots contain the most active medicinal qualities (Cott & Fugh, 1998).

MATERIALS AND METHODS

Hypericium (*Hypericum perforatum* L.) was collected from the West Anatolia region of Turkey and the flowers and leaves were separated mechanically. Acid phosphatase was purified from the flowers and leaves

Extraction and purification of enzyme. Hypericiums were cut and each 0.5 kg of plant was suspended in 1 L of buffer (0.05 M sodium phosphate, 0.01 M β-mercaptoethanol, pH 7.0). All these procedures were performed at 4°C in a cold room. The suspension was filtered twice through a fiberglass window screen. The filtrate was centrifuged and the precipitate was discarded. The supernatant fluid was brought to 45% saturation by the addition of solid ammonium sulfate (Sugiura *et al*., 1981). The mixture was stirred for 1 hour at 4°C and then centrifuged for 5 minutes at 10,000 rpm. The supernatant was further cleared by filtration through a filter pad. The enzyme precipitated was dialyzed overnight against distilled water. It was then dialyzed for 3 hours against 2 L of the buffer (0.1 M sodium-acetate, 0.01 M β-mercaptoethanol, pH 7.0). Insoluble material solution was cleared by ammonium sulfate, and then the solution was centrifuged

for 20 minutes (8,000 rpm) at room temperature; it was later centrifuged at 8,000 rpm for 5 minutes at 0°C. Protein concentrations and activities were determined at each step.

The enzymes present in the extracts were purified with ion exchange chromatography on a 3x50 cm column that contained DEAE-Cellulose. Elution was carried out with 0.2 M sodium-acetate, and 0.01 M β-mercaptoethanol, at pH 6, 5 (Sugiura *et al*., 1981).

Determination of acid phosphatase activity. Acid Phosphatase ACP activity was determined using pNPP tablets (1 g/mL). Enzyme activity was assayed in a reaction mixture (0.5 mL) containing 4.6 mM pNPP and 100 mM CH_3COONa, pH 5.5, at 15°C. Assays were started by the addition of enzyme, and after 20 min 0.2 M NaOH (0.5 mL) was added to the reaction mixture to stop the reaction. Nonenzymatic hydrolysis of pNPP was corrected by including control assays without added enzyme. The concentration of liberated p-nitrophenol was determined from the absorbance at 410 nm. A molar extinction coefficient (Δ_E) of 17,800 $M^{-1}cm^{-1}$ for pNPP was used in the calculations. One unit of activity was defined as the amount of enzyme providing the formation of 1 μmol of p-nitrophenol/min at 15°C (Olczak *et al*., 1997).

Protein determination. After scanning at 280 nm, the tubes with significant absorbance were pooled and a quantitative protein was determined by the Coomassie Blue G-250 method (Bradford, 1976).

The effect of MoO^{2-} and Cu^{2+} on acid phosphatase. The effects of sodium molybdate and cupper sulphate on acid phosphatase were investigated. It was prepared using three distinct solution as 10^{-2}, 10^{-4} and 10^{-6} M. 50, 100, 150, 200 and 250 μL effectors of this solution were added in cuvette consist of 0.5 mL substrate and 0.5 mL enzyme. The effect of effectors on activity of enzyme was investigated by measuring increased absorbance a 410 nm.

SDS-page. The purity of ACP enzyme was analyzed by 3-10% discountinuous SDS-polyacrylamid gel electrophoresis (Laemmli *et al*., 1970).

Determination of molecular weight with gel filtration. For this purpose, a column (3x70 cm) was filled with Sephacryl S-200. The column was balanced for 24 h with the buffer (0.1% Triton X-100, 0.2 M KCl, pH 5.5) until no absorbance was obtained at 280 nm. A protein standard solution composed with lysozyme (14 kDa), β-Lactoglobulin (Mr. 18 kDa), tripsinojen HMSF (Mr. 24 kDa), pepsin (Mr. 34 kDa), albumin egg (Mr. 45 kDa) and albumin bovine (Mr. 66 kDa) was added to the column and standard graphics were obtained. The protein solution concentration was 0.2 mg/mL. The standard proteins and AP isoenzymes were eluted under the same conditions in separate steps. The flow rate through the column was 20 mL/h (Demir *et al*., 2001).

RESULTS AND DISCUSSION

Our aim was to purify and to determine, biochemical characteristics of acid phosphate from hypericium. For this purpose, acid phosphatase of hypericium was purified by DEAE-celulose ion-change chromatography. The phosphatase activity of acid phosphatase was detected with p-nitrophenyl phosphate as substrate. In our research, the acid phosphatase of plant was purified 10 fold with ammonium sulfate precipitate at saturation 45% and 73 fold with DEAE-celulose (Table I).

As the acid phosphatase of hypericium exhibited phosphatase activities, enzyme K_M and V_{max} values were determined to be 0.03 mM and 9.3 μmol/L*min. respectively. Many others have reported that during chromatography, acid phosphatase activity was eluated in more than one protein peak. As a rule, only the fractions containing the highest activity were collected and used for further purification steps (Olczak *et al*., 1997). Graphs of activity-absorbance were drawn for the acid phosphatase (Fig. 1).

Optimal pH was 5 in the hypericium (Fig. 2). This value was not very different from all of plant acid phosphatase. pH has been noted to be 5.8 for sweet potato tubers and 5.6 for tobacco cells (Sugiura *et al*., 1981, Takako *et al*., 1990).

Four cytoplasmic isoforms of acid phosphatases, purified from mature soybean seeds showed high activities at temperatures above 80°C (Ferreira *et al*., 1998). In contrast to other, the optimum temperature was 10°C acid phosphatase in the hypericium, (Fig. 3). The range of activity temperature was from 0 to 85°C for this enzyme. The enzyme conserved 21% of its activity at 80°C.

The mammalian enzymes are closely related with respect to size (35 kDa). The well-characterized red kidney bean is a homodimer, each subunit of 50 kDa. Acid phosphatase from yellow lupin seeds is a dimmer with the 50 kDa and 44 kDa subunits. The moleculer mass of acide phosphatase from white lupin has been reported to be 68 and 61 kDa According to the results of gel filtration chromatography; the molecular weights of hypericium acid phosphatase were 57 and 18 kDa as revealed from electrophoresis (Schenk *et al*., 1999) (Fig. 4).

Table I. Acid phosphatase from hypericium (*Hypericum perforatum* L.)

Enzyme Fraction	Volume mL	Activity EU/mL	Total Activity EU %		Protein Mg/mL	Specific Activity EU/mg	Purification Fold
Crude extract	413	16.8	2940	100	57	0.29	
$(NH4)_2SO_4$%35	35	13.4	1273	43.2	4.3	3.12	10.9
After DEAE-Cellulose column	52	7.46	522	17	0.35	46.63	73

Fig. 1. The purification of acid phosphatase from hypericium (*Hypericum perforatum* L.)

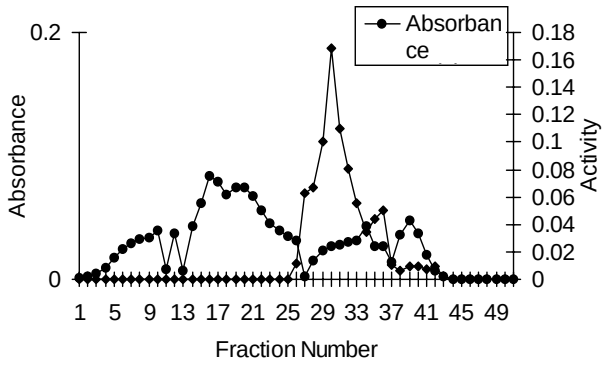


Fig. 2. The effect of pH on the purified acid phosphatase

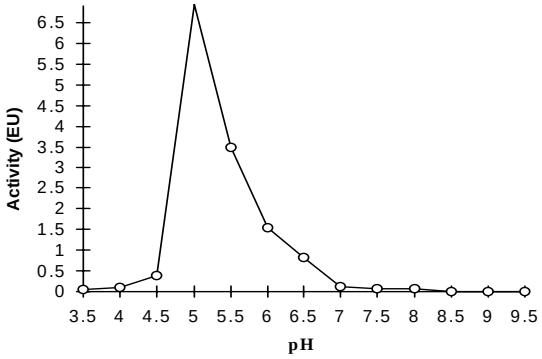


Fig. 3. The effect of temperature on the purified acid phosphatase

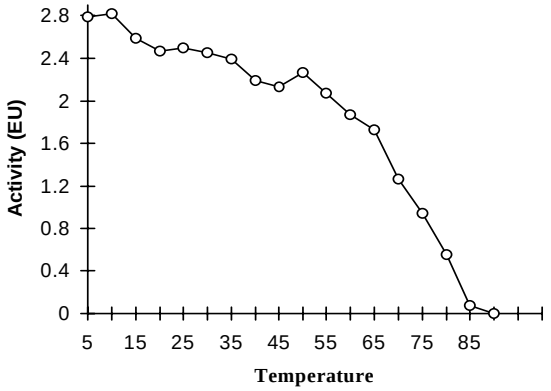


Fig. 5A. The effects of 10-2 M molybdate and cupper on acid phosphatase

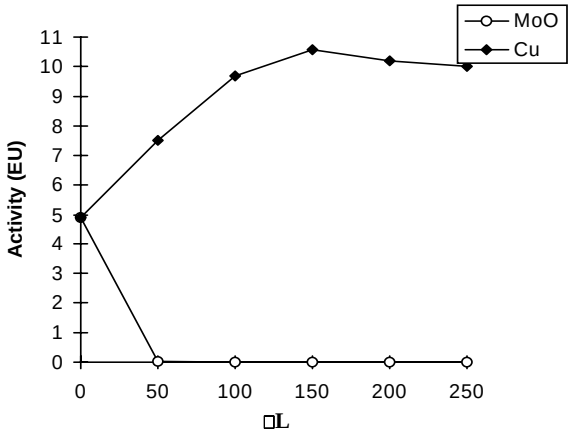


Fig. 5B. The effects of 10^{-4} M molybdate and cupper on acid phosphatase

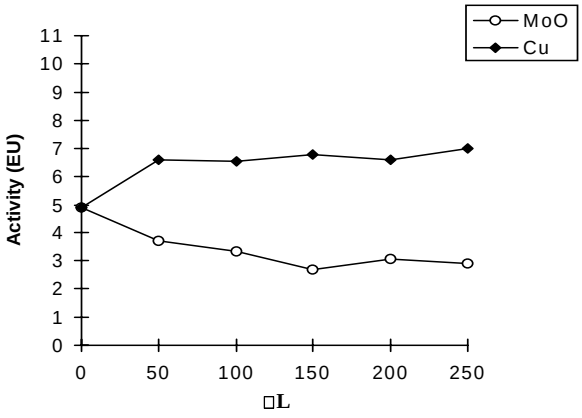


Fig. 5C. The effects of 10^{-6} M molybdate and cupper on acid phosphatase

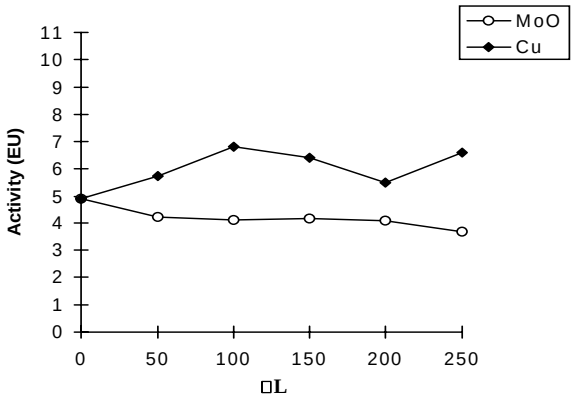
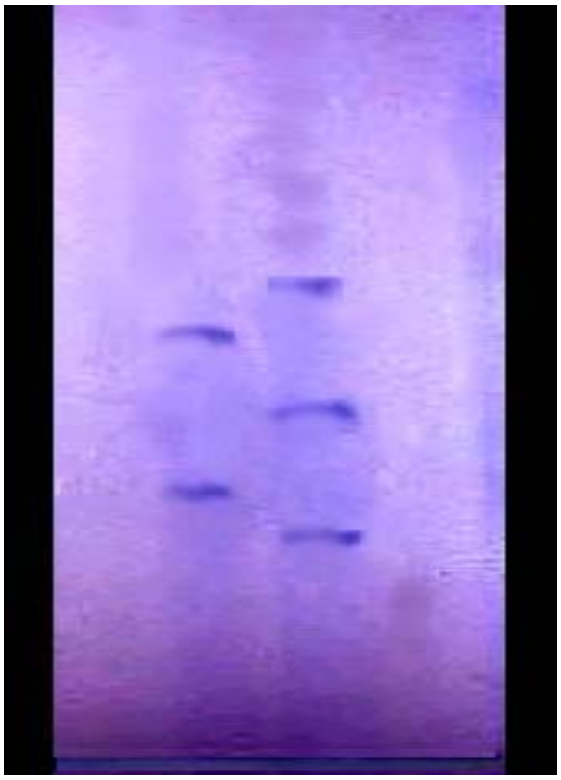


Fig. 4. SDS-polyacrylamide gel electrophoresis of acid phosphatase(Mr: 57, 18kDa)(I) purified from Hypericium(*Hypericum Perforatum* L.), and bovine serum albumin (66 kDa), pepsine (34 kDa) and lysozyme (14 kDa)(II)



I II

The activities of purified acid phosphatase were determined against the effect of Cu^{2+} and MoO_4^{2-}, all of which are known effectors of plant acid phosphatase. Copper ions inhibited the acid phosphatase of sweet potato (Sugiura *et al*., 1981). In contrast, acid phosphatase of the hypericium remained active under cupper ions. Acid phosphatase from yellow lupin seeds was inhibited by molybdate (Olczak *et al*., 1997). Sodium molybdate was inhibited acid phosphatase acitivity. Each inhibition and activition are much more upper concentration than lower concentration.

The acid phosphatase activity was decrease to be competitive by cupper ions and its Ki value was found to be $3{,}96x10^{-5}$M (Fig. 5A, B & C).

The characterization of acid phosphatase is important, as it is believed to play a role in germination. As a result of our study, acid phosphatase was purified and characterized from the hypercium, which is a plant thought to have healing qualities.

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