

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Isolation and Characterization of Phosphatase Enzyme from the Freshwater Macroalga *Cladophora glomerata* Kützing (Chlorophyta)

A.M. El-Shahed, H. Ibrahim and M. Abd-Elnaeim
Department of Botany, Faculty of Science, Minia University, Minia, 61519, Egypt

Abstract: Acid phosphatase enzyme (acPase) has been isolated from *Cladophora glomerata* Kützing and its kinetic properties were investigated. The optimum pH for activity of both the two isolated fractions; the cell wall-bound and the secreted acPases was found to be 4.5. Both fractions exhibited another peak of activity at 9.5 which was attributed to an alkaline phosphatase. The optimum protein concentration that resulted in maximal enzyme activities for both fractions was 8 mg mL⁻¹. The Km value determined with p-nitrophenyl phosphate (pNPP) was 24 and 14 mM for the cell wall-bound and the secreted acPases, respectively. The enzyme was inhibited by phosphate in the form of KH₂PO₄, molybdate in the form of K₂MoO₄, zinc in the form of ZnCl₂.4H₂O and iodine as KI. Zinc ions were the most potent inhibitor among all the tested inhibitors (max. % inhibition was 96 and 80% for the cell wall-bound and the secreted acPases, respectively). Molybdate had the least inhibitory effects (72 and 50%) on the cell wall-bound and the secreted acPases respectively at 40 mM concentration.

Key words: Phosphatase, enzyme isolation, kinetic characterization

INTRODUCTION

Phosphatases are enzymes that are believed to have an essential function in nutrient dynamics in aquatic habitats since they promote the degradation of complex phosphate compounds into orthophosphates and organic moieties (Jansson *et al.*, 1988). Synthesis of phosphatases with external function has been demonstrated in algal cultures (Healey, 1973; Healey and Hendzel, 1979; Wynne, 1981). Phosphatases are located either on the cell surface (Brandes and Elston, 1965) or in cell membrane (Kuenzler and Perras, 1965; Moller *et al.*, 1975) as well as released as extracellular secretions (Aaronson and Panti, 1976; Wynne, 1981).

Phosphatase activity has been found in all major groups of algae (Jansson *et al.*, 1988). Phosphatases typically have maximum hydrolyzing capacity at different pH values and are commonly divided into alkaline and acid phosphatases. Both alkaline and acid phosphatases have been found as external and internal enzymes in algae (Siuda, 1984). There have been a number of reports describing the purification and characterization of phosphatases from green algae, however, most of which dealt with alkaline phosphatases. *Chlamydomonas reinhardtii* was found to produce an extracellular phosphatase which was biochemically estimated and characterized in response to phosphorus deprivation (Quisel *et al.*, 1996). An alkaline phosphatase activity has been described in two planktonic desmid species;

Cosmarium abberiatum and *Staurastrum chaetoceras* (Spijkerman and Coesel, 1995). Red algae were also found to produce extracellular alkaline phosphatases in axenic cultures (Walther and Fries, 1976; Cashikar and Rao 1996a, b).

Plant acid phosphatases have been characterized in seeds (Tapan *et al.*, 1996; Olczak *et al.*, 1997); seedlings (Bhargava and Sacher, 1987; Haraguchi *et al.*, 1990); roots (Armienta-Aldana and Gonzalez De La Vara, 2004); leaves (Tanaka *et al.*, 1990) and pollen grains (Ibrahim *et al.*, 2002; Ibrahim, 2005).

Despite the fact that phosphorus is an important and often limiting nutrient, we have little knowledge on the nature and regulation of phosphatases in fresh water algae. An understanding of the properties of these enzymes may help to identify the types of organic phosphates that are available to different organisms and the role that these organisms play in facilitating the turnover of organic phosphates in the environment. Additionally, by studying the regulation of algal phosphatases, we may elucidate the mechanisms by which photosynthetic organisms respond to change the levels of phosphorus in their environments.

In this study, we aimed at isolation of acid phosphatases, from the fresh water macroalga *Cladophora glomerata*, their biochemical characterization and defining their kinetic properties in order to accumulate data which may help in understanding the physiological role played by these enzymes in aquatic environments.

MATERIALS AND METHODS

Isolation of fractions enriched in bound and secreted acid phosphatases: Samples of *Cladophora glomerata* were collected from a stream in Minia town, Egypt and stored at -80°C after washing several times with distilled water. Frozen tissue was used for preparing fractions of the cell wall bound and the secreted acid phosphatases. About 0.6 g fresh weight of the alga were suspended in 5 mL of Chu 10 medium (Bold and Wynne, 1978), shaken gently and pelleted by centrifugation at 13,000 g for 15 min. at 4°C . The supernatant was collected and the algal tissue pellets were resuspended in the same medium. Washing was repeated five times. The first and the second supernatants were pooled and represented the fraction of cell wall-bound acid phosphatase. The algal pellet was grown in the growing medium for 4 days at room temperature ($24\pm 2^{\circ}\text{C}$) to obtain the secreted fraction of the enzyme. The secretion of the enzyme was followed according to Pfeiffer (1996).

Assay of acid phosphatase activity: The activity of the enzyme was determined by measuring the release of p-nitrophenol (pNP) from p-nitrophenyl phosphate (pNPP). Samples of 200 μL containing enzyme were incubated with 200 μL reaction buffer containing 40 mM Mes/Tris, pH 4.5, 5 mM pNPP and 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ for 45 min at 30°C . The reaction was stopped and the colour was developed by addition of 800 μL of 400 mM borate buffer, pH 9.8. The concentrations of pNP were determined using a linear regression of calibration standards. All experiments were performed in ice as triplicates.

Biochemical tests: To characterize the enzyme biochemically, samples of bound and secreted fractions of the enzyme were adjusted to the same protein content in a volume of 200 μL . The concentration of the enzyme was measured according to Lowry *et al.* (1951).

The pH dependence of the enzyme was assayed using the reaction buffer at various pH values ranging from 3 to 10 at 0.5 pH unit steps. The activity was measured as mentioned above.

For studying the effect of substrate concentration [pNPP] on the activity of acid phosphatase, samples of both the two fractions containing the same concentration of protein were added to 200 μL reaction buffer (pH 4.5) containing various concentrations of p-nitrophenyl phosphate. To obtain the Michaelis-Menten constant (K_m) of p-nitrophenyl phosphate, 50% of the enzyme activity was estimated.

The effect of some inhibitory ions on the activity of the enzyme at various concentrations of the inhibitors were tested in the reaction mixture. The activity of the enzyme was measured as mentioned before using methods of Pfeiffer (1996). Inhibition of the enzyme activity (%) was calculated using the formula:

$$\% \text{ inhibition} = \frac{[(\text{Activity of blank} - \text{Activity of sample}) / \text{activity of blank}] \times 100.}$$

The tested inhibitors were: phosphate as KH_2PO_4 , molybdate as K_2MoO_4 , Zinc as $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$ and iodide in the form of KI. Potassium and chloride salts of the tested ions are used since they are known to be of no effect on enzyme activity.

RESULTS AND DISCUSSION

Both fractions of the isolated acid phosphatase from *Cladophora glomerata*, the cell wall bound and the secreted, had the same pH optimum for activity at 4.5 (Fig. 1A). These results were typically identical to the results obtained by Kruskopf and Du Plessis (2004) for *Chlorella* sp. and *Chlamydomonas* sp. isolated from an European reservoir. Similar results were obtained for different plant species such as white clover roots (Zhang and McManus, 2000) and garlic seedlings (Yenigun and Guvenilir, 2003). The appearance of a second peak of activity for both protein fractions indicates the presence of an alkaline phosphatase with an optimum pH at 9.5 in the isolated protein fraction (Fig. 1A). In accordance with these results, alkaline phosphatase activity was obtained from the dinoflagellate *Peridinium cinctum* (Carpene and Wynne, 2002), the chrysophyte *Synura petersenii* which was isolated from an eutrophic lake in Israel (Strojsova *et al.*, 2003) and from phosphorus-deprived *Chlamydomonas reinhardtii* (Quisel *et al.*, 1996).

The optimal protein concentration that resulted in maximal enzyme activities for both the two fractions is presented in Fig. 1B which indicates that protein concentration of 8 mg mL^{-1} produced about the maximal enzyme activity for both acid phosphatases.

It is clearly shown that substrate concentration of 30 mM resulted in a maximal enzyme activity for both the two fractions (Fig. 1C). Both acid phosphatase activities showed similar substrate concentration dependence with a K_m of 24 and 14 mM for the cell wall-bound acPase and the secreted one, respectively. Also in accordance with our results, the apparent K_m for pNPP was determined by Yenigun and Guvenilir (2003) for the enzyme isolated from garlic seedlings as 21.3 and 12.9 mM for the bound and secreted acPases, respectively.

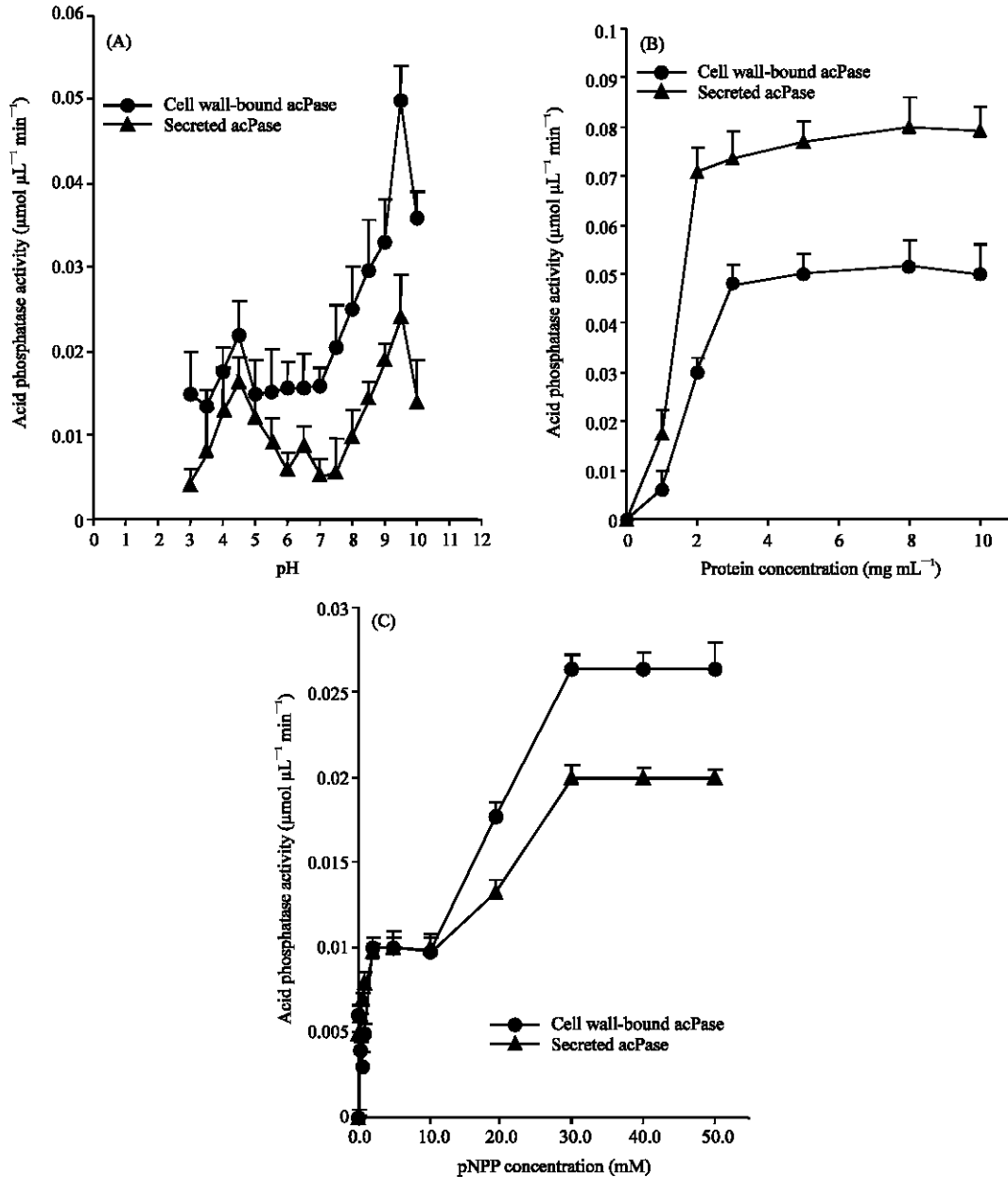


Fig. (1A-C): Biochemical characterization of an acid phosphatase from the freshwater macroalga *Cladophora glomerata* after 4 days of incubation in growth medium, A; pH dependence, B; enzyme concentration dependence, C; substrate concentration dependence. Data are mean values of three experiments ±SD

Testing the effect of phosphatase inhibitors was performed to further characterize the properties of both acPase activities. Figure (2A-D) shows that all the tested inhibitory ions caused maximal inhibition at 40 μM. Zinc ions inhibited the activity of the cell wall-bound and the secreted fractions by 96 and 80%, respectively at the highest concentration 40 mM. Phosphate ions inhibited the cell wall-bound by 95% and the secreted one by 50%.

Molybdate ions showed a slight inhibition on acPase fractions when compared to other ions since the cell wall-bound and the secreted acPase activities were reduced by 72 and 50%, respectively at the highest ion concentration. Iodide ions caused a detectable inhibition of both enzyme activities by 95 and 55% for the cell wall-bound and the secreted acPases, respectively (Table 1). The studied inhibitory ions on enzyme activity

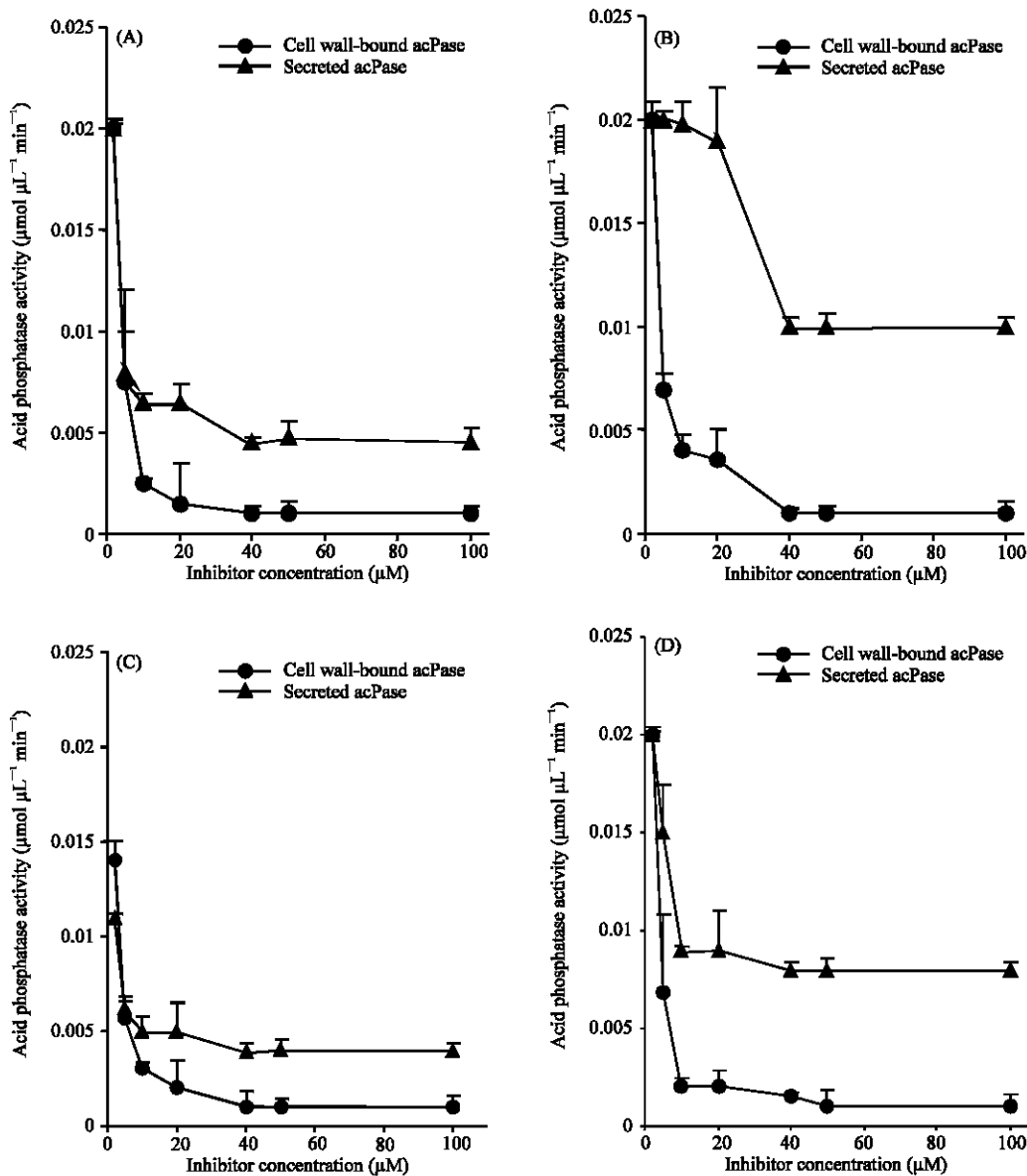


Fig. (2A-D): Effect of some inhibitory ions at various concentrations on acid phosphatase from the freshwater macroalga *Cladophora glomerata* after 4 days of incubation in growth medium, A; ZnCl₂ · 4H₂O, B; KH₂PO₄, C; K₂MoO₄, D; KI. Data are mean values of three experiments±SD

Table 1: Inhibition of the cell wall-bound and the secreted acid phosphatases isolated from the macroalga *Cladophora glomerata* by various compounds

Inhibitor	Maximum% inhibition	
	Cell wall-bound	Secreted
Zinc	96±2.2	80±1.3
Phosphate	95±3.6	50±2.0
Molybdate	72±2.7	50±1.8
Iodide	95±4.3	55±2.2

Data represent mean values of three±SD. All inhibitory ions were added as potassium or chloride ions

in the present investigation showed that: First; the acid phosphatase activity of the fraction bound to the cell surface was strongly inhibited by phosphate, zinc and iodide ions. These results in this respect are similar to those obtained by Sugiura *et al.* (1981), Srivastava *et al.* (1995), Olczak *et al.* (1997), Zhang and McManus (2000) and Carpenne and Wynne (2002) but in contrast with Tanaka *et al.* (1990) who stated that the divalent ion zinc enhanced the activity of the enzyme isolated from tomato leaves. Second; lesser inhibitory effect was detected with

molybdate ions on both fractions and this is in contrast with the results obtained by Vescaia and Chance, (1958), Van-Etten and Hickey (1977), Van-Etten *et al.* (1974) who reported that molybdate is a competitive inhibitor and its inhibition effect is caused by the ability of the ion to function as a transition state analog at the active site on the enzyme.

Since acid phosphatases are very heterogeneous family of enzymes, classification of these enzymes needs further biochemical analyses like SDS-PAGE analysis in addition to the kinetic and biochemical behavior for more accurate identification.

REFERENCES

- Aaronson, S. and N.J. Pantl, 1976. The role of surface and extracellular phosphatases in the phosphorus requirement of *Ochromonas*. *Limnol. Oceanogr.*, 21: 838-845.
- Armienta-Aldana, E. and L.E. Gonzalez De La Vara, 2004. Acid phosphatase from beet root (*Beta vulgaris*) plasma membrane. *Physiol. Plant*, 121: 223-230.
- Bhargava, R. and R.C. Sacher, 1987. Induction of acid phosphatase in cotton seedlings: Enzyme purification, subunit structure and kinetic properties. *Phytochemistry*, 26: 1293-1297.
- Bold, H. and M. Wynne, 1978. Introduction to the Algae. In: *Cultivation of Algae in the Laboratory*. Bold, H. and M. Wynne (Eds.), Prentice-Hall, Inc., Englewood Cliffs, New Jersey, pp: 571-578.
- Brandes, D. and N. Elston, 1965. An electron microscopical study of the histochemical localization of alkaline phosphatase in the cell wall of *Chlorella vulgaris*. *Nature*, 17: 274-277.
- Carpene, E. and D. Wynne, 2002. Properties of acid and alkaline phosphatase from the dinoflagellate *Peridinium cinctum*. *Hydrobiology*, 38: 73-84.
- Cashikar, A.G. and N.M. Rao, 1996a. Role of the inter-subunit disulphide bond in the unfolding pathway of dimeric red kidney bean acid phosphatase. *Biochim. Biophys. Acta*, 1296: 76-84.
- Cashikar, A.G. and N.M. Rao, 1996b. Unfolding pathway in red kidney bean purple acid phosphatase is dependent on ligand binding. *J. Biol. Chem.*, 271: 4741-4746.
- Haraguchi, H., D. Yamauch and T. Minamikawa, 1990. Multiple forms of acid phosphatase in cotyledons of *Vigna mungo* seedlings: Immunological detection and quantification. *Plant Cell Physiol.*, 31: 917-923.
- Healey, F.P., 1973. Characterization of phosphorus deficiency in *Anabaena*. *J. Phycol.*, 9: 383-394.
- Healey, F.P. and L.L. Hendzel, 1979. Fluorometric measurement of alkaline phosphatase activity in algae. *Freshwater Biol.*, 9: 429-439.
- Ibrahim, H., H. Pertl, K. Pittertschatscher, E. Fadl-Allah, A. El-Shahed, F.W. Bentrub and G. Obermeyer, 2002. Release of an acid phosphatase during lily pollen tube growth involves components of the secreted pathway. *Protoplasma*, 219: 176-183.
- Ibrahim, H., 2005. Biochemical characterization of acid phosphatase from germinating pollen grains of *Zea mays*. *J. Assui. Univ. Bot.*, 34: 371-282.
- Jansson, M., H. Olsson and K. Pettersson, 1988. Phosphatases: Origin, characteristics and function in lakes. *Hydrobiology*, 170: 157-175.
- Kruskopf, M.M. and S. Du Plessis, 2004. Induction of both acid and alkaline phosphatase activities in two green-algae (Chlorophyceae) in low N and P Concentrations. *Hydrobiology*, 513: 59-70.
- Kuenzler, E.J. and P. Perras, 1965. Phosphatases of marine algae. *Biol. Bull. Woods Hole*, 128: 271-284.
- Lowry, O.H., N.J. Rosbrough, A.L. Farr and R. Randall, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- Moller, M., S. Myklestad and A. Haug, 1975. Alkaline and acid phosphatases of the marine diatoms *Chaetoceras affinis* var. *Willei* (Gran.) Hustedt and *Skeletonema costatum* (Grev.). *Cleve. J. Exp. Mar. Biol. Ecol.*, 19: 217-226.
- Olczak, M., W. Watorek and B. Morawiecka, 1997. Purification and characterization of acid phosphatase from yellow lupin (*Lupinus luteus*) seeds. *Biochim. Biophys. Acta*, 1341: 14-25.
- Pfeiffer, W., 1996. Auxin induces exocytosis of acid phosphatase in coleoptiles from *Zea mays*. *Physiol. Plant*, 98: 773-779.
- Quisel, J.D., D.D. Wykoff and A.R. Grossman, 1996. Biochemical characterization of the extracellular phosphatases produced by phosphorus-deprived *Clamydomonas reinhardtii*. *Plant Physiol.*, 111: 839-848.
- Siuda, W., 1984. Phosphatases and their role in organic phosphorus transformation in natural waters. *Pol. Arch. Hydrobiol.*, 31: 207-233.
- Spijkerman, E. and P.F.M. Coesel, 1995. Alkaline phosphatase activity in two planktonic desmid species and the possible role of the extracellular envelope. *Freshwater Biol.*, 3: 1-11.
- Srivastava, R., Y.S. Rajput, S.K. Khare and M.N. Gupta, 1995. Purification and characterization of an acid phosphatase from *Arachis hypogaea*. *Biochem. Mol. Biol. Intl.*, 35: 949-956.

- Strojsova, A., J. Vrba, J. Nedoma, J. Komarkova and P. Znachor, 2003. Seasonal study of extracellular phosphatase expression in the phytoplankton of an eutrophic reservoir. *Eur. J. Phycol.*, 38: 295-306.
- Sugiura, Y., H. Kawabe, H. Tanaka, S. Fujimoto and A. Ohara, 1981. Purification, enzymatic properties and active site environment of a novel manganese-containing acid phosphatase. *J. Biol. Chem.*, 256: 10664-10670.
- Tanaka, K.R., Masuda, T. Sugimoto, K. Omasa and T. Sakaki, 1990. Water deficiency-induced changes in the contents of defensive substances against active oxygen in spinach leaves. *Agric. Biol. Chem.*, 54: 2629-2634.
- Tapan, K., M. Promo and B. Biswas, 1996. Purification of acid phosphatase from germinating seeds of *Vigna sinensis*. *Phytochemistry*, 41: 1457-1458.
- Van-Etten, R.L., P.P. Waymack and D.M. Rehkop, 1974. Transition metal ion inhibition of enzyme catalyzed phosphate ester displacement reactions. *J. Am. Chem. Soc.*, 96: 6782-6785.
- Van-Etten, R.L. and M.E. Hickey, 1977. Phosphohistidine as a stoichiometric intermediate in reactions catalyzed by isoenzymes of wheat germ acid phosphatase. *Arch. Biochim. Biophys.*, 183: 250-259.
- Vescia, A. and E.K. Chance, 1958. Evidence of different active groups for hydrolytic and transphosphorylating activities of acid phosphatase. *Biochim. Biophys. Acta*, 30: 446-447.
- Walther, K. and L. Fries, 1976. Extracellular alkaline phosphatase in multicellular marine algae and their utilization of glycerophosphate. *Physiol. Plant*, 36: 118-122.
- Wynne, D., 1981. The role of phosphatases in the metabolism of *Peridinium cinctum* from lake Kinneret. *Hydrobiology*, 83: 93-99.
- Yenigun, B. and Y. Guvenilir, 2003. Partial purification and kinetic characterization of acid phosphatase from garlic seedlings. *Applied Biochem. Biotechnol.*, 107: 677-688.
- Zhang, C. and M.T. McManus, 2000. Identification and characterization of two distinct acid phosphatases in cell walls of roots of white clover. *Plant Physiol.*, 38: 259-270.