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**RESEARCH ARTICLE**

# PURIFICATION OF ACID PHOSPHATASE FROM GINGER (*ZINGIBER OFFICINALE*) RHIZOMES

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## *Abstract*

Acid phosphatase has been obtained from the rhizomes in an apparently homogenous form following ammonium sulphate (60%) fractionation, DEAE-cellulose ion exchange chromatography and sephadex G-100 gel filtration. The final step in the purification on sephadex G-100 gel filtration provided an yield of 27% with 5.8 fold purification of the enzyme. Acid phosphatase isolated by the purification procedure described here appears to be homogeneous by PAGE, SDS-PAGE and gel filtration on G-200. The specific staining for the enzyme activity gave a single band corresponding to protein staining by coomassie blue or silver. The molecular weight of acid phosphatase was determined to be 46kDa by SDS-PAGE and on sephadex G-200 gel filtration, the enzyme gave a molecular weight of 45.5kDa , a value very close to that of obtained by SDS-PAGE. The enzyme was found to be a monomeric protein active at pH 5.6 and temperature $40^0$C preferentially using sodium β-glycerophosphate as the substrate.



## INTRODUCTION

Acid phosphatases (Orthophosporic-monoester phosphohydrolase, EC 3.1.3.2) are ubiquitous in nature, found in animals, plants and microorganisms. In plants, they are abundant in storage organs such as seeds and tubers and distributed in different parts of the plants- bulbs, roots, stems, leaves, fruits, coleoptiles and pollen (Biswas *et al.,* 1991; Ferreira *et al.,* 1998; Granjeiro *et al.,* 1999; Gellatly *et al.,* 1994; Guo *et al.,* 1997; Shinano *et al.,* 2001; Penheiter *et al.,* 1997; Zhang *et al.,* 2000; Staswick *et al.,* 1994; Gabard *et al.,* 1986; Pasqualini *et al.,* 1997; Cirkovic, 2002.).

Acid phosphatases are involved in the metabolic processes of germination and maturation of plants and are constitutively expressed in seeds during germination, and their activities increase with germination to release the reserve materials for the growing embryo (Biwas and Cundiff, 1991; Thomas, 1993). Plant acid phosphatases have been reported to be induced under developmental conditions such as flowering, fruit ripening and seed germination (Turner and Plaxton, 2001; Bozzo *et al.,* 2002). Some acid phosphatases could be involved in protein dephosphorylations and therefore in signalling pathways (Duff *et al.* 1994).

Ginger, a herb, belongs to the family- *Zingiberaceae* is native to Asia, is a common cooking spice and can be found in a variety of foods and drinks, including ginger bread, ginger snaps, ginger sticks, and ginger ale. It is also used as a medicine in Asian, Indian, and Arabic herbal traditions since ancient times and has been used to help treat arthritis, colic, diarrhoea, common cold, flu-like symptoms, headaches, painful menstrual periods and heart conditions.

Although the functional significance of phosphatases is well known in prokaryotes and animals, it may as well be applicable for plants. Extensive studies have been carried out on this enzyme from plant storage organs such as seeds and tubers, cotyledons, roots, leaves, fruits. However, not much work has been carried out on phosphatases of rhizomes for understanding their role. The present study, therefore, focuses on the purification and partial characterization of acid phosphatase of ginger rhizome.

## Materials and Methods

**Source:** Fresh ginger root *(Zingiber officinale)* was procured from local market, washed with distilled water several times and then stored in an air tight container placed in the refrigerator at $5^0$C.

**Chemicals:** Sodium β- glycerophosphate was obtained from Kochlight Laboratories, England. Molecular markers-soyabean trypsin inhibitor (SBTI), chymotrypsinogen A, bovine serum albumin, ovalbumin, lysozyme, phosphorylase-b were procured from Sigma Chemical Company, U.S.A. Sephadex G-100 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide and N,$N^1$- methylene bisacrylamide were purchased from Central Drug House, Mumbai, India. Ammonium molybdate, 1-Amino -2-naphthal -4-sulphonic acid (ANSA) were of highest purity commercially available in India. All other chemicals used were of analytical grade.

**Preparation of ginger extract**

Ginger extract was prepared by grinding small pieces of 10g of ginger with acid washed sand in a mortar and pestle with ice cold 100ml of 0.1M citrate buffer, pH 5.6 at $5^0$C using tissue to buffer ratio of 1:10. The extract was then centrifuged at 12,000×g for 15 min at $4^0$C.

**Purification of acid phosphatase from ginger rhizomes**

A procedure has been devised for the purification of acid phosphatase from the ginger. 20g of ginger rhizome was homogenized with acid washed sand in a mortar and pestle with ice cold 200ml of 0.1M sodium citrate buffer, pH 5.6 at $4^0$C using tissue to buffer ratio of 1:10. The extract was then centrifuged at 8,000 rpm for 15 min at $4^0$C and the supernatant was collected and then subjected to ammonium sulphate fractionation.

To the enzyme solution (200ml), solid ammonium sulphate was added gradually with constant stirring at $4^0$C to obtain 60% saturation. The mixture was allowed to stand overnight at $5^0$C.The precipitate was collected by centrifugation at 8,000 rpm for 20min at $4^0$C,dissolved in 50 ml of 0.1M sodium citrate buffer pH 5.6 and dialyzed against the same buffer. The dialyzed and concentrated ammonium sulphate fraction was loaded on DEAE-cellulose (2.2×34 cm) previously equilibrated with 0.01M Tris-HCl buffer, pH 7.4. The unbound proteins were eluted with 100ml of the equilibration buffer and the bound material was then eluted with 75ml of same buffer each containing 0.1M NaCl and 0.2M NaCl. Fractions of 5 ml were collected at a flow rate of 30ml/h. The fractions were assayed for protein by measuring their absorbance at 280 nm as well as for the enzyme activity. The active fractions were pooled, dialyzed at $4^0$C and lyophilized and then loaded on Sephadex G-100 column (1.9 x 63 cm) which was previously equilibrated with 0.01 M Tris-HCl buffer, pH 7.4 and eluted with the same buffer. 2 ml fractions were collected at a flow rate of 12 ml/h and the protein was monitored by measuring the absorbance at 280 nm. The enzyme activities of the fractions were assayed using sodium β-glycerophosphate as the substrate. The active fractions containing the enzyme activities were pooled, dialyzed against distilled water at 4 °C and then lyophilized.

**Assay of acid phosphatase(EC 3.1.3.2)**

Acid phosphatase activity was determined according to the method of Naganna *et al.,* (1955) using β-glycero phosphate as the substrate.

To 5ml of buffered substrate (0.1M sodium β-glycerophosphate in 0.1M sodium citrate buffer, pH 5.6) preincubated for 5 min at $37^0$C, 1ml of enzyme extract was added and the reaction was continued for 30min and then terminated by adding 2.5 ml of 10%TCA. The contents were filtered in cold condition and the amount of inorganic phosphate liberated was estimated by the modified method of Fiske and Subbarow (1925). In the controls, TCA was added prior to the addition of the extract. One unit of acid phosphatase activity was expressed as µmoles of phosphate liberated for 30 min under the experimental conditions.

**Optimum pH and optimum temperature**

Acid phosphatase activity was determined using different buffers in a pH range of 3-9 by the method described earlier. Buffers used were sodium acetate, 0.1M pH (3-4), sodium citrate, 0.1 M pH (5-6) and Tris- HCl, 0.1M pH (7-9).

Optimum temperature was determined for acid phosphatase by measuring the activity in the temperature range $20^0$-$70^0$ C using the activity assay procedure.

**Protein estimation**

Protein was estimated by the method of Lowry *et al*., (1951) using bovine serum albumin as the standard.

**Polyacrylamide gel electrophoresis (PAGE)**

PAGE, at pH 8.3, was performed in 10% slab gels according to the method of Reisfeld *et al.*, (1962) as followed by Gabriel (1971).

**Silver staining of proteins**

This was done according to the method of Blum *et al*., (1987).After the electrophoretic run, the gel was fixed overnight in 100-200ml of fixative solution (50% methanol, 12% glacial acetic acid and 50µl of formaldehyde). Then the gel was washed thrice with 50% ethanol each time 20 min and then pretreated with sodium

thiosulphate (40mg /200ml water) for one min. The gel after rinsing with water was treated with silver nitrate solution (200mg silver nitrate and 75µl formaldehyde in 100ml water) for 20 min and again rinsed with water. The gel was then covered with freshly prepared developing solution (6g sodium carbonate and 50µl of formaldehyde in100ml water) until the protein bands reached the desired intensity of staining.

**Specific staining for acid phosphatase**

Visualisation of acid phosphatase in acrylamide gels was performed according to the method of Tono and Korenberg (1967). The slab gel was rinsed with distilled water, incubated with assay mixture (0.1M glycerophosphate in sodium citrate buffer, pH 5.6) for 5-10 min at $37^0$C. After incubation, the gel was rinsed with distilled water, then immersed in triethylamine-molybdate reagent of Sugino and Miyoshi (1964) which specifically precipitates inorganic phosphate. A sharp discrete zone corresponding to the enzyme activity in the gel appears in a few seconds.

**Molecular weight by SDS-PAGE**

SDS-PAGE was carried out by the method of Laemmli (1970) in slab gels. After electrophoretic run, proteins were fixed in glacial acetic acid 10% (v/v), methanol 30% (v/v) and were visualized using coomassie brilliant blue.

**Molecular weight determination by gel filtration on Sephadex G-200**

The method of Andrews (1964) was used to determine the molecular weight of the enzyme by molecular sieve chromatography on Sephadex G-200. Sephadex was swollen in 0.01 M Tris-HCl, pH 7.4 and packed in a column of dimensions 1.6 x 94 cm (bed volume 85 ml). The column was equilibrated and developed with the same buffer. The void volume (Vo) was determined using Blue Dextran. Fractions of 2 ml were collected at a flow rate of 10ml/h. Elution volume (Ve) for the molecular weight standards, phosphorylase b (97 kDa), BSA (67 kDa), ovalbumin (44 kDa), chymotrypsinogen A (25 kDa), SBTI (20.1 kDa) and lysozyme (14 kDa) was plotted against log molecular weight to obtain the calibration curve. The molecular weight of the enzyme was estimated from the calibration curve.

## Results and Discussion

**Isolation and purification**

Preliminary studies revealed that the acid phosphatase from ginger was stable at pH 6-9 and at temperatures below $60^0$C. All the purification steps, therefore, were carried out under controlled conditions i.e. pH 5.6, temperature $4^0$C.

Acid phosphatase from the ginger was purified by ammonium sulphate fractionation and chromatography on DEAE-cellulose and Sephadex G-100. The dialyzed and concentrated ammonium sulphate fraction (150mg) was loaded on DEAE- cellulose previously equilibrated with 0.01M Tris-HCl buffer, pH 7.4. The unbound proteins were eluted with 100ml of the equilibration buffer and the bound material was then eluted with 75ml of same buffer each containing 0.1M NaCl and 0.2M NaCl. The active fractions were pooled, dialyzed at $4^0$C and lyophilized. The elution profile of DEAE-cellulose chromatography for acid phosphatase is shown in Fig 1. The enzyme activity was, however, associated with a protein eluted from the column with the starting buffer containing 0.1M NaCl. Eluants from 0.3M, 0.4M and 0.5M NaCl did not exhibit acid phosphatase activity. About 60% of the protein present in ammonium sulphate fraction was unbound to DEAE-cellulose and appeared in the fractions 5-13.

Then the lyophilized DEAE sample (45mg) was dissolved in 0.01 M Tris-HCl buffer, pH 7.4 and was loaded on to Sephadex G-100 column. 2 ml fractions were collected at a flow rate of 12 ml/h and the protein was monitored by measuring the absorbance at 280 nm. The elution profile of this gel permeation chromatography is shown in Fig 2. The fractions (30-37) containing the enzyme activities were pooled, dialyzed against distilled water at 4 °C and then lyophilized (protein yield 25 mg).

Recoveries and relative purification at each step for a typical purification from 20 g of ginger is shown in Table -1. By this procedure, about 25 mg of the enzyme was obtained and the final yield of enzyme was about 27%.

**Homogeneity and physico-chemical properties of acid phosphatase**

**Polyacrylamide gel electrophoresis**

PAGE was carried out under non-denaturing conditions using slab gels. A single protein band was obtained at alkaline pH in 10% slab gels. Specific staining of the acid phosphatase also showed a single band corresponding to the coomassie blue and silver stainable bands (Fig 3). The enzyme was not positive towards PAS stain suggesting the absence of carbohydrate moieties in the protein.

**Molecular weight determination by SDS-PAGE and Gel filtration on Sephadex G-200**

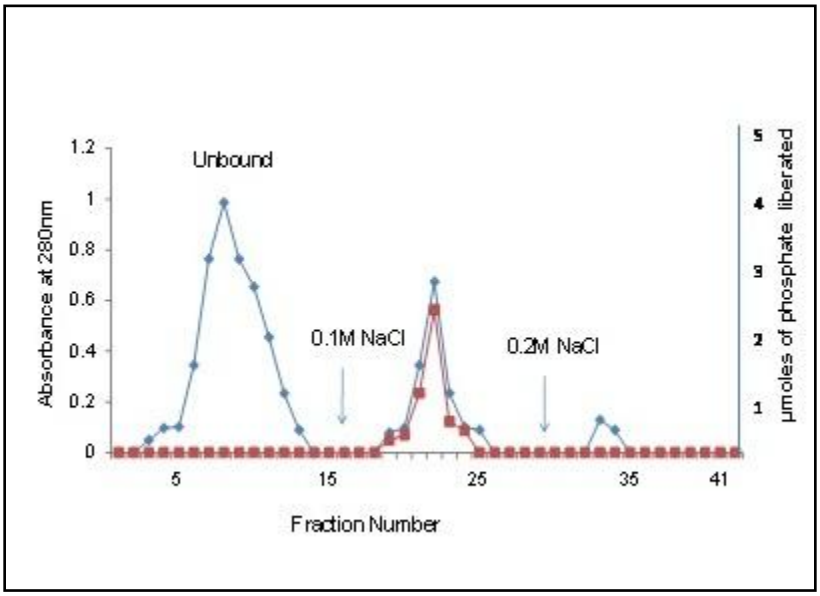
When subjected to SDS-PAGE, acid phosphatase showed single band on 10% slab gels. The presence or absence of 2-mercaptoethanol in the system had no effect on the band pattern of the enzyme on the gel (Fig 4).

Calibrating proteins were also used for determining the molecular weight of the enzyme. The plot of $V_e/V_0$ versus log molecular weight for these proteins was shown in Fig 5. The molecular weight of the enzyme, as calculated from the plot, was 46kDa.

**Effect of pH and temperature on acid phosphatase**

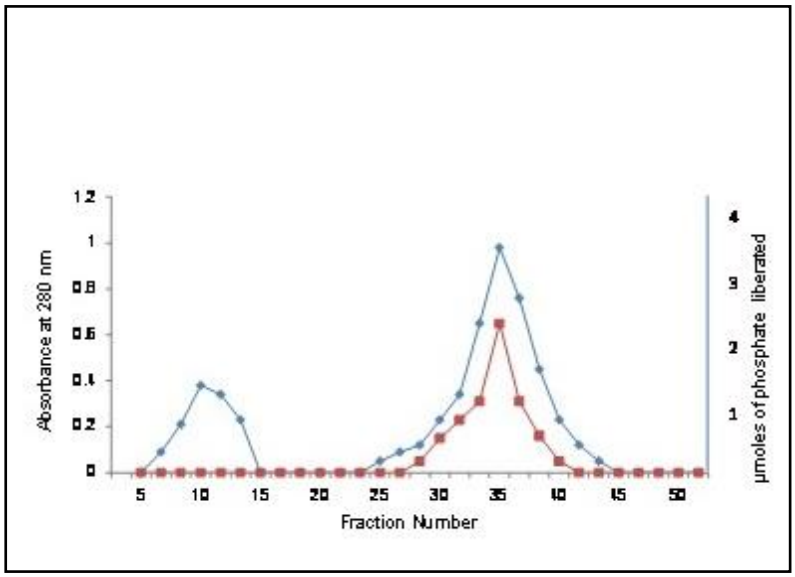
The purified enzyme showed maximum activity at a pH 5.6 (Fig 6) and at temperature $40^0$C (Fig 7). The results are in agreement with those obtained with the crude preparation. Compared to the pure enzyme, the one in the crude preparation showed more activity at $60^0$C possibly due to the protection provided by other proteins present in the extract against thermal inactivation.

**Figure 1: Ion exchange chromatography on DEAE-cellulose**




150mg of Ammonium sulphate fraction (0-60%) was loaded on to the column (2.2×34 cm) in 0.01 M Tris–HCl buffer, pH 7.4 and the adsorbed proteins were eluted with 0.1M and 0.2M NaCl in the buffer. Fractions, each 5 ml were collected at a flow rate of 30ml/h. Protein was monitored through absorbance at 280nm (♦---------♦).
Acid phosphatase activity **(■---------■)**

**Figure 2: Gel filtration on Sephadex G-100 of the DEAE-cellulose preparation**




45mg of the lyophilized preparation was applied to the column (1.9 x 63 cm) in 0.01M Tris- HCl buffer, pH 7.4 and eluted with the same buffer. Fractions, each 2 ml, were collected at a flow rate of 12 ml/h.
Protein was monitored by measuring the absorbance at 280nm (♦---------♦).
Acid phosphatase activity **(■---------■)**

**Figure 3: Polyacrylalmaide gel electrophoretic pattern at pH 8.3 in 10% slab gel**

1) Coomassie blue staining
2) Silver staining
3) Specific staining

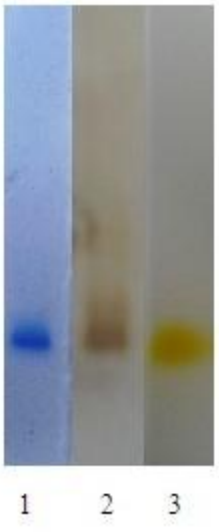
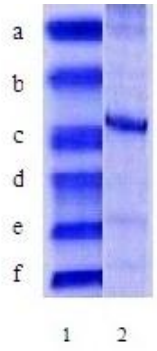


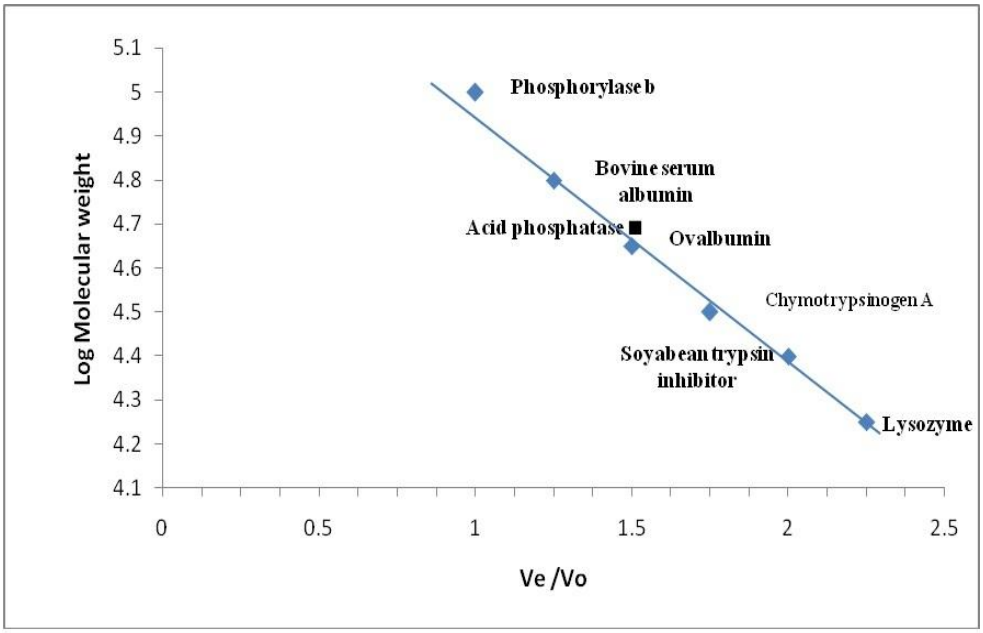
**Figure 4: Molecular weight determination of ginger acid phosphatase by SDS-PAGE on 5-20% gradient slab gel**




1. Standard proteins
   a) Phosphorylase b , 97 kDa
   b) Bovine serum albumin, 67 kDa
   c) Ovalbumin, 44kDa
   d) Chymotrypsinogen A, 25 kDa
   e) Soyabean trypsin inhibitor, 20.1 kDa
   f) Lysozyme,14kDa

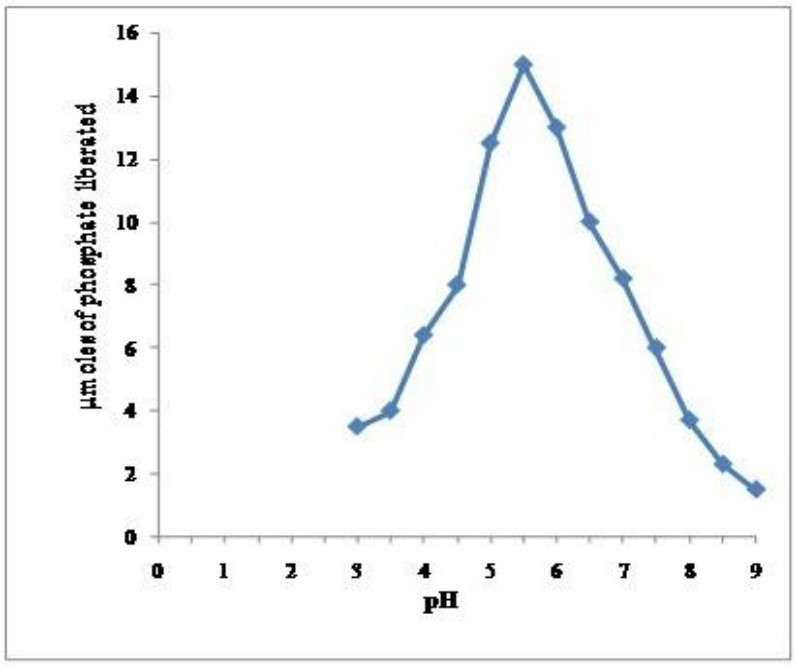
2.Acid phosphatase kept at 100 $^{0}$ C for 2 min with SDS and 2-mercaptoethanol

**Figure 5: Molecular weight determination of acid phosphatase by gel filtration on Sephadex G-200**




Plot of elution volume against log molecular weight of standard proteins (♦)
and acid phosphatase (■)

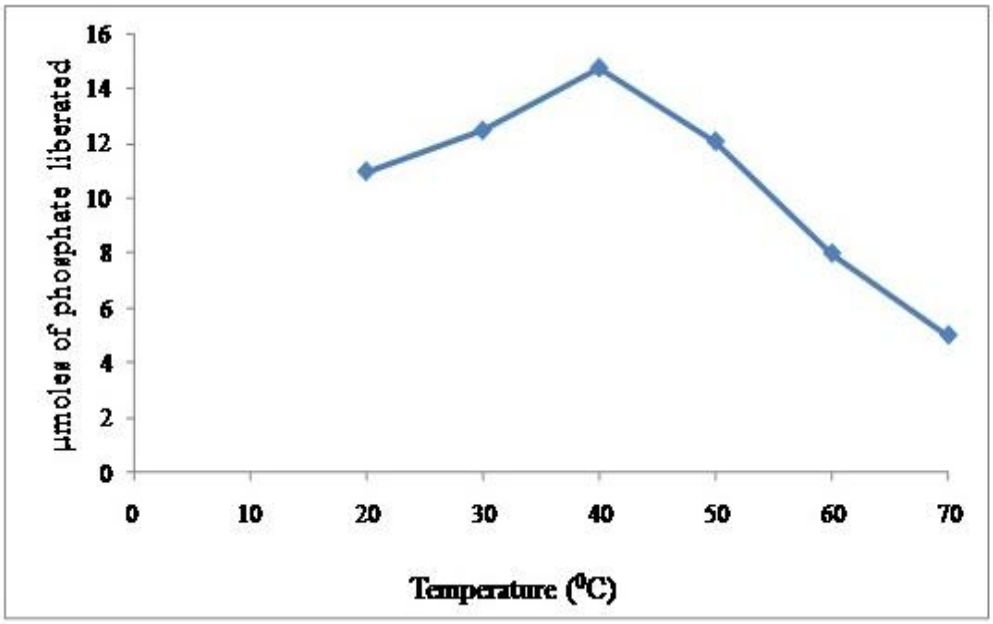
**Figure 6: Effect of pH on the activity of purified acid phosphatase**




To 5.9 ml buffered substrate, 0.1 ml of purified acid phosphatase preparation was added and incubated at 37 $^{0}$C for 30 min and the reaction was terminated by the addition of 2.5 ml of 10% TCA. The contents were filtered and inorganic phosphate was estimated in the filtrate as described in Methods. Inorganic phosphate values are expressed as µmoles of phosphate liberated per mg protein.

Buffers used were sodium acetate, 0.1M, pH (3-4), sodium citrate, 0.1 M, pH (5-6) and Tris- HCl, 0.1M, pH (7-9).

**Figure 7: Effect of temperature on the activity of purified acid phosphatase**




To 5.9 ml buffered substrate, 0.1 ml of purified acid phosphatase preparation was added and incubated at different temperatures (20-70$^0$C) for 30 min and the reaction was terminated by the addition of 2.5 ml of 10% TCA. The contents were filtered and inorganic phosphate was estimated in the filtrate by the method described in Methods. Inorganic phosphate values are expressed as µmoles of phosphate liberated per mg protein.

**Table 1: Summary of purification of ginger acid phosphatase (20 g of ginger)**

| Preparation | Volume ml | Total protein mg | Total activity units | Specific activity units/mg protein | Yield% | Fold purification |
|---|---|---|---|---|---|---|
| Crude extract | 200 | 530 | 112.9 | 0.21 | 100 | 1 |
| 60%Ammonium Sulphate | 50 | 150 | 82 | 0.54 | 72.63 | 2.57 |
| DEAE-Cellulose | 20 | 45 | 50 | 1.11 | 44.28 | 5.28 |
| Sephadex G-100 | 15 | 25 | 30.5 | 1.22 | 27.01 | 5.80 |

*Yield and fold purification were calculated on the basis of total activity units and specific activity units respectively.

## Discussion

Acid phosphatase has been obtained from the rhizomes in an apparently homogeneous form. The enzyme has been isolated and purified following ammonium sulphate (60%) fractionation, DEAE-cellulose ion exchange chromatography and sephadex G-100 gel filtration. Ammonium sulphate (60%) saturation removed 71% of unwanted proteins and gave an yield of 73% of the enzyme. The enzyme in this fraction was adsorbed on to DEAE-cellulose column and was eluted at 0.1M NaCl concentration where in a 44% yield was achieved. The final step in the purification on sephadex G-100 gel filtration provided an yield of 27% with 5.8 fold purification of the enzyme. The steps involved employed for the purification of acid phosphatase from ginger rhizome are similar to those employed for the purification of enzyme from chick pea (Kaur *et al.,* 2011).

Acid phosphatase isolated by the purification procedure described here appears to be homogeneous by PAGE and gel filtration on G-200. The specific staining for the enzyme activity gave a single band corresponding to protein staining by coomassie blue or silver. It is to be noted that the enzyme bound to DEAE-cellulose is eluted completely with 0.1M NaCl and no other enzyme activity was detected in the eluates with 0.2M - 0.5M NaCl. The presence of a single band on the gels also suggests that the preparation is pure and free from any isoenzymic forms.

It is well known that acid phosphatase from *Vigna mungo* seedlings, *Vigna sinensis*, rice plant, soya bean seeds, buckwheat seeds and *Arachis hypogea* seeds occurs in tissues in isoenzymic forms (Haraguchi *et al.,* 1990; Biswas *et al.,* 1991; Tso *et al.,* 1997; Ferreira *et al.,* 1998; Greiner *et al.,* 2002; Gonnety *et al.,* 2006).

The enzyme was devoid of carbohydrate moieties. This is in contrast to the enzyme isolated from marine algae, *Poa pratensis* seeds, lupin roots, potato tuber, triticale seeds (Kuenzler *et al.,* 1965; Kubis *et al.,* 1980; Kozulic et al., 1984; Gellatly *et al.,* 1994; Ching *et al.,* 1987) which were reported to be glycoproteins with a carbohydrate content of 5-15%. Acid phosphatase isolated from sweet potato and black gram seeds (Uehera *et al.,* 1974; Asaduzzaman *et al.,* 2011) were found to be homogeneous and free from carbohydrate moieties. The purified enzyme gave a single band on SDS-PAGE both under denaturing and denaturing conditions suggesting it to be a monomeric protein.

The molecular weight of acid phosphatase was determined to be 46kDa by SDS-PAGE and on sephadex G-200 gel filtration, the enzyme gave a molecular weight of 45.5kDa , a value very close to that of obtained by SDS-PAGE. The molecular weight of the enzyme was found to be similar to that isolated from triticale seeds, soybean seeds, buck wheat seeds (Ching *et al.,* 1987; Ferreira *et al.,* 1998; Greiner *et al.,* 2002 ) and lower than those purified from other plant sources i.e, 53 kDa for soybean (Ullah and Gibson 1988), 58 kDa for wheat germ (Waymack and van Etten 1991), 66 kDa for barley (Greiner *et al.,* 2000), 67 kDa for faba bean (Greiner *et al,.* 2001) and oat (Greiner and Larsson Alminger, 1999). Acid phosphatase was found to exist in polymeric form in maize seedlings and *Lens esculenta* seeds (Laboure *et al.,* 1993; Roknabadi *et al.,* 1999) with molecular weights of 76-120kDa.

The enzyme showed an optimum pH of 5.6 similar to the enzyme from *Brassica nigra* suspension cells, pea nut seeds, castor bean seeds, garlic seedlings (Ferreira *et al.,* 1998; Gonnety *et al.,* 2006; Granjeiro *et al.,* 1999; Yenigun *et al.,* 2003). Acid phosphatases from plant sources have been reported to exhibit maximal activity either in the range pH 3.5 - 4 or in the range pH 5-6. Several isoenzymic forms of acid phosphatase are reported to exhibit different pH optima apart from showing differences in their kinetic parameters, substrate specificity, activation or inactivation by metal ions.

Acid phosphatases from peanut seedlings, soybean seeds, black gram seedlings, chick pea seeds showed maximal activity at temperatures 50-60$^0$C (Gonnety *et al.,* 2006, Ferreira *et al.,* 1998 and Ullah and Gibson,1988; Kaur *et al.,* 2011).The optimum temperature, 40$^0$C, of the enzyme was similar to the one reported from duck weed (Hoehamer *et al.,* 2005), but lesser than those reported from garlic seedlings, *Vigna sinensis* seeds, castor bean seeds (Yenigun *et al.,* 2003; Biswas *et al.,* 1991; Granjeiro *et al.,* 2004). The fall in enzyme activity beyond 50$^0$C could be due to thermal inactivation of the enzyme or inability of sodium β-glycerophosphate to protect the enzyme at higher temperatures.

## Conclusion

In conclusion, an acid phosphatase from ginger rhizomes has been isolated and purified following conventional methods of protein purification and appears to be homogeneous by PAGE, SDS-PAGE and gel filtration on G-200. The molecular weight of acid phosphatase was determined to be 46kDa by SDS-PAGE. The enzyme was found to be a monomeric protein active at pH 5.6 and temperature 40$^0$C preferentially using sodium β-glycerophosphate as the substrate.

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**References**

1. Andrews P. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem J., 91 (2): 222-233.
2. Asaduzzaman A.K.M, Rahman M. Habibur and Yeasmin Tanzima (2011). Purification And characterization of acid phosphatase from a germinating black gram (*Vigna mungo* L.) seedling, Arch. Biol. Sci., Belgrade, 63 (3): 747-756.
3. Biwas, T.K. and C. Cundiff (1991). Multiple forms of acid phosphatases in germination seeds of *Vigna sinensis*, Phytochem., 30: 2119-2125.
4. Blum, H., Beier, H. and Gross, H.J. (1987). Improved silver staining of plant proteins, RNA and. DNA in polyacrylamide gels. Electrophoresis., 8: 93-99.
5. Bozzo, G. G., Raghothama, K. G. and Plaxton, W. C. (2002) Purification and characterization of two secreted purple acid phosphatase isozymes from phosphate-starved tomato (*Lycopersicon esculentum*) cell cultures. Eur. J. Biochem., 269 (24): 6278-6286.
6. Ching Te May, Lin Tsan-Piao, and Metzger Robert J. (1987). Purification and Properties of Acid Phosphatase from Plump and Shriveled Seeds of Triticale, Plant Physiol., 84(3): 789- 795.
7. Cirkovic . Tanja D.,. Gavrovi Marijadj-Jankulovi, Mirjanan. Bukilica, Ljubamandi,. Petrovic Spomenkaz and Jankov Ratko M. (2002). Isolation and partial characterization of an acid phosphatase from *Artemisia vulgaris* pollen extract. J.Serb.Chem.Soc., 67(8),567-572.
8. Duff, S.M.G., Sarath, G. and Plaxton, W.C. (1994). The role of acid phosphatase in plant phosphorus metabolism. Physiol. Plant., 90(4): 791- 800.
9. Ferreira C.V,. Granjeiro J.M,. Taga E.M. and Aoyama H. (1998). Purification and characterization of multiple forms of soybean seed acid phosphatase, Plant Physiol. Biochem., 36(7): 487-494.
10. Fiske, C.H. and Subbarow, Y. (1925). The colorimetric determination of phosphorus. J. Biol. Chem., 66: 375-400.
11. Gabard K.A., Jones R.L. (1986). Localization of phytase and acid phosphatase isoenzymes in aleurone layers of barley, Physiol. Plant., 67:182-192.
12. Gabriel, O. (1971). Methods Enzymol., 22: 565-578.
13. Gellatly, K. S., Moorehead, G. B. G., Duff, S. M. G., Lefebvre, D. D. and Plaxton, W. (1994). Purification and characterization of a potato tuber acid phosphatase having significant phosphotyrosine activity. Plant Physiol., 106(1): 223-232.
14. Gonnety, J.T. and L.P. Kouame. (2006). Purification and characterization of three low-molecular weight acid phosphatase from peanut (*Arachis hypogaea*) seedlings. AJB 5: 35-44.
15. Granjeiro Paulo Afonso., Cavagis Alexandre Donizeti Martins., Leite Luciana de Campos., Carmen Ferreira Ver´ıssima., Granjeiro Jos´e Mauro and Aoyama Hiroshi. (2004). The thermal stability of a castor bean seed acid phosphatase, Molecular and Cellular Biochemistry., 266**:**11-15.
16. Granjeiro, P.A, Ferreira, C.V, Taga, E.M., and H. Aoyama. (1999). Purification and kinetic properties of a castor bean seed acid phosphatase containing sulfhydryl groups. Plant physiol., 107: 151-158.
17. Greiner, R and Klaus-Dieter, J. (2002). Purification and characterization of homogenous acid phosphatase from non germinated buckwheat (*Fagopyrum esculentum*) seeds. J. Food Biochem., 27: 197-220.
18. Greiner, R. and Larsson Alminger, M. (1999). Purification and characterization of a phytate-degrading enzyme from oat *(Avena sativa).* J. Sci. Food Agric., 79: 1453-1460.
19. Greiner, R., Jany, K.D. and Larsson Alminger, M. (2000). Identification and properties of myo-inositol hexakisphosphate phosphohydrolase (phytase) from barley *(Hordeurn vulgure).* J. Cereal Sci., 31: 127-139.

20. Greiner, R., Muzquiz, M., Burbano, C., Cuadrado, C., Pedrosa, M.M. and Goyoaga, C**.** (2001). Purification and characterization of a phytate-degrading enzyme from germinated faba beans **(***Vicia faba* Var. Alameda). J. Agric. Food Chem., 49: 2234-2240.
21. Guo, J., and J.C. Pesacreta. (1997). Purification and characterization of an acid phosphatase from the bulb of *Allium cepa* L. J. Plant physiol., 151: 520-527.
22. Haraguchi, H., Yamauchi D. and Minamikawa T. (1990). Multiple forms of acid phosphatase in cotyledons of *Vigna mungo* seedlings: immunological detection and quantitation, Plant Cell Physiol., 31: 917-923.
23. Hoehamer, C.F., Mazur, C.S., and N.L. Wolfe. (2005). Purification and partial characterization of an acid phosphatase from S*pirodela oligorrhiza* and its affinity for selected organophosphate pesticides. J. Agrc. Food Chem., 53: 90-97.
24. Kaur Parminder, Sharma Arun Dev and Rakhra Gurmeen. (2011). Purification and characterization of a heat-stable acid phosphatase from chickpea, Research in Plant Biology., 1(4):1- 6.
25. Kozulic, B., Barbaric, S., Ries, B. and Mildne, P. (1984). Study of the yeast carbohydrate part of the yeast acid phosphatase. Biochem. Biophys. Res. Commun, 122: 1083-1090.
26. Kubis Irena Lorenc and Morawiecka Bronislawa. (1980). Purification and some properties of the main polymorphic form of acid phosphatase from *Poa pratensis* seeds., Acta Biochemica Polonica, 27: 345- 352.
27. Kuenzler, E. J. and Perras, J. P. (1965). Phosphatases in marine algae. Biol. Bull. Mar. Biol. Lab., Woods Hole, 128: 271-284.
28. Laboure, A.M., Gagnon, J. and Lescure, A.M. (1993). Purification and characterization of a phytase **(**myo-inositolhexakisphosphate phosphohydrolase) accumulated in maize *(Zea mays)* seedlings during germination. Biochem. J., 295: 413-419.
29. Laemmli, U.K. (1970). Nature., 227 (5259): 680–685.
30. Lowry O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J Biol Chem., 193: 265-275.
31. Naganna, B., Raman, A., Venugopal, B.R. and Sripathi, C. E.(1955). Biochem. J., 60(2): 215-223.
32. Pasqualini, S., Panara, F., Ederli L., Batini, P. and Antonielli, M. (1997). Multiple acid phosphatase in barley coleoptiles: Isolation and characterization of the 63kDa soluble enzyme form. Plant Physiol Biochem., 35: 95-101.
33. Reisfeld, R.A., Lewis.U.J. and Williams D.E. (1962). Nature., 195: 281-283.
34. Roknabadi Sadroddin Mohammadi, Bose Sudeep Kumar and Taneja Veena. (1999). A histidine thiol 100 kDa, tetrameric acid phosphatase from lentil, *Lens esculenta*, seeds with the characteristics of protein tyrosine phosphatases, Biochimica et Biophysica Acta., 1433: 272-280.
35. Staswick, P.E., Papa. C., Hung, J., and Y. Rhee. (1994). Purification of major soybean leaf acid phosphatase that is increased by seed-pod removal. Plant Physiol. 104(1): 49-57.
36. Sugino, Y. and Miyoshi, Y. (1964). J. Biol. Chem., 239(7): 2360-2364.
37. Thomas, T.L. (1993). Gene expression during plant embryogenesis and germination: an overview plant cell. 5(10): 1401-1410.
38. Tono, N. and Kornberg, A. J. (1967). Biol. Chem., 242(10): 2375-2382.
39. Tso, S.C. and Chen, Y.R. (1997). Isolation and characterization of a group I11 isozyme of acid phosphatase from rice plants. Bot. Bull. Acad. Sin., 38: 245-250.
40. Turner, W. L. and Plaxton, W. C. (2001). Purification and characterization of banana fruit acid phosphatase. Planta., 214: 243-249.
41. Uehera, K., Fujimoto, S., Taniguchi, T. and Nakai, K. (1974). Studies on violet coloured acid phosphatase of sweet potato. II, Enzymatic properties and amino acid composition. J.Biochem., 75(3): 639-649.
42. Ullah, A.H.J. and Gibson, D.M. (1988). Purification and characterization of acid phosphatase from cotyledons of germinating soybean seeds. Arch. Biochem. Biophys., 260: 514-520.
43. Waymack, P.P. and Van Eiiten, R.L. (1991). Isolation and characterization of a homogeneous isoenzyme of wheat germ acid phosphatase. Arch. Biochem. Biophys., 288: 621-633.
44. Yenigun Begum and Yuksel Guvenilir. (2003). Partial purification and kinetic characterization of acid phosphatase from garlic seedling, Applied Biochemistry and Biotechnology., 107(1): 677-687.