Purification and partial characterization of two forms of acid phosphatase from seeds of *Erythrina indica*

Uzgare Ashish[1]* and Bhide Shobhana[2]
1. Department of Chemistry, Wilson College, Chowpatty, Mumbai 400007, INDIA
2. Department of Chemistry, Fergusson College, Pune 411004, INDIA
*ashishuzgare@yahoo.com

Abstract

Erythrina indica seeds showed high activity of acid phosphatase. Purification of the enzyme was achieved by combination of chromatographic techniques and two forms namely AP-I and AP-II were resolved by affinity chromatography on ConA-CL Seralose column. Both AP-I and AP-II are glycoproteins with molecular mass of 126 and 102 kDa respectively. K_m values for p-nitrophenyl phosphate were 86.9 µM and 80 µM for AP-I and AO-II respectively.

Both the enzymes can hydrolyze broad range of phosphorylated substrates. Although the two forms showed similar pH optima, optimum temperature for both the forms showed significant variation. V_{max} values of AP-II with various phosphate esters as substrates were greater than that of AP-I.

Keywords: Acid phosphatase, affinity chromatography, *Erythrina indica*, purification.

Introduction

Enzymes which catalyze the hydrolysis of phosphoric acid monoesters and anhydrides have been purified and characterized from a number of plant and animal sources. Based on substrate specificities and pH optima, these enzymes have been termed as acid phosphatases, alkaline phosphatases, phosphoprotein phosphatases or ATPases. The best characterized members of this group are acid phosphatases (E.C. 3.1.3.2) from bovine spleen and pig allantoic fluid which have been shown to be very similar in both catalytic and molecular properties[1]. Purple acid phosphatases purified from sweet potato[2], soybeans[3], spinach[4] and rice cell walls [5] have been reported to contain essential manganese. The widely studied of these enzymes is from sweet potato, a dimeric glycoprotein of approximately 110 kDa, which contains two[2] or one[6] atom of manganese per molecule. Multiple forms of acid phosphatase have been reported in various plant sources[7,8].

Phosphate is a major plant nutrient as well as key regulatory component both in respiratory and photosynthetic metabolism in plants, with acid phosphatases playing a important role in phosphate metabolism[9,10]. Elevated levels of acid phosphatases were observed during stress and nutritional deprivation especially phosphorous deprivation [23]. Most of the plant acid phosphatases show broad substrate specificities as well as can act as phytases[10].

Erythrina indica also known as Indian coral tree belongs to the family Fabaceae. Its foliage and seeds have medicinal value and are not being consumed by grazing animals [11]. During isolation of lectin and glycosidases from seeds of *Erythrina indica*, high activity of acid phosphatase was detected.

In view of important role of acid phosphatase in plants and its abundance in *E. indica* seeds, present work was undertaken

Material and Methods

The mature seeds of *E. indica* were collected from the trees on Pune University campus. All other chemicals and bio-chemicals were of analytical grade. Con A-Cl Seralose was from Bangalore Genei, Bangalore, India.

Enzyme assay: Enzyme activity assays were performed using 100 µl p-nitro-phenyl-phosphate (pNPP,2 mM), 100 µl citrate buffer (pH 4.8, 0.1 M) 25-100 µl enzyme extract made to a final volume of the 1.50 ml. The incubation time was 30 min and the reaction was stopped by addition of 0.05M borate buffer pH 9.0. The liberated p-nitro-phenol was estimated at 405 nm using spectrophotometer (Shimadzu model uv-1601). Enzyme activity unit (IU) was defined as µmole of p-nitro-phenol released per min per ml of reaction mixture at 30 ^{O} C[12].

For determination of phosphatase activity using natural substrates, the enzyme solution was incubated with the substrate solution in presence of citrate buffer (0.05 M, pH 4.6) for 60 minutes. The reaction was arrested by adding 10 % w/v SDS solution. The total volume of the reaction mixture was adjusted to 1.0 ml with distilled water. 10 % w/v TCA was added to it and the reaction mixture was centrifuged to remove the precipitate, if any. The clear supernatant was then used to estimate the liberated phosphate (Pi) by Fiske – Subbarao method[13].

One unit of enzyme is defined as the amount of enzyme that produces one micromole of phosphate (i.e. 31 µg of phosphorous) per minute per milliliter.

Protein estimation: Protein concentrations during purification were determined by absorbance measurements at 280 nm [14] as well as by using Lowry's method [15].

Carbohydrate estimation: Carbohydrate content was estimated by Phenol-Sulphuric acid method[16].

Preparation of Fraction A: All operations were carried out at 4^{O}C. Fraction A was prepared according to the protocol[11].

Gel Filtration and Lectin separation: An aliquot (approx. 50 mg protein) was applied to gel filtration column (2.5 cm X 60 cm) packed with Sephadex G 100, equilibrated with physiological saline (saline I) and having a flow rate of 9 ml/hr. Gel filtration was done using saline I as a developer. Fractions of 2 ml were collected and analyzed for protein, lectin and enzyme activities. Enzyme activity rich fractions were pooled and were passed through Lactamyl Seralose affinity column (2.5 cm X 40 cm) to remove the lectin.

Detection of glycoprotein nature of enzyme: When an aliquot of lectin free enzyme rich fraction was mixed with ConA, it led to precipitation and the precipitate contained all the phosphatase activity. On adding solid glucose, the precipitate was dissolved with release of enzyme activity in the solution, thus confirming glycoprotein nature of the enzyme.

Affinity chromatography using ConA-CL Seralose: The lectin free enzyme rich fraction was loaded on ConA CL Seralose packed in a 5ml syringe, recycled twice and left overnight. The column was washed exhaustively with saline I to remove any unbound protein and enzyme activity. The column was then eluted with a stepwise glucose gradient (0.001-1 M in saline I). 3 ml fractions were collected and analysed for protein and enzyme activity.

The active fractions were pooled and dialyzed against saline I and then concentrated using a concentration unit (Amicon) having a membrane filter of 20,000 D cut off range. Homogeneity and purity of the enzyme were checked by gel filtration of the pure sample on Sephadex G 100 and by Polyacrylamide gel electrophoresis (PAGE) at pH 8.3 (Fig.1).

Determination of molecular mass: The homogeneity and purity of the purified forms of the enzymes were checked by electrophoresis in native PAGE at both pH 8.3 and 4.5[17,18].

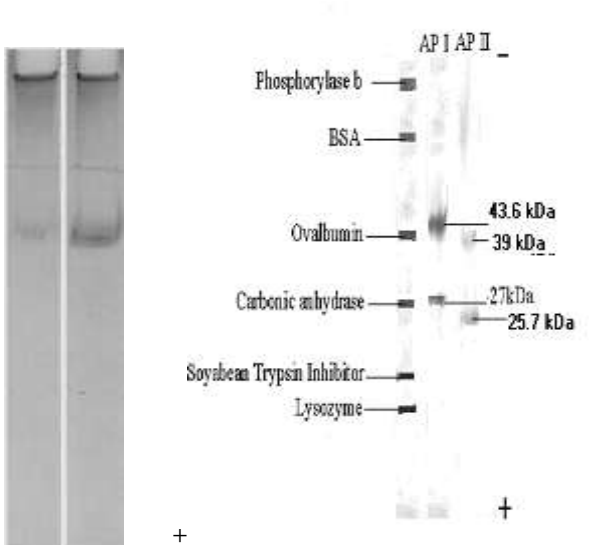
Molecular mass of pure protein was determined by gel filtration on Sephadex G100 column calibrated with proteins namely Bovine serum albumin (dimmer) (1, 32,000 Da), Bovine serum albumin (monomer) (66,000 Da), Ovalbumin (45,000 Da), Pepsin (37, 000 Da) and Trypsin (23,800 Da).

Molecular mass and subunit composition for both the forms were determined by SDS-PAGE under both reducing[19,20] and non reducing condition using phosphorylase b (97,400 Da), bovine serum albmin (66,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), soybean trypsin inhibitor (20,100 Da) and lysozyme (14,300 Da) as standards.

Substrate specificity: The ability of the two forms of the enzyme to hydrolyze a range of natural substrates was checked. Following substrates were used: Adenosine di phosphate (0.1mM – 0.5mM), Pyridoxal phosphate (0.1mM – 0.5mM), Glucose 6 phosphate (0.1mM – 0.5mM), Phosphoenol pyruvate (0.04 – 0.2mM), Sodium beta glycerophosphate (0.04 – 0.2mM),Sodium phenyl phosphate (0.6 – 3.0m,M), Casein (2 % w/v) and Histone (2 % w/v) were screened to check the substrate specificity. This was done essentially according to Fiske and Subbarao method[13].

Influence of pH on enzyme activity: For the determination of the optimum pH for the enzyme, the following buffers were used: (a) Citrate buffer (pH 3.0 – 6.0) (b) Tris - HCl buffer (pH 7.0 – 8.0). A system containing buffer and enzyme was incubated at 37°C (±0.2°C) for 30 minutes.

Influence of temperature on enzyme activity: For the study of the effect of temperature on the enzyme activity using pNPP, the reaction was carried out at temperatures ranging from 5 - 95°C (±0.2°C). Enzyme was incubated at the desired temperatures for 30 minutes.



A: PAGE (pH 8.3) Minor Form (AP-I)

B: PAGE (pH 8.3) Major Form (AP-II)

M: Markers

C: SDS-PAGE Minor Form (AP-I)

D: SDS-PAGE Major Form (AP-II)

Fig. 1: Electrophoretic pattern of pure enzyme

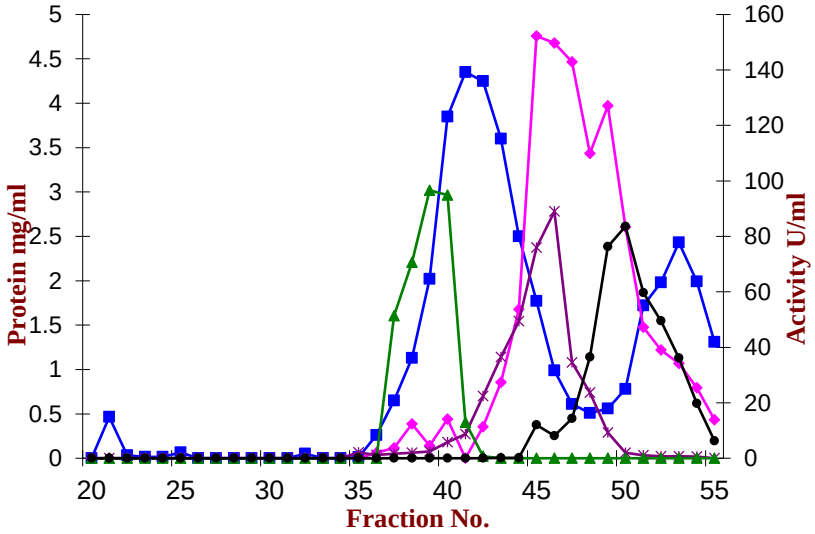
Influence of substrate concentration: For determining the influence of substrate concentration, p-nitro phenyl phosphate concentrations ranging from 0.035mM to 0.165mM were used.

Influence of metal ions on the enzyme activity: Effect of metal ions on enzyme activity was checked by pre-incubating the dialyzed enzyme (against de-ionized water) with various metal ions at 20mM for 30 minutes. Corresponding controls without metal ions were also assayed.

Determination of metal ions in AP-I and AP-II: The purified enzymes were dialyzed exhaustively against EDTA solution (1 %). Both the forms of the enzyme were then analyzed for the presence of various metal ions by using Perkin Elmer model 3100 atomic absorption spectrophotometer.

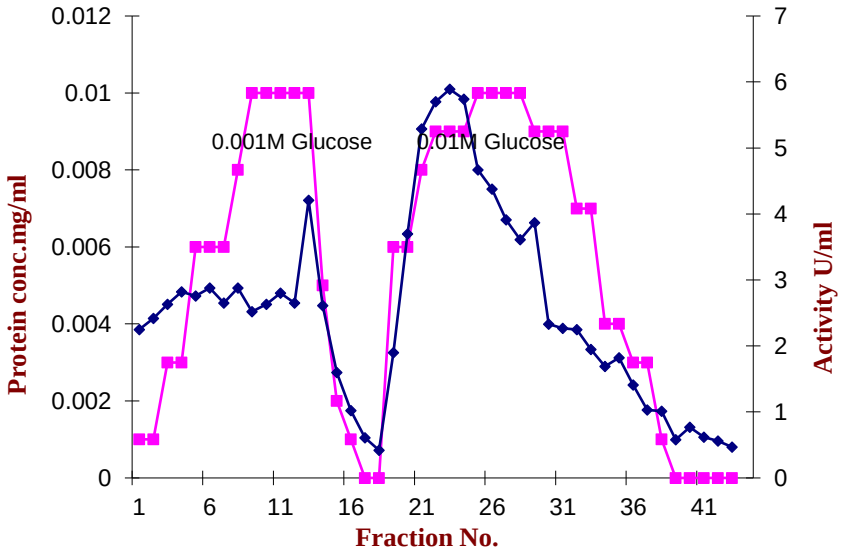
Results and Discussion

Preparative gel filtration of fraction A on Sephadex G -100 resolved the protein into two peaks. All the enzyme activities as well as lection were found in peak 1 (Fig. 2A). Lectin was removed from peak I by passing through lactamyl seralose column. Affinity chromatography of lectin free phosphatase rich fraction on Con A -CL Seralose column (Fig. 2B) and subsequent elution of the retained enzyme with stepwise gradient of glucose resolved enzyme into two forms of acid phosphatase, one eluting at 0.001M glucose (AP-I the minor form) and the other at 0.01M glucose (AP-II the major form) concentration.



■: Protein mg/ml ♦: Acid phosphatase ●: N-acetyl β D-glucosamine
▲: ά D-mannosidase ■: β-D galactosidase

Fig. 2A: Elution profile on Sephadex G100



■: Protein mg/ml ♦: Activity U/ml

Fig. 2B: Elution Profile on ConA Affinity Matrix

Homogeneity of the two forms was confirmed by gel filtration on Sephadex G-100 column. Carbohydrate content of the minor and major form was estimated to be 5.2 and 7.1 %, respectively which is in accordance with their elution behavior from affinity column. Table 1 gives summary of purification of two forms of the enzyme.

Gel filtration yielded a molecular mass of 126± 2.71 kDa for minor form whereas it was 102± 2.71kDa for the major form. The SDS profile of both the purified forms showed the presence of two bands indicating that both forms are heterodimers. The molecular mass of the subunits was found to be 43.6 kDa and 27 kDa for AP I and 39 kDa and 25.7 kDa for AP II respectively. Two forms of acid phosphatases have also been reported from seeds of cucurbitaceae edible seeds with molecular weights of 70 kDa and 55 kDa, respectively[21]. pH optima, temperature optima, K_m and V_{max} values using pNPP as the substrates are shown in table 2. Both the forms showed maximum activity at pH 4.8 and were stable in the pH range 4-6 whereas the optimum temperature was 55 ^{O}C for minor form, major form showed 45 ^{O}C as the optimum temperature.

A logical explanation for this could be that the major form is the prominent one and controls the phosphate metabolism largely and at higher temperature, the minor form takes over. Both the forms were stable in the temperature range 20-75 ^{O}C. The effect of varying pNPP concentration yielded a K_m value of 86.95 µM and 80.0 µM for minor and major forms respectively whereas the V_{max} values were 7.4 and 12.5 I.U. respectively. Properites similar to these observations are also reported for acid phosphatases from germinating soybean seed cotyledon[22]. The V_{max}/K_m values for the different substrates indicate that the two forms exhibited similar affinity for them as seen from table 3.

Table 1
Summary of purification

	Specific activity	Percent Recovery	Fold purification
Saline extract	0.689	100	1
Fraction A	0.922	23.22	1.33
Sephadex G-100	11.83	22.64	17.16
Lactamyl Seralose	12.02	15.67	17.44
Con-A Cl agarose form I	93.88	2.10	136.25
Con-A Cl agarose form II	200.66	11.27	291.23

Table 2
Characterization of the minor and major form of acid phosphatase

Form	Molecular Mass kDa	Optimum pH	pH stability	Optimum Temperature ^{o}C	Thermal stability ^{o}C	K_m M	Other substrates
AP-1	126 ± 2.71	4.8	3-5	55	15-65	1.08 x 10^{-4}	Casein, Histone, ADP, G-6-P, SβGpi, PyrPi, PEP, SPP
AP-2	102 ± 2.71	4.8	3-5	45	15-55	0.5 x 10^{-4}	Casein, Histone, ADP, G-6-P, SβGlyP, PyrPi, PEP, SPP

ADP: Adenosine di phosphate
G-6-P: Glucose 6 phosphate
SβGpi: Sodium β glycerophosphate
PyrPi: Pyridoxal phosphate
PEP: Phosphoenol pyruvate
SPP: Sodium phenylphosphate

Table 3
K_m and V_{max} / K_m values of AP –I and AP –II for various substrates

Substrate	Minor Form K_m (µM)	Major Form K_m ((µM)	V_{max} / K_m Minor form	V_{max} / K_m Major form
p-Nitrophenyl phosphate	87	80	1.0 X 10^{-1}	1.5 X 10^{-1}
Adenosine-di-phosphate	163	163	1.0 x 10^{-3}	1.3 X 10^{-3}
Glucose-6-phosphate	78	77	1.0 X 10^{-3}	1.0 X 10^{-3}
Pyridoxal phosphate	68	73	1.0 X 10^{-3}	1.1 X 10^{-3}
Phospho-enol-pyruvate	51	60	1.0 X 10^{-3}	1.0 X 10^{-3}
Sodium phenyl phosphate	46	47	1 X 10^{-3}	1 X 10^{-3}
Sodium-β-glycero phosphate	198	196	1 X 10^{-3}	1.2 X 10^{-3}

Of the various substrates screened, it is noteworthy that casein and histone, both phosphoproteins were cleaved of their phosphate by both the forms, though to a lesser extent than other substrates. Casein (Minor: 0.10 U and Major: 0.266 U) was dephosphorylated to larger extent as compared to histone (Minor: 0.01 U and Major: 0.02 U).

Incubation of the demetallized enzymes with different metal ions and the effect on their activity are depicted in table 4A. The two forms show different results on incubation with metal ions and exhibit different behavior for the same metal ion e.g. optimum activation for the major form was observed by Ca^{++} while in minor form Mg^{++} had maximum effect. Hg^{++} totally inactivated both the enzymes. The metalloprotein nature of the two forms is evident from the results of atomic absorption studies (Table 4B).

Acid phosphatases are reported to be metalloenzymes and so are the two purified forms In addition to iron and zinc, presence of calcium and magnesium is a notable feature for acid phosphatase from *Erythrina indica*. An interesting feature noted was that Ca^{++} brought about maximum activation of major form while it was Mg^{++} in case of minor form.

Conclusion

In conclusion, existence of two forms of acid phosphatases in *Erythrina indica* seeds and their ability to hydrolyze broad range of phosphorylated substrates is in accordance with acid phosphatases reported from other plant species. Thus, acid phosphatases from *Erythrina indica* suggest their possible role in phosphorous metabolism.

Table 4A
Determination of Metal ion content in AP-I and AP-II

Metal ions (20mM)	Minor	Major
$CuSO_4$	128	94.
$ZnCl_2$	84	118.
$MgSO_4$	179	91
$MnCl_2$	112	143.
$CaCl_2$	108	172
$HgCl_2$	0.0	0.0
$FeCl_3$	No change	No change
Control	100	100
Demetallization by EDTA	22	20

Table 4B
Influence of metal ions on the AP-I and AP-II activity

Metal ion	Minor Form (ppm)	Major Form (ppm)
Copper	0.014	0.028
Magnesium	0.405	0.630
Manganese	0.027	0.016
Calcium	2.460	3.717
Zinc	0.103	0.168
Iron	0.058	0.038

References

1. Campbell H.D., Dionysius D.A., Keough D.T., De Jersey Wilson B.E. and Zerner B., *Biochem. Biophys. Res. Commun*., **82**, 615 – 620 **(1978)**

2. Fujimoto S., Muraakami K. and Ohara A., *J. Biochem.*, **97**, 1777–1784 **(1985)**

3. Fujimoto S., Nakagawa T. and Ohara A., *Agric. Biol. Chem*., **41**, 599 – 600 **(1977)**

4. Fujimoto S., Ishimitsu S., Nakagawa T. and Ohara A., *Chem. Pharma. Bull*., **25**, 1459 – 1462 **(1977)**

5. Igaue I., Morota A., Takahashi K., Takekoshi M. and Watabe H., *Agric. Biol. Chem*., **40**, 823 – 825 **(1976)**

6. Fujimota S., Kawabe H., Ohara A., Sugiura Y. and Tanaka H., *J. Biol. Chem*., **256**, 10664 – 10670 **(1981)**

7. Bozzo G.G., Plaxton W.C. and Raghothama K.G., *Eur. J. Biochem*., **269(24)**, 6278 – 6286 **(2002)**

8. Aoyama H., Ferreira C.V. and Taga E.M., *J. Enzyme. Inhib*., **15(4)**, 403 – 410 **(2000)**

9. Brenden A.H., Marty N.J., Mullen R.T., Plaxton W.C., Snedden W.A. and Tran H.T., *Plant Physiology*, **153**, 1112–1122 **(2010)**

10. Duff S.M.G., Plaxton W.C. and Sarath G., *Plant Physiology*, **90**, 791–800 **(1994)**

11. Bhide S.V. and Kestwal R.M., Purification and partial characterization of α-D-mannosidase from *Erythrina indica* seeds, *Indian J. Biochem. Biophys*., **42**, 156 – 160 **(2005)**

12. Li Y.T., *J. Biol. Chem*., **242**, 5474 – 5480 **(1967)**

13. Fiske C.H. and Subbarow Y., The colorimetric determination of phosphorus, *J. Biol. Chem*., **66**, 375– 400 (**1925**)

14. Bhide S.V. and Sawhney S.Y., *J. Chromatogr*., **503**, 272 – 276 **(1990)**

15. Farr A.L., Lowry O.H., Randall A.J. and Rosebrough N.J., *J Biol. Chem.*, **193**, 265 – 275 **(1951)**

16. Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A. and Smith F., *Anal. Chem.*, **28**, 350-356 **(1956)**

17. Ornstein L., *Annal NY. Acad Sci*, **121**, 321 – 349 **(1964)**

18. Reisfeld R.A., Lewis U.J. and Williams D.E., *Nature*, **195**, 281 – 283 **(1962)**

19. Reisfeld R.A. and Williams D.E., *Annal N Y Acad Sci*, **121**, 373 – 381 **(1964)**

20. Laemmli U.K., *Nature*, **227**, 680 – 685 **(1970)**

21. Koffi D.M. et al, *JAPS*, **7**, 860- 875 **(2010)**

22. Ferreira C.V., Granjeiro J.M., Taga E.M. and Aoyama H., *Plant Physiology and Biochemistry* , **36**, 487-494 **(1998)**

23. Catherine E.L. et al, *New Phytologist*, **195 (2)**, 306-320 **(2012)**.

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