Global Advanced Research Journal of Agricultural Science (ISSN: 2315-5094) Vol. 5(6) pp. 195-203, June, 2016 Issue.
Available online http://garj.org/garjas/home


*Full Length Research Paper*

# Purification and Characterization of Acid Phosphatase (*AcPase*) from Cotyledon of *Erythrina indica*

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Accepted 07 June, 2016

**Acid phosphatase (AcPase) (EC 3.1.3.2) is a ubiquitous lysosomal enzyme that hydrolyzes phosphate esters at an acid pH and therefore, plays critical role in plant development and growth. AcPase from seeds of *Erythrina indica*, a leguminous plant, was extracted and purified 61 folds to apparent electrophoretic homogeneity in 5 purification steps which involved; ammonium sulphate fractionation, ConA-seralose 4B, acidification to pH 5 and finally DEAE-cellulose. The native molecular weight of the enzyme as estimated by calibrated gelfiltration was 89kDa while 38 and 42kDa were obtained for subunits under reduced denatured SDS-PAGE conditions. AcPase had a broad pH optima from 4.5 to 5.5 with a maxima centered at around pH 5. The enzyme, as well, had a wide range of temperature optima ranging from 30°C to 55°C. AcPase activity was totally inhibited with $Zn^{++}$, while 80% increase in activity was noticed in the presence of $Cu^{++}$. The enzyme kinetics data, km and Vmax values, for hydrolysis of *p*-nitrophenyl phosphate were 3.3 and 13, respectively. AcPase was present in at least three isoforms, had a glycoprotein nature with mannose as terminal putative sugar.**

**Keywords:** Acid phosphatase, *Erythrina indica*, leguminousae, purification, characterization.

## INTRODUCTION

Phosphorus in its soluble salts represents one of the main micro-elements required for plant growth and development (Yuan and Liu, 2008). It is not only critical for metabolic regulation, energy transfer and metabolic regulation, but it is also critical for many metabolites construction such as nucleotides, sugar phosphates and phospholipids (Vance *et al*., 2003). Inorganic phosphate, which is freely, absorbed from soil remains the first choice for plant phosphorus reservoir. However, during nutritional oxidative stress plant leads various biochemical adaptive responses, of which increases expression of enzymes that assist in mobilization of phosphates (Ticconi *et al*., 2001). Among these enzymes are acid phosphatase (EC 3.1.3.2) and alkaline phosphatases (EC 3.1.3.1) which work optimally on acidic or alkaline *p*Hs respectively (Makoi *et al*., 2010). These two enzymes are ubiquitously spread in the globe, being found from microorganisms to humans (BO, 2010).In humans, the enzyme is predominantly detected as lysosomal enzyme (Yajima, 1988). Though of the ubiquitous nature of acid phosphatase (AcPase), its exact physiological significance, in animal cell, remains mysterious (Muniyan *et al*., 2013). Nevertheless, paradoxically, expression of the enzyme is routinely used as biomarker to follow surgical progress (Hong *et al*., 2015)or disease situation (Chen *et al*., 2015; Chou *et al*., 2015). Monitoring expression of which is linked

with diagnostic pathological situations. In men, the enzyme activity increases sharply in blood in cases of prostate diseases (Muniyan *et al.*, 2013). In our laboratory, we detected strong AcPase activity in seeds of *Erythrina indica* that could hydrolyze the chromogenic pseudosubstrate *p*-nitrophenyl phosphate. Therefore, our main objectives of this investigation were to isolate, purify and characterize this enzyme.

## Experimental

### Chemicals

*Erythrina indica* season fresh good quality seeds were collected from the Botanical garden, Pune University, Pune, India. Gel filtration molecular weight marker kit (MWGF70- IKT 098k6082) was purchased from Sigma-Aldrich, USA. Sephadex G-100 was from Pharmacia; Uppsala, Sweden. Biogel-100 was generously donated by the late Dr. Khan M. I., NCL, Pune. All other materials and chemicals were either of analytical grade or highest grade available.

### Protein Extraction and Fractionation

*Erythrina indica* seeds were ground to fine powder, defatted and extracted for protein as stated by (Konozy and Bhide 2012). In brief: 50g *Erythrina indica* seeds were soaked overnight in warm water, outer shells were peeled off, softened seeds cotyledons were mixed in fruit mixer and defatted by n-butanol-1 (5mL/1g powder); lipid and pigments layer was removed by centrifugation. Protein was precipitated with chilled acetone, the precipitant was filtered through cheesecloth, completely dehydrated with several folds of chilled acetone and left to dry at room temperature. Thus obtained powder is termed Acetone Dried Powder. The acetone dried powder was then extracted for 3h with 10mM sodium phosphate buffer pH 7.2 prepared in 0.145M NaCl. Extract was filtered through cheesecloth, turbid supernatant was centrifuged at 6000rpm for 45 min at 10$^{o}$C, clear supernatant was collected and denoted as Fraction A (FrA). Protein fractionation of FrA was done by addition of 80% of solid ammonium sulfate (Am-SO4). Precipitated protein by salting out was dissolved in minimal amount of 0.145M NaCl, dialyzed exhaustively against the same saline to remove excess of Am-SO4. The fraction obtained by 80% Am-SO4 were preserved at -20$^{o}$C till further use

### Protein quantification

Protein contents were determined either by UV at 280nm or colorimetrically as performed by Lowry et al (Lowry *et al.*, 1951) using Bovine Serum Albumin (BSA) as the standard.

### Purification of AcPas

Fractions rich in AcPase activity obtained upon fractionation of FrA ConA Seralose 4B column, were pooled, pH of the solution was adjusted to 5.0. Solution was centrifuged to remove precipitated proteins and clear supernatant was dialyzed against Tris-HCl buffer pH 7.2 and this was loaded on DEAE-cellulose column equilibrated with Tris-HCl buffer pH 7.2. Elution with discontinuous gradient of NaCl20 mM resulted in desorption of only active AcPase. Fraction with AcPase was dialyzed exhaustively against distilled water, lyophilized to dryness.

### Native molecular weight determination on gelfiltration

The molecular weights of AcPase was estimated by gel elution on Sephadex G-100, in 0.145 M, NaCl as described by konozy (Konozy, 1999).
The molecular weights were calculated by plotting a graph of log MW x 104 Vs Ve/V0. Where: Ve: is the volume at which the protein peak is obtained.
The following protein molecular weight markers were initially used to calibrate the column: Bovine serum albumin-(BSA), ovalbumin, pepsin, trypsin and cytochrome C.

### Purity confirmation

Purity of AcPase was assessed on native electrophoresis essentially as described (Williams and Reisfeld, 1964).
V0: Void volume, as determined by using Blue Dextran 2000.

### Subunits molecular weight estimation by SDS-PAGE

This was done essentially according to the procedures of Lammeli (Laemmli, 1970). The electrophoresis was carried out on 8% acrylamide gel with Tris glycine buffer pH 8.3, at a constant current.

### Preparation of ConA-Seralose 4B affinity resin

Coupling of ConA to Seralose 4B was done essentially as previously shown (Konozy and Bhide, 2012). 10mg protein were loaded onto ConA-Seralose 4B column, recycled several times to ensure maximum retention, unbound proteins were washed off till OD280 < 0.02. Bound enzyme was eluted with 50 and 100mM mannose. Fractions of 2mL were collected at a flow of 1mL/2min.

### Influence of pH

50 µL of the enzyme (~10 Units) was incubated with 200 µL of buffers of different pH ranging between pH 3.0 to pH

7.2 for 30 min. and 250 µL of p.nitrophenylphosphate was added. Reaction was arrested after suitable time period by addition of 1 ml of 0.1 M borate buffer, pH 9.5. The released p-nitrophenol was monitored spectrophotometerically at 405 min.

**Influence of temperature**

50 µL of the enzyme (~10 Units) was incubated with 200 µL of 0.2 M citrate puffer pH 4.5, at different temperatures ranging between 25 to 70$^{o}$C for 10 min. 250 µL of substrate was added. The reaction mixture was incubated for suitable time period and then arrested by addition of 1 ml 0.1 M borate buffer pH 9.5. The released p-nitrophenol was monitored spectrophotometerically at 405 nm.

**Influence of substrate concentration**

50 µL of the enzyme (~ 50 Units) solution was incubated with 200 µL of 0.1 M citrate buffer pH 4.5, to this mixture different concentrations of the corresponding pseudo-substrate ranging between 1 mM to 7 mM were added, test mixtures were incubated for suitable time period, the reactions were arrested by addition of 1 ml 0.1 M borate buffer pH 9.5. The released p. nitrophenol was measured spectrophotometerically at 405 nm.

**Influence of metal ions on enzyme activity**

Three mM solutions of metal ions namely $Zn^{++}$, $Mg^{++}$, $Mn^{++}$, Hg. $Ca^{++}$ and $Cu^{++}$ were prepared from their corresponding salts. The assay was done by incubating 50 uL of each metal ion solution with a suitably diluted enzyme (~ 10 Units) for suitable time period under the standard conditions of pH and temperature. The substrate was added, and the reaction mixture was re-incubated for suitable time period. The reaction was arrested by addition of 1 ml 0.1 M borate buffer pH 9.5.

## RESULTS AND DISCUSSION

***Purification of AcPase and molecular weight determination:***

The cotyledon of *Erythrina indica* seeds contains high enzymatic activity that could hydrolyze the chromogenic pseudosubstrate *p*.nitrophenyl phosphate under acidic condition; therefore this enzyme is referred to as acid phosphatase (AcPase). Our trials to purify this activity started by extraction of overnight soaked softened seeds. The seeds coats were peeled off and whitish cotyledons were grinded in fruit mixture with ample amount of 0.145M NaCl till moderately viscous slurry was obtained. Treatment of this slurry with butanol was aimed to remove fats and pigments which are likely to interfere with purification and protein estimation (Konozy, 1999) which followed by dehydration with acetone and the resultant acetone dried powder was extracted with 0.145M NaCl, the obtained saline extract was the first step in our purification strategy. Soluble proteins were precipitated with 80% ammonium sulphate saturation to obtained Fraction A (FrA), this step led to increase in the specific activity of the enzyme by three times. Our next step was to try fractionating FrA proteins according to their molecular weights by using gelfiltration. The use of both Sephadex G-100 and Biogel P-100 were, unfortunately, futile and did not result in any appreciable AcPase fractionation. AcPase along with several other proteins that did exhibit neither AcPase activity nor other glycosidases activity, were eluted near the void volume of the columns (data not shown). Upon loading of FrA onto the affinity matrix ConA-Seralose 4B and subsequent elution of bound proteins with 50 mM mannose followed by 100 mM of the same sugar, two discrete protein peaks were obtained, testing these eluted fractions for AcPase activity, four AcPase activity peaks were noticed. These results besides being as a conclusive proof for the glycoprotein nature of this enzyme and that it possesses mannose unit at its putative glycan chain, it also indicates the presence of this enzyme in, at least, four forms in which two were eluted with 50mM mannose while others were firmly bound to the column and required higher concentration of thehaptenic sugar mannose to release the enzyme (Figure 1 ) Presence of AcPase.

in multiform or isozymes was not surprising to us, since several publications highlighted this fact (Chu *et al*., 1978; Panara *et al*., 1990; Waymack and Van Etten, 1991; Ferreira *et al*., 2000; Bozzo *et al*., 2002; de la Fuente van Bentem *et al*., 2003). Though this step did not result in pure preparation, however, 11 folds purification were obtained. Eluents rich in AcPase activity were pooled and dialyzed exhaustively against 50mM acetate buffer pH 5.0. Upon subsequent removal of precipitated protein by centrifugation, 48 folds purification of enzyme was obtained. The supernatant which was rich in AcPase was then loaded onto the cationic ion exchanger DEAE-cellulose. Elution the column with discontinuous gradient of 20mM NaCl resulted in fraction that was solely rich in AcPase protein. This fraction was then dialyzed against double distilled water and lyophilized to dryness (Figure 2).Native polyacrylamide gel electrophoresis (Native-PAGE) of this fraction resulted in single discrete band (Figure 3C), indicating the purity of the preparation. While two bands of around 42 and 38kDa were obtained by SDS-PAGE under reduced conditions, indicating the heterodimeric nature of the enzyme (Figure 2B). On loading the pure enzyme on the previously calibrated Bio-gel P-100, 89kDa was obtained as native molecular weight of the enzyme (not shown). Quite a few of plant AcPase are shown to be heterodimer; however,

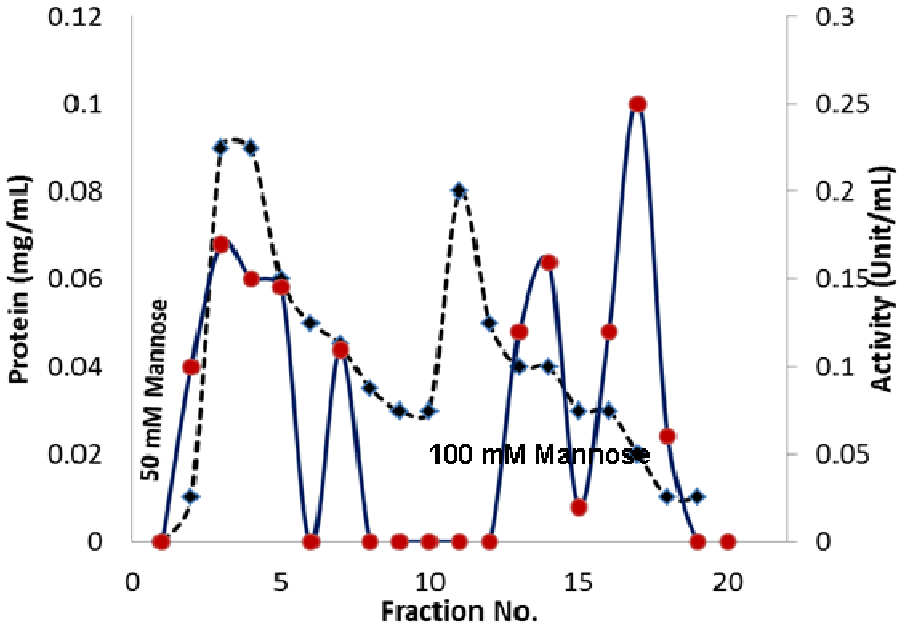



Figure 1:**FrA on the affinity matrix ConA-Seralose 4B.** 10 mg protein were loaded onto the affinity column and recycled several times to obtain maximum retention. Column was washed till free of unbound proteins. Bound protein was eluted by using 50 followed by 100mM mannose. Fractions of 2mL were collected and monitored for protein content and enzyme activity.
Solid line: Protein, Dashed line: AcPase activity.

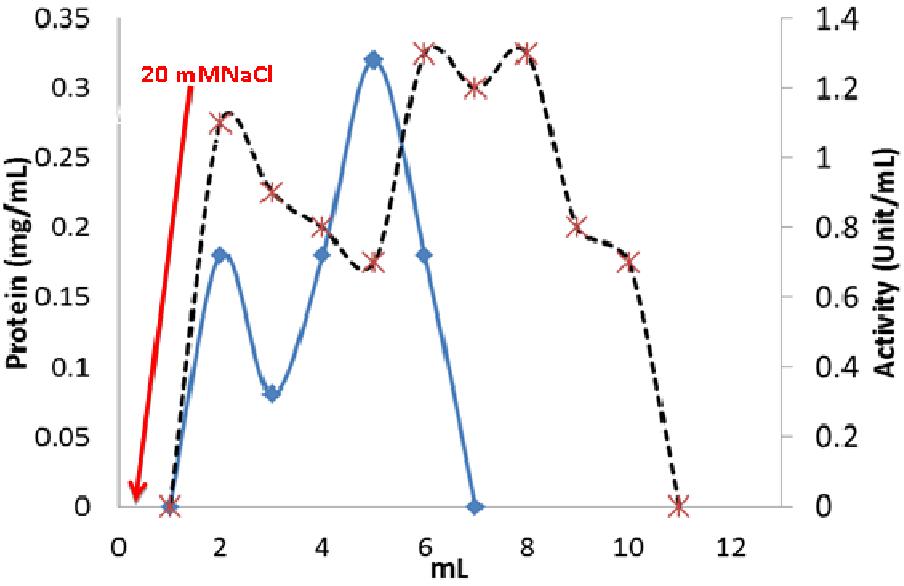



**Figure 2:** Purification of AcPase on DEAE-cellulose column. Column was made free of unbound proteins after loading. Elution of enzyme was made with 20mM NaCl. Solid line: Protein; Dashed line: AcPase

**Table 1:** Purification steps of AcPase

| Purification Stage | Total Protein (mg) | Activity (Unit/mL)* | Specific Activity (Unit/mg) | Fold Purification |
|---|---|---|---|---|
| **Saline Extract** | 2070 | 17 | 2 | 1 |
| **Fraction A** | 432 | 68 | 6 | 3 |
| **ConA- Seralose** | 2 | 7.2 | 20 | 11 |
| **pH 5 Acidification** | 0.54 | 17 | 92 | 48 |
| **DEAE-Cellulose** | 2 | 1 | 116 | 61 |

* **One Unit** of enzyme is defined as the amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute.

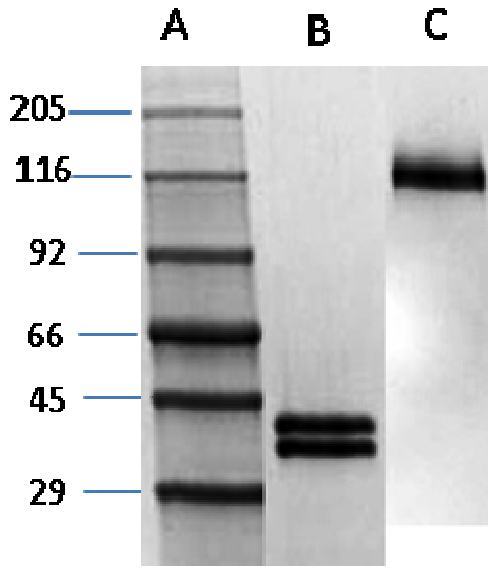



**Figure: Eletrophoresis of purified AcPase**
A: Protein molecular weight markers (Myosin 205 kDa; β-galactosidase 116 kDa; Phosphorylase B 92 kDa; Bovine serum albumin 66 kDa; Egg albumin 45 kDa; Carbonic anhydrase 29kDa.
B: SDS-PAGE of pure AcPase: 5µg of protein was loaded, protein was stained with coomassie brilliant blue G-250
C: Native-PAGE at pH 8.3 of pure AcPase

homodimer proteins were also reported. Interestingly all of the reported purified enzyme, from their variable source of purifications, exhibit dramatic variabilities in native molecular weights range from as small as 18kDa to more than 100 kDa (Schell *et al*., 1990; Joh *et al*., 1996; Demir *et al*., 2004; Gonnety; *et al*., 2006).

### *pH and temperature optima*

Like other AcPases, our preparation exhibited pH optima falling in the lower side of the pH, with maximum activity at around pH 5 (Figure 3A). As far as our result with the pH optima is concern, it's in agreement with Ullah et al (Ullah and Gibson, 1988) and Surchandra and his colleagues (Surchandra T. H *et al*., 2012) who purified the enzyme from mungs beans and soybean seeds, respectively.

AcPase was found to be active over a wide range of temperature (30 to 60°C). Maximum enzyme activity was observed at 45°C. After 55 °C the enzyme activity decreased sharply but was not completely abolished even at 75°C when 70% of total activity was lost (Figure 3B). These results confirm the high thermostability of AcPase isolated from cotyledon of *Erythrina Indica* seeds. These results are again in accordance with Surchandra et al who reported broad pH optima and thermostable AcPase from mungs been (Surchandra T. H *et al*., 2012). It is noticeably that majority of the so far characterized AcPases, from varying sources, possesses temperature optima above 40 °C and so the current study (Wannet *et al*., 2000; Leitao *et al*., 2010). Interesting exception was reported for the aquatic plant *Spirodela oligorrhiza* AcPase which had a pH

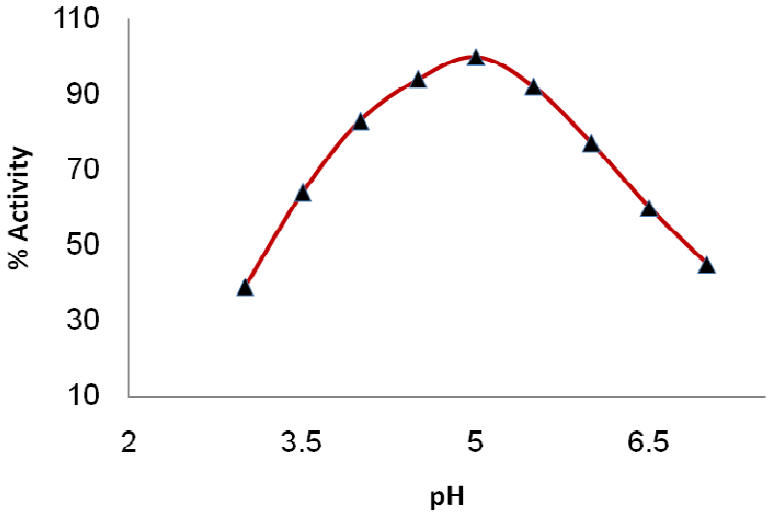
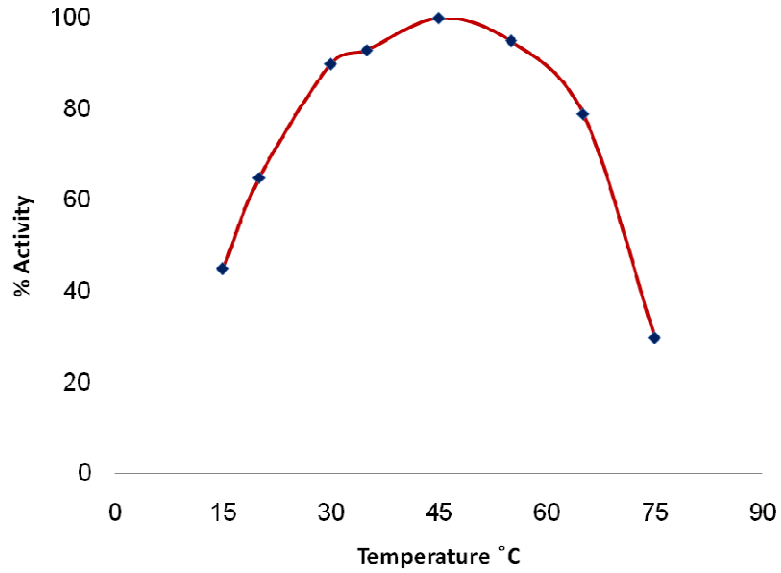



**Figure 3: Effect of pH and Temperature on AcPase catalyzed reaction**
A. Effect of pH




B. Effect of temperature

optimum of 6.0 and borad temperature optima from 10-40 °C (Hoehamer *et al*., 2005).

### *Effect of metal ions on AcPase activity*

The effect of $Hg^{+++}$,$Mn^{++}$, $Zn^{++}$, $Cu^{++}$and $Mn^{++}$ and Mg metals on *Erythrina indica* seeds AcPase was studied using a *p*-NPP (6 mM) as substrate. Among these metal ions; $Zn^{++}$ was the most potent inhibitor, followed by $Hg^{+++}$. Whereas 82% and 38% increase in enzyme activity was noticed in the presence of Cu and Ca, respectively. Metals like Mg and Mn had no or very minor change in AcPase activity (Figure 4). Interestingly, castor seeds and banana fruit acid phosphatase were significantly inhibited by copper, while mild inhibition was observed by $Zn^{++}$(Srivastava and Anand, 2015). Inhibitory effect of $Zn^{++}$ was reported with soybean seeds, however, in the same paper $Cu^{++}$, which acted as activator for AcPase in the current study, had inhibitory effect (Ferreira *et al*., 1998).

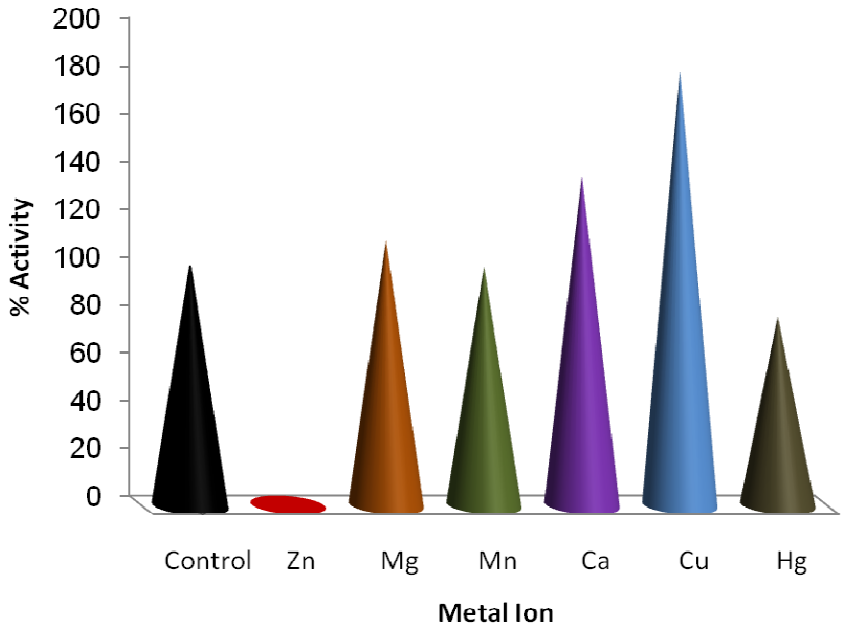



**Figure 4: Effect of metal ions on AcPase activity**
**Control: Indicates enzyme activity in absence of any metal ion**

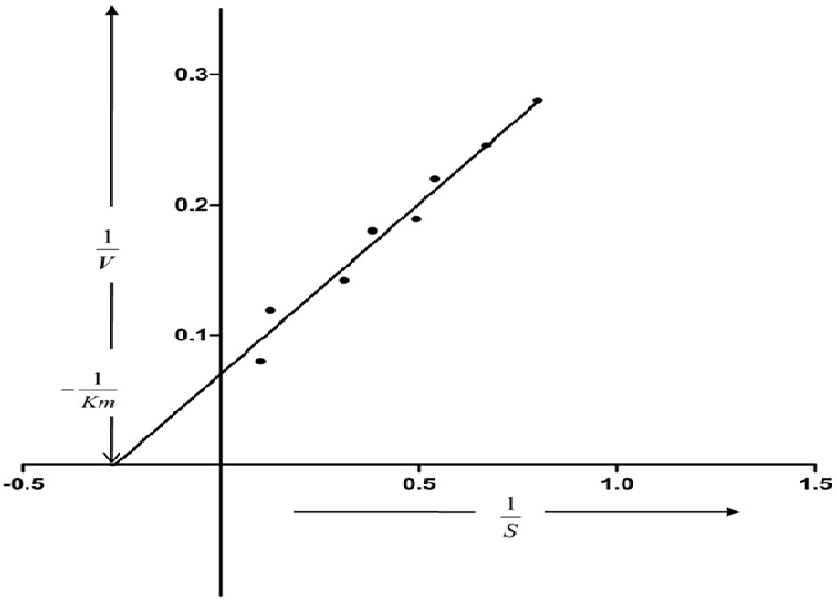



**Figure 5: Effect of substrate concentrations on AcPase**

***Effect of substrate concentration on AcPase activity:***

AcPase $K_M$ and $V_{max}$ values were determined to be 3.3 and 13.3 mM, respectively (Figure 5). The kinetic values obtained in this investigation seem to be different from AcPase reported in several other periodicals. In fact, none of the reports in the literature indicated similar or near to similar data (Ullah and Gibson, 1988; Jonsson and Aoyama, 2009; Surchandra T. H *et al.*, 2012).

## CONCLUSION

Though the current investigation adds a mosaic piece of information to our current knowledge on these interesting enzymes, however, their variable native and subunit molecular weights and kinetics data may complicate any trial to assign for them any conclusive physiological role(s).

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