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Isolation and characterization of alkaline phospatase from the hepatopancreatic wastes of brown shrimp, Parapeneopsis stylifera

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ABSTRACT

Marine environment contains a large pool of diversified species adapted to a variety of habitat conditions and enzymes with unique properties for industrial usage can be recovered as by-product from fish processing wastes. Shrimp alkaline phosphatase (SAP) was isolated and purified to homogeneity from the hepatopancreatic wastes of brown shrimp (Parapenaeopsis stylifera). Alkaline phosphatase was extracted from the tissues by homogenization at 3000 rpm for 10 minutes, followed by centrifugation at 10000 rpm for 30 minutes, precipitation at 55% ammonium sulphate saturation level, and dialysis for 12 hours against tris buffer. Further purification was carried out by column chromatography through silica gel and DEAE-sephadex. Even though 37°C was the optimum temperature for catalytic activity, alkaline phosphatase retained 91% of its activity after incubation at 65°C for 15 minutes and 75% of its activity after incubation at 100°C for 15 minutes. Optimum pH for catalytic activity of alkaline phosphatase was 9. Catalytic activity of the shrimp alkaline phosphatase was enhanced by Ca^{2+} or Zn^{2+} at lower concentration, inhibited above 10mM concentration of Ca^{2+} or Zn^{2+}, inhibited by increasing concentration of EDTA and K^{+}, activated by Na^{+} and Mg^{2+}, and reactivated the Ca^{2+} inhibited alkaline phosphatase by Mg^{2+}.

KEYWORDS

Alkaline phosphatase;
Brown shrimp;
Enzyme;
Temperature;
Inhibitor;
pH;
Activators.

INTRODUCTION

Alkaline phosphatase (EC 3.1.3.1) are non-specific phosphomonoesterases that are found widely in species ranging from bacteria to man. Marine environment contains a large pool of diversified species adapted to a variety of habitat conditions and exploitations of the unique properties of fish enzymes are only at the beginning stage. Enzymes with unique properties for industrial usage can be recovered as by-product from fish processing wastes. Alkaline phosphatase isolated from arctic shrimps completely and irreversibly inactivates at 65°C for 15 minutes[1,2] and are cold adopted[3]. While alkaline phosphatases isolated from the warm water shrimp, Penaeus japonicus and clam, Meretrix lusoria are unique with their thermostability at 65°C for

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30 minutes[4]. Alkaline phosphatase from Penaeus monodon can be differentiated by its pH optimum at 9.0, while alkaline phosphatases from human placenta, bovine milk, and clam studied have their optimal pH at 10.0. Alkaline phosphatase (SAP) from the hepatopancreas of Macrobrachium nipponense (Crustacean: Palaemonidae) showed increased activity gradually with increasing pH value from 7.6 to 9.8[5]. Alkaline phosphatases with a pH optimum around 9 and 10 are now widely used to clean the stock, increase water adsorption, remove hair and, get clean and relaxed pellet from the hide or skin. Alkaline phosphatase from Jawala shrimps (Acetes indicus) showed the pH optima of 9.5, optimal temperature of 40^0C, inhibition by 1, 10-phenanthroline and EDTA, and reactivation by Zn^{2+} and Mn^{2+} salts[6]. Alkaline phosphatase (SAP) from the cold water shrimp, Pandalus borealis is completely inhibited by EDTA, but the activity can be restored to a large degree by Zn^{2+}[7]. Green crab (Scylla serrata) alkaline phosphatase gets inactivated by Zn^{2+}, a complex scheme where it first reversibly and quickly binds Zn^{2+} and then undergoes a slow reversible course to inactivation and slow conformational change[8]. Even though alkaline phosphotase were isolated and characterized from different cold adopted shrimp, not much attempt has been made to study shrimp alkaline phosphatase from the warm water zone where fish processing industry discard amounts to about quarter of the value added seafood products. There is a considerable demand for enzymes with the right combination of properties for specific application in Industry. Attempt is been made to isolate and characterize alkaline phosphates from the hepatopancreatic tissues of marine shrimp discard from Mangalore region.

EXPERIMENTAL

Enzyme preparation: Brown shrimp caught using trawl nets from the Arabian Sea were obtained from the fishing boats landed in 'Bunder area', Mangalore between November and December month. The time lapsed between catching and landing may not exceed over four to six hours. A 10 kg portion of the freshly caught brown shrimp belongs to size group of 7 to 8 cm long; each weighing around 7 to 9 grams was selected for the present study. The material was brought in an insulated container after adequately icing them in the proportion of 1:1 shrimp to ice, to the laboratory within two hours. The head portion was collected, cleaned, packed in plastic bags, labeled, frozen at -40^0C and stored at -20^0C in a deep freezer until further use. Shrimp head wastes were thawed at room temperature of 28^0C. The hepatopancreas and attached tissues were selected, weighed and homogenized using the potter-elvehjem homogenizer (Rotek Instruments, Kerala,) using 0.1 M-Tris buffer, pH 8.2 at homogenization speed of 600, 1200, 1800, 2400, or 3000 revolutions per minutes for 10 minutes. Resulting homogenates were filtered through Hyflo Super-Cel and stored at 4^0C. Filtered crude homogenate obtained were assayed for Total protein and alkaline phosphatase as cited elsewhere. The crude homogenate with highest protein were centrifuged at 2000, 4000, 6000, 8,000, or 10,000 revolutions per minute for 30 minutes at 4^0C in a cooling centrifuge (REMI Instruments Ltd. Mumbai). Each supernatant and precipitate collected was assayed for protein content and alkaline phosphatase activity. Extract with highest specific activity for alkaline phospahtase were fractionated with ammonium sulphate at 25, 35, 45, or 55 % saturation levels as described by Deutscher[9], followed by dialysis against 0.1M-Tris buffer, pH 8.2 at 4^0C using cellulose dialysis tubing of 35000 MWCO (Hi Media Laboratories Ltd, Mumbai) and assayed for Total protein[10] and alkaline phosphatase[11] at different time intervals of 6, 12, 18 and 24 hours with the change of buffer on every 2 hours. Further purification was carried out in silica gel chromatography, followed by DEAE-Sephadex (GE Healthcare, Bangalore) chromatography at 28^0C. The buffer for DEAE-Sephadex chromatography was 0.05M-tris-HCl, pH 7.6, and the enzyme was eluted from the DEAE-Sephadex column, which measured 40cm×2cm of 250ml capacity, by an increasing concentration of NaCl.

Electrophoresis

SDS-PAGE was performed by Laemmli[12] gel method utilizing vertical gel electrophoresis (Bhat-Biotech, Bangalore) apparatus using ready to mix SDS-PAGE gel kit (Bhat-Biotech, Bangalore) as per the manufacturers instructions. The SDS gel was run at 100 volts for 1 hour. Purified alkaline phosphatase was identified in gel lanes through visualization coomassie blue

staining procedure (Bhat-Biotech, Bangalore).

Assay of alkaline phosphatase

The measurement of alkaline phosphatase activity was based upon the work of Brandenberger and Hanson[11]. The catalytic effect of the enzyme on the initial rate of hydrolysis of p-nitrophenylphosphate was determined by measuring an increase in absorbance at 410nm resulting from the hydrolysis of p-nitro phenylphosphate to p-nitrophenol using a UV-Visible double beam spectrophotometer 2201 (Systronics, Mumbai). One unit releases one micromole of p-nitrophenol per minute under the specified conditions. Alkaline phosphatase activity was expressed as, μmoles of p-nitrophenol released/min/mL of enzyme solution.

Effect of temperature on enzyme activity

Temperature effect upon alkaline phosphatase activity was examined by establishing the temperature of the enzyme assay solution[11], within each, individual test tubes at 25, 35, 45, 55, 65, 75, 85, 95 and 100^{0}C. As temperature was the only parameter being changed in this line of analysis, all other aspects of the assay were identical to those already described for the standard enzyme assay. Reaction temperature was obtained through use of either a water bath or in cooling BOD incubator (Rotex, Kerala).

Effect of pH on enzyme activity

Analysis of pH effect upon hydrolysis of artificial substrate by alkaline phosphatase was determined[11] at pH values ranging from 2.0 to 10.0. Measurements of pH were made on a pH-meter (Systronics, Mumbai). All other aspects of the alkaline phosphatase assay were as described in standard assay conditions.

Effect of inhibitors or activators on enzyme activity

Alkaline phosphatase activity[11] in the presence of ethylene diamine tetra acetic acid (EDTA) at 0.1mM, 1.0mM and 10.0mM concentrations was conducted. Effects of cations like $CaCl_2$, KCl, $ZnCl_2$ and $MgCl_2$, NaCl (Merck, Mumbai) upon alkaline phosphatase activity were examined at 0.1, 1, 10, and 100mM concentrations of cations. All other aspects were identical as described for our standard assay conditions.

Chemicals

All the chemicals used were of analytical grade and were obtained from Merck Limited (Mumbai, India).

Statistical analysis

One- and two-way ANOVA was performed using Statographics 2.1 (STSC Inc., Rock vile, MD). The difference in means was analyzed using a Turkey HSD test ($p<0.05$).

RESULTS AND DISCUSSIONS

Alkaline phosphatase was isolated from the hepatopancreatic tissues of brown shrimp, Para peneopsis stylifera, through various purification steps. It is interesting to note that increase in homogenization speed increased release of protein (Figure 1). The absence of rigid cell wall makes the homogenization of the hepatopancreatic tissue of shrimp easy. The homogenization speed of 3000 rpm for 10 minutes resulted in homogenate with highest specific activity of 17.46±0.025 units/mg of protein for alkaline phosphatase. But it is instructive to note here that the thaw drip of frozen shrimp before homogenization showed a specific activity of 9.79±0.025 units/mg of protein and contributes significant ($p<0.01$) portion of the specific activity and hence care should be taken so as to not to exclude these portions on or before homogenization. Usually during cell disruption yield is measured in terms

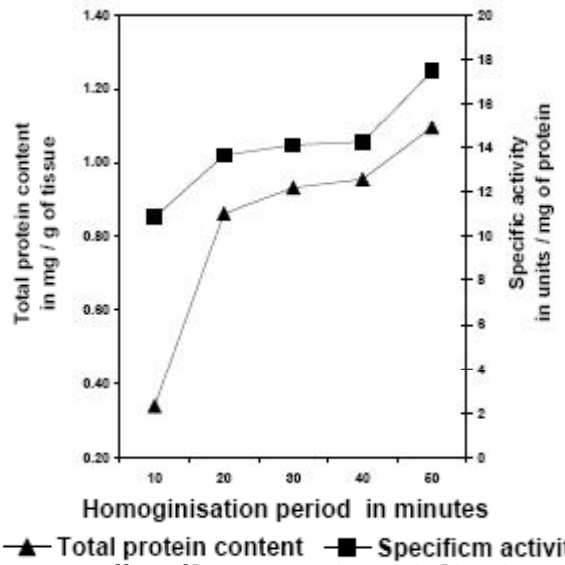

Figure 1 : Effect of homogenization period in minutes at 45 revolution per second on extraction of alkaline phosphatase and total protein content of hepatopancreatic tissues of brown shrimp (parapeneopsis stylifera)

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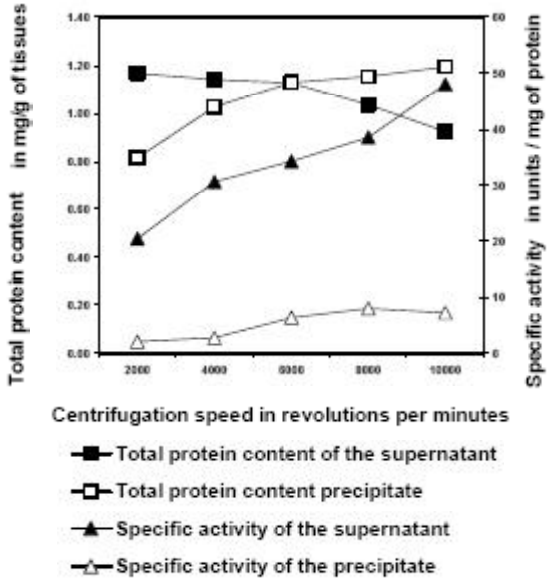


Figure 2: Effect of centrifugation speed in revolutions per minutes at 4°C on the sepeartion of alkaline phosphatase and total protein content of hepatopancreatic tisue of Brown shrimp (Parapeneopsis stylifera)

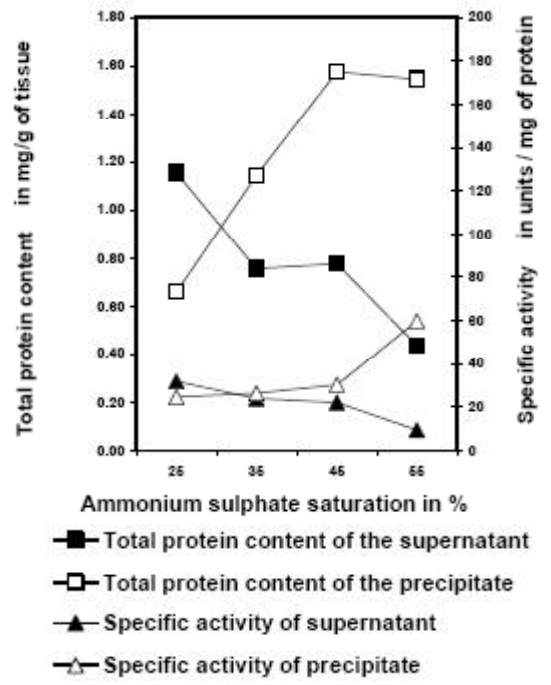


Figure 3: Effect of ammonium sulphate saturation on the extractability of alkaline phosphatase and total protein in the hepatopancreatic tissues of brown shrimp (Parapeneopsis stylifera)

of the total protein releases, rather than the specific activity of the enzyme[13]. We have selected a homogenization speed of 3000 rpm for 10 minutes for further

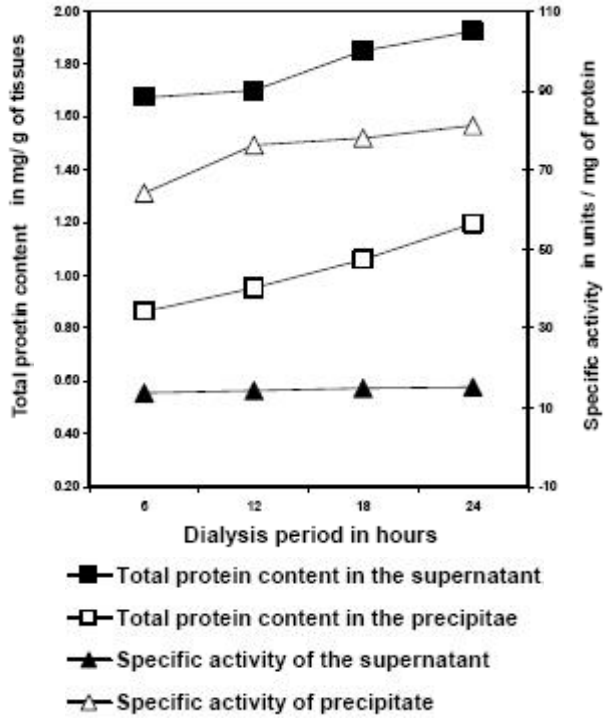


Figure 4: Total protein content and alkaline phosphatase activity in the ammonium sulphate precipitated hepato pancreatic tissue extract of brown shrimp (Parapeneopsis stylifera) at different intervals of dialysis period

purification as maximum ($p<0.01$) quantity of protein released by this method. At a centrifugal speed of 10,000 rpm for 30 minutes at 4°C, quantity of protein sedimented was more. While specific activity of alkaline phosphatase in the supernatant increased significantly ($p<0.01$) with the increase of centrifugation speed from 2000 to 10000 rpm and in the sediment specific activity decreased proportionately (Figure 2). Centrifugation speed of 10000 revolutions per minutes for 30 minutes at 4°C was optimum in removing the impurities and proceeded for further purification. It is interesting to note here that ammonium sulphate at 55% saturation level extracted maximum ($p<0.01$) enzymes in to precipitate with the specific activity of the precipitate for alkaline phoshatase was 59.71±0.184 units/mg of protein, and retained minimum ($p<0.01$) enzymes in the supernatant with specific activity of the supernatant for alkaline phosphatase was 9.33±0.016 units/mg of protein (Figure 3). Dialysis of the buffer resuspended pellets which were precipitated at 55% ammonium saturation levels, against 0.01M-tris buffer, pH 8.2 at 4°C

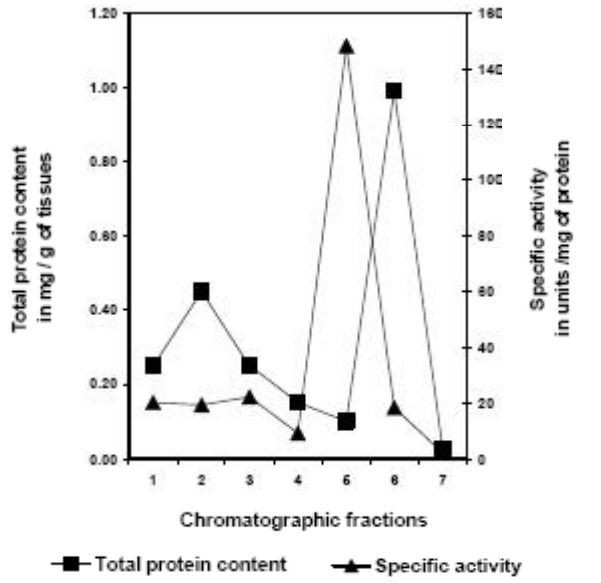


Figure 5: Total protein content and specific activity of alkaline phosphatase in different fractions during chromatographic seperation in silica gel of extract of hepato pancreatic tissue of brown shrimp (Parapeneopsis stylifera)

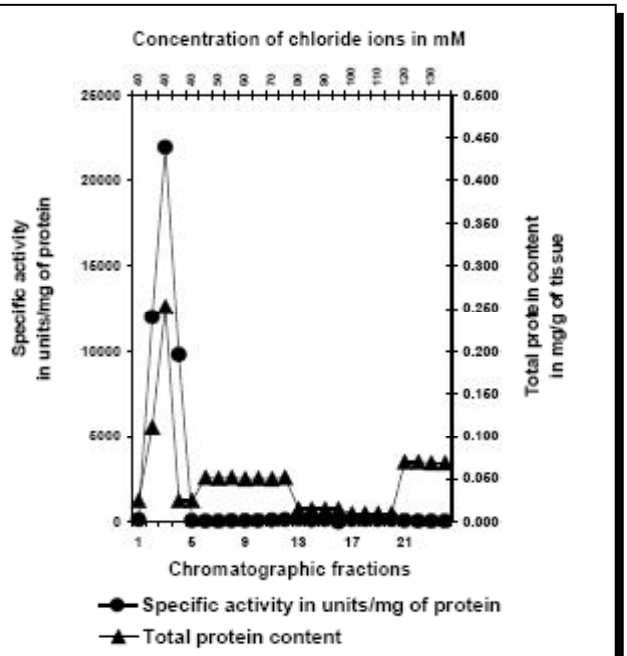
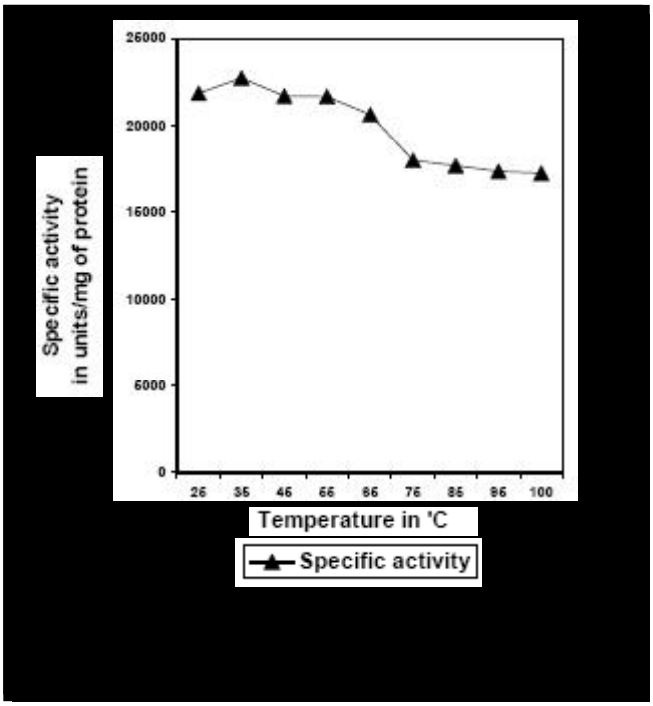


Figure 6: DEAE-Sephadex chromatogram of shrimp alkaline phospatase isolated from hepatopancreatic tissues of brown shrimp (Parapeneopsis stylifera)

for 24 hours recovered samples with highest specific activity for alkaline phosphatase (Figure 4). Since there was no significant ($p>0.05$) difference in the specific activity of alkaline phosphatase was observed between 12th and 24th hours of dialysis, dialysis for 12 hours was chosen for further purification. Shrimp alkaline phosphatase was eluted from silica gel as single peak (Figure 5). Separation of this peak on DEAE-Sephadex was judged as pure because symmetrical peaks of activity and protein were obtained when the purified enzyme was chromatographed (Figure 6). The sharp peak of alkaline phosphatase eluted from DEAE-Sephadex in the initial fractions. These data were confirmed by the behavior of the enzyme during electrophoresis on SDS-PAGE. Chromatographic fractions constituting the sharp peak were pooled to provide the source of enzyme used in the characterization.



The experiment was performed where the purified alkaline phosphatase was assayed at various temperatures (Figure 7). The temperature optimum was at 35^0C although the enzyme showed activity over an unusual broad range of temperature. The reaction mixture contained 0.1M-tris buffer, pH 8.2 and 1mM p-nitrophenyle phosphate. Since there is a small amount of nonenzymatic hydrolysis, especially at the higher temperature a control without enzyme was run at each temperature. In our study alkaline phosphatase demonstrated as a very thermostable enzyme, retaining about 91% of its activity after being incubated at 65^0C for 15 minutes and 75% of its activity after being incubated at 100^0C for 15 minutes when we compared it to the specific activity of alkaline phosphatase at 35^0C for 15 minutes. A heat stable alkaline phosphate was also iso-

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lated from Penaeus japonicus by Chuang[4] and the alkaline phosphatase from bivalve, Meretrix lusoria as reported by Chuang and Yang[14] is unique with its thermostability at 65^{0}C for 30 min.

Exposure of alkaline phosphatase to low pH led to a progressive loss of enzyme activity at 37^{0}C (Figure 8). Above pH 8 catalytic activity of alkaline phosphatase increased slowly, but as the pH was lowered the rate of inactivation was markedly ($p<0.01$) increased. Wang and others[5] showed that the alkaline phosphatase activity of shrimp (Macrobrochium nipponense) increased gradually with increasing pH value from 7.6 to 9.8. In our study the optimum pH for alkaline phosphatase was found to be 9. This pH value is comparable with alkaline phospatase of Penaeus monodon reported by Chuang and Yang[14] in the whole homogenate of the hepatopancreas by the same procedure. It is instructive to note that sharp decline ($p<0.01$) the catalytic activity observed at pH 6 and reached the lowest at pH 3.

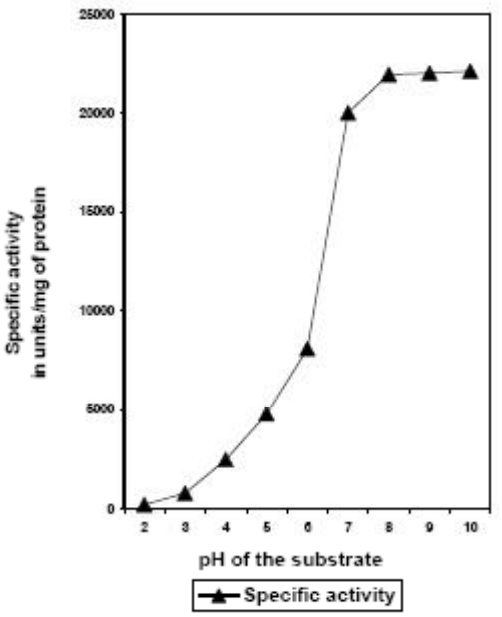
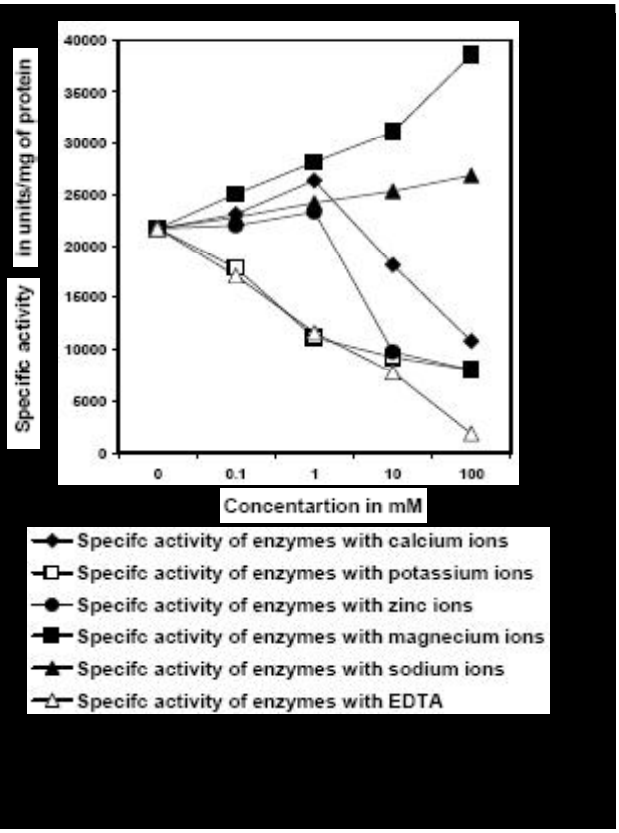


Figure 8 : Effect of pH of the substrate on the enzyme activity of alkaline phosphatase isolated from the hepatopan creatic tissue of brown shrimp (Parapeneopsis stylifera)

The catalytic activity of alkaline phosphatase was measured for different concentrations of CaCl2, KCl, ZnCl2 and MgCl2, NaCl and EDTA (Figure 9). Catalytic activity of the alkaline phosphatase increased as the concentration of calcium or zinc ions increased ($p<0.01$), but further increase in concentration of calcium or zinc ions to 10 and 100mM significantly ($p<0.01$) decreased specific activity of the enzyme, but increase in specific activity was 21% and 7% and decrease was 49% and 37% respectively in samples with calcium and zinc ions when compared to those samples without metal ions. Calcium increases the activity of the enzyme at lower concentrations[13] and calcium ions inhibit the enzyme activity at hepatopancreas at higher concentrations[15]. This is due to the kinetic and structural changes of intestinal alkaline phosphatase as a function of calcium concentration and enzymatic activity increases as a linear function, but above 10 mM calcium concentration enzyme activity decreases as shown by Brun and others[16]. It is interesting to note that increase in concentration of potassium ions in the reaction mixture significantly ($p<0.01$) inhibited alkaline phosphatase. It is instructive to note that 63% of alkaline phosphatase was inhibited in those samples incubated with 100mM concentration of potassium ions for 15 minutes. Increase in the concentration of the magnesium or sodium ions increased ($p<0.01$) the catalytic activity of the alkaline phosphatase and highest ($p<0.01$) specific activity of alkaline phosphatase was observed at 100mM concentration of magnesium or sodium ions



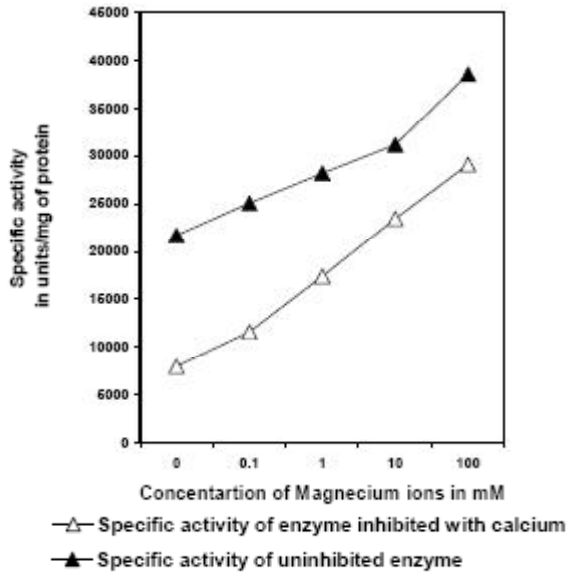


Figure10 : Effect of magnesium ions (Mg^{2+}) on the enzyme activity of inhibited and uninhibited alkaline phosphatase isolated from the hepatopancreatic tissue of brown shrimp (Parapeneopsis stylifera)

and the increase in the activity is nearly 77% for the former and 23% for the latter. Exogenous magnesium ions have been shown to activate the enzyme activity of the alkaline phosphatase[13]. Even in samples shrimp alkaline phosphatase treated with 100mM concentration of calcium ions for 15 minutes were incubated with increasing concentration of magnesium ions and found that specific activity of the inhibited alkaline phosphatase increased to nearly one and half fold. Catalytic activity of the alkaline phosphatase inhibited by calcium can be restored by magnesium ions[5]. Zinc ion induces a slow conformational change of the enzyme, which locks the enzyme in a conformation having an extremely high affinity for the Zn^{2+}. Enzyme activity changed at lower concentrations of substrate, indicating a complex cooperativity between Zn^{2+} and pNPP [17]. Both Mg^{2+} activation and Zn^{2+} inhibition of the enzyme are reversible processes (Figure 10) and magnesium ion may play an important modulatory role in the cell for protecting the enzyme by retaining a favorable geometry of the active site needed for catalysis [18].

In our present study increase in concentration of EDTA significantly ($p<0.01$) decreased the catalytic activity of shrimp alkaline phosphatase. Almost 92% decrease in the catalytic activity of the alkaline phosphatase was observed. The enzyme alkaline phosphatase was inhibited by EDTA indicating that it is a metalloprotein[5]. Alkaline phosphatase from the hepatopancreas of cold water shrimp (Pandalus borealis) as reported by Olsen and others[7] completely inhibited by EDTA, but the activity was restored to a large degree by zinc. Competitive complexing mechanism and process of inactivation by EDTA composed of the rapid initial formation of an enzyme-EDTA complex and change in the conformational structure of alkaline phoshatase[19].

CONCLUSIONS

Homogenization speed of 3000 rpm for 10 minutes is ideal for the extraction of the enzyme alkaline phosphatase from the hepatopancreatic tissue of brown shrimp, Parapeneopsis stylifera, as optimum protein extractability was obtained at this level. The centrifugation speed of 10000 rpm for 30 minutes at 4^0C gave maximum specific activity for alkaline phosphatase and this speed was found to be optimum for separation of impurities from the homogenate. The ammonium sulphate saturation of 55% followed by dialysis period of 12 hours was found to be ideal for extracting alkaline phosphatase enzyme from the crude homogenate of the hepatopancreatic tissue of shrimp. Calcium and zinc ions were found to be an activator of the alkaline phosphatase activity, but at higher than 10mM concentration of calcium or zinc ions were found to decrease the catalytic activity. Magnesium and sodium ions are shown to have an enhancing effect on the catalytic activity alkaline phosphatase. Specific activity decreased drastically at increasing concentration of EDTA and potassium ions. The enzyme extracted from Brown shrimp is found to be heat resistant. The optimum pH for the alkaline phosphatase was pH 9.

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