〈Original Article〉

Characteristics of alkaline phosphatase isolated from the hepatopancreas of Indian white shrimp, (*Penaeus indicus*)

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Summary Alkaline phosphatase of novel characteristic was optimally isolated from the hepatopancreas of Indian white shrimp. The optimum temperature of the enzyme was at 50℃, and was thermally stable up to 40℃ for 30 min. The enzyme hydrolysed paranitrophenyl phosphate (*p*NPP) optimally at pH 9 and 37℃, and was stable between pH 7-10 for 30 min. Calcium ion activated the enzyme up to 10 mmol/L, and inhibited beyond this level. The enzyme lost less than 90% of the activity at 0.1 mmol/L of zinc, and beyond this level loss of the activity was substantial. Magnesium ions up to 1.0 mmol/L activated the enzyme. The K_m values of *p*NPP for the enzyme were 0.05±0.0009 mmol/L and V_{max} of the enzyme during the hydrolysis of *p*NPP as substrate was 50.00±0.99 mmol/L/min. Hence, shrimp industrial waste can be converted into commercially important, environmental friendly and socially responsible product.

Key words: Alkaline phosphatase, Indian white shrimp, Anion exchange chromatography, Enzyme purification, DEAE-cellulose

1. Introduction

Alkaline phosphatase with unique physico-chemical properties of industrial importance can be recovered from the hepatopancreas of shrimps as they are adapted to various oceanic conditions[1, 2]. Heat-stable alkaline phosphatase was isolated from *Penaeus japonicus* with molecular weight of 55,000 and an isoelectric point (pI) of 7.6[3]. Alkaline phosphatase isolated from calm, *Meretrix lusoria* showed thermostability at 65℃ for 30 min and pH optimum at 10, whereas the alkaline phosphatase isolated from *Penaeus monodon* were inactivated at 65℃ for 30 min and showed optimum pH at 9.0[4]. Alkaline phosphatase isolated from Jawala shrimp, *Acetus indicus* showed optimum pH of 9.5 and optimum temperature of 40℃, and completely inhibited by 1, 10-phenanthroline and EDTA, but the activity of inhibited enzyme was restored by Zn^{2+} and Mn^{2+} salts[5]. Three alkaline phosphatase isomers resistant to the action of bacterial sialidase was isolated from the hepatopancreas of *Penaeus monodon* were resolved with an molecular weight of 31,000, 31,000 and 43,000 in SDS-PAGE, and these isomers were differentiated by inhibitions with EDTA, inactivation with heating, its Michaelis constant (K_m) or its pI[1]. Heat-

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stable alkaline phosphatase isolated from *Penaeus japonicus* was stable to heating at 65℃ for 5 min, and was found to consist of deglycosylated monomers of molecular weight 40,000 in SDS-PAGE and has a pI of 7.6[6]. Alkaline phosphatase isolated from *Penaeus penicillatus* was reported to have the molecular weight of 82,000 Da, optimum temperature of 47℃, optimum pH of 8.2, and K_m value of 8.0×10^{-4} mol/L, and further reported to have activated by magnesium and inhibited by copper ion, mercury ion, methanol, ethanol and ethylene glycol[7].

Since their discovery in 1923, though there has been a great deal of interest in the alkaline phosphatases over 90 years of study, there is much about the enzyme that remains unknown. Physico-chemical characteristics of the alkaline phosphatase isolated from various sources have reported to show distinct in some aspects, and even alkaline phosphatase isolated from different species of shrimps are known to vary in their physico-chemical properties[3-7]. This is being told, the enzyme kinetics of alkaline phosphatase of hepatopancreas from Indian white shrimp described here provide valuable insights concerning the kinetic behavior of the enzyme available abundantly along Mangalore coastal region that on the other hand dumped as a waste from seafood processing plant.

2. Materials and Methods

1. Chemicals and reagents

Analytical grade chemicals and reagents procured form Merck Limited (Mumbai, India) and Himedia (Mumbai, India) and were used according to the current American Chemical Society specifications[8]. Sodium acetate buffer with buffering capacity of pH 4 or 5 was obtained by combining 146 mL or 59 mL of 0.1 mol/L acetic acid, and 36 mL or 141 mL of 0.1 mol/L sodium acetate, respectively. Citrate acetate buffer with buffering capacity of pH 6 was obtained by combining 9.5 mL of 0.1 mol/L citric acid and 41.5 mL of 0.1 mol/L sodium acetate solutions. Tris(hydroxymethyl)-aminomethane buffer of buffering capacity of pH 7 or 8 was obtained by combining 50 mL 0.1 mol/L Tris, with 46.6 and 29.2 mL of 0.1 mol/L HCl, and diluting it to 100 mL. Glycine buffer of pH 9 or 10 were obtained by combining 25 mL glycine stock of 0.2 mol/L solution with 4.4 or 16 mL of 0.2 mol/L NaOH, respectively, and then diluted with deionised water to make a 100 mL solution[9]. Alkaline phosphatase assay buffer used for the present study was 2-amino 2-methyl 1-propanol (AMP) buffer of pH 10.3, which was prepared by dissolving 78 g of AMP in 500 mL of deionised water, and then 200 mL of 1 mol/L hydrochloric acid (HCl) was added, and subsequently made up to 1000 mL in 1 L volumetric flask using deionised water. Then, 0.1 mol/L Tris-HCl buffer of pH 8.4 was prepared by adding 1000 mL of 0.1 mol/L Tris Base to 500 mL of 0.1 mmol/L Tris HCl. Here, 0.1 mol/L Tris (hydroxymethyl) aminomethane solution was prepared by taking 12.114 g of the Tris (hydroxymethyl) aminomethane in 1000 mL volumetric flask and making up the volume to 1000 mL using deionised water.

2. Raw material

Indian white shrimp was caught near FAO fishing area, 51, Indian Ocean Western between the months of July and December using trawl nets. Freshly caught Indian white shrimp were transported to the laboratory within two hours in an insulated container maintained at less than 4℃ using ice at 1:1 shrimp to ice ratio, and identified according to Racek[10]. The shrimps of the size group of 105-140 mm in length and weighing around 39-64 g were sorted, washed, and dissected to remove pancreas for further processing.

3. Isolation of alkaline phosphatase

The hepatopancreatic tissues were homogenised (RH-2 model Homogenizer, Rotek Instruments, India) at the speed of 3,000 rpm for 10 min using working buffer at 4℃. The homogenate was clarified of insolubles (C-24BL/CRP24 model centrifuge, Remi Laboratory Instruments, India) at relative centrifugal force (RCF) of 1681.1 x g for 5 min in at 4℃. The clarified homogenate was subjected to ammonium sulfate precipitation at 65% saturation level at 0℃ and pellets obtained at RCF of 15,124.8 x g for 30 min at 0℃ were reconstituted using working buffer at 1:1 pellets to buffer ratio. Reconstituted pellets were

dialysed to remove the dissolved salts using 10,000 Da cellophane tubes (Himedia, Mumbai, India) in working buffer at 1:10 sample to buffer at 4℃ for 24 h at constant stirring[11].

4. Purification of alkaline phosphatase

Dialysed homogenates were subsequently purified in DEAE-cellulose chromatographic columns of size 8×80 mm (internal diameter × height) using binding buffer of pH 8.4 with 0.10 mol/L NaCl at flow rate of 1 mL/min. Bound proteins were eluted from the column using elution buffer of 0.10-0.35 mol/L NaCl gradiance at flow rate of 1.5 mL/min. Homogeneity of the enzyme preparation was determined using Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli[12].

5. Effect of temperature on enzyme activity

Temperature effect upon the enzyme activity was done according to Bower Jr. and McComb[13] but at temperature 20, 30, 40, 50, 60, 70, 80, 90, or 100℃ using AMP buffer of pH 10.3. Thermal stability of the alkaline phosphatase was determined by estimating remaining activity of the enzyme at 37℃ for 15 min at pH 10.3 of the samples that has been pre-incubated at 20, 30, 40, 50, 60, 70, 80, 90, or 100℃ using AMP buffer of pH 10.3 for 30 min using substrate control.

6. Effect of pH on enzyme activity

Analysis of pH effect upon hydrolysis of *p*NPP by alkaline phosphatase was determined according to Bower Jr. and McComb[13] but at pH values 4, 5, 6, 7, 8, 9, 10, or 11 at 37℃ using pH-meter (Systronics, Mumbai). The activity of the alkaline phosphatase was determined at pH 4-11 range and 37℃ temperature. Stability of the enzyme was determined by estimating remaining activity at 37℃ and pH 10.3 in those samples that were pre-incubated at 37℃ for 30 min at varying pH of 4, 5, 6, 7, 8, 9, 10, and 11.

7. Effect of effectors on enzyme activity

Analysis of effectors on the hydrolysis of *p*NPP at 37℃ and pH 10.3 by shrimp alkaline phosphatase was determined according to Bower Jr. and McComb[13] but replacing $MgCl_2$ and $ZnCl_2$ of the assay buffer with 0.1, 1, 10, or 100 mmol/L concentrations of EDTA, calcium chloride ($CaCl_2$), potassium chloride (KCl), zinc chloride ($ZnCl_2$) and magnesium chloride ($MgCl_2$), and these activities obtained were compared with the activities of those respective samples performed using assay buffers incorporated only with $MgCl_2$ and $ZnCl_2$ at 1 mmol/L concentration levels.

8. Determination of K_m value and V_{max} value

The rate of hydrolysis of *p*NPP by the enzyme was measured at substrate concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, or 5.0 mmol/L at pH 10.3. Maximum enzyme catalysed reaction (V_{max}) of *p*NPP by shrimp alkaline phosphatase and Michaelis constant (K_m) of alkaline phosphatase for *p*NPP will be measured at 37℃ in working buffer. Respective kinetic parameters were evaluated from Lineweaver-Burk plot[14].

9. Proximate analysis

The protein content was estimated by the Folin-Ciocalteu method of Lowry et al.[15], using bovine serum albumin (BSA) as a standard and expressed as mg/mL. Moisture content and solid content of the samples were estimated as per the guidelines of FAO[16], and expressed as percentage moisture.

10. Enzyme assay

The procedure used for alkaline phosphatase analysis was based on the method of Bowers and McComb[13] using the substrate *p*NPP. Alkaline phosphatase activity in units/L is the liberation of 1 mmol/L of paranitrophenol per min at 37℃ incubation temperature per liter of tissue homogenate in respective buffers.

11. Statistical analysis

The analysis of samples was carried out in quadruplicate. The results were treated by analysis of variance (ANOVA), followed by Tukey's test, using the software Statistica 6.0 (Statsoft, Tulsa, OK, USA). The results were expressed as averages±standard deviations followed by corresponding letters which indicates the significant differences. All analyses were performed considering a confidence level of 95% (p<0.05).

3. Results

The overall increase in the volume of the hepatopancreatic tissue homogenate in working buffer during the entire recovery process of the alkaline phosphatase was around 2.51±0.22 folds. Here, specific activity of the tissue homogenate increased by 14.27±0.02 folds with final specific activity of 0.414±0.01 units/mL even after achieving 53.29± 0.03% of final yield. Purified alkaline phosphatase of Indian white shrimp following DEAE-Cellulose chromatography had a single band with molecular weight of 43,000 Da on SDS-PAGE gel and no minor bands were observed. During the entire process of the enzyme recovery we have considered as a criteria of purity, not only the increment of specific activity but also the electrophoretic analysis of the sample collected at that unit step. Initial tissue homogenate showed numerous distinct protein bands and subsequent recovery steps reduced the number of distinct protein bands.

1. Effect of temperature on enzyme activity

Alkaline phosphatase isolated from the Indian white shrimp hydrolysed *p*NPP over an unusual broad range of temperature (Fig. 1). However, optimum hydrolysis of *p*NPP by alkaline phosphatase was registered at 50℃ with an optimum activity of 136.50±5.34 units/L. Hydrolytic activity of the alkaline phosphatase at 10, 20, 30, 40, 60, 70, and 80℃ was 50.73±3.13, 57.42±4.23, 80.59±4.11, 88.37±5.23, 40.48±3.11, 34.43±4.11, or 23.63± 5.23% of its optimum activity shown at 50℃. Here, increase in the temperature of the incubation significantly ($p<0.05$) increased the activity of the enzyme up to 50℃ and subsequent increase in the temperature reduced ($p<0.05$) the activity, and beyond 80℃ no enzyme activity was registered.

Stability of the alkaline phosphatase at different temperature of pre-assay exposure is shown in the Figure 2. The residual activity of the alkaline phosphatase after pre-assay exposure of the samples at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100℃ for a period of 30 min was, respectively, 99.97±0.03, 99.40±1.13, 98.99±1.23, 99.20±1.11, 91.05±2.12, 76.46±0.99, 50.10±1.99, 27.47±2.76, 18.61±4.87, and 0.00±0.00% of the activity registered in those samples without any pre-assay exposure. During this experiment, rise in the pre-assay exposure to temperature did not have any significant ($p>0.05$) effect on the residual activity of alkaline phosphatase of shrimp up to 40℃, and further increase in the pre-assay exposure to temperature sharply ($p<0.05$) decreased the residual activity of the alkaline phosphatase. In our study, the alkaline phosphatase lost less than 10% of

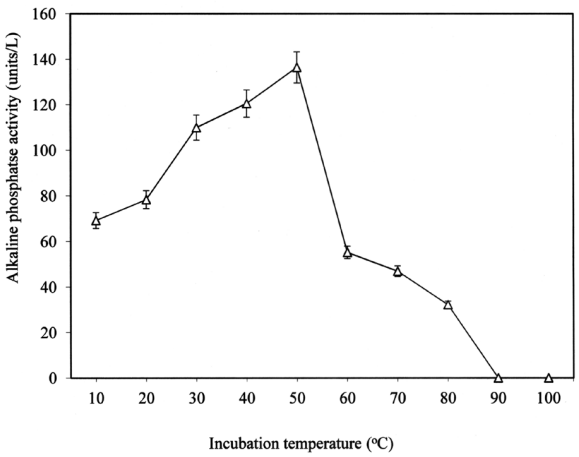

Fig. 1 Alkaline phosphatase activity of Indian white shrimp at various temperature of incubation.

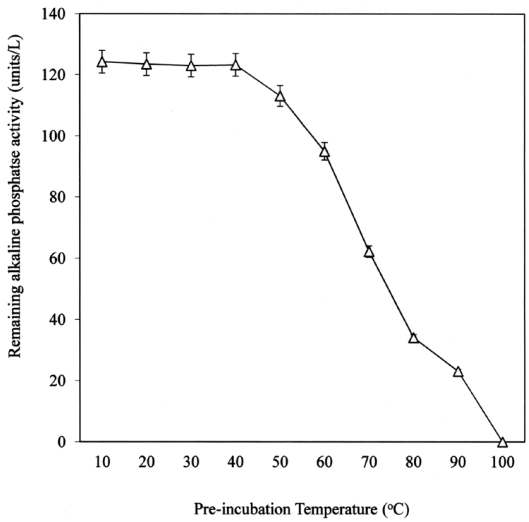

Fig. 2 Residual alkaline phosphatse activity in samples of Indian white shrimps preincubated at various temperature for 30 min.

its activity after being exposed to 60℃ for 30 min prior to assay, more than 80% of its activity after being exposed to 60℃ for 30 min prior to assay, more than 50% of its activity after being exposed to 70℃ for 30 min prior to assay, and nearly 25% of its activity beyond 80℃ in comparison to the remaining activity of alkaline phosphatase in the respective samples without pre-assay exposure. White shrimp lost less than 10% of its activity after being exposed to 50℃ for 30 min prior to assay, in comparison to the remaining activity of the samples without pre-assay exposure.

2. Effect of pH on enzyme activity

Activity of alkaline phosphatase was determined using buffers of different pH (Fig. 3). Here, alkaline phosphatase showed the maximum activity of 141.50±21.55 units/L at pH 9. In one hand, at pH level of 8, 7, 6, 5, and 4, the activity was only 91.60±1.19, 53.80±1.43, 37.28±2.12, 22.17±1.96, and 13.07±1.98% of the activity shown at pH 9, respectively. On the other hand, at 10 and 11 pH levels the enzyme activity was 98.41±1.19 and 73.14±1.43% of its activity at pH 9, respectively. During this period, reducing the pH to 8 resulted in loss of activity nearly by 10% of the activity shown at pH 9. At pH level 11, the samples were showing only three fourths of the activity and at pH 7 the samples were showing nearly half of the activity shown at pH 9. Here below the pH level of 7 samples were exhibiting only one third of the activity.

Stability of the enzyme exposed to pH levels of 11, 10, 9, 8, 7, 6, 5, and 4 was determined by estimating remaining activity of the enzyme in sample (Fig. 4). Indian white shrimp alkaline phosphatase has shown stability at a broad range of pH ranging from 7 to 10. On pre-assay exposure at pH 6, 5, and 4, residual alkaline phosphatase activity was only 71.58±2.09, 70.31±1.09, and 60.02±1.48% of the activity shown at pH 9, respectively.

3. Effect of EDTA on enzyme activity

Alkaline phosphatase activity was 137.25±3.22 units/L when the assay was conducted using *p*NPP as an artificial substrate in the assay buffer without EDTA (Fig. 5). When the $ZnCl_2$ and $MgCl_2$ of the assay buffer was replaced with EDTA the activity of alkaline phosphatase isolated from the hepatopancreatic tissues of Indian white shrimp was 95.40±1.12%

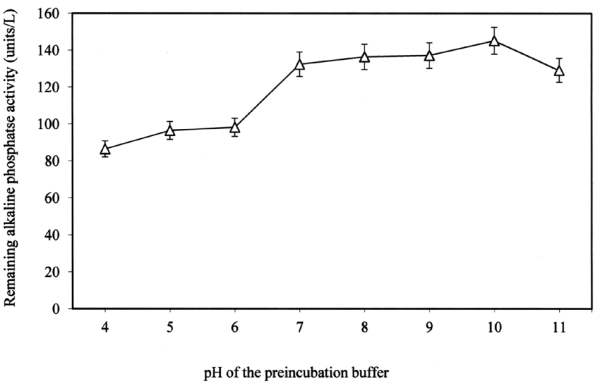

Fig. 4 Stability of alkaline phosphatse of Indian white shrimp at various pH levels for 30 min.

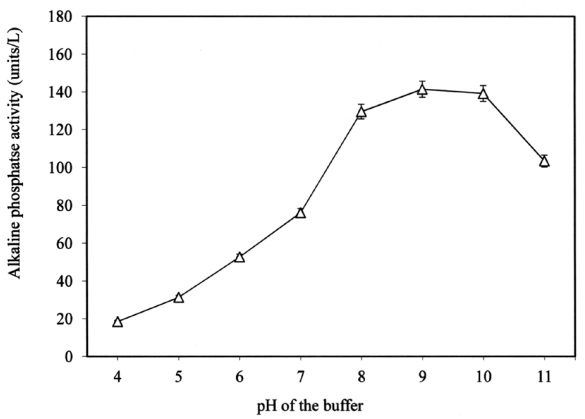

Fig. 3 Alkaline phosphatase activity of Indian white shrimp in buffers of different pH.

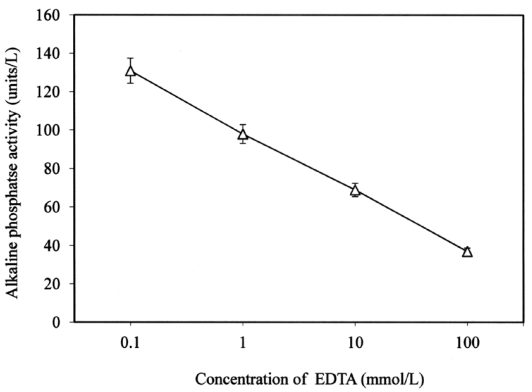

Fig. 5 Activity of alkaline phosphatse of Indian white shrimp at different concentration of EDTA.

at 0.1 mmol/L of EDTA, 71.40±4.12% at 1.0 mmol/L of EDTA, 50.27±2.52% at 10 mmol/L of EDTA, and 26.92±4.12% at 100 mmol/L of EDTA, in comparison to the activity in the samples incubated using AMP assay buffer without EDTA. In this study, increase in the concentration of EDTA significantly (p<0.05) decreased the catalytic activity of alkaline phosphatase, in comparison to the activity in the samples incubated without EDTA, as estimated by one-way ANOVA with *post hoc* Tukey's test.

4. Effect of calcium ion on enzyme activity

Alkaline phosphatase activity of Indian white shrimp when the assay was conducted using *p*NPP as an artificial substrate in assay buffer without $CaCl_2$ was 85.60±0.28 units/L (Fig 6). When $ZnCl_2$ and $MgCl_2$ of the assay buffer was replaced with $CaCl_2$, the activity was 114.49±0.98% at 0.1 mmol/L concentration of $CaCl_2$, 128.94±2.08% at 1.0 mmol/L concentration of $CaCl_2$, and 149.53±4.18% at 10 mmol/L concentration of $CaCl_2$ in comparison to the activity registered in the samples assayed using AMP assay buffer without $CaCl_2$ Indian white shrimp samples. However, further increase in concentration of $CaCl_2$ to 100 mmol/L, no alkaline phosphatase activity was registered and we have observed a complete precipitation of the reaction mixture. Here, one-way ANOVA with *post hoc* Tukey's test was able to establish significant (p<0.01) difference amongst the samples in shrimps incubated with assay buffer incorporated with 0.1, 1.0 and 10 mmol/L concentration of $CaCl_2$, and also between the samples incubated with and without $CaCl_2$.

5. Effect of zinc ion on enzyme activity

Catalytic activity of the alkaline phosphatase decreased in comparison to the activity in the reaction mixture containing assay buffer without any metal ions as the concentration of zinc increased (p<0.01) from 0.1 mmol/L to 100 mmol/L (Fig.7). Hydrolysis of *p*NPP by alkaline phosphatase of Indian white shrimp in the presence of zinc was 93.31±0.18% at 0.1 mmol/L of zinc, 74.47±4.58% at 1 mmol/L of zinc, 53.74±1.32% at 10 mmol/L of zinc, and it is interesting to note that no activity was observed at 100 mmol/L of zinc in comparison to the activity in the reaction mixtures without any metal ions. At 0.1 mmol/L concentration of zinc, alkaline phosphatase purified from the shrimps showed only less than 10% of the activity in comparison to the activities in those samples incubated without zinc. Alkaline phosphatase of the shrimps exhibited one third falls in the activity at 1 mmol/L concentration of zinc in comparison to the samples incubated without zinc. As the concentration of the zinc ion in the buffer increased up to 10 mmol/L, activity of the enzyme reduced to half of the activity in the samples shown without zinc.

6. Effect of magnesium ion on enzyme activity

Purified alkaline phosphatase isolated from the hepatopancreatic tissues of Indian white shrimp registered enzyme activity of 65.60±0.28 units/L, when

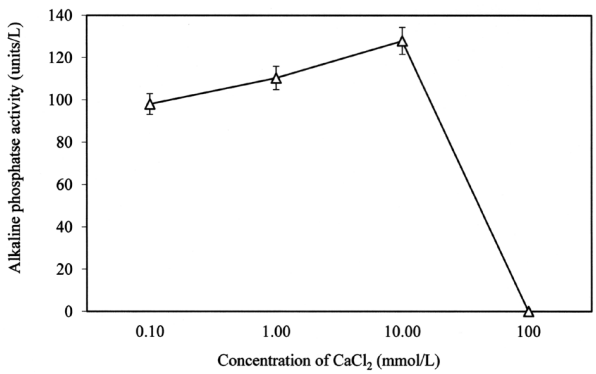

Fig. 6 Activity of alkaline phosphatse of Indian white shrimp at different concentration of $CaCl_2$.

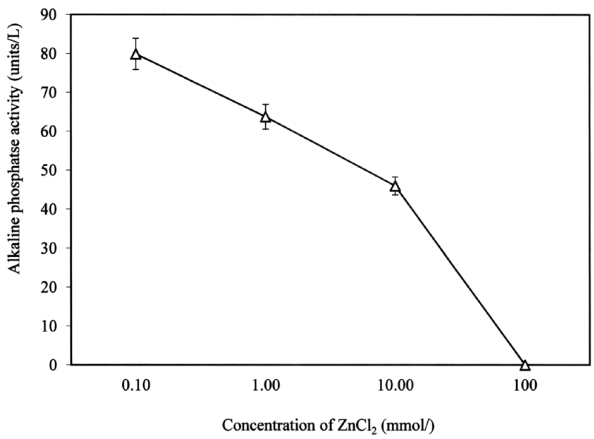

Fig.7 Activity of alkaline phosphatse of Indian white shrimp at different concentration of $ZnCl_2$.

the assay was carried out using assay buffer without $MgCl_2$ (Fig. 8). Alkaline phosphatase activity of Indian white shrimp samples was, respectively, 191.31±2.29% at 0.1 mmol/L concentration of $MgCl_2$, and 200.27±2.19% at 1.0 mmol/L concentration of $MgCl_2$, in comparison to the activity shown in the assay buffer without any metal ions. Increase in the concentration of the magnesium ions from 0.0 mmol/L to 1.0 mmol/L resulted in significant ($p<0.05$) increase in the catalytic activity of the alkaline phosphatase. However, one-way ANOVA with *post hoc* Tukey's test was not able to establish any significant difference ($p>0.05$) in enzyme activity amongst the samples maintained at 1.0, 10, and 100 mmol/L concentration of $MgCl_2$, but significant difference ($p<0.05$) in enzyme activity was registered between the samples maintained at 0.0 mmol/L, 1.0 mmol/L, and more than 1.0 mmol/L concentration of $MgCl_2$.

7. Determination of K_m value and V_{max} value

Alkaline phosphatase isolated from the hepatopancreatic tissues of Tiny shrimp, Indian white shrimp, Red shrimp and Brown shrimp were measured for its affinity for *p*NPP at incubation temperature of 37℃ (Fig. 9). The initial velocities of hydrolysis of *p*NPP were measured at substrate concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 mmol/L at pH 10.3. Kinetic constants of alkaline phosphatase for *p*NPP were measured at 37℃ in working buffer. Respective kinetic parameters were evaluated from Lineweaver-Burk. As represented in the Figure 9, the pseudo R-squared value indicates that more than 99.50% of the variation in $1/v_o$ (*y*-axis) in Indian white shrimp due to the variation in 1/[S] (*x*-axis). On taking the square root of r^2 we have determined that the correlation coefficient, r, is almost 1, indicating an excellent fit between the data points and the regression line. These results show that as 1/[S] increases, $1/v_o$ increases. K_m and V_{max} values for alkaline phosphatase isolated from the Indian white shrimp were calculated and recorded on the spreadsheet by using this equation of the lines.

The K_m values of *p*NPP for alkaline phosphatase isolated from the hepatopancreatic tissues of Indian white shrimp were 0.05±0.0009 mmol/L, respectively. Here, V_{max} of alkaline phosphatase isolated from the hepatopancreatic tissues of Indian white shrimp during the hydrolysis of *p*NPP as substrate were 50.00±0.99 mmol/L/min, respectively.

4. Discussion

Entire recovery process purified of alkaline phosphatase from the hepatopancreatic tissues from shrimps by 14.27±0.02 folds yielding 59.94±0.04% of the protein with alkaline phosphatase activity. Samples collected between 76-96 fractions from DEAE-cellulose chromatography showed as major single band at 43,000 Da regions. Various workers reported molecular weight value for various other vertebrate alkaline phosphatases ranging between 55,000-70,000 Da[3-7]. During every unit step both the incremental specific activity and the electrophoretic analysis of the samples was considered as the criteria of purity. High specific activity and electrophoresis analysis result lead us to suppose that the final sample is practically pure.

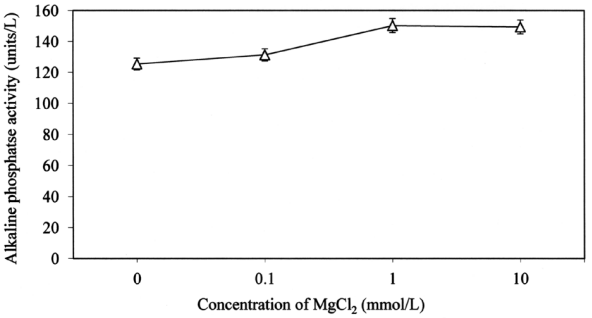

Fig. 8 Activity of alkaline phosphatse of Indian white shrimp at different concentration of $MgCl_2$.

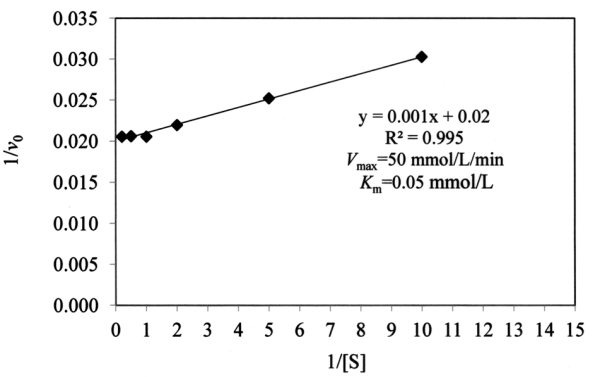

Fig. 9 Lineweaver and Burk plot of alkaline phosphatase of Indian white shrimp for *p*NPP.

Even though, the alkaline phosphatase showed activity over an unusual broad range of temperature ranging from 10℃ to 80℃, temperature optimum of the enzyme was at 50℃. Chandar and Thomas[5] reported that 40℃ is the temperature optimum of the purified alkaline phosphatase isolated from hepatopancreas of Jawala shrimps (*Acetes indicus*). According to Qingxi et al.[7] alkaline phosphatase isolated from *Penaeus penicillatus* hydrolyses *p*NPP optimally at 47℃. In our study, increase in temperature from 10℃ to 50℃ steadily increased the activity, which is supported by the report that increase in temperature speeds up movement of molecules and decrease in temperature slows molecules down. However, increase in the temperature beyond its optimum temperature reduced enzyme activity sharply due to the denaturation[17]. Nevertheless, the enzyme did not show any significant activity both at 90℃ and 100℃.

Thermal stability study proved that the alkaline phosphatase was thermally stable up to 40℃ for 30 min. However, thermal stability study indicated that the alkaline phosphatase lost less than 10% of its activity at 50℃, less than one fourth of the activity at 60℃, nearly half of the activity at 70℃, nearly 75% of its activity at 80℃, and no activities at 100℃. A heat stable alkaline phosphate was also isolated from Penaeus japonicus by Chuang[3] and the alkaline phosphatase from bivalve, *Meretrix lusoria* as reported by Chuang and Yang[4] is unique with its thermostability at 65℃ for 30 min. Chuang and Shih[6] reported that the alkaline phosphatase purified from the hepatopancreas of *Penaeus japonicus* was stable to heating at 65℃ for 5 min.

Alkaline phosphatase of the shrimps hydrolysed *p*NPP optimally at pH 9 at incubation temperature of 37℃, which is comparable with alkaline phosphatase of *Penaeus monodon* reported by Chuang and Yang[4]. The optimum pH of 10 was reported in alkaline phosphatase isolated from *Meretrix lusoria*[4], 8.2 in *Penaeus penicillatus*[7], 9.5 in *Acetes indicus*[5], and 9.8 in *Macrobrachium* nipponense[18]. Exposure of alkaline phosphatase to low pH led to a progressive loss of enzyme activity at 37℃. Above pH 8 catalytic activity of alkaline phosphatase increased slowly, but as the pH was lowered the rate of inactivation was markedly increased.

During pH stability study, The alkaline phosphatase of Indian white shrimp lost only less than 10% of activity on pre-assay exposure at pH 11 for 30 min and lost only around one third of its activity on pre-assay exposure below pH 7 for 30 min. The alkaline phosphatase showed pH stability from pH 7 to pH 10. Irreversible inactivation of the enzyme is particularly evident at the lower and higher ranges of acidic and alkaline conditions[19]. The values so obtained in those samples that were incubated for 30 min in the buffers of the pH 4, 5, 6, 7, 8, 9, 10, or 11 at 37℃ were not true initial rate compared to the samples without pre-incubation, and the optima pH are significantly lower than the true values are probably due to the partial irreversible loss of enzyme activity in respective pH at 37℃ due to denaturation, since the incubation period was 30 min. Alkaline phosphatase gets reversibly inactivated at lower pH at 0℃ and inactivated alkaline phosphatase can be partially reactivated by treatment with buffers of neutral or alkaline pH, and the amount of inactivation depends of the degree of pH treatment[20].

The enzyme alkaline phosphatase was inhibited by EDTA indicating that it is a metalloprotein as stated by Wang et al[18]. Alkaline phosphatase from the hepatopancreas of cold water shrimp (*Pandalus borealis*) as reported by Olsen et al[21] was completely inhibited by EDTA, but the activity was restored to a larger degree by zinc. However, we have not registered complete inhibition of the alkaline phosphatase, probable that the activity in the absence of activator is due to the retention by the enzyme of a small amount of magnesium, even after treatment with EDTA. This supports the view expressed by previous work that purified intestinal phosphatase binds magnesium so strongly that it is not removed by extensive dialysis against water[22]. Calcium increases the activity of the enzyme from 0.1 mmol/L to 10 mmol/L[23] and calcium ions inhibit the enzyme beyond 10 mmol/L due to kinetic and structural changes[21]. The shrimp alkaline phosphatase activity loss in the presence of less than 0.1 mmol/L concentration on zinc was slow and at higher concentrations is substantial. Exogenous magnesium ions have been shown to activate the

mammalian alkaline phosphatase[24].

The general properties of the alkaline phosphatase given in the plot of $1/v_o$ versus 1/[S] using *p*NPP as a substrate, and the correlation coefficient indicates that there exists a linear relationship between the increase in the concentration of the substrate and the rate of hydrolysis of *p*NPP by alkaline phosphatase. Qingxi et al.[7] isolated alkaline phosphatase from Penaeus penicillatus and he reported that at pH 8.3 and temperature 37℃ the Michaelis constant (K_m) was 8.0×10^{-4} mol/L. Lee and Chuang[6] isolated alkaline phosphatase from the hepatopancreas of *Penaeus monodon* that was resolved into three forms, AP-A, AP-B1 and AP-B2, with a molecular weight of 31,000, 31,000 and 43,000 Da in SDS-PAGE, and stated that they were differentiated by Michaelis constant (K_m).

5. Conclusion

Alkaline phosphatase isolated from hepatopancreas of Indian white shrimp hydrolyses *p*NPP optimally at the temperature of 50℃ and thermally stable up to 40℃. The alkaline phosphatase has optimum pH of 9 at 37℃ and has high stability to pH for 30 min at pH values from 7 to 10. Inhibitory activity of EDTA on the enzyme indicated that it is a metalloenzyme, but did not completely inhibited alkaline phosphatase even at 100 mmol/L. Calcium and zinc ions activate the alkaline phosphatase at lower concentrations, but inhibit the enzyme activity at higher concentrations. Magnesium at lower concentration increases the catalytic activity of the alkaline phosphatase and at higher concentrations does not affects the enzyme activity. The general properties of the alkaline phosphatase indicate that there exists a linear relationship between the increase in the concentration and the rate of hydrolysis of *p*NPP. Hence, alkaline phosphatase isolated from the hepatopancreatic tissues of Indian white shrimp has unique properties that can be exploited commercially as per our requirement. Recovery and characterization of the commercially important alkaline phosphatase from the seafood industrial waste paves the way for the commercial exploitation of such catalyst of unique industrial properties leading to the generation of ancillary industry, creation of employment, improvement of socioeconomic aspects of fishing folks, and uplift of already stressed fish processing industry due to successive international ban, competition and rejections.

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