ALKALINE PHOSPHATASE FROM JAWALA SHRIMP (*ACETES INDICUS*)

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Received for Publication March 24, 1999
Accepted for Publication July 31, 2000

ABSTRACT

Alkaline phosphatase was isolated from Jawala shrimps (Acetes indicus), *a tiny crustacean. The hepatopancreas (head region) is a good source of this enzyme. pH optima of the partial purified enzyme was found to be 9.5 and optimal temperature for maximal activity was 40C. Jawala phosphatase was completely inhibited by 1,10-phenanthroline and EDTA, indicating that it is a metalloprotein. The activity of inhibited enzyme was restored by Zn^{2+} and Mn^{2+} salts. Feed back inhibition of the enzyme by inorganic phosphate was also observed.*

INTRODUCTION

Alkaline phosphatases are widely distributed in nature (McComb *et al.* 1979). These enzymes, which hydrolyze a broad class of phosphomonoesters, have been studied in detail from mammalian tissues and *Escherichia coli* (Olafsdottir and Chlebowski 1989; Harris 1989; Kim and Wyckoff 1989). The enzymes are dimeric Zn-metalloproteins (McComb *et al.* 1979; Reid and Wilson 1971) and the molecular weights of mammalian enzymes vary from 130,000 to 170,000 while the value for *E. coli* enzyme is 94,000 (Olsen *et al.* 1991). Although alkaline phosphatase have been detected in most animal species, detailed information on the enzyme from invertebrates is limited. The enzyme has been shown to be present in crustaceans such as spring lobster (Travis 1955), hermit crab (Chockalingam 1971) and cray fish (Denuce 1967). Recently, we have shown Jawala to be good source of commercially important enzyme such as alkaline proteinase (Sherekar *et al.* 1997).

Jawala (*Acetus indicus*) is a low priced, nonpenaeid shrimp species. Although the annual catch is 1,74000 metric ton (MPEDA 1999), representing 65% of nonpenaeid shrimps, only a fraction is used for human consumption. Jawala, a tiny crustacean (1 to 3 cm in length) is impossible to deshell or devein. It fetches a very low price in the market; however, processing of Jawala for enzymes may add a good value to otherwise cheap and trash crustacean. The present investigations were undertaken to determine the alkaline phosphatase activity from Jawala. The enzyme is of industrial importance. It has been conjugated with either antibodies to detect food pathogens such as *Salmonella* or to oligonucleotides to identify enterohaemorrhagic *E. coli*, *Shigella.*, *Vibrio cholera* and *Vibrio parahaemolyticus* (Yoh *et al.* 1997). It also finds its application in recombinant technology. The enzyme has not been reported from Jawala earlier. The present paper describes the isolation, partial purification, characterization and localization of alkaline phosphatase from Jawala.

MATERIALS AND METHODS

Materials

p-Nitrophenyl phosphate, and 4-nitrophenol were obtained from Sisco Research Laboratories, Mumbai India. Azocasein, bovine serum albumin (BSA), Tris, 1,10-phenanthroline, and disodium ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. St. Louis (USA). Sephacryl S-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of Crude Enzyme Extract

Jawala was obtained from Mandala harbor, Mumbai. Being tiny, Jawala is sold in lumps and often smaller varieties of fish are mixed in the lump. These were separated out. Since peeling and deveining operations are not possible, Jawala was thoroughly washed in tap water and homogenized (10 g) with cold 0.2 M KCl (100 mL) in a Waring blender for 3 min. The homogenate (10%) was centrifuged at 12,000 x g for 20 min in Sorvall RC-2 refrigerated centrifuge at 0-4C. The supernatant was filtered through muslin cloth. The filtrate was used for assaying alkaline phosphatase activity.

Jawala was also dissected under a magnifying glass into two portions, namely head region and abdomen region. Extracts of head and body region were prepared as described above and the enzyme activities were determined.

Determination of Alkaline Phosphatase

The enzyme activity was measured by the method described by Olsen *et al.* (1991). Briefly the enzyme was incubated with 6 mM *p*-nitrophenylphosphate at 30C in 0.1 M glycine/NaOH buffer, pH 10.5 with 1 mM $MgCl_2$ in the total volume of 1.0 mL. After 15 min, the reaction was stopped by 10.0 mL of 0.02 N NaOH and the amount of *p*-nitrophenylphosphate released was determined by measuring absorbance at 405 nm. One unit of enzyme activity will produce 1 μ mole of *p*-nitrophenylphosphate/min.

Proteinases Activity

Proteolytic activity of the extracts was determined using azocasein, as substrate in a 2.0 mL reaction system containing 0.5% azocasein, an aliquot of enzyme extract and borate buffer (0.1M, pH 8.5). After incubation at 50C for 10 min, reaction was terminated by adding 2 mL of 10% TCA. Absorbance of TCA filtrate was measured at 366 nm (Sherekar *et al.* 1997). A unit of activity corresponds to change in absorbance of 0.001 per min.

Protein Determination

The protein concentration was determined by Lowry's method (Lowry *et al.* 1951) with BSA as a standard.

Ammonium Sulfate Fraction

Ammonium sulfate was slowly added to the 10% extract (at 0-4C) with continuous stirring till 60% saturation was achieved. The mixture was then left at 0-4C overnight, and the precipitated protein was collected at 12,000 x g for 20 min. The precipitate was suspended in 10 mM Tris-HCl (pH 7.4) buffer and dialyzed against the same buffer with several changes. It was then centrifuged at 12,000 x g for 20 min to obtain a clear supernatant.

Ultrafiltration

The dialyzed enzyme sample was concentrated 10 times by ultrafiltration with a 30,000 molecular weight cut off membrane in an Amicon Diaflo stirred cell.

Gel Filtration

The concentrated enzyme preparation was applied on Sephacryl S-200 column (1.6 x 95cm) equilibrated with 10 mM Tris HCl buffer. The elution was carried with the same buffer at flow rate of 12.0 mL/h and fractions of 3.0 mL. Fractions showing alkaline phosphatase activity were pooled.

Optimum pH

The enzyme activity in the pH range of 6.5-10.5 was determined. The buffers used for the assay were 0.1 M phosphate buffer (pH 6.5-7.5), 0.1 M borate buffer (pH 8.5), and 0.1 M glycine/NaOH buffer (pH 9.5-10.5).

Optimum Temperature

Temperature optima of the enzyme was determined by carrying out the assay at various temperatures (25 - 60C).

Temperature Stability

Aliquot of the enzyme was incubated at different temperatures (25-60C) for 30 min and the residual enzyme activity was then determined.

Effect of Inhibitors

The enzyme was incubated with inhibitors (with concentration mentioned in the experiments) at 25C for 30 min and the residual enzyme activity was then determined. Restoration of 1,10-phenanthroline-inhibited enzyme activity by metal ions was also examined. The enzyme was incubated first with1,10-phenanthroline then with metal ions at 25C for 30 min following which enzyme activity was determined.

Effect of Metal Ions

Aliquots of the enzyme were incubated with metal ion $Hg,^{2+}$ $Fe,^{2+}$ $Ba,^{2+}$. $Li,^{2+}$ $Cu,^{2+}$ $Mn,^{2+}$ and $Ca,^{2+}$ solutions (final concentration being 1 mM) at 25C for 30 min and the enzyme activity was then determined.

Effect of Phosphate

The enzyme was incubated with different concentrations (2×10^{-1} to 2×10^{-4}M) of phosphate (K_2HPO_4) at 25C for 30 min and the enzyme activity was then measured.

RESULTS

Distribution of Alkaline Phosphatase in Jawala

Jawala was dissected under magnifying glass into head and abdomen regions to investigate the distribution of the enzyme. Figure 1 shows enzyme activities in these regions as well as that of whole Jawala. As can be seen, the enzyme is mainly localized in the head region. The enzyme activity in this region was 66.52 units/mg protein which was 3.5 times more than that in the abdomen region. Diffusion of some activity from the head region to abdomen region cannot be ruled out, considering the small size and the manner in which Jawala is handled after the catch. Only 50% of the enzyme activity was observed by the omission of Mg^{2+} from the assay.

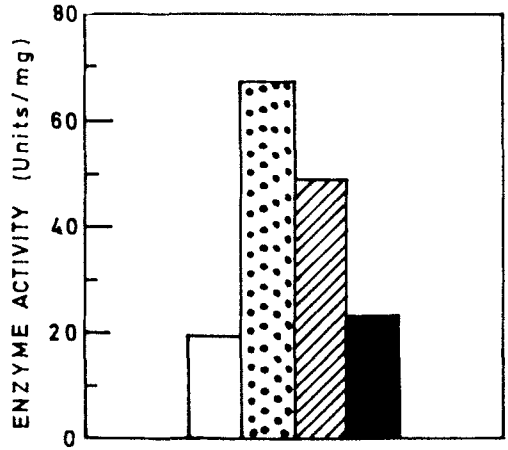

FIG. 1. ALKALINE PHOSPHATASE ACTIVITY IN DIFFERENT REGIONS OF JAWALA
Alkaline phosphatase activity of 0.2M KCl extract of abdomen region □, head region ▦, and whole Jawala ▨ was determined in presence 1 mM of Mg^{2+} as given in materials and methods. Enzyme activity from whole Jawala was also determined without Mg^{2+} in the assay ■. Enzyme activity was expressed as units/mg protein.

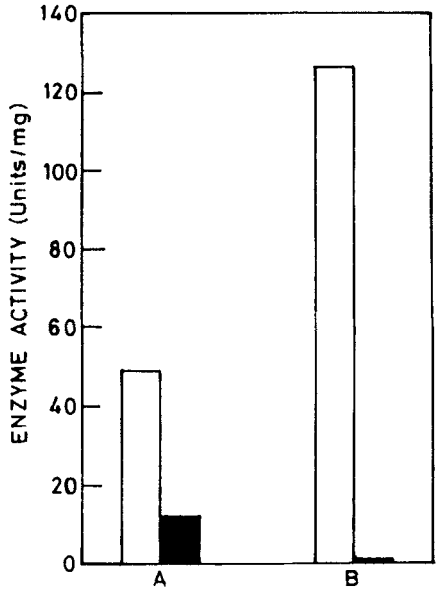

FIG. 2. ALKALINE PHOSPHATASE □ AND AZOCASEINOLYTIC ACTIVITY ■ FROM JAWALA
Alkaline phosphatase and azocaseinolytic activity of 0.2 M KCl extract (A) and partially purified preparation (B) was determined as given in materials and methods.

Separation of Protease Activity from Alkaline Phosphatase

Crude extract contained large amounts of alkaline protease which could interfere with characterization of alkaline phosphatase. Therefore, partial purification involving ammonium sulfate fractionation, ultrafiltration and gel filtration chromatography through Sephacryl S-200 was carried out to separate the two enzymes. The results are shown in Fig. 2. The pooled fractions had no detectable protease activity. Azocaseinolytic activity of crude extract was 11.14 units/mg protein. Further alkaline phosphatase activity from the pooled Sephacryl S-200 fractions was 125.63 units/mg protein with 2.5 fold purification. This preparation of the enzyme was used for further characterization.

Optimum pH and Temperature

The maximum activity was observed between pH 9.5-10.5 (Fig. 3). At pH lower than 9.0, rapid loss of the activity was observed. The effect of temperature on enzyme activity is shown in Fig. 4. The enzyme showed maximum activity at 40C.

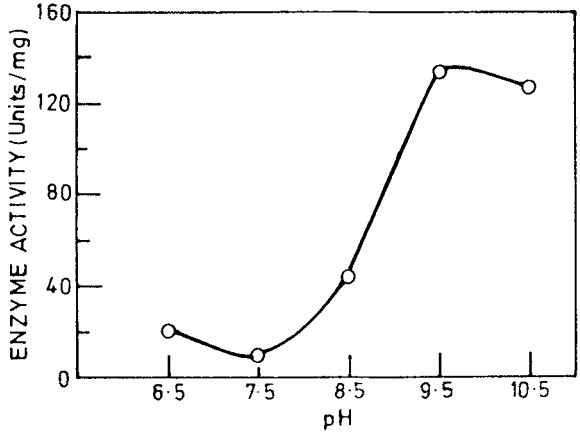

FIG. 3. EFFECT OF pH ON THE ACTIVITY OF JAWALA ALKALINE PHOSPHATASE
The enzyme activity was determined at different pH using the assay system described in materials and methods. Enzyme activity was expressed as units/mg protein.

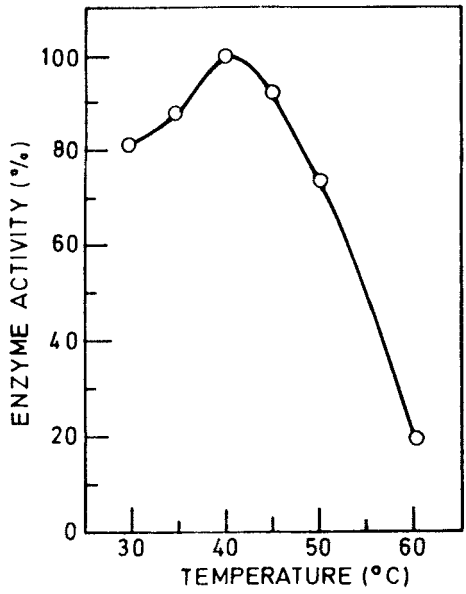

FIG. 4. EFFECT OF TEMPERATURE ON THE ACTIVITY OF JAWALA ALKALINE PHOSPHATASE
The enzyme activity was determined at different temperatures using the assay system described in materials and methods.

Thermal Stability

The enzyme was very sensitive to exposure to high temperatures. Incubation of the enzyme above 50C resulted in thermal inactivation (Fig. 5).

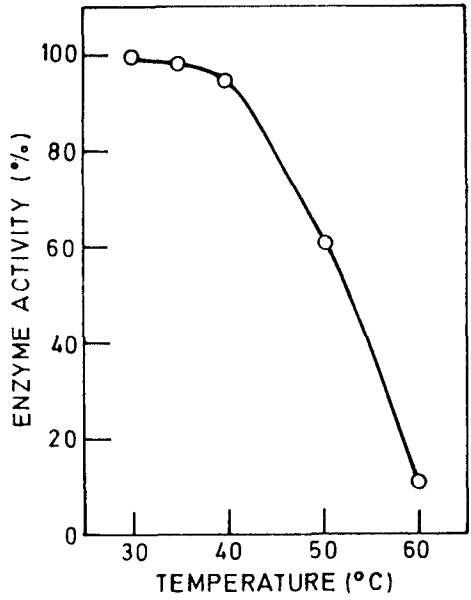

FIG. 5. EFFECT OF TEMPERATURE ON THE STABILITY OF JAWALA ALKALINE PHOSPHATASE
After incubating the enzyme for 30 min activity at different temperatures in 10 mM Tris-HCl buffer, pH 8.0, the samples were cooled, and the residual activity was determined as described in materials and methods.

Effect of Inhibitors

The influence of inhibitors is presented in Table 1. Alkaline phosphatase was strongly inhibited by 1,10 phenanthroline or EDTA and the inhibition was nearly complete in the presence of these inhibitors. The activity of 1:10 Phenanthroline incubated enzyme was restored by the addition of Zn^{2+} and Mn^{2+} ions and not by Mg^{2+}. In contrast, enzyme inhibition with EDTA was restored to 45% by Mg^{2+}.

TABLE 1.
EFFECT OF INHIBITORS ON THE ACTIVITY OF JAWALA ALKALINE PHOSPHATASE

Inhibitor	Final concentration	Residual activity [a] (%)
None		100
EDTA	5 mM	10.2
1,10-phenanthroline	2 mM	2.1
EDTA and	5 mM	45.4
Mg^{2+}	1 mM	
1,10-phenanthroline and	2 mM	100
Zn^{2+}	1 mM	
1,10-phenanthroline and	2 mM	100
Mn^{2+}	1 mM	

[a]Enzyme was preincubated with inhibitor at 25C for 30 min and the remaining activity was determined as described in materials and methods.

Effect of Metal Ions

Enzyme was incubated with a number of metal ions and the influence of these on alkaline phosphatase is shown in Table 2. Hg^{2+} completely inhibited the enzyme followed by near complete inhibition by Fe^{2+} (residual enzyme activity was 7.83%). There was 80% inhibition by Ba^{2+} and Li^{2+} and Cu^{2+} moderately inhibited giving 41% and 37% residual enzyme activity, respectively. No significant effect was seen with Mn^{2+} and Ca^{2+}. The enzyme was inhibited by some bications while Mg^{2+} and Zn^{2+} were required for its activity. Similar observations have been made by other researchers (Fernley 1971; Olsen *et al.* 1991).

Effect of Phosphate

The effect of inorganic phosphate on the alkaline phosphatase was tested. Enzyme was inhibited by phosphate and inhibition was nearly complete (residual enzyme activity, 6.13%) in the presence of 0.2 M phosphate.

TABLE 2.
EFFECT OF METAL IONS ON THE ACTIVITY OF JAWALA ALKALINE PHOSPHATASE

Metal ion	Residual activity [a] (%)
None	100
Hg^{2+}	0
Fe^{2+}	7.83
Ba^{2+}	20.14
Li^{2+}	41.79
Cu^{2+}	36.19
Mn^{2+}	92.53
Ca^{2+}	73.44

[a]Enzyme was incubated with metal ion solution (1mM) at 25C for 30 min, and the remaining activity was determined as described in materials and methods.

TABLE 3.
EFFECT OF INORGANIC PHOSPHATE ON THE ACTIVITY OF JAWALA ALKALINE PHOSPHATASE

Concentration of phosphate(M)	Residual activity [a] (%)
None	100
0.0002	89.7
0.002	75.7
0.02	28.2
0.22	6.13

[a]Enzyme was incubated with different concentrations of phosphate at 25C for 30 min, and the remaining activity was determined as described in materials and methods.

DISCUSSION

Our data show high activity of soluble alkaline phosphatase in Jawala, a tiny crustacean. The enzyme activities in the head region were higher than in the abdomen region, due to the location of the hepatopancreas of shrimp (Olsen *et al.* 1991). Jawala has a very small hepatopancreas which could not be easily removed, so we determined the enzyme activity of the head region after dissection. In contrast to membrane bound mammalian alkaline phosphatase, the enzyme from Jawala could be extracted without n-butanol. Similar soluble form of enzyme has been reported in some other marine invertebrates (Pricipeto *et al.* 1985; Malik and Low 1986; Miki *et al.* 1986). Alkaline phosphatase from Jawala needed divalent ions as low activity was observed without Mg^{2+} and after dialysis. This is similar to the mammalian and *E. coli* enzymes (Reid and Wilson 1971).

Alkaline phosphatase could be separated from the alkaline protease by using classical fractionation technique. pH optima was 9.5 similar to the mammalian and prawn enzymes (Garattini *et al.*1987; Olsen *et al.* 1991). Jawala alkaline phosphatase was susceptible to higher temperatures and its thermal inactivation was observed on incubation at temperatures above 40C. This is similar to alkaline phosphatases from other sources (Reid and Wilson 1971; Fernley 1971). Our results indicate the enzyme to be a metalloprotein as it was completely inhibited by 1,10 phenanthroline and EDTA. Including Zn^{2+} and Mg^{2+} in the assay system led to reactivation. Similar observations have been reported for mammalian and prawn enzymes (Fernley 1971; Olsen *et al.* 1991). Jawala enzyme was inhibited by phosphate and in this respect resembled the enzyme from *E. coli* (Reid and Wilson 1971).

In this work, we showed the presence of high activity of alkaline phosphatase in Jawala indicating that this small crustacean may be a convenient and cheap source for large preparations of this enzyme.

ACKNOWLEDGMENT

We wish to thank Ms. Anupama Menon and Ms. Aishwarya Sane for their help.

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