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Purification and enzymatic characterization of alkaline phosphatase from *Pinctada fucata*

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Abstract

An alkaline phosphatase was purified from *Pinctada fucata*, a kind of pearl oyster, by chromatography on DEAE-32 cellulose, Sephadex G-150 and DEAE A-25. The specific activity of the enzyme was 2040 U mg^{-1}. The kinetics characteristics of the enzyme have been studied. The product HPO_4^{2-} and the product-analog WO_4^{3-} competitively inhibited the enzyme activity. Positive monovalent cations had no effect on the enzyme activity, while positive bivalent cations had different effects on the enzyme: Mg^{2+}, Ca^{2+}, Co^{2+} and Mn^{2+} activated the enzyme while Zn^{2+}, Cu^{2+} and Cd^{2+} inhibited the enzyme.

Keywords: Alkaline phosphatase; Purification; Kinetics; Mollusc; *Pinctada fucata*

1. Introduction

Being efficient, economical, and clean catalysts, enzymes are enjoying increasing applications ranging from laundry detergents and paper processing to fine chemical synthesis and diagnostic/research reagents [1]. Great efforts have been taken to screen new enzymes from various kinds of organisms to improve existing enzymes, optimize existing processes, or acquire marketable pieces of intellectual property. Marine life as a rich reservoir for enzymes has long been neglected. Since marine organisms live in an environment quite different from land organisms, enzymes in these organisms are expected to have novel characteristics.

Alkaline phosphatase (ALP, EC 3.1.3.1) is a non-specific phosphomononoesterase that functions through a phosphoseryl intermediate to produce free inorganic phosphate or to transfer the phosphoryl group to other alcohols [2–4]. In the last decade, ALPs from mammalian sources and microorganisms have been extensively investigated and widely used as diagnostic and research reagents [5–8]. The wide distribution of ALP in organisms suggests their essential roles in life. ALPs in marine organisms play important roles in cell phosphate metabolism, which is related to the absorption of phosphate and calcium from seawater and the biomineralization process in marine organisms. As a first step in screening new ALP from marine organisms, we have thoroughly investigated ALP from green crab *Scylla serrata*

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in previous studies [9]. Here, we report the purification of the ALP from the bivalve mollusk *Pinctada fucata* and the enzymological properties of this enzyme.

2. Materials and methods

2.1. Materials

ALP was prepared from the viscera of adult *Pinctada fucata*, which was obtained from the Beihai Oyster Culture Co., Guangxi Province, China. *p*-Nitrophenyl phosphate (*p*-NPP) was from GIBCO. DEAE-32 cellulose was a Whatman product. Sephadex G-150 and DEAE A-25 were Pharmacia products. All other reagents were local products of analytical grade.

2.2. Assay of ALP activity

The enzyme activity was determined in 0.05 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.7) at 40 °C by following the absorbance change at 405 nm accompanying the hydrolysis of the substrate (*p*-NPP). One enzyme activity unit represents the amount of enzyme catalyzing the formation of 1 μmol *p*-nitrophenol per minute from *p*-NPP.

2.3. Enzyme purification

The purification process was carried out as described by Chen et al. [9] with some modifications. All procedures were carried out at 4 °C. The standard buffer used throughout the purification procedure was 10 mM Tris–HCl (pH 7.5). Briefly, 200 g oyster viscera was homogenized in 100 ml cold Tris–HCl buffer (20 mM, pH 7.5) and incubated for 4 h. Then 20% (v/v) cold *n*-butyl alcohol was added and the solution was incubated for another 2 h. The water phase was separated by centrifugation at 4000 × *g* for 20 min. The enzyme solution was fractioned with ammonium sulfate at 35–70% saturation. The active precipitate was dissolved in the standard buffer and dialyzed against the same buffer until no sulfate could be detected. The dialyzed solution was loaded onto DEAE-32 column pre-equilibrated with the standard buffer. Elution was carried out with a linear gradient of 0–1 M NaCl. The active fractions were combined and applied to Sephedex G-150 column. The column was eluted with the standard buffer containing 0.2 M NaCl. The active peaks was combined and dialyzed against the standard buffer. The dialyzed solution was concentrated by lyophylization. The concentrated solution was loaded onto a DEAE-Sephacel A-25 and eluted with a linear-gradient of 0–1 M NaCl. The final preparation was applied to polyacrylamide gel electrophoresis. Protein concentrations were measured with a BCA protein assay reagent (Pierce) using bovine serum albumin as standard.

2.4. Assay of the temperature and pH optima

The temperature optimum of the enzyme was determined by measuring the activity at different temperatures in the buffer described earlier. The reaction temperatures ranged from 25 to 60 °C.

The optimal pH was determined by measuring the activities at the different pH values at 40 °C. The pH range was varied from 8.8 to 10.6 by changing the ratio of Na_2CO_3 to $NaHCO_3$.

2.5. Assay of the Michaelis–Menten constant

The kinetics assay method was used in these experiments to investigate the Michaelis–Menten constants (K_m and V_m).

2.6. Assay of the p-NPP enzyme hydrolysis activation energy

The enzyme activation energy was determined by measuring the reaction velocity at different temperatures (0, 10, 20, 30, 40, 45 °C). At each temperature, the maximum velocity (V_m) was assayed and then the Arrhenius formula was used to calculate the activation energy by making a plot of $\log_{10} V_m$ versus $1/T$. The slope of the line was the enzyme activation energy to catalyze the hydrolysis of *p*-NPP.

2.7. Effects of the product (HPO_4^{2-}) and the product-analog (WO_4^{3-}) on the enzyme

The effects of the product HPO_4^{2-} and its analog WO_4^{3-} on the enzyme were analyzed using assays

in 0.05 M Na_2CO_3–$NaHCO_3$ (pH 9.7), 4 mM *p*-NPP, 2 mM Mg^{2+} with different concentrations of HPO_4^{2-} or WO_4^{3-} at 40 °C. The concentrations ranged from 1.0 to 10 mM. The HPO_4^{2-} and WO_4^{3-} inhibition types on the enzyme was determined by changing the substrate *p*-NPP concentrations in the presence of HPO_4^{2-} or WO_4^{3-}.

2.8. Effects of metal ions on the enzyme

Experiments were performed in the buffer described earlier with different concentrations of metal ions. An amount of 10 μl of enzyme solution was incubated at 40 °C with the reaction ended by adding NaOH solutions.

3. Results

3.1. Purification of ALP

Purified enzyme with a specific activity of 2040 U mg^{-1} was obtained using chromatography on DEAE-32 cellulose, Sephadex G-150 and DEAE A-25. SDS-PAGE analysis of the purified ALP showed a single band with a molecular mass of ~40 kDa (Fig. 1). The purification steps are summarized in Table 1.

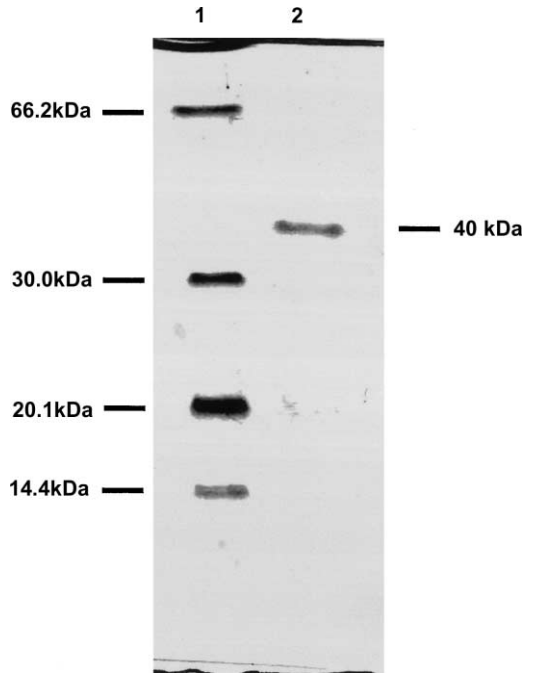

Fig. 1. Purification of ALP from *Pinctada fucata*. SDS-PAGE analysis was carried out on 12% gels. The gel was stained with Coomassie Brilliant Blue R250. Lane 1 was molecular markers (kDa) and lane 2 was purified ALP.

3.2. Temperature and pH optima

The enzyme activities measured at various temperatures and pH values are shown in Fig. 2. The optimal catalysis reaction temperature was 45 °C with the activity decreasing rapidly at temperatures higher or lower than the optimum.

The optimal pH for the enzyme in Fig. 2b was 9.7. The optimum pH is lower than that for the green crab ALP which was 10.0.

3.3. Michaelis–Menten constants

The initial reaction velocity of the enzyme catalysis of the hydrolysis of *p*-NPP calculated from the product concentration changed with reaction time was 6.1 μmol l^{-1} min^{-1}. The Michaelis–Menten

Table 1
Purification of ALP from *Pinctada fucata*

Steps	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude extract	665	3100	57700	18.6	1	100
$(NH_4)SO_4$ fraction	139	630	34800	55.3	3.0	60.4
DEAE-32 fraction	92	63.4	13200	208	11.2	22.9
G-150 fraction	101	17.3	10800	623	33.5	18.7
A-25 fraction	15	4.5	9200	2040	101	15.9

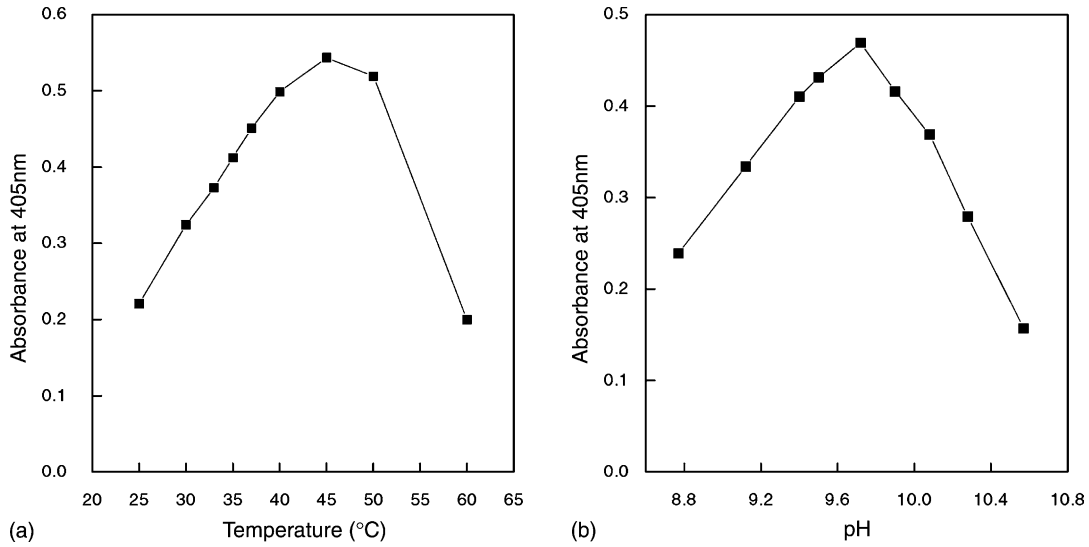

Fig. 2. Effect of temperature (a) and pH (b) on ALP activity.

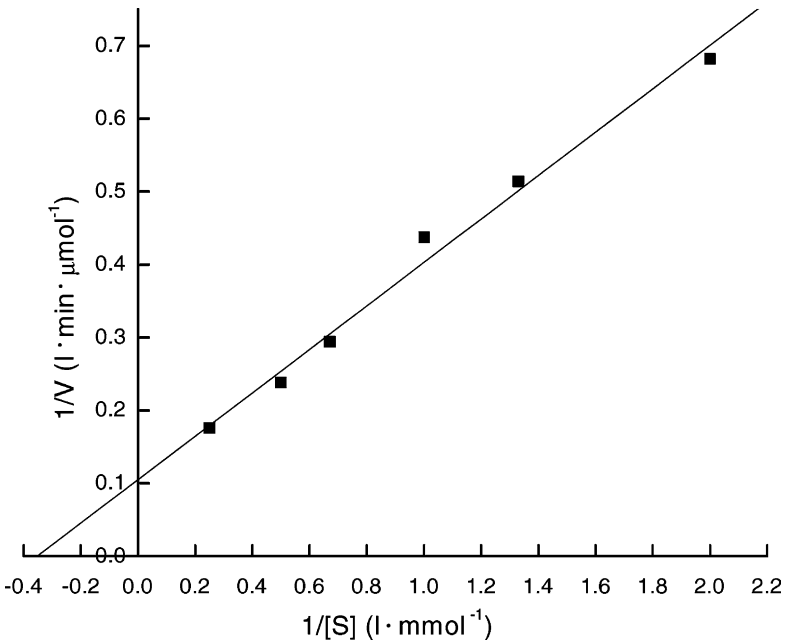

Fig. 3. Lineweaver–Burk plot for the Michaelis–Menten constant K_m and the maximum velocity V_m. The enzyme is incubated for 10 min at 37 °C in 1 ml of activity assay system containing 50 mM Na_2CO_3/$NaHCO_3$ buffer (pH 9.7) and 2 mM *p*-NPP. Final enzyme concentration was 2 mg l^{-1}.

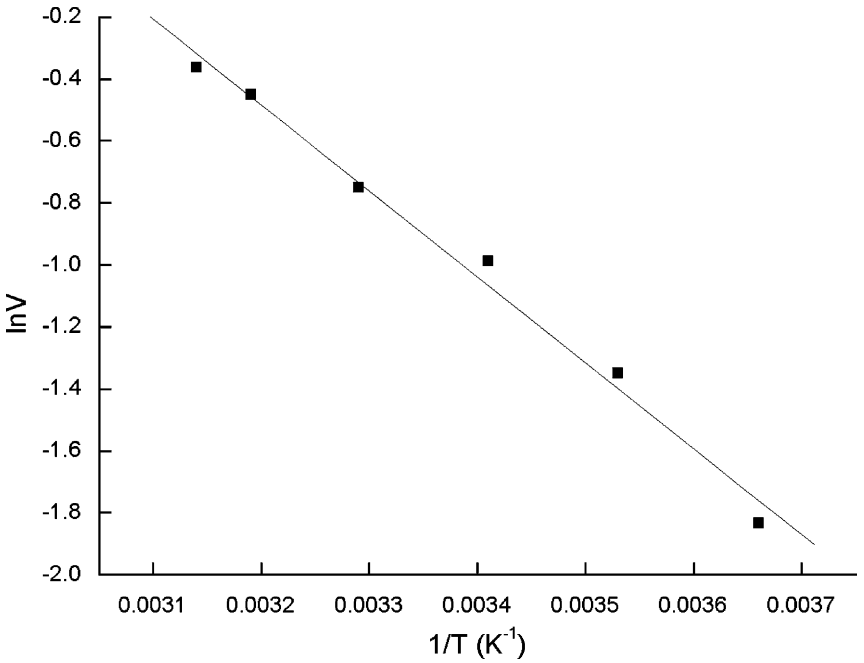

Fig. 4. The enzyme activation energy for the hydrolysis of *p*-NPP. The experimental conditions were as for Fig. 3. The reactions were carried out at 0, 10, 20, 30, 40 and 45 °C.

constants calculated from the Lineweaver–Burk plot (Fig. 3) were: $K_m = 2.86$ mM and $V_m = 9.09$ μmol l^{-1} min^{-1}.

3.4. The enzyme activation energy

The enzyme activation energy was assayed by measuring the maximum velocity of the catalysis reaction at different temperatures. The results showed in Fig. 4 were used to determine the activation energy of 22.62 kJ mol^{-1}.

3.5. Effects of HPO_4^{2-} and WO_4^{3-} on ALP

Increasing concentrations of Na_2HPO_4 or Na_3WO_4 in the assay system reduced the enzyme activity relative to the control as shown in Fig. 5. The concentrations of the effectors were increased from 1 to 10 mM in the assay system with only about 33% activity left at concentrations of 10 mM. The inhibitory effect of HPO_4^{2-} was a little stronger than that of WO_4^{3-}.

The Lineweaver–Burk plots (Fig. 6) showed that both HPO_4^{2-} and WO_4^{3-} could competitively

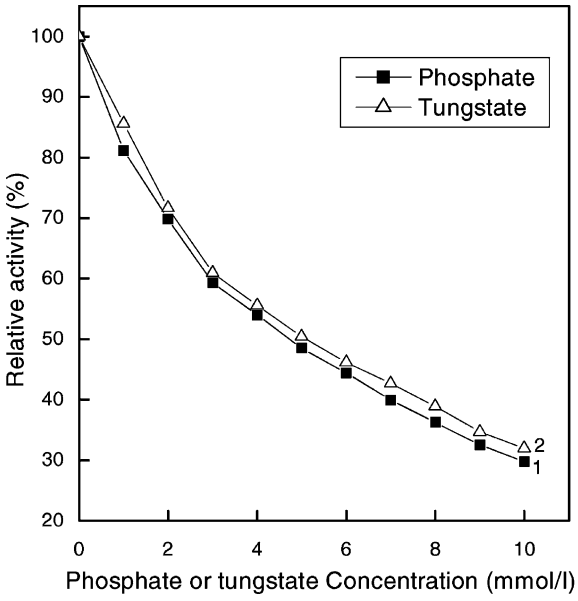

Fig. 5. Effect of the product HPO_4^{2-} and the product-analog WO_4^{3-} on the enzyme activity. The experimental conditions were as for Fig. 3. Curve 1 represents the effect of HPO_4^{2-} and curve 2 represents the effect of WO_4^{3-}.

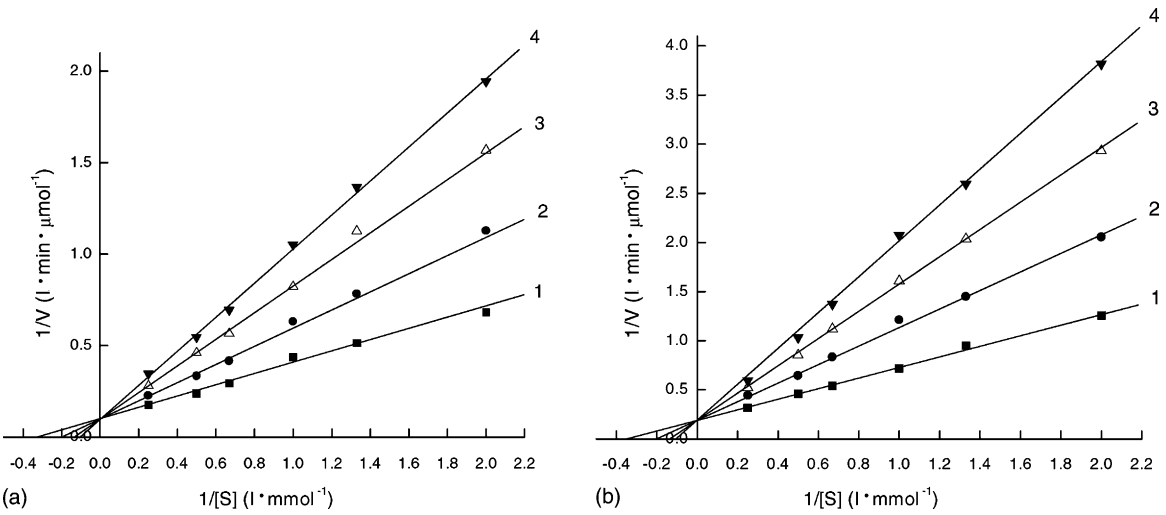

Fig. 6. Lineweaver–Burk plots for inhibition by HPO_4^{2-} and WO_4^{3-}. The experimental conditions were as for Fig. 3. The HPO_4^{2-} (a) or WO_4^{3-} (b) concentrations for curves 1–4 were 0, 2, 4 and 6 mM, respectively.

inhibit the enzyme with inhibition constants of 0.89 and 1.20 mM, respectively, which also shows that HPO_4^{2-} more strongly inhibits the enzyme activity than WO_4^{3-}.

3.6. Effects of metal ions on ALP

At concentrations ranging from 5 to 50 mM, positive monovalent alkali metal ions such as Li^+, Na^+ and K^+ had no obvious effect on the ALP activity from *Pinctada fucata* (data not shown). However, treatment with positive divalent alkali metal ions such as Mg^{2+}, Mn^{2+}, Co^{2+} and Ca^{2+} increased the enzyme activity as shown in Fig. 7.

With 2 mM Mg^{2+}, the enzyme activity increased by 60% (Fig. 7a). However, in contrast to Mg^{2+}, the effect of Ca^{2+} on the activity of *Pinctada fucata* ALP was relatively low (Fig. 7d) with an enzyme activity increase with 2 mM Ca^{2+} of 27% compared with the control. Mn^{2+} and Co^{2+} substantially increased the enzyme activity at micromole concentrations (Fig. 7b and c). However, the Lineweaver–Burk analysis showed that the mechanisms of these two ions were different. The active effect of Mg^{2+} on ALP (Fig. 9a) is uncompetitive while the active effect of Co^{2+} is competitive (Fig. 9c). The active effect of Mn^{2+} on ALP activity showed mixed uncompetitive and competitive effects (Fig. 9b).

Some heavy metal ions such as Zn^{2+}, Pb^{2+}, Cd^{2+} and Cu^{2+} were shown to inhibit the ALP activity (Fig. 8). As shown in Fig. 8a, treatment with 40 μM Zn^{2+}, substantially reduced the ALP activity to only 30.8% of the control. Further increases of the Zn^{2+} concentration had a small additional inhibitory effect on the enzyme. With an activity of 24% at a Zn^{2+} concentration of 200 μM, the Lineweaver–Burk analysis using Zn^{2+} showed that the inhibition effect of Zn^{2+} on the enzyme was anti-competitive (Fig. 9d).

Pb^{2+} and Cd^{2+} are two elements in marine pollution. As shown in Fig. 8b and c, 11 μM Pb^{2+} and 55 μM Cd^{2+} inhibited the enzyme activity to 82.3 and 73.2%, respectively. The results also showed that Cu^{2+} could reduce the enzyme activity to 30.9% at a concentration of 200 μM (Fig. 8d).

4. Discussion

It was well known that metal ions play important roles in the catalysis of a large number of enzymes. ALP is a metalloenzyme which catalyzes the non-specific hydrolysis of phosphate monoesters [2]. The X-ray crystal structure of *Escherichia coli* ALP has been reported to 2.0 angstroms with the presence of inorganic phosphate [10]. The active site is a pocket containing a tight cluster of two zinc ions (3.9 Å

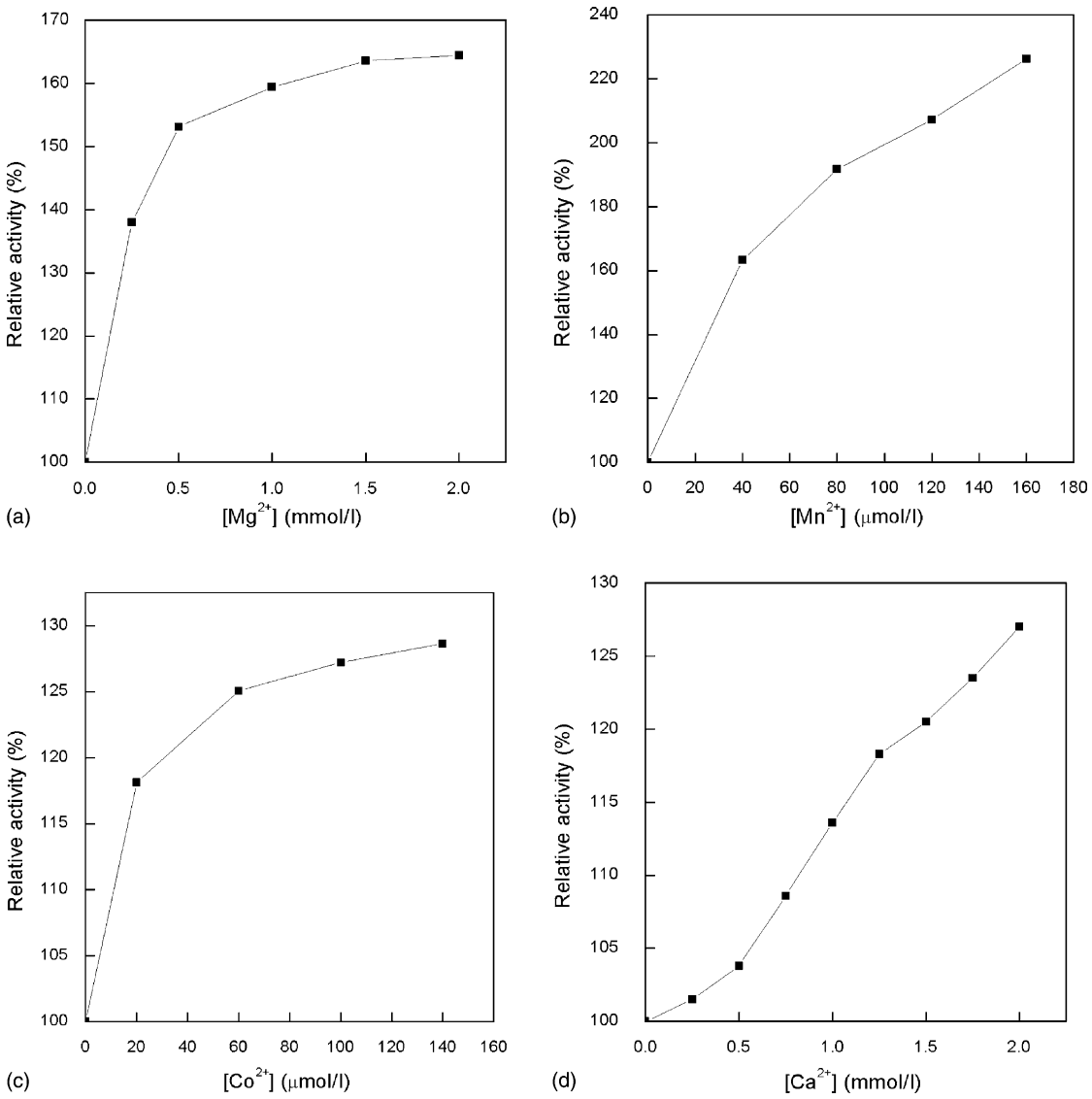

Fig. 7. Activation effects of metal ions on ALP activity. The experimental conditions were as for Fig. 3: (a) Mg^{2+}, (b) Mn^{2+}, (c) Co^{2+}, (d) Ca^{2+}.

separation) and one magnesium ion (5 and 7 Å from the two zinc ions). The structure of *Escherichia coli* ALP was recently refined to 1.75 Å and a revised catalysis mechanism was suggested [11]. The three metal ions were all participated in the catalytic reaction. ALP from pearl oyster (*Pinctada fucata*) is believed also a metalloenzyme containing zinc and magnesium ions, and the structure of its active site is probably similar to that of bacterial ALP.

ALP from the mantle of *Pinctada fucata* has some features that differ from those of freshwater mollusks. For example, the optimal pH of the enzyme from *Pinctada fucata* is 9.7, while the optimum pH values of enzyme from *Cristaria plicata* and *Anodonta*

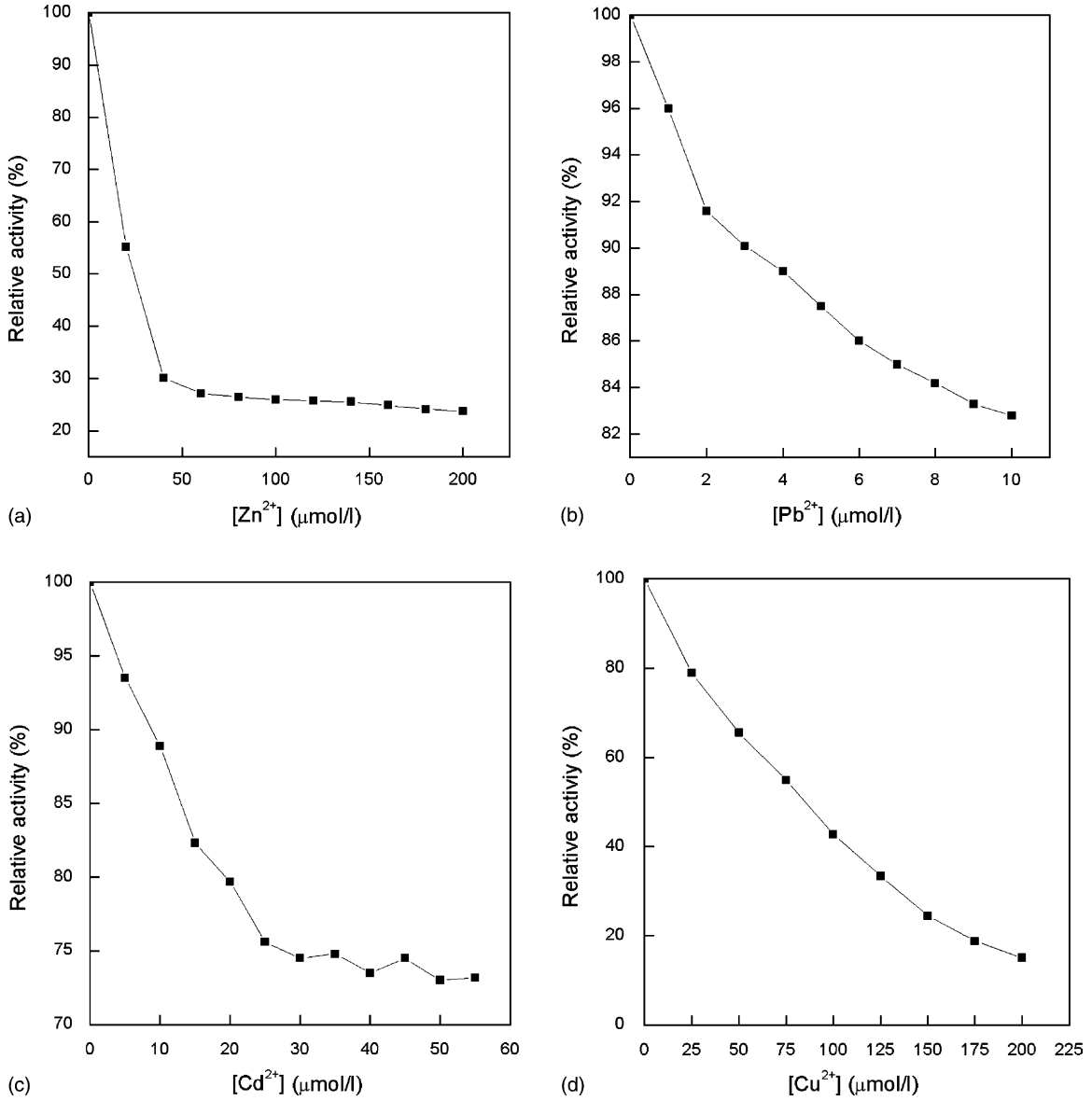

Fig. 8. The inhibition effects of metal ions on the enzyme activity. The experimental conditions were as for Fig. 4: (a) Zn^{2+}, (b) Pb^{2+}, (c) Cd^{2+}, (d) Cu^{2+}.

woodiana are 9.1 and 9.5, respectively [12,13]. These differences may be related to their environment. The optimal temperatures are also different. The optimum temperature of the enzyme from *Pinctada fucata* is 45 °C, but the optimum temperature of the enzymes from both *Cristaria plicata* and *Anodonta woodiana* are 40 °C [12,13].

Inorganic phosphate (P_i), which is a substrate for the reverse reaction, is also a competitive inhibitor of the catalytic reaction [10]. P_i can be fitted in the active site pocket and form covalent/non-covalent intermediate with the enzyme. The kinetics study of the hydrolysis of *p*-NPP in the presence of the product HPO_4^{2-} and the product-analog WO_4^{3-} showed that

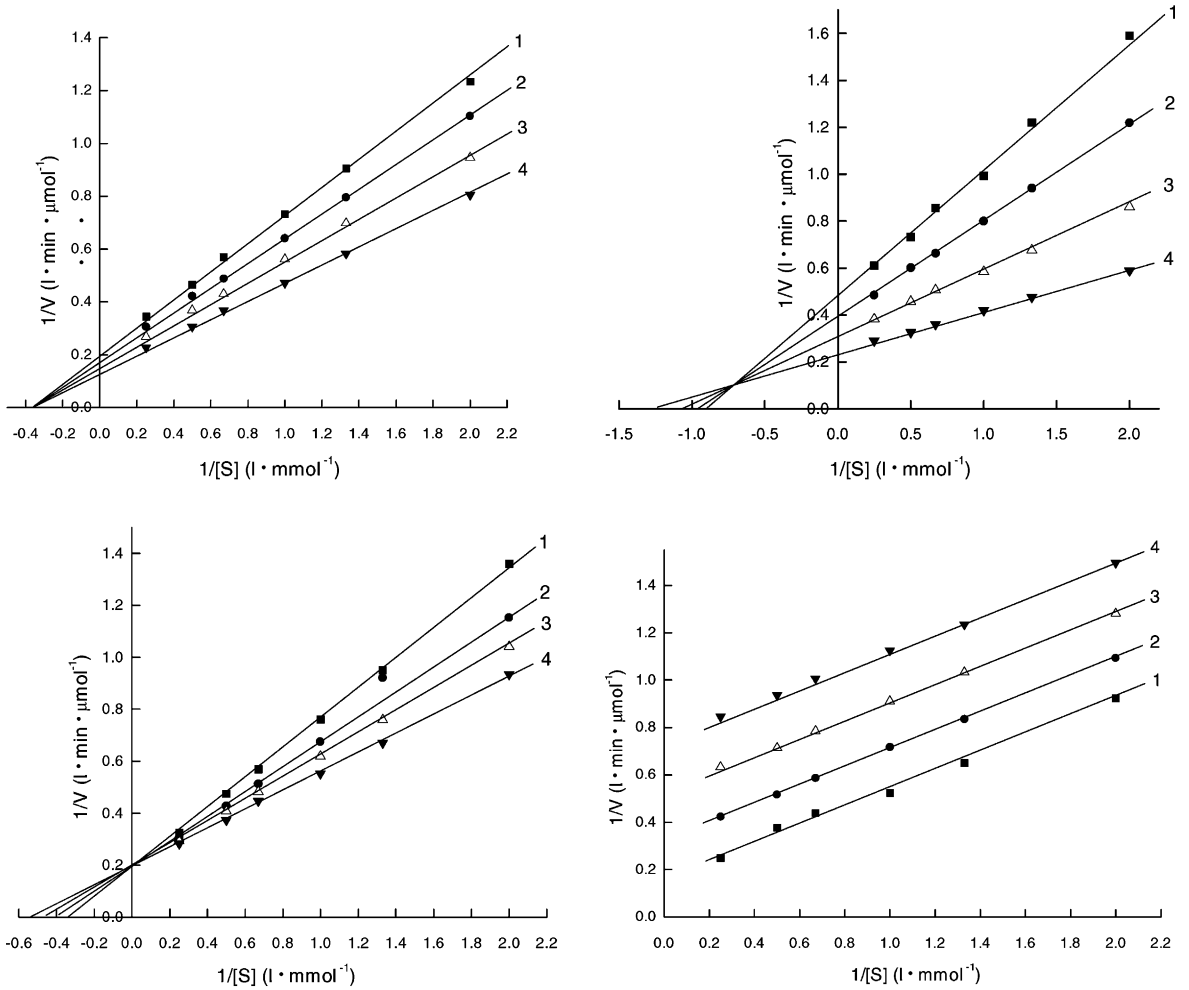

Fig. 9. Lineweaver–Burk plots for the effect of metal ions on *p*-NPP hydrolysis. The experimental conditions were as for Fig. 3. The Mg^{2+} concentrations for curves 1–4 in (a) were 0, 0.1, 0.2 and 0.3 mM, respectively. The Mn^{2+} concentrations for curves 1–4 in (b) were 0, 40, 80 and 120 μM, respectively. The Co^{2+} concentrations for curves 1–4 in (c) were 0, 15, 30 and 45 μM, respectively. The Zn^{2+} concentrations for curves 1–4 in (d) were 0, 10, 20 and 30 μM, respectively.

both competitively inhibited the enzyme activity, but the degrees of inhibition differed with the inhibitory ability of HPO_3^{2-} being greater than that of WO_4^{3-}. The results agree with similar findings for the enzyme from green crab [14,15].

In this investigation, we studied the effects of various metal ions added to the reaction solutions on the enzyme activity. Lead ions, cadmium ions, and copper ions are major marine pollutants which inhibit various enzymes. The experimental results showed that all these metal ions also inhibited the activity of ALP from pearl oyster. Therefore, marine pollution might disrupt the production of artificially cultured pearls.

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