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INHIBITORS OF GREEN CRAB (*SCYLLA SERRATA*) ALKALINE PHOSPHATASE

QING-XI CHEN[a,b,†] and HAI-MENG ZHOU[a,b,*]

[a]*Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, P.R. China;* [b]*National Laboratory, Institute of Biophysics, Academia Sinica, Beijing 100101, P.R. China*

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Green crab (*Scylla serrata*) alkaline phosphatase (EC 3.1.3.1) is a metalloenzyme which catalyzes the nonspecific hydrolysis of phosphate monoesters. The effects of several inhibitors on its activity have been studied. The results show that Na_2HPO_4, Na_2HAsO_4 and Na_2WO_4 are competitive inhibitors, L-phenylalanine is an uncompetitive inhibitor, while L-cysteine is a mixed-type inhibitor. The equilibrium constants were determined for inhibitor binding. The order of potency was Na_2HPO_4 ($K_I = 0.95$ mM) > Na_2AsO_4 (1.10 mM) > Na_2WO_4 (1.55 mM).

Keywords: Alkaline phosphatase; Green crab; Enzymatic/catalytic mechanism; Inactivation; Inhibition

Abbreviations: ALP, Alkaline phosphatase; *p*NPP, *p*-Nitrophenyl phosphate

INTRODUCTION

Alkaline phosphatases (EC 3.1.3.1) are widely distributed in nature and are characterized by a high pH optimum and a broad substrate specificity.[1,2] Alkaline phosphatase is a zinc-containing metalloenzyme which catalyzes the transfer of a phosphate group to water (hydrolysis) or alcohol (transphosphorylation) from a wide variety of phosphomonoesters. The enzyme from *Escherichia coli* has been extensively studied.[3] The X-ray crystal structure of bacterial alkaline phosphatase has been recently reported at

* Corresponding author. Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, P.R. China.
† Present Address: Department of Biology, Xiamen University, Xiamen 361005, P.R. China.

2.0 Å resolution in the presence of inorganic phosphate.[4] The active site is a tight cluster two zinc ions (3.9 Å separation) and one magnesium ion (5 and 7 Å from the two zinc ions). Alkaline phosphatase from green crab (*Scylla serrata*) is also a dimeric metalloenzyme containing zinc and magnesium ions, and the structure of its active site is probably similar to that of bacterial alkaline phosphatase. It is well known that green crab alkaline phosphatase is inactivated by EDTA. The complete kinetic course of EDTA inactivation has been determined by monitoring the hydrolysis of *p*-nitrophenyl phosphates. This paper reports the effects of several inhibitors on the activity of green crab alkaline phosphatase.

MATERIALS AND METHODS

Alkaline phosphatase was prepared from green crab (*Scylla serrata*) viscera according to the method of Yan and Chen[6] to the step of ammonium sulfate fractionation. This crude preparation was further chromatographed by ion-exchange with DEAE-cellulose, then by gel filtration through Sephadex G-150 followed by DEAE-Sephadex A-50. The final preparation was homogeneous on polyacrylamide gel isoelectric focusing and on HPLC chromatography. The specific activity of the purified enzyme was 3320 μmol mg protein^{-1}min^{-1}. *p*-Nitrophenyl phosphate (*p*NPP) was from E. Merck. All other reagents were local products of analytical grade.

The protein concentration was determined as described by Lowry *et al.*,[7] and the green crab alkaline phosphatase was assayed as described by Chen *et al.*[8] Absorbance and kinetics measurements were carried out on a Perkin-Elmer Lambda Bio spectrophotometer.

Inhibitor studies were performed by dissolving the inhibitors in 50 mM $Na_2CO_3/NaHCO_3$ buffer (pH 10.0) and incubating with the enzyme for 10 min. The assay system consisted of *p*-nitrophenyl phosphate (2 mM), $MgCl_2$ (2 mM) and $Na_2CO_3/NaHCO_3$ buffer (50 mM, pH 10.0). An aliquot (5 μl) of the mixture of the enzyme and inhibitor was added to 1 ml of the above assay system at 30°C and the rate of substrate hydrolysis was monitored for 2 min after a lag period of 20 s.

RESULTS

Effects of Inorganic Salts on the Activity of Green Crab Alkaline Phosphatase

The effects of various inorganic salts on *p*NPP hydrolysis by the enzyme were studied. The inhibition of green crab alkaline phosphatase by

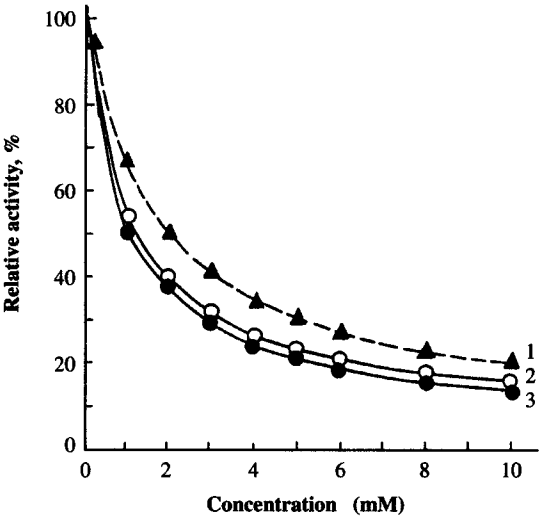


FIGURE 1 Effects of the substrate and its analogues on the activity of green crab alkaline phophatase. Enzyme was incubated for 10 min in 50 mM Na_2CO_3–$NaHCO_3$ buffer at pH 10.0 containing effectors at the different concentrations before determination of the remaining activity. Curves 1–3 represent Na_2HPO_4, Na_2HAsO_4 and Na_2WO_4 respectively.

Na_2HPO_4, Na_2HAsO_4 and Na_2WO_4 was concentration dependent (Figure 1) results in inactivation of the enzyme. Lineweaver–Burk plots (Figure 2(a)) showed that Na_2HPO_4 was a competitive inhibitor since increasing the Na_2HPO_4 concentration resulted in a family of lines with a common intercept on the $1/v$ axis but with different slopes. The equilibrium constant for inhibitor binding, K_I, was obtained from a plot of the apparent Michaelis–Menten constant (K_{mapp}) versus [Na_2HPO_4] (Figure 2(b)) Na_2HAsO_4 and Na_2WO_4 were also competitive inhibitors (not shown). The K_I values are summarized in Table I. It can be seen that Na_2HPO_4 (K_I = 0.95 mM) is a slightly more potent inhibitor than Na_2HAsO_4 (K_I = 1.1 mM), which was a more potent inhibitor than Na_2WO_4 (K_I = 1.55 mM).

Neither NaCl nor KCl were found to result in any significant inhibition of green crab alkaline phosphatase at concentrations up to 10 mM (data not shown). This observation is in marked contrast with the studies of Cyboron and Wuthier.[9]

Effects of Various Amino Acids on the Activity of Green Crab Alkaline Phosphatase

The inhibition of green crab alkaline phosphatase by different amino acids is used to classify different human isozymes.[10] The inhibition of green crab

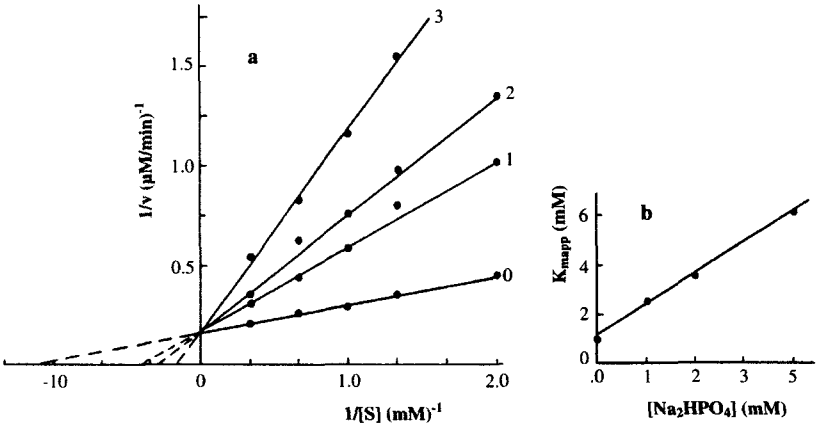


FIGURE 2 Inhibition of *p*-nitrophenylphosphate hydrolysis by Na_2HPO_4. The experimental conditions are described in Figure 1. (a) Lineweaver–Burk plots of *p*-nitrophenylphosphate hydrolysis data. The concentration of Na_2HPO_4 for curves 0–3 were 0, 1.0, 2.0 and 3.0 mM, respectively. (b) Plot of apparent Michaelis constant (K_{mapp}) versus the concentration of Na_2HPO_4. The lines are drawn using linear least squares fit.

TABLE I Inhibition types and inhibition constants for several inhibitors of green crab alkaline phosphatase

Inhibitor	*Inhibition type*	*Inhibition constant* (mM)	
		K_I	K_{IS}
Na_2HPO_4	Competitive	0.95	
Na_2HAsO_4	Competitive	1.10	
Na_2WO_4	Competitive	1.55	
L-Phenylalanine	Uncompetitive		9.80
L-Cysteine	Mixed*	1.50	5.70

*Mixed uncompetitive and noncompetitive inhibition.

alkaline phosphatase by various amino acids was concentration dependent (Figure 3). After preincubation for 10 min with 10 mM glycine, tyrosine, tryptophan, methionine, phenylalanine or cysteine, the extent of the inhibition of the enzyme was 8.5%, 16.0%, 24.5%, 42.5%, 58.0% and 93.5%, respectively.

A set of double-reciprocal plots for the inhibition by L-phenylalanine at different concentrations (Figure 4(a)) indicated that L-phenylalanine was an uncompetitive inhibitor which binds at a site distinct from the substrate and binds only to the ES complex. The K_{IS} value was 9.8 mM as obtained from a plot of $1/V_{max}$ versus [L phenylalanine] (Figure 4(b)).

Inhibition by L-cysteine was of a mixed, non- and uncompetitive type (Figure 5(a)) as shown by a Lineweaver–Burk plot. The equilibrium

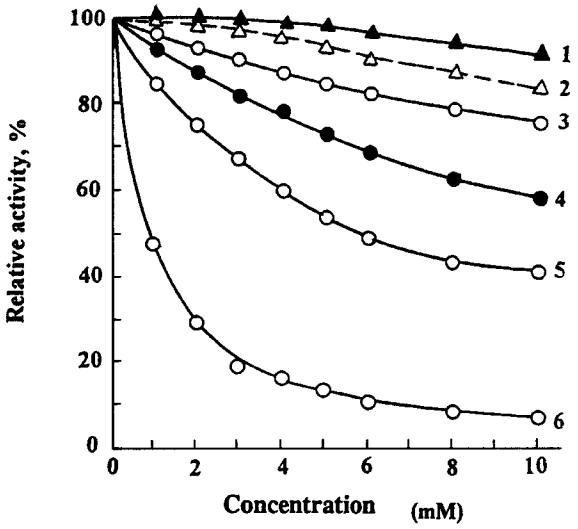


FIGURE 3 Effects of various amino acids on the activity of green crab alkaline phosphatase. The experimental conditions are described in Figure 1. The amino acids for curves 1–6 were glycine, tyrosine, tryptophan, methionine, phenylalanine and cysteine, respectively.

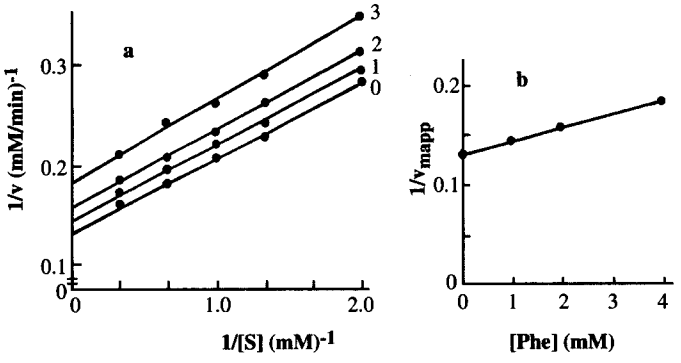


FIGURE 4 Inhibition of *p*-nitrophenylphosphate hydrolysis by L-phenylalanine. The experimental conditions are described in Figure 1. (a) Lineweaver–Burk plots of *p*NPP hydrolysis. Concentrations of L-phenylalanine for curves 0–3 were 0, 1.0, 2.0 and 3.0 mM, respectively. (b) Plot of $1/V_{max}$ versus L-phenylalanine concentration. The lines are drawn using linear least squares fit.

constant for inhibitor binding with the free enzyme (E), K_I, was obtained from a plot of the slopes of the straight lines versus the L-cysteine concentrations (Figure 5(b)). The equilibrium constant for inhibitor binding with the enzyme–substrate complex (ES), K_{IS}, was obtained from a plot of

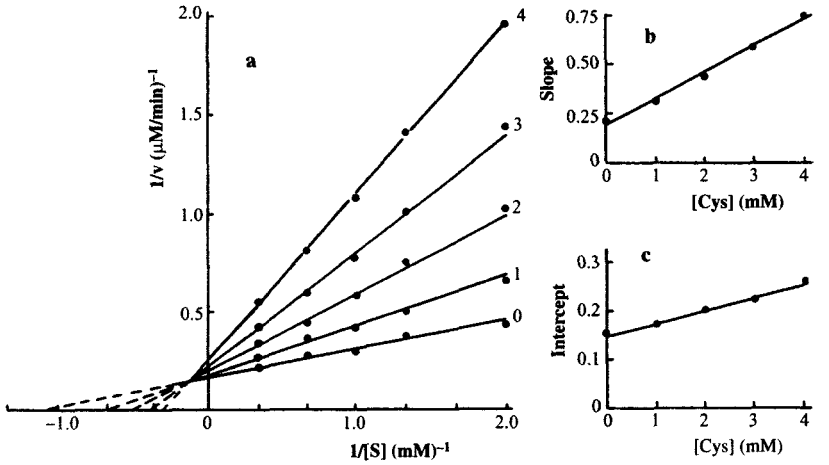


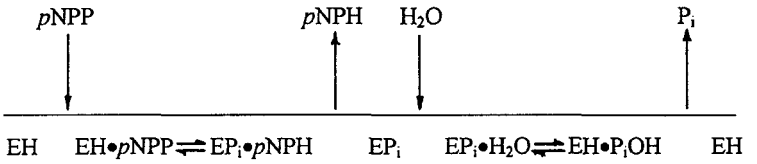
FIGURE 5 Inhibition of *p*-nitrophenylphosphate hydrolysis by L-cysteine. The experimental conditions are as described in Figure 1. (a) Lineweaver–Burk plots of *p*NPP hydrolysis. Concentrations of L-cysteine for curves 0–4 were 0, 1.0, 2.0, 3.0 and 4.0 mM, respectively. (b) Secondary plot of the slopes of the straight lines versus L-cysteine concentration. (c) Secondary plot of the intercept of the straight lines versus L-cysteine concentration. The lines are drawn using linear least squares fit.

the intercepts versus the L-cysteine concentrations (Figure 5(c)). The values obtained are summarized in Table I.

DISCUSSION

This paper reports the effects of various inhibitors on *p*NPP hydrolysis by green crab alkaline phosphatase. The results clearly show that Na_2HPO_4, Na_2HAsO_4 and Na_2WO_4 are competitive inhibitors of the enzyme. Control experiments showed that neither NaCl nor KCl has any significant effect on the activity of the enzyme at concentrations up to 10 mM, suggesting that Na_2HPO_4, Na_2HAsO_4 and Na_2WO_4 do not act as salt-inhibitors. It is likely that Na_2HPO_4, Na_2HAsO_4 and Na_2WO_4 server as structural analogues of the product (P_i) of *p*NPP hydrolysis and combine with the enzyme to form an EI complex. It can be seen from the K_I values that the structural analogues of P_i (Na_2HAsO_4 and Na_2WO_4) are weaker inhibitors than P_i. The results also show that L-phenylalanine is an uncompetitve inhibitor and binds only to the ES at a site distinct from the substrate. The inhibition by cysteine is of a mixed, non- and uncompetitive type. Moreover, the first product, *p*-nitrophenol, has no observable effect on the activity of green crab

alkaline phosphatase (data not shown), whereas the secondary product, HPO_4^{2-}, is a competitive inhibitor of the enzyme. This kinetic behavior suggests that the reaction mechanism is of the Bi Bi Ping-pong type that can be represented as shown below:



where EH, *p*NPP, *p*NPH and P_i represent the enzyme, *p*-nitrophenyl phosphate, *p*-nitrophenol and inorganic phosphate, respectively. EH · *p*NPP, EP_i · *p*NPH, EP_i, $EP_i \cdot H_2O$ and EH · P_iOH are the respective complexes.

Acknowledgments

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References

[1] Fernley, H.N. (1971). *The Enzymes* (Boyer, P.D. (Ed.)) 3rd ed. Vol. IV, pp. 417–447. Academic Press; New York.
[2] McComb, R.B., Bowers, G.N. and Posen, S. (1979). *Alkaline Phosphatase*. Plenum Press; New York.
[3] Coleman, J.E. (1992). *Ann. Rev. Biophys. Biomol. Struct.*, **24**, 441–483.
[4] Kim, E.E. and Wyckoff, H.W. (1991). *J. Mol. Biol.*, **281**, 449–464
[5] Chen, Q.X., Zhang, W., Wang, H.R. and Zhou, H.M. (1996). *Int. J. Biol. Macromol.*, **19**, 257–261.
[6] Yan, S.X. and Chen, Q.X. (1985). *J. Xiamen University*, **24**, 367–372 (in Chinese).
[7] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). *J. Biol. Chem.*, **193**, 265–275.
[8] Chen, Q.X., Zhang, W., Zheng, W.Z., Zhao, H., Yan, S.X., Wang, H.R. and Zhou, H.M. (1996). *J. Protein Chem.*, **15**, 345–350.
[9] Cyboron, G.W. and Wuthier, R.E. (1981). *J. Biol. Chem.*, **256**, 7262–7268.
[10] Harris, H. (1989). *Clin. Chim. Acta*, **186**, 133–150.
[11] Xie, W.Z., Wang, H.R., Chen, Q.X. and Zhou, H.M. (1996). *Biochem. Mol. Biol. Int.*, **40**(5), 981–991.