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Inhibitory Kinetics of β-*N*-Acetyl-D-glucosaminidase from Green Crab (*Scylla serrata*) by Zinc Ion

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Heavy metal pollution such as chromium and zinc in the seawater has been increasing in recent years in the China Sea. Marine shellfish such as prawn and crab are sensitive to this pollution. β-*N*-Acetyl-D-glucosaminidase (NAGase, EC 3.2.1.52) catalyzes the cleavage the oligomers of *N*-acetylglucosamine (NAG) into the monomer. In this study, taking *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNP-NAG) as substrate, the effects of Zn^{2+} on NAGase from green crab (*Scylla serrata*) have been studied. The results showed that appropriate concentrations of zinc could lead to reversible inhibition on the enzyme, and the IC_{50} has been estimated to be 0.5 ± 0.012 mM. Furthermore, it has been shown that Zn^{2+} could reduce the thermal stability of NAGase depending on the concentration of Zn^{2+}. The inhibitory kinetics of zinc on the enzyme in the appropriate concentrations has been studied using the kinetic method of substrate reaction. The inhibition model has been set up, and the rate constants have been determined. The results showed that Zn^{2+} was a mixed-type inhibitor of NAGase and that it could combine at the free enzyme and the enzyme−substrate active sites.

KEYWORDS: ***Scylla serrata***; **β-*N*-acetyl-D-glucosaminidase; zinc ion; inhibition; kinetics**

INTRODUCTION

In China, chemical pollution such as chromium and zinc in the seawater has been increasing in recent years, due to the continuous discharge of wastewater from rural industries such as printing, manufacturing, dyeing, textile production, electroplating, meat-processing, and tanning enterprises (*1*, *2*). It was proved that marine shellfish such as prawn and crab were sensitive to both chromium and zinc (*3*). The rising mortality and decreasing yield of marine fisheries and shellfish have taken place in recent years in China sea. Therefore, it is important to investigate the risk of chemical pollution to marine organisms and establish the model of risk forecast.

β-*N*-Acetyl-D-glucosaminidase (NAGase, EC 3.2.1.52), widely distributed in microorganisms, animals, and plants, can disassemble the dimer or trimer of NAG into a monomer. It is a composition of the chitinase and cooperates with exochitinase and endochitinase to disintegrate chitin. NAGases from microorganisms and animals have been well documented for their important roles in defense systems against parasites, molting, and digestion of chitinous foods (*4*−*6*). NAGases from crustaceans such as Antarctic krill (*5*), fiddler crab (*Uca pugilator*)(*6*), and prawn (*Penaeus vannamei*) (*7*) have also been studied for their purification, concentrations in different growth stages, and distribution in different organs. However, NAGase from green crab (*Scylla serrata*) has hardly been known. In our previous studies, we purified NAGase from green crab and discussed the enzymatic characterization and the effects of some metal ions on the enzyme activity (*8*). Because the metal ions of the aquatic environment can affect the activity and conformation of enzyme from aquatic animals, it can affect the growth or survival of aquatic animals. Up to the present, the effects of metal ion on NAGase have hardly been reported. In our continuous investigation, zinc was found to have a strong effect on enzyme activity; therefore, the aim of this paper is to carry out a kinetics study on the inhibition of NAGase by zinc, which will be valuable to marine shellfish and other aquatic animals against pollutants. Furthermore, the inhibition model and rate constants determined will be valuable to establish the model of risk forecast for marine organisms to zinc.

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MATERIALS AND METHODS

Materials. *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNP-NAG) was purchased from the the Biochemistry Lab of Shanghai Medicine Industry Academy (China). All other reagents were of analytical grade. The water used was redistilled and ion-free.

NAGase was prepared from green crab (*S. serrata*) according to the method of Zhang et al. (*8*) by extraction with 0.01 M Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl and ammonium sulfate fractionation. The crude preparation was further chromatographed on gel filtration through Sephadex G-100 and then by ion-exchange with DEAE-cellulose. The final preparation was homogeneous on PAGE, and the specific activity of the purified enzyme was 7990 U/mg.

Inhibitory Kinetics of Zinc on Green Crab NAGase. The progress of substrate reaction method previously described by Tsou (*9*) was used to study the inhibitory kinetics of zinc on green crab NAGase. In this

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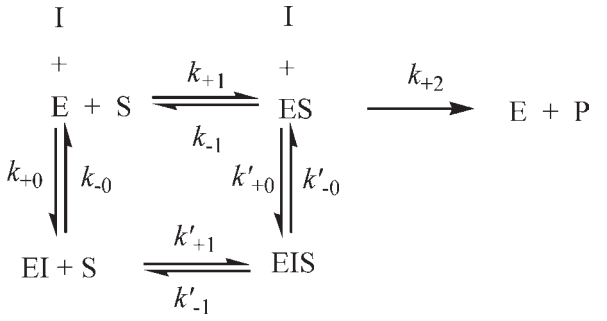
Scheme 1



method, 10 μL of NAGase was added to a 2.0 mL reaction system containing 0.05 M NaAc−HAc buffer (pH 5.8), different concentrations of pNP-NAG (0.25, 0.33, 0.40, 0.45, 0.50 mM), and zinc (0.25, 0.50, 1.0, 2.0, 4.0 mM). The enzyme activity was calculated from the linear increase in optical density at 405 nm with the pNP molar absorption coefficient (at pH 5.8) of 1.77×10^3 M^{-1} cm^{-1} (*10*). The substrate reaction progress curve was analyzed to obtain the reaction rate constants as detailed below. The time course of NAGase hydrolysis of pNP-NAG in the presence of different zinc concentrations showed that, at each concentration of zinc, the reaction rate decreased with increasing time until a straight line was approached. The results showed that the inhibition was a reversible reaction with fractional residual enzyme activity. This can be written as shown in **Scheme 1** (*11*), where E, S, I, and P denote enzyme, substrate, inhibitor (zinc), and product, respectively. EI, ES, and EIS are the respective compounds. As a rule, [S] ≫ [E_0] and [I] ≫ [E_0], and the product formation can be written as

$$[P]_t = \frac{Bv}{A[I]+B}t + \frac{A[I]v}{(A[I]+B)^2} - \frac{A[I]}{(A[I]+B)^2}e^{-(A[I]+B)t} \quad (1)$$

$$A = \frac{k_{+0}K_m + k'_{+0}[S]}{K_m + [S]} \quad (2)$$

$$B = \frac{k_{-0}k_{-1}/k_{+1} + k_{-0}[S]}{k_{-1}/k_{+1} + [S]} \quad (3)$$

where $[P]_t$ is the concentration of the product formed at time t, which is the reaction time; A and B are the apparent rate constants; k_{+0} is the microscopic rate constant for the forward reaction of zinc ion and E; k'_{+0} is the microscopic rate constant for the forward reaction of zinc ion and ES; k_{-0} and k'_{-0} are the microscopic rate constants for the reverse reactions of E and ES, respectively; $(A[I] + B)$ is the apparent rate constant of inhibition; [S] and [I] are the concentrations of the substrate and inhibitor, respectively; and v is the initial rate of reaction in the absence of the inhibitor ($v = V_m[S]/K_m + [S]$, which is the Michaelis−Menten equation). When t is sufficiently large, the curves become straight lines and the product concentration is written as $[P]_{calcd}$:

$$[P]_{calcd} = \frac{Bv}{A[I]+B}t + \frac{A[I]}{(A[I]+B)^2} \quad (4)$$

Combining eqs 1 and 4 yields

$$[P]_{calcd} - [P]t = \frac{A[I]}{(A[I]+B)^2}e^{-(A[I]+B)t} \quad (5)$$

$$\ln([P]_{calcd} - [P]_t) = \text{constant} - (A[I]+B)t \quad (6)$$

where $[P]_{calcd}$ is the product concentration to be expected from the straight-line portions of the curves as calculated from eq 4 and $[P]_t$ is the product concentration actually observed at time t. Plots of $\ln([P]_{calcd} - [P]_t)$ versus t give a series of straight lines at different concentrations of inhibitor ([I]) with slopes of $-(A[I] + B)$. A secondary plot of the slopes versus [I] gives a straight line. The apparent forward and reverse rate constants, A and B, can be obtained from the slope and intercept of this straight line. The value of B directly gives the microscopic rate constant k_{-0}.

From eq 2 and the Michaelis−Menten equation we can get

$$\frac{A}{v} = \frac{K_m k_{+0}}{V_m} \times \frac{1}{[S]} + \frac{k'_{+0}}{V_m} \quad (7)$$

A plot of A/v versus 1/[S] gives a straight line with $(k_{+0}K_m/V_m)$ as the slope of the straight line and k_{+0}/V_m as the intercept on the y-axis; K_m and V_m are known quantities from measurements of the substrate reaction in the absence of zinc at different substrate concentrations, and the rate constant k_{+0} can be easily determined.

Effect of Zn^{2+} on Thermal Stability of the Enzyme. The effect of Zn^{2+} on the thermal stability of NAGase was determined according to the method described by Zhang (*10*) in the presence of different Zn^{2+} concentrations (0, 0.5, 1.0 mM).

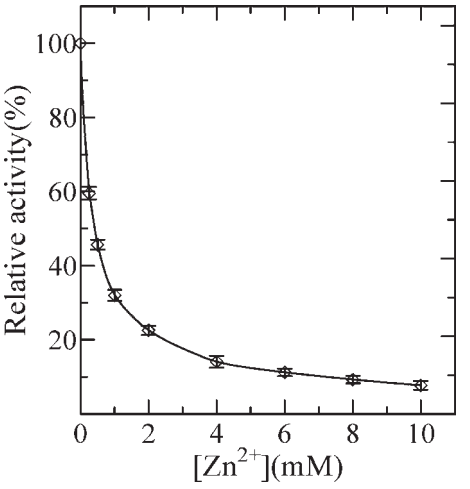


Figure 1. Effect of Zn^{2+} on the activity of enzyme.

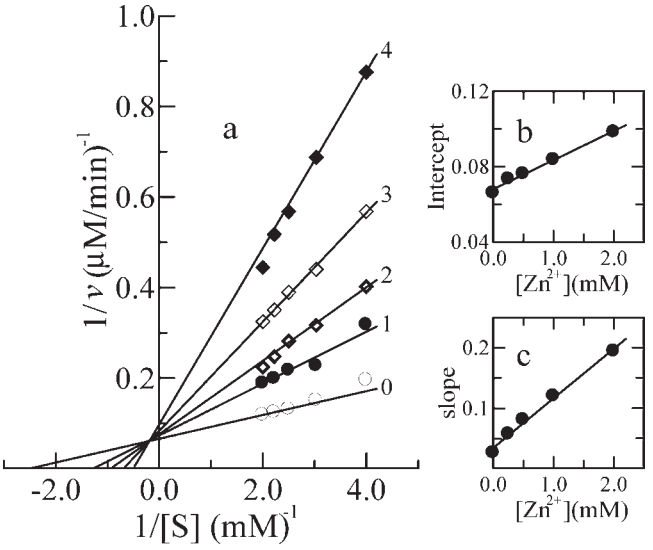


Figure 2. Inhibition kinetics of Zn^{2+} on the enzyme by Lineweaver−Burk plots (**a**). Concentrations of Zn^{2+} for curves 0−4 are 0, 0.25, 0.5, 1.0, and 2.0 mM, respectively. Insets **b** and **c** represent the secondary plot of the slope and the intercept of the straight lines versus concentration of Zn^{2+}, respectively.

RESULTS AND DISCUSSION

Determination of the Kinetic Parameters of NAGase. The kinetic behavior of NAGase from green crab in catalyzing the hydrolysis of pNP-NAG was studied. Under the conditions

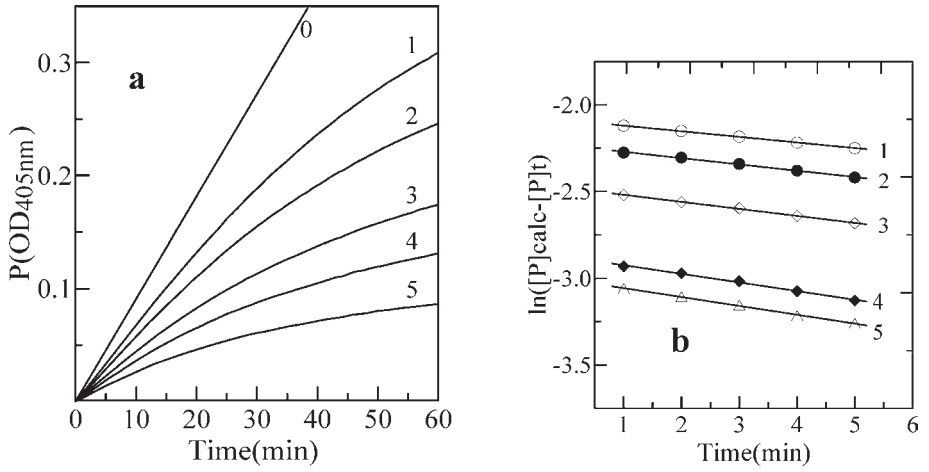


Figure 3. Course of substrate reaction in the presence of different concentrations of Zn^{2+}: (**a**) substrate reaction course (the concentrations of Zn^{2+} for curves 0−5 were 0, 0.25, 0.5, 1.0, and 2.0 mM, respectively); (**b**) semilogarithmic plots of $\ln([P]_{calcd} - [P]_t)$ against time. Data were taken from curves 1−5 in (**a**). The assay conditions were the same as in **Figure 2** with the exception of 0.4 mM pNP-NAG.

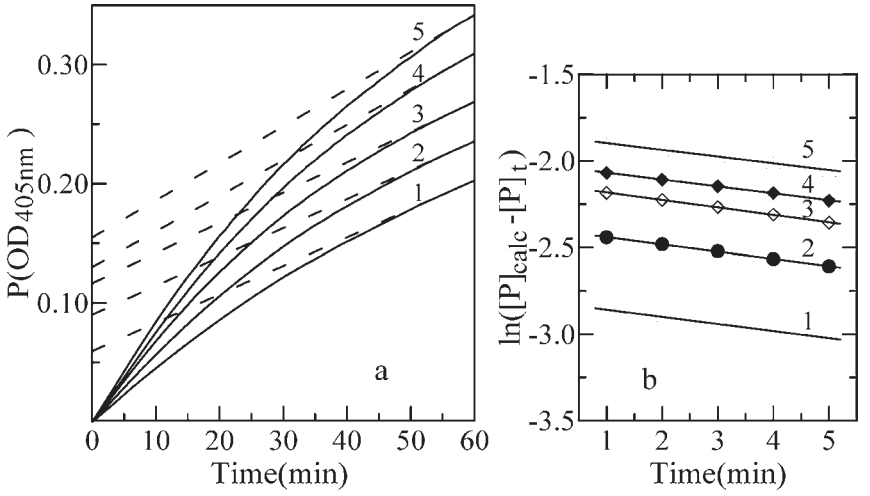


Figure 4. Course of reaction at different substrate concentrations in the presence of Zn^{2+}: (**a**) curves 1−5 are progress curves with 0.25, 0.33, 0.40, 0.45, and 0.5 mM substrate, respectively; (**b**) semilogrithmic plot of ln $([P]_{calcd} - [P]_t)$ against time. Data were taken from curves 1−5 in (**a**). Experimental conditions were as described for **Figure 3** except for the substrate concentrations. Final concentrations were 0.25 mM Zn^{2+} and 0.1 μg/mL enzyme.

employed in the present investigation, the hydrolysis reaction by NAGase follows Michaelis−Menten kinetics. The kinetic parameters obtained from a Lineweaver−Burk plot showed that K_m was 0.404 ± 0.025 mM and V_m was 14.98 ± 1.05 μM/min.

Effect of Zn^{2+} on the Activity of NAGase. The effect of zinc on the hydrolysis of pNP-NAG by NAGase was studied. The relationship between residual enzyme activity and the concentrations of zinc is shown in **Figure 1**. The result showed that zinc could inhibit the enzyme activity, and the inhibition of zinc on the enzyme was concentration-dependent. As the concentrations of zinc increased, the residual enzyme activity decreased. The IC_{50} value, the zinc concentration leading to 50% enzyme activity lost, was estimated to be 0.5 ± 0.012 mM.

Inhibition Mechanism of Zn^{2+}on the Enzyme. The inhibition kinetics of Zn^{2+} on NAGase has been determined by Lineweaver−Burk plots. The results in **Figure 2** show that Zn^{2+} is a mixed-type inhibitor. The equilibrium constants for the inhibitor binding with free enzyme (E), K_I, and with enzyme−substrate (ES) complex, K_{IS}, are obtained from the second plots of the K_m/V_m and $1/V_m$ versus concentration of Zn^{2+}, respectively, which are inset in **Figure 2**. The value of K_{IS} (4.4 mM) is larger than that of K_I (0.328 mM), indicating that the affinity of inhibitor to free enzyme is stronger than that to the enzyme−substrate complex.

Table 1. Result of Microscopic Inhibition Rate Constants of Zn^{2+} on the *Scylla serrata* NAGase

K_m		0.404 ± 0.02 mM
V_{max}		14.98 ± 0.12 μM min^{-1}
IC_{50}		0.5 ± 0.012 mM
E	k_{+0}	2.18×10^{-3} mM^{-1} s^{-1}
	k_{-0}	0.71×10^{-3} s^{-1}
ES	k'_{+0}	0.15×10^{-3} mM^{-1} s^{-1}
	k'_{-0}	0.71×10^{-3} s^{-1}

Kinetics of the Substrate Reaction in the Presence of Different Concentrations of Zn^{2+}. The time course of NAGase hydrolysis of pNP-NAG in the presence of different Zn^{2+} concentrations is shown in **Figure 3a**. At each concentration of Zn^{2+}, the reaction rate decreased with increasing time until a straight line was approached, the slope of which decreased with increasing zinc concentration. The above results analyzed according to Tsou's method (*9*) and that of Xie et al. (*12*) suggested that the formation of the enzyme−zinc complex was a slow, reversible reaction in the

curves 1−5 of **Figure 3a**. According to eq 6, plots of $\ln([P]_{calcd} - [P]_t)$ versus t give a family of straight lines at different concentrations of zinc with slopes of $-(A[I] + B)$ (**Figure 3b**).

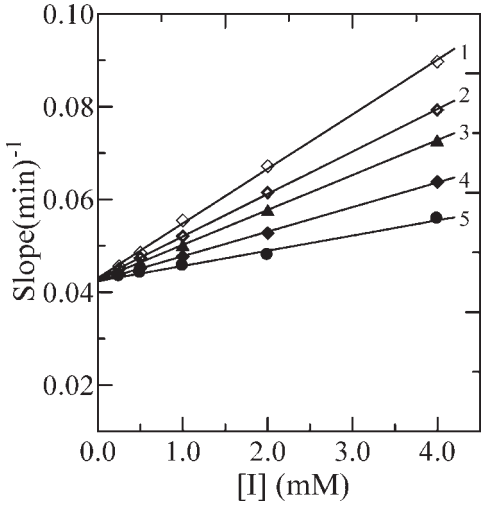


Figure 5. Secondary plots of the slopes of the semilogarithmic plots versus $[Zn^{2+}]$ for a series of different substrate concentrations. Curves 1−5 are curves with 0.25, 0.33, 0.40, 0.45, and 0.5 mM substrate.

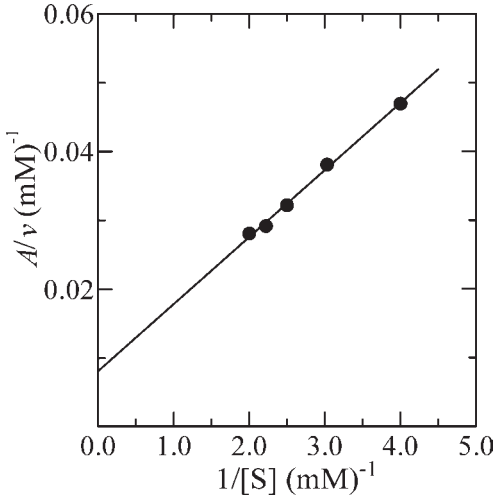


Figure 6. Plot of A/v versus 1/[S]. A values were obtained from the slopes of the straight lines in **Figure 5**.

Kinetics of the Reaction at Different Substrate Concentrations in the Presence of Zn^{2+}. **Figure 4a** shows the kinetic courses of the reaction at different pNP-NAG concentrations in the presence of 0.25 mM Zn^{2+}. It can be seen from **Figure 4a** that when t is sufficiently large, both the initial rate and the slope of the asymptote increase with increasing substrate concentration. Similarly, plots of $\ln([P]_{calcd} - [P]_t)$ versus t give a family of straight lines at different concentrations of the substrate with slopes of $-(A[I] + B)$ (**Figure 4b**). The apparent forward and reverse rate constants, A and B, can be obtained through suitable plots.

Determination of the Microscopic Rate Constants of Inhibition of the Enzyme by Zn^{2+}. A plot of the slopes of the straight lines in **Figure 4b** against the zinc concentration [I] gave the straight line, curve 3, in **Figure 5**. Similarly, data collected for other concentrations gave the other straight lines in **Figure 5**. All of the straight lines have a common intercept on the ordinate. The apparent reverse rate constant B can be obtained from the ordinate intercept, and the value of B is invariable through the change of [S]. Therefore, the value of the microscopic rate constant k_{-0} is equal to k'_{-0} and equal to B given in **Table 1**. **Figure 6** shows that the plot of A/v versus 1/[S] gives a straight line, which had an intercept on the ordinate. According to eq 7, the intercept of the straight line is equal to the k'_{+0}/V_m, so the microscopic rate constant k'_{+0} could be obtained (**Table 1**). At the same time, the slope of the straight line gave the value of $K_m k_{+0}/V_m$. Because K_m is a quantity known from measurements, k_{+0} also could be obtained from the slope (**Table 1**).

Effect of Zn^{2+} on Thermal Stability of Green Crab NAGase. The effect of Zn^{2+} on the thermal stability of NAGase was determined. The result in **Figure 7** shows that Zn^{2+} had an obvious effect on the thermal stability of NAGase. The enzyme was stable below 45 °C in the absence of Zn^{2+} (**Figure 7a**, curve 1), with the relative activity >90% (**Figure 7b**, curve 1). When the zinc concentration was increased to 0.5 mM, the enzyme was only stable below 35 °C (**Figure 7a**, curve 2), and the activity of the enzyme was almost lost at 45 °C (**Figure 7b**, curve 2). Furthermore, the enzyme was stable below 30 °C only in the presence of 1.0 mM Zn^{2+}, and the activity of the enzyme was almost lost at 35 °C (**Figure 7b**, curve 3). The results show that Zn^{2+} could decrease the thermal stability of NAGase by binding to enzyme and changing the conformation of NAGase.

Effect of Zn^{2+} on Fluorescence Emission Spectra of the Enzyme. The fluorescence emission spectra of the enzyme in different concentrations of Zn^{2+} are shown in **Figure 8**. The emission peak at 338.2 nm decreased in intensity with the increasing concentrations of Zn^{2+} (**Figure 8**, curves 0−4). The results indicate that

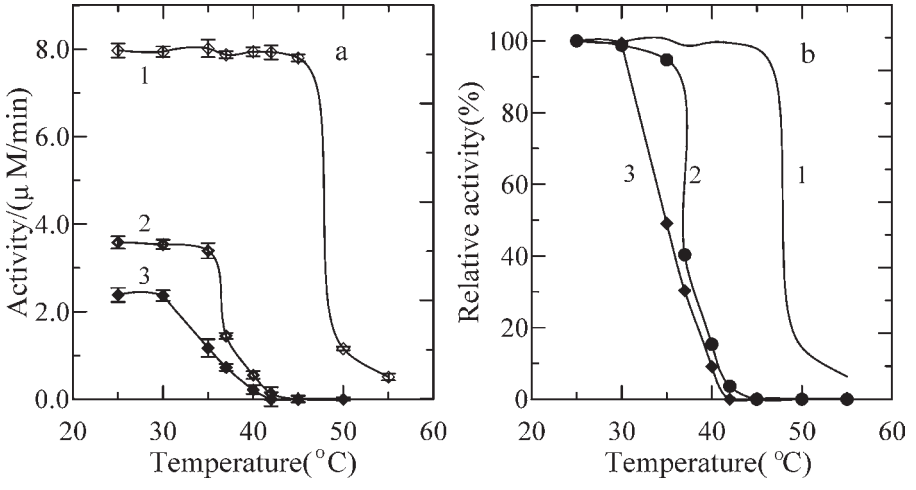


Figure 7. Effect of Zn^{2+} on thermal stability of the enzyme. Curves 1−3 are curves with 0, 0.5, and 1.0 mM Zn^{2+}, respectively.

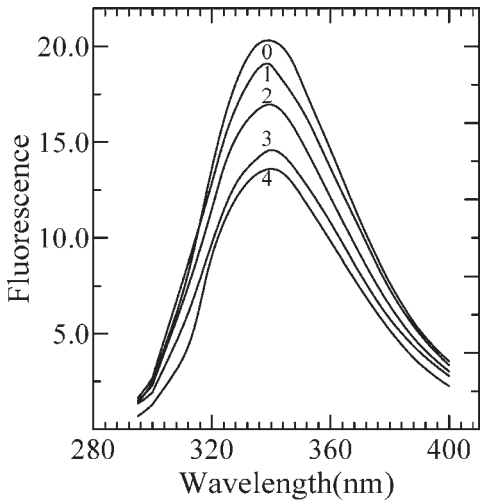


Figure 8. Fluorescence emission spectra of NAGase effect in buffer solution containing Zn^{2+}. Curves 0−4 are curves with 0, 20, 50, 100, and 300 mM Zn^{2+}, respectively.

Zn^{2+} could induce the enzyme conformation to change, which is in agreement with the result of **Figure 7**. At the same time, when the concentration of Zn^{2+} was increased to 10 mM, the residual enzyme activity only remained at 15% in **Figure 1**, whereas the fluorescence emission peak intensity decreased little even when the concentration of Zn^{2+} was increased to 300 mM; this result indicated that the change of NAGase activity was faster than the change of conformation.

Enzymes and proteins in metal ion solvents have received expanding attention in the past decade. In this paper, the effect of zinc on the green crab NAGase activity has been studied, and the results showed that zinc could inhibit the enzyme activity obviously with IC_{50} of 0.5 ± 0.012 mM . The reaction of zinc with the free enzyme molecule was shown to be reversible with residual enzyme activity because the product increasing trend was found to be a straight line at each concentration of zinc in **Figure 3a**. **Scheme 1** shows the model of the action of the mixed-type inhibitor with the free enzyme (E) and enzyme−substrate (ES) in the presence of substrate. The progress of substrate reaction catalyzed by the enzyme was used to study the inhibitory kinetics of green crab NAGase by zinc. According to the theoretical deduction, a plot of A/v versus 1/[S] must be a straight line with an intercept on the ordinate as k'_{+0}/V_m and with the slope $K_m k_{+0}/V_m$ (eq 7). The results obtained from **Figures 2** and **6** showed that zinc was a mixed-type inhibitor of the enzyme. Furthermore, Zn^{2+} could bind to enzyme and change the conformation of it, which could induce some change of enzyme such as the decrease of the thermal stability. Fluorescence emission spectra, CD spectra, and UV absorbance spectra are usually used to discuss the conformational changes of proteins after denaturation by denaturants, organic solvents, or heat (*13*, *14*). In this paper, we used the fluorescence emission spectra to assay the conformational changes of NAGase inactivated by Zn^{2+}. The results showed that the change of NAGase activity was faster than the change of conformation.

ABBREVIATIONS USED

pNP-NAG, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide; NAGase, β-*N*-acetyl-D-glucosaminidase; NaAc-HAc, sodium acetate−acetate buffer.

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