Inactivation Kinetics of β-N-Acetyl-D-Glucosaminidase from Prawn (*Penaeus vannamei*) by Formaldehyde

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Abstract: β-N-Acetyl-D-glucosaminidase (NAGase, EC.3.2.1.52) is a composition of chitinolytic enzymes and disintegrate dimmer and trimer oligomers of N-acetyl-β-D-glucosamine (NAG) into monomer. Prawn (*P. vannamei*) NAGase is involved in digestion and molting processes. Some pollutants in seawater affect the enzyme activity causing loss of the biological function of the enzyme, which affects the exuviating shell and threatens the survival of the animal. The effect of formaldehyde on prawn (*P. vannamei*) β-N-acetyl-D-glucosaminidase activity for the hydrolysis of pNP-NAG has been studied. The results show that formaldehyde, at appropriate concentrations, can lead to reversible inactivation of the enzyme, and the IC_{50} is estimated to be 1.05 mol • L^{-1}. The inactivation mechanism obtained from Lineweaver-Burk plots shows that the inactivation of the enzyme by formaldehyde belongs to the competitive type. The inactivation kinetics of the enzyme by formaldehyde has been studied using the progress-of-substrate-reaction method described by Tsou, and the rate constants have been determined. The results show that k_{+0} is much larger than k_{-0}, indicating the free enzyme molecule is fragile in the formaldehyde solution.

Keywords: *P. vannamei*; β-N-acetyl-D-glucosaminidase; inactivation; kinetics; formaldehyde

Introduction

Chitin, one of the most abundant organic compounds in nature, is a structural polysaccharide composed of N-acetyl-β-D-glucosamine (NAG) residues. The polysaccharide, sometime referred to as "animal cellulose", is a major component of the crustacean exoskeleton. Crustacean growth and development are achieved by ecdysis, the periodic shedding of the rigid exoskeleton [1]. Three chitinolytic enzymes, exo-chitinase, endo-chitinase and N-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.52), are necessary for degradation of chitin to NAG. Chitinases cleave chitin into dimmer and trimer oligomers of NAG, which are further hydrolyzed by NAGase to monomer NAG. In marine species, chitinolytic enzymes, particularly associated with the moulting processes of arthropods and crustaceans, are not only involved in the moulting process but also act as hatching enzymes in brine

Received on Mar. 23, 2006

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shrimp [2]. The NAGases from the crustaceans such as Antarctic krill [3, 4], lobster (*Homarus americanus*) [5], fiddler crab (*Uca pugilator*) [6] and Northern shrimp (*Pandalus borealis*) [7] have been reported about their purification, concentrations in different growth stage and distribution in different organs. NAGase from Antarctic krill exists in two isoenzymes which are involved in digestion and molting processes, respectively [3, 4]. Their simultaneous occurrence may indicate a physiological adaptation utilizing a mechanism of altering isoenzyme concentrations. The investigation of enzymatic properties is an essential mission to study physiological adaptations expressed by organisms in response to different environmental conditions. However, the NAGase from prawn (*Penaeus vannamei*) has hardly been known. Recently, we purified a NAGase from prawn and discussed the enzymatic characterization and the effects of metal ions on the enzyme activity [8].

The life environment of prawn is a complex system containing not only lots and lots of necessary organic and inorganic compounds but also all varieties of pollutants such as heavy metal ions or organic solvents and so on. While pollutants are assimilated, the activity and conformation of prawn NAGase can be affected, and growth and survival of the animal is threatened. Formaldehyde is a kind of organic solution widely used in current industry. So formaldehyde is a sort of main organic pollutant. Absorbing superfluous formaldehyde might induce various forms of distant-site toxicity [9]. It is very important to research the influence of formaldehyde solvents on the enzyme activity and the enzyme performance change in formaldehyde solvents. No information is available on the effects of formaldehyde on the NAGase activity. In our investigation, we found that the prawn NAGase activity could be affected by formaldehyde, and the inactivation of the enzyme in formaldehyde solutions was showed to be reversible. The aim of this paper is, therefore, to carry out a kinetics study on the inactivation of NAGase in the formaldehyde solvent. It is of great significance to inspect the pollution of the breeding aquatic environment by applying a high delicacy of enzyme to organic solvents.

1 Materials and methods

1.1 Materials

Preparation of prawn NAGase (EC3.2.1.52) was as described previously [8]. The crude preparation was further chromatographed on a Sephadex G-100 column following on the DEAE-cellulose column. The final preparation was homogeneous on PAGE and SDS-PAGE. *p*-Nitrophenyl-N-acetyl-β-D-glucosaminide (pNP-NAG) was purchased from the Biochemistry Lab of Shanghai Medicine Industry Academy (China). Sephadex G-100 was Pharmacia products. DEAE-cellulose (DE-32) was from Whatman. Formaldehyde and all other reagents were local products of analytical grade. The water used was re-distillated and ion-free.

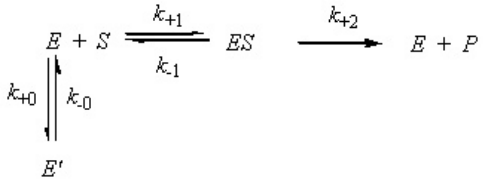
1.2 Assay methods

Enzyme concentration was measured by the Lowry method [10] (Lowry et al., 1951). Enzyme activity was determined at 37 ^{o}C by following the increasing absorbance at 405 nm accompanying the hydrolysis of the substrate (pNP-NAG) [11]. A portion of 10 μl of enzyme solution was added to the

reaction media (2.0 mL) containing 0.5 mmol·L^{-1} pNP-NAG in 0.1 mol·L^{-1} sodium phosphate buffer (pH6.2). Absorption was carried out using a Beckman UV-650 spectrophotometer. The pNP molar absorption coefficient was determined to be of 5.22×10^3 mol^{-1}·L·cm^{-1} at pH 6.2 condition.

1. 3 Microscopic inactivation rate constants of the enzyme

The progress-of-substrate-reaction theory previously described [12, 13] was applied to the current study of the inactivation kinetics of prawn NAGase by formaldehyde. In this method, 10 μl of enzyme (2.13 μmol·L^{-1}) was added to 2.0 ml of assay system containing different concentrations of substrate in 0.1 mol·L^{-1} sodium phosphate buffer (pH 6.2) with different concentrations of formaldehyde. The substrate reaction progress curve was analyzed to obtain the reaction rate constants as detailed below. The reaction was carried out at a constant temperature of 37 °C. The time course of hydrolysis of the substrate in the presence of different formaldehyde concentrations showed that, at each concentration of formaldehyde, the rate decreased with increasing time until a straight line was approached. The results showed that the inactivation reaction was a reversible reaction with fractional residual activity. This can be written as [13, 14]:



E, E', S and P denote native enzyme, denatured native, substrate, formaldehyde and product, respectively; ES are enzyme-substrate compounds. The product formation can be written as [14]:

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

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

$$B = k_{-0} \quad (3)$$

where $[P]$t is the concentration of the product formed at the time t, which is the reaction time; A and B are the apparent rate constants for the forward and reverse reactions of inactivation, respectively; $[S]$ are the concentrations of the substrate; v is the initial rate of reaction in the absence of formaldehyde, and $v = \frac{V_m\cdot[S]}{K_m+[S]}$. When t is sufficiently large, the curves become straight lines and the product concentration is written as $[P]$calc:

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

Combining Eqs. (1) and (4) yields

$$[P]_{calc} - [P]_t = \frac{Av}{(A+B)^2} \cdot e^{-(A+B)\cdot t} \quad (5)$$

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

where $[P]_{calc}$ 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 the time t. Plots of $\ln([P]_{calc}-[P]_t)$ versus reaction time (t) give a series of straight lines at different concentrations of formaldehyde with slopes of $-(A+B)$. From Eq. (4), a plot of $[P]_{calc}$ against time, t, gives a straight line with a slope of $\frac{Bv}{(A+B)}$. According to the values of $\frac{Bv}{(A+B)}$ and $(A+B)$ gotten from the above plots, and $v = \frac{V_m \cdot [S]}{K_m + [S]}$ obtained from experiment of the substrate reaction in the absence of formaldehyde at different substrate concentrations, A and B can be obtained. The rate constant $k_{-0} = B$ can be easily determined.

Combining Eq. (2) and $v = \frac{V_m \cdot [S]}{K_m + [S]}$, we can get

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

A plot of A/v versus 1/[S] gives a straight line with $\frac{K_m \cdot k_{+0}}{V_m}$ as the slope of the straight line, which passes through the origin. As K_m and V_m are known quantities from measurements of the substrate reaction in the absence of formaldehyde at different substrate concentrations, the rate constant k_{+0} can be easily determined.

2 Results

2.1 Determination of the kinetic parameters of the enzyme

The kinetic behavior of NAGase in catalyzing the hydrolysis of pNP-NAG was studied. Under the condition employed in the present investigation, the hydrolysis of pNP-NAG by NAGase follows Michaelis-Menten kinetics (the inset of Fig. 1). The kinetic parameters for NAGase obtained from a Lineweaver-Burk plot (Fig. 1) showed that K_m was equal to 0.182 mmol·L^{-1} and V_m was equal to 17.12 μmol·L^{-1}·min^{-1}.

2.2 Effect of formaldehyde on the enzyme activity

The effect of formaldehyde on the hydrolysis of pNP-NAG by prawn NAGase was first studied.

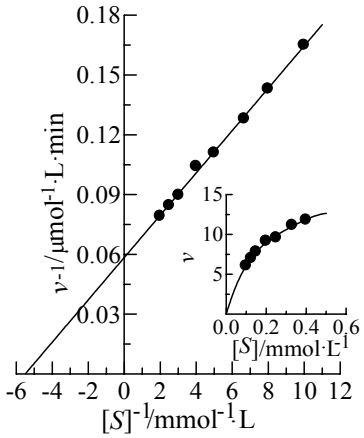


Fig. 1 Lineweaver-Burk plot for the determination of K_m and V_m for NAGase on the hydrolysis of pNP-β-D-GlcNAc. Conditions were 2 mL system containing 0.1 mol • L^{-1} sodium phosphate buffer (pH6.2) at 37 °C. The enzyme final concentration was 0.01 μmol • L^{-1}. The inset showed the relationship between the intial rate and the substrate concentration.

The relationship between the residual enzyme activity and the concentrations of formaldehyde was shown in Fig. 2. The effect of formaldehyde on the enzyme was concentration dependent. The residual enzyme activity rapidly decreased with the increasing concentrations of formaldehyde. The formaldehyde concentration leading to the loss of 50 % activity (IC_{50}) was estimated to be 1.05 mol·L^{-1}. The inactivation mechanism of formaldehyde on the enzyme was studied. Fig. 3 showed the relationship of enzyme activity with its concentration in the presence of different concentrations of formaldehyde. The plots of the remaining enzyme activity *versus* the concentrations of enzyme in the presence of different concentrations of formaldehyde gave a family of straight lines, which all passed through the origin.

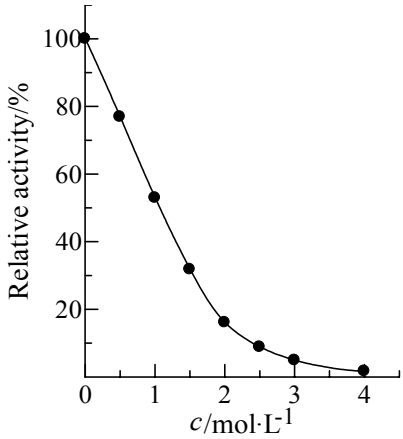


Fig. 2 Inactivation of the enzyme in different concentrations of formaldehyde solutions. Assay conditions were as described for Fig.1 except that the substrate concentration was 0.5 mmol • L^{-1}.

Increasing the formaldehyde concentration resulted in the descending of the line slope, indicating that the inactivation of formaldehyde on the enzyme was reversible. The presence of formaldehyde did not bring down the amount of the efficient enzyme, but just resulted in the inactivation and the descending of the activity of the enzyme.

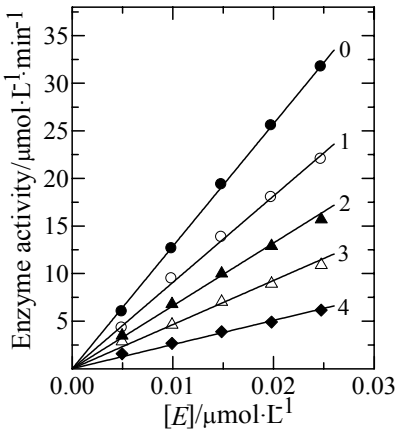


Fig. 3 Effects of the enzyme concentration on its activity at different concentrations of formaldehyde. The concentrations of formaldehyde for curves 0-4 were 0, 0.5, 1.0, 1.5 and 2.0 mol·L^{-1}, respectively. Assay conditions were the same as in Fig.2.

2.3 Determination of the inactivation type of formaldehyde on the enzyme

In the presence of formaldehyde, the inactivation kinetics of the enzyme was showed in Fig. 4 as the plots of Lineweaver-Burk. The results showed that the inactivation of formaldehyde on the enzyme

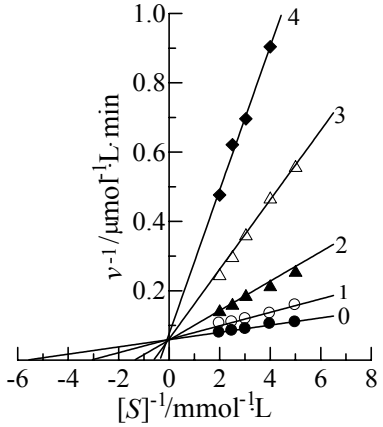


Fig. 4 Lineweaver-Burk plots for the hydrolysis of pNP-β-D-GlcNAc by the enzyme in different concentrations of formaldehyde. The formaldehyde concentration for lines 0-4 was 0, 0.5, 1.0, 1.5 and 2.0 mol • L^{-1}, respectively. The assay conditions were the same as Fig.1.

was a competitive type since increasing the formaldehyde concentration resulted in a family of lines with different slope and same intercept. This behavior observed indicated that only free enzyme inactivated in formaldehyde solution, and the enzyme-substrate complex was not affected by formaldehyde. Substrate had a protective function for enzyme in the formaldehyde solution.

2.4 Kinetics of the substrate reaction in the presence of different concentrations of formaldehyde

The temporal variation of the product concentration during the substrate hydrolysis in the presence of different formaldehyde concentrations is shown in Fig. 5a. At each concentration of formaldehyde, the rate decreases with the increasing time until a straight line is approached, the slope of which decreases with increasing formaldehyde concentration. The results as analyzed by Tsou′s method [12], suggest that the denatured NAGase still has partial residual activity (curves 1-4). According to Eq. (6), plots of $\ln([P]_{calc} - [P]_t)$ versus t give a series of straight lines shown in Fig. 5b.

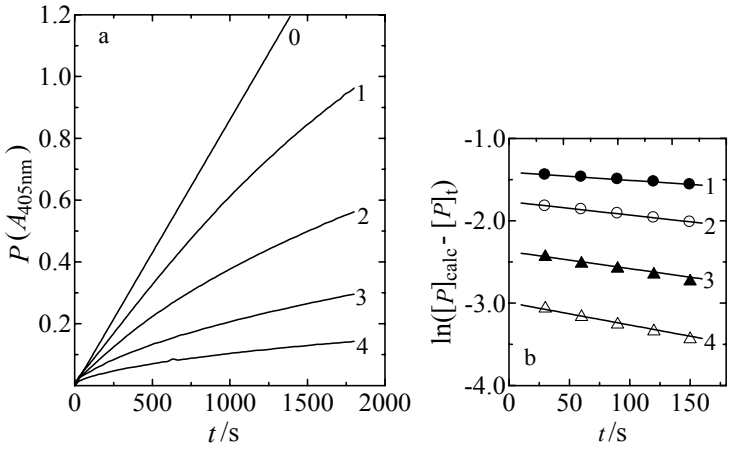


Fig. 5. Course of inactivation of enzyme in different concentrations of formaldehyde. The assay conditions were the same as in Fig. 1 with the exception of 0.4 mmol·L^{-1} pNP-β-D-GlcNAc. (a) Substrate reaction course. The final formaldehyde concentrations for curves 0-4 were 0, 0.5, 1.0, 1.5, and 2.0 mol·L^{-1}, respectively. (b) Semilogarithmic plots of $\ln([P]_{calc}-[P]_t)$ against time. Data were taken from curves 1-4 in (a).

2.5 Kinetics of the reaction at different substrate concentrations in the presence of formaldehyde

Fig. 6a showed the kinetic courses of the reaction at different substrate concentrations in the presence of 1.0 mol·L^{-1} formaldehyde. It can be seen from Fig. 6a that when t is sufficiently large, both the initial rate and the slope of the asymptote increase with the increasing substrate concentration. Similarly, plots of $\ln([P]_{calc} - [P]_t)$ versus t give a family of straight lines at different concentrations of the substrate with slopes of $-(A + B)$ as shown in Fig. 6b.

2.6 Determination of the microscopic rate constants of inactivation of the enzyme by formaldehyde

The kinetic course of the hydrolysis reaction at different substrate concentrations in the presence of

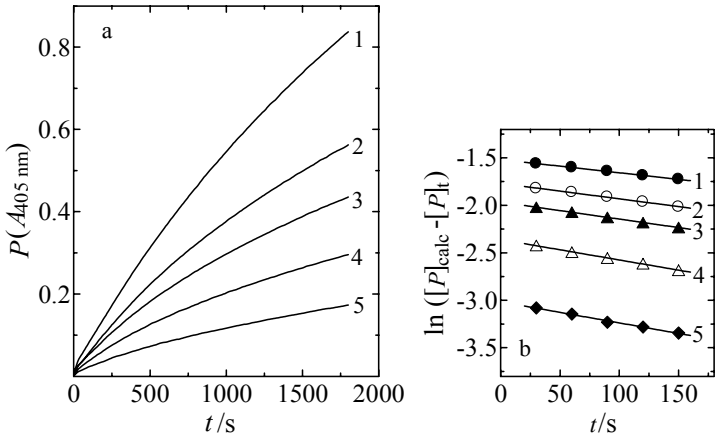


Fig. 6 Determination of the inactivation rate constants for enzyme inactivation in 1.0 mol·L-1 of formaldehyde solution. (a) Substrate reaction courses of the enzyme. Curves 1-5 are progress curves with 0.50, 0.40, 0.33, 0.25 and 0.20 mmol·L-1 of substrate, respectively. The assay conditions were the same as Fig. 1. (b) Semilogrithmic plot of ln ($[P]_{calc} - [P]_t$) against time. Data were taken from curves 1-5 in (a).

formaldehyde was studied. When the time is sufficiently large, a straight line is approached at each concentration of substrate. From Eq. (4), a plot of $[P]_{calc}$ against time t gives a straight line with a slope of $Bv/(A + B)$. Both the initial rate and the slope of the asymptote $(\frac{Bv}{A+B})$ increase with the increasing substrate concentration (Fig. 6a). From Eq. (6), plots of $\ln([P]_{calc} - [P]_t)$ versus t give a series of straight lines at different concentrations of substrate, whose slopes are equal to the apparent rate constant $(A + B)$ (Fig. 6b). According to the values of $\frac{Bv}{(A+B)}$ and $(A + B)$ obtained from the above plots, and v obtained from the experiment on the substrate reaction in the absence of formaldehyde at different substrate concentrations, A and B can be calculated.

According to B values as calculated above, plots of B versus $[S]$ at different formaldehyde concentrations give a series of straight lines shown in Fig. 7. The result showed that all lines paralleled the X-axis, indicating the value of B was not affected by the substrate concentrations. The value of B, equal to the microscopic rate constant, k_{-0}, was determined and the results were given in Tab. 1.

From Eq. (7), a plot of $\frac{A}{v}$ versus $1/[S]$ gives a series of straight lines which passes through the origin, Fig. 8, where the slope gives the value of $\frac{k_{+0} \cdot K_m}{V_m}$, which were used to determine the inactivation rate constants k_{+0}. These rate constants were also shown in Tab. 1.

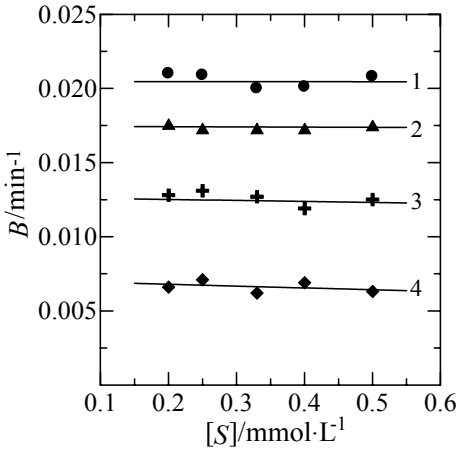


Fig. 7 Plot of *B* versus [*S*]. The final formaldehyde concentration for curves *1-4* are 0.5, 1.0, 1.5 and 2.0 mol·L^{-1}, respectively.

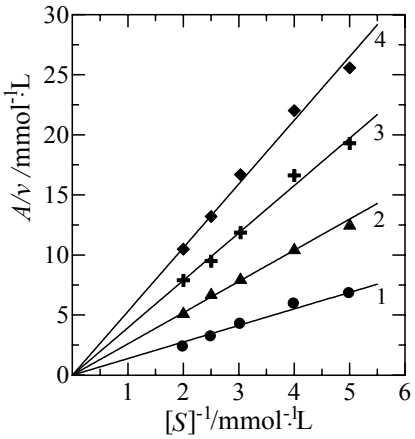


Fig. 8 Plot of *A*/*v* versus 1/[*S*]. The final formaldehyde concentration for curves *1-4* are 0.5, 1.0, 1.5 and 2.0 mol·L^{-1}, respectively.

Tab. 1 Microscopic Rate Constants of the Inactivation of *P. vannamei* NAGase in Formaldehyde Solutions

c(formaldehyde) / mol·L^{-1}	Rate constants / × 10^3 s^{-1}		Residual activity / %
	k_{+0}	k_{-0}	
0	0	0	100.0
0.5	2.16±0.25	0.35±0.05	76.9±0.80
1.0	4.07±0.30	0.29±0.02	52.9±0.45
1.5	6.18±0.45	0.21±0.025	31.8±0.30
2.0	8.31±0.30	0.11±0.02	16.1±0.15

3 Discussion

Enzymes and proteins in organic solvents have received more and more attention in the past decade, and some novel properties have been reported while enzymes work in organic solvents, which benefit both biotechnology and pharmaceutical industry. Formaldehyde can chiefly modify the amino groups in protein in acidic condition [15]. Lin et al reported formaldehyde could modify the amino groups of *T. cornutus* NAGase [16]. The chemical modification of formaldehyde on the enzyme induced enzyme inactivation, which showed the amino group was the enzyme′s functional group [16]. When the amino groups of the protein are modified, the protein conformation and its function may change and easily induces disease or endangers the animal's survival. The investigation of the effect of formaldehyde on the prawn NAGase is of important significance for breeding *Penaeus vannamei*.

However, the inactivation of *Penaeus vannamei* NAGase in the formaldehyde solution has not been investigated. In order to further study its inactivation mechanism in detail, the present paper investigates the inactivation kinetics of the enzyme activity by formaldehyde. The results indicate that formaldehyde is a competitive inactivator of the enzyme, which is different from dioxane. The inactivation mechanism of enzyme in the dioxane solution is a mixed type [17]. Formaldehyde can only bind with the native enzyme and can not bind with the enzyme-substrate. High concentration substrate can protect the native enzyme from inactivation in the formaldehyde solution. Formaldehyde is widely used in current industry. The increasing content of formaldehyde in breed seawater from industrial pollution thus could affect the growth and reproduction of prawns, which are an important economic marine animal nowadays. The studies in this field are carried out in our lab and have got some interesting positive evidences, which will be valuable to the seawater aquiculture of other marine economic animals against pollutants.

Acknowledgments

This work was supported by the Scientific and Technological Innovation Fund for the Young Scholars of Fujian Province (No. 2004J054) and Plan Project of the Education Bureau of Fujian Province (No. JA04260).

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甲醛对南美白对虾(*Penaeus vannamei*)β-N-乙酰-D-氨基葡萄糖苷酶的失活动力学研究

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摘 要：β-N-乙酰-D-氨基葡萄糖苷酶与南美白对虾的食物消化吸收、蜕壳生长有着密切关系。海水里存在的有机污染物将影响酶生理功能，从而进一步影响虾的正常蜕壳，严重将导致对虾的死亡。甲醛是常用的有机溶剂，故本文应用动力学方法研究了南美白对虾 β-N-乙酰-D-氨基葡萄糖苷酶在甲醛溶液中以 pNP-NAG 为底物时酶活力的变化规律。表明酶在甲醛浓度低于 2.0 mol·L^{-1}，酶的失活过程是可逆的，测得甲醛对酶抑制的 IC_{50} 为 1.05 mol·L^{-1}。用双倒数作图法测定甲醛对酶的抑制类型，结果显示甲醛是酶的竞争性抑制剂，甲醛只能与游离酶(E)结合，底物对酶的失活具有保护作用。用底物反应动力学方法观测在不同底物浓度下酶在 0.0、0.5、1.0、1.5、2.0 mol·L^{-1} 的醋酸酐溶液中的失活过程，分别测定了酶的微观失活速度常数 k_{+0} 及复活速度常数 k_{-0} 并加以比较，结果表明甲醛对酶的影响是缓慢结合同时导致失活的过程，比较微观失活速度常数 k_{+0} 及复活速度常数 k_{-0}，结果暗示在高浓度的甲醛溶液中，酶将完全失活。

关键词：南美白对虾；β-N-乙酰-D-氨基葡萄糖苷酶；失活；动力学；甲醛