Inactivation Kinetics of β-N-Acetyl-D-glucosaminidase from Pacific White Shrimp (*Penaeus vannamei*) by Dimethyl Sulfoxide

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Abstract   β-N-Acetyl-D-glucosaminidase (NAGase, EC 3.2.1.52) catalyzes the cleavage of N-acetylglucosamine polymers. We previously purified this enzyme from pacific white shrimp (*Penaeus vannamei*). The effects of dimethyl sulfoxide(DMSO) on the enzyme activity for the hydrolysis of pNP-β-D-GlcNAc have been studied. The results show that DMSO, at appropriate concentrations, can lead to reversible inactivation of the enzyme, and the IC_{50} is estimated to be 1.2 mol/L. The kinetics of inactivation of NAGase in DMSO solution has been studied using the kinetic method of the progress-substrate-reaction described by Tsou. The rate constants of inactivation have been determined. The results show that k_{+0} is much higher than k'_{+0}, indicating the free enzyme molecule is more fragile than the enzyme-substrate complex in the DMSO solution. These data suggested that the presence of the substrate offers marked protection of the enzyme against inactivation by DMSO.

Key words   β-N-acetyl-D-glucosaminidase, *Penaeus vannamei*, inactivation, kinetics, dimethyl sulfoxide

(*Penaeus vannamei*) β-N-　　-D-

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应用动力学方法研究了太平洋白对虾(*Penaeus vannamei*) β-N-乙酰-D-氨基葡萄糖苷酶在二甲亚砜溶液中以 pNP-β-D-GlcNAc 为底物时酶活力的变化规律. 表明酶在 DMSO 浓度低于 4.20 mol/L,酶的失活过程是可逆的, DMSO 并不造成酶绝对量的减少,仅对酶的活力发生可逆的下降. 测得 DMSO 对酶抑制的 IC_{50} 为 1.2 mol/L. 观测了在不同底物浓度下 NAGase 在 0 0.35 0.70 1.05 1.40 1.75 mol/L 的 DMSO 溶液中的失活过程,分别测定了游离酶(E)和酶-底物络合物(ES)的微观失活速度常数 k_{+0} 和 k'_{+0} 比较结果(k_{+0} 值远远大于 k'_{+0})表明, 在 DMSO 溶液中游离酶比酶-底物络合物更易失活,即底物的存在对于酶被 DMSO 的失活具有明显的保护作用. 随着 DMSO 浓度的增加,游离酶的逆向微观复活速度常数 k_{-0} 却不断降低,说明在高浓度 DMSO 环境中,NAGase 可逆恢复的能力逐渐微弱.

β-N-乙酰-D-氨基葡萄糖苷酶,太平洋白对虾,失活,动力学,二甲亚砜

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Chitin, an insoluble linear β-1,4-linked polymer of N-acetylglucosamine (GlcNAc)[1], is second abundant carbohydrates on earth. Naturally occurring chitin varies in its degree of deacetylation and in its crystalline form (α, β, γ). In most organisms, chitin is found cross-linked with specific proteins and glucans to form structural units[2]. For chitin is so complex, its biological decomposition needs a system of enzymes containing exo-chitinase, endo-chitinase and N-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.52). Chitinases cleave chitin into dimmer and trimer or oligomers of NAG, which are further hydrolyzed by NAGase to monomer NAG[3]. It is not surprising that chitinolytic enzymes are widely distributed in nature. They are found in bacteria, fungi, plants and invertebrates such as protozoans, arachnids, insects, crustaceans and nematodes[4].

Pacific white shrimp (*Penaeus vannamei*) originates on the Western Pacific coast of Latin America from Peru in the south to Mexico in the north. Now, it has become the one of three primary cultured shrimp species in the world. NAGase from *P. vannamei* have been purified and characterized in our previous studies[5]. As the breeding aquatic environment always effect by the external factor, such as metal ions, organic compounds and some other pollutant, it is important to investigate the influence of these ingredients on the enzyme activity and the enzyme performance change[6,7]. For this purpose, we have studied the effects of metal ions on the enzyme activity and made a kinetics study on the inactivation of NAGase in the dioxane solvent[8].

In this paper, we studied the impact of dimethyl sulfoxide (DMSO) on enzyme. DMSO is widely applied in organic chemistry, chemical technology, cell biology and medicine because of its many important biological properties[9]. A important fact of DMSO is that it has been used as a vehicle due to its miscibility with water and ability to increase membrane permeability, thereby facilitating the absorption of compounds that are otherwise insoluble in water. In other words, we can use it to accelerate the absorption of the medicine. As DMSO itself is a medicine, and at the same time a powerful solvent, before it use in the shrimp cultivation it is very meaningful to understand DMSO function on the enzyme.

1 Materials and Methods

1.1 Materials

Preparation of *P. vannamei* NAGase was made as previously described[5]. The crude preparation was further purified by gel filtration chromatography through Sephadex G-100, then by ion exchange with DEAE-cellulose. The final enzyme preparation was homogeneous as judged by electrophoresis on polyacrylamide gel in the absence and the presence of SDS. The specific activity of t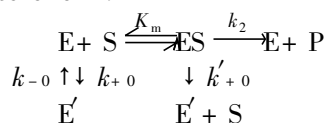he enzyme is 1 560 U/mg protein. *p*-Nitrophenyl-N-acetyl-β-D-glucosaminide (pNP-β-D-GlcNAc) was purchased from the Biochemistry Lab of Shanghai Medicine Industry Academy (China). Sephadex G-100 was Pharmacia products. DEAE-cellulose (DE-32) was from Whatman. The chitin was from the Third Institute of Oceanography, SOA of China. DMSO was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade. The water used was re-distillated and ion-free.

1.2 Determination of enzyme activity

Protein concentration was determined as described by Lowry et al[10]. Enzyme activity was assayed at 37 ℃ by following the increasing absorbance at 405 nm accompanying the hydrolysis of the substrate (pNP-β-D-GlcNAc)[11]. 10 μl of enzyme solution was added to 2.0 ml of activity assay system containing 0.5 mmol/L pNP-β-D-GlcNAc, 0.15 mol/L NaAc-HAc buffer (pH 5.2). After reaction for 10 min at 37 ℃, 2 ml of 0.5 mol/L NaOH was added into the reaction mixture to stop the reaction. The enzyme activity was calculated by the increased absorption of the reaction mixture at 405 nm using a molar absorption coefficient $1.73 \times 10^4 \text{ mol}^{-1} \cdot \text{L} \cdot \text{cm}^{-1}$[12]. One unit (U) of enzymatic activity was defined as the amount of enzyme required to produce 1 μmol pNP-β-D-GlcNAc per min under these conditions. Absorption measurements were recorded using a Beckman UV-650 spectrophotometer.

1.3 Inactivation rate constants of *P. vannamei* NAGase in DMSO solutions

The progress-of-substrate-reaction method as previously described[12,13] was used to study the inactivation kinetics of *P. vannamei* NAGase in DMSO solutions. In this method, 10 μl of NAGase (0.14 mg/ml) was added to 2.0 ml of assay system containing different concentrations of substrate in 0.1 mol/L NaAc-HAc buffer (pH 5.2) with different concentration of DMSO. 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 ℃. The time course of the hydrolysis of the substrate in the presence of different DMSO concentrations showed that the rate decreased with increasing time until a straight line was approached. The results showed that the inactivation was a reversible reaction with fractional residue activity. This can be written as scheme[14]:

$$\mathrm{E + S} \underset{K_m}{\rightleftharpoons} \mathrm{ES} \xrightarrow{k_2} \mathrm{E + P}$$

$$k_{-0} \uparrow\downarrow k_{+0} \qquad \downarrow k'_{+0}$$

$$\mathrm{E'} \qquad \mathrm{E' + S}$$

where S, P, E, E' denote substrate, product, and the natural free enzyme and inactivated enzyme, respectively; ES is the enzyme-substrate complex. k_{+0} and k_{-0} are rate constants for forward and reverse inactivation of free enzyme, respectively, and k'_{+0} is inactivation rate

constant of enzyme-substrate complex. The scheme means that the inactive enzyme (E') can not combine with the substrate (S); however, the inactive enzyme (E') can reversibly turn into the active native enzyme (E), so the inactive process is still reversible. As is usual the case $[S] \gg [E_0]$, the product formation can be written as:

$$[P]_t = \frac{v \cdot k_{-0}}{A} \cdot t + \frac{v}{A^2}(A - k_{-0})(1 - e^{-A \cdot t})$$

(1)

and

$$A = \frac{k_{+0}K_m + k'_{+0}[S]}{K_m + [S]} + k_{-0}$$

(2)

where $[P]_t$ is the concentration of the product formed at time (t) which is the reaction time; A is the apparent forward rate constant of inactivation; $[S]$ is the concentration of the substrate; and v is the initial rate of reaction in the absence of denaturant (DMSO), where $v = \frac{V_m \cdot [S]}{K_m + [S]}$. When t is sufficiently large, the curves become straight lines and the product concentration is written as $[P]_{cal}$:

$$[P]_{cal} = \frac{vk_{-0}}{A} \cdot t + \frac{v}{A^2}(A - k_{-0})$$

(3)

Combining Eqs (1) and (3) yields

$$[P]_{cal} - [P]_t = \frac{v}{A^2}(A - k_{-0}) \cdot e^{-A \cdot t}$$

(4)

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

(5)

where $[P]_{cal}$ is the product concentration to be expected from the straight-line portions of the curves as calculated from Eq (3) and $[P]_t$ is the product concentration actually observed at time t. Plots of $\ln([P]_{cal} - [P]_t)$ versus t give a series of straight lines at different concentrations of denaturant with slopes of $-A$. The apparent forward rate constant A can be obtained from such graphs. From Eq (3), a plot of $[P]_{cal}$ against time, t, gives a straight line with a slope of $\frac{vk_{-0}}{A}$. From the slope of the straight line, k_{-0} can be obtained.

Combining Eq (2) and the Michaelis-Menton equation gives

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

(6)

A plot of A/v versus $1/[S]$ gives a straight line with $\frac{K_m}{V_m}(k_{+0} + k_{-0})$ and $\frac{k'_{+0} + k_{-0}}{V_m}$ as the slope and intercept, respectively. As K_m and V_m are known quantities from measurements of the substrate reaction in the absence of DMSO at different substrate concentrations and k_{-0} can be obtained from a suitable plot as above, the rate constants k_{+0} and k'_{+0} can be obtained from the slope and intercept of the straight line, respectively.

2   Results

2.1   Determination of the kinetic parameters of *P. vannamei* NAGase

The kinetic performance of NAGase in the hydrolysis of pNP-β-*D*-GlcNAc was studied. Under the condition employed, the hydrolysis of pNP-β-*D*-GlcNAc by NAGase follows Michaelis-Menton kinetics. The kinetic parameters for NAGase obtained from a Lineweaver-Burk plot (Fig. 1) showed that K_m was 0.266 mmol/L and V_m was 15.20 μmol·L^{-1}·min^{-1}.
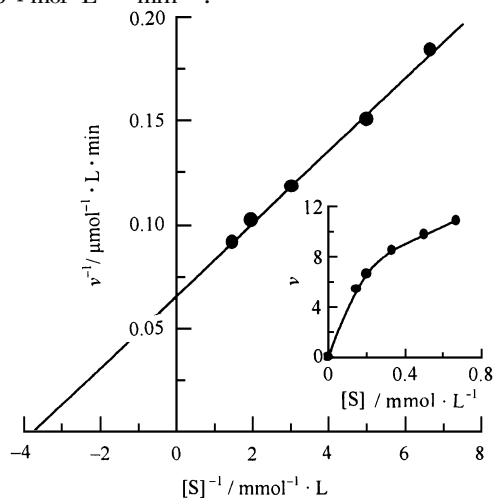

Fig.1   Lineweaver-Burk plot for the determination of K_m and V_m for *P. vannamei* NAGase on the hydrolysis of pNP-β-*D*-GlcNAc
Conditions were 2 ml system containing 0.15 mol/L NaAe-HAc buffer(pH 5.2) and different concentration of pNP-β-*D*-GlcNAc at 37℃. The final concentration of enzyme was 0.70 μg/ml. The inset showed the relationship between the initial velocity and the substrate concentration

2.2   Effect of DMSO on the enzyme activity

The effect of DMSO on the hydrolysis of pNP-β-*D*-GlcNAc by NAGase was first studied. The relationship between residual enzyme activity and the concentrations of DMSO was shown in Fig. 2. The effect of DMSO on NAGase was concentration dependent. As the concentrations of DMSO increased, the residual enzyme activity rapidly decreased. The DMSO concentration leading to 50% activity lost (IC_{50}) was estimated to be 1.2 mol/L. The inactivation mechanism of the enzyme in DMSO solution for the hydrolysis of pNP-β-*D*-GlcNAc was studied. Fig. 3 showed the relationship of enzyme activity with its concentration in the presence of different concentrations of DMSO. The plots of the remaining enzyme activity *versus* the concentrations of enzyme in the presence of different concentrations of DMSO gave a family of straight lines, which all passed through the origin. Increasing the DMSO concentration resulted in the descending of the slope of the line, indicating that the inactivation of DMSO on the enzyme was reversible reaction course. The presence of DMSO did not bring down the amount of the efficient enzyme, but just resulted

in the inhibition and the descending of the activity of the enzyme. DMSO is reversible inactivator of NAGase for hydrolysis of pNP-β-D-GlcNAc.
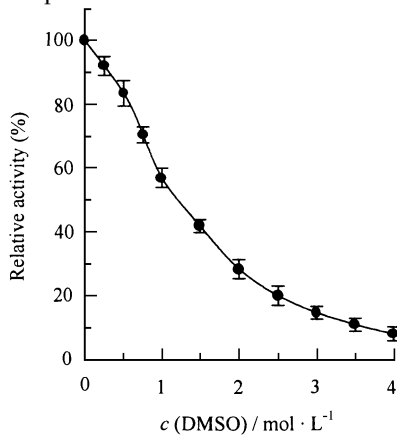

Fig. 2 Effect of DMSO on the activity of *P. Vannamei* NAGase for the hydrolysis of pNP-β-D-GlcNAc

Assay conditions were as described for Fig. 1 except that the pNP-β-D-GlcNAc concentration was 0.5 mmol/L with different concentrations of DMSO
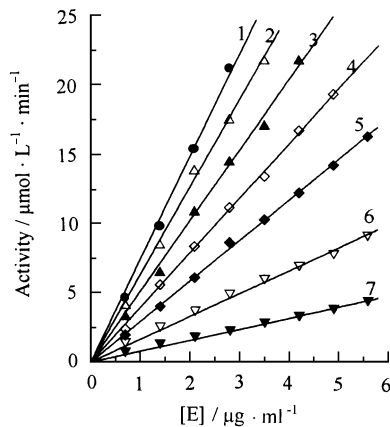

Fig. 3 Effects of *P. Vannamei* NAGase concentration of its activity for the hydrolysis of pNP-β-D-GlcNAc at different concentrations of DMSO

The concentrations of DMSO for curves 1—7 were 0, 0.35, 0.75, 1.05, 1.40, 2.80 and 4.20 mol/L, respectively. Assay conditions were the same as Fig. 1

2.3   Kinetics of the substrate reaction in the presence of different concentration of DMSO

The temporal variation of the product concentration during the substrate hydrolysis in the presence of different DMSO concentrations was shown in Fig. 4A. At each concentration of DMSO, the rate decreases with increasing time until a straight line is approached, the slope of which decreases with increasing DMSO concentration. The results suggest that denatured NAGase still had partial residue activity (curves 1—5). According to Eq (5), plots of $\ln([P]_{cal} - [P]_t)$ versus t give a series of straight lines were shown in Fig. 4B. From the slopes of the straight line, the apparent forward rate constant of inactivation, A, can be obtained.

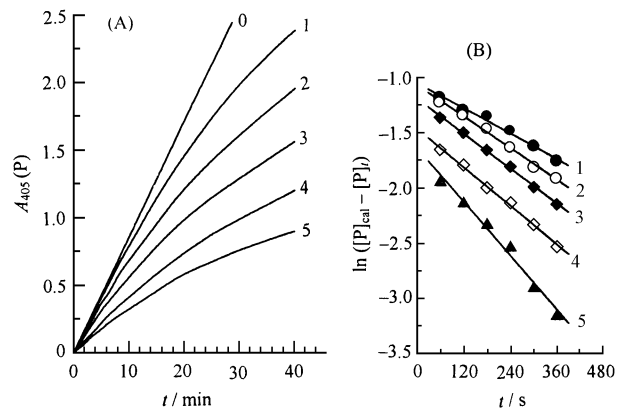## 2.4   Kinetics of the reaction at different substrate

Fig. 4   Course of substrate reaction in the presence of different concentrations of DMSO

The assay conditions were the same as Fig. 1 with exception of 0.5 mmol/L pNP-β-D-GlcNAc. (A) Substrate reaction course. The final DMSO concentrations for curves 0—5 were 0, 0.35, 0.7, 1.05, 1.40 and 1.75 mol/L, respectively. (B) Semilogarithmic plots of $\ln([P]_{cal} - [P]_t)$ against time. Data were taken from curves 1—5 in (A)

concentrations in the presence of DMSO

For the substrate reaction in the presence of DMSO, when the time is sufficiently large, a straight line is approached at each concentration of substrate. Both the initial rate and the slope of the asymptote increase with increasing substrate concentration. Fig. 5A shows the kinetic course of the hydrolysis reaction at different substrate concentrations in the presence of 1.05 mol/L DMSO. Similarly, plots of $\ln([P]_{cal} - [P]_t)$ versus t give a series of straight lines at different concentrations of substrate, whose slopes are equal to the apparent forward
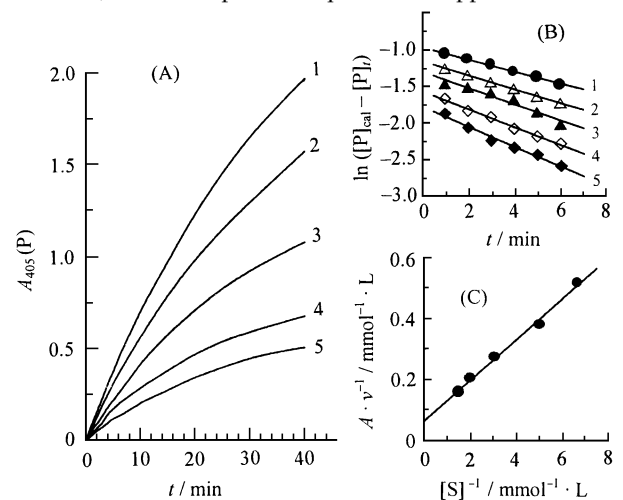

Fig. 5   Determination of the inactivation rate constants of the enzyme in 1.05 mol/L of DMSO solution

(A) Substrate reaction courses of the enzyme in the presence of 1.05 mol/L DMSO

Curves 1—5 are progress curves with 0.75, 0.5, 0.33, 0.2 and 0.15 mmol/L of substrate, respectively. Conditions were the same as Fig. 1.

(B) Semilogarithmic plot of $\ln([P]_{cal} - [P]_t)$ against time for data given in (A).

(C) Plot of A/v versus $1/[S]$. The A were obtained from the slopes of the straight lines in (B)

rate constant A (Fig. 5B). From Eq (3), a plot of $[P]_{calc}$ against time t gives a straight line with a slope of $\frac{v \cdot k_{-0}}{A}$. From the slope of the straight line, k_{-0} can be obtained. According to Eq (6), a plot of A/v versus $1/[S]$ gives a straight line with $\frac{K_m}{V_m}(k_{+0}+k_{-0})$ and $\frac{k'_{+0}+k_{-0}}{V_m}$ as the slope and intercept, respectively. Since K_m and V_m are quantities known, the values of k_{+0} and k'_{+0} can be obtained from the slope and the intercept of the straight line in Fig. 5C, respectively. The results obtained are listed in Table 1. Similarly, the inactivation rate constants of the enzyme at other DMSO concentrations were also obtained and listed in Table 1 for comparison.

Table 1　Microscopic rate constants of the inactivation of *P. vannamei* NAGase in DMSO solutions

$c(DMSO)$ /mol·L^{-1}	Rate constants/10^{-3}·s^{-1}			Residual activity %
	k_{+0}	k_{-0}	k'_{+0}	
0				100.00
0.35	2.078±0.08	0.450 1±0.010	0.102 5±0.002	88.06±0.50
0.70	2.357±0.05	0.391 7±0.005	0.255 0±0.004	68.99±0.80
1.05	2.817±0.04	0.340 1±0.004	0.468 5±0.005	56.74±0.50
1.40	3.057±0.07	0.300 5±0.006	0.539 7±0.008	43.50±0.50
2.00	NM*	NM*	NM*	28.36±0.60
2.50	NM*	NM*	NM*	20.01±0.44

NM* : Not measured

3　Discussion

　　Pacific white shrimp (*P. vannamei*) NAGase catalyzes the hydrolysis of *N*-acetyl-β-*D*-glucosamine residues from the terminal nonreducing ends of chitooligosaccharides. Due to the confining exoskeleton, pacific white shrimp must undergo discontinuous growth, expanding their body dimensions only after the shedding of the exoskeleton. In the shrimp cultivation, sometimes the shrimp will got sick and can not exuviate and will die in a large amount without treatment in time. Some medicines are organic compounds and not dissolved in water, so they can not be absorbed effectively. Owing to an amphipathic molecule, DMSO can work as a vehicle, contributing to the absorption of the medicine. In order to use DMSO as a solvent for some organic compounds, the effects of DMSO on NAGase must be first understood.

　　In our investigation, we found that DMSO had inhibitory effect upon the activity of *P. vannamei* NAGase. The enzyme activity decreased exponentially with the DMSO concentration increasing. When the concentration of DMSO reached 1.2 mol/L, the enzyme activity lost 50%. In order to know well the influence of DMSO on the enzyme, we used the substrate-reaction-kinetic method described by Tsou to analyze the inhibitory kinetics of the enzyme in DMSO solution. The results showed that the enzyme was inactivated by DMSO reversibly. The presence of DMSO did not bring down the

amount of the efficient enzyme, but just resulted in the descending of the activity of the enzyme. The inactivation rate constants were determined and the results were listed in Table 1. The values of k_{+0} and k'_{+0} increased apparently with increasing the DMSO concentration while the value of k_{-0} decreased, illuminating the reversible intensity of enzyme was weakened with increasing the DMSO concentration. That the value of k_{+0} was much larger than k'_{+0} was much larger than k'_{+0} indicates the free enzyme molecule was more fragile than the enzyme-substrate complex in the DMSO solution.

　　Because DMSO is an amphipathic molecule with a polar domain and two apolar groups, it may interfere with the microenvironment of some amino acid residues and weaken the non-covalent interaction of the enzyme molecular. This process will causes the incompactness of the enzyme in a certain degree, which may lead the activity decrease. When the concentration of DMSO was lower than 0.24 mol/L, the enzyme activity inhibited lightly and just lost by 5%. Within this range, the use of DMSO will be acceptable for solvent the organic compound.

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