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ARTICLE

Inhibitory Kinetics of Betaine on β-*N*-Acetyl-D-glucosaminidase from Prawn (*Litopenaeus vannamei*)

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The effects of betaine on prawn β-*N*-acetyl-D-glucosaminidase (NAGase) activity for the hydrolysis of *p*-nitrophenyl-*N*-acetyl- β-D-glucosaminide (pNP-NAG) have been studied. The results showed that appropriate concentrations of betaine could lead to reversible inhibition against NAGase, and the IC_{50} value was estimated to be 15.00 ± 0.30 mM. The inhibitory kinetics assay showed that betaine was a mixed type inhibitor with a K_I value of 9.17 ± 0.85 mM and a K_{IS} value of 45.58 ± 2.52 mM. The inhibitory model was set, and the microscopic rate constants were determined using the kinetic method of the substrate reaction. The time course of the hydrolysis of pNP-NAG catalyzed by NAGase in the presence of different betaine concentrations showed that at each betaine concentration, the rate decreased with an increase in time until a straight line was approached, indicating that the inhibition of NAGase by betaine is a slow, reversible reaction with fractional residual activity. The fact that k_{+0} is much larger than k_{+0}' indicated that the free enzyme molecule is more fragile than the enzyme−substrate complex against betaine. It is suggested that the presence of the substrate offers marked protection of NAGase against inhibition by betaine.

KEYWORDS: ***Litopenaeus vannamei*; β-*N*-acetyl-D-glucosaminidase; inhibition kinetics; betaine**

INTRODUCTION

β-*N*-Acetyl-D-glucosaminidase (NAGase, EC3.2.1.52), one of the chitinases that cooperates with chitinases to catalyze the cleavage of *N*-acetylglucosamine (NAG) polymers into NAG, is widely distributed in animal tissues, in plants, and in microorganisms (*1*−*3*). NAGase in crustaceans plays an important role in molting and digestion of chitinous foods. The purification, concentrations in different growth stages, and distribution in different organs of NAGases from Antarctic krill (*4*, *5*), lobster (*Homarus americanus*) (*6*), fiddler crab (*Uca pugilator*) (*7*), and Northern shrimp (*Pandalus borealis*) (*8*) have been reported. NAGase from Antarctic krill exists in two isoenzymes that are involved in digestion and molting processes, respectively. Their simultaneous occurrence may indicate a physiological adaptation that utilizes a mechanism of altering isoenzyme concentrations (*4*, *5*). Prawn (*Litopenaeus vannamei*) is one of the most popular farmed prawns in the world. At present, systematical studies of NAGase from *L. vannamei* are taking place in our laboratory (*9*−*12*).

Betaine (chemical name *N*,*N*,*N*-trimethylglycine) is a kind of methylated amino acid with a carboxylate group and quaternary ammonium group. The compound is produced by a wide variety of organisms (bacteria, plants, invertebrates, and mammals). It was first isolated from the sap of sugar beet, *Beta vulgaris*, hence, the name betaine (*13*). It has a dual role as an osmolyte and as a methyl group donor in the living body (*14*). Hence, betaine has been well-studied in both its purification from natural resources or chemical preparations and its variety of applications in feed additives, the medicine industry, agriculture, etc. At present, betaine is considered a flavor additive in the diets or rearing water of crustaceans, and it can promote the growth and survival of crustaceans (*15*, *16*). However, investigations of the effects of betaine on the NAGase activity of crustaceans are rare. So, our laboratory has been interested in this problem. In our continuous investigation, betaine was found to have a strong inhibitory effect on the enzyme activity. The aim of this paper is, therefore, to carry out the mechanism and kinetics study on the inhibition of NAGase by betaine using Lineweaver−Burk plots and the substrate reaction kinetics method described by Tsou (*17*). The studies have revealed some interesting and positive evidence, which will significantly enhance the knowledge of the effect of betaine on crustacean animals.

MATERIALS AND METHODS

Materials. NAGase was prepared from prawn (*L. vannamei*) as described previously (*9*). The specific activity of the purified NAGase was 1560 U/mg. *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNP-NAG) was purchased from the Biochemistry Lab of Shanghai Medicine Industry Academy (China). Betaine hydrochloride and all other reagents were local products of analytical grade. The water used was redistilled and ion-free.

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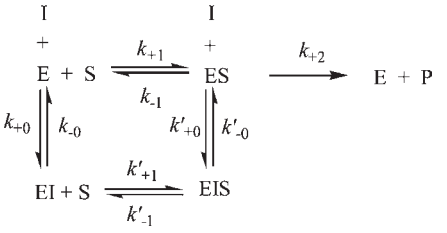
Assay of the Concentration and Enzymatic Activity. The NAGase concentration was measured by the method of Lowry et al. (*18*). The NAGase activity was determined at 37 °C by following the increase in absorbance at 405 nm that accompanied the hydrolysis of the substrate (pNP-NAG) (*9*). A 10 μL portion of NAGase solutions was added to the reaction media (2.0 mL) containing 0.25 mM pNP-NAG in 0.1 M sodium phosphate buffer (pH 6.2). Absorption was carried out using a Beckman UV-650 spectrophotometer. The molar absorption coefficient of product (pNP) was determined to be 5.22×10^3 (M^{-1} cm^{-1}) at pH 6.2.

Inhibition Rate Constants of NAGase by Betaine. The progress of substrate reaction theory previously described (*17*) was applied to the current study of the inhibition kinetics of prawn NAGase by betaine. In this method, 10 μL of enzyme was added to 2.0 mL of assay system containing different concentrations of substrate in 0.1 M sodium phosphate buffer (pH 6.2) with different concentrations of betaine. 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 betaine concentrations showed that, at each concentration of betaine, the rate decreased with increasing time until a straight line was approached. The results showed that the inhibition reaction was a reversible reaction with fractional residual activity. This can be written as (*19*)



where E, S, I, and P denote enzyme, substrate, betaine, and product, respectively; ES, EI, and EIS are the respective compounds. $[E_0]$ represents initial enzyme concentration. As is usual, for the case $[S] \gg [E_0]$ and $[I] \gg [E_0]$, the product formation can be written as (*19*)

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

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

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

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 for the forward and reverse reactions of inhibition, respectively; [S] and [I] are the concentrations of the substrate and betaine, respectively; v is the initial rate of reaction in the absence of betaine; and $v = (V_m[S])/(K_m + [S])$. When t is sufficiently large, the curves become straight lines, and the product concentration is written as $[P]_{calcd}$:

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

Combining eqs **1** and **4** yields

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

where $[P]_{calcd}$ is the product concentration to be expected from the straight-line portions of the curves as calculated from eq **4** and

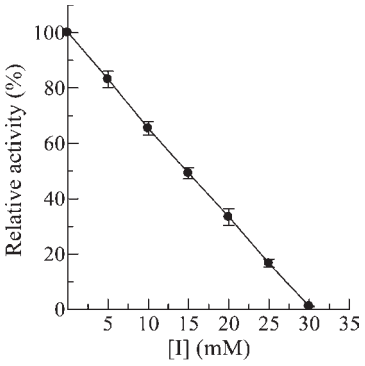


Figure 1. Effect of betaine on the activity of NAGase for the hydrolysis of pNP-NAG. Conditions were a 2 mL system containing 0.1 M sodium phosphate buffer (pH 6.2) and 0.25 mM substrate at 37 °C. The enzyme final concentration was 0.01 μM.

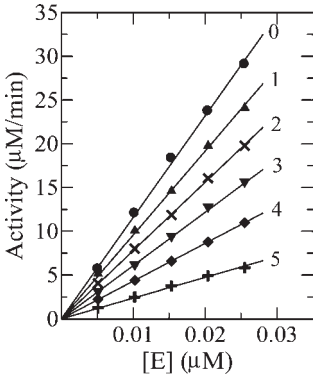


Figure 2. Effects of NAGase concentration on its activity for the hydrolysis of pNP-NAG at different betaine concentrations. The concentrations of betaine for curves 0−5 were 0, 5, 10, 15, 20, and 25 mM, respectively. Assay conditions were the same as **Figure 1**.

$[P]_t$ is the product concentration actually observed at time t. Plots of $\ln([P]_{calcd} - [P]_t)$ versus reaction time (t) give a series of straight lines at different concentrations of betaine 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.

Combining eqs **2** and $v = (V_m[S])/(K_m + [S])$, we can get

$$\frac{A}{v} = \frac{k_{+0}K_m}{V_m}\frac{1}{[S]} + \frac{k'_{+0}}{V_m} \quad (6)$$

A plot of A/v versus $1/[S]$ gives a straight 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. As K_m and V_m are known quantities from the measurements of the substrate reaction in the absence of the inhibitor at different substrate concentrations, the rate constants k_{+0} and k_{+0}' can be obtained from the slope and intercept of the straight line.

RESULTS

Effect of Betaine on the Activity of Prawn NAGase. The effect of betaine on the hydrolysis of pNP-NAG by prawn NAGase was first studied. The relationship between the residual enzyme activity and the concentrations of betaine is shown in **Figure 1**. The effect of betaine on NAGase activity was concentration dependent. The residual enzyme activity rapidly decreased with increasing concentrations of betaine. The IC_{50} value, the betaine concentration leading to 50% activity lost, was estimated to be 15.00 ± 0.30 mM. The inhibition mechanism of betaine on

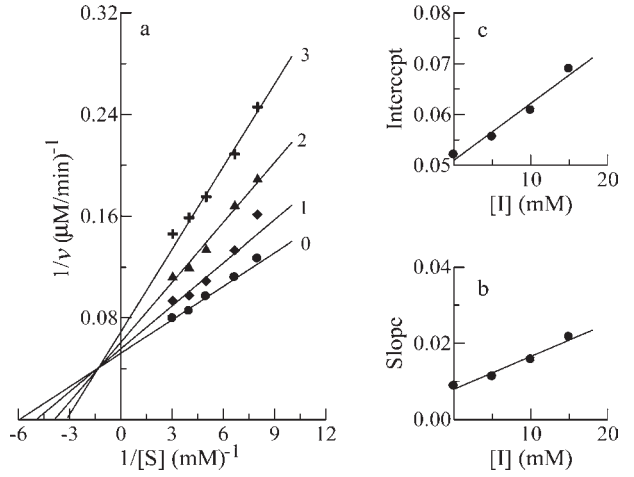


Figure 3. Inhibitory type of betaine for NAGase. Assay conditions were the same as **Figure 1** except that the substrate concentration was variable. (**a**) Lineweaver−Burk plots for the inhibition of betaine. The concentrations of betaine for curves 0−3 were 0, 5, 10, and 15 mM, respectively. (**b**) Plot of the slopes of the lines in panel **a** versus concentrations of betaine. (**c**) Plot of the intercepts of the lines in panel **a** versus concentrations of betaine.

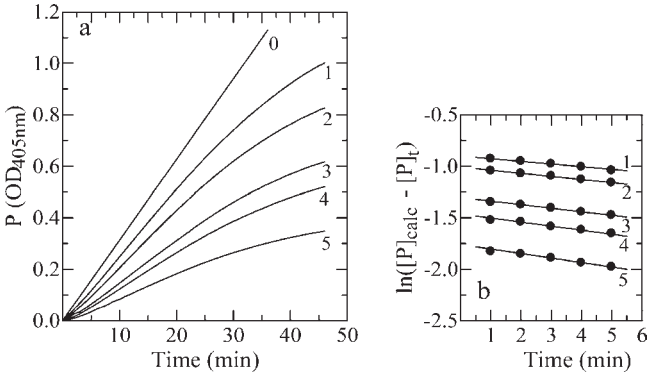


Figure 4. Course of inhibition of NAGase in different concentrations of betaine. The enzyme final concentration was 0.005 μM. The assay conditions were the same as **Figure 1** except that the concentration of pNP-NAG was 0.33 mM. (**a**) Substrate reaction course. The final betaine concentrations for curves 0−5 were 0, 5, 10, 15, 20, and 25 mM, respectively. (**b**) Semilogarithmic plots of $\ln([P]_{calcd} - [P]_t)$ against time. Data were taken from curves 1−5 in panel **a**.

NAGase for the hydrolysis of pNP-NAG was studied. **Figure 2** showed the relationship of enzymatic activity with its concentration in the presence of different concentrations of betaine. The plots of the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations of betaine gave a family of straight lines, which all passed through the origin. Increasing the betaine concentration resulted in a descending line slope, indicating that the inhibition of betaine on NAGase was a reversible reaction course. The presence of betaine did not bring down the amount of the efficient enzyme but just resulted in the inhibition and the descending activity of the enzyme.

Determination of the Inhibition Type of Betaine on NAGase. In the presence of betaine, the inhibition kinetics of NAGase is shown in **Figure 3a** as Lineweaver−Burk plots. The results showed that the inhibition of betaine on NAGase was a mixed type since an increase in the betaine concentration resulted in a family of lines with different slopes and intercepts, and they intersected one another in the second quadrant. This observed behavior indicated that the activities of both the free enzyme and

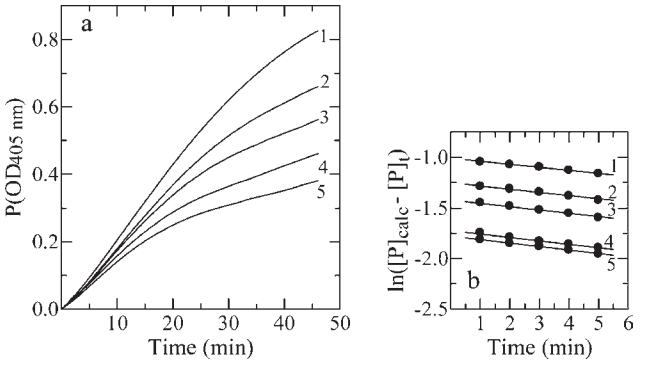


Figure 5. Course of inhibition at different substrate concentrations in the presence of 10 mM betaine. (**a**) Curves 1−5 are progress curves with 0.33, 0.25, 0.2, 0.15, and 0.125 mM pNP-NAG, respectively. (**b**) Semilogrithmic plot of $\ln([P]_{calcd} - [P]_t)$ against time. Data were taken from curves 1−5 in panel **a**.

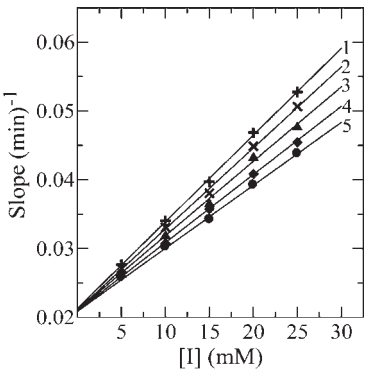


Figure 6. Secondary plots of the slopes of the semilogarithmic plots versus betaine concentrations for a series of fixed substrate concentrations. The data for curve 5 are from **Figure 4b** (at 0.33 mM substrate). Substrate concentrations for curves 1−5 were 0.125, 0.15, 0.20, 0.25, and 0.33 mM, respectively.

the enzyme−substrate complex were inhibited by betaine. The equilibrium constant for the inhibitor binding with free enzyme, K_I, can be obtained from the plot of the slope from **Figure 3a** versus the betaine hydrochloride concentration, as shown in **Figure 3b**. The obtained inhibition constant (K_I) for betaine was 9.17 ± 0.85 mM. The equilibrium constant for the inhibitor binding with enzyme−substrate complex, K_{IS}, can be obtained from the plot of the intercept from **Figure 3a** versus the betaine concentration, as shown in **Figure 3c**. The obtained inhibition constant (K_{IS}) for betaine was 45.58 ± 2.52 mM.

Kinetics of the Substrate Reaction in the Presence of Different Concentrations of Betaine. The temporal variation of the product concentration during substrate hydrolysis in the presence of different betaine concentrations is shown in **Figure 4a**. At each concentration of betaine, the rate decreased with an increase in time until a straight line was approached, the slope of which decreased with an increase in the betaine concentration. The results suggested that inhibited NAGase still had a partial residue activity (curves 1−5). The above results, as analyzed by Tsou's method (*17*), suggested that the formation of the inhibited EI or EIS complex was a slow and reversible reaction. According to eq **5**, plots of $\ln([P]_{calcd} - [P]_t)$ versus t give a series of straight lines with slopes of $-(A[I] + B)$ and are shown in **Figure 4b**.

Kinetics of the Reaction at Different Substrate Concentrations in the Presence of Betaine. Figure 5a shows the kinetic courses of the reaction at different substrate concentrations in the presence of

Table 1. Inhibitory Equilibrium Constants and Microscopic Rate Constants for NAGase by Betaine Hydrochloride

enzyme forms	rate constants		inhibitory equilibrium constants	
native enzyme	k_{+0}	0.31×10^{-4} mM^{-1} s^{-1}	K_I	9.17 ± 0.85 mM
	k_{-0}	0.35×10^{-3} s^{-1}		
enzyme−substrate complex	k_{+0}'	0.72×10^{-5} mM^{-1} s^{-1}	K_{IS}	45.58 ± 2.52 mM
	k_{-0}'	0.35×10^{-3} s^{-1}		

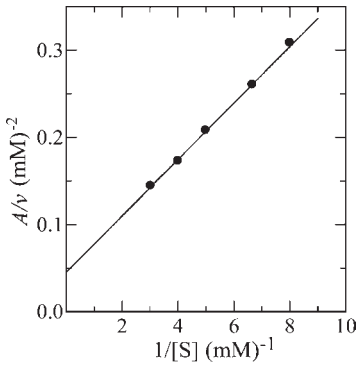


Figure 7. Plot of A/v versus 1/[S].

10 mM betaine. It can be seen from **Figure 5a** that when t is sufficiently large, the curves become approximately straight lines. Both the initial rate and the slope of the asymptote increase with an increase in 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 $-(A[I] + B)$ as shown in **Figure 5b**.

Determination of the Microscopic Rate Constants of Betaine on NAGase. A plot of the slopes of the straight lines in **Figure 4b** versus betaine concentration [I] gives the straight line, as line 5 in **Figure 6**. Similarly, data collected for other substrate concentrations give the other straight lines in **Figure 6**. All of the straight lines have a common intercept on the ordinate, indicating that k_{-0} and k_{-0}' have the same values. The apparent reverse rate constant B can be obtained from the ordinate intercept. The value of B is then equal to the microscopic rate constant k_{-0} and k_{-0}' given in **Table 1**. From eq **6**, a plot of A/v versus 1/[S] gives a straight line, **Figure 7**, where the slope and the intercept of the straight line give the value of $k_{+0}K_m/V_m$ and k_{+0}'/V_m, which were used to determine the inhibition rate constants k_{+0} and k_{+0}'. Because K_m and V_{max} are quantities known from measurements of the substrate reaction in the absence of betaine, K_m is equal to 0.169 mM and V_m is equal to 9.610 μM/min, these rate constants are also shown in **Table 1**.

DISCUSSION

Because the structure of betaine contains three methyl groups, betaine is presumed to work as a methyl donor and to play an important role in homocysteine metabolism. It provides homocysteine with a methyl group for remethylation to methionine and reduces the intravital homocysteine level (*20*). Betaine is also known to accumulate in the cytoplasm as an osmolyte and may have a stabilizing and protective action on proteins, enzymes, and membranes under unfavorable and stressful conditions including extreme temperature, high salinity, and low water content (*21*, *22*).

Betaine entices some aquatic animals to eat, and absorbed betaine accumulates in muscle cells, may be beneficial in fish or crustaceans exposed to changing salinities, and is an important component in the sulfur amino acid catabolic pathway (*23*). Betaine is a flavor additive in diets fed to some species of fish and crustaceans, either alone or in combination with other amino acids (*23*, *24*).

In our studies, the results in **Figure 1** show that the NAGase activity can be strongly inhibited by betaine hydrochloride, and the IC_{50} value is estimated to be 15.00 ± 0.30 mM. At a sufficiently large concentration of betaine, NAGase can be completely inactivated. On the other hand, when betaine is at low concentrations, a slow and reversible reaction is observed. In this paper, we discuss the inhibition mechanism and the inhibition kinetics of prawn NAGase at lower betaine concentrations. Using Lineweaver−Burk plots, the inhibition of NAGase by betaine is a mixed type; the inhibitory equilibrium constant for betaine binding with free enzyme, K_I, is 9.17 ± 0.85 mM; and the inhibitory equilibrium constant for betaine binding with enzyme−substrate complex, K_{IS}, is 45.58 ± 2.52 mM. The results indicate that the free enzyme is more easily inhibited by betaine than the enzyme−substrate complex. The inhibition kinetics of NAGase by betaine has been studied using the kinetic method of the substrate reaction, and the microscopic rate constants have been determined and are listed in **Table 1**. The results show that the forward inactivation rate constant of the free enzyme (k_{+0}), which is 0.31×10^{-4} mM^{-1} s^{-1}, is about four times as much as that of the enzyme−substrate complex (k_{+0}'), which is 0.72×10^{-5} mM^{-1} s^{-1}, indicating that the free enzyme molecule is more fragile than the enzyme−substrate complex by betaine.

ABBREVIATIONS USED

NAGase, β-*N*-acetyl-D-glucosaminidase; *p*NP-NAG, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide; NAG, *N*-acetylglucosamine; pNP, *p*-nitrophenol; IC_{50}, the inhibitor concentration leading to 50% of enzyme activity lost.

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