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Inactivation Kinetics of β-N-Acetyl-D-glucosaminidase from Prawn (*Penaeus vannamei*) in Dioxane Solution

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Abstract—β-N-Acetyl-D-glucosaminidase (NAGase, EC 3.2.1.52) catalyzes the cleavage of N-acetylglucosamine polymers. It is in the composition of the chitinases and cooperates with endo-chitinase and exo-chitinase to disintegrate chitin into N-acetylglucosamine. In this work, the effects of dioxane on the enzyme activity for the hydrolysis of *p*-nitrophenyl-N-acetyl-β-D-glucosaminide from the prawn (*Penaeus vannamei*) have been studied. The results show that appropriate concentrations of dioxane can lead to reversible inactivation of the enzyme, and the IC_{50} is estimated to be 1.1 M. The kinetics of inactivation of NAGase in the appropriate concentrations of dioxane solution has been studied using the kinetic method of the substrate reaction. The rate constants of inactivation have been determined. The results show that the free enzyme molecule is more fragile than the enzyme–substrate complex in the dioxane solution. It is suggested that the presence of the substrate offers marked protection of this enzyme against inactivation by dioxane.

Key words: β-N-acetyl-D-glucosaminidase, *Penaeus vannamei*, dioxane, inactivation kinetics

The β-N-acetyl-D-glucosaminidase (NAGase, EC 3.2.1.52) widely distributed in microorganisms, animals, and plants disassembles the dimer or trimer of NAG into monomer. It is a component of chitinase and cooperates with exo-chitinase and endo-chitinase to disintegrate chitin. The NAGases from microorganisms and animals have been well documented for their important roles in defense systems against parasites, molting, and digestion of chitinous foods [1-4]. The NAGases from the crustaceans such as Antarctic krill (*Euphasia superba*) [5, 6], lobster (*Homarus americanus*) [7], fiddler crab (*Uca pugilator*) [8], and Northern shrimp (*Pandalus borealis*) [9] have also been reported about their purification, concentrations in different growth stage, and distribution in different organs. NAGase from Antarctic krill exists as two isoenzymes, which are involved in digestion and molting processes, respectively [5, 6]. Their simultaneous occurrence may indicate a physiological adaptation utilizing a mechanism of altering isoenzyme concentrations. The investigation of enzymatic properties is essential to the study of physiological adaptations expressed by organism in response to different environmental conditions. However, the NAGase from prawn (*Penaeus vannamei*) is poorly known. In our previous studies, we purified a NAGase from prawn and discussed the enzymatic characterization and the effects of metal ions on the enzyme activity.

The natural environment of most proteins is a complex system containing all varieties of organic compounds such as sugar, fat, organic acid, alcoholate, and so on. Meanwhile, because of pollution of the breeding aquatic environment, such as a shift in acidity and alkalinity and heavy metal ions or organic solvents, the enzyme activity and its conformation can be affected, and the growth and survival of the animal is threatened.

So it is very important to research the influence of organic solvents on the enzyme activity and the enzyme performance change in organic solvents. At present, the effect of organic solvents on NAGase is little reported. In our investigation, we found that the prawn NAGase activity could be affected by dioxane, and the inactivation of the enzyme in dioxane solutions was shown to be reversible. The aim of the current work was, therefore, to carry out a kinetics study on the inactivation of NAGase in dioxane solvent. It has significance in the inspection of the pollution of the breeding aquatic environment by utilizing high sensitivity of the enzyme to organic solvents.

Abbreviations: NAGase) β-N-acetyl-D-glucosaminidase; pNP-β-D-GlcNAc) *p*-nitrophenyl-N-acetyl-β-D-glucosaminide; NaAc-HAc) sodium acetate/acetate buffer.

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MATERIALS AND METHODS

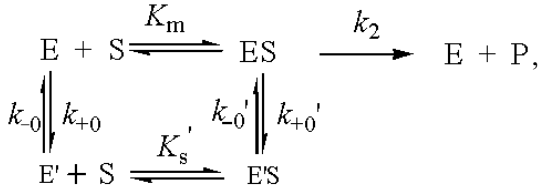
Reagents. *p*-Nitrophenyl-N-acetyl-β-D-glucosaminide (pNP-β-D-GlcNAc) was purchased from the Biochemistry Laboratory of the Shanghai Medicine Industry Academy (China). Sephadex G-100 was a Pharmacia (Sweden) product. DEAE-cellulose (DE-32) was from Whatman (UK). The chitin was from the Third Ocean Academy of China. Dioxane was purchased from Sigma (USA). All other reagents were of analytical grade. The water used was redistilled and ion-free.

Enzyme preparation. The NAGase was prepared from prawn (*Penaeus vannamei*) according to the method of Koga et al. [1] to the step of ammonium sulfate fractionation. The crude preparation was further chromatographed on a Sephadex G-100 column following on a DEAE-cellulose column. The final preparation was homogeneous on PAGE and SDS-PAGE. The specific activity of the purified enzyme was 1560 U/mg. The protein concentration was measured by the method of Lowry et al. [10].

Enzyme activity was determined at 37°C by following the increasing absorbance at 405 nm accompanying the hydrolysis of the substrate pNP-β-D-GlcNAc [11] with the molar absorption coefficient of $1.73 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [12]. A portion of 10 μl of enzyme solution was added to 2 ml of reaction system containing 0.15 M NaAc-HAc buffer (pH 5.2) and 0.5 mM pNP-β-D-GlcNAc. After reaction for 10 min at 37°C, 2 ml of 0.5 M NaOH was added into the reaction mixture to stop the reaction. Absorption and kinetic measurements were carried out using a Beckman UV-650 spectrophotometer.

Fluorescence spectra were measured with a Hitachi 850 (Japan) spectrophotometer. NAGase (45 μg) was dissolved in 1.0 ml of 0.15 M NaAc-HAc buffer (pH 5.2) with different concentrations of dioxane and preincubated at 37°C for 2 min before fluorescence spectra measurements with an excitation wavelength of 278.2 nm.

The progress-of-substrate-reaction method as previously described [12-16] was used to study the inactivation kinetics of NAGase in dioxane solutions. With this method, 10 μl of NAGase (0.156 mg/ml) is added to 2.0 ml of assay system containing different concentrations of substrate in 0.1 M NaAc-HAc buffer (pH 5.2) with different concentration of dioxane. The substrate reaction progress curve was analyzed to obtain the reaction rate constants. The reaction was carried out at a constant temperature of 37°C. The substrate reaction progress curve was analyzed to obtain the rate constants as detailed below. The time course of the hydrolysis of the substrate in the presence of different dioxane 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 residual activity. This can be written as following scheme [17]:



where S, P, E, and E′ denote substrate, product, and the native and inactivated enzyme, respectively; ES is the native enzyme–substrate complex; and E′S is the inactivated enzyme–substrate complex. k_{+0} and k_{-0} are rate constants for forward and reverse inactivation of free enzyme, respectively; k'_{+0} and k'_{-0} are forward and reverse inactivation rate constants of enzyme–substrate complex. As is usual for the case [S] >> [E_0], the product formation can be written as:

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

with

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

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

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; B is the apparent reverse rate constant of inactivation; [S] is the concentration of the substrate; and v is the initial rate of reaction in the absence of denaturant (dioxane), where

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}.$$

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 time t. Plots of $\ln([P]_{calc} - [P]_t)$ versus t give a series of straight lines at different concentrations of denaturant with slopes of $-(A + B)$. The apparent rate constant $(A + B)$ can be obtained from such graphs. From Eq. (4), a plot of $[P]_{calc}$ against time, t, gives a straight line with a slope of $B \cdot v/(A + B)$. According to the values of $B \cdot v/(A + B)$ and $(A + B)$ gotten from the above plots, and

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

obtained from experiment of the substrate reaction in the absence of dioxane at different substrate concentrations, A and B can be obtained.

Combining Eq. (2) and the Michaelis–Menten equation gives:

$$\frac{A}{v} = \frac{K_m}{V_{max}} k_{+0} \frac{1}{[S]} + \frac{k'_{+0}}{V_{max}} . \qquad (7)$$

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

RESULTS

Determination of the kinetic parameters of NAGase from *Penaeus vannamei*. The kinetic behavior of NAGase in catalyzing the hydrolysis of pNP-β-D-GlcNAc was studied. Under the condition employed in the present investigation, the hydrolysis of pNP-β-D-GlcNAc by NAGase follows Michaelis–Menten kinetics. The kinetic parameters for NAGase obtained from a Lineweaver–Burk plot (Fig. 1) showed that K_m was equal to 0.254 mM and V_{max} was equal to 9.438 μM/min.

Effect of dioxane on NAGase activity for the hydrolysis of pNP-β-D-GlcNAc. The effect of dioxane on the hydrolysis of pNP-β-D-GlcNAc by NAGase was first studied. The relationship between residual enzyme activity and the concentrations of dioxane is shown in Fig. 2. The effect of dioxane on NAGase was concentration dependent. As the concentrations of dioxane increased, the residual enzyme activity rapidly decreased. The dioxane concentration leading to 50% activity lost (IC_{50}) was estimated to be 1.1 M. The inactivation mechanism of the enzyme in dioxane solution for the hydrolysis of pNP-β-D-GlcNAc was studied. Figure 3 shows the relationship of enzyme activity with its concentration in the presence of different concentrations of dioxane. The plots of the remaining enzyme activity versus the con-

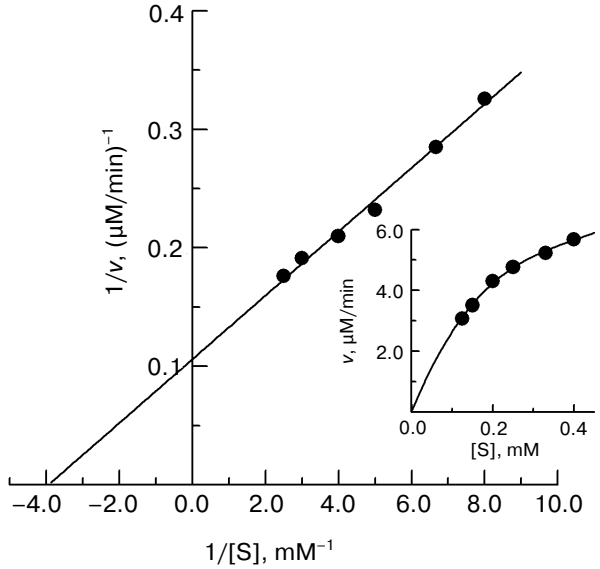


Fig. 1. Lineweaver–Burk plot for the determination of K_m and V_{max} for NAGase on the hydrolysis of pNP-β-D-GlcNAc. Conditions were: 2 ml system containing 0.15 M NaAc-HAc buffer (pH 5.2) and different concentrations of pNP-β-D-GlcNAc at 37°C. The final enzyme concentration was 0.78 μg/ml. The inset shows the relationship between the initial rate and the substrate concentration.

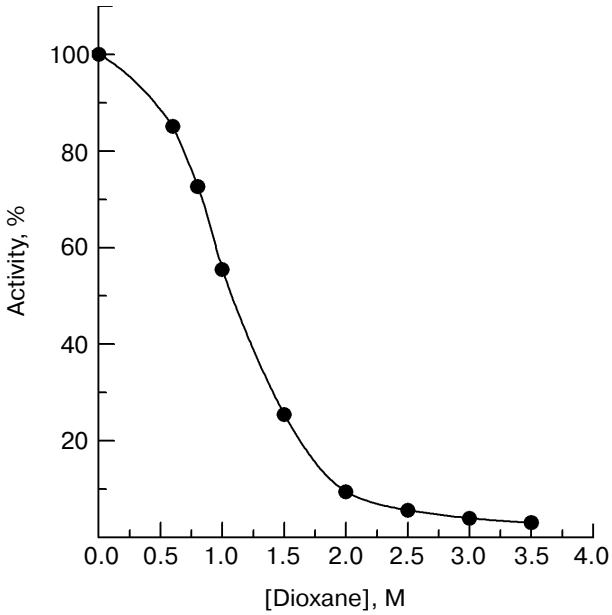


Fig. 2. Effect of dioxane on the activity of NAGase for the hydrolysis of pNP-β-D-GlcNAc. Assay conditions were as described for Fig. 1 except that the pNP-β-D-GlcNAc concentration was 0.5 mM with different concentrations of dioxane.

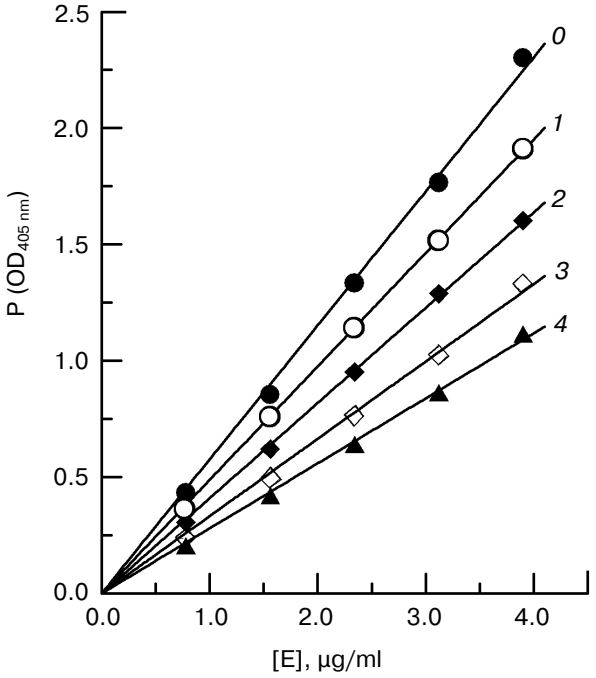


Fig. 3. Effects of NAGase concentration on its activity for the hydrolysis of pNP-β-D-GlcNAc at different concentrations of dioxane. The concentrations of dioxane for curves *0-4* were 0, 0.6, 0.8, 1.0, and 1.2 M, respectively. Assay conditions were the same as for Fig. 1.

centrations of enzyme in the presence of different concentrations of dioxane gave a family of straight lines, which all passed through the origin. Increasing the dioxane concentration resulted in a decrease in the slope of the line, indicating that the inactivation of the enzyme by dioxane was a reversible reaction. The presence of dioxane did not bring down the amount of the efficient enzyme, but just resulted in the inhibition and the decrease in the activity of the enzyme. Dioxane is a reversible inactivator of NAGase for hydrolysis of pNP-β-D-GlcNAc.

Measurement of forward inactivation rate constants of NAGase in dioxane solutions. The temporal variation of the product concentration during the substrate hydrolysis in the presence of different dioxane concentrations is shown in Fig. 4a. At each concentration of dioxane, the rate decreases with increasing time until a straight line is approached, the slope of which decreases with increasing dioxane concentration. The results suggest that 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. 4b. From the slopes of the straight line, the apparent rate constant of inactivation, $A + B$, can be obtained.

The kinetic course of the hydrolysis reaction at different substrate concentrations in the presence of 0.8 M dioxane is shown in Fig. 5a. In the presence of 0.8 M dioxane, when the time is sufficiently large, a straight line is

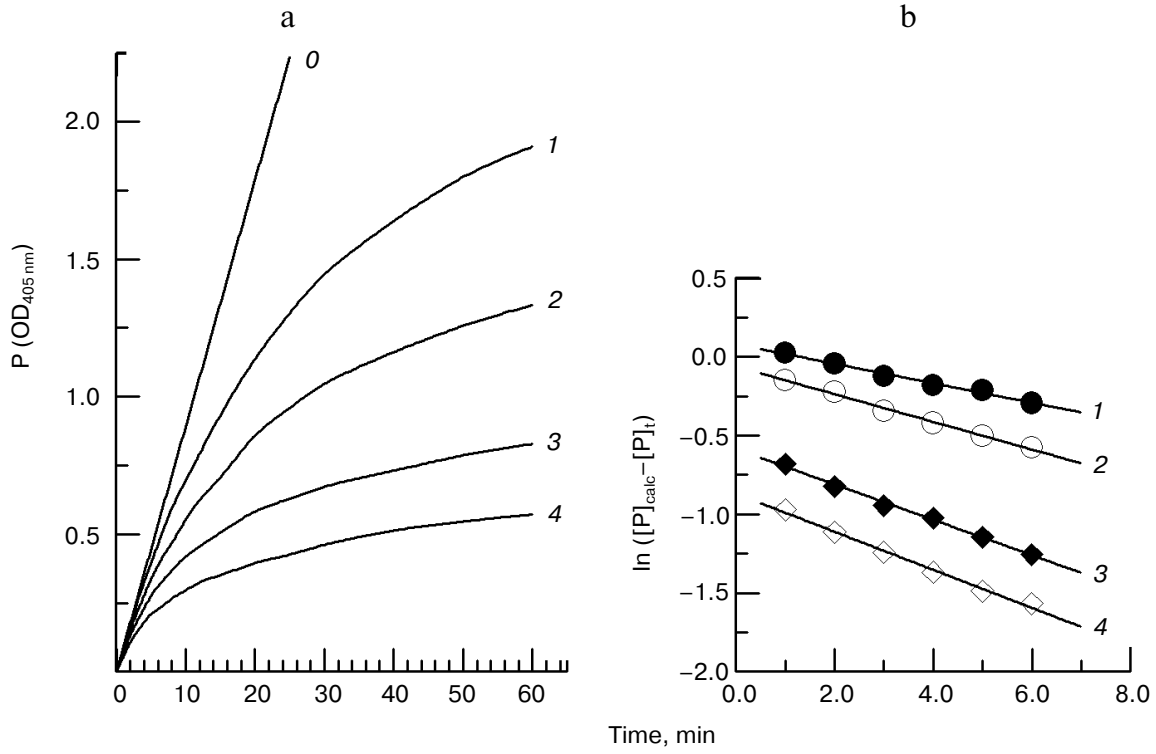


Fig. 4. Course of inactivation of NAGase on incubation in different concentrations of dioxane. The assay conditions were the same as for Fig. 1 with exception of 0.4 mM pNP-β-D-GlcNAc. a) Substrate reaction course. The final dioxane concentrations for curves *0-4* were 0, 0.6, 0.8, 1.0, and 1.2 M, respectively. b) Semilogarithmic plots of $\ln([P]_{calc} - [P]_t)$ against time. Data were taken from curves *1-4* in panel (a).

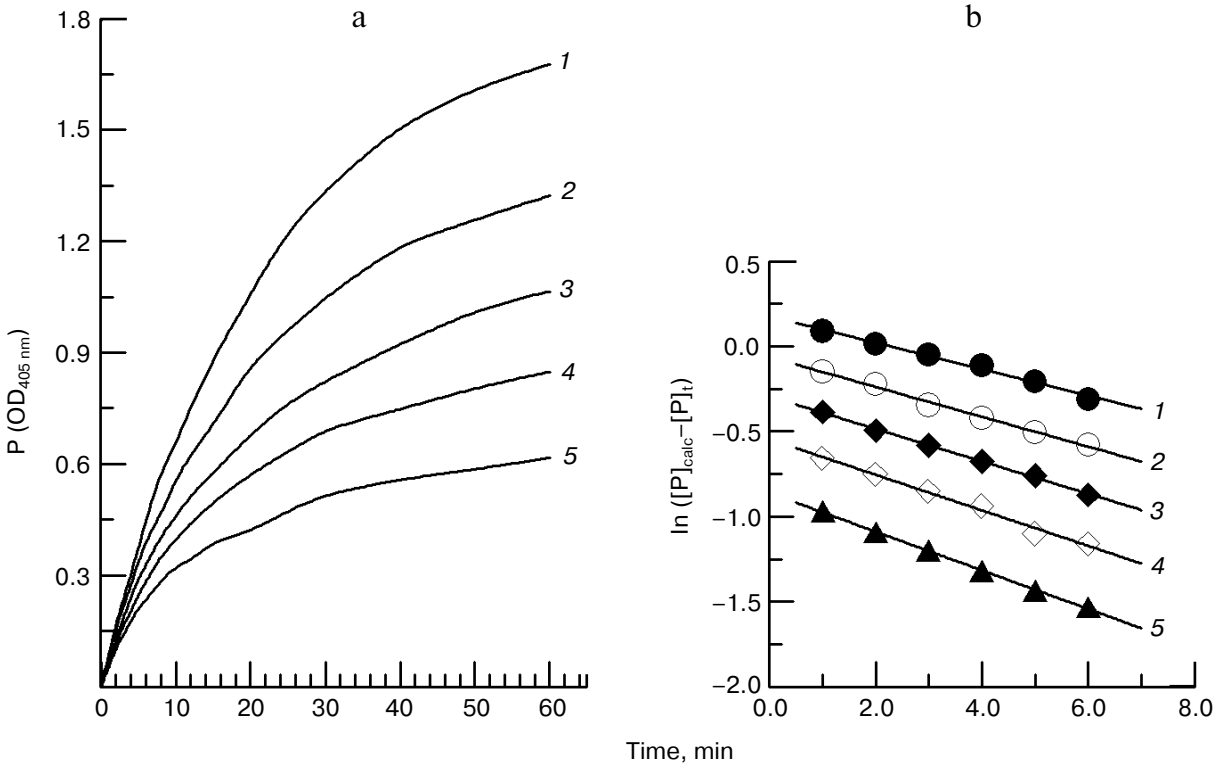


Fig. 5. Determination of the inactivation rate constants for NAGase in 0.8 M dioxane solution. a) Substrate reaction courses of the enzyme in the presence of 0.8 M dioxane. Curves *1-5* are progress curves with 0.5, 0.4, 0.33, 0.25, and 0.2 mM of substrate, respectively. The assay conditions were the same as for Fig. 1. b) Semilogarithmic plot of $\ln([P]_{calc} - [P]_t)$ against time. Data were taken from curves *1-5* in panel (a).

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, $Bv/(A + B)$, increase with increasing substrate concentration (Fig. 5a). 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. 5b). According to the values of $Bv/(A + B)$ and $(A + B)$ obtained from the above plots and v obtained from experiment of the substrate reaction in the absence of dioxane at different substrate concentrations, A and B can be calculated. Since K_m and V_{max} are quantities known from measurement of the substrate reaction in the absence of dioxane at different substrate concentrations, the values of k_{+0} and k'_{+0} can be obtained from the slope and the intercept of the straight line in Fig. 6, a plot of A/v versus $1/[S]$ according to Eq. (7). These results are shown in the table. Similarly, the inactivation forward rate constants of NAGase at other dioxane concentrations were also obtained (table).

Measurement of reverse rate constants of NAGase in dioxane solutions. According to B values as calculated

Microscopic rate constants of the inactivation of NAGase in dioxane solutions

Dioxane concentration, M	Rate constants, $k \times 10^3$, min^{-1}				Residual activity, %	Fluorescence intensity, %
	k_{+0}	k_{-0}	k'_{+0}	k'_{-0}		
0.0					100.00	100.00
0.6	121.49 ± 1.50	8.60 ± 0.40	10.67 ± 0.30	8.60 ± 0.40	85.06 ± 0.80	92.62 ± 1.50
0.8	184.28 ± 2.00	6.28 ± 0.30	15.74 ± 0.50	6.28 ± 0.30	72.59 ± 0.80	85.12 ± 1.00
1.0	252.51 ± 2.50	5.03 ± 0.25	19.75 ± 0.50	5.03 ± 0.25	55.41 ± 0.50	78.42 ± 1.20
1.2	281.62 ± 2.50	3.94 ± 0.25	25.37 ± 0.60	3.94 ± 0.25	42.50 ± 0.50	60.62 ± 0.60

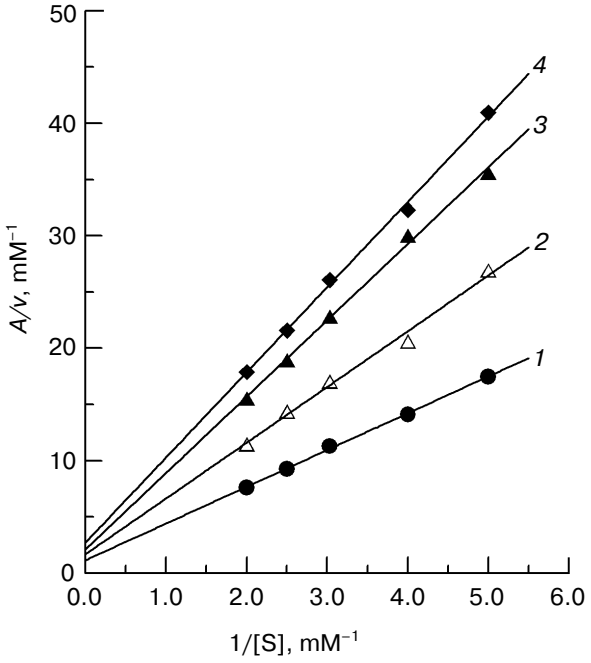


Fig. 6. Plot of A/v versus 1/[S]. The final dioxane concentrations for curves *1-4* are 0.6, 0.8, 1.0, and 1.2 M, respectively. The A values were obtained from the slopes of the straight lines in Fig. 5b.

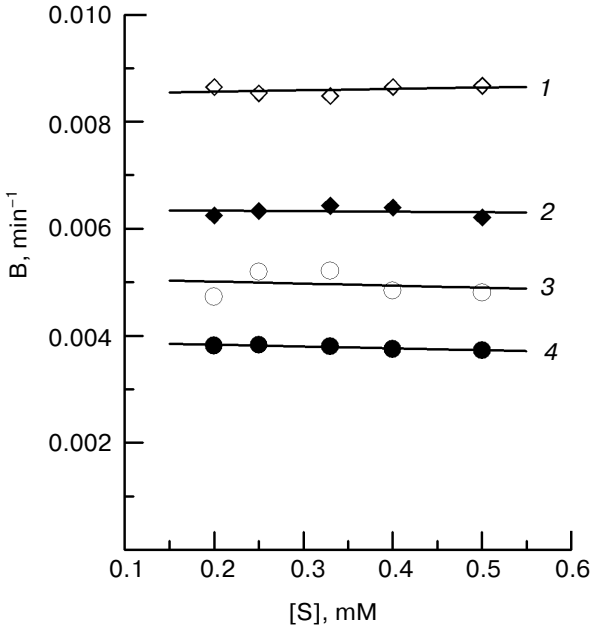


Fig. 7. Plot of B versus [S]. The final dioxane concentrations for curves *1-4* are 0.6, 0.8, 1.0, and 1.2 M, respectively.

above and Eq. (3), plots of B versus [S] at different dioxane concentrations give a series of straight lines shown in Fig. 7. The result showed that all lines paralleled the X-axis, indicating the values of B at different concentrations

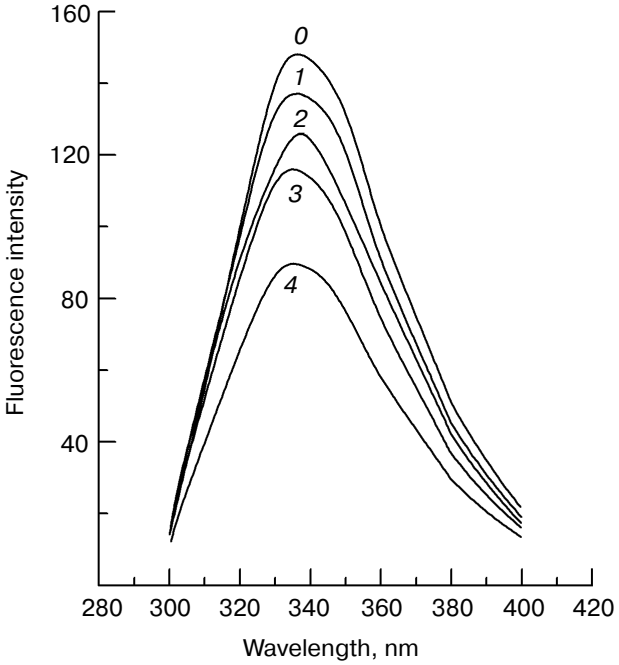


Fig. 8. Fluorescence emission spectra of NAGase inactivated in dioxane solutions. Enzyme (50 μl of 906 μg/ml) was added into 1 ml of 0.15 M NaAc-HAc buffer, pH 5.2, containing different concentrations of dioxane before determination of the fluorescence spectra. The excitation wavelength was 278.2 nm. The final concentration of the enzyme was 45 μg/ml. The dioxane concentrations for curves *0-4* were 0, 0.6, 0.8, 1.0, and 1.2 M, respectively.

of substrate were not affected by the substrate concentrations. From Eq. (3),

$$B = \frac{k_{-0}K'_s + k'_{-0}[S]}{K'_s + [S]},$$

we could draw the conclusion that $B = k_{-0} = k'_{-0}$, the values of k_{-0} and k'_{-0} could be determined, and the B values of the enzyme in different concentrations of dioxane were determined and listed in the table for comparison.

Fluorescence emission spectra of NAGase in dioxane solutions. The fluorescence emission spectra of NAGase in different concentrations of dioxane are shown in Fig. 8. The emission peak of the native enzyme is at 337.8 nm; it may contain contributions from both Trp and Tyr residues. Increasing the dioxane concentration caused the fluorescence emission intensity to be markedly decreased and the emission peak (at 337.8 nm) a little red-shifted. When the concentration of dioxane reached 1.2 M, the fluorescence intensity decreased from 147.8 to 89.6, decreasing by 39.4%. And the red shift of the emission maximum was only 3 nm. The result indicated that when the dioxane binds to the enzyme molecule, it can induce the enzyme conformation to change and leads to the inactivation of the enzyme.

DISCUSSION

Enzymes and proteins in organic solvents have received increased 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. In this paper, we not only used the substrate reaction kinetic method to analyze the inhibitory kinetics of NAGase in dioxane solution, but also elucidated the relationship between the changes in conformation and function of the enzyme at different concentrations of dioxane. The results showed that the enzyme inactivated reversibly by dioxane, increasing the dioxane concentrations, the enzyme activity decreased by an exponent. When the concentration of dioxane reached 1.1 M, the enzyme activity lost 50%. The results listed in table show that the values of k_{+0} and k'_{+0} increase apparently with increasing the dioxane concentration, but the values of k_{-0} and k'_{+0} decrease, illuminating that the reversible intensity of the enzyme is weakened with increasing the dioxane concentration. The value of k_{+0} is much larger than k'_{+0}, indicating the free enzyme molecule is more fragile than the enzyme–substrate complex in the dioxane solution. Accompanying the increase in dioxane concentration, both residual activity and fluorescence intensity decrease. The fluorescence intensity decreases more slowly than the residual activity, which means that the whole conformation of the enzyme changes more slowly than the conformation of the active center of the enzyme in the dioxane solution.

The change in the fluorescence intensity of NAGase in dioxane solution is very different from that of mushroom tyrosinase in dimethylsulfoxide, whose fluorescence intensity increased with increasing concentration of dimethylsulfoxide [18]. However, accompanying the loss of activity resulting from the increase in dioxane concentration, the fluorescence intensity decreased and the emission peak was red-shifted indistinctly. This may be because the adding of dioxane changes the pH or dielectric constant, or the presence of organic reagent, dioxane, could modify the nature and the number of noncovalent interactions, so the microenvironments of NAGase change and then its conformation is affected, and its activity decreases.

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