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Studies on the Chemical Modification of the Essential Groups of *N*-Acetyl-β-D-Glucosaminidase from Viscera of Green Crab (*Scylla Serrata*)

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Abstract The chemical modification of *N*-acetyl-β-D-glucosaminidase (EC3.2.1.30) from viscera of green crab (*Scylla serrata*) has been first studied. The modification of indole groups of tryptophan of the enzyme by *N*-bromosuccinimide can lead to complete inactivation, accompanying the absorption decreasing at 275 nm and the fluorescence intensity quenching at 338 nm, indicating that tryptophan is essential residue to the enzyme. The modification of histidine residue, the carboxyl groups, and lysine residue inactivates the enzyme completely or incompletely. The results show that imidazole groups of histidine residue or sulfhydryl residues, the carboxyl groups of acidic amino acid, amino groups of lysine residue, and indole groups of tryptophan were essential for the catalytic activity of enzyme, while the results demonstrate that the disulfide bonds and the carbamidine groups of arginine residues are not essential to the enzyme's function.

Keywords *N*-Acetyl-β-D-glucosaminidase · *Scylla serrata* · Essential groups · Chemical modification

Abbreviation

pNP-NAG	*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide
DTT	dithiothreitol
BrAc	bromoacetic acid
EDC	1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride

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NBS	*N*-bromosuccinimide
NaAc-HAc buffer	sodium acetate-acetate buffer
NaOH	sodium hydroxide
Tris–HCl buffer	Tri (hydroxymethyl) aminomethane-hydrochloric acid buffer
Na_2HPO_4–NaH_2PO_4 buffer	sodium phosphate buffer
TEMED-HCl buffer	tetramethylethylenediamine-hydrochloric acid buffer

Introduction

Chitinases, defined as enzymes cleaving a bond between the C_1 and C_4 of two consecutive *N*-acetylglucosamines of chitin, are widely distributed in nature and play important roles in degradation of chitin. Chitinases are composed of three kinds of enzymes: endochitinases, exochitinases, and *N*-acetyl-β-D-glucosaminidase (NAGase, EC3.2.1.30). They cooperate to disintegrate chitin into *N*-acetylglucosamine (NAG). Chitinase cleaves chitin into dimer and trimer oligomers of NAG which are further hydrolyzed by NAGase to monomer NAG [1]. Chitinases have been well investigated in some insects [2], bacteria [3], and plants [4] for their important roles in molting, digestion of chitinous foods, and defense systems against parasites. Meanwhile, chitinases have been characterized and reported in marine invertebrates, molluscs, and crustaceans about their purification, concentrations in different growth stage, and distribution in different organs, such as oysters [5], prawns [6], lobsters [7], krills [8], topshell (*Turbo cornutus*) [9], and prawn (*Penaeus vannamei*) [10].

However, the knowledge about functional groups and catalytic mechanism of NAGase are yet limited. Amutha et al. [11] had found that the residues of tryptophan, histidine, and acidic amino acids were the functional groups of NAGase from thermotolerant *Bacillus* sp. NCIM 5120 by chemical modification. Prag et al. [12] had also found that both Asp539 and Glu540 residues are essential for activity of chitobiase from *Serratia marcescens* by site-directed mutagenesis, biochemical characterization, and structural analyses of chitobiase-substrate co-crystals. Recently, Lin et al. [9] reported that the residues of tryptophan, histidine, lysine, and acidic amino acids were the essential groups of NAGase from *T cornutus* Solander by chemical modification. Functional groups of NAGase from different organisms are variable to adapt to various life environments and exert different physiological functions.

The green crab (*Scylla serrata*) is a most important species for mariculture in China. However, The NAGase from green crab (*S. serrata*) has hardly been known. The research of the crab NAGase has very important significance to the breeding and the growth of crab. Systematical studies of NAGase from green crab (*S. serrata*) are currently taking place in our laboratory. In our previous studies, we have reported the purification and some enzymatic characterization of the enzyme [13]. In the present paper, we carry out chemical modification of green crab NAGase to study the essential groups of the enzyme catalytic activity.

Materials and Methods

Preparation of green crab NAGase (EC3.2.1.52) from viscera was as described previously [13]. The final enzyme preparation was homogeneous on polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate-PAGE. The specific activity of the purified enzyme was 7990 U/mg. *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNP-NAG) was purchased from the Biochemistry Lab of Shanghai Medicine Industry Academy (China). Dithiothreitol (DTT),

N-bromosuccinimide (NBS), and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma. Bromoacetic acid (BrAc), acetic anhydride, acetyl acetone, and all other reagents were local products of analytical grade.

The protein concentration was measured by the method of Lowry et al. [14], with bovine serum albumin as the standard. Enzyme activity was measured by following the increasing absorbance at 405 nm accompanying the hydrolysis of the substrate (pNP-β-D-GlcNAc) with the molar absorption coefficient of $1.73 \times 10^4\ M^{-1}\ cm^{-1}$. A portion of 10 μl of enzyme solutions was added to the reaction media (2.0 ml) containing 0.2 mM pNP-NAG in 0.05 M NaAc-HAc buffer (pH 5.8). 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 was recorded using a Beckman DU-650 spectrophotometer. One unit (U) of enzymatic activity was defined as the amount of enzyme catalyzing the hydrolysis of pNP-NAG to form 1 μM pNP per minute at 37°C [13].

The enzyme was modified by specific modifier in the certain buffer with the given value of pH for given time, and 20 μl portions were taken for activity determination in the assay system.

The changes of absorbance and the fluorescence spectra modified by NBS were measured with a Beckman DU-650 and Hitachi 4010 spectrophotometer. Approximately 20 μl of NAGase was dissolved in 1.0 ml of 0.1 M NaAc-HAc buffer (pH 5.5) with different concentrations of NBS. The mixture was preincubated at 37°C for 30 min before determination of absorbance and the fluorescence spectra. The excitation wavelength was 280 nm.

Results

The Chemical Modification of Enzyme with BrAc

BrAc can react with the imidazole groups of histidine residues in the acidic condition, while it can react with sulfhydryl residues under the weak-acid reaction conditions [15]. In the present investigation, BrAc was used to modify the enzyme. The chemical modification was performed in various concentrations of BrAc solutions in 0.1 M NaAc-HAc buffer (pH 5.8) for 30 min at room temperature. The chemical modification by BrAc was shown in Fig. 1. The increase in BrAc concentration led to the rapid drop in enzyme activity. When BrAc concentration was 60 mM, the enzyme activity was lost 50%. When BrAc concentration was increased to 120 mM, the enzyme had almost no activity. The result illuminated that the imidazole group of histidine residue or sulfhydryl residues was essential for the activity of enzyme.

The Chemical Modification of Amino Groups

Acetic anhydride can chiefly modify the amino groups of lysine residues in protein in alkaline condition [16]. The chemical modification of acetic anhydride on the enzyme was studied in 0.01 M Tris–HCl buffer (pH 8.0) for 30 min at 4°C. The enzyme activity was inactivated by acetic anhydride dependently on the concentrations as shown in Fig. 2. By increasing the concentrations of acetic anhydride, the remaining enzyme activity was rapidly decreased. When acetic anhydride concentration reached 0.4 mM, the enzyme activity was lost 50%. When acetic anhydride concentration was increased to be 1.0 mM, the enzyme activity was almost lost. This result demonstrated that the amino group of lysine residue was the enzyme's functional group.

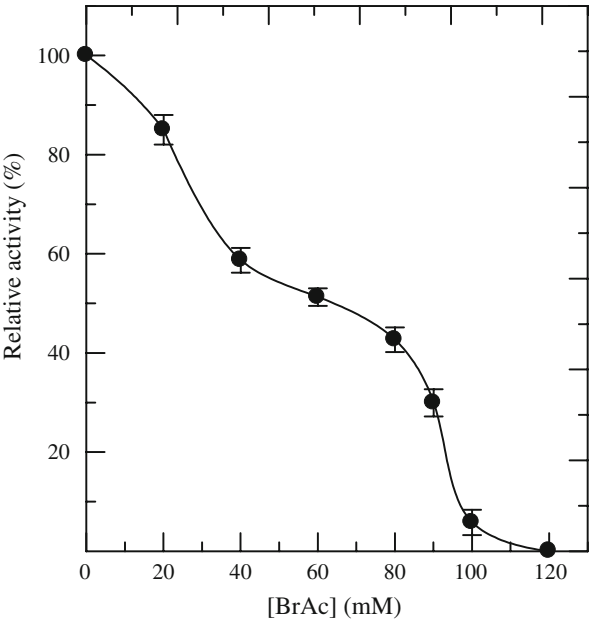

Fig. 1 Inactivation of NAGase from viscera of green crab (*Scylla serrata*) by BrAc. The enzyme was mixed with different concentrations of BrAc in 0.1 M NaAc-HAc buffer (pH 5.8) for 30 min at room temperature. Approximately 20 μl portions were taken for activity determination in the assay system. The final enzyme concentration was 0.0076 μM

The Chemical Modification of Carboxyl Groups

EDC can effectively react with the carboxyl groups of the protein in acid condition [9]. In this paper, we used EDC as modifier of the carboxyl groups of the enzyme. The chemical modification by EDC was carried out in 0.1 M tetramethylethylenediamine (TEMED)-HCl buffer (pH 4.5) for 60 min at room temperature. The result showed that the increase in EDC

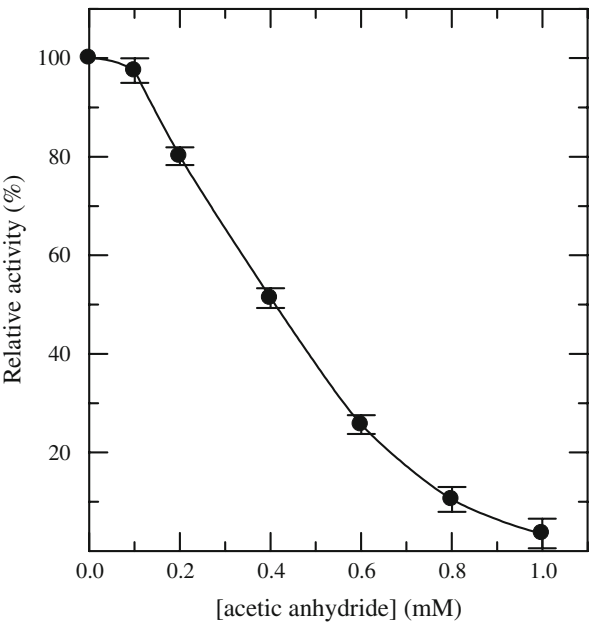

Fig. 2 Inactivation of NAGase from viscera of green crab (*Scylla serrata*) by acetic anhydride. The enzyme was mixed with different concentrations of acetic anhydride in 0.01 M Tris–HCl buffer (pH 8.0) for 30 min at 4°C. Approximately 20 μl portions were taken for test. The final enzyme concentration was 0.0076 μM

concentration led to the rapid drop in enzyme activity (shown in Fig. 3). When EDC concentration reached 150 mM, the enzyme's remaining activity was 20%, indicating that the carboxyl group was essential to the enzyme.

The Chemical Modification of Tryptophan Residues

NBS can react with indole groups of tryptophan for a number of proteins in the certain condition [17]. We can use NBS to modify the tryptophan residue of the enzyme. The enzyme was treated with different amounts of NBS in 0.1 M NaAc-HAc buffer (pH 5.5) at 37°C for 30 min. The result (shown in Fig. 4) showed that by increasing the concentration of NBS, the enzyme activity decreased to reach the complete inactivation when the concentration of NBS was 20 μM.

Most of proteins have the typical UV-absorbance peaks at the range from 270 to 280 nm approximately. It has been reported that some proteins with tryptophan residue modified by NBS has a decrease in absorption at 278 nm [17]. The UV-absorbance spectra of green crab NAGase modified by NBS were shown in Fig. 5. The UV-absorbance peak of the native enzyme is at 275 nm. The absorbance peak at 275 nm rapidly decreased in intensity with increasing NBS concentrations. While the NBS concentration was increased to be 20 μM, the absorbance peak had basically disappeared. The results showed that modification of the tryptophan residue resulted in a decrease in absorption at 275 nm.

The intrinsic emission spectra of the native enzyme and the modified enzyme were determined. The results obtained were shown in Fig. 6. With the increase in the concentration of NBS, the fluorescence emission intensity at 338 nm decreased in magnitude and disappeared at 20 μM of NBS. It suggested that the tryptophan residues relatively close to the surface of the molecule have been modified.

From the above results, we can conclude that the tryptophan residue is one of the essential groups of the enzyme activity, and it is situated on the surface of the enzyme and easily modified by NBS.

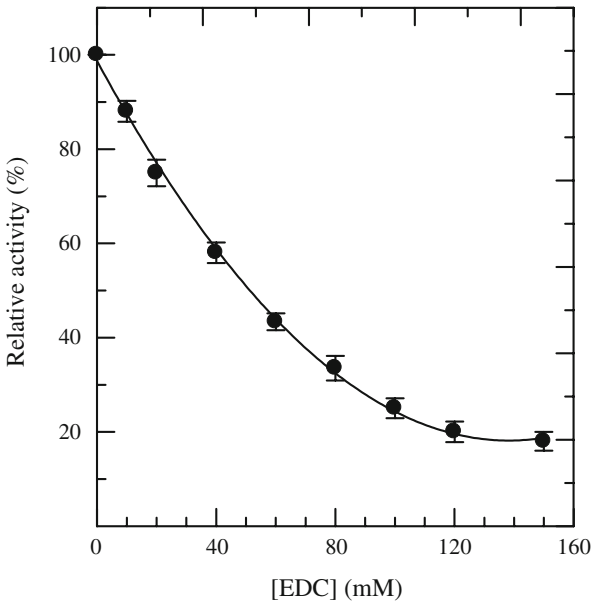

Fig. 3 Effect of the activity of NAGase from viscera of green crab (*Scylla serrata*) modified by EDC in 0.1 M TEMED-HCl buffer (pH 4.5) for 60 min at room temperature. Approximately 20 μl portions were taken for activity determination in the assay system. The final enzyme concentration was 0.0076 μM

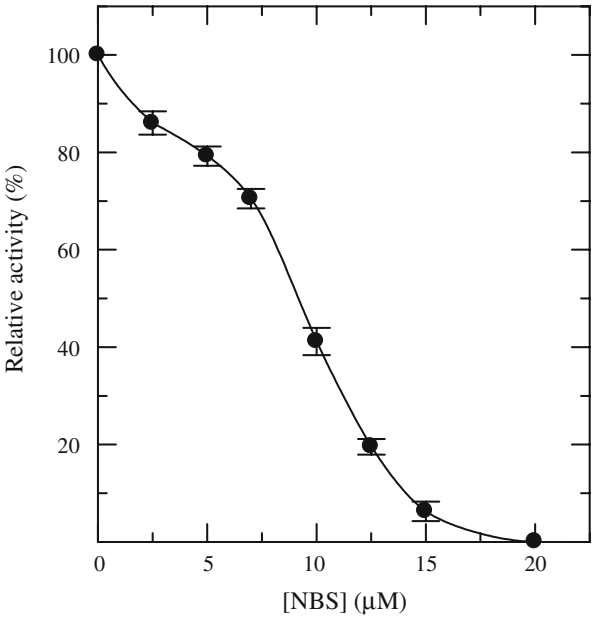

Fig. 4 Inactivation of NAGase from viscera of green crab (*Scylla serrata*) by NBS. The enzyme was mixed with different concentrations of NBS in 0.1 M NaAc-HAc buffer (pH 5.5) at 37°C for 30 min. Approximately 20 μl portions were taken for activity determination in the assay system. The final enzyme concentration was 0.0076 μM

The Chemical Modification of Disulfide Bond and Arginine Residues

DTT and acetyl acetone are among the specific modifiers for the disulfide bond formed between cysteine residues [18] and the carbamidine groups of arginine residue in alkaline condition [19], respectively. Chemical modification of the enzyme by the three modifiers has been studied. The enzyme was treated with different concentrations of the three

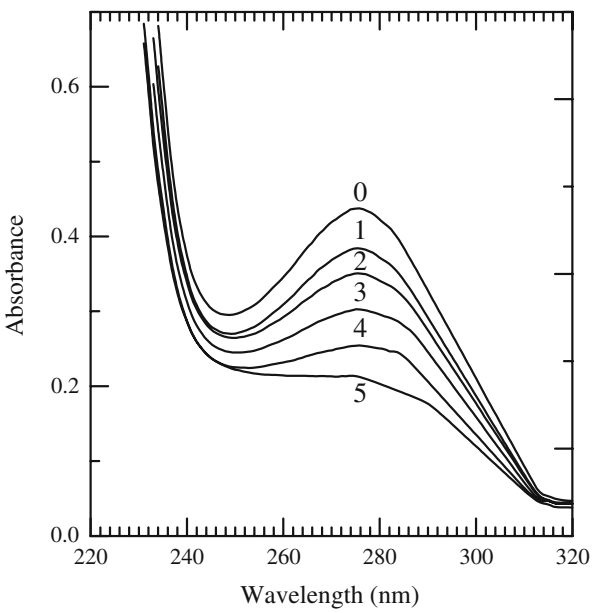

Fig. 5 Absorption spectra of NAGase from viscera of green crab (*Scylla serrata*) modified by NBS. The enzyme solutions were incubated in different NBS concentrations at 37°C for 30 min before determination of absorbance. The final enzyme concentration was 0.0076 μM. The concentration of NBS for curves 0–5 was 0, 5, 7.5, 10, 15, and 20 μM, respectively

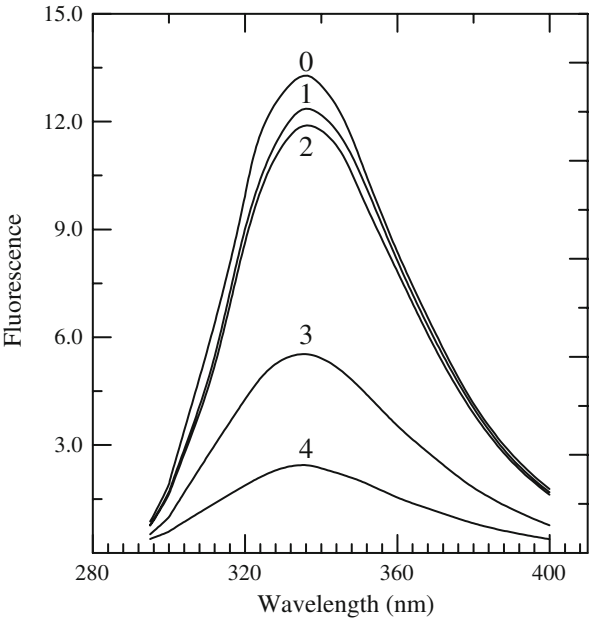

Fig. 6 Fluorescence emission spectra of NAGase from viscera of green crab (*Scylla serrata*) modified by NBS. The enzyme solutions were incubated in different NBS concentrations at 37°C for 30 min before determination of the fluorescence spectra. The final enzyme concentration was 0.0076 μM. The excitation wavelength was 280 nm. The concentration of NBS for curves 0–4 was 0, 2.5, 7.5, 15, and 20 μM, respectively

modifiers and determined the residual activity, respectively: DTT in 0.01 M Tris–HCl buffer (pH 8.0) for 30 min at 37°C; acetyl acetone in 0.01 M Tris–HCl buffer (pH 8.0) for 30 min at 25°C. The results (not shown in figure) showed that enzyme activities were kept unchanged, respectively, and indicated that disulfide bonds and the carbamidine groups of arginine residue were unrelated to the enzyme-catalyzing function.

Discussion

BrAc is a specific modifier for the histidine residue [15, 20], although it can react with sulfhydryl residues under the weak-acid reaction conditions used in this experiment. Chen et al. [21] used BrAc as a modifier to study the histidine residue of the active site of β-glucosidase from *Ampullarium crossean*. They suggested that BrAc can selectively modify the histidine residue of the enzyme on this prerequisite that it is not a sulfhydryl enzyme. The enzyme can be completely inactivated at a sufficiently large BrAc concentration, which is reasonable as BrAc covalently modifies the histidine residue of the enzyme irreversibly. On the other hand, when BrAc is at low concentrations, a slow and reversible reaction was observed. In this paper, we used BrAc to modify the NAGase from viscera of green crab (*S. serrata*). The result in Fig. 1 showed that the enzyme activity, almost lost in the high concentration of BrAc, indicated that the histidine residue or sulfhydryl residues played an important role in the enzyme action. It probably had connection with the maintenance of the conformation of the enzyme active center.

In our investigation, the enzyme activity decreased when enzyme was modified by BrAc, acetic anhydride, EDC, and NBS, while the modification of enzyme with DTT and acetyl acetone did not result in any loss of enzyme activity, respectively. Therefore, we suggest that the carboxyl groups of acidic amino acid, imidazole groups of histidine residue or sulfhydryl residues, amino groups of lysine residue, and indole group of tryptophan were

essential for the activity of enzyme. However, the disulfide bond and carbamidine groups of arginine residue were unrelated to the enzyme-catalyzing function. All previous research on NAGase from different species unanimously agreed that the carboxyl residue of aspartic acid or glutamic acid was involved in the active site of the enzyme [10–12]. Both Lin and Amutha reported that the histidine and tryptophan residue were essential groups for NAGase activity, respectively [9, 11]. Amutha et al. [11] reported that one tryptophan residue of NAGase was involved with binding substrate, and one histidine residue and one acidic amino acid residue of NAGase were involved with catalysis. The investigation of Lin et al. [9] showed clearly that the modification of tryptophan residues of NAGase from *T. cornutus* by high concentrations of NBS led to the complete inactivation of this enzyme. The absorption decreased at 278 nm and the florescence intensity quenched at 335 nm indicated the loss of tryptophan residues of the enzyme. The loss of enzyme loss owed to the modification of enzyme by NBS. Our study also showed that the activity, the UV-absorbance intensity at 276 nm, and the florescence intensity at 338 nm of green crab NAGase from viscera rapidly decreased with increasing NBS concentrations, which suggested that tryptophan residue was essential for NAGase activity.

Our results and other reports suggest that the carboxyl groups of acidic amino acid, indole group of tryptophan, imidazole groups of histidine residue, or sulfhydryl residues were relatively conservative functional residues of NAGase without species variation, while the relationship between the enzyme activity and disulfide bond and carbamidine groups of arginine residue of NAGase were different from the species. The conservative functional residues could ensure NAGase to accomplish its catalysis, and the changes of some essential residues might be caused by adaptability for habitat according to different species.

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