Property of Midgut $\alpha$-Amylase From *Mythimna separata* (Lepidoptera: Noctuidae) Larvae and Its Responses to Potential Inhibitors In Vitro

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**ABSTRACT.** Midgut $\alpha$-amylase is an important digestive enzyme involved in larval energy metabolism and carbohydrate assimilation. In this article, the properties of midgut $\alpha$-amylase from the Oriental armyworm, *Mythimna separata* (Lepidoptera: Noctuidae), larvae were characterized, and its in vitro responses to chemical inhibitors were also determined. The kinetic parameters $K_m$ and $V_{\text{max}}$ of midgut $\alpha$-amylase were 0.064 M, 4.81 U mg$^{-1}$ in phosphate buffer, and 0.128 M, 1.96 U mg$^{-1}$ in barbiturate-acetate buffer; $\alpha$-amylase activity linearly increased as starch concentration increased. $\alpha$-Amylase activity was not influenced by amino acids such as Pro, Met, Try, His, Ala, and Phe but was strongly activated by antioxidants. Reduced glutathione, 1,4-dithiothreitol, $\beta$-mercaptoethanol, and ascorbic acid improved the activity of $\alpha$-amylase about 2.06, 3.46, 3.37, and 6.38 times, respectively, relative to the control. Ethylenediaminetetraacetic acid, sodium dodecyl sulfate, and N-bromosuccinimide (NBS) strongly inhibited $\alpha$-amylase. $\alpha$, $\beta$, and $\gamma$-cyclodextrin were not the preferred substrates for $\alpha$-amylase. Kinetic analysis showed that $IC_{50}$ value of NBS against $\alpha$-amylase was 1.52 ($\pm$0.26) $\mu$M, and the mode of action of NBS with $K_m$ as 2.53 (0.35) $\mu$M was a mixed-type inhibition that indicated a combination of partial competitive and pure noncompetitive inhibition. The midgut $\alpha$-amylase of armyworm larvae may be a potential target for novel insecticide development and pest control.

**Key Words:** midgut $\alpha$-amylase, *Mythimna separata*, chemical inhibitor

Herbivorous insects utilize a complement of extracellular enzymes to digest their food. One of these is $\alpha$-amylase, which cleaves $\alpha$-1,4-glycosidic bonds in starch, glycogen, oligosaccharides, and polysaccharides and thereby promotes larval carbon assimilation from the diets (Daone et al. 1975, Buonocore et al. 1976). Several functional proteins such as lectin-like, knottin-like, cereal-type, Kunitz-like, c-purothionin-like, and thaumatin-like, which are often found in the seeds of leguminous and graminaceous plant, have been reported to inhibit $\alpha$-amylase. One of these is $\alpha$-amylase in the midgut of armyworm larvae and to screen nonproteinaceous chemical inhibitors that might be used as precursors for developing novel insecticides to reduce losses caused by armyworm pests.

**Materials and Methods**

**Insect.** An insecticide-susceptible strain of the armyworm, *M. separata*, was reared continuously in our laboratory on artificial diet (wheat bran 14.3 g, ale yeast powder 3.4 g, vitamin C 0.5 g, dried corn leaves 6.8 g, sorbic acid 6.8 g, nipagin 0.3 g, agar 2.4 g, and water 120 ml) (Bi and Wang 1989). The rearing conditions were maintained at 23 ± 1°C, 70–80% relative humidity, and a photoperiod of 16:8 (L:D) light regime. The larvae were held without food for 4 h before the experiments.

**Chemicals.** Bovine serum albumin and Coomassie brilliant blue G-250 were purchased from Sigma Chemical (St. Louis). Soluble starch, 3,5-dinitrosalicylic acid, disodium hydrogen phosphate, and sodium dihydrogen phosphate were purchased from Aladdin Co., Ltd. (Shanghai, China).

The candidate inhibitors were selected based on the previous reports (Buonocore et al. 1976, Timmins and Reynolds 1992, Mohamed 2000, Zeng and Cohen 2000, Roger et al. 2001, Sliva and Terra 2001, Sliva and Terra 2001, 2014).

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Solved in ddH2O to prepare 10.0 mg/ml of stock aqueous solutions. 

ured, and the 50% inhibitory concentration (IC50) values for NBS were 0.01, 0.1, 1, 10, and 100 μM.

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Preparation of α-Amylase From the Midgut of Armyworm Larvae. 

Midguts dissected from actively feeding fifth instar 2nd day armyworm larvae in the gluttony stage were homogenized in ice-cold normal saline solution containing 20 mM NaCl and 0.1 mM CaCl2 (50 μl per midgut), then centrifuged at 10,000 × g, 4°C for 20 min. The supernatant was filtered through a 0.2 μm nitrocellulose membrane filter to remove small suspended particles and bacteria, freeze-dried to a powder, and then frozen (−35°C). This served as an enzyme source (Bergmeyer et al. 1984, Li et al. 1997).

Assays of α-Amylase Activity. α-Amylase activity was assayed with 3,5-dinitrosalicylic acid using soluble starch as substrate (Nagaraju and Abraham 1995, Ishimoto et al. 1999). The enzyme powder was dissolved in 0.03 M phosphate buffer containing 20 mM NaCl and 0.1 mM CaCl2 (pH 8.0, PBS). Twenty microliters of α-amylase solution containing 180.4 ng of protein were used and preincubated with 20 μl of PBS at 37°C for 15 min. After preincubation, 200 μl of PBS containing 17.5, 23.4, 29.2, 58.4, and 116.9 mM soluble starch was added, respectively. The resulting mixture was kept at 37°C for 3 min. The reaction was stopped by the addition of 3.0 ml of 3,5-dinitrosalicylic acid (43.8 mM) and then held in boiling water for 5 min, cooled by running water, and diluted by adding 6.0 ml of distilled water. Absorbance was measured at 520 nm on a ultraviolet spectrophotometer; 0.03 mM barbiturate-acetate buffer containing 20 mM NaCl and 0.1 mM CaCl2 (pH 5.4, BBA) was used instead of PBS (pH 8.0) as the negative control. Concentration–activity relationships of α-amylase were determined using concentrations of 0.16, 0.32, 0.65, and 1.30 mg/ml in the presence of 0.029 M soluble starch as substrate. α-Amylase activity was determined in two sets of triplicates each. Maltose hydrate was used as standard. The unit of α-amylase activity was expressed as U mg protein−1.

Effects of Inhibitors on α-Amylase Activity. Twenty microliters of α-amylase in PBS (pH 8.0) containing 180.4 ng of protein were used and preincubated with 20 μl of 5.0 μg/ml of the candidate inhibitors at 37°C for 15 min. After preincubation, 200 μl of PBS containing 29.2 mM soluble starch was added, and resulting mixture was kept at 37°C for 3 min. The next step for determining α-amylase activity was the same as for the above protocol. Twenty microliters of PBS buffer were used instead of 5.0 μg/ml candidate inhibitors as the control. The inhibitory activity of 0, 0.01, 0.1, 1, 10, and 100 μM NBS on midgut α-amylase was also measured, and the 50% inhibitory concentration (IC50) values for NBS were determined in two sets of triplicate measurements. The activity of α-amylase relative to the control was calculated as enzyme activity in treatment divided by enzyme activity in control.

Kinetics Assays of α-Amylase Inhibitors. The kinetic behavior of α-amylase inhibitors was determined using Lineweaver–Burk plots according to the method of Kakkar et al. (1999) with modifications. Twenty microliters of α-amylase in PBS (pH 8.0) containing 180.4 ng of protein were used and preincubated with 20 μl of candidate inhibitor at concentrations of 0, 10, and 100 μM at 37°C for 15 min. After preincubation, 200 μl of PBS containing 17.5, 23.4, 29.2, 58.4, and 87.6 mM soluble starch was added, respectively, and the resulting mixture was incubated at 37°C for 3 min. The kinetic parameters Km and Vmax of midgut α-amylase in the presence of 10 and 100 μM potential inhibitors were calculated by using Lineweaver–Burk plots. The Ki value was obtained by incubating 0.7, 1.5, 5.8, and 29.2 mM soluble starch and 0, 10, 20, 40, 80, and 100 μM inhibitors, respectively, and the initial velocities versus concentration of inhibitors was established as Dixon plots. Data were obtained by using two sets of three-parallel replicates.

Protein Determination. Protein content was determined by the Bradford method using bovine serum albumin as the standard and staining with Coomassie brilliant blue G250 (Bradford 1976).

Data Statistics. All the experimental data are presented as the mean ± SE and subjected to analysis of variance by Microsoft Excel 2007. Means separation used the t-test for significance of difference analysis (P < 0.05).

Results

Properties of α-Amylase in Armyworm Midgut. Midgut α-amylase activity on the starch was much more active in PBS (pH 8.0) than in BBA (pH 5.4) (Fig. 1A). The kinetic parameters Km and Vmax of α-amylase obtained from the Lineweaver–Burk plot were 0.064 M and 4.81 U mg protein−1 in PBS and 0.128 M and 1.96 U mg protein−1 in BBA, respectively. The production of maltose from soluble starch was significantly greater in PBS than in BBA over the same time period. The α-amylase activities were increased as the concentration of substrate starch was increased. Moreover, α-amylase activity increased when the enzyme
concentration was gradually increased up to 1.30 mg/ml in the presence of starch with concentration as 0.029M (Fig. 1B).

**Response of Midgut α-Amylase to Amino Acids and Antioxidants.** Among the amino acids and antioxidants tested (Fig. 2), α-amylase activity was little influenced by Pro, Met, Try, His, Ala, and Phe at concentrations of 5.0 μg/ml (P > 0.05) but was inhibited with 46.8 ± 3.2% and 25.3 ± 1.9% in the presence of 5.0 μg/ml Gly and Tyr, respectively. However, antioxidants strongly promoted midgut α-amylase activity. RED, DTT, and βME increased the activity of α-amylase by about 2.1, 3.5, and 3.4 times relative to the control, respectively, whereas ASC enhanced α-amylase activity >6.4-fold when compared with the control.

**Response of Midgut α-Amylase to Exogenous Chemicals and Cyclodextrins.** After exposure to 5.0 μg/ml of exogenous chemicals, the activities of midgut α-amylase were inhibited on different degree (Fig. 3). EDTA, SDS, CIT, and NBS showed the strongest inhibitory effect on α-amylase with inhibitory rate of 85.5 ± 3.1%, 91.5 ± 3.6%, 72.2 ± 2.6%, and 84.9 ± 2.8%, respectively; CIT and TBAA showed medium inhibitory effect with inhibitory rate of 53.6 ± 2.4 and 56.7 ± 2.1%, respectively; TART, TFAH, TCAA, ACAH, and OCBZ showed slight inhibitory effect with inhibitory rate about 35%. Other chemicals showed no significant inhibitory effect (P > 0.05). αCYC, βCYC, and γCYCn were not the preferred substrates for midgut α-amylase.

**Inhibition Kinetics of NBS on Larval Midgut α-Amylase.** α-Amylase activity gradually decreased in the presence of increasing concentration of NBS from 0.01 to 100 μM, indicating that NBS possesses concentration-dependent inhibitory potential against α-amylase (Fig. 4), mean inhibitory concentration (IC50) of NBS against larval midgut α-amylase was 1.52 (± 0.26) μM.

Kinetic analysis of NBS inhibition utilized Lineweaver–Burk and Dixon plots and their secondary plots (Fig. 5). In presence of 10 and 100 μM NBS, Km of midgut α-amylase increased from 0.146 to 0.174M, and Vmax values decreased from 1.99 and 1.33 U mg pro⁻¹, respectively. The secondary plots of both the Dixon and Lineweaver–Burk plots were also studied. Based on the kinetic analysis, the mode of action of NBS can be described as a mixed-type inhibition with the Ki value of 2.53 (±0.35) μM. This mixed-type inhibition was a combination of partial competitive and pure noncompetitive inhibition.

**Discussion**

Inefficient food digestion and nutrient utilization greatly retard larval growth and development (Slansky et al. 1985), and the inhibition of intestinal proteases and amylases directly leads to low digestive efficiency in most lepidopteran (Timmins and Reynolds 1992, Terra and Ferreira 2005). α-Amylase is an important digestive enzyme that hydrolyzes starch, glycogen, or polysaccharides to soluble monosaccharides, disaccharides maltose, fructose, and even glucose. It helps regulate larval energy metabolism. Ishimoto et al. (1999) reported that the inhibition on the activity of α-amylase could reduce the carbohydrate assimilation, thereby inhibiting the growth of insects. When α-amylase inhibitor from common bean is expressed in peas, the peas no longer allow the survival of cowpea bruchid larvae (Shade et al. 1994).

Insect α-amylase has been the subject of much study. Prigent et al. (1998) and Kundu and Das (1970) reported that insect α-amylase is a metalloenzyme that contains at least one Ca²⁺ ion to maintain its stable conformation and catalytic activity, but Gupta et al. (2003) reported that the presence of Ca²⁺ ion might induce the inactivation of α-amylase, whereas Ca²⁺ chelation by EDTA could preserve its enzymatic activity. Biggs and McGregor (1996) used amylase from grass beetles Costelytra zealandica as a target enzyme to screen for inhibitors. Titarenko and Chrispeels (2000) cloned cDNA encoding α-amylase from corn rootworm Diabrotica virgifera by RT-polymerase chain reaction technology to screen α-amylase inhibitors. Prigent et al. (1998) purified α-amylase from Drosophila virilis and Drosophila repleta to study the metabolic evolutionary relationships of Drosophila living in different habitats. Huang et al. (2003) reported that oxadiazoles
inhibited α-amylase activity from the midgut of *M. separata* larvae, an inhibition that decreased in presence of increasing levels of α-amylase protein. Here, the results show that α-amylase activity from the midgut of *M. separata* larvae is higher in a rich starch system. EDTA, SDS, and NBS strongly inhibited midgut α-amylase, just as has been observed for *H. dromedarii* (Mohamed 2000) and for *Bacillus licheniformis* (Ivanova et al. 1993). Moreover, αCYC, βCYC, and γCYC showed weaker inhibitory effects against α-amylase from the midgut of armyworm larvae; this was probably caused by substrate-competitive interaction between cyclodextrin and soluble starch. This was similar to the results outlined by Roger et al. (2001) and Hur et al. (2001), who showed that αCYC, βCYC, and γCYC were capable of combining to the active centre of porcine pancreas α-amylase to form enzyme—cyclodextrin complexes.

Antioxidants such as ASC as well as the reductive thios, RED, DTT, and βME strongly activated α-amylase from the midgut of *M. separata* larvae. Podoler and Appleaum (1971) reported that glutathione and βME were capable of activating α-amylase in bean weevil *Callosobruchus chinensis*. The similar activation of βME on α-amylase was also noticed by Lee et al. (1997). These antioxidants may preserve the optimal catalytic conformation of the enzyme and enhance Ca$^{2+}$ activation of α-amylase (Podoler and Appleaum 1971). This activation mechanism needs to be further investigated. Moreover, Tris was also found to activate the activity of α-amylase, opposite to the reports of Ghalanbor et al. (2008) and Aghajari et al. (1998) that the Tris molecule inhibits a number of bacterial α-amylases and that Tris buffer should not be used in assays of bacterial α-amylases.

In this article, data further revealed that EDTA strongly inhibits armyworm larval α-amylase activity, and a result was consistent with the study reported by Zeng and Cohen (2000) that competitive chelation of Ca$^{2+}$ ion was a very important factor in the inhibition of EDTA on larval α-amylase in salivary glands of *Lygus hesperus* and *L. lineolaris*. Moreover, SDS, TAPS, and CITS also showed strong inhibition on α-amylase, a result similar to that of Mohamed (2000) who showed that NaCl inhibited α-amylase from *H. dromedarii*. We speculate that the inhibitory effects of these compounds on α-amylase are due to the destruction of the Ca$^{2+}$-enzyme-stable conjunctive status, so as to demolish the active conformation of α-amylase.

Our experiments further showed that IAM slightly inhibited the armyworm midgut α-amylase, a result contrary to that reported by Lu et al. (2004) that IAM could specifically react with cys-residue in poly-peptides and cause the alkylation of hydrosulfide groups, thereby leading to the structural modification of urease and the degradation of the enzymatic activity of urease.

Analysis of the inhibition kinetics of NBS on α-amylase activity in the midgut of armyworm larvae showed that the mixed-type action of NBS on α-amylase was a combination of partial competitive and pure noncompetitive inhibition. According to previous reports, NBS may modify a tryptophan residue(s) involved in maintaining structural integrity of the α-amylase of an acidophilic bacterium *Bacillus aciditilicola* (Sharma and Satyanarayana 2013). Four tryptophan residues of saccharifying α-amylase from *Bacillus subtilis* were found to be reactive toward NBS, and these tryptophan residues were confirmed to be located on the surface of α-amylase, not in the substrate binding site (Haruko et al. 1978). The α-amylase from the midgut of armyworm larvae may be similar to the saccharifying α-amylase from *B. subtilis* based on the mixed-type inhibition of NBS.

Analysis of the inhibition kinetics of NBS on α-amylase activity in the midgut of armyworm larvae exhibits positive responses to different chemical inhibitors. Inhibition of midgut α-amylase can strongly affect the growth and development of larvae. Because inhibitors of digestive enzymes could be used to protect plants against insects (Shade et al. 1994), further research on the interactions of α-amylase and its inhibitors will not only facilitate the screening of effective inhibitors but also potentially provide new targets for pest control.
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