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Research Paper

Purification and biochemical characterization of a novel transglutaminase from *Mythimna separata* larvae (Noctuidae, Lepidoptera)

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A B S T R A C T

A novel transglutaminase (MsTGase) from *Mythimna separata* larvae was separated and purified; its biochemical property and enzymatic catalytic activities were investigated. MsTGase was obtained chromatographically by the precipitation of Sephadex G-100 gel and DEAE-Cellulose-52 ion-exchange column with 48-fold purification and a reproducible yield of approximately 12%. Molecular weight of the MsTGase was 63.5 *K*Da and its *N*-terminal amino acid sequence was GKIEEG-LVI. Michaelis constant of the MsTGase for the substrate N-CBZ-Gln-Gly was 12.83 m*M* with a V_{max} of 7.99 U/mL. Optimum conditions for MsTGase activity were at 42 °C and pH7.5. The enzyme didn't possess metal ion at its catalytic active site; its activity could be significantly inhibited by Mg^{2+}, but activated by Ca^{2+}. Chlorpyrifos and spinosad showed a strong potential to increase MsTGase activity, supporting the view that MsTGase was a novel target. Moreover, the formation of intermolecular cross-links of casein and bovine serum albumin polymerized by MsTGase in the presence of DTT was observed. These findings pave the way for future studies on the physiological role of MsTGase and the potential impact of its regulation on MsTGase-associated pest management.

1. Background

Transglutaminase (protein-glutamine gamma-glutamyltransferase, EC 2.3.2.13, TGase) that catalyzes the formation of isopeptide bonds between protein-bound glutamine residues and primary amine groups in animal tissues and body fluids, involves in proteid protein cross-links or amine/protein conjugates (Beninati et al., 2013; Eckert et al., 2014). TGase that presents in most animal tissues and body fluids, responds to several biological processes, including blood clotting, wound healing, epidermal keratinization, erythrocyte membrane stiffening (Theopold et al., 2002; Cortez et al., 2004). TGases in human even have been the target of drug development for the immobilization and/or treatment of various diseases (Yokoyama et al., 2004; Iannaccone et al., 2013; Keillor and Apperley, 2016).

Because of the relatively small quantity obtained and the complex separation and purification procedure required for the enzyme from tissues, TGase derived from insects have been seldom reported and its biochemical property was also limited understanding (Zhou and Yang, 2006; Kaufmann et al., 2012). Recently, TGase expression in the vector *Anopheles gambiae* was found to play a crucial role for mosquito immune responses in restricting *Plasmodium* development (Silveira et al., 2012), and silencing of TGase aborted the wounding-induced mosquito killing of *P. falciparum* (Nsango et al., 2013). The seminal transglutaminase AgTG3 coagulates male *A. gambiae* seminal fluid, and an AgTG3 inhibitor dihydroisoxazole led to a 15% reduction of *A. gambiae* males in mating plug transfer (Le et al., 2014). TGase from *Drosophila melanogaster* that shares similar attributes with the Factor XIIIa from guinea pig liver enacts the hemostatic response of the *Drosophila* immune system (Theopold et al., 2002). Thus, the uses of TGase for haemolymph coagulation, immune responses, even mating reproduction in insects inform a new target for vector control strategies (Shibata et al., 2010; Ichikawa et al., 2011; Lin et al., 2015).

The armyworm *Mythimna separata* Walker, a major pest of grain crops, causes huge losses in food production because of its large population. The application of insecticides is the most important method for decreasing armyworm populations, but the emergence of resistance and resurgence declined incrementally the efficiency of traditional insecticides, such as organophosphate and carbamate (Kikuchi et al., 2012). Therefore, to find new functional targets to improve the controlling effect has become a major issue to be solved. In this paper, a novel MsTGase was separated from *M. separata* tissues and purified chromatographically. Biochemical properties such as the molecular

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weight, the *N*-terminal amino acid sequence and the kinetic parameters were analyzed. The enzymatic property of the MsTGase was clarified under different conditions of pH and temperature as well as fetal ions. The effect of two common-used insecticide chlorpyrifos and spinosad on the MsTGase activity was determined and the polymerization effect of the MsTGase on casein and bovine serum albumin (BSA) was further estimated. The aim was to get details about the property of MsTGase so as to provide an alternative new target for efficient pest management.

2. Materials and methods

2.1. Insect

The armyworm *M. separata* Walker was a laboratory-acclimatized strain that was fed continuously with freshly cut maize seedlings in a conditioned room maintained at 27 ± 1 °C, 70–80% relative humidity, and a photoperiod of 14:10 (L:D) light regime. The fourth instar larvae were collected with starvation for 4 h before the experiments.

2.2. Chemicals and reagents

The gel filtration packing Sephadex G-100 and the ion-exchange packing DEAE-cellulose-52 were purchased from Shanghai Yuanye Co. LTD (China). N_α-carboxybenzoy-L-glutaminyl glycine, L-Glutamic acid γ-monohydroxamate (N_α-CBZ-GIn-Gly), tris, 1,4-dithiothreitol (DTT), bovine serum albumin (BSA), casein, chlopyrifos (98% purity) and spinosad (98% purity) were from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) and other chemicals were commercially available from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All the chemicals used were of analytical grade unless otherwise mentioned.

2.3. MsTGase activity assay

MsTGase activity was determined using hydroxamate formation with N-CBZ-Gln-Gly as substrate (Grosswicz et al., 1950; Cui et al., 2007). A 100 μl substrate solution (pH 8.0) containing 0.2 M Tris-HCl buffer, 0.1 M hydroxylamine, 0.01 M reduced glutathione and 0.4 M N-CBZ-Gln-Gly, was mixed with 50 μl of enzyme solution. The reaction mixture was incubated at 37 °C for 10 min followed by adding 100 μl of ferric chloridetrichloracetic acid reagent (consisting of 1 vol 12% HCl, 1 vol 12% trichloracetic acid and 1 vol 5% ferric trichloride solution). The mixtures were centrifuged at 8000 g and 4 °C for 5 min, and the supernatants were used to measure the absorbance at 525 nm under the microplate reader (Biotek Synergy). The calibration was performed using L-glutamic acid c-monohydroxamate as standard. One unit of transglutaminase was defined as the amount of enzyme which causes the formation of 1.0 μM L-glutamic acid γ-monohydroxamate per minute at 37 °C. The amount of protein was determined by the Bradford method with bovine serum albumin as the standard (Bradford, 1976).

2.4. Purification of M. separata MsTGase

Enzyme preparation was carried out according to Singh and Mehta (1994) with some modification. After weighted, five hundred of armyworm larvae were homogenized in 20 mM Tris·HCl buffer containing 2 mM l,4-dithiothreitol (pH 8.0) for 20 min on ice, and the crude extract was then sonicated continuously for three 1-min periods with 5-min intervals using a pre-chilled small probe of the W-225 ultrasonic processor (Michigan, USA). The homogenates were centrifuged at 12,000*g* and 4 °C for 20 min and the supernatant was precipitated at a 55% ammonium sulfate overnight. The precipitate was collected by centrifugation (12,000*g*, 4 °C) for 30 min and then dissolved in Tris·HCl buffer (pH 8.0) for dialysis overnight at 4 °C until no SO_4^{2-} could be detected out by using 1.0% $BaCl_2$.

Table 1
Purification of *M. separata* MsTGase in procedure **I.**

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Cumulative purification (n)	Yield (%)
Crude enzyme	211.186	2111.969	0.100	/	100
Ammonium sulfate precipitation	190.032	912.948	0.208	2.082	89.98
Sepadex G-100	85.750	150.896	0.568	5.683	40.60
DEAE-cellulose-52	26.794	5.541	4.836	48.362	12.69

2.4.1. Purification procedure I

The dialyzed enzyme solution was applied to a Sephadex G-100 chromatography column (1.6 × 100 cm) pre-equilibrated with 20 mM Tris·HCl buffer A (pH 8.0). The column was washed with buffer A at a flow rate of 0.5 ml/min and the active fractions were collected. Then the active fractions was applied to a DEAE-cellulose-52 chromatography column (2.6 × 20 cm) pre-equilibrated with 20 mM Tris·HCl buffer B (pH 8.5). The column was washed extensively with buffer B and eluted with a linear gradient of 0.0–1.0 M NaCl in buffer B at a flow rate of 1.0 ml/min. All steps were performed at 4 °C. Fractions with MsTGase activity were collected and then freeze-dried to a powder.

2.4.2. Purification procedure II

The dialyzed enzyme solution was applied to a DEAE-cellulose-52 chromatography column (3.9 × 20 cm) previously equilibrated with 20 mM Tris·HCl buffer C (pH 7.8). The column was washed extensively with buffer C and eluted with a linear gradient of 0.0-1.0 M NaCl in buffer C at a flow rate of 1.0 ml/min. The active fractions was then applied to a DEAE-cellulose-52 chromatography column (2.6 × 20 cm) pre-equilibrated with 20 mM Tris·HCl buffer D (pH 8.25). The column was washed extensively with buffer D and eluted with a linear gradient of 0.0-1.0 M NaCl in buffer D at a flow rate of 1.0 ml/min. The next fractions with TGase activity were pooled and further purified by gel filtration on a column of Sephadex G-100 (1.6 × 100 cm) pre-equilibrated with buffer A. The column was washed with buffer A at a flow rate of 0.5 ml/min. All steps were performed at 4 °C. The active fractions were collected and then freeze-dried to a powder.

2.5. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE)

The purified MsTGase was analyzed by SDS–PAGE (Yazdani et al., 2016). A 12.0% separating gel was used. The proteins were stained with a 0.1% solution of Coomasiie brilliant blue R-250. The gel image was photographed using the Gel Image System of Vilber Lourmat (Shanghai, China). The *N*-terminal sequence of the purified enzyme was analyzed by Sangon Bioteck Co., LTD (Shanghai, China).

2.6. Biochemical characterization on M. separata MsTGase

Thermal stability of MsTGase was determined by pre-incubating the enzyme in substrate solution at different temperatures in the range 27–57 °C for 120 min. The pH stability of MsTGase was determined by pre-incubating the enzyme in substrate solution with pH values as 3.5–8.5 at 37 °C for 30 min and 120 min, respectively. The effect of metal ions and EDTA on MsTGase activity was determined by pre-incubating the enzyme in substrate solution with the different concentrations of metal ions and EDTA at 37 °C for 30 min. The remaining activity of MsTGase was measured using the above standard protocol. MsTGase activity that was measured using the normal substrate solution was used as the negative control. Moreover, kinetic parameters were further determined in the reaction mixtures containing different

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