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Functional analysis of five trypsin-like protease genes in the oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae)☆

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ABSTRACT

Insect midgut proteases catalyze the release of free amino acids from dietary proteins and are essential for insect normal development. To date, digestive proteases as potential candidates have made great progress in pest control. To clarify the function of trypsin-like protease genes in the digestive system of *Bactrocera dorsalis*, a serious pest of a wide range of tropical and subtropical fruit and vegetable crops, five trypsin genes (*BdTry1, BdTry2, BdTry3, BdTry4* and *BdTry5*) were identified from transcriptome dataset, and the effects of feeding condition on their expression levels were examined subsequently. RNA interference (RNAi) was applied to further explore their function on the growth of *B. dorsalis*. The results showed that all the *BdTrys* in starving midgut expressed at a minimal level but up-regulated upon feeding (except *BdTry3*). Besides, RNAi by feeding dsRNAs to larvae proved to be an effective method to cause gene silencing and the mixed dsRNAs of the five *BdTrys* slowed larvae growth of *B. dorsalis*. The current data suggest that trypsin genes are actively involved in digestion process of *B. dorsalis* larvae and thereafter play crucial roles in their development.

1. Introduction

The oriental fruit fly, *Bactrocera dorsalis* (Hendel) is considered as one important agricultural and economical pest in most of the tropical and sub-tropical areas of the world. It is a typical polyphagous pest with the larval stages feeding on a wide range of >250 kinds of fruits and vegetables, such as guava, carambola, citrus, mango, coffee and chili pepper [1,2]. Direct fruit damage, fruit drop, and loss of export markets through quarantine restrictions have made *B. dorsalis* as an important invader [3].

Serine proteinases (SPs) are a supergene family including chymotrypsin, trypsin, thrombin, subtilisin, plasmin, elastase and other related enzymes, which are also the most important digestive enzymes in insects [4,5]. They are generally active in the pH 7.0–10.0 range and can be inhibited by soybean inhibitors Bowman-Birk and Kunitz [6]. In *Drosophila melanogaster*, a total of 204 SPs-related genes have been identified, which undertake the hydrolysis of proteins and other critical roles in diverse biological processes, such as development and immune responses [7]. The evolution of SPs in highly conserved amino acids that form crucial structural and chemical elements of catalytic apparatus results in functional diversity [8]. Therefore, aside from their involvement in protein digestion, blood coagulation, immune responses, signal transduction and hormone activation [9], SPs also play major roles in embryo development, tissue reconstruction and cell differentiation. Thus, research on SPs is of great value and interest in insect SPs has primarily focused on their functions.

Trypsin protease is one of the major members in SPs family, and its function reflects in two aspects, including the major alkaline proteolytic enzyme in animal digestive tract and one signal media activating other pepsinogens secreted by pancreas to maintain the digestive function [10]. In *Daphnia magna*, trypsin and chymotrypsin activities accounted for 75–83% of the proteolytic activity of gut contents [11]. Besides, examination of digestion mechanism of the cutworm *Spodoptera litura* indicated that trypsin inhibitor AeTI could result in the decrease for both the larval body weight and survival of early instar larvae [12].

In dipteran insect, transcriptomic analysis of the third instar larval midgut of *D. melanogaster* showed that serine proteases were the main proteolytic enzymes detected [13]. As for *Ceratitis capitata*, the larvae mainly utilized serine proteinases, such as trypsin/chymotrypsin, and the adult only used chymotrypsin-like enzymes in their digestive process [14]. In *Aedes aegypti*, the female-specific early trypsin protease could transcriptionally activate the late trypsin form, which was the major midgut endoprotease involved in the blood meal digestion [15]. The further study confirmed that endoproteolytic serine type proteases within the S1.A subfamily undertook the blood-meal digestion in *A. aegypti* [16]. Therefore, among all the endoproteolytic enzymes, serine proteases may be the principle enzymes in dipteran digestive system.

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Although genomic and transcriptomic research on proteolytic enzymes have made great achievements in a diversity of insect species, relatively little work has been done on *B. dorsalis* digestive system. In our previous study about trypsin-like protease genes in *B. dorsalis*, all the five genes were significantly up-regulated in the larvae midgut after the ingestion of artificial diet mixed with cypermethrin and the larval development was delayed, which showed their indirect function of *BdTrys* in insecticide tolerance [17]. In the current study, the functions of trypsin genes in the digestive system of *B. dorsalis* were further explored, and their possible roles in diet digestion and growth development were assessed subsequently. Serine proteases activity using BApNA as substrate in different life stages and tissues of *B. dorsalis* was measured, and the transcript levels of these five *BdTrys* in larval stage and under feeding condition were examined by quantitative real time PCR (qRT-PCR). Furthermore, RNAi by dsRNA ingestion was applied to knock down the *BdTrys* in *B. dorsalis* larvae and the results of RNAi would be an attempt to better characterize this pest for improving control strategies. Taken together, the current study should help to clarify the functions of trypsin-like protease genes in the digestive system of *B. dorsalis*, and provide useful information on using RNAi strategy for the control of this pest.

2. Materials and methods

2.1. Insect and chemicals

The laboratory stock of *B. dorsalis* (Hendel), originally collected in 2008 from Hainan province, China, was cultured in the insectary at 27 ± 1 °C, 70% ± 5% relative humidity, and 14 h light: 10 h dark photoperiod. Larvae and adults were reared on artificial diets as described previously [18].

Synthetic substrates N-α-Benzoyl-DL-arginine *p*-nitroanilide (BApNA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemicals for artificial diets and the rest of the insect rearing materials were purchased locally.

2.2. Preparation of enzyme extracts

Enzyme extracts were prepared from different life stages (larvae, pupae and adults) and different tissues (midgut, fatbody and integument) of 5-d-old larvae. Samples in different life stages included 10 larvae (5-d-old), 10 pupae (5-d-old), and 10 adults (2-day-old), respectively, and three biological replications were carried out for each sample. Different tissues were achieved by dissecting flies on ice under a stereomicroscope (Olympus SZX12, Tokyo, Japan). A total of 40 larvae tissues were collected in each sample and were frozen in liquid nitrogen, homogenized in 0.1 M Tris-HCl (pH 8.2, containing 10 mM $CaCl_2$). Subsequently, the homogenate was centrifuged at 12,000 g for 5 min at 4 °C, then filtered through a 0.25 μm filter membrane. The supernatant was centrifuged again at 17,500 g for 25 min at 4 °C, and 2 mL of the final supernatant was used as the enzyme source.

2.3. Protein content and protease activity

Protein content was determined using bovine serum albumin as the standard [19]. Enzymatic assays using BApNA as substrate were performed in order to estimate the serine proteases activity. Serine proteases activity were measured at a final concentration of 1.7 mM glycine sodium-hydroxide buffer (pH 10.5) containing 7.4% dimethyl sulfoxide (DMSO, *v*/v) at 37 °C. The absorbance of the solution was measured at 410 nm [20].

2.4. RNA isolation and first-strand cDNA synthesis

The midguts of *B. dorsalis* larvae were dissected under a stereomicroscope (Olympus SZX12, Tokyo, Japan) and placed in a 1.5 mL centrifuge tube containing RNA store reagent (Tiangen, Beijing, China). RNAs from whole bodies of larvae in different ages and midgut in control and treatment were extracted, respectively, using TRIzol kit (Invitrogen, Carlsbad, CA). Three biological replicates were conducted for every treatment. RNAs were quantified by measuring the absorbance at 260 nm using a NanoVue UV–Vis spectrophotometer (GE Healthcare Bio-Science, Uppsala, Sweden). The purity of all RNA samples were assessed based on absorbance ratio at $OD_{260/280}$ and $OD_{260/230}$, and the integrity of RNA was checked by a 1% agarose gel electrophoresis.

The genomic DNAs from the total RNA samples were removed by using DNase I (Promega, Madison, WI, USA) and then first strand cDNA was synthesized using PrimeScript® RT reagent Kit (Takara, Dalian, China) according to the manufacturer's protocols.

2.5. Expression of digestive proteases genes

Expression patterns of five trypsin genes through larval stage of *B. dorsalis* and under both feeding and starvation conditions were analyzed by qRT-PCR. The α-tubulin was selected as reference gene for the analysis [21].

qRT-PCR was conducted on a Mx3000P thermal cycler (Stratagene, La Jolla, CA, USA) in a total reaction volume of 20 μL containing 500 ng cDNA, 10 μL GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), 10 pmol of each primer (Table S1), and double distilled water. All reactions were performed under the following conditions: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. To verify the specificity of the amplicon for each primer pair, a dissociation curve was included from 60 to 95 °C at the end of each qRT-PCR run. A cDNA dilution series (1, 1/3, 1/9, 1/27, and 1/81) with the sample cDNA was used to construct the standard curve and calculate the efficiency of amplification. The PCR efficiencies of each gene and the reference were calculated using Mxpro-Mx3000P version 3.20 (Agilent-Stratagene, Santa Clara, CA, USA). Relative expression levels were calculated by the comparative CT method [22].

2.6. 'Starvation and feeding' treatment

In the 'starvation and feeding' experiment, about 800 4-d-old larvae were collected and placed into culture dishes for starvation. Twenty-four hours later, 120 of these hungry larvae were chilled on ice for 30 min and dissected under a stereomicroscope (Olympus SZX12, Tokyo, Japan). The midguts were achieved on ice (40 larvae midguts per sample, three biological replicates), placed in a 2.0 mL diethyl pyrocarbonate (DEPC)-treated centrifuge tube containing RNA storage reagent (Tiangen, Beijing, China), and immediately frozen in liquid nitrogen and stored at −80 °C. The corresponding expression levels of five *BdTrys* were quantified as control. Other larvae after starvation were resumed to feed on artificial diet, and the expression levels of these five *BdTrys* were determined at 6 h, 12 h, 18 h and 24 h post-meal, respectively.

2.7. dsRNA synthesis and RNAi treatment

Primers used to synthesize dsRNA are listed in Table S2. PBS (pH 7.3) was used as control. All the reagents and enzymes used for the dsRNA synthesis were from MEGAscript® RNAi kit (Ambion, Carlsbad, CA, USA). The size and integrity of the dsRNA products were confirmed by electrophoresis on a 1% agarose gel. The final concentration of dsRNAs used in this experiment was about 3 μg/μL. For the mixture treatment, a high concentration (about 10 μg/μL) of each *BdTry* dsRNA was achieved and all the dsRNA were subsequently mixed together, so that

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