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Biochemical and kinetic characterization of the digestive trypsin-like activity of the lesser grain borer *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae)

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ABSTRACT

The digestive trypsin-like activity of the lesser grain borer *Rhyzopertha dominica* was characterized in some of its biochemical and kinetic properties. The enzyme activity from insect midguts was isolated using hydrophobic interaction chromatography with phenyl-sepharose CL-4B. Eight bands (identified from A through H) with caseinolytic activity and molecular weights in the range of 22–51.3 kDa were detected by zymography in casein-polyacrylamide gels. The strongest bands were D, G, and H, and showed estimated molecular weights of 33.6, 25.4, and 22 kDa, respectively. In-gel inhibition of caseinolytic activity showed that the serine protease inhibitors TLCK and SBTI inhibited all the proteases, except E. In-vitro inhibitory assays showed that SBTI and TLCK suppressed the BA*p*NAase activity by 92.3% and 79.2%, respectively, indicating the presence of serine proteases. Wheat hexaploid albumin extracts were highly effective in inhibiting all the proteolytic activity. The chymotrypsin inhibitor TPCK did not affect the BA*p*NAase activity, indicating that the proteolytic activity in *R. dominica* belongs to the trypsin-like type. With BA*p*NA as the substrate, the proteolytic activity was high across a broad pH range of 6–11 with two peaks of maximum activity at pH 8 and 10 with an optimum temperature of 50 °C. SBTI inhibited the BA*p*NAase activity with IC_{50} and K_i values of 0.02 μM and 1.17×10^{-8} M, respectively. The kinetic constants K_m and V_{max} were 0.07 mM and 2.8 mM/min, respectively. The activation energy (E_a) for BA*p*NA hydrolysis was 33.5 kJ/mol. The results of this study confirm that *R. dominica* rely on serine protease activity for food digestion.

1. Introduction

Insect proteases have attracted attention due to their role in plant–insect interactions (Reeck et al., 1999). Significant advances have been made in characterizing the different properties of the proteases from different stored-product pests, which have contributed to a better understanding of the way they work on hydrolyzing proteins from different sources (Houseman and Thie, 1993; Elpidina et al., 2005; Hosseininaveh et al., 2007; Oppert et al., 2010; Vinokurov et al., 2006). The coleopteran *Rhyzoperta dominica* is a primary pest that feeds in intact wheat kernels under storage causing significant economical losses (Zhu and Baker, 1999; Cinco-Moroyoqui et al., 2008). The developing larva feeds inside grain kernels and cause weight loss and damage to the germ and endosperm in wheat (Janarthanan et al., 2008). The female *R. dominica* lays eggs on the exterior of the kernels, and the larvae bores through the hull and actively feeds inside the kernel where they reach the adult stage creating a large exit hole in the kernel causing significant damages to the kernels (Chanbang et al., 2007). Several studies have related certain physical characteristics of the wheat kernel with its susceptibility to the feeding activity of *R. dominica* (McGaughey et al., 1990; Toews et al., 2000). Proteinaceous amylase inhibitors found in the wheat kernel are effective in suppressing the amylase activity of *R. dominica* (Baker, 1991) and variations in amylase inhibitory activity of infested wheat cultivars were found to negatively correlate with F-1 progeny sizes (Cinco-Moroyoqui et al., 2006).

Proteases from stored-product pests play an important role in the digestion process of dietary proteins and some have been studied in detail (Houseman and Thie, 1993; Reeck et al., 1999;

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Elpidina et al., 2005; Oppert et al., 2005; Vinokurov et al., 2006; Hosseininaveh et al., 2007). Although some coleopterans such as *Tenebrio molitor* (Vinokurov et al., 2006), *Tribolium castaneum* (Murdock et al., 1987; Oppert et al., 2003) and *Callosobruchus maculatus* (Kitch and Murdock, 1986; Silva et al., 2001) rely mainly on cysteine proteases for protein digestion, others like *Trogoderma granarium* (Hosseininaveh et al., 2007) and *R. dominica* (Zhu and Baker, 1999) does it on serine proteases. Other stored-product pests such as *Sitophilus zeamais* (Silva et al., 2010a, 2010b), *Prostephanus truncatus* (Houseman and Thie, 1993), and *Zabrotes subfasciatus* (Silva et al., 2001) use a combination of cysteine and serine proteases for food digestion.

Studies have demonstrated that the presence of protease inhibitors in the diet of stored-product pests lead them to produce insensitive proteases as a mechanism of adaptation to overcome the effect of those inhibitors (Oppert et al., 2005; Amorim et al., 2008). Serine proteases are common in many insects and are involved in the digestion of dietary protein (Reeck et al., 1999; Terra and Ferreira, 1994), protein activation in the melanization cascade (Terra and Ferreira, 1994; Lee et al., 2002; Kan et al., 2008) and antibacterial activity and insect immune response (Ma and Kanost, 2000). Among the digestive enzymes of *R. dominica*, only the amylases of this coleopteran has been studied in detail (Cinco-Moroyoqui et al., 2006, 2008), whereas those belonging to the proteolytic activity has been relatively little studied (Zhu and Baker, 1999). The objectives of the present study were to characterize certain biochemical and kinetic properties of the *R. dominica* digestive trypsin-like proteolytic activity.

2. Materials and methods

2.1. Insect rearing

A population of 100 unsexed adults of *R. dominica* was used to infest 1 kg of hard winter wheat and held at 27 °C and 70% RH (12:12 photophase period) in a rearing chamber for a 15-day oviposition period. The adults were sieved and the wheat samples were incubated under the same conditions for 45 days. The progeny emerged was collected and used as the source of proteolytic activity.

2.2. Preparation of proteolytic extracts

Intestinal tracts were removed from *R. dominica* adults by pressing their abdomen and removing the head with the help of forceps. The tracts were placed in a mortar seated in an ice-water bath containing chilled assay buffer (100 mM phosphate buffer, pH 8) and homogenized. The mixture was centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was removed and further filtered through 0.45 μm Cameo 17N nylon syringe filters (Osmonics Laboratory Products, Minnetonka, MN). The protease homogenate was saturated with 25% ammonium sulfate and left for 1 h. The precipitate formed was pelleted by centrifugation at 10,000 × *g* for 60 min, re-dissolved in 40 mL of assay buffer, and filtered through 0.45 μm nylon filters. Ten milliliters (equivalent to approximately 45 mg protein) of the resulting fraction was loaded onto a 1.2 × 40 cm phenyl sepharose CL-4B column (Sigma Aldrich Chemical Co., St. Louis, MO, USA) equilibrated with assay buffer containing 25% (w/v) saturation of ammonium sulfate. After all the unbound protein was removed from the column with equilibration buffer, the proteolytic activity was eluted with a linear gradient of ethylene glycol (0–50%; 400 mL) in assay buffer without ammonium sulfate. The flow rate was 24 mL/h and fractions of 3 mL were collected. Fractions showing azocaseinolytic and BA*p*NAase activity were pooled and concentrated in an Amicon ultrafiltration cell (Fisher Co.) under a stream of nitrogen to a final volume of approximately 50 mL. The flow rate was 1.0 mL/min at 4.0 psi through a YM-10 Millipore ultrafiltration membrane 44.5 mm in diameter and a nominal MW limit of 10 kDa.

2.3. Protease assay

Total proteolytic activity in crude extracts and in chromatographic fractions was determined using 2% (w/v) azocasein as substrate (Araújo et al., 2008). The reaction mixture contained 100 μL enzyme extract, 100 μL of phosphate buffer (0.01 M phosphate buffer, pH 8, containing 1 mM dithiothreitol or 1 mM β-mercaptoethanol), and 300 μL substrate. The mixture was incubated at 37 °C for 60 min for azocasein hydrolysis. The reaction was stopped by adding 500 μL of 25% (w/v) trichloroacetic acid. The samples were rested on ice for 15 min for protein precipitation and centrifuged for 5 min at 10,000 × *g* at 4 °C for protein removal. The supernatants were combined with 300 μL of 1 N NaOH and their absorbance was read at 428 nm. One unit of azocaseinolytic activity was defined as the change in 0.01 absorbance units under the conditions of the assay.

Trypsin-like activity in crude extracts and in chromatographic fractions was determined using N-α-benzoyl-DL-arginine *p*-nitroanilide (BA*p*NA) as substrate (Hosseininaveh et al., 2007). Usually, 75 μL of the enzyme preparation was combined with 325 μL of 1 mM phosphate buffer, pH 8, at 37 °C. The reaction was started by adding 200 μL of 1 mM BA*p*NA and stopped after 10 min by the addition of 150 μL of 30% acetic acid. The released *p*-nitroaniline was estimated at 410 nm. One unit of proteolytic activity was defined as the amount of enzyme causing an increase in absorbance of 0.01 at 410 nm under the conditions of the assay.

2.4. Protein determination

Protein content of the insect proteolytic crude extract and wheat albumin extracts were determined using the dye binding assay of Bradford (1976) with bovine serum albumin as standard.

2.5. Polyacrylamide gel electrophoresis (PAGE)

Crude proteolytic homogenates and pooled fractions from hydrophobic interaction chromatography were analyzed under non-denaturing conditions (Laemmli, 1970). Bands of protein were visualized by a silver nitrate method (Blum et al., 1987).

2.6. Zymography analysis

For zymography, protease aliquots (approximately 2 units of BA*p*NAase activity) were combined with equal volumes of sample buffer containing no β-mercaptoethanol and were not heat-treated. Thirty microliters of the mixture was loaded onto a polyacrylamide gel system consisting of a 12% acrylamide separation gel and a 3.83% acrylamide stacking gel, both containing 1.35% bisacrylamide (Laemmli, 1970) without sodium dodecyl sulfate. The protein separation was done under non-denaturing conditions at a constant voltage of 200 V for 45 min. The running buffer was 25 mM Tris–HCl and 192 mM glycine, pH 8.3. After electrophoresis, the gel containing the proteases was recovered and placed onto a polyacrylamide gel containing 0.33% (w/v) casein co-polymerized with the acrylamide matrix following the procedure of Heussen and Dowdle (1980). Both gels were transferred into a plastic container that was floated for 2 h in a water bath set at 37 °C to allow casein digestion. After that, the gel containing the co-polymerized casein was recovered and stained for 2 h with 0.1% (w/v) Amido black in 7% acetic acid (v/v). Destaining was