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CHARACTERIZATION OF TRYPSIN-LIKE ENZYMES FROM THE MIDGUT OF *MORIMUS FUNEREUS* (COLEOPTERA: CERAMBYCIDAE) LARVAE

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Abstract — The pH along the midgut of *M. funereus* larvae had different values, being acidic in the anterior section and basic in the middle and posterior sections. Elastase- and chymotrypsin-like activities were highest in the middle, low in the anterior, and negligible in the posterior section of the midgut. Trypsin-like activities were detected along the whole midgut, with more than 90% of activity in the anterior section. The level of elastase- and chymotrypsin-like activity was very low compared to trypsin-like activity. In the anterior section of the midgut, two isoforms of trypsin-like enzymes were found, both being basic and almost completely inhibited by benzamidine.

Key words: Cerambycidae, *Morimus funereus*, trypsin, midgut peptidases, synthetic substrate, zymogram

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INTRODUCTION

All classes of digestive peptidases that have been identified in verterbrates also occur in insects (Reeck et al., 1999). Among serine peptidases, trypsin- and chymotrypsin-like enzymes have been most frequently detected in Lepidoptera and Coleoptera, while elastase-like activity is less presented. Trypsins (3.4.21.4) are serine peptidases that preferentially cleave protein chains on the C- terminus of basic amino acids such as arginine or lysine. Digestive enzymes, trypsin-like, have been found in almost all insect species tested (Terra and Ferreira, 1994). Most insect trypsins are 20-35 kDa. These enzymes are most active at alkaline pH, are not activated by calcium ions, and are sensitive to natural trypsin inhibitors.

Peptidases has been the least studied in xylophagous insects, which usually belong to different families of the order Coleoptera, including Buprestidae, Cerambycidae, Tenebrionidae, Platypodidae, Ipidae, and Bostrychidae. Some of these insects are severely damaging, high-density pests. However, there are also generalists that are interesting from a theoretical point of view due to higher plasticity of behavioral and dietary ecology.

Of these generalists, the cerambycid beetle *M. funereus* inhabits an environment rich in deciduous and coniferous trees and has a long life span with development over a 3-4 year period. Tree mortality is normally not associated with long-horned beetle infestation, although damage to oak lumber may be economically important throughout its range.

Earlier studies performed on *M. funereus* larvae focused primarily on the role of protein and amino acid metabolism during thermal and diet-induced stress (Ivanović et al., 1975, 1988, 2002; Nenadović et al., 1994). The diversity of peptidases in the midgut of *M. funereus* larvae was described previously (Đurđević et al., 1997; Božić et al., 2003, 2008a). Purification and properties of midgut α-amylase were also described (Dojnov et al., 2008), as was purification of the major LAP and its enzymological characteristics and molecular properties (Božić et al., 2008b).

The present study was performed to extend our knowledge of the distribution of trypsin-like pepti-

dases along the midgut in order to better understand the biochemical organization of digestive process in *M. funereus* larvae.

MATERIALS AND METHODS

Reagents

All reagents and solvents used were of the highest available purity and at least analytical grade. Unless otherwise stated, they were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St.Louis, MO, USA). Gelatin was from Kemika (Zagreb, Croatia).

Insects

Actively feeding sixth-instar *M. funereus* larvae were used in the experiments. Larvae were continuously reared at 23°C and at approximately 80% humidity (Ivanović et al., 1989) using a modified artificial diet for *Drosophila* sp. (Roberts, 1986).

Preparation of crude midgut extracts

After decapitation, the midgut was dissected out on ice and intestinum was cut into three equal pieces: anterior, middle, and posterior. The pH value was determined in all three sections of midgut using a universal pH detector (Merck, Darmstadt, Germany). Midgut sections were weighed and homogenized using a pre-chilled mortar and pestle in 2 volumes (g/ml) of ice-cold 0.9% NaCl and 20 mM acetate buffer, pH 6.0, with the addition of quartz powder. The homogenate was centrifuged for 2 min at 14,500 rpm at 4°C. Lipids were removed by combining the resulting supernatant with an equal volume of carbon tetrachloride followed by centrifugation for 2 min at 14,500 rpm at 4°C. This procedure was repeated twice. The concentration of proteins was determined by the Bradford assay with bovine albumin as a standard (Bradford, 1976) before dividing the concentrated supernatant into smaller aliquots for storage at -20°C.

Peptidase activity in the presence of specific chromogenic substrates

The activities of trypsin-, chymotrypsin-, and elastase-like enzymes were determined in crude extracts of different sections of midgut using specific chromogenic substrates (Lee and Anstee, 1995). Reaction mixtures contained 5 µl of the fraction in 0.5 ml of the corresponding buffer: 1.0 mM BapNA in 3% DMF for trypsin-like; 1.0 mM BTpNA in 3% dimethylformamide (DMF), 1.0 mM SFpNA in 3% DMF, and 1.0 mM GFpNA in 3% DMF for chymotrypsin-like; and 1.0 mM SA_3pNA in 3.5% DMF for elastase-like enzymes. Assays were performed at 30°C. All enzymatic reactions were stopped by adding 0.1 ml of 30% acetic acid. The concentration of the resulting p-nitroaniline was estimated by measuring absorbance at 410 nm (Erlanger et al., 1961).

Trypsin-like activity in the presence of specific inhibitors

Peptidase inhibitors were prepared as stock solutions in dimethylsulfoxide (DMSO) and added individually to crude extract prior to the addition of substrates. The crude extract of the anterior section of intestinum was treated with specific inhibitors of trypsin (benzamidine) and cysteine peptidases E-64 [trans-epoxysuccinyl-L-leucylamido(4-guanidinio)-butane]. Reaction mixtures contained 5 µl of crude extract in 0.5 ml of the corresponding buffer, 1.0 ml BApNA in 3% DMF, and inhibitor in concentrations of 10 µM (E64) and 2 mM (benzamidine).

Zymogram analysis after isoelectric focusing of anterior midgut

Isoelectric focusing (IEF) was performed using the Multiphor II electrophoresis system (GE Healthcare) according to the maunfacturer's instructions. Focusing was carried out in 7.5% acrylamide gel with ampholytes in a pH range of 3.0-10.0 at 7 W constant power for 1.5 h at 10°C. After focusing, gels were printed onto 7.5% acrylamide gel containing 0.1% gelatin (Božić et al., 2008a) for 1 h. Gels were stained with CBB R-250, after which peptidase activities were visible as clear bands on a dark background.

After focusing, gels were also printed onto nitrocellulose mebrane for 20 min. Trypsin-like enzymes were detected on the membrane using BApNA by the zymogram procedure (Božić and Vujčić, 2005).

RESULTS

The pH along the midgut of the *M. funereus* larvae had different values, being acidic in the anterior and basic in the middle and posterior (Table 1).

Proteolytic activities of different peptidases in different sections of the midgut were obtained using specific chromogenic substrates (Table 2). Enzyme activity was expressed in *U*, defined as the amount of enzyme hydrolyzing 1 μmol of p-nitroanilide at 30°C.

Determination of trypsin-like enzyme activity in crude anterior midgut extract was performed in the presence of specific peptidase inhibitors, viz., benzamidine and E64 for serine-peptidases and cysteine peptidases, respectively (Table 3).

By zymogram analysis of peptidase activities in the crude anterior midgut extract after IEF on PAA-gelatin gel, several peptidases throughout the broad range of pI values were detected (Fig. 1). Peptidases appeared as clear bands of enzyme activity on a dark background.

Table 1. pH values of homogenized sections of midgut extracts of *M. funereus* larvae.

Section of midgut	anterior	middle	posterior
pH	5.5-6.0	8.0-8.5	8.5-9.0

Table 2. Activities of midgut peptidases of *M. funereus* larvae determined using specific chromogenic substrates.

Substrate	Section of midgut		
	anterior	middle	posterior
	(U/L)	(U/L)	(U/L)
BA*p*NA	1172.2	97.3	18.2
SA_3*p*NA	9.1	54.4	0
BT*p*NA	1.6	9.8	0.7
SF*p*NA	0	0	0
GF*p*NA	0	0	0

Table 3. Activity of peptidases from the anterior midgut of *M. funereus* larvae in the presence of inhibitors.

	Enzyme activity (U/L)	Realative activity (%)
Control	951.4	100
Benzamidine	124.4	13
E-64	903.8	95

Zymogram analysis of trypsin-like enzymes on nitrocellulose membrane using BApNA as a substrate showed two bands of unequal intensity in the basic region of IEF gel (Fig. 2).

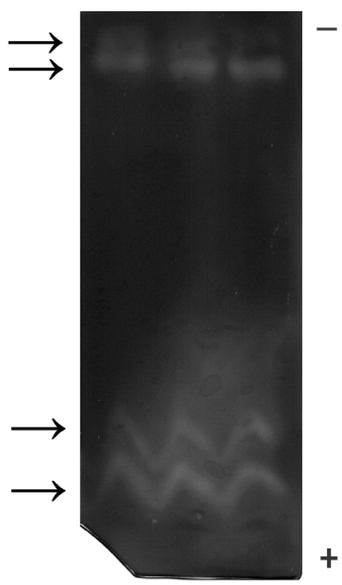

Fig. 1. Detection of peptidases of crude anterior midgut extracts of *M. funereus* larvae after IEF and transfer onto PAA-gelatin gel. The arrows indicate the position of peptidases. (-) pI 10, (+) pI 3.

Fig. 2. Zymogram detection of trypsin-like enzymes in crude anterior midgut extract of *M. funereus* larvae after IEF and transfer onto NC membrane. The arrows indicate the position of basic trypsin-like enzymes. (-) pI 10, (+) pI 3.

DISCUSSION

Peptidases are the major digestive enzymes in the insect gut. They are responsible for providing a continuous supply of essential amino acids and energy from the food source for development. Insects are known to use enzymes like serine peptidases, cysteine peptidases, aspartyl-peptidases, metallo-peptidases, aminopeptidases, and carboxypeptidases, for digestion of food (Terra and Ferreira, 2004).

Midgut contents are acidic in the anterior ventriculus and nearly neutral or alkaline in the posterior ventriculus in most families of Coleoptera (Terra and Fereira, 1994). In contrast, the middle and posterior sections of the *M. funereus* midgut were both alkaline, and the pH gradient was similar to that observed in *Tenebrio molitor* larvae (Vinokurov et al., 2006a). Associated with the observed pH gradients is the occurence of high carbohydrase activity in the anterior midgut and a high peptidase activity in the posterior midgut, probably as a result of instability of carbohydrases in the presence of peptidases (Terra, 1990).

Application of specific chromogenic substrates enabled us to identify trypsin-like, chymotrypsin-like, and elastase-like enzymes in different sections of the midgut of *M. funereus* larvae. Trypsin-like activities were detected along the whole midgut, with more than 90% of activity in the anterior region. As α-amylase was also found in the anterior midgut, Terra's theory of carbohydrase instability in the presence of peptidases needs to be revised. Colepicolo-Neto (1986) published data on trypsin-like enzymes from the midgut of *Pyrearinus termitilluminans* larvae, such enzymes being predominate in the anterior midgut. Similarly, trypsin with Mm 59 kDa was found mainly in the anterior midgut of *T. molitor* larvae (Vinokurov et al., 2006b). The level of chymotrypsin-like and especially elastase-like activity was very low compared to that of trypsin-like activity. These results differ from those published by Božić et al. (2008a) concerning *M. funereus* collected in the field during the autumn season. They reveal the capability of *M. funereus* to modify peptidase expression. In relation to different diets, adequate peptidase with maximum food consumption capacity seems to have prevalence. The diversity of peptidase activities observed in the midgut of *M. funereus* larvae and the flexibility in their expresion depending upon the diet and temperature (Ivanović et al., 1992) provide a basis for selection of proper peptidase inhibitors for insect resistance in transgenic plants.

Class-specific inhibitors enabled us to confirm that trypsin-like serine peptidase has prevalence in the anterior section of the midgut.

In terms of pI value, the *M. funereus* trypsin-like enzyme bears resemblance to other coleopteran trypsin-like enzymes reported thus far (Levinsky et al., 1977). Several trypsin isoforms as well have been found in other colepteran species. Three trypsin-like activities were found in the midgut lumen of *P. termitilluminans* larvae (Colepicolo-Neto et al., 1986) and four trypsin-like enzymes were detected in the midgut of *Costelytra zealandica* (Christeller et al., 1989) and *T. molitor* (Vinokurov et al., 2006a).

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КАРАКТЕРИЗАЦИЈА ТРИПСИНИМА-СЛИЧНИХ ЕНЗИМА СРЕДЊЕГ ЦРЕВА ЛАРВИ *MORIMUS FUNEREUS* (COLEOPTERA: CERAMBYCIDAE)

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pH вредност дуж средњег црева ларви *M. funereus* се разликује, кисела је у региону предњег дела, док је у средњем и задњем делу базна. Еластазама и химотрипсинима слична активност је највећа у средњем делу средњег црева док је у предњем делу детектована мала вредност, а у задњем делу занемарљива. Трипсинима слична активност је детектована дуж целог средњег црева, с тим да се више од 90 % активности детектује у предњем делу средњег црева. Заступљеност еластазама и химотрипсинима сличних ендопептидаза је занемарљиво мала у поређењу са заступљеношћу трипсинима сличних ензима. У предњем делу средњег црева налазе се две изоформе трипсинима сличних ензима, са базним pI вредностима, које су скоро у потпуности инхибиране бензамидином.