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CHARACTERIZATION OF ENDOPEPTIDASES FROM THE MIDGUT OF *MORIMUS FUNEREUS* (COLEOPTERA: CERAMBYCIDAE) LARVAE

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Abstract — Application of specific chromogenic substrates, use of class-specific inhibitors, and zymogram analysis enabled us to identify several peptidase classes in extracts of the midgut of *Morimus funereus* larvae. Zymogram analysis with gelatin as a peptidase substrate and phenylmethylsulfonyl fluoride as an inhibitor showed that serine peptidases were the most abundant endopeptidases in the midgut of *M. funereus* larvae. By zymogram analysis with gelatin as a peptidase substrate and 1,10-phenanthroline as an inhibitor, metallopeptidases were also detected. Analyses of serine peptidases with specific chromogenic substrates revealed dominance of elastase-like peptidases in extracts of the midgut of *M. funereus* larvae, with less pronounced chymotrypsin- and trypsin-like activities.

Key words: Cerambycid beetle, *Morimus funereus,* midgut peptidases, protease inhibitors, synthetic substrate, zymogram

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INTRODUCTION

Peptidases have been the least studied in xylophagous insects, which usually belong to different families of the order Coleoptera, including Buprestidae, Cerambycidae, 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 environments rich in deciduous and coniferous trees and has a long life span with development over a 3- to 4-year period. Tree mortality is normally not associated with infestation by long-horned beetles, although damage to oak lumber may be economically important throughout their range.

All classes of digestive peptidases that have been identified in vertebrates 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 represented. Cysteine peptidases are found in Heteroptera and Coleoptera (Wieman and Nielsen, 1988), aspartic peptidases are rarely detected (Blanco-Labra et al., 1996), and among metallopeptidases, aminopeptidases are the most frequently identified (Novillo et al., 1997).

During evolution, phytophagous insects have adapted to many different trophic niches. Niches such as plant organs differ not only in the content and quality of nutrients, but also in the quantity of secondary metabolites and enzyme inhibitors appearing naturally in response to insect attack (Ryan, 1980; Ahmad et al., 1986; Bernays and Chapman, 1994). In addition to abiotic factors such as temperature, biotic factors such as second-

ary metabolites and enzyme inhibitors influence to a great extent the survival, development, and reproduction of phytophagous insects (Mattson, 1980; Jongsma and Bolter, 1997; Ivanović and Nenadović, 1999).

Previous 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). Purification and properties of midgut α-amylase were also described recently (Dojnov et al., 2008), as was purification of the major LAP and its enzymological characteristics and molecular properties (Božić et al., 2008).

The present study was performed to extend our previous work on the diversity of peptidases in the midgut of *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). Gelatins were from Kemika (Zagreb, Croatia) and Serva (Heidelberg, Germany).

Insects

Three-year-old M funereus larvae with body weight of 1.0-1.5 g were collected in November from oak trees (Quercus species) in the Fruška Gora Mountains. Their midguts were full of food particles when the larvae were dissected, which indicates that they were still feeding. The full length of midguts was between 10.0 and 11.5 cm, and 75% of midgut was mesenteron with epithelial cells that secrete digestive proteinases.

Preparation of crude midgut extracts

After decapitation, the midguts were dissected out on ice, weighed, and homogenized using a pre-chilled mortar and pestle in 5 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 5 min at 5,000 x g 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 5,000 x g at 4ºC. This procedure was repeated three times before the final supernatant was concentrated two-fold by ultrafiltration using Amicon P10 membranes (Millipore, Billerica, MA, USA). 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.

Zymogram analysis after sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Peptidases were assayed by 10% SDS-PAGE with 0.1% gelatin incorporated into the gel as substrate (Heussen and Dowdle, 1980). Samples, crude midgut extracts, were prepared with non-reducing sample buffer [0.0625 M Tris (pH 6.8), 2% SDS, 10% glycerol, and 0.002% bromophenol blue] without heating. Electrophoresis was carried out at a constant current of 10 mA at 4°C. After treatment with Triton X-100 to remove SDS and restore enzyme activity and subsequent CBB R-250 staining, peptidase activities were visible as clear bands on a dark background.

Peptidase inhibitors were prepared as stock solutions in dimethyl sulfoxide (DMSO) and added individually to crude midgut extracts before electrophoresis (Alcala-Canto et al., 2007). To characterize the crude midgut proteolytic activity of *M. funereus* larvae, the following inhibitors were tested: phenylmethylsulfonyl fluoride (PMSF; 5 mM); and N-[N-(L-3-trans-carboxirane-2-carbonyl)-L-leucyl]-agmatine (E64; 10 μM).

Zymogram analysis after isoelectric focusing (IEF)

IEF was performed using the Multiphor II electrophoresis system (GE Healthcare) according to the manufacturer's instructions. Focusing was carried out on 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 1% agarose gel containing 0.5% gelatin and 7.5% PAA gel containing 0.1% gelatin according to Bruno et al. (2002) for 1 h. Gels were then stained with CBB R-250, after which peptidase activities were visible as clear bands on a dark background.

Peptidase inhibitors were prepared as stock solutions in DMSO and added individually to crude midgut extracts before electrophoresis (Alcala-Canto et al., 2007). To characterize the crude midgut proteolytic activity of *M. funereus* larvae, the following inhibitors were tested: PMSF (5 mM), N-α-tosyl-L-lysine chloromethyl ketone (TLCK; 500 μM); tosyl-L-phenylalanine chloromethyl ketone (TPCK; 500 μM); E64 (10 μM); and 1, 10-phenanthroline (10 mM).

Protein fractionation

A 2-ml volume of concentrated midgut extract of *M. funereus* larvae (26.7 mg protein/ml) was loaded onto a Sephadex G-100 column (1.6 x 60 cm, Pharmacia, Uppsala, Sweden) equilibrated with 0.15 M NaCl in 20 mM acetate buffer, pH 6.0, and calibrated with molecular weight markers (BSA, 67 kDa; β-LGA, 36 kDa; and lysozyme, 14.4 kDa). Fractions of 3.4 ml were collected, and A_{280} was monitored. These fractions were also checked for proteolytic activity.

Peptidase activity assays

Total proteolytic activity in fractions obtained by gel filtration was assayed using 1% gelatin dissolved in 50 mM carbonate-bicarbonate buffer, pH 8.5. Reaction mixtures contained 50 μl of gel filtration fractions and 0.5 ml of gelatin solution, with total volume adjusted to 1.0 ml with buffer. After incubation for 2 h at 30°C, the reaction was stopped by adding 0.5 ml of 15% trichloroacetic acid (TCA). The supernatants were used to determine the content of free amino groups by the trinitrobenzenesulfonic acid (TNBS) method (Kwan et al., 1983). Briefly, 0.9 ml of 0.2 M borate buffer, pH 9.2, and 0.5 ml of TNBS (1.18 mg in 1.0 ml of water) were added to 0.2 ml of the sample. The reaction was stopped after 30 min by adding 0.5 ml of 2 M NaH_2PO_4 and 18 mM Na_2SO_3, and A_{420} was measured using a Philips UV-VIS-NIR PU 8630 spectrophotometer. Data are the means of triplicate measurements. Standard errors were within 5% of the means.

Proteolytic activity in the presence of specific chromogenic substrates

The activities of trypsin-, chymotrypsin-, and elastase-like enzymes were determined in gel filtration fractions using specific chromogenic substrates (Lee and Anstee, 1995). Reaction mixtures contained 25 μl of fractions in 0.5 ml of the corresponding buffer: 1.0 mM BApNA in 3% DMF for trypsin-like; 1.0 mM BTpNA in 3% DMF and 1.0 mM SFpNA in 3% DMF for chymotrypsin-like; and 1.0 mM SA_2PLpNA in 4% DMF and 1.0 mM SA_3pNA in 3.5% DMF for elastase-like enzymes. Reaction times for the different substrates were 150, 150, 150, 90, and 150 min, respectively, 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).

RESULTS

Zymogram analysis of peptidase activities in the crude midgut extract from *M. funereus* larvae after SDS-PAGE showed differences in sensitivity according to the type of gelatin used. Gelatin from Kemika (Fig. 1B) was more sensitive to these peptidases than gelatin from Serva (Fig. 1A). Peptidases appeared as clear bands of enzyme activity on a dark background with molecular masses above 60 kDa. The inhibitor of cysteine peptidases E64 (Fig. 1, lane 1) did not inhibit gelatin degradation. In contrast, the serine peptidase inhibitor PMSF (Fig. 1, lane 2) reduced this degradation.

Zymogram analysis of peptidase activities in the crude midgut extract of *M. funereus* larvae after IEF showed differences in sensitivity according to the type of transfer used. Better results, in the terms of clarity, were achieved in printing onto PAA-gelatin (Fig. 2B) gel than in printing onto agarose-gelatin gel (Fig. 2A). Peptidases were detected throughout the whole acid region of IEF gel; still, the most

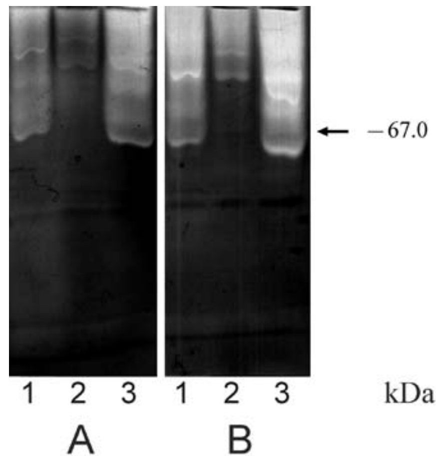

Fig. 1. Zymogram of peptidase activities after gelatin-SDS PAGE and inhibitor treatments. A: Gelatin from Serva. B: Gelatin from Kemika. Lane 1: E-64. Lane 2: PMSF. Lane 3: crude midgut extract of *M. funereus* larvae. Lane kDa: position of standard protein molecular mass.

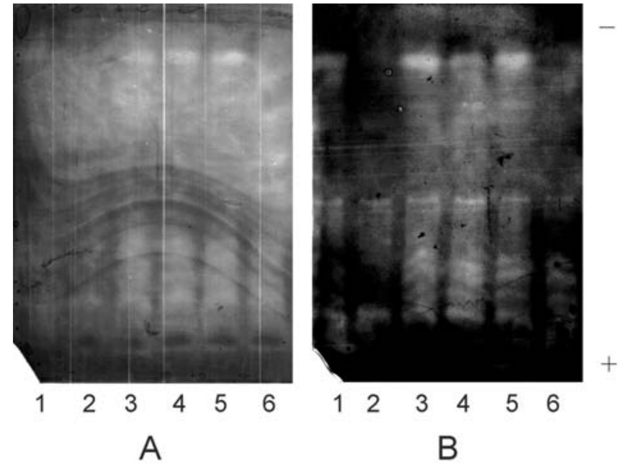

Fig. 2. Zymogram of peptidase activities after IEF and inhibitor treatments. A: Agarose-gelatin gel. B: PAA-gelatin gel. Lane 1: crude midgut extract of *M. funereus* larvae. Lane 2: PMSF. Lane 3: E-64. Lane 4: 1, 10-Phenanthroline. Lane 5: TPCK. Lane 6: TLCK.

intense one was found in the base part of the gel. The serine peptidase inhibitor PMSF (Fig. 2, lane 2) and inhibitor of trypsin activities TLCK (Fig. 2, lane 6) reduced peptidase activity. Peptidase activities were also slightly reduced by the metallo peptidase inhibitor 1,10-phenanthroline (Fig. 2, lane 4).

Elastase-like enzymes were very active in both crude midgut extracts of M. funereus larvae and the fractions obtained by gel filtration on Sephadex G-100 (Fig. 3). There was a minor BApNA-ase activity, but no chymotrypsin-like activity was detected. Peptidases in the fraction with maximal proteolytic activity toward gelatin as a substrate had molecular masses that ranged from 30 to 35 kDa.

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 cysteine peptidases, aminopeptidases, serine peptidases, carboxypeptidases, aspartyl- peptidases, and metallo- peptidases for digestion of food (Terra and Ferreira, 2004).

Applications of specific chromogenic substrates and class-specific inhibitors enabled us to identify several peptidase classes in midgut extracts of *M. funereus* larvae. Among metallo-peptidases, leucyl aminopeptidase was detected (Božić et al., 2003). In order to extend our previous work on the diversity of endopeptidases in the midgut of *M. funereus* larvae and to better understand effects of inhibitors on specific peptidases, zymogram analyses were performed. The method depends upon the fact that gelatins, when incorporated into the polyacrylamide matrix at the time of casting, are retained during subsequent electrophoresis of enzyme samples and serve as satisfactory sequential, *in situ* substrates for the localization of peptidase bands by negative staining.

Peptidases detected in this way had molecular masses above 60 kDa. Low-molecular-weight enzymes, like elastase-like enzymes were not detected, probably due to non-reductive electrophoretic conditions that might cause S-S protein multimerization. Since E-64 did not inhibit the degradation of gelatin and PMSF reduced this degradation, it can be concluded that serine-peptidases are the major endopeptidases in the midgut of *M. funereus* larvae. Peptidase activities were also slightly reduced by 1, 10-phenanthroline, implying that metallopeptidases are likewise part of the peptidase pool of *M. funereus* larvae. TLCK also reduced peptidase activity, but

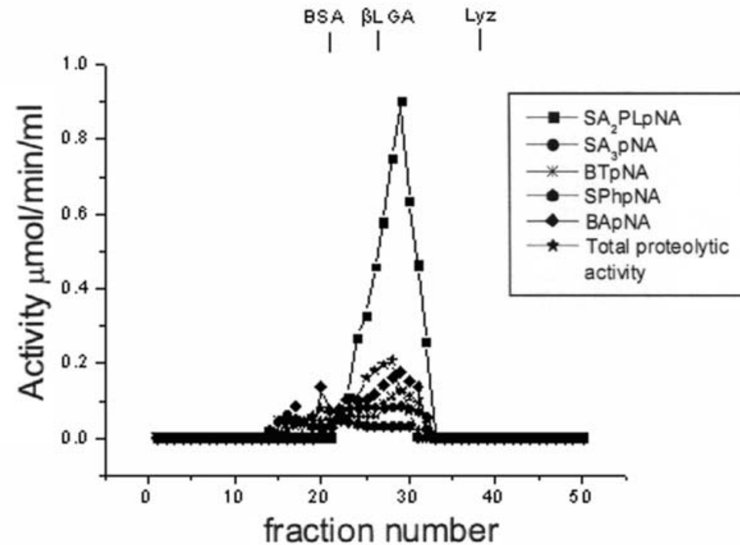

Fig. 3. Endopeptidase activities after fractionation of a homogenized midgut extract of *M. funerues* larvae on Sephadex G-100 column. Elution of molecular weight markers BSA (67 kDa), lactoglobulin β-LGA (36 kDa) and lysozyme (14.4 kDa) occurred where indicated.

since trypsin-like activity was low in the midgut of *M. funereus* larvae, results obtained using the synthetic substrate BapNA suggest that it is not impossible that these "gelatinase" enzymes have some unique characteristics that distinguish them from other trypsins described.

Analyses of serine peptidases revealed the dominance of elastase-like peptidases in the midgut extract of *M. funereus* larvae, with fewer chymotrypsin- and trypsin-like activities. These data agree with results obtained on *Costelytra zealandica* (Christeller et al., 1989), *Melolontha melolontha* (Wagner et al., 2002) and *Helicoverpa armigera* (Telang et al., 2005), insects that have elastase- and trypsin-like proteinases in larval midgut extracts. However, *C. zealandica* and *M. melolontha* had more trypsin-like activity than was found during the present study in midgut extracts of *M. funereus* larvae collected in the field during the autumn season.

In terms of molecular masses, the *M. funereus* elastase-like enzyme bears resemblance to other insect elastase-like enzymes reported thus far. Low molecular masses (24-26 kDa) of elastase were described for *Lymantria dispar* (Valaitis, 1995) and *H. armigera* (Telang et al., 2005). Also, three elastase-like enzymes (with molecular masses of 33, 36, and 38 kDa) were found in the midgut of *M. melolontha* (Wagner et al., 2002).

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КАРАКТЕРИЗАЦИЈА ЕНДОПЕПТИДАЗА СРЕДЊЕГ ЦРЕВА ЛАРВИ *MORIMUS FUNEREUS* (COLEOPTERA: CERAMBYCIDAE)

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Применом специфичних хромогених супстрата, класно-специфичних инхибитора и зимограмске анализе идентификовано је неколико класа пептидаза у сировом екстракту средњег црева ларви колеоптере *Morimus funereus*. Зимограмском анализом са желатином као супстратом и фенилметилсулфонил-флуоридом као инхибитором утврђено је да су серин-пептидазе најзаступљеније пептидазе у екстракту средњег црева ларви *M. funereus*. Зимограмском анализом са желатином као супстратом и 1,10-фенантролином као инхибитором такође су детектоване металопептидазе. Анализом серин-пептидаза, употребом специфичних хромогених супстрата, доказано је да су доминантни еластази-слични ензими у сировом екстракту средњег црева ларви *M. funereus,* док су химотрипсинима- и трипсинима-слични ензими мање заступљени.