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Expression and distribution of cellulase, amylase and peptidase isoforms along the midgut of *Morimus funereus* L. (Coleoptera: Cerambycidae) larvae is dependent on nutrient substrate composition

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ABSTRACT

The influence of diet composition – two substrates, wheat bran and sawdust – on isoform expression of digestive enzymes (cellulase, amylase and peptidase) in the midgut of *Morimus funereus* larvae was examined. Their impact on larval development was demonstrated by measuring the increase of larval weight during development and by analysis of digestive enzymes zymographic profiles, where the expression of cellulase isoforms from *M. funereus* larvae midgut has been examined for the first time in this study. Larvae reared on wheat bran had higher body weight between day 60 and day 100 than larvae reared on sawdust; however, both groups achieved similar body weight after day 110. Wheat bran as substrate induced different cellulase and amylase isoforms. Oak sawdust in substrate acted as inducer of peptidases. The highest cellulase activity and the greatest isoform variability were detected in the midgut extracts of larvae reared on wheat bran. From our results it can be assumed that *M. funereus* endocellulase, amylase and peptidase are secreted in the anterior midgut, and their concentration gradually decreases towards the hindgut.

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1. Introduction

Cellulose, starch and peptides are the most common polymers in the diet of inner bark saproxylic insects. The digestive enzymes responsible for their hydrolysis (cellulase, amylase and peptidase) are very important for the digestion process.

Enzymatic activity against cellulose substrates was detected in digestive fluids of insects belonging to different insect orders (Martin, 1983; Oppert et al., 2010). Cerambycidae survival depends on their ability to digest cellulose. It can be assumed that they have, over the course of evolution, developed one of the most efficient cellulose digestion systems. The origin of cellulase activity in xylophagous ('wood-feeding') coleopteran larvae has been attributed to: fermentative hindgut bacteria, acquired fungal enzymes and endogenous enzyme activity (Scrivener et al., 1997). Cellulases were detected for the first time in *Morimus funereus* larvae midgut long time ago (Ivanović and Barbič, 1966).

From the pool of enzymes known to act on long α-1,4-glucan chains, only α-amylases have been found in insects (Terra et al., 1996). Little is known about the presence and properties of α-amylase in the Cerambycid family (Weber et al., 1985; Dojnov et al., 2008). Amylase from *M. funereus* (Coleopterae, Cerambycidae) larvae midgut has been purified and biochemically characterized previously (Dojnov et al., 2008).

All classes of digestive peptidases that have been identified in vertebrates also occur in insects (Reeck et al., 1999). Serine-type endopeptidases are the primary digestive peptidases in most insect groups (Terra et al., 1996), with the exception of some hemipteran (Houseman and Downe, 1983) and coleopteran species (Božić et al., 2003) in which cysteine, aspartic and aminopeptidases are predominant. It is known from our previous work on *M. funereus* that leucyl aminopeptidases are the major peptidases and serine peptidases of the elastase type are the major digestive endopeptidase in the midgut of *M. funereus* larvae (Božić et al., 2003), while trypsin-like enzymes are the major digestive endopeptidases in the anterior midgut of *M. funereus* larvae (Lončar et al., 2009). We have purified to homogeneity and characterized a leucyl aminopeptidase from the midgut (Božić et al., 2008) and a novel cationic form of trypsin-like activity from the anterior section of the midgut of *M. funereus* larvae (Lončar et al., 2010).

M. funereus (Coleoptera: Cerambycidae) inhabits a relatively narrow geographical zone (forests of southeastern Europe), and infests deciduous and coniferous trees (IUCN, 2012). It is listed as vulnerable

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on the IUCN Red list of threatened species (IUCN, 2012). This cerambycid beetle develops on host plants of the families *Tiliaceae*, *Fagaceae*, *Corylaceae*, *Salicaceae*, *Fabaceae* and *Pinaceae*, using either physiologically debilitated trees, tree stumps or recently cut logs. The larvae are inner bark (phloem) feeders, found in the first stage of decomposition of trees (Ivanović, 1968). Due to the extended period of larval development *M. funereus* is a good model for the study of impacts of various types of stress on insect metabolism (Nenadović et al., 1986; Đorđević et al., 1999; Ivanović et al., 2002).

Previous studies indicate great plasticity of *M. funereus* larvae, and the resulting variability is reflected in different enzyme isoforms among other physiological characteristics (Ivanović et al., 1975; Božić et al., 2003; Lončar et al., 2009; Dojnov et al., 2012). Variability in enzymes (isoenzymes) may be important for insects as it can provide increased capability to digest different foods and to overcome the activities of plant origin inhibitors (Wagner et al., 2002). Nutrient substrate affects the entire larval development, including duration of larval instars and body weight (Dojnov et al., 2012). This article describes the production of three major groups of hydrolytic digestive enzymes — cellulase, amylase, and peptidase on isoenzyme level and in relation to different nutrient substrates. A major reason for selection of a diet rich in cellulose is the fact that *M. funereus* cellulases are still poorly investigated. Wheat bran was chosen as endocellulase inducer because it is a naturally rich source of cellulose and contains a number of other substances that are essential for the larval development. Sawdust was used as rearing substrate for *M. funereus* larvae in this study because it is very similar with the substrate in the wild (wood material that *M. funereus* larvae inhabit). Medium containing polenta, Brewer's yeast and sugar was chosen as control medium during the first four larval instars because it appears to be well balanced in nutrients (Dojnov et al., 2012). The effect of substrates on digestion was monitored by detection of different cellulase isoforms in the midgut of larvae grown on different substrates. To complete the picture, that shows the influence of substrate on the digestive process in larvae, amylase and peptidase isoforms were also analyzed. Substrate impact on whole organism development was monitored by measurement of the growth rate of larvae.

The secretion of cellulases, amylases and peptidases along the larval gut (anterior midgut, middle midgut, rear midgut and hindgut) was mapped in this work, in order to better understand the biochemical organization of the digestive process in *M. funereus* species.

2. Material and methods

2.1. Reagents

All reagents were of the highest available purity and were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO, USA), unless otherwise stated. Polenta was produced from corn grits and purchased from Mitrosrem (Sremska Mitrovica, Serbia). Wheat bran was produced from wheat shells and purchased from Vega (Belgrade, Serbia). Sawdust was made from oak wood by milling.

2.2. Experimental animals

M. funereus adults collected from nature ("Fruska Gora" mountain) were bred in the laboratory during the first oviposition cycle. Deposited eggs were collected daily and placed on dietary media in Petri dishes. Eggs were examined on a daily basis for hatching.

Four groups of *M. funereus* larvae were examined in this study. Wild larvae (WL) were collected from nature ("Fruska Gora" mountain), from recently cut logs of oak trees (Quercus sp.). The number of adults and larvae taken from the field was confined to the number proposed in permits to work with protected species, obtained from relevant institutions — Institute for Nature Conservation of Serbia and Ministry of Environment and Spatial Planning, Serbia. Other three groups of larvae have hatched in the laboratory from eggs deposited by adults taken from nature and were subsequently reared in the laboratory. Artificial diet with polenta was used for rearing during the first four instars (Dojnov et al., 2012). At the start of the fifth instar, larvae were divided into three groups and reared on different types of diets. The diet composition and description of larvae groups are presented in Table 1.

The media were prepared by cooking all ingredients in water, with the exception of antibiotics, nipagin and gentamicin, which were added to the medium after cooling to a temperature below 70 °C. Warm medium was poured into round-bottomed plastic boxes.

During development, larvae were kept in the dark at 23 °C and RH 50%. Fresh dietary medium was provided each week and total body weight of larvae was measured. Larvae were examined daily for molting.

2.3. Preparation of crude midgut extracts

Larvae were dissected during the sixth instar, when they were feeding actively. After decapitation and removal of the adhering unwanted tissues, the guts were dissected on ice and cut into four pieces: anterior midgut (I), middle midgut (II), posterior midgut (III) and hindgut (IV) as shown in Fig. 1. Parts of guts and the whole midgut were weighed and homogenized, using a pre-chilled mortar and pestle, in 2 vol. (g/mL) of ice-cold 0.9% NaCl in 0.1 M acetate buffers, pH 5, with addition of quartz powder. The crude extracts were prepared as described by Božić et al. (2003).

2.4. Cellulase activity assay

Cellulolytic activity (cellulase, EC 3.2.1.4) was determined using carboxyl methyl cellulose (CMC) as a substrate and dinitrosalicylic acid (DNS) reagent for reaction termination (Bernfeld, 1955). Samples (50 μL) were incubated in 450 μL 50 mM acetate buffer pH 5.0 containing 2.0% (w/v) CMC, for 60 min at 35 °C. Glucose was used as standard. One unit (U) of cellulolytic activity was defined as the amount of enzyme required to produce 1 μmol glucose per min at 35 °C. Each data point represents the mean of three independent assays (standard errors were less than 5% of the means) in all three assays (cellulose, amylase and peptidase).

2.5. Amylase activity assay

A-Amylase (EC 3.2.1.1) activity was assayed by the (DNS) procedure (Bernfeld, 1955) using soluble starch as substrate. Samples (50 μL) were incubated in 450 μL 50 mM acetate buffer pH 5.0 containing 1.0% (w/v) starch, 2.0 mM NaCl and 0.1 mM $CaCl_2$ for 10 min at 35 °C.

Table 1
Composition of artificial diets used for rearing of larvae.

Larvae group	Designation of larvae groups	Components of all diets	Additional components of diets
First group	PL (polenta larvae)	Polenta 20 g, agar-agar 4 g, sucrose 10 g, dry brewer's yeast 10 g,	polenta ("mitrosrem"*) 20 g
Second group	OSL (oak sawdust larvae)	water 400 mL, methyl ester-*p*-hydroxyl benzoic acid (nipagin) 0.2 g/1 mL	milled oak sawdust 20 g
Third group	WBL (wheat bran larvae)	ethanol, gentamicin (10 mg/mL) 100 μL	wheat bran ("Vega") 20 g

* Composition of product: moisture 13-15%, fat 0.5–1.5%, cellulose 0.35–0.7%, ash 0.4–0.8%, proteins 7–9%, and starch 70–80%.

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