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INFLUENCE OF NUTRIENT SUBSTRATES ON THE EXPRESSION OF CELLULASES IN *CERAMBYX CERDO* L. (COLEOPTERA: CERAMBYCIDAE) LARVAE

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Abstract - The expression and distribution of digestive cellulases along the midgut of *Cerambyx cerdo* larvae were analyzed for the first time and are presented in this article. Four groups of larvae were examined: larvae developed in the wild; larvae taken from the wild and successively reared on an artificial diet based on polenta; and larvae hatched in the laboratory and reared on two different artificial diets. Seven endocellulase and seven β-D-glucosidase isoforms were detected in all midgut extracts of *C. cerdo* with a zymogram after native PAGE. We observed that *C. cerdo* larvae are capable of producing cellulase isoforms with different PAGE mobilities depending on the nutrient substrate. From our findings it can be assumed that, depending on the distribution of endocellulase and β-D-glucosidase, cellulose molecules are first fragmented in the anterior and middle midgut by endo-β-1,4-glucanase; subsequently, the obtained fragments are broken down by β-D-glucosidase mostly in middle midgut.

Key words: Cerambycidae, *Cerambyx cerdo*, cellulase, β-D-glucosidase, endocellulase, isoforms, zymogram, midgut, Serbia

INTRODUCTION

Enzymatic activity against cellulose substrates was detected in the digestive tract of insects belonging to different insect orders (Martin, 1983; Watanabe and Tokuda, 2010, Oppert et al., 2010). Cellulose digestion occurs in insects that have, as a rule, nutritionally poor diets (Terra and Ferreira, 1994). Cerambycid larvae survival depends on their ability to digest cellulose. Cellulase enzyme production is a key process in the enzymatic hydrolysis of lignocellulosic materials (Sun and Cheng, 2002). The first studies on Cerambycidae cellulases in Serbia began in 1966 (Ivanović and Barbič, 1966).

A combination of three categories of enzymes completes the digestion of native cellulose to glucose: endocellulase, exocellulase and cellobiase or β-D-glucosidase. There are no published data on cellulases from longhorn beetle *C. cerdo*, and only limited information is available on its other digestive enzymes (Janković et al., 1967; Ivanović and Milanović, 1967; Nenadović et al., 1982; 1994; 1999; Božić et al., 2001; 2004; Dojnov et al., 2010).

C. cerdo is polyphagous and can be found on deciduous, mature, weakened trees and occasionally on young and healthy trees, especially those growing in open and sunny locations. The larval development

of *C. cerdo* lasts 3-4 years and takes place in the inner bark, sapwood and heartwood along the stem. *C. cerdo* is listed as vulnerable on the IUCN Red list of threatened species (1996), in an Annex II Species of the EU Habitats and Species Directive (2009), and by the Decree on Protection of Natural Rarities of Serbia (1993). It is distributed in Europe, the Caucasus, Asia Minor and northern Africa (Kimoto and Duthie-Holt, 2006). In Central Europe, only trees of the genus *Quercus* (oaks) are hosts to *C. cerdo*, while outside Europe *C. cerdo* can be found on *Carpinus*, *Castanea*, *Ceratonia*, *Fagus*, *Fraxinus*, *Juglans*, *Pyrus*, *Robinia*, *Salix* and *Ulmus* (Bense, 1995, Kimoto and Duthie-Holt, 2006). *C. cerdo* is an important saproxylic beetle because it can alter its own habitat to create favorable conditions for other threatened beetle species (Buse et al., 2008).

Hydrolysis of carbohydrates occurs in the anterior midgut of many insects (Cristofoletti et al., 2001; Zverlov et al., 2003; Vinokurov et al., 2007). Localization of the site of secretion of endocellulase and β-D-glucosidase can be useful to explain the digestion process of cellulose, one of the most important carbohydrates in the diet of *C. cerdo* larvae.

The present study was performed to extend our knowledge of the digestive enzymes present in the midgut of *C. cerdo* larvae, in order to improve the understanding of the biochemical organization of the digestive process and to determine the role of cellulases in the adaptability of the species *C. cerdo*. It has already been demonstrated that the expression of amylolytic (on the isoenzyme level) and proteolytic enzymes depends on environmental conditions and nutrient substrate (Nenadović et al., 1994, Dojnov et al., 2010). The goal of this investigation was to examine the expression of endocellulase and β-D-glucosidase on the isoform level in larvae fed on two different artificial diets, and to detect these enzymes along the midgut of *C. cerdo* larval.

MATERIALS AND METHODS

Experimental animals

C. cerdo adults and larvae from the wild were collected from recently cut logs of oak trees (*Quercus* sp.) from Fruška Gora Mountain. 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 the relevant institutions; the Institute for Nature Conservation of Serbia and the Ministry of Environment and Spatial Planning,

Adults were bred in the laboratory. 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 *C. cerdo* larvae were examined in this study. The first larvae group contained larvae collected in the wild (LW – larvae, wild). The second group of larvae was collected in the wild and then reared in the laboratory on an artificial diet consisting of polenta (LWP – larvae, wild, polenta). The third and fourth groups of larvae hatched in the laboratory from eggs deposited by adults taken from the wild. An artificial diet with polenta was used for rearing during the first three instars. During the fourth instar, larvae were divided into two groups. The third group was reared on the same artificial diet (EP – egg, polenta – larvae that have hatched in the laboratory), while the fourth group of larvae was reared on an artificial diet consisting of oak sawdust (EOS – egg, oak sawdust).

Rearing conditions of larvae

Individual larvae were reared as described in our previous work (Dojnov et al., 2011). The composition of the polenta-containing artificial diet was: 40 g polenta, 4 g agar-agar, 10 g sucrose, 10 g dry Brewers' yeast, 400 mL water, 0.2 g methyl ester-p-hidroxy benzoic acid (Nipagin). The composition of the oak sawdust-containing artificial diet was: 20 g polenta, 20 g milled oak sawdust, 4 g agar-agar, 10 g sucrose, 10 g, dry Brewers' yeast, 400 mL water, 0.2 g methyl ester-p-hidroxy benzoic acid (Nipagin). The media were prepared by cooking all the ingredients in water, except Nipagin, which was added to the medium after cooling to a temperature below 70°C. The warm media was spread in round plastic boxes.

Preparation of crude midgut extracts

Larvae were dissected in accordance with the requirements of the Ethics Committee of the Faculty of Biological Science, University of Belgrade, during the eighth instar, when they were feeding actively. After decapitation and removal of the adhering unwanted tissues, the midguts were dissected on ice and cut into three pieces: anterior midgut, middle midgut and rear midgut as shown in Fig. 4. Parts of the midgut and the whole midgut were homogenized and crude extracts were prepared as described by Božić et al. (2003).

Cellulase activity assay

Endocellulolytic activity was determined using carboxymethyl cellulose (CMC) as a substrate and dinitrosalicylic acid (DNS) reagent as stop reagent (Bernfeld, 1955). Samples (50 μL) were incubated in 450 μL 50 mM acetate buffer pH 5.0 containing 2.0% (w/v) CMC, at 35°C for 60 min. Reaction was stopped by the addition of DNS reagent and boiling for 5 min. Absorbance of the reaction mixture was measured at 540 nm. Glucose was used as standard. One unit (U/mL) of cellulolytic activity was defined as the amount of enzyme required to produce 1 μmol glucose in 1 min at 35°C.

β-D-glucosidase activity was determined using p-nitrophenyl-β-D-glucopyranoside (pNPG) (ε = 18,5 $cm^2/\mu mol$) as substrate and Na_2CO_3 as stop reagent. Samples (25 μL) were incubated in 500 μL 50 mM acetate buffer pH 5.75 containing 0.55 mM pNPG, at 37°C for 10 min. The reaction was stopped by the addition of 1M sodium carbonate (75 μL), and the absorbance of the reaction mixture was measured at 405 nm. One unit (U/mL) of β-D-glucosidase activity was defined as the amount of enzyme required to produce 1 μmol p-nitrophenol per 1 min under the defined reaction conditions.

Determination of protein concentration

Protein concentrations were determined by Bradford (1976) using bovine serum albumin as the protein standard. Specific enzyme activities were calculated as U/mg of proteins.

Zymographic detection

Endocellulases were detected using in-gel activity staining following the electrophoretic separation of the intestinal extracts of *C. cerdo* larvae according to Davis (1964). The electrophoretic gel was printed on an AA (7.5%) gel with copolymerized CMC (0.1%) for 30 min. CMC gel was colored with 0.1% Congo-red for 10 min. 1M NaCl was used to rinse the gel. Endocellulases appeared as lighter bands on a red background. Acetic acid was used to change the color of the gel from red to blue in order to obtain better resolution.

β-D-glucosidases were detected using in-gel activity staining with 0.1% esculin and 0.03% $FeCl_3$ in 50 mM acetate buffer pH 5.7 as substrate, following native PAGE according to Kwon (1994).

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 – "Palenta" was made from corn grits and purchased from Mitrosrem (Sremska Mitrovica, Serbia). Sawdust obtained from oak wood was milled to flour using a laboratory mill (IKA).

Statistical analysis

Each data point for enzyme assays and for protein concentration represents the mean of three independent assays ± SEM (standard errors were less than 5% of the means). Larval weights during laboratory rearing were statistically analyzed using one-way analysis of variance (ANOVA) with Tukey post-hoc tests for analysis inside the larval groups and t-tests with 95% confidence intervals were used for comparing the groups. The GraphPad Prism 5 program was used for statistical analysis.

RESULTS

Growth rate of larvae depending on the composition of artificial diet

Average larval weight curves are presented in Fig. 1. Larvae had similar body weights during four instars, while both groups were on diet medium with polenta, Brewer's yeast and sugar. Larvae from the EOS group reared on the diet consisting of oak sawdust had higher body masses than larvae from the EP group reared only on polenta, from the fifth instar.

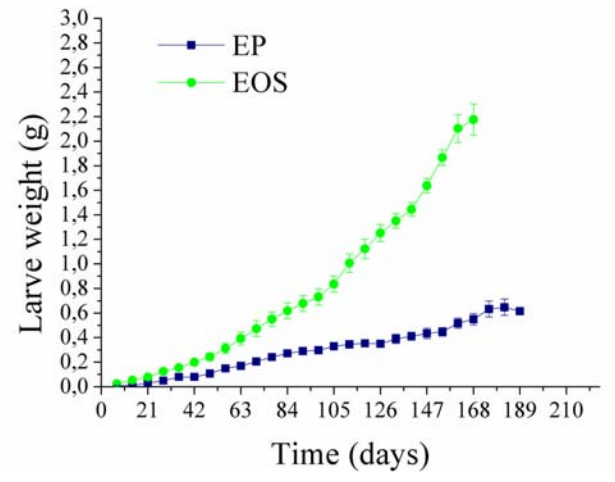

Fig.1. Body weights of *C. cerdo* larvae reared on different substrates. Average curves of body weights from larvae hatched in the laboratory reared on oak sawdust – EOS (circles) and larvae hatched in the laboratory reared on polenta–EP (boxes), are presented as average value ± SEM.

Larval body weights were very different between two groups after the 100th day, with significant differences within the larval group reared on oak sawdust (ANOVA results $F_{19,428}$=2.933, P<0.0001***) while there were no significant differences within the larval group reared on polenta (ANOVA results $F_{4,127}$=1.098, P=0.3604 ns). There were significant differences observed between the EP and EOS groups using the t-test (P<0.05) P=0.0004***.

Influence of substrate on expression of endocellulase

The activity of endocellulase (1.22 U/mL) was highest in the crude midgut extract of *C. cerdo* larvae reared on oak sawdust (EOS) (Fig. 2a), while specific endocellulase activity (0.46 U/mg) was highest in the crude midgut extract of wild larvae (LW).

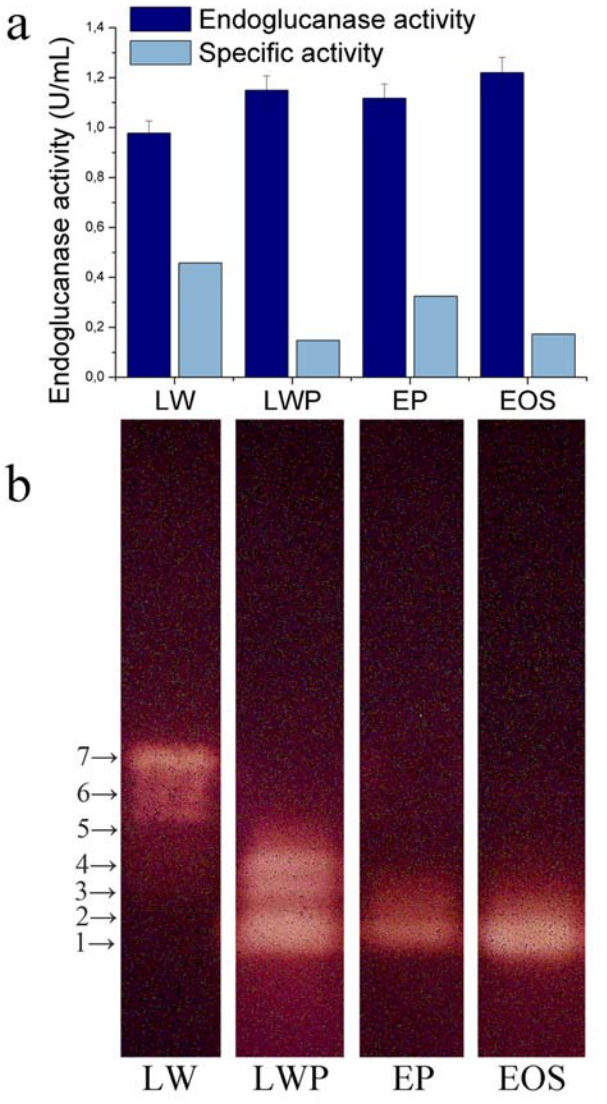

Fig. 2. Endocellulase activity in midgut extracts of *C. cerdo* larvae reared on different diets. a) Histogram of endocellulase activities; b) Zymogram detection of endocellulase after native PAGE; LW – wild larvae, LWP – wild larvae reared on diet with polenta, EP – larvae that hatched in the laboratory and were reared on a diet with polenta, EOS – larvae that hatched in the laboratory and were reared on a diet with oak sawdust, endocellulase *C. cerdo* isoforms (ECC) are indicated by arrows.

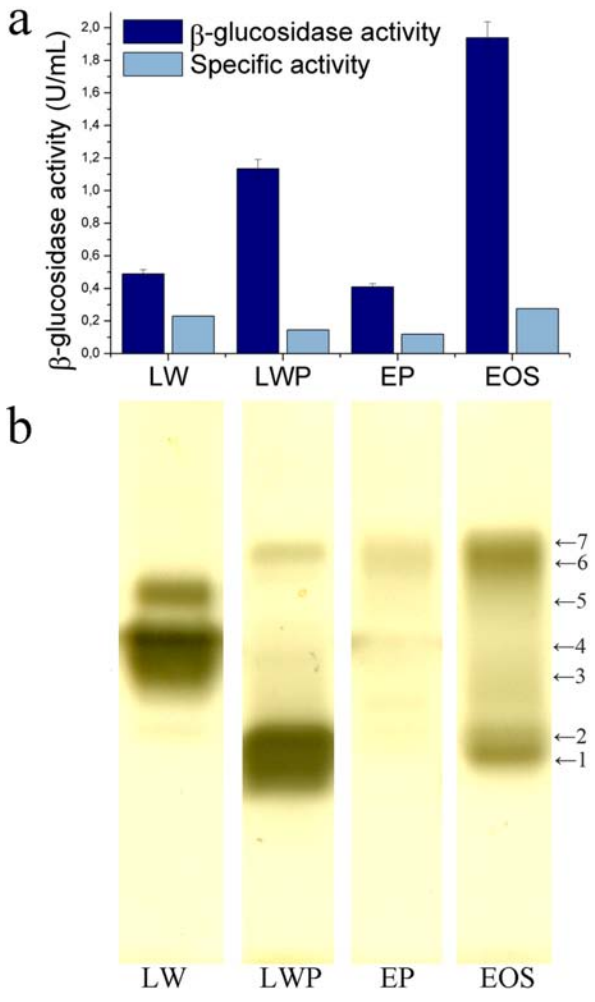

Fig. 3. β-D-glucosidase activity in midgut extracts of *C. cerdo* larvae reared on different diets. a) Histogram of β-D-glucosidase activities; b) Zymogram detection of β-D-glucosidase after native PAGE; LW – wild larvae, LWP – wild larvae reared on a diet with polenta, EP – larvae that hatched in the laboratory and were reared on a diet with polenta, EOS – larvae that hatched in the laboratory and were reared on a diet with oak sawdust, β-D-glucosidase *C. cerdo* isoforms (BCC) are indicated by arrows.

Midgut extracts of larvae taken from the wild and subsequently reared on a diet of polenta (LWP) had the greatest variety of endocellulase isoforms, as detected on the zymogram after native PAGE (four isoforms) (Fig. 2b). Endoglucanase *C. cerdo* (ECC) 1 to 4 were present in the midgut of laboratory-reared larvae. ECC 5, 6 and 7 were detected only in LW midgut extract.

Influence of substrate on the expression of β-D-glucosidase

The activity of β-D-glucosidase (1.94 U/mL) and specific β-D-glucosidase activity (0.28 U/mg) were highest in the midgut extract of *C. cerdo* larvae reared on oak sawdust (Fig. 3a).

Larvae reared in the laboratory had β-D-glucosidase isoforms with different mobility from the larvae taken from the wild. Midgut extracts of larvae reared on the diet with oak sawdust (EOS) had the greatest variety of β-D-glucosidase isoforms, as detected on the zymogram after native PAGE – intensive β-D-glucosidase *C. cerdo* (BCC) 1, 2 and 6, 7; and week isoforms BCC 3 and 5) (Fig. 3b). BCC 4 and 5 were very intensive in midgut of the wild larvae (LW).

Distribution of endocellulases and β-D-glucosidase along C. cerdo larvae midgut

Endocellulase and β-D-glucosidase activities were measured and isoforms were detected in three parts of the *C. cerdo* larvae midgut using a zymogram after native PAGE: anterior midgut, middle midgut and rear midgut (as indicated in Fig. 4).

Cellulases were localized in the midguts of larvae developed in the wild (LW) and in the midguts of larvae developed in the laboratory on oak sawdust (EOS).

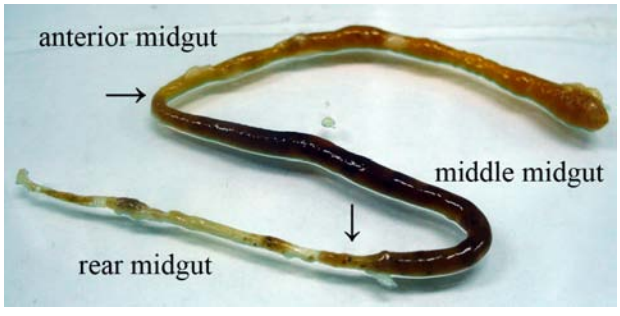

Fig. 4. Midgut of *C. cerdo* larvae. Arrows indicate cutting position of midgut.

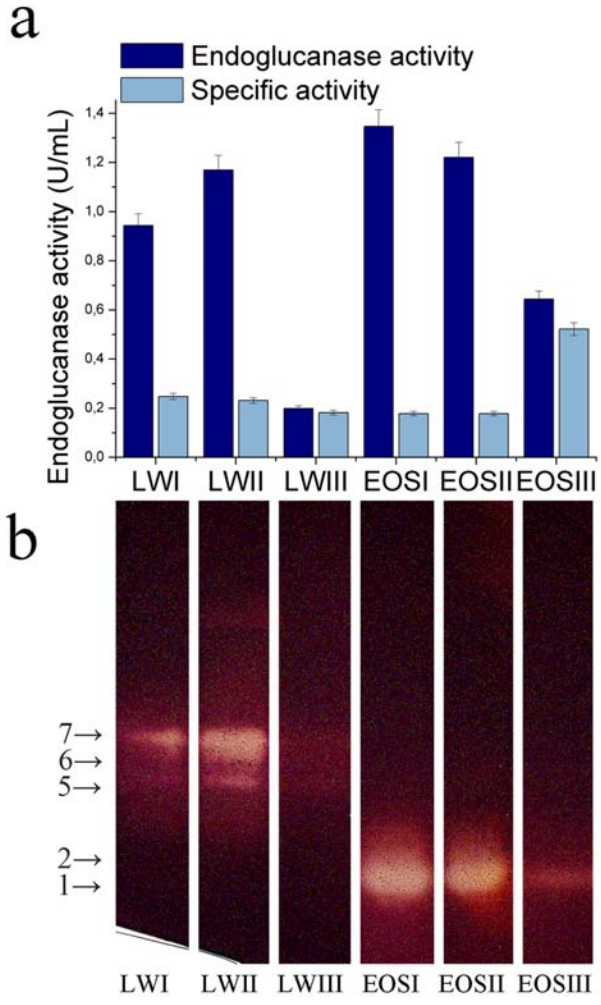

Fig. 5. Endocellulase activity in midgut extracts of *C. cerdo* larvae reared on different diets. a) Histogram of endocellulase activities; b) Zymogram detection of endocellulase after native PAGE; LW – wild larvae, EOS – larvae that hatched in the laboratory and were reared on a diet with oak sawdust, 1 – anterior, 2 – middle and 3 – rear part of midgut, endocellulase *C. cerdo* isoforms (ECC) are indicated by arrows.

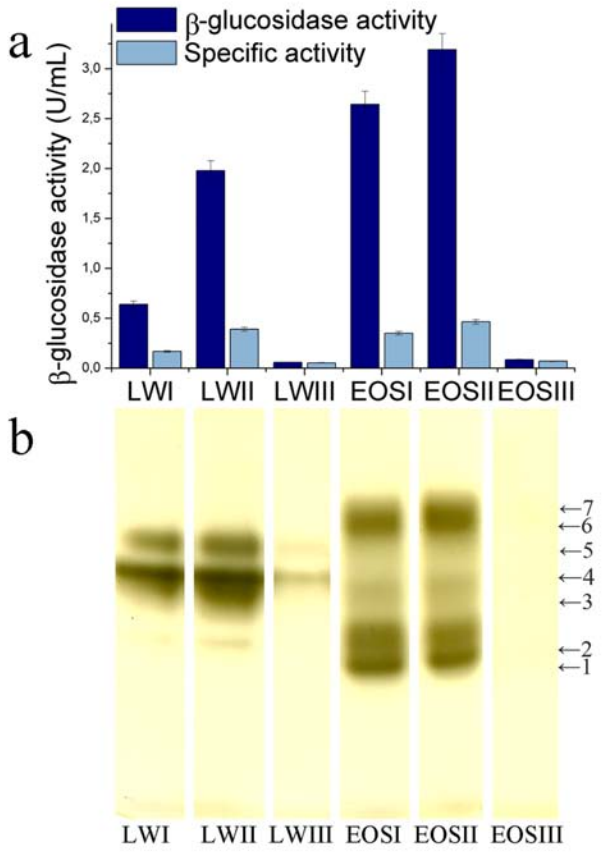

Fig. 6. β-D-glucosidase activity in midgut extracts of *C. cerdo* larvae reared on different diets. a) Histogram of β-D-glucosidase activities; b) Zymogram detection of β-D-glucosidase after native PAGE; LW – wild larvae, EOS – larvae that hatched in the laboratory and were reared on a diet with oak sawdust, 1 – anterior, 2 – middle and 3 – rear part of midgut, β-D-glucosidase *C. cerdo* isoforms (BCC) are indicated by arrows.

Distribution of endocellulases along the midgut of C. cerdo larvae

Results presented in Fig. 5 demonstrate that the larvae taken from the wild (LW) had the highest endocellulase activity in the middle part of the midgut, while larvae developed in the laboratory on a diet with oak sawdust (EOS) had the highest endocellulase activity in anterior midgut. Both examined larvae groups had high endocellulase activities in the anterior and middle midgut, while rear midguts were poor in this digestive enzyme.

The greatest number of endocellulase isoforms was detected in the middle midgut of wild larvae, LWII in Fig. 5b.

Distribution of β-D-glucosidases along the midgut of C. cerdo larvae

The highest β-D-glucosidase activity for both examined larvae groups was detected in the middle

midgut, as shown by enzymatic assays and zymography detection (Fig. 6). β-D-glucosidase activity was significantly lower in the anterior midgut and it was lowest in the rear midgut.

DISSCUSION

The polyphagy of *C. cerdo* comprises the plasticity of the species that enables it to inhabit new hosts. In this work, the presence of multiple endocellulase and β-D-glucosidase isoforms in *C. cerdo* midgut is shown. *C. cerdo* leucyl aminopeptidase and α-amylase also occur in multiple forms (Božić et al., 2004; Dojnov et al., 2010), which indicates high plasticity. Cellulases are detected in multiple isoforms in other Cerambycidae as well (Chararas et al., 1983; Geib S., 2010).

According to Marović (1973), it is possible to rear *C. cerdo* larvae under laboratory conditions; it should be noted that larval development is approximately one third shorter than in the wild. The medium containing polenta, Brewer's yeast and sugar was chosen as the control medium during the first larval instars because it appeared to be well-balanced in nutrients for the Cerambycid larvae *M. funereus* (Dojnov et al. 2011), and, moreover, because it induced peptidases and amylase in *M. funereus* larvae (Lončar et al., 2009; Dojnov et al., 2010) and also in *C. cerdo* larvae (Božić et al., 2004; Dojnov et al., 2010). It induces new cellulase isoforms (with different positions on native PAGE) in *C. cerdo* larvae compared with larvae developed in the wild, as shown in this work.

The diet consisting of oak sawdust proved to be a better choice for the rearing of *C. cerdo* larvae than the diet with polenta because of faster growing larvae and a higher induction of cellulase (endocellulase and β-D-glucosidase). The decrease in food quality could be compensated by the increase of food ingestion (Perić-Mataruga et al., 2011). Due to the increased amount of low nutritional-value food consumed, digestion is less efficient, which can be one of the reasons for the lower growth rates of the EP group compared to the EOS group. *C. cerdo* larvae move toward the center of a tree, thus changing the composition of the natural substrate during their development in the wood. Oak sawdust was obtained by milling the wood that comes from all parts of the timber section. Its composition is most similar to the composition of the substrate on which larvae develop in the wild. It seems that oak sawdust has some substances necessary for larval growth and development that is lacking in the diet with polenta. On the other hand, the positions of induced cellulase isoforms correspond to the isoforms of larvae reared on the polenta diet, indicating that all substances from the diet in combination with environmental factors affect enzyme induction.

The results shown in this work are important because they contribute to our understanding of Cerambycidae cellulases. Cellulases have been studied in long-horned beetles larvae of *Anoplophora glabripennis* (Geib, 2010) and *Ergates faber* (Chararas et al., 1983). One of the most cited and studied β-D-glucosidases is that from *Tenebrio molitor* larvae (Fereira et al., 2001).

From the results obtained for the distribution of endocellulase and β-D-glucosidase along the midgut, it can be assumed that cellulose molecules are first fragmented in the anterior and middle midgut by endo-β-1,4-glucanase. The products of this reaction are hydrolyzed by β-D-glucosidase, mostly in the middle midgut. The digestion of cellulose has been the subject of considerable controversy (Scrivener, 1997). Exogenous cellulases are usually located in the rear gut, while endogenous cellulases are in the anterior gut, pharynx, midgut and salivary glands (Martin, 1983; Tokuda et al., 2009). The foregut and midgut are either devoid of microorganisms or contain them in very small numbers (Slaytor and Scrivener, 1994). Treatment with antibiotics removes endosymbionts (Heddi et al., 1999; Shen et al., 2003). The use of antibiotics and the fact that the lowest cellulase activity is in the rear midgut supports the assumption that the detected *C. cerdo* cellulases from laboratory reared larvae are of endogenous origin.

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