

## COMPARISON OF $\alpha$ -AMYLASE ISOFORMS FROM THE MIDGUT OF *CERAMBYX CERDO* L. (COLEOPTERA: CERAMBYCIDAE) LARVAE DEVELOPED IN THE WILD AND ON AN ARTIFICIAL DIET

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**Abstract** -  $\alpha$ -Amylase isoforms of *Cerambyx cerdo* larvae from the wild (ML and SL) and reared in the laboratory (ADL) were compared. Three amylase isoforms were presented in the SL and ML extracts while two isoforms were presented in the ADL according to zymogram after isoelectric focusing (IEF). All *C. cerdo* amylase isoforms were acidic proteins (pI < 3.5). Seven amylase isoforms (ACC 1-7) from the midgut of *C. cerdo* larvae were found in the ML midgut extract, six in the SL extract, and four in the ADL extract according to native PAGE zymogram. The ADL amylase had the highest activity. All crude midgut extracts of *C. cerdo* larvae were fractionated on a Superose 12 HR column. The molecular mass of the ACC was estimated to be 34 kDa.

**Keywords:** Cerambycidae, *Cerambyx cerdo*, midgut,  $\alpha$ -amylase, isoform, zymogram, xylophagous larvae

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### INTRODUCTION

Carbohydrates are essential energy-producing nutrients required for both optimal larval growth and for the maintenance of adult longevity for the majority of insects (Dadd, 1985). The hydrolysis of starch and other carbohydrates occurs in the anterior midgut in many insects (Cristofolletti, 2001; Zverlov et al., 2003; Vinokurov et al., 2007). From the pool of enzymes known to act preferentially on long  $\alpha$ -1,4-glucan chains, only  $\alpha$ -amylase have been found in insects (Terra et al., 1996).  $\alpha$ -Amylases ( $\alpha$ -1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) catalyze the hydrolysis of (1,4)- $\alpha$ -D-glucan linkages in both starch and glycogen. Little information is available concerning the presence and properties of such enzymes in the Cerambycid family (Janković et al., 1967; Weber et al., 1985; Zverlov et al., 2003; Dojnov et al., 2008). There is limited literature data about the amylolytic enzymes of *C. cerdo* (Janković

et al., 1967; Ivanović and Milaković, 1967). The activity of *C. cerdo* amylase has been thoroughly examined in studies of the metabolic response of larvae to temperature and food quality (Nenadović et al., 1982; 1994; 1999).

*C. cerdo* larvae feed on the inner bark, sapwood and heartwood along the stem. Their development lasts 3 – 4 years in the wild while in the laboratory the period of development is approximately one third shorter (Nenadović et al., 1999).

*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. It is distributed in Europe, the Caucasus, Asia Minor and northern Africa (Kimoto and Duthie-Holt, 2004). In Central Europe, only trees of the genus *Quercus* (oaks) are hosts to *C. cerdo*, while in more southern parts they can develop in some

other trees ([www.arkive.org](http://www.arkive.org)). Outside Europe *C. cerdo* can be found on *Carpinus*, *Castanea*, *Ceratonia*, *Fagus*, *Fraxinus*, *Juglans*, *Pyrus*, *Robinia*, *Salix* and *Ulmus* (Kimoto and Duthie-Holt, 2004). From this point of view, it was interesting to find out how many isoforms of amylase are present in the midgut of *C. cerdo* larvae and clarify whether there is any connection between this enzyme and polyphagy in *C. cerdo*. It was also interesting to establish if there are differences in the amylase isoforms between the *C. cerdo* larvae developed in nature and those reared in the laboratory since it is possible that all insects have the full complement of ordinary digestive enzymes, the relative amounts of which change in response to diet composition (Terra and Ferreira, 1994).

In the present paper the amylase isoforms of *C. cerdo* larvae from nature and those reared in the laboratory were compared. Two groups of *C. cerdo* larvae were examined to find out whether seasonal changes in the environment have an impact on this enzyme.

## MATERIAL AND METHODS

### *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. "Palenta" ("Mitrosrem", Sremska Mitrovica, Serbia) was produced from corn grits.

### *Insects*

Tree groups of *C. cerdo* larvae were examined in this study. The first larvae group was collected from the wild in March – ML (March larvae). The second larvae groups was also collected from the wild in September – SL (September larvae); the third group was collected from the wild in March and then reared under laboratory conditions and fed an artificial diet – ADL (artificial diet larvae). All the larvae were collected from oak trees (*quercus* species) in the Fruška Gora Mountains in Serbia.

### *Rearing conditions of larvae*

Individual larvae were confined in round-bottomed plastic boxes (diameter 6 cm) with ventilation holes on top. During development, the larvae were kept in the dark at 23°C and RH 50%. Dietary media was replaced with a fresh one weekly, and the weight of the larvae was measured. Larval molting was examined daily.

The artificial diet was "Palenta" 40 g, agar-agar 4 g, sucrose 10 g, dry Brewers' yeast 10 g, water 400 mL, methyl ester-p-hydroxy benzoic acid (nipagin) 0,2 g. Raw corn starch was converted to digestible dextrin chains (soluble starch) by swelling, heating and mechanically milling in industry process. The chemical composition of "Palenta" is: moisture 13-15%, fat 0.5-1.5%, cellulose 0.35-0.70%, proteins 7-9%, soluble starch 70-80% and ash 0.4-0.8%. The medium was prepared by cooking all the ingredients in water, except the nipagin, which was added to the medium after cooling at a temperature below 70°C. Warm medium was distributed in round-bottomed plastic boxes.

### *Preparation of crude midgut extracts*

After decapitation the midguts were dissected on ice, weighed and homogenized using a pre-chilled mortar and pestle in ice-cold 0.9% NaCl, 0.1 M acetate buffer pH 6.0 with the addition of quartz powder. The homogenates were centrifuged for 10 min at 5000 x g (Božić *et al.*, 2003).

### *Protein fractionation*

The crude midgut extracts of the *C. cerdo* larvae (0.2 mL) were loaded onto a Superose 12 HR 10/30 column on a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). The column was calibrated using bovine serum albumin (66.5 kDa), ovalbumin (44.0 kDa), chymotrypsinogen (28.5 kDa) and lysozyme (14.4 kDa) (GE Healthcare). The flow rate was 0.5 mL/min. Fractions of 0.5 mL were collected and assayed for amylase activity.

### *$\alpha$ -Amylase activity assay*

$\alpha$ -Amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955) using soluble starch as a substrate. Samples (50  $\mu$ l) were incubated in 450  $\mu$ l 50 mM acetate buffer pH 5.0 containing 1.0% (w/v) starch, 2.0 mM NaCl and 0.1 mM CaCl<sub>2</sub> at 35°C for 10 min. Maltose was used as a standard. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means). One unit of  $\alpha$ -amylase activity was defined as the amount of enzyme required to produce 1  $\mu$ mol maltose in 1 min at 35°C.

### *Zymograms*

Midgut  $\alpha$ -amylases were detected using in-gel activity staining following native polyacrylamide gel electrophoresis or PAGE (Davis, 1964) and isoelectric focusing (IEF). For native PAGE, the samples were diluted twice in an electrophoresis sample buffer and subjected to electrophoresis. IEF was carried out on a 7.5% polyacrylamide gel with ampholytes in the 2.5-4.5 pH range at 3 W constant power for 2 h at 10°C. H<sub>3</sub>PO<sub>4</sub> (0.25 M) and Hepes (0.4 M) were used as electrode buffers. Pepsinogen (pI 2.80), amyglucosidase (pI 3.50), methyl red (pI 3.75), glucose oxidase (pI 4.15), trypsin inhibitor (pI 4.55),  $\beta$ -lactoglobulin A (pI 5.20), carbonic anhydrase B – bovine (pI 5.85), carbonic anhydrase B – human (pI 6.55) (Low pI kit, GE Healthcare) were used as pI markers.

After the electrophoretical separations,  $\alpha$ -amylase activity was detected according to a previously published method (Dojnov *et al.*, 2008). The gels were transferred first to a buffered substrate solution (1.0% (w/v) starch, 50 mM acetate, 2.0 mM NaCl and 0.1 mM CaCl<sub>2</sub> pH 5.0) for 30 min at 30°C and then incubated in a buffer solution (50 mM acetate, 2.0 mM NaCl and 0.1 mM CaCl<sub>2</sub> pH 5.0) at 30°C for 30 min. After rinsing in water, amylolytic activity was stopped by adding a staining solution (1.3% (w/v) I<sub>2</sub>, 3% (w/v) KI). The  $\alpha$ -Amylase activity appeared as clear bands on a dark background.

### *Determination of protein concentrations*

Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the protein standard. Each data point represents the mean of three independent assays  $\pm$  SEM (the standard errors were less than 5% of the means).

### *SDS PAGE*

Proteins from the midguts were examined by SDS-PAGE according to Laemmli (1970). The samples were diluted twice in an electrophoresis sample buffer and subjected to electrophoresis.  $\alpha$ -Lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), albumin (67 kDa) and phosphorylase B (97.4 kDa) (components within the LMW-SDS marker kit, GE Healthcare) were used as molecular mass standards. After electrophoresis, the gel was stained with Coomassie brilliant blue (CBB).

## RESULTS

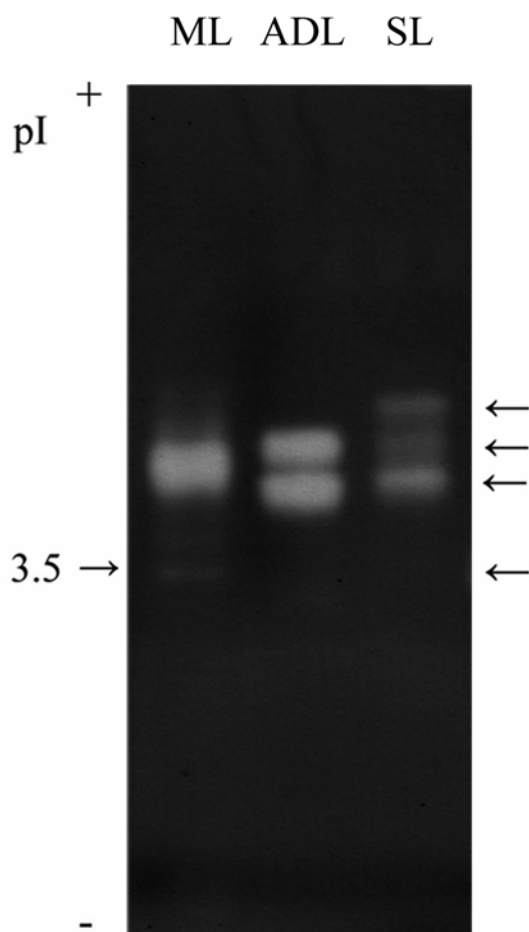
The  $\alpha$ -Amylase activity and protein concentration in *C. cerdo* midgut extracts were compared and the results are shown in Table 1. The highest amylase activity and specific activity was observed in the ADL midgut extract, while the ML extract had a higher amylase activity than the SL extract.

Zymography detection of the amylase isoforms of *C. cerdo* midgut extracts after IEF is shown in Fig. 1. Three amylase isoforms were found in the SL midgut extract as well as in the ML midgut extract, with one intensive and two less visible bands with higher pI values in the latter. Two strong bands of amylase isoforms were detected in the ADL extract, both had similar pI values (near to that as the major isoform of ML). The pI values of all the *C. cerdo* amylase isoforms were less than 3.5.

Seven amylase isoforms of *C. cerdo* (ACC 1-7) larvae were found in the ML midgut extract, six isoforms in the SL midgut extract, while in the ADL

**Table 1.** Amylase activity, protein concentration and specific activity in midgut extracts of *C. cerdo* larvae. ML – larvae collected from nature in March, SL – larvae collected from nature in September, ADL – larvae from artificial diet. Values are represent as Mean  $\pm$  SEM (n=3).

Larvae group	Amylase activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg proteins)
ML	24.14 $\pm$ 0.97	11.54 $\pm$ 0.43	2.09 $\pm$ 0.07
SL	9.06 $\pm$ 0.23	10.58 $\pm$ 0.34	0.86 $\pm$ 0.02
ADL	35.53 $\pm$ 1.27	7.84 $\pm$ 0.23	4.53 $\pm$ 0.19



**Fig. 1.**

extract there were four major isoforms (ACC 1-4) according to the native PAGE zymogram (Figs 2/B, 3/B and 4/B). The Amylase isoform on the ACC 6 position in the ML extract was missing in the SL crude extract.

Fractionation of proteins from the ML midgut extract is shown in Fig. 2. Amylase activity was detected in fractions 9 – 13 with a maximum in 11 fraction ( $V_e = 13.5$  mL). ACC 1-4 were found in all eluted fractions, while ACC 5 and 7 were presented in fractions 11 and 12 (Fig. 2/B). ACC 5 was the most visible in fraction 11, while ACC 6 was not present in the eluted fractions, only in the crude extract.

Fractionation of the proteins from the SL midgut extract is shown in Fig. 3. Amylase activity was detected in fractions 9 – 13 with a maximum in the 12 fraction ( $V_e = 14.0$  mL). ACC 1-4 isoforms were detected in fractions 10, while all isoforms (6) were presented in fractions 11 and 12 (Figure 3/B).

Fractionation of the proteins from the ADL midgut extract is shown in Fig. 4. There were two amylase activity peaks in the elution profile. The first peak corresponded to fraction 5 ( $V_e = 10.5$  mL), where only one amylase isoform was found (Fig. 4/B). The second amylase activity peak corresponded to fraction 11 and contained all isoforms presented in the ADL crude midgut extract.

The molecular mass of the ACC was estimated to be 34 kDa, calculated from the plot of  $\log$  molecular mass versus  $K_{av}$  using standard proteins as markers after elution from a Superose 12 HR FPLC column.

## DISCUSSION

*C. cerdo* is classified as “Vulnerable” on the list of protected species by the World Conservation Monitoring Centre from 1996 (In: IUCN 2009. IUCN Red List of Threatened Species). The polyphagy of *C. cerdo* comprises the plasticity of the species which enables it to inhabit new hosts. Nevertheless *C. cerdo* can occasionally inhabit

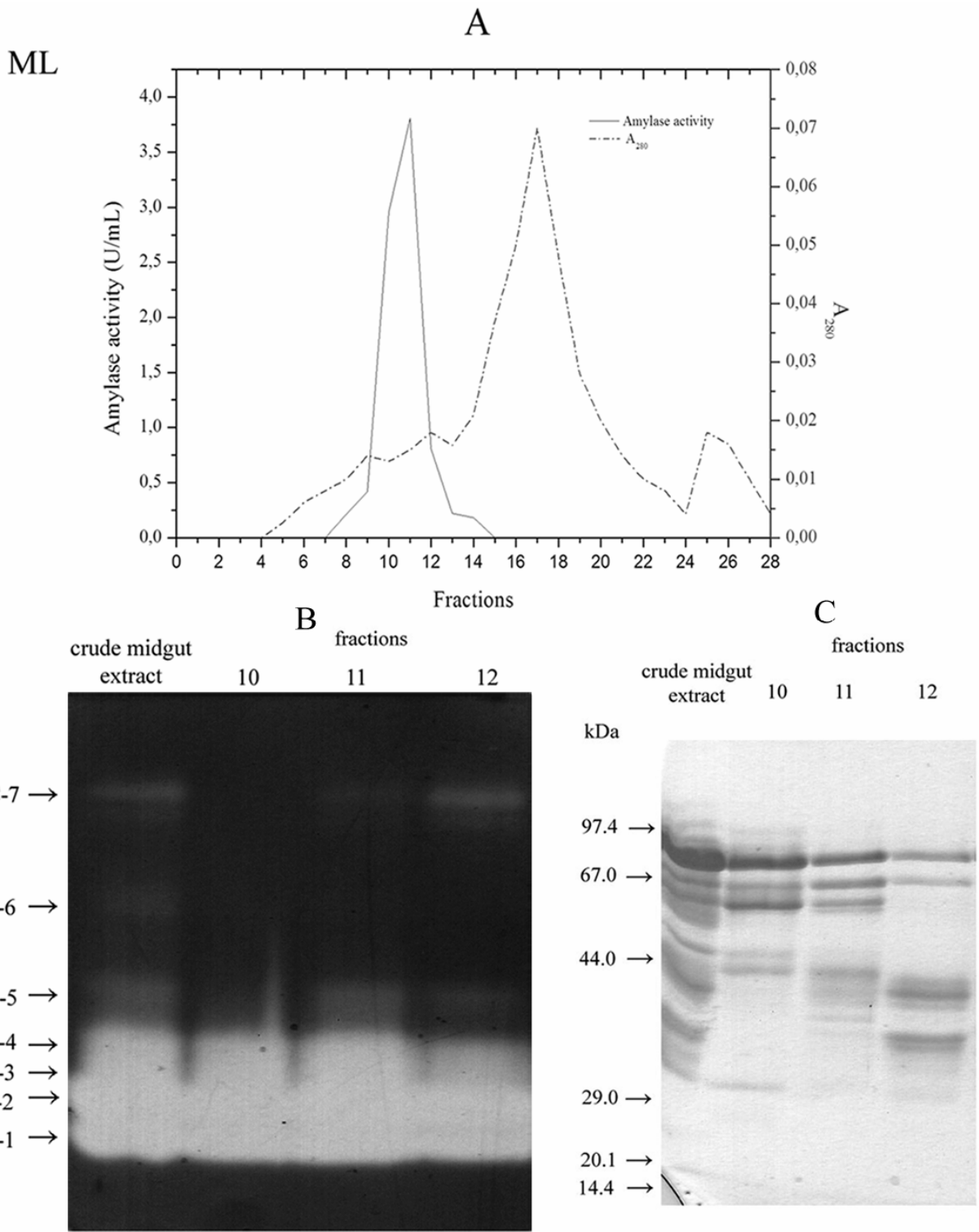


Fig. 2.

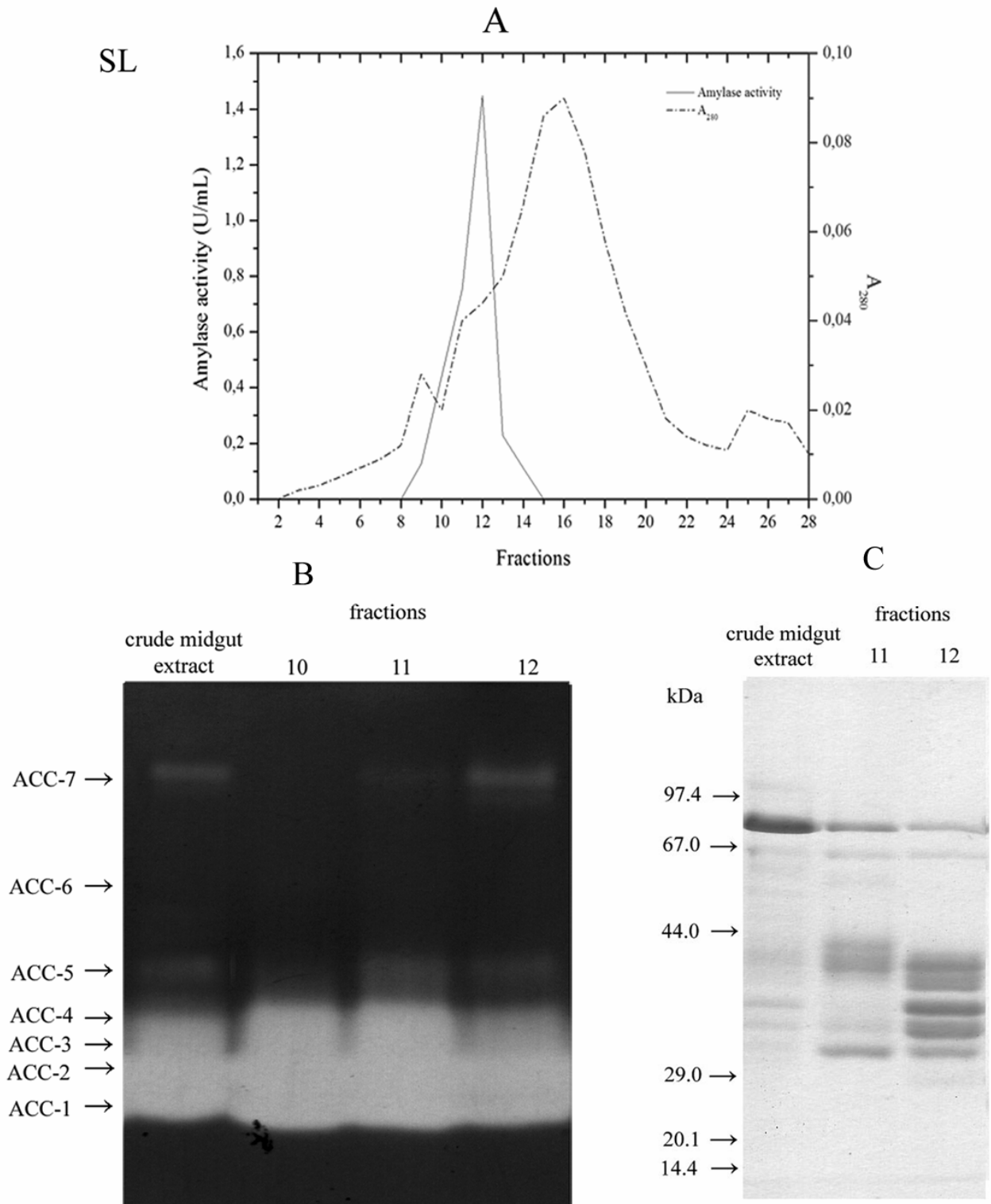


Fig. 3.

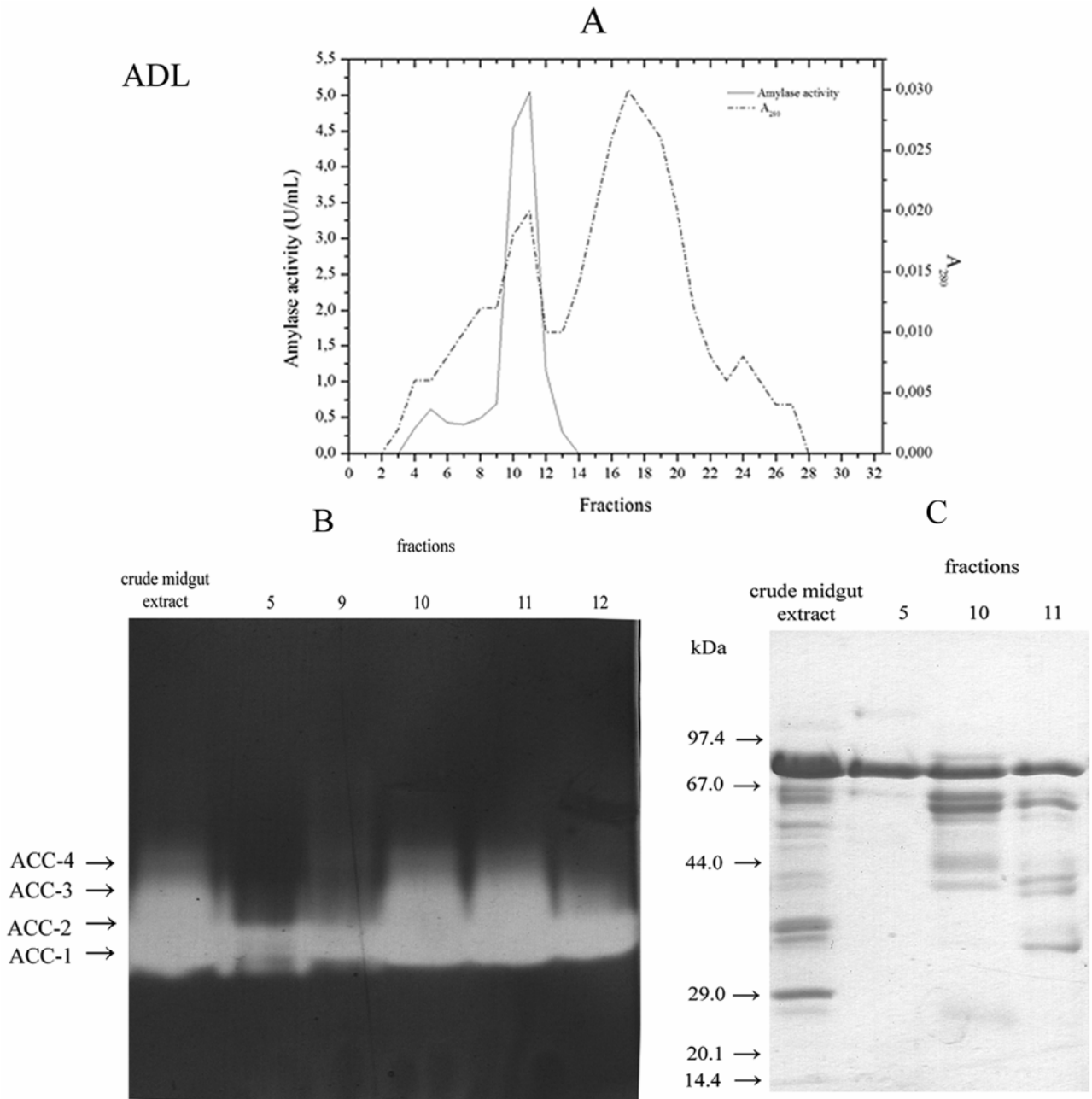


Fig. 4.

healthy trees and this is a characteristic of pest insects. The tunnels that are produced in a tree by the action of *C. cerdo* larvae are places of fungal infection by *Biscogniauxia mediterranea* which can cause charcoal disease (Martin et al., 2005). Although not a pest in its original form, *C. cerdo*

might have negative economical and medical impact.

It has been postulated that isoenzymes may be important for insects as they could provide an increased capability to adapt to different sources of

food and to overcome the activities of inhibitors of plant origin (Wagner et al., 2002). In all the larvae groups of *C. cerdo* examined, multiple forms of amylase were detected. Seven amylase isoforms were detected in the ML midgut extracts of *C. cerdo*. Similarly, five amylase isoforms were detected in the midgut of *M. funereus* larvae from the wild (Dojnov et al., 2008). Since *C. cerdo* can be found on wider geographical region and the larvae feed on the inner bark, sapwood and heartwood along the stem, the existence of more amylase isoforms in the midgut extracts of the *C. cerdo* larvae from the wild might be due to this. Two major isoforms of  $\alpha$ -amylase have been reported in *Prostephanus truncatus* (Mendiola-Olaya et al., 2000), three isoforms in *Rhyzopertha dominica* (Cinco – Moroyoqui et al., 2008) and four isoforms of  $\alpha$ -amylase have been identified in *Callosobruchus maculatus* (Campos et al., 1989). *C. cerdo* leucyl – aminopeptidase also exist in multiple forms (Božić et al., 2004).

The seasonal variability in amylase isoform presence in *C. cerdo* larvae presented in this paper is in agreement with other Cerambycidae, and was observed in *M. funereus* larvae as well (Ivanović and Nenadović, 1999; Ivanović et al., 1987; and in our previous work: Nenadović et al., 1982). Amylase activity is in correlation with the overall higher activity of larvae in spring.

Induction of this enzyme by different diets has been described in *Zabrotes subfasciatus* larvae (Silva et al., 2001). The artificial diet used in this work induced *C. cerdo* larval midgut amylases. Although there were fewer isoforms in the ADL group, the amylases had a higher overall activity as well as specific activity. The artificial diet used in this work induced amylase (results not published) and peptidases in *M. funereus* larvae as well (Lončar et al., 2009). Thus, *C. cerdo* larvae reared in the laboratory can be used for the purification and characterization of  $\alpha$ -amylase and other digestive enzymes.

The effects of food on the expression of proteins can be seen not only by the levels and presence of amylase isoforms but also the overall protein profiles after SDS-PAGE. The obtained differences

between the protein profiles of the ML, SL and ADL midguts are in correlation with results obtained for *M. funereus* protein profiles reared on different diets (Ilijin et al., 2004).

In terms of molecular weights, *C. cerdo*  $\alpha$ -amylase bears a resemblance to *M. funereus* amylase (31 kDa) (Dojnov et al., 2008) and to the  $\alpha$ -amylase isoforms from *C. maculatus* midgut (Campos et al., 1989). We found that *C. cerdo*  $\alpha$ -amylase was an acidic protein which compares well with the pI values of  $\alpha$ -amylases from other Coleoptera (Terra and Ferreira, 1994).

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УПОРЕДНА АНАЛИЗА ИЗОФОРМИ  $\alpha$ -АМИЛАЗЕ ИЗ СРЕДЊЕГ ЦРЕВА ЛАРВИ *CERAMBYX CERDO* L. (COLEOPTERA: CERAMBYCIDAE) ИЗ ПРИРОДЕ И ГАЈЕНИХ НА ВЕШТАЧКОЈ ПОДЛОЗИ

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Упоређене су изоформе  $\alpha$ -амилазе ларви *Cerambyx cerdo* сакупљених из природе (МЛ и СЛ) и гајених на вештачкој подлози у лабораторији (АДЛ). Зимограмском детекцијом после ИЕФ-а по три изоформе су детектоване у МЛ и СЛ екстрактима, а у АДЛ две изоформе. Све амилазне изоформе из *C. cerdo* су биле киселе ( $pI < 3.5$ ). Зимограмском де-

текцијом после нативне електрофорезе седам изоформи је детектовано у МЛ екстракту, шест у СЛ екстракту и четири у АДЛ екстракту. Највећа амилазна активност је детектована у АДЛ екстракту. Сви екстракти средњих црева ларви *C. cerdo* су фракционисани на колони Superose 12 HR. Молекулска маса АЦЦ-а је била 34 kDa.