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Digestive proteases during development of larvae of red palm weevil, *Rhynchophorus ferrugineus* (Olivier, 1790) (Coleoptera: Curculionidae)

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Abstract

The evolution of digestive proteases during larval development of *Rhynchophorus ferrugineus* (Olivier, 1790) has been studied. A progressive increase of protease activity has been found. The optimum pH for proteolytic activity against azocasein was determined. Caseinograms revealed an active complex of alkaline proteases from the early stages of the development. From the apparent molecular masses, three groups of proteases have been found — high molecular-mass proteases, medium molecular-mass proteases, and low molecular-mass proteases. Studies using specific protease inhibitors showed the major presence of serine proteases in gut extracts. The results obtained from larvae reared on different substrates have made possible a comparative assessment of the influence of diet on the development of the digestive enzymatic system. Larvae fed on an artificial diet showed a complete pattern of digestive proteases. Data suggest that this diet seems to be suitable for future research with this insect pest. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Various species belonging to the genus *Rhynchophorus* are wide-distribution plagues in temperate and tropical areas. These insects cause significant negative economic effects on palm trees and coconut palms. Among these species, is *Rhynchophorus ferrugineus* from Asia (Lepesme, 1947; Hill, 1987). Recently it has been accidentally introduced into southeast Spain through imported palms (Barranco et al., 1996a,b). Because of a suitable climate and other conditions, this species has infested ornamental palms. Palm trees are some of the most important ornamental trees in the middle south of Spain, especially those belonging to the *Phoenix* genus.

Currently some of the major aspects in pest control are to achieve the selective inhibition of the digestive enzymes of many insect pests. These attempts have as

their main aim, the production of detrimental effects on the growth of larvae to prevent the digestion and assimilation of nutrients to retard their development and cause their death. There are some evidences that secondary compounds within plants, like protease inhibitors, have defensive functions against phytophagous animal (Ryan, 1981). In general, three approaches have been used to evaluate the biological activity of protease inhibitors against insect pests. The most direct approach measures the ability of protease inhibitors to inhibit enzyme activity from the gut of a given species, in vitro (Coppedge et al., 1994; Rymerson and Bodnaryk, 1995). The second approach measures the target organism's response to protease inhibitors added to artificial diets (Elden, 1995; Kuroda et al., 1996). A third approach is to evaluate the response of insects to protease inhibitors in planta (Girard et al., 1998a,b; Cloutier et al., 1999). The aim of these approaches is to enhance the resistance of plants economically by transferring into them selected plant genes whose products affect the growth or development so that only insects that eat the transgenic plants would be affected (Confalonieri et al., 1998).

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As a first step to achieving this it is necessary to characterize gut proteolytic enzymes. There are no studies on the characterization of the digestive enzymes of this species, a characterization that could allow the use of protease inhibitors as is done with other insect pests.

Our aims were to determine the proteolytic enzymes that occur during the ontogenetic development of *R. ferrugineus*, identify inhibitors that block activity of these enzymes, and examine the effect of different larval diets on proteolytic activity.

2. Materials and methods

2.1. Chemicals

All substrates, enzymes, and protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO). Electrophoresis chambers and reagents were obtained from Bio-Rad Laboratories (Richmond, CA).

2.2. Insects

A population of *R. ferrugineus* has been established in our laboratory since 1997 from wild specimens caught in Almuñecar (Granada, Spain), along the southern coast of Spain. The larvae were reared on an artificial diet from hatching to the ultimate instar (Table 1, Barranco et al., 1997). Under these conditions, the larval cycle was completed in about 90 days. At the same time, three different groups of *R. ferrugineus* larvae were reared to the seventh instar on different fresh plant substrates obtained from ornamental palms (*Phoenix canariensis* and *Trachycarpus fortunei*) and sugar cane (*Saccharum officinarum*). During the experiments, larvae were maintained in a growth chamber in the dark at $25 \pm 0.5^\circ\text{C}$ and $65 \pm 5\%$ relative humidity. Larvae were reared individually in transparent clear plastic containers ($8 \times 3 \text{ cm}^2$ diameter) to ensure precise control of feeding and data

collection. Five day old (L-I), 10 day old (L-III), 15 day old (L-V), 20 day old (L-V), 25 day old (L-VI), 30 day old (L-VI) and 35 day old (L-VII) larvae were used. The different larvae instars were identified according to Barranco et al. (1999).

2.3. Preparation of samples

R. ferrugineus larvae were sampled two days after head-capsule slippage when an active feeding behavior of animal was observed. Larvae at different instar stages were dissected following the procedure described by Broadway and Duffey (1985). In brief, larvae were dissected in 0.15 M NaCl at 4°C (Jungreis et al., 1973), and entire guts and contents were removed and stored frozen (-20°C) until use. Ten guts of each larvae instar were homogenized in 0.15 M NaCl by sonication (MICROSON XL200, Misonix Inc., Farmingdale, NY), and active extracts were obtained after further centrifugation ($12\,500g$ for 20 min at 4°C) of the homogenates. The supernatant was recovered and frozen (-20°C) for enzymatic assays. Extracts of five-day-old larvae were obtained using decapitated whole-body larvae. Whole insects have been used in several studies on coleopteran larvae to show the proteolytic pattern in whole larvae extracts matched the proteolytic pattern in the gut (Overnay et al., 1997; Girard et al., 1998a,b). Soluble protein of the larvae homogenates was measured by the method of Bradford (1976) using bovine serum albumin as standard.

2.4. Enzyme assays

Total protease activity of extracts was assayed according to the method described by García-Carreño and Haard (1993) with some modifications. Azocasein (0.5% w/v) dissolved in 50 mM Tris-HCl, 10 mM L-cysteine, buffer, pH 9.0, was used as substrate. In brief, 20 μl of the enzyme preparation was mixed with 0.5 ml of 0.1 M Tris-HCl, pH 9.0, at 37°C . Reaction was started by the addition of 0.5 ml of 1% azocasein and stopped 30 min later by adding 0.5 ml of 20% TCA. Precipitation was caused by cooling (4°C for 10 min) and the reaction mixture was centrifuged at $15\,000g$ for 5 min and the $\text{ABS}_{366 \text{ nm}}$ recorded. Blanks, in which TCA was added before the substrate, were prepared for each assay. Units of protease activity (UA) were calculated by using the equation $\text{UA} = \Delta \text{ABS}_{366 \text{ nm}} / \text{time}(\text{min}) \cdot \text{volume of enzyme}(\text{ml})$. All determinations were done in triplicate.

Trypsin-like activities were assayed using BAPNA (*N*-benzoyl-DL-arginine-*p*-nitroanilide) as substrate. About 1 mM BAPNA was dissolved in 1 ml dimethylsulfoxide (DMSO) and then made up to 100 ml with Tris, pH 7.5, containing 20 mM CaCl_2 . Chymotrypsin-like activities were determined using 0.1 mM SAPNA (*N*-succinyl-L-Ala-L-Ala-L-Pro-Phe-*p*-nitroanilide) in

Table 1
Semi-artificial diet composition

Agar	20 g
Distilled water	880 ml
Brewers yeast	50 g
Wheat germ	50 g
Corn meal	50 g
M-nipagine	1.8 g
Benzonic acid	1.8 g
Ascorbic acid	4.5 g
Chloramphenicol	0.5 g
Coconut fiber	8 g
Vitamin and amino acid additive ¹	50 ml
% Crude protein (dry weight)	15

¹ Hidro Rex Vital Aminoácidos. S.P. Veterinaria, S.A., Tarragona (Spain).

0.1 M Tris, pH 7.8, containing 0.01 M CaCl₂. Reactions were started by the addition of 10 µl enzyme extract to the buffered substrate solutions and then incubated at 37°C. The enzymatic reactions were stopped by the addition of 30% acetic acid. The solution was centrifuged at 12 000g for 4 min and the supernatant monitored spectrophotometrically at 410 nm. Each triplicate assay included blanks in which the changes in absorbance were recorded without the enzyme extract.

2.5. Effect of pH on proteolytic activity

The optimum pH for protease activity was determined using Universal Buffer (Stauffer, 1989) ranging from 2 to 12 in proteolytic assays.

2.6. Protease inhibition assays

Unknown proteases in gut extracts were classified according to their sensitivity to specific protease inhibitors of each of the main four protease classes, following the procedures described by Dunn (1989) and García-Carreño (1992). The four groups are: (a) serine protease; (b) sulfhydryl proteases (the names thiol proteases and cysteine proteases are also used); (c) the metal-containing proteases, and (d) the aspartic proteases (the name carboxyl proteases and acidic proteases are also used). The names indicate one of the key catalytic groups in the active site. The inhibitors used and their concentrations were: PMSF (phenylmethylsulfonyl fluoride, a serine protease inhibitor), 100 mM; SBTI (soybean trypsin inhibitor, a serine protease inhibitor), 12 mM; LBTI (lima bean trypsin inhibitor, a serine protease inhibitor), 12 mM; OVO (ovomucoid, a serine protease inhibitor), 12 mM; TLCK (*N*-*p*-tosyl-L-lysine chloromethyl ketone, a trypsin inhibitor), 10 mM; TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone, a chymotrypsin inhibitor), 5 mM; ZPCK (*N*-CBZ-L-phenylchloromethyl, a chymotrypsin inhibitor), 10 mM; IA (iodoacetamide, a cysteine protease inactivator), 0.1 M; E64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, a cysteine protease inhibitor), 500 mM; LP (leupeptin, a serine and cysteine protease inhibitor), 10 mM; PA (pepstatin A, a aspartic protease inhibitor), 1 mM; EDTA (a metalloprotease inactivator), 10 mM; EGTA (ethylene glycol bis(β-aminooethyl ether) *N,N'*-tetra acetate, a metalloprotease inactivator), 10 mM.

Enzyme extracts (20 µl of 30-day-old larvae) were mixed with 0.5 ml of 0.1 M Tris-HCl, 10 mM cysteine buffer, pH 9.0, and 10 µl of different inhibitors and incubated for 60 min at 25°C. The mixture was assayed for protease activity as detailed before. The protease assay includes an internal control for inhibition caused by solvents, and control enzymes (porcine trypsin, a serine protease; bovine chymotrypsin, a serine protease; papain, a cysteine protease; thermolysin from *Bacillus*

thermoproteolyticus, a metalloprotease and, porcine pepsin, an acid protease) to ensure the chemical integrity of the different commercial inhibitors assayed. Average inhibition is expressed as a percentage, with 0% the inhibition obtained in proteolytic assays from extracts incubated without inhibitors.

2.7. Electrophoresis

SDS-PAGE of the proteins in the enzyme preparations was done according to Laemmli (1970), using 12% polyacrylamide and 8×10×0.075 cm³ gels. About 5 µg of molecular weight marker (MWM) were loaded on each plate. Preparation of samples and zymograms of protease activities were done according to García-Carreño et al. (1993). The amount of protein sample loaded was 15 µg in all cases. Electrophoresis was done for 60 min at a constant voltage of 100 V per gel at 5°C. After electrophoresis, the gels were placed in 2.5% (v/v) Triton X-100 for 30 min (Lacks and Springhorn, 1980), then washed and incubated in substrate solution (0.25% w/v hemoglobin in 0.1 M glycine-HCl, pH 5.0, containing 10 mM L-cysteine or in 0.5% w/v casein Hammerstein, pH 9.0) for 30 min at 5°C with gentle agitation. Then, they were transferred for 90 min to the same solution at 37°C without agitation. Finally, gels were washed and proteolysis was stopped by transferring the gels into 12% TCA, prior to staining with 0.1% (w/v) Coomassie brilliant blue (R-250) in methanol-acetic acid-water (35:10:55). Destaining was done in methanol-acetic acid-water (35:10:55).

Characterization of protease classes in SDS-PAGE zymograms using specific inhibitors was done according to García-Carreño and Haard (1993). About 20 µl of the extracts were mixed with 5 µl of inhibitor stock solutions (see above) and incubated for 1 h at 25°C. Then they were mixed in a 1:1 ratio with sample buffer, and 25 µl of the final solution loaded on SDS-PAGE plates. Extracts incubated without inhibitor were used as controls. Electrophoresis and zymograms were done as described in the previous paragraph. After electrophoresis, gels were washed for 15 min using 0.1 M Tris-HCl, 10 mM L-cysteine buffer, pH 9.0, at room temperature, before incubation with the substrate (casein).

Changes in the relative intensity of caseinolytic bands in zymograms were estimated by scanning the gels directly (Scanjet 4c; Hewlett Packard) and studying the images by densitometry with specific software (Diversity One™; Huntington Station, New York). Data of image analysis were expressed as percentage of optical density.

2.8. Statistical analysis

Results are given as mean±SD (*n*=3). Data were compared by one-way analysis of variance followed by a

Newman Keuls multiple range test when significant differences were found at a $p < 0.05$ level.

3. Results

3.1. Protease activity in *R. ferrugineus* gut

Fig. 1A shows changes in values of total protease activity contained within the larvae gut from five days post-hatch until day 35. The activity pattern showed two sharp peaks around 15 and 30 days and a marked decrease of activity in 20-day-old larvae. In the same figure, proteolytic activity, expressed as U/gut, is also shown. The general trend was an increase of activity with age up to 25 days, after which noticeable decrease

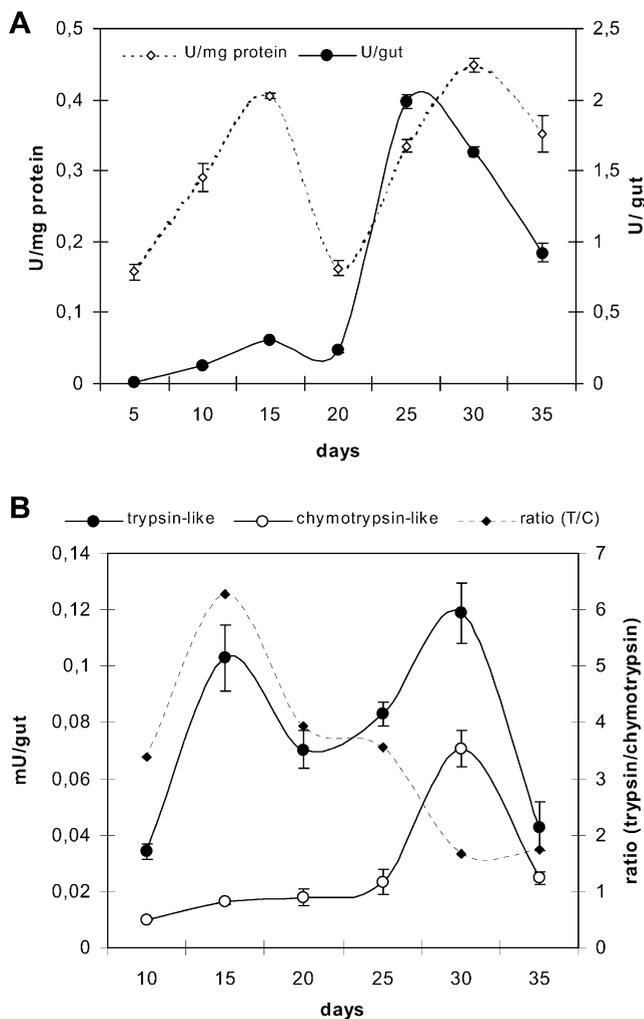


Fig. 1. Evolution of digestive protease activity during larval development of *R. ferrugineus*. (A) Azocasein hydrolysis by enzymatic extracts. The solid line expressed activity in U/gut, the dashed line was U/mg protein. (B) Tryptic and chymotryptic activity of digestive extracts. Dark dots represented BAPNA hydrolysis, open dots were SAPNA hydrolysis. The ratio trypsin/chymotrypsin was also represented (dashed line). Each data point was three replicates.

of the enzymatic activity was found. Total protease activity existing in 35-day-old larvae was clearly higher than those measured at day 5.

Results obtained when assaying digestive extracts with specific substrates showed the presence of both trypsin-like and chymotrypsin-like activities (Fig. 1B). The amount of trypsin activity in individual larval was from 2 to 6 times higher than chymotrypsin activity.

Hydrolysis of the azocasein by the gut extracts from 30-day-old *R. ferrugineus* larvae showed an alkaline optimum pH range (9.0–10.0, Fig. 2). Gut homogenate activity was relatively inactive below a pH of 5.0 (15% of maximum activity) whereas at pH 12.0 proteases showed 70% of the maximum activity recorded.

3.2. Inhibition studies

Crude gut extracts of 30-day-old larvae were incubated with specific inhibitors of the four main protease classes (Dunn, 1989). The extent of the inhibition of azocasein hydrolysis provided an indication about the relative contribution of the inhibited class of protease to total protease activity in the gut (Table 2). The serine protease inhibitors SBTI reduced gut activity by 78% and ovomucoid 83%. Gut homogenate protease activity showed a slight inhibition by LBTI (32%). The differences in effectiveness between serine protease inhibitors are probably because of the different specificity of these inhibitors for proteases. The synthetic inhibitor PMSF inhibits 61% of gut protease activity but it was less active than the SBTI and OVO proteinaceous inhibitors. When testing peptidyl chloromethyl ketones, TLCK (specific inhibitor to trypsin-like enzymes) inhibits only the 18% of protease activity, whereas ZPCK (inhibitor

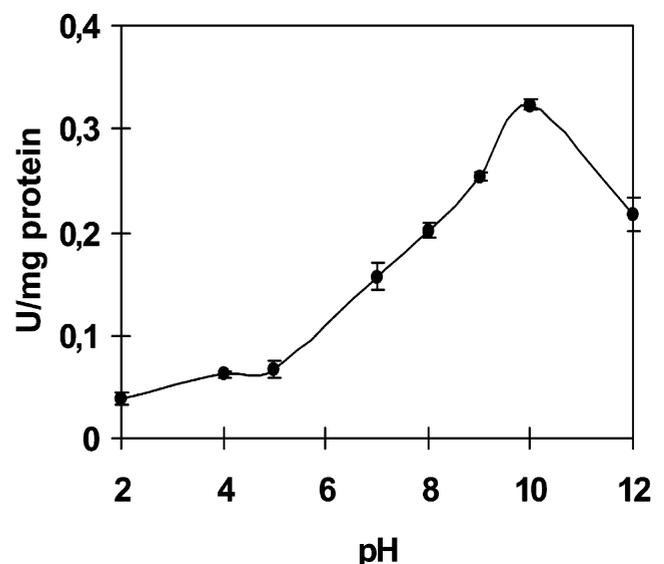


Fig. 2. The effect of pH on proteolysis of azocasein by *R. ferrugineus* gut extracts. Extracts were incubated with azocasein buffered solution for 60 min at 37°C. Buffers used are listed in Section 2.

Table 2

Effect of different inhibitors on proteolytic activity of gut extracts. Values are mean of three determinations \pm SD (ND, not detected)

Inhibitor	Percentage of inhibition
PMSF	61.3 \pm 9.2
SBTI	78.3 \pm 2.3
LBI	31.8 \pm 1.3
OVO	82.5 \pm 3.2
TLCK	17.7 \pm 3.6
TPCK	2.9 \pm 1.9
ZPCK	34.5 \pm 5.4
EDTA	39.3 \pm 3.8
EGTA	15.4 \pm 4.1
IA	3.3 \pm 1.6
LEU	ND
E-64	ND
PA	ND

for chymotrypsin-like enzymes) yielded 35% inhibition. TPCK (inhibitor for chymotrypsin-like enzymes) did not reduce proteolytic activity. The cysteine protease inhibitors E-64, LP, and IAA did not alter the activity of enzymes, which suggests that cysteine protease-like enzymes did not contribute to total protease activity to a great extent. The fractions of the proteases inhibited by EDTA (39%) and EGTA (15%) may be attributed to metalloprotease-like activity, but some chelating agents affect others classes of proteases that require divalent ions. Pepstatin A, an aspartic acid protease inhibitor from microbial source had no significant effect on protease activity, indicating that aspartic acid proteases are not active in the gut extracts.

3.3. Characterization of digestive proteases by SDS-PAGE

Individual protease fractions during larval stages of *R. ferrugineus* from 5 to 35 days were separated on SDS-PAGE, and visualized by incubating gels in hemoglobin at pH 5.0 or casein at pH 9.0. None of the gut larvae extracts showed active fractions when using hemoglobin as substrate (data not shown). However, the incubation of gels in an alkaline casein solution yielded several bands of protease activity (Fig. 3). Protease activity was shown early in the development (day 5) in the form of three clear bands with relative molecular masses of 20, 66, and 100 kDa. A more complex set of active bands was observed from day 15 on (lane 3), with the composition of proteases practically constant throughout larval development, although some differences in the intensity of specific bands were found (Fig. 4). Digestive proteases ranged from 16 to 100 kDa, and they could be classified into three different groups according to their apparent molecular mass. High molecular-mass proteases (HMP) ranging from 80 to 100 kDa are more evident from day 15 on and vary their intensity as the age

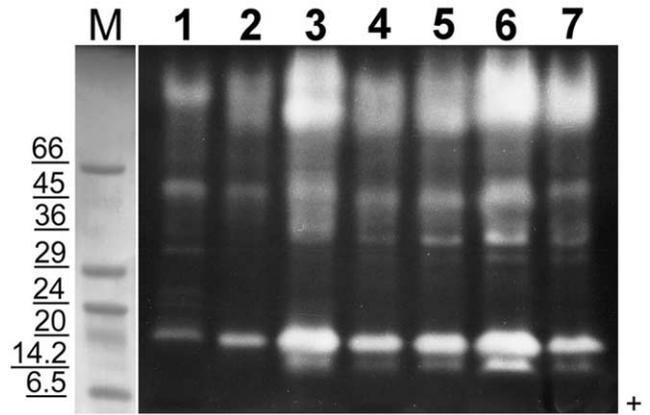


Fig. 3. Substrate-SDS-PAGE of extracts obtained from *R. ferrugineus* larvae at different days after hatching. Columns: 1–7=15 μ g of total protein from larvae extracts at 5, 10, 15, 20, 25, 30 and 35 day post hatching. M is 5 μ g of molecular weight markers; bovine serum albumin (66 kDa), chicken egg ovalbumin (45), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36), bovine erythrocytes carbonic anhydrase (29), bovine pancreas trypsinogen (24), soybean trypsin inhibitor (20), bovine milk α -lactoalbumin (14.2) and bovine lung aprotinin (6.5). The larval proteins were resolved in 12% polyacrilamide gels and incubated in 0.5% casein solution at 37°C. Protease forms were grouped according to their apparent molecular weight as HMP (100–80 kDa), MMP (66–30 kDa) and LMP (24–16 kDa).

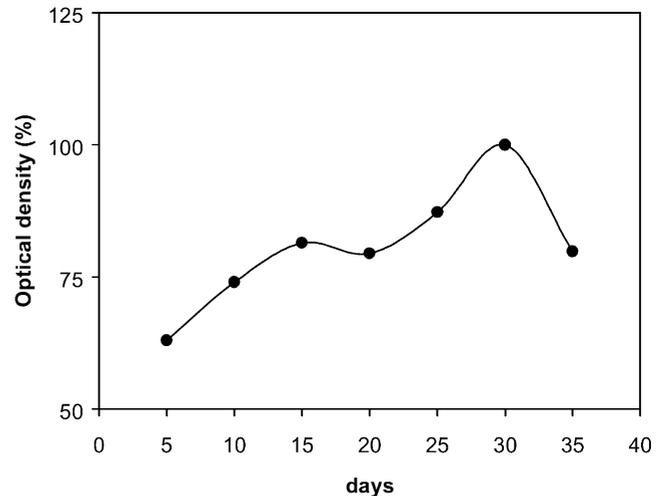


Fig. 4. Changes in total optical density obtained from zymogram showed in Fig. 3. Dots were the sum of total optical density of active fractions expressed as percentage.

of larvae increases. Medium molecular-mass proteases (MMP) ranged from 30 to 66 kDa. Low molecular-mass proteases (LMP) of 16–24 kDa were present until day 35. Values of densitometry (intensity of active bands) obtained from zymograms agreed with results of enzymatic activity obtained by biochemical assays (Fig. 4).

Fig. 5 shows the results of zymogram image analysis to determine the contribution of HMP, MMP and LMP protease group to the total protease activity detected in gel electrophoresis. It was found that, with the exception of five-day-old larvae, the HMP and MMP protease

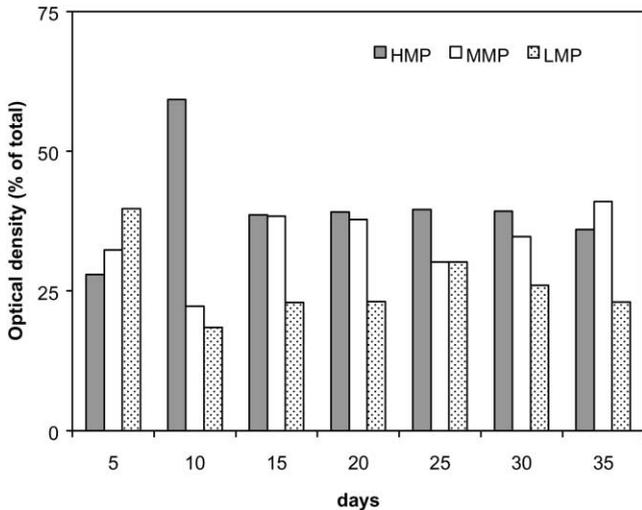


Fig. 5. Contribution of HMP (100–80 kDa), MMP (66–30 kDa) and LMP (24–16 kDa) active fractions to total protease activity in larval extracts. Data were expressed as percentage of optical density and calculated from Fig. 3.

groups produced more of 60% total protease activity revealed in zymograms. Besides, 15–35-days-old larvae showed a similar distribution pattern of protease groups.

Enzyme extracts were incubated with protease inhibitors before electrophoresis to directly identify inhibited proteases. The use of inhibitors demonstrated that alkaline protease activity was affected by PMSF and OVO, as shown by a 30-day-old larvae (Fig. 6, lane 1 and 2). Inhibition caused by proteinaceous inhibitors (lane 2) produces differences in mobility of bands in relation to those present in the control extracts (lane C). This indicates a modification in the apparent molecular masses of active proteases, likely because of the formation of a reversible protein complex between enzyme and inhibi-

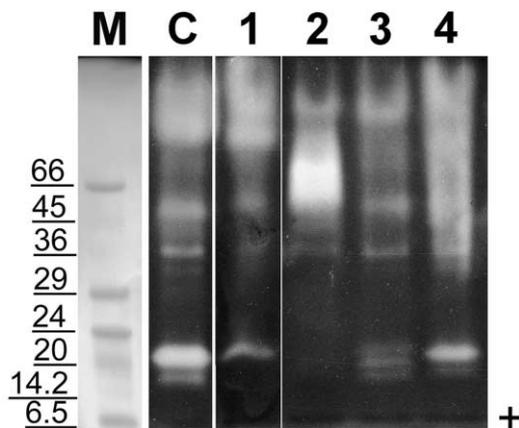


Fig. 6. Substrate-SDS-PAGE of gut extracts of *R. ferrugineus* larvae treated with different specific inhibitors. The protein extract (15 μ g total protein) was incubated with water (control lane) or 5 μ l of different protease inhibitors. M=5 μ g of molecular weight markers as Fig. 3, Lane C=control without inhibitor, lane 1=2 mM PMSF, lane 2=240 μ M OVO, lane 3=100 μ M TLCK, and lane 4=10 mM EDTA.

tor. TLCK (specific for trypsin-like enzymes) inhibits the HMP and LMP groups of active bands (lane 3). The presence of chelating-sensitive proteases was also detected in zymograms (lane 4).

3.4. Effect of substrates on the digestive proteases of *R. ferrugineus* larvae

Values of protease activity from the gut extracts of 35-day-old larvae reared on different substrates are shown in Fig. 7. Comparison on an individual gut basis revealed that the highest activity was measured in larvae fed an artificial diet, with those larvae fed on *P. canariensis* the most similar. Larvae fed on *T. fortunei* showed lower values for protease activity. When a full analysis of variance was carried out, differences were found between larvae fed on artificial diet and larvae fed on *T. fortunei* or sugar cane. The protease activity measured in larvae fed on *S. officinarum* is significantly reduced when compared to those fed on other substrates, meanwhile *Phoenix canariensis* showed no significant effects on protease activity of enzymatic extracts when compared to *T. fortunei* and artificial diet. A similar result was obtained when using specific substrates to measure tryptic activity. Proteases were visualized on casein zymograms at pH 9.0 (Fig. 8). In insects reared on artificial diets, the three groups of proteases described above were present through the whole larval development. Similar pattern of proteases in insects reared on *P. canariensis* and *T. fortunei* were detected, although some variations in the intensity of bands (lanes 2 and 3) were observed. In contrast, *S. officinarum* decreased both the intensity and the number of active band in gut

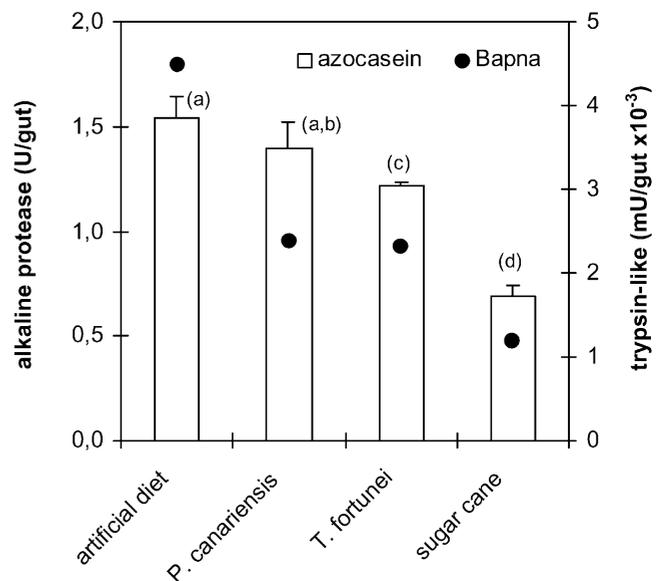


Fig. 7. Total protease and trypsin-like activities from 35-day-old *R. ferrugineus* larvae reared on different substrates. Data with different superscript were significantly different ($p < 0.05$). Azocasein and Bapna were used for total protease and tryptic determinations, respectively.

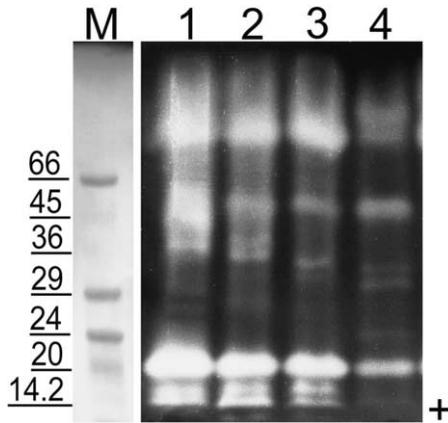


Fig. 8. Substrate-SDS-PAGE of gut extracts (15 μ g of total protein) of 35-day-old *R. ferrugineus* larvae reared on different substrates. M=5 μ g of molecular weight markers as Fig. 3, lane 1=artificial diet, lane 2=*Phoenix canariensis*, lane 3=*Trachycarpus fortunei* and, lane 4=sugar cane.

extracts. Results are consistent with values of protease activity obtained in biochemical assays.

4. Discussion

Results obtained in the present work showed the presence of digestive proteases in the early stages of the larval development of *R. ferrugineus*. Activity of digestive proteases assayed in the active feeding period of larvae, expressed as U/mg protein, showed a noticeable increase between day 5 and day 35. However, on day 20 specific protease activity showed a noticeable decrease, sharing values with five-day-old larvae. This decline may result from a greater degradation or a lower synthesis of digestive proteases produced by a quantitative decrease of the feed intake when larvae is near of the next molt stage. In this sense, the diminution in enzymatic activity could be related with some anatomical and physiological modifications of larvae gut. In this study, there was no quantitative control of ingestion. Nevertheless, feed intake was evident in both 15- and 20-day-old larvae. Investigations into ingestion and digestive enzyme activities within an instar are required for further interpretations.

Similar patterns in specific activity of digestive proteases were also reported throughout the larval development of Lepidoptera (Broadway and Duffey, 1985) and other groups of Arthropoda, like Crustacea (Lemos et al., 1999). However, the expression of data in this way (U/mg prot) could not seem to make any sense from a physiological point of view, because variations in specific activities are highly influenced by variations in other soluble proteins, without proteolytic activity, taking place during larval growth. One way to avoid this effect is to express the activities in relation to individuals. In effect, values of protease activity expressed as

U/gut showed a progressive increase of digestive proteases from day 5 to day 25. In this case, the decrease of gut protease activity was recorded on, and after, day 30. Results showed that proteolytic activities of digestive enzymes were high, although the dietary protein contents were relatively low (artificial diet 15%protein, palms 4%protein and sugar cane 3%protein). According with our results, protease activity in gut extracts includes both tryptic and chymotryptic activities, although the contribution of trypsin-like enzymes was more evident (Fig. 1B). A survey of the literature of insect proteases reveals that trypsin-like activity is the commonest, highest activity found in total protease activity in insect guts (Baker, 1977), and chymotrypsin-like activity may (Gooding, 1975) or may not be present (Yang and Davies, 1971). The presence of protease activity in the gut of Coleoptera larvae was also reported in several species: *Costelytra zealandica* (Christeller et al., 1989; Biggs and McGregor, 1996); *Phyllotreta cruciferae* (Rymerson and Bodnaryk, 1995); *Sophrorhinus insperatus* (Adedire, 1994); *Baris coerulea* (Bonadé-Bottino et al., 1999); *Phaedon cochleariae* (Girard et al., 1998a), *Tribolium castaneum* (Blanco-Labra et al., 1996); and *Leptinotarsa decemlineata* (Michaud et al., 1995; Novillo et al., 1997). With some exceptions, most of these works study only digestive proteases in the last instar of the larval development, and they do not provide data on changes in enzymatic activities during ontogenetic development.

Gut homogenates of 30-day-old larvae displayed substantial activity only at alkaline pH with maximum values recorded at pH 10.0 (Fig. 2). This high value is not the same as the optimum pH reported for several species of Coleoptera, which show a neutral (pH 7.0) or even slightly acid (pH 5.0) optimum pH (Coppedge et al., 1994; Rymerson and Bodnaryk, 1995; Terra and Cristofolletti, 1996; Kuroda et al., 1996; Novillo et al., 1997). However, an alkaline optimum pH was also reported in other related species of the Curculionidae family (Adedire, 1990; Girard et al., 1998a; Bonadé-Bottino et al., 1999). In this family, the existence of two optimum pHs is well documented, one at 5.0–6.0 and the other at 9.0–10.0, corresponding to the pH range of cysteine and serine proteases (Girard et al., 1998b; Bonadé-Bottino et al., 1999). These authors have confirmed the presence of both types of proteases in larval gut homogenates. Our results — obtained for *R. ferrugineus* larvae — suggest a major involvement of alkaline proteases in protein digestion. It is well known that insects are capable of finely regulating the gut pH to provide optimum conditions for the digestive process. For example, Biggs and McGregor (1996) have reported a variation of three units of pH along the digestive tract of the Coleoptera *Costelytra zealandica*. For *R. ferrugineus*, an alkaline gut could provide some additional positive effects to larvae by avoiding tannin–protein complexes, inactivating harmful plant substances, and

helping to extract alkali-soluble plant protein or cell-wall polysaccharides, as described for other species (Martin et al., 1987; Terra, 1988; Felton and Duffey, 1991; Felton et al., 1992). To confirm this hypothesis, it will be necessary to accurately measure the pH along digestive system of larvae because preparation of homogenates will mask local variation on gut pH.

Results of SDS-PAGE are in close agreement with those obtained by using test tube assays. The use of electrophoresis allows greater accuracy in determining and classifying protease activities, because the use of substrate SDS-PAGE makes it possible to determine exactly when an activity appears during ontogeny. Zymograms made using casein as substrate showed the presence of alkaline protease from the first-instar larvae. The enzymatic profile was fully developed in 15-day-old larvae and no differences could be observed until an age of 35 days, except in the variation of intensity in some active bands. According to their apparent molecular masses, we could classify digestive proteases into three different groups; HMP, MMP, and LMP containing proteases ranging from 80 to 100 kDa, 30 to 66 kDa and 16 to 24 kDa. The number of total active fractions in larval extracts was between four and seven. From the first-instar larvae, at least one active band of each group of proteases was observed. A similar number of active fractions were described in other species of Curculionidae. Girard et al. (1998b) described six proteases ranging from 22 to 96 kDa in digestive extracts of *Ceutorhynchus assimilitis* larvae and the *Baris coerulescens* L4-instar showed six different digestive proteases on zymograms (Bonadé-Bottino et al., 1999). However, *R. ferrugineus* showed proteases only at alkaline pH, whereas in other species of Coleoptera proteases were found both at pH 5.0 and 9.0. From a physiological perspective, and taking into account that larvae feed mainly on carbohydrates, it is surprising to find this complex protease profile in gut extracts. It could be assumed that this helps larvae digest the scarce protein of their feed.

Gut extracts were incubated with protease inhibitor specific for different classes of proteases. The percentage of inhibition of azocasein digestion provided an indication of the relative contribution of the inhibited class of protease to total gut protease activity. *R. ferrugineus* digestive proteases were not inhibited by cysteine protease inhibitors, but more than 50% reduction in activity was detected in the presence of serine protease inhibitors (Table 2). This data established that the gut of larvae contains mainly serine proteases. The evidence is based on the alkaline pH optimum of gut protease activity (Fig. 2) and its inhibition by diagnostic protease inhibitors. It is well documented that cysteine and serine proteases are the principal families of proteases involved in protein digestion by Coleoptera (Murdock et al., 1987; Terra and Cristofolletti, 1996; Girard et al., 1998b). The lack of cysteine proteases in some Coleoptera species was

reported and would indicate a secondary loss of these enzymes in phytophagous species fed on vegetables without serine protease inhibitors (Terra and Cristofolletti, 1996). This seems to be true for *R. ferrugineus*. In addition, the lack of serine protease inhibitors in palms that are usually infected by this insect pest is confirmed (data not shown). Results of inhibition obtained by zymograms also showed the major presence of serine proteases in larval extracts (Fig. 6, lanes 1–3).

We have examined the effect of different rearing substrates on the digestive activities of larval *R. ferrugineus*. The digestive development of *R. ferrugineus* larvae seems to be influenced by the kind of feed utilized during insect rearing. The highest level of protease activity was found in larvae reared on artificial diets (Fig. 7), whereas larvae reared on sugar cane showed half of this value. This fact could be derived from both the reduced growth of larvae reared on sugar cane and the higher protein content of artificial diet (15%; Kjeldahl Nx6.25). On the one hand, results obtained in our laboratory showed that the weight of larvae fed on artificial diets or *P. canariensis* was four times higher than those reared on sugar cane (unpublished data). On the other, the synthesis of digestive proteases could be modulated by protein intake (Baker et al., 1984).

Caseinograms obtained from digestive extracts showed the presence of the three groups of proteases in larvae reared on artificial diet and palms. Larvae reared on sugar cane showed both lower levels of protease activity and a reduced number of active fractions in zymograms. The proteinograms found in digestive extracts seems to be influenced by the protein source used, revealing only quantitative changes in the protein profile of each group of larvae. The endogenous origin of these proteins is supported by the similar electrophoretic profile of all the gut extracts, independently of the substrate (data not shown).

According with the literature of *Rhynchophorus* rearing, larvae of this weevil were reared in different substrates as natural palm (Genty et al., 1978), coconut stem (Hagley, 1965), sugar cane (Rahalkar et al., 1972; Zagatti et al., 1993) and artificial diets (Rahalkar et al., 1978; Weissling and Giblin-Davis, 1995). Results obtained here pointed out that larvae reared on different substrates showed differences in the relative contribution of the different protease active fractions revealed in zymograms. This fact was more evident in sugar cane reared larvae, curiously one substrate widely used to maintain laboratory populations. In the light of the data, we can conclude that the artificial diet allows a similar ontogenetic development of *R. ferrugineus* larvae digestive proteases, when comparing with their natural feed. The possibility of future evaluations of antimetabolites as potential insect defensive compounds depends on a reliable insect bioassay system. As in other insect pests, artificial diets provide a powerful tool for testing these

compounds against *R. ferrugineus* (Allsopp, 1994). Modern pest strategies will require continuous supplies of test insects for research. Insect reared in laboratory conditions will be used to test the activity of several compounds on individuals and populations. However, the insect rearing management needs an efficient production of insects of standardized quality to meet programmed goals (Singh and Ashby, 1985). The artificial diet employed in this work seems to be a suitable substrate to establish a good-quality red weevil laboratory population for future researches.

To sum up, studies on digestive development of *R. ferrugineus* provides both a basic knowledge of the digestive capacities of this insect to degrade nutrients and helpful knowledge to understand the potential target for future control of this insect pest.

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