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Characterization and Distribution of Chymotrypsin-Like and Other Digestive Proteases in Colorado Potato Beetle Larvae

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Chymotrypsin-like, carboxypeptidase A-like and leucine aminopeptidase-like activities have been detected in the midgut of Colorado potato beetle larvae, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), in addition to the previously identified cathepsin B, D, and H. We have characterized a new chymotrypsin-like activity using the specific substrates N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide and N-benzoyl-L-tyrosine p-nitroanilide. This novel proteinase, with a pH optimum of 5.5–6.5, was neither activated by thiol compounds nor inhibited by cysteine proteinase inhibitors. Among several serine proteinase inhibitors tested, PMSF was the most effective. Gelatin-containing SDS-PAGE gels and activity staining after gel electrophoresis indicated that chymotrypsin-like activity was associated with a major band of about 63 KDa and a minor band of about 100 KDa. The major exopeptidases found in the larval midgut extracts were leucine aminopeptidase and carboxypeptidase A. Most endo- and exoproteolytic activities studied were evenly distributed among the midgut sections, indicating that there is no clear regional differentiation in the digestion of proteins. Chymotrypsin and cathepsin B, D, and H were mainly located in the endoperitrophic and ectoperitrophic spaces, with only a small activity associated with the midgut epithelium. In contrast, leucine aminopeptidase was mainly located on the wall tissue, although some activity was distributed between the ecto- and endoperitrophic spaces. The potential roles of Colorado potato beetle digestive chymotrypsin in the proteolytic activation of the δ-endotoxin from *Bacillus thuringiensis*, and in the use of protease inhibitors to disrupt protein digestion, are discussed. Arch. Insect Biochem. Physiol. 36:181–201, 1997. © 1997 Wiley-Liss, Inc.

Key words: ***Leptinotarsa decemlineata*; proteases; chymotrypsin; *Bacillus thuringiensis***

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INTRODUCTION

The majority of coleopteran larvae and adults have slightly acidic midguts, and cysteine proteinases provide the major midgut endoproteolytic activity (Murdock et al., 1987; Wolfson and Murdock, 1990). Nevertheless, aspartic and/or serine proteinases are detected in some of these species (Wolfson and Murdock, 1990; Thie and Houseman, 1990a), and protein digestion is provided by serine proteinases at alkaline pH in other coleopterans (Baker, 1982; Ferreira and Terra, 1989). Aminopeptidase and carboxypeptidase activities have been reported in several coleopteran species (Baker, 1982; Christeller et al., 1989; Ferreira and Terra, 1989).

Based on the use of family-specific protease inhibitors, Wolfson and Murdock (1987) indicated that the larval midgut of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) contains cysteine and aspartyl proteinases. More precisely, Thie and Houseman (1990b) identified and characterized the cysteine proteinases, cathepsin B and H, and the aspartyl proteinase, cathepsin D. Apart from a higher specific proteinase activity in larvae as compared to adults, no qualitative differences in the proteinase spectrum have been noted between developmental stages (Michaud et al., 1995). Incidentally, Reuveni et al. (1993) detected, but did not characterize, leucine aminopeptidase activity in crude homogenates and brush border membrane vesicles from midguts of CPB larvae. There have been no reports of serine proteinases or carboxypeptidases in this species. This proteolytic profile suggest that disruption of protein digestion by dietary cysteine proteinase inhibitors might be an alternative to pesticides for CPB control. However, previous studies have shown that growth and development of CPB larvae were not severely affected when they were reared on potato leaves that contain high levels of cystatins (Bolter and Jongsma, 1995; Michaud et al., 1995, 1996).

We report here the characterization of chymotrypsin-like, carboxypeptidase A-like and leucine aminopeptidase-like activities in the midgut of CPB larvae. In addition, the spatial organization of these and previously characterized digestive proteases along the gut sections and among the different midgut regions were also examined. The presence of chymotrypsin-like activity in CPB may have important implications for its control, either by using protease inhibitors or the δ-endotoxin from *Bacillus thuringiensis*.

MATERIALS AND METHODS

Insects

A colony of Colorado potato beetle was established by collecting over 80 adults from a potato field located in Toledo (Spain) during the fall of 1995. The laboratory population was reared on potato plants, *Solanum tuberosum* cv. Kennebec, at 24 ± 1°C, 90 ± 10 % rh and 16:8h (L:D) photoperiod in an environmental chamber.

Chemicals and Equipment

All substrates, protease inhibitors and electrophoretic reagents were purchased from Sigma Chemical Co., (St. Louis, MO). Sephadex G-25 was

from Pharmacia (Uppsala, Sweden), black India drawing ink was from Pelikan (Hanover, Germany), and the nitrocellulose membrane used was Hybond-C from Amersham Life Science (Buckinghamshire, UK). Spectrophotometric measurements were made using a Hitachi U-2000 spectrophotometer. The pH of each midgut region was determined using a microprocessor pH/mv/°C system with isfet sensor from UniFet Inc. (San Diego, CA).

Gut Extracts

Last instar larvae were dissected in 0.15 M NaCl, and the midguts and contents removed and stored frozen (–20°C) until needed. Each midgut was subsequently homogenized in 500 µl 0.15 M NaCl, centrifuged at 10,000*g* for 5 min, and the supernatants pooled and kept on ice for enzymatic activity assays.

For the distribution of digestive proteases along the gut sections, the gut was quick frozen and then dissected to obtain the anterior, middle, and posterior portions of the midgut and the hindgut. To separate the different midgut regions, the gut wall of the middle and posterior portions was split longitudinally to obtain the peritrophic membrane and its contents (endoperitrophic space), the midgut epithelium, and the washings of these two regions (ectoperitrophic space). Each fraction was homogenized in 500 µl 0.15 M NaCl as above, and the supernatants kept individually on ice for enzymatic activity assays.

Enzyme Assays

For the characterization of digestive proteases, the assays were done in triplicate using three different pooled gut extracts. For the distribution of digestive proteases, the assays were done using 15 independent gut extracts. In all cases, blanks were used to account for spontaneous breakdown of substrates. Reaction buffers were: 0.1 M citric acid-NaOH (pH 2.0–3.0); 0.1 M citrate (pH 3.0–6.0); 0.1 M phosphate (pH 6.0–7.0); 0.1 M tris-HCl (pH 6.5–9.0); and 0.1 M glycine-NaOH (pH 9.0–10.0). All buffers contained 0.15 M NaCl and 5 mM $MgCl_2$. Unless otherwise stated, protease assays were performed in 1 ml of reaction buffer at their optimum pH of activity and met initial rate conditions.

Non-specific protease activity was determined by adding 1 ml of 0.1% sulfanilamide-azocasein solution to 50 µl of midgut extract, as described by Ortego et al. (1996). Likewise, the activity of aspartyl proteinases was measured with 0.2% hemoglobin solution as substrate (Houseman and Downe, 1982). In both cases, the reaction mixture was incubated at 30°C for 1 h (hemoglobin) or 4 h (azocasein) and the reaction stopped by the addition of 500 µl of 10% ice-cold trichloroacetic acid. The solution was centrifuged at 10,000*g* for 5 min and the absorbance of the supernatant measured at 420 nm and 280 nm for azocasein and hemoglobin, respectively.

Cethepsin B and cathepsin H activities were determined according to Barrett

(1976), using as substrates 50 μM ZAA_2MNA* (N-carbobenzoxy-alanine-arginine-arginine 4-methoxy-β-naphthyl amide) and 1mM ArgNA (L-arginine β-naphthyl amide), respectively. For ArgNA hydrolysis, the reaction was started by the addition of the substrate solution to the reaction buffer containing 50 μl midgut extract, and incubated at 30°C for 60 min. In the case of ZAA_2MNA hydrolysis, 15 μl of one fourth diluted midgut extract was used, and the incubation time was 15 min. In both cases, the reaction buffer contained 1 mM L-cysteine. The reactions were stopped by the addition of 1 ml of mersalyl-Fast Garnet-Brij reactive solution (10 mM mersalyl; 2% Brij-35; 0.2 mg/ml Fast Garnet), and the absorbance monitored at 520 nm. The activity was measured as nmol substrate hydrolyzed per minute per mg protein. A molar extinction coefficient of 24,000 M^{-1} cm^{-1} was obtained under our experimental conditions.

Chymotrypsin-like activity using 0.25 mM SA_2PPpNa (N-succinyl-alanine-alanine-proline-phenylalanine p-nitroanilide) and 1 mM BTpNa (N-benzoyl-L-tyrosine p-nitroanilide), elastase-like activity with 0.5 mM SA_3pNa (N-succinyl-alanine-alanine-alanine p-nitroanilide), and leucine aminopeptidase-like activity with 1 mM LpNa (L-leucine p-nitroanilide) as substrates, were surveyed as described by Ortego et al. (1996). The reaction was started by the addition of the substrate solution to the reaction buffer containing 50 μl midgut extract, and incubated at 30°C for 30 min (LpNa), 1 h (SA_2PPpNa), 2 h (BTpNa) or 24 h (SA_3pNa). In all cases, the reaction was stopped by the addition of 500 μl of 30% acetic acid. The solution was centrifuged at 10,000*g* for 5 min and the absorbance of the supernatant measured at 410 nm.

Total cysteine proteinase activity was measured using 1 mM BApNa (Nα-benzoyl-DL-arginine p-nitroanilide) as substrate, according to Houseman (1978). The assay was performed adding the substrate solution and 50 μl midgut extract to the reaction buffer containing 1 mM L-cysteine. The reaction was incubated at 30°C for 3 h, stopped by the addition of 500 μl of 30% acetic acid, and the absorbance measured at 410 nm as above described for other p-nitroanilide substrates.

Carboxypeptidase A activity was assayed with 1 mM HPA (hippuryl-phenylalanine) and carboxypeptidase B activity with 1 mM HA (hippuryl-L-arginine), according to Ortego et al. (1996). The reaction was started by the addition of the substrate solution to the reaction buffer containing 30 μl midgut extract, incubated at 30°C for 24 h, and stopped by the addition of 500 μl of 30% acetic acid. The rate of the reaction was calculated from the amount of liberated aminoacids by the ninhydrin procedure (Moore, 1968). The reac-

*Abbreviations used: ArgNA = L-arginine β-naphthyl amide; BApNa = Nα-benzoyl-DL-arginine p-nitroanilide; BTpNa = N-benzoyl-L-tyrosine p-nitroanilide; DMSO = dimethylsulfoxid; DTT = dithiothreitol; E-64 = L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane; EDTA = ethylenediamine tetraacetic acid; HA = hippuryl-L-arginine; HPA = hippuryl-phenylalanine; IAA = iodoacetamide; LpNa = L-leucine p-nitroanilide; PCPI = potato tuber carboxypeptidase inhibitor; PMSF = phenylmethylsulfonyl fluoride; SA_2PPpNa = N-succinyl-alanine-alanine-proline-phenylalanine p-nitroanilide; SA_3pNa = N-succinyl-alanine-alanine-alanine p-nitroanilide; SBBI = Soybean Bowman-Birk inhibitor; TLCK = Nα-p-tosyl-L-lysine chloromethyl ketone; TPCK = N-tosyl-L-phenylalanine chloromethyl ketone; ZAA_2MNA = N-carbobenzoxy-alanine-arginine-argine 4-metoxy-β-naphthyl amide; and ZPCK = N-carbobenzoxy-L-phenylalanine chloromethyl ketone.

tion buffer 0.1 M borate-NaOH (pH 9.0–10.0) was used instead of the corresponding glycine buffer, which react with ninhydrin.

Total protein in the midgut extracts was determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

Protease Inhibitors and Activators

The proteolytic activities of midgut extracts were assayed in the presence of the following specific protease inhibitors: the serine protease inhibitors, SBBI (Soybean Bowman-Birk inhibitor), PMSF (phenylmethylsulfonyl fluoride) and chymostatin; the trypsin inhibitor, TLCK (Nα-p-tosyl-L-lysine chloromethyl ketone); the chymotrypsin inhibitors, TPCK (N-tosyl-L-phenylalanine chloromethyl ketone) and ZPCK (N-carbobenzoxy-L-phenylalanine chloromethyl ketone); the cysteine protease inhibitors, E-64 (L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane), IAA (iodoacetamide) and leupeptin; the aspartic protease inhibitor, pepstatin-A; the metalloprotease inhibitors, EDTA (ethylenediamine tetraacetic acid) and 1,10 phenanthroline; the carboxypeptidase inhibitor, PCPI (potato tuber carboxypeptidase inhibitor); and the divalent heavy metal ions, $CdCl_2$ and $CuCl_2$. The cysteine protease activators L-cysteine and DTT (dithiothreitol) were also tested.

Protease inhibitors and activators were preincubated at 30°C with the midgut extract for 15 min, prior to addition of substrate. All compounds were added in 100 µl of 0.15 M NaCl, except PMSF, TPCK, chymostatin, 1,10-phenanthroline and pepstatin-A that were added in 20 µl of DMSO and ZPCK, in 30 µl of DMSO. The inhibitors and activators were used to determine the catalytic type of proteases according to the effective concentrations recommended by Beynon and Salvesen (1989).

Zymograms

Electrophoretic detection of proteolytic forms was performed using 0.1% (w/v) gelatin-containing 0.1% (w/v) SDS-12% (w/v) polyacrylamide slab gels according to Michaud et al. (1993a). The ratio of acrylamide to N,N´-methylenebisacrylamide was adjusted to 29.0:1.1. Samples of endoperitrophic contents from CPB larval midgut extracts were homogenized in 0.15 M NaCl, centrifuged at 10,000*g* for 5 min, and the supernatants passed through a Sephadex G-25 column to remove low molecular weight compounds. Samples were subjected to treatment with specific inhibitors before electrophoresis. To inhibit cysteine proteinases, 25 µl extract was incubated with 5 µl of 0.5 mM E-64 in 0.15 M NaCl and 20 µl of incubation buffer (100 mM citrate, pH 6.0), for 15 min at 30°C. To inhibit serine proteinases, 25 µl extract was incubated with 4 µl of 50 mM PMSF in DMSO and 21 µl of incubation buffer, for 15 min at 30°C. Controls were prepared by replacing the inhibitor solutions by their corresponding solvents. Samples containing approximately 10 µg of protein were diluted two-fold in electrophoresis sample buffer to obtain 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 0.05% (w/v) bromophenol blue, and subjected to electrophoresis using a Bio-Rad Mini-Protein II Electrophoresis Cell system. After migration at 4°C, gels were transferred into a 2.5% (v/v) aqueous solution of Triton X-100 for 30 min at

room temperature, to allow renaturation of the proteinases. Gels were then placed in an activation buffer (100 mM citrate, pH 6.0, 5 mM L-cysteine) for 2 h at 35°C. Proteolysis was stopped by transferring the gels into a staining solution (0.3% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol, 10% (v/v) acetic acid). The gels were destained in 25% (v/v) methanol, 10% (v/v) acetic acid.

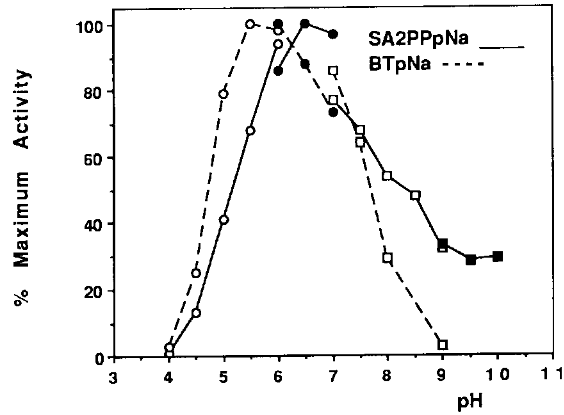
Activity Staining After Gel Electrophoresis

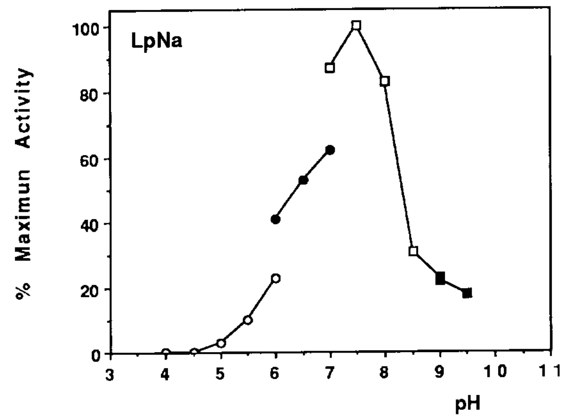
Samples of endoperitrophic contents from CPB larval midgut extracts (containing approximately 15 μg of protein) were submitted to SDS-PAGE electrophoresis, performed as previously described. After electrophoresis, the gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% (v/v) methanol) for 15 min at 4°C. The proteins on the gel were then blotted onto nitrocellulose paper using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell at 100 volt for 1 h at 4°C. The proteases blotted in the nitrocellulose membrane were incubated with 20 μM E-64 or 2 mM PMSF in 100 mM citrate, pH 6.0, for 15 min at 30°C. Controls were incubated in the same buffer. Chymotrypsin-like activity staining was performed according to Ohlsson et al. (1986). The nitrocellulose membrane was soaked in 100 mM citrate, pH 6.0, for 5 min and then placed in a 0.1 mM SA_2PPpNa solution (1% DMSO (v/v) 100 mM citrate, pH 6.0) for 90 min at 30°C. After incubation with the substrate, the nitroanilide liberated was diazotized by subsequent incubations of 5 min each in 0.1% sodium nitrite in 1 M HCl, 0.5% ammonium sulphamate in 1 M HCl, and 0.05% N-(1-naphthyl)ethylene diamine dihydrochloride in 47.5% ethanol. General proteolytic activity was performed soaking the membrane in 100 mM citrate, pH 6.0, for 5 min, and then placing it in a 1% (w/v) azocasein solution (0.15 M ClNa) for 45 min at 4°C to let the protein substrate diffuse into the membrane. The membrane was then incubated with 100 mM citrate, pH 6.0, at 30°C. After incubation overnight, the protein substrate embedded in the membrane was stained with India ink (Hancock and Tsang, 1983). Bands of proteolytic activity were visualized against the black background of the membrane.

RESULTS

Characterization of Proteolytic Activities

The use of specific substrates and inhibitors has allowed the identification of chymotrypsin-, leucine aminopeptidase-, and carboxypeptidase A-like activities in CPB midgut extracts. The effect of pH on these proteolytic activities is summarized in Figure 1. Optimal hydrolysis of the chymotrypsin substrates SA_2PPpNa and BTpNa occurred at pH 6.5 and 5.5–6.0, respectively. The hydrolysis of the leucine aminopeptidase substrate LpNa has a pH optimum of 7.5. Carboxypeptidase A activity was characterized using HPA as substrate and presented an optimum pH of 8.0. Elastase activity against the specific substrate SA_3pNa and carboxypeptidase B activity using HA as substrate were too low to characterize them. Table 1 shows the specific activities of both, novel and previously characterized, midgut proteases at their optimum pH of activity under our experimental conditions.





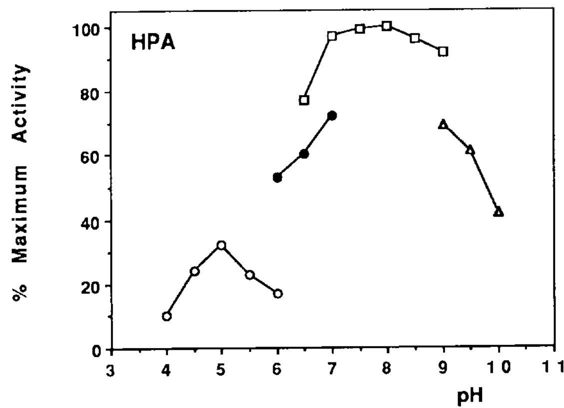


Fig. 1. The effect of pH on the rate of hydrolysis of the chymotrypsin substrates SA_2PPpNa and BTpNa, the leucine aminopeptidase substrate LpNa, and the carboxypeptidase A substrate HPA by larval midgut extracts of *Leptinotarsa decemlineata*. Data are the means of triplicate measurements, with standard errors within 5% of the means. Reaction buffers were 0.1 M citrate (○), 0.1 M phosphate (●), 0.1 M tris-HCl (□), 0.1 M glycine-NaOH (■), and 0.1 M borate-NaOH (△).

TABLE 1. Properties of Midgut Proteases From *L. decemlineata* Against Specific Substrates

Enzyme	Substrate	Optimum pH	Specific activity[a]
Proteases	Azocasein	5.5	24.4 ± 1.2
Cysteine proteases	BApNa	6.5	5.4 ± 0.5
Cathepsin B	ZAA_2MNA	7.5	221.4 ± 18.3
Cathepsin H	ArgNA	6.5	14.8 ± 0.6
Cathepsin D	Hemoglobin	3.5	48.5 ± 2.6
New activities			
Chymotrypsin	SA_2PPpNa	6.5	9.8 ± 0.5
	BTpNa	5.5–6.0	3.8 ± 0.4
Leucine aminopeptidase	LpNa	7.5	28.1 ± 1.9
Carboxypeptidase A	HPA	8.0	8.6 ± 0.5

[a]Specific activities as nmoles of substrate hydrolyzed/min/mg protein, except for general proteolytic activity against azocasein, and cathepsin D activity against hemoglobin, as mU Δ Abs 420 and 280 nm/min/mg protein, respectively. Figures are the mean ± standard error of triplicate measurements.

The effect of specific protease inhibitors and activators on the hydrolysis of protein and synthetic substrates is presented in Table 2. Hydrolysis of the chymotrypsin substrates SA_2PPpNa and BTpNa was not activated by thiol compounds (L-cysteine, DTT) or inhibited by aspartyl (pepstatin-A) or cysteine (E-64, IAA, leupeptin) specific proteinase inhibitors. Among the several serine proteinase inhibitors tested, PMSF was the most effective, and inhibition was not reversed by the addition of sulphydryl reagents. Divalent heavy metal ions and 1,10-phenanthroline inhibited hydrolysis of LpNa. Hydrolysis of the carboxypeptidase A substrate HPA was inhibited by 1,10-phenanthroline and DTT, but not by divalent heavy metal ions or by the specific carboxypeptidase protease inhibitor from potato, PCPI. Cysteine proteinases were enhanced by thiol compounds and inhibited by E-64, IAA, leupeptin, chymostatin, TLCK, TPCK, ZPCK, and heavy metal ions, whereas the aspartic proteinase cathepsin D was inhibited by pepstatin-A.

The contribution of chymotrypsin-like cysteine, and aspartyl proteinases to protein digestion was estimated by testing the effect of PMSF, E-64, and pepstatin-A on azocasein hydrolysis (Table 3). According to our results above, these inhibitors were specific for CPB chymotrypsin-like, cysteine, and aspartyl digestive proteinases, respectively. We also verified that the inhibitor concentrations used were adequate to permit the titration of the different types of proteinases. In the absence of thiol compounds, E-64 inhibits 42% of activity, whereas with PMSF or pepstatin-A, about 35% inhibition was obtained. However, when 5 mM L-cysteine was added to the reaction buffer, the inhibition with E-64 increased to 67%, whereas those with PMSF and pepstatin-A were reduced to 7 and 18%, respectively. In both cases, with or without L-cysteine, the inhibitory effect was additive when mixtures of the inhibitors were tested, showing inhibitions up to 90% when all three of them were added.

Gelatin-containing SDS-PAGE gels of digestive proteases is shown in Figure 2A. At least 9 bands with proteolytic activity (b1–b9) were resolved from endoperitrophic contents of CPB larval midgut extracts. Incubation of the

TABLE 2. Effect of Proteases Inhibitors and Activators on the Hydrolysis of Specific Substrates by Larval Midgut Extracts From *L. decemlineata* at Their Optimum pH of Activity

		% Relative activity[a]								
							New activities			
	Concentration	Protease (azocasein) pH 5.5	CTD (hemoglobin) pH 3.5	CEP (BApNa)[c] pH 6.5	CTH (ArgNA)[c] pH 6.5	CTB (ZAA$_2$MNA)[c] pH 7.5	CHY (SA$_2$PPpNa) pH 6.5	CHY (BTpNa) pH 6.0	LAP (LpNa) pH 7.5	CPA (HPA) pH 8.0
Inhibitor (protease type)[b]										
PMSF (SEP)	1 mM	58±2	ne	ne	ne	ne	10±1	29±2	ne	ne
SBBI (SEP)	10 µM	ne	ne	ne	ne	ne	ne	ne	ne	ne
Chymostatin (SEP > CEP)	10 µM	74±3	ne	56±2	ne	32±3	62±4	ne	ne	ne
TLCK (TRY > CEP)	1 mM	68±2	ne	4±4	(†)	(†)	75±2	ne	ne	ne
TPCK (CHY > CEP)	1 mM	66±6	ne	49±1	(†)	(†)	72±1	ne	ne	ne
ZPCK (CHY > CEP)	0.5 mM	43±3	ne	4±1	(†)	(†)	65±2	34±1	ne	ne
E-64 (CEP)	10 µM	56±2	ne	12±5	55±3	6±0	ne	ne	ne	ne
IAA (CEP)	1 mM	69±2	ne	18±8	54±3	11±2	ne	ne	ne	ne
Leupeptin (CTB > TRY)	10 µM	ne	ne	32±1	67±2	4±2	ne	ne	ne	ne
Pepstatin-A (AEP)	10 µM	68±3	10±2	ne	ne	ne	ne	ne	ne	ne
EDTA (MP)	1 mM	ne	ne	ne	ne	ne	ne	ne	ne	ne
1, 10 Phenanthroline (MP)	1 mM	ne	(†)	ne	20±1	ne	ne	ne	42±1	36±3
PCPI (CPA & CPB)	1 µM	ne	ne	ne	ne	ne	ne	ne	ne	ne
$CdCl_2$	1 mM	53±2	ne	7±3	18±1	6±1	ne	ne	1±1	ne
$CuCl_2$	1 mM	38±1	ne	0±0	4±1	5±0	ne	ne	8±1	ne
Activator										
L-cysteine (CEP)	1 mM	233±6	ne	446±12	409±18	642±16	ne	ne	ne	(†)
DTT (CEP)	1 mM	229±3	ne	510±12	617±30	735±21	ne	ne	ne	42±3

[a]The percentage of relative activity represents the mean ± standard error of three different pooled midgut extracts treated with an inhibitor or activator with respect to their corresponding controls without them. No effect (ne) was considered for activities between 80 and 120%. (†) It chemically interferes with the assay.
[b]Protease type abbreviations: AEP, aspartyl proteases; CEP, cysteine proteases; CHY, chymotrypsin-like proteases; CPA and CPB, carboxypeptidases A and B; CTD, CTH, and CTB, cathepsin D, H, and B-like proteases; LAP, leucine aminopeptidase; MP, metallo proteases; SEP, serine proteases; and TRY, trypsin-like proteases.
[c]The reaction buffer contained 1mM L-cysteine, except when L-cysteine and DTT were used as activators.

TABLE 3. Effect of Protease Inhibitors, in the Presence or Absence of L-Cysteine, on the Hydrolysis of Azocasein by Larval Midgut Extracts From *L. decemlineata*

	% Inhibition[a]	
Inhibitor	No thiol compounds	L-cysteine (5mM)
PMSF (1 mM)	35 ± 2	7 ± 1
E-64 (10 μM)	42 ± 2	67 ± 1
Pepstatin-A (10 μM)	37 ± 3	18 ± 2
PMSF (1 mM) + E-64 (10 μM)	67 ± 1	79 ± 1
PMSF (1 mM) + E-64 (10 μM) + Pepstatin-A (10 μM)	88 ± 1	93 ± 1

[a]Azocasein hydrolysis was performed at pH 5.5. The percentage of inhibition was calculated as in Table 2.

samples with E-64 inhibited the proteases b4, b6, b7, b8, and b9, whereas incubation with PMSF inhibited the proteases b1, b2, b3, and b5, and activated b6. Chymotrypsin-like enzymes were detected by incubating Western blots of SDS-PAGE gels with azocasein or SA_2PPpNa (Fig. 2B). At least seven bands were resolved after incubation with azocasein. Their mobility was higher than that of the corresponding bands in the zymograms, because the addition of gelatin to SDS-PAGE gels in the zymograms reduce the migration of proteins by 15–20% (Hummel et al., 1996). Chymotrypsin-like activity was associated with a major band of about 63 KDa and a minor band of about 100 KDa. Both bands of SA_2PPpNa hydrolytic activity were totally inhibited by PMSF, unaffected by E-64, and have their corresponding bands of azocaseinolytic activity.

Digestive Compartmentalization of Proteolytic Activities

The pH of the lumen of the anterior midgut was 5.7, whereas that of the endoperitrophic space of the middle and posterior portions of the mid-

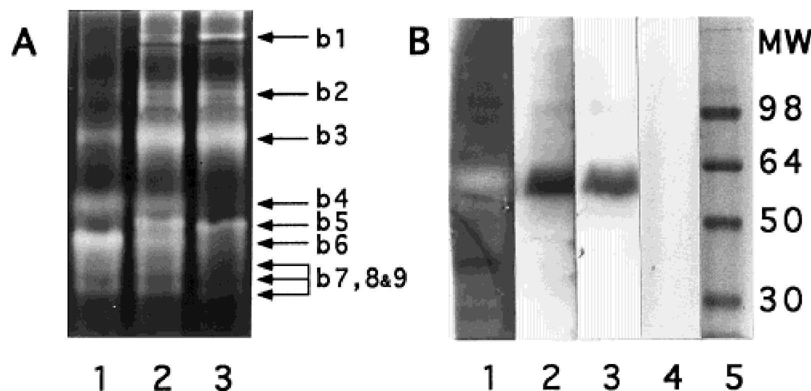


Fig. 2. **A:** Zymogram showing gelatinolytic activity of larval midgut extracts of *Leptinotarsa decemlineata* treated with PMSF (1), control (2), and E-64 (3). Proteinase forms (b1-b9) are named from lowest to highest mobility. **B:** Activity staining of proteases from larval midgut extracts of *L. decemlineata* subjected to SDS-PAGE, Western blotting, and incubation with azocasein (1), SA_2PPpNa (2), SA_2PPpNA plus E-64 (3), and SA_2PPpNa plus PMSF (4). Molecular weight standards (5).

TABLE 4. Distribution of Total Protein and Proteolytic Activities Along the Gut Sections of *L. decemlineata* larvae

	Whole gut[a]	Anterior midgut[b]	Middle midgut[b]	Posterior midgut[b]	Hindgut[b]
Total protein	1.2 ± 0.1	30.0 ± 2.3	30.7 ± 1.4	34.8 ± 2.0	4.1 ± 0.3
Chymotrypsin (SA_2PPpNa)	10.8 ± 0.9	27.0 ± 3.4	38.8 ± 1.9	30.3 ± 2.6	4.0 ± 0.3
Aminopeptidase (LpNa)	89.3 ± 5.5	22.6 ± 2.3	32.5 ± 1.3	39.5 ± 2.5	5.7 ± 0.7
Cysteine proteases (BApNa)	4.2 ± 0.3	26.2 ± 3.2	35.9 ± 2.8	37.5 ± 3.2	0.4 ± 0.1
Cathepsin H (ArgNA)	10.6 ± 0.7	32.5 ± 2.4	32.1 ± 1.9	29.2 ± 2.0	6.2 ± 1.1
Cathepsin B (ZAA_2MNA)	192.3 ± 12.2	26.2 ± 3.5	38.2 ± 2.9	35.6 ± 3.7	0.1 ± 0.0
Cathepsin D (hemoglobin)	59.8 ± 4.5	17.9 ± 4.0	35.1 ± 2.9	38.1 ± 3.6	9.0 ± 2.0

[a]Activities as nmoles of substrate hydrolyzed/min/gut, and total protein as mg protein/gut. Figures are the mean ± standard error (n = 15).
[b]Percentage of activity ± standard error in each gut section with respect to the activity of the whole gut.

TABLE 5. Distribution of Total Protein and Proteolytic Activities in Different Midgut Regions of *L. decemlineata* larvae

	Whole midgut[a]	Endoperitrophic space[b]	Ectoperitrophic space[b]	Midgut epithelium[b]
Total protein	1.0 ± 0.1	47.8 ± 2.6	25.8 ± 2.5	26.2 ± 2.7
Chymotrypsin (SA_2PPpNa)	10.1 ± 0.8	59.2 ± 2.1	39.0 ± 2.1	1.8 ± 0.1
Aminopeptidase (LpNa)	28.9 ± 2.4	25.7 ± 2.3	13.5 ± 1.6	60.9 ± 3.2
Cysteine proteases (BApNa)	5.4 ± 0.5	64.3 ± 2.7	32.2 ± 2.8	3.5 ± 0.2
Cathepsin H (ArgNA)	15.1 ± 0.4	47.5 ± 2.4	35.8 ± 2.8	16.7 ± 2.6
Cathepsin B (ZAA_2MNA)	215.1 ± 20.7	69.2 ± 3.0	30.7 ± 3.0	0.1 ± 0.0
Cathepsin D (hemoglobin)	49.0 ± 3.3	60.7 ± 3.0	30.9 ± 2.8	8.3 ± 1.1

[a]Activities as nmoles of substrate hydrolyzed/min/midgut, and total protein as mg protein/midgut. Figures are the mean ± standard error (n = 15).
[b]Percentage of activity ± standard error in each gut section with respect to the activity of the whole midgut.

gut was 5.9. Most endo-and exoproteolytic activities studied were evenly distributed among the midgut sections (Table 4). Only cathepsin D activity appears to be slightly more concentrated in the middle and posterior sections of the midgut. A small percentage (0.1–9%) of the activity was present in the hindgut.

Chymotrypsin-like and cathepsin B-, D- and H-like activities were mainly located in the endoperitrophic and ectoperitrophic spaces with only minor activities in the midgut epithelium, except for cathepsin H that represented 16.7% of activity (Table 5). By contrast, leucine aminopeptidase was mainly located in the midgut wall tissues, although some activity was distributed in the ecto- and endoperitrophic spaces.

Cathepsin H is usually distinguished by hydrolysis of the specific substrate ArgNA. However, aminopeptidases are able to hydrolyze several aminoacyl-β-naphthylamides, including ArgNA. To further distinguish between the hydrolytic activities due to leucine aminopeptidase and the cysteine aminopeptidase cathepsin H, we tested the effects of specific protease inhibitors and activators on the hydrolysis of LpNa and ArgNA by endoperitrophic and midgut epithelium extracts (Table 6). The hydrolysis of ArgNA by endoperitrophic extracts was inhibited by E-64, IAA, and heavy metal ions and enhanced by DTT, whereas only the metal ions inhibit the hydrolysis by midgut epithelium extracts, and the addition of DTT only slightly increased the activity. In contrast, there were no differences in the hydrolysis of LpNa by endoperitrophic and midgut epithelium extracts. In both cases, they were inhibited by heavy metal ions, but were not affected by E-64, IAA, and DTT.

DISCUSSION

Characterization of Proteolytic Activities

The results of this study indicate the presence of chymotrypsin-like, carboxypeptidase A-like and leucine aminopeptidase-like activities in the midgut of Colorado potato beetle larvae, in addition to cathepsin B, D, and H.

A novel chymotrypsin-like activity was characterized with SA_2PPpNa and BTpNa as substrates, that presented a pH optimum in the range 5.5–6.5, and exhibited different sensitivity towards inhibitors and activators than all other proteolytic activities previously described for this species. It was inhibited by some serine proteinase inhibitors, PMSF being the most effective. On the other hand, it was not activated by thiol compounds or inhibited by cysteine- and aspartyl-specific proteinase inhibitors. The optimum pH of activity is consistent with the slightly acidic pH of the midgut lumen in CPB larve, indicating that this protease is physiologically adapted to the low pH environment. Furthermore, azocasein hydrolysis was inhibited by PMSF at similar levels to those obtained with E-64 and pepstatin-A. Our data reveal that these inhibitors were specific for CPB chymotrypsin-like, cysteine and aspartyl digestive proteinases, respectively, suggesting that chymotrypsin-like proteinases may play an important role in the digestion in CPB larvae.

In previous studies with CPB, inhibition of protein substrate hydrolysis by E-64 was in the range 70–90% and by pepstatin-A about 10–30%. A combina-

TABLE 6. Effect of Protease Inhibitors and Activators on the Hydrolysis of LpNa and ArgNA by Endoperitrophic and Midgut Epithelium Extracts From Larval Midguts of *L. decemlineata*

	% Relative activity[a]			
	LpNa[b]		ArgNA[c]	
	Endoperitrophic space	Midgut epithelium	Endoperitrophic space	Midgut epithelium
E-64 (50 μM)	ne	ne	18 ± 2	ne
IAA (1 mM)	ne	ne	20 ± 3	ne
$CdCl_2$ (1 mM)	3 ± 1	6 ± 1	17 ± 1	19 ± 1
$CuCl_2$ (1 mM)	3 ± 1	10 ± 2	5 ± 2	3 ± 1
DTT (1 mM)	ne	ne	409 ± 19	132 ± 5

[a]The percentage of relative activity was calculated as in Table 2. No effect (ne) was considered for activities between 80 and 120%.
[b]LpNa hydrolysis was performed at pH 7.5
[c]ArgNA hydrolysis was performed at pH 6.5 and the reaction buffer contained 1mM L-cysteine, except when DTT was tested.

tion of both the inhibitors almost completely inhibited the activity, suggesting that only cysteine and aspartyl proteinases contribute to total proteolysis (Bolter and Jongsma, 1995; Michaud et al., 1995, 1996). The only major difference between the methodology used by these authors and us was that they performed the assays in the presence of thiol compounds. Purcell et al. (1992) reported complete inhibition of CPB gut azocaseinolytic activity by E-64, but they did not determine the effect of the presence or absence of reducing agents. Therefore, we tested the effect of PMSF, E-64, and pepstatin-A on azocasein hydrolysis in the presence or absence of L-cysteine. Our results indicate that in the presence of 5 mM L-cysteine, cysteine proteinases appear to account for two-thirds of the total proteolytic activity, aspartyl proteinases are responsible for the most part of the remaining activity, and chymotrypsin-like proteinases are of minor importance (Table 3). However, these are not necessarily the physiological conditions in which these proteinases may act. The addition of L-cysteine to the reaction buffer probably overwhelms the endogenous levels of reducing agents present in the digestive tract of CPB larvae. When no thiol compounds were added to the midgut extracts, each of the three types of proteinases accounts for about one-third of the total proteolytic activity. Thus, when using protein substrates, the presence in the reaction buffers of thiol compounds, that specifically enhance the activity of cysteine proteinases, may disguise the presence of other types of proteinases. This may be one of the reasons as to why chymotrypsin-like activity was not previously identified in CPB.

When characterizing the protease activity in an insect gut homogenate, synthetic or protein substrates can be used. Synthetic substrates might be hydrolyzed by dipeptidases and esterases present in gut extracts. On the other hand, most protein substrates are subjected to hydrolysis by the different proteases present in the digestive tract. However, specific protein substrates for chymotrypsin-like proteases are not available and, hence, we have used an alternative approach that combines activity staining with both types of substrates: protein (azocasein) or synthetic (SA_2PPpNa), after gel electrophoresis. A major band of SA_2PPpNa hydrolytic activity of about 63 KDa and a minor band of about 100 KDa were resolved, that have the same mobility as the two bands with azocaseinolytic activity, indicating that the enzymes that hydrolyze SA_2PPpNa are the same as the ones that hydrolyze azocasein, and, therefore, proteases. In addition, both bands were totally inhibited by PMSF and unaffected by E-64, as previously described for the novel chymotrypsin-like activity.

We have found at least nine protease forms (b1–b9) in the endoperitrophic contents of CPB larvae. Incubation of the samples with E-64 inhibited the protease forms b4, b6, b7, b8, and b9, indicating that they may be of the cysteine proteinase type. Similarly, b1, b2, b3, and b5 were inhibited by PMSF, suggesting that they may correspond to the two chymotrypsin-like proteinases identified with the activity staining gels, and some other not characterized serine proteases. Surprisingly, Michaud et al. (1995) resolved nine digestive proteinases from larvae and adults of CPB using similar zymograms, all of them inhibited by E-64 and activated in the presence of PMSF. The authors suggest that the putative existence of serine proteases in CPB mid-

gut extracts more likely explains the activation of cysteine proteinases in the presence of PMSF. Our results prove one of the premises of this hypothesis, the presence in the digestive tract of CPB of serine proteinases of the chymotrypsin-like type, but we have only observed activation of one cysteine proteinase form (b6) by PMSF. Furthermore, we have not obtained activation in the rate of hydrolysis of azocasein or any of the cysteine-specific substrates. Nevertheless, a comprehensive study should be accomplished in order to discern the important implications of the complementation among serine and cysteine proteinases suggested by Michaud et al. (1995).

The proteolytic features of cysteine and aspartyl proteinases under our experimental conditions were similar to those previously described for this species (Thie and Houseman, 1990b). The aspartic proteinase cathepsin D, detected using hemoglobin as substrate, had maximal activity at pH 3.5 and was inhibited by pepstatin-A. Cysteine proteinases presented mildly acidic to neutral optimum pH of activity, and were enhanced by thiol compounds and inhibited by E-64, IAA, leupeptin, chymostatin, TLCK, TPCK, and heavy metal ions. Cathepsin B and H were distinguished by hydrolysis of the specific substrates ZAA_2MNA and ArgNA, respectively.

We have characterized leucine aminopeptidase and carboxypeptidase A activities in CPB larvae. Aminopeptidase and carboxypeptidase activities have been reported in midgut extracts of several coleopteran species (Baker, 1982; Christeller et al., 1989; Ferreira and Terra, 1989; Terra and Ferreira, 1994). The absence of carboxypeptidase A inhibition by PCPI, an inhibitor purified from potato tuber (Ryan et al., 1974), that has been reported to inhibit digestive carboxypeptidases A and B from several insect species (Terra and Ferreira, 1994; Ortego et al., 1996), suggest that CPB has adapted its carboxypeptidases to the presence of this inhibitor in potato.

Distribution of Proteolytic Activities

The digestive tract of CPB larvae is characterized by a very short foregut, followed by a long and twisted midgut, and the hindgut. We have observed that the anterior region of the midgut is stretched and presents a peritrophic membrane of fluid consistency, whereas the middle and posterior regions are thinner and the peritrophic membrane is always well defined. Our results indicate that all endo- and exoproteolytic activities studied are evenly distributed among the midgut sections, indicating that there is no clear regional differentiation in the digestion of proteins along the midgut in CPB larvae. Taking into account that the pH prevailing along the midgut is uniform, and in agreement with the optimum pH for chymotrypsin-like and cysteine proteinases, a regional differentiation for the digestion of proteins might not be necessary. This distribution contrasts with that of *Tenebrio molitor* larvae, whose midgut also contains both serine (trypsin- and chymotrypsin-like) and cysteine proteinases (Terra et al., 1985; Thie and Houseman, 1990a; Terra and Cristofoletti, 1996). In the case of *T. molitor*, the differences in pH requirements for maximal activity of its proteinases appear to be accommodated by the presence of cysteine proteinases in the acidic anterior midgut, while the serine proteinases are mostly located in the alkaline posterior region.

We have found minor amounts (0.1–10%) of proteolytic activity in the hind-

gut. This small activity may be due to contamination during dissection, or because some active enzymes pass with the food bolus to the hindgut. A recycling mechanism of luminal digestive enzymes to conserve secreted enzymes with no loss in the feces is provided by an endo-ectoperitrophic circulation of digestive enzymes in several insects (Terra, 1990). However, the necessary midgut fluid fluxes have not been established in Coleoptera. Cheeseman and Gillott (1987) questioned this mechanism on the grounds that some beetles do not have a peritrophic membrane in the anterior midgut and suggest that a pyloric valve may restrict the movement of midgut contents into the hindgut. However, Ferreira and Terra (1989) reported that the presence of a fluid peritrophic membrane in the anterior midgut of *Pheropsophus aequinoctialis* makes it possible for the existence of an endo-ectoperitrophic circulation of digestive enzymes in Coleoptera.

According to our results with CPB larvae, chymotrypsin-like, aspartyl and cysteine digestive proteinases were mainly located in the endoperitrophic and ectoperitrophic spaces, with only a small activity in the midgut epithelium. On the contrary, leucine aminopeptidase was mainly located in the wall tissues, although some activity was distributed between the ecto- and endoperitrophic spaces. This spatial organization supports the hypothesis that proteolytic digestion starts in the midgut lumen under the action of chymotrypsin-like, cysteine and aspartyl endoproteases. The resulting peptides are then hydrolyzed to aminoacids by aminopeptidases bound to the membranes of the midgut epithelium. A similar compartmentalization has been suggested for other coleopteran larvae, such as *T. molitor* (Terra et al., 1985; Thie and Houseman, 1990a) and *Costelytra zealandica* (Christeller et al., 1989).

It is remarkable that 17% of cathepsin H activity was present in the midgut epithelium, whereas only minor amounts of other endoproteases were located in the wall tissues. Cathepsin H is an aminopeptidase, activated by thiol compounds, that is usually distinguished by hydrolysis of the specific substrate ArgNA. However, an aminopeptidase isolated from larval midguts of the dermestiid *Attagenus megatoma* has been shown to hydrolyze several aminoacyl-β-naphthylamides, including ArgNA (Baker and Woo, 1981). We attempted to determine whether ArgNA hydrolysis in the midgut epithelium was due to cathepsin H or aminopeptidases, testing the effects of specific protease inhibitors and activators on the hydrolysis of LpNa and ArgNA by endoperitrophic and midgut epithelium extracts separately. Hydrolysis of ArgNA by endoperitrophic extracts was inhibited by E-64, IAA, and heavy metal ions, and enhanced by DTT, whereas only the metal ions inhibit the hydrolysis by midgut epithelium extracts. These results indicate that the hydrolysis of ArgNA observed in the midgut epithelium is mainly due to the action of aminopeptidases. On the contrary, hydrolysis of LpNa was inhibited in both tissues by heavy metal ions and was not affected by cysteine proteinase inhibitors or activators, confirming the presence of leucine aminopeptidase activity in the endoperitrophic and midgut epithelium extracts.

Implications of the New Protease Activities for CPB Control

Studies dealing with the significance of protease inhibitors to disrupt CPB protein digestion have concentrated on the use of cystatins (Michaud

et al., 1993b, 1995, 1996; Bolter and Jongsma, 1995). Recently, transgenic potato plants expressing the rice cysteine proteinase inhibitor oryzacystatin-I have been developed (Benchekroun et al., 1995). However, the real potential of cystatins for the control of CPB has not been clearly demonstrated. Wolfson and Murdock (1987) showed that chronic ingestion of E-64 retarded larval growth and development, but they were not severely affected when reared on potato leaves that contain high levels of cystatins (Bolter and Jongsma, 1995; Michaud et al., 1995, 1996). Two interpretations have been proposed to explain the apparent inefficiency of cystatins to affect CPB development: the presence of non-target proteinases in CPB midgut extracts that hydrolyze the proteinaceous inhibitors (Michaud et al., 1996); and that the inhibited proteolytic activity is compensated by synthesis of cysteine proteinases insensitive to the cystatin (Bolter and Jongsma, 1995). Our study suggests that a physiological complementation by the non-target chymotrypsin-like and aspartyl proteinases may also be possible. The complexity of the proteolytic system used by CPB for protein digestion suggests that it may be necessary to use a combination of chymotrypsin, aspartyl, and cysteine proteinase inhibitors to obtain significant detrimental effects on the growth and development of CPB.

Digestive proteases in insects play an essential role in the proteolytic activation of the δ-endotoxin from *Bacillus thuringiensis* (Bt) (Gill et al., 1992). The CryIIIA δ-endotoxin synthesized by *B. thuringiensis* subsp. *tenebrionis* is active against coleopteran insects (Krieg et al., 1983). CryIIIA is soluble in alkaline and acid buffer solutions, but the toxin precipitates when returned to neutral pH conditions (Koller et al., 1992). However, the midgut pH of susceptible CPB larvae is neutral to slightly acidic, a pH environment in which the CryIIIA toxin is insoluble. To investigate this paradox, Carroll et al. (1997) studied the CryIIIA toxin after various proteolytic treatments. In many cases, the toxin was cleaved into polypeptides that remained associated in non-denaturing conditions. Interestingly a chymotrypsinized CryIIIA product was soluble at neutral pH conditions, retained full activity against susceptible beetle larvae and exhibited specific binding to CPB midgut membranes (Carroll et al., 1997). Our discovery that CPB larvae possess chymotrypsin-like proteinases, which are physiologically active at the slightly acidic pH environment of the larval midgut, may help to interpret their results and to explain the paradox. These digestive chymotrypsins may be responsible for the proteolytic activation of the CryIIIA endotoxin in CPB larvae. Nevertheless, this hypothesis must be confirmed when CPB chymotrypsin will be purified.

Serine protease inhibitors were shown to increase the toxicity of *B. thuringiensis* endotoxins towards four species of Lepidoptera and CPB (MacIntosh et al., 1990). Although the mechanism by which the proteinase inhibitors potentiate Bt insecticidal activity is unknown, whatever is the correct explanation, it implies the existence of digestive proteases of the serine type. Once again, our discovery of chymotrypsin-like activity in the digestive tract of CPB larvae confers significance to previous results that were difficult to explain, assuming a proteolytic profile exclusively based on aspartyl and cysteine proteinases.

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