Entomologia Experimentalis et Applicata **88:** 265–274, 1998.

Characterization of digestive proteases in the weevil *Aubeonymus mariaefranciscae* and effects of proteinase inhibitors on larval development and survival

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Accepted: August 18, 1998

Key words: Aubeonymus, Curculionidae, *Beta vulgaris*, sugar beet, digestive proteases, proteinase inhibitors

Abstract

The major digestive proteinase activities of a new sugar beet pest, *Aubeonymus mariaefranciscae* Roudier (Coleoptera: Curculionidae), were characterized. Both larvae and adults of *A. mariaefranciscae* were found to use a complex proteolytic system for protein digestion based on at least trypsin-, chymotrypsin-, elastase-, cathepsin D, leucine aminopeptidase-, carboxypeptidase A- and carboxypeptidase B-like activities. An azocaseinolytic activity at pH 5.0–7.0 was identified, that was not affected by specific inhibitors and activators, making its classification in any of the mechanistic classes established not possible. According to this proteolytic profile, several serine proteinase inhibitors were tested *in vitro* and *in vivo* to establish their potential as resistance factors against *A. mariaefranciscae*. Larvae fed from neonate to pupation on diets containing 0.2% (w/w) soybean Bowman-Birk trypsin-chymotrypsin inhibitor, soybean Kunitz trypsin inhibitor, turkey egg white trypsin inhibitor, or lima bean trypsin inhibitor endure lower survival rates and display significant delays in the developmental time to pupation and to adult emergence. Interestingly, the most significant levels of mortality (about 90%) occurred with larvae fed on diets containing a combination of two or three inhibitors, suggesting a synergistic toxicity.

Introduction

The weevil *Aubeonymus mariaefranciscae* Roudier was first recorded in 1979 as a new pest of sugar beet in Southern Spain (Santiago-Alvarez et al., 1982), and formally described as a new species two years later (Roudier, 1981). The damage caused by the autumn adult populations of this curculionid is particularly devastating, because they feed on leaves, petioles and roots of the sugar beet seedlings about the time of emergence. Adults and larvae also can produce severe damage during the spring by feeding on sugar beet leaves and storage roots.

Current control strategies focus on the use of neurotoxic chemical pesticides, chiefly parathion-methyl and soil applications of the carbamate aldicarb. The risk of these broad-spectrum insecticides to the environment makes the search for biorational environmentally friendly control technologies of great economical and ecological interest.

Disruption of insect protein digestion by transformation of plant genomes with proteinaceous proteinase inhibitors represents an alternative approach to pest control (see Reeck et al., 1997, for a review). However, because of the variability in proteinases between insects (Wolfson & Murdock, 1990), and the limited spectrum of activity of these inhibitors (García-Olmedo et al., 1987), the expression in plants of a particular proteinase inhibitor may not result in a broad spectrum control. It is, therefore, necessary to select the appropriate inhibitors for the digestive proteinases of each particular pest species. This requires knowledge of the proteinases present in the insect gut and of the way they interact with the various inhibitors. Furthermore, it has been demonstrated that insects are physiologically adapted to circumvent plant protease inhibitors by secreting 'inhibitor-resistant' enzymes

and by the proteolysis of proteinase inhibitors by non-target digestive proteases (Broadway, 1996; Jongsma & Bolter, 1997). It may be, therefore, necessary to use transgenic plants with complementary protease inhibitors to improve their usefulness (Jongsma et al., 1996; Michaud, 1997).

The aim of this study is to characterize the digestive proteases in the weevil *A. mariaefranciscae* and to determine the effects of proteinase inhibitors on larval development and survival, as a basis for selecting appropriate inhibitors for this species.

Materials and methods

Insects. Adults of *A. mariaefranciscae* were collected in Cordoba (Spain) by removing them from sugar beet root wastes of the previous crop. They were maintained on sugar beet plants in a growth chamber at 23±2 °C, 90±10% r.h. and a L16:D8 photoperiod. Neonate larvae were obtained from the laboratory colony by peeling off the epidermis of sugar beet leaves under a microscope to expose the eggs, which were then transferred onto moistened filter paper inside plastic containers and incubated under identical conditions as above until they hatched. Larvae were fed with a semi-artificial diet that contains sugar beet root homogenate (Marco et al., 1997).

Chemicals and equipment. All substrates and protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, USA). Spectrophotometric measurements were made using a Hitachi U-2000 spectrophotometer.

Gut extracts. Adults and fourth instar larvae were dissected in 0.15 M NaCl, and the guts and contents removed and stored frozen (−20 °C) until needed. Guts from adults (10.2±0.3 μg protein/gut) and larvae (75±1.4 μg protein/gut) were subsequently homogenized in 0.15 M NaCl (5 guts/100 μl for adults and 5 guts/500 μl for larvae), centrifuged at 10 000 g for 5 min, and the supernatants pooled and kept on ice for enzymatic activity assays.

Enzyme assays. All assays were carried out in triplicate and 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); 0.1 M glycine-NaOH (pH 9.0–10.0); 0.05 M sodium borate-NaOH (pH 9.0–10.5); and 0.05 M Na_2HPO_4–NaOH (pH 11.0–12.0). All buffers contained 0.15 M NaCl and 5 mM $mgCl_2$ except Na_2HPO_4–NaOH that only contained 0.15 M NaCl, since $MgCl_2$ is not maintained in solution at high alkaline pHs.

Unless otherwise stated, all protease activities were performed at their optimum pH of activity in 1 ml of reaction mixture that contains 20 μl of midgut extract. Non-specific protease activity was assayed with 0.1% sulfanilamide-azocasein solution and the incubation time was 24 h; trypsin-like activity using 1 mM BApNa (Nα-benzoyl-DL-arginine p-nitroanilide) and incubating for 2 h; chymotrypsin-like activity with 0.25 mM SA_2PPpNa (N-succinyl-alanine-alanine-proline-phenylalanine p-nitroanilide) and incubating for 30 min; elastase-like activity with 0.5 mM SA_3pNa (N-succinyl-alanine-alanine-alanine p-nitroanilide) and incubating for 6 h; carboxypeptidase A-like activity with 1 mM HPA (hippuryl-phenylalanine) and incubating for 24 h; carboxypeptidase B-like activity with 1 mM HA (hippuryl-L-arginine) and incubating for 24 h; and leucine aminopeptidase-like activity with 1 mM LpNa (L-leucine p-nitroanilide) and incubating for 1 h; as described by Ortego et al. (1996). Cathepsin D-like activity was measured with 0.2% hemoglobin solution and the incubation time was 24 h; cathepsin B-like activity with 50 μm ZAA_2MNA (N-carbobenzoxy-alanine-arginine-arginine 4-methoxy-β-naphthyl amide) and incubating for 3 h; and cathepsin H-like activity with 1 mM ArgNA (L-arginine β-naphthyl amide) and incubating for 30 min; as described by Novillo et al. (1997 a,b). Total protein in the midgut extracts was determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

Effects of protease inhibitors and activators in vitro. 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), CEOM (Chicken Egg White Ovomucoid), TEI (Turkey Egg White Inhibitor), STI (Soybean Trypsin Inhibitor), LBI (Lima Bean Inhibitor), and chymostatin; the trypsin inhibitor, TLCK (Nα-p-tosyl-L-lysine chloromethyl ketone); the chymotrypsin inhibitor, TPCK (N-tosyl-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 (ethilenediamine tetraacetic acid) and 1,10 Phe (1,10 phenanthroline); the carboxypeptidase inhibitor, PCPI (potato tuber carboxypeptidase inhibitor); and the heavy metal ions $CdCl_2$ and $CuCl_2$, inhibitors of aminopeptidases and some other metalloproteases. 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, TPCK, chymostatin, 1,10-phenanthroline and pepstatin-A that were added in 20 μl of DMSO. The doses tested (Table 1) were selected according to the effective concentrations recommended by Beynon & Salvesen (1989).

Effects of protease inhibitors in vivo. Larvae were fed from neonate to pupation with an artificial diet (Marco et al., 1997) containing 0.2% (w/w) albumin (control), 0.2% (w/w) of one of the following proteinase inhibitors: SBBI, CEOM, TEI, STI and LBI, and 0.2% (w/w) of the mixtures LBI/TEI (contains 0.1% (w/w) LBI and 0.1% (w/w) TEI) and SBBI/STI/TEI (contains 0.066% (w/w) of each inhibitor). Larvae were placed singly in cylindrical cages of 1.2 cm height and 2.5 cm diameter that contain about 0.4 g of treated diet placed on dry filter paper. They were maintained during the assay in an environmental chamber (Sanyo MLR-350H, Sanyo, Japan) at 26±1 °C, 80±10% r.h. and a L16:D8 photoperiod. Changes of diet were made every 5–7 days to determine insect development and survival.

Results

The pH profiles for proteolytic activities of gut extracts from *A. mariaefranciscae* adults are presented in Figure 1. The protein substrate azocasein was hydrolyzed over a broad range of pHs, with two peaks of optimum activity at pH 6.0–7.0 and 9.0–10.5. Hydrolysis of hemoglobin gave an optimum of activity at pH 2.5. Maximal hydrolysis of BApNa, SA_2PPpNa, and SA_3pNa occurred at pH 11.5, 11.0, and 10.5, respectively. ArgNA and ZAA_2MNA presented similar pH profiles, with maximum activity at pH 7.5 and 8.0, respectively. A maximum of activity was observed against HPA at pH 8.5, whereas the activity against HA gave maximum activity at pH 7.5. Optimal hydrolysis of LpNa occurred at pH 7.5.

The proteolytic activity of midgut extracts from *A. mariaefranciscae* adults was further characterized by reaction with specific protease inhibitors (Table 1). The azocaseinolytic activity was inhibited by SBBI, chymostatin, TLCK, leupeptin and DTT at pH 10.0; and by $CdCl_2$ and $CuCl_2$ at pH 8.0; whereas none of the inhibitors was able to inhibit it at pH 6.0. The hydrolysis of hemoglobin was only inhibited by pepstatin-A. BApNa hydrolysis was inhibited by SBBI, chymostatin, TLCK, leupeptin, DTT and L-cysteine; whereas SA_2PPpNa by SBBI, chymostatin, TLCK, DTT and L-cysteine; and SA_3pNa only by SBBI. Both, HPA and HA, were inhibited by IAA, EDTA, 1,10-phenanthroline, DTT, $CdCl_2$, $CuCl_2$ and PCPI. The hydrolysis of LpNa, ArgNA and ZAA_2MNA was inhibited by 1,10-phenanthroline, $CdCl_2$, $CuCl_2$and L-cysteine.

Midgut extracts from larvae of *A. mariaefranciscae* presented the same pH profile of azocaseinolytic activity (data not shown) and were able to hydrolyze the same substrates as adults. Table 2 shows the specific activities of midgut extracts from adults and last instar larvae against the substrates tested at their optimum pH. The specific activity for general proteolytic activity against azocasein was 1.5 times higher in larvae than in adults. Likewise, the hydrolysis of synthetic substrates was higher in larvae, except for HA hydrolysis which was higher in adults.

Based on its digestive proteolytic profile, we tested the *in vitro* effect of several serine proteinase inhibitors on the hydrolysis of BApNa, SA_2PPpNa, and SA_3pNa by gut extracts of *A. mariaefranciscae* last instar larvae (Table 3). SA_2PPpNa hydrolysis was the most susceptible to inhibition, as determined from the ID50 values (SBBI < LBI ≤ STI < TEI < CEOM). A similar pattern was obtained with SA_3pNa, that presented an identical ID50 for SBBI, but the ID50 values for the other inhibitors were one or two orders of magnitude higher. The order of ID50 values for BApNa hydrolysis was CEOM ≤ SBBI < STI ≤ TEI < LBI.

The survival and development of *A. mariaefranciscae* larvae fed from neonate to pupation with protease inhibitor-containing artificial diet are reported in Table 4. Larvae fed on a control diet containing 0.2% (w/w) albumin gave 25% mortality. Larvae fed on diet containing 0.2% (w/w) CEOM gave levels of mortality, larval duration and pupal and adult weights, similar to that of the control group. Consumption of diets containing 0.2% (w/w) SBBI, TEI, STI, and LBI

Table 1. Effect of protease inhibitors and activators on the hydrolysis of protein and synthetic substrates by gut extracts from *A. mariaefranciscae* adults at their optimum pH of activity

Inhibitor (concentration)	% Relative activity[a]											
	Azocasein			Hemoglobin	ZAA_2MNA	ArgNA	LpNa	HPA	HA	BApNa	SA_2PPpNa	SA_3pNa
	pH 6.0	pH 8.0	pH 10.0	pH 2.5	pH 8.0	pH 7.5	pH 7.5	pH 8.5	pH 7.5	pH 10.5	pH 10.5	pH 10.5
SBBI (10 μM)	ne	ne	36±1	ne	ne	ne	ne	ne	ne	25±1	12±1	25±3
Chymostatin (2 μM)	ne	ne	57±2	ne	ne	ne	ne	ne	ne	65±1	15±1	ne
TLCK (1 mM)	ne	ne	69±4	ne	(†)	(†)	ne	ne	ne	24±3	27±2	ne
TPCK (1 mM)	ne	ne	ne	ne	(†)	(†)	ne	ne	ne	ne	ne	ne
E-64 (10 μM)	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne
IAA (1 mM)	ne	ne	ne	ne	ne	ne	ne	52±5	33±4	ne	ne	ne
Leupeptin (10 μM)	ne	ne	63±2	ne	ne	ne	ne	ne	ne	17±1	ne	ne
Pepstatin-A (10 μM)	ne	ne	ne	23±2	ne	ne	ne	ne	ne	ne	ne	ne
EDTA (1 mM)	ne	ne	ne	ne	ne	ne	ne	38±3	54±1	ne	ne	ne
1,10 Phe (1 mM)	ne	ne	ne	(†)	24±1	43±3	21±1	7±2	22±1	ne	ne	ne
PCPI (1 μM)	ne	ne	ne	ne	ne	ne	ne	56±4	57±3	ne	ne	ne
$CdCl_2$ (1 mM)	ne	48±2	ne	ne	25±1	32±1	31±1	12±1	17±1	ne	ne	ne
$CuCl_2$ (1 mM)	ne	51±2	ne	ne	10±1	10±1	29±2	12±1	34±4	ne	ne	ne
Activator												
DTT (1 mM)	ne	ne	75±2	ne	ne	ne	ne	41±5	28±2	52±4	33±4	ne
L-cysteine (1 mM)	ne	ne	ne	ne	79±3	61±2	73±3	(†)	(†)	64±2	77±4	ne

[a]Values are mean ± SE of three different pooled midgut extracts treated with an inhibitor or activator versus their corresponding controls without them. No effect (ne) was considered for activities between 80% and 120%. (†) It chemically interferes with the assay.

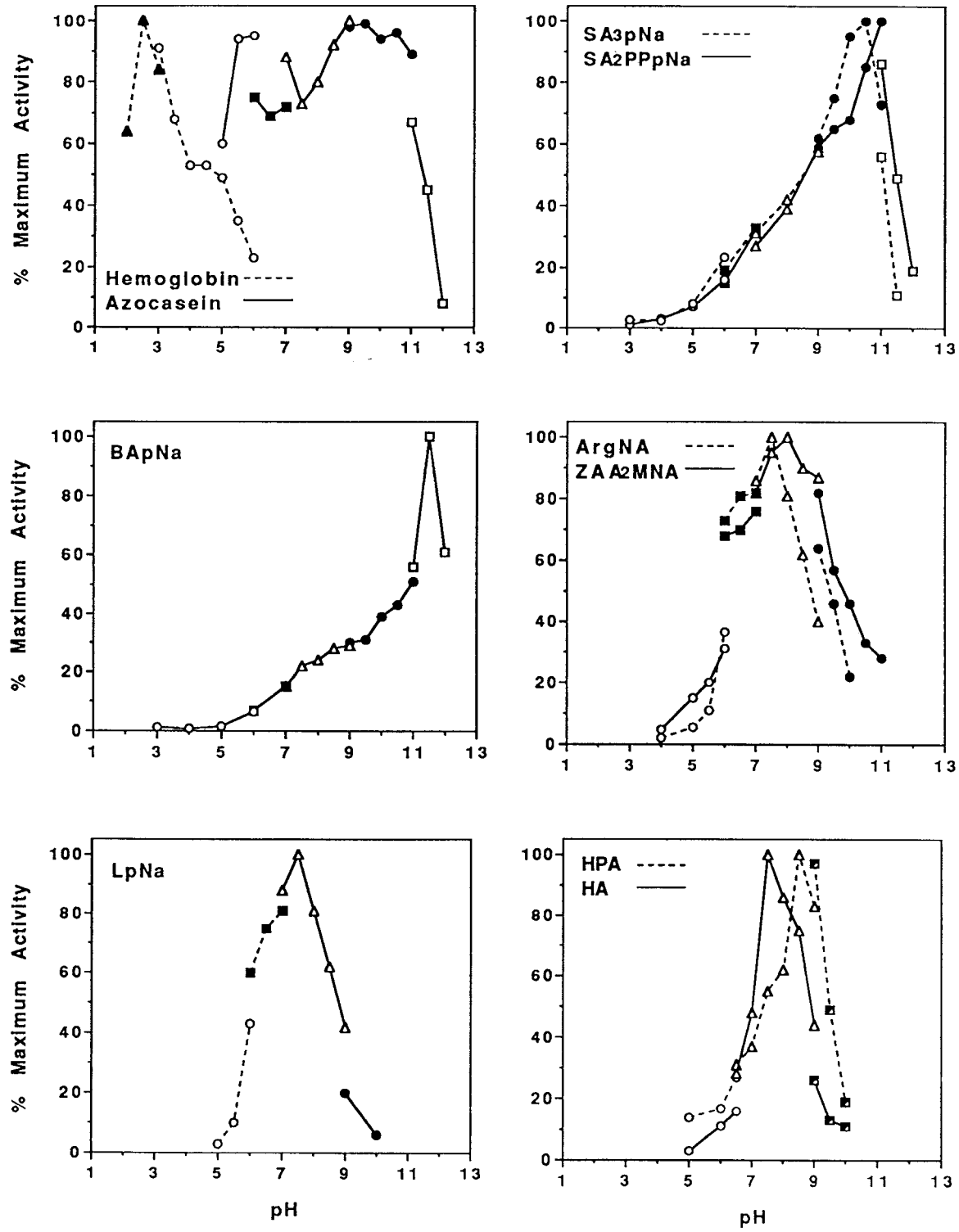

Figure 1. The effect of pH on the rate of hydrolysis of the protein substrates azocasein and hemoglobin, and the synthetic substrates SA_2PPpNa, SA_3pNa, BApNa, ZAA2MNA, ArgNA, LpNa, HPA, and HA by adult midgut extracts from *A. mariaefranciscae.* Data are mean of triplicate measurements, with standard errors within 5% of the means. Reaction buffers were 0.1 M citric acid-NaOH (▲), 0.1 M citrate (○), 0.1 M phosphate (■), 0.1 M tris-HCl (△), 0.1 M glycine-NaOH (●), 0.1 M Na_2HPO_4-NaOH (□) and 0.1M borate-NaOH (◩).

Table 2 Properties of midgut proteases from adults and last instar larvae of *A. mariaefranciscae* against protein and synthetic substrates

Substrate	Optimum pH[b]	Specific activity[a]	
		Adults	Last instar larvae
Azocasein	6.0 & 9.0	15.2±0.1 & 16.0±0.2	24.8±0.3 & 24.4±0.2
BApNa	11.5	62.9±0.7	92.5±0.6
SA2PPpNa	11.0	409±4	940±5
SA3pNa	10.5	16.1±0.2	48.4±0.3
Hemoglobin	2.5	21.0±0.5	30.1±0.7
ZAA2MNA	8.0	17.1±0.9	19.1±1.3
ArgNA	7.5	105±1	128±3
LpNa	7.5	126±4	185±6
HPA	8.5	19.2±0.3	33.2±1.7
HA	7.5	33.7±2.0	24.8±0.3

[a]Specific activities as nmoles of substrate hydrolyzed/min/mg protein, except for proteolytic activity against azocasein as mU Δ Abs 420 nm/min/mg protein and against hemoglobin as mU Δ Abs 280 nm/min/mg protein. Figures are mean ± SE of triplicate measurements.
[b]Optimum pH of activity determined with midgut extracts from adults (see Figure 1).

Table 3 Effect of protease inhibitors on the hydrolysis of synthetic substrates by gut extracts from *A. mariaefranciscae* last instar larvae at their optimum pH of activity

Inhibitor[b]	ID50 (μM)[a]		
	BApNa pH 10.5	SA_2PPpNa pH 10.5	SA_3pNa pH 10.5
SBBI	0.97	0.007	0.007
STI	3.2	0.2	3.1
CEOM	0.6	3.0	35
TEI	3.9	0.5	18
LBI	24	0.07	6.7

[a]Figures are the estimated doses of inhibitor that inhibits 50% of the activity (see Table 2) of gut extracts (15±0.3 μg protein/20μl gut extract).
[b]SBBI (Soybean Bowman-Birk inhibitor), CEOM (Chicken Egg White Ovomucoid), TEI (Turkey Egg White Inhibitor), STI (Soybean Trypsin Inhibitor), LBI (Lima Bean Inhibitor).

increased the mortality to 60–70%, whereas in diets containing the mixtures 0.2% (w/w) LBI/TEI (contains 0.1% (w/w) of each inhibitor) and 0.2% (w/w) SBBI/STI/TEI (contains 0.066% (w/w) of each inhibitor) the mortality was about 90%. With all of these proteinase inhibitors, either singly or in combination, the developmental time to pupation and to adult emergence were significantly ($P \leq 0.05$) delayed. The pupal and adult weights in all these treatments were lower than in the control, but not significantly different.

Discussion

Characterization of digestive proteases. We have found that midgut extracts of *A. mariaefranciscae* adults have azocaseinolytic activity within a broad range of pH values, from acid to alkaline, suggesting that this species has a digestive system based on proteases of different mechanistic classes. The ability of midgut extracts to hydrolyze specific synthetic substrates, the elucidation of the pH at which maximal hydrolysis occurs and their sensitivity to protease inhibitors confirmed the presence of trypsin-, chymotrypsin-, elastase-, cathepsin-D, leucine aminopeptidase- and carboxypeptidase A- and B-like activities. Midgut extracts from larvae of *A. mariaefranciscae* exhibited higher specific proteinase activity rates than those measured for adult extracts. However, apart from these quantitative variations, no qualitative differences were noted. Thus, we can conclude that larvae and adults possess a similar proteolytic profile, as expected in an insect with the same feeding habits and exploiting the same ecological niches at the larval and adult stages.

Complex proteolytic systems for protein digestion, based on the presence of several proteinases of different mechanistic classes and exopeptidases, appear to be widespread among curculionids. A wide pH range of proteolytic activity against proteinaceous substrates has been reported for the boll weevil, *Anthonomus grandis* (Wolfson & Murdock, 1990), the black vine weevil, *Otiorhynchus sulcatus* (Michaud et

Table 4 Effect of protease inhibitors on survival and development of *A. mariaefranciscae* larvae

Treatment[a]	Dose (% w/w)	N	Mortality (%)	Larval duration (days)	Pupal weight (mg)	Immature length (days)	Adult weight (mg)
Control (Albumin)	0.2	40	25.0	53.5 ± 1.7	8.9 ± 0.3	64.9 ± 1.7	6.7 ± 0.2
CEOM	0.2	40	22.5	53.3 ± 1.6	8.9 ± 0.2	64.4 ± 1.8	6.6 ± 0.2
SBBI	0.2	40	70.0	71.9 ± 1.5*	8.2 ± 0.3	81.0 ± 1.7*	5.9 ± 0.3
TEI	0.2	40	70.0	68.7 ± 1.7*	8.3 ± 0.4	79.8 ± 1.6*	5.8 ± 0.3
STI	0.2	40	67.5	61.5 ± 2.5*	8.3 ± 0.3	72.8 ± 2.1*	6.1 ± 0.3
LBI	0.2	40	60.0	66.8 ± 2.3*	8.5 ± 0.4	78.3 ± 2.2*	5.9 ± 0.3
LBI/TEI	0.2	40	90.0	61.9 ± 1.8*	8.5 ± 0.4	73.4 ± 1.7*	6.1 ± 0.3
SBBI/STI/TEI	0.2	40	87.5	67.1 ± 2.2*	7.9 ± 0.3	78.0 ± 2.3*	5.9 ± 0.2

[a]SBBI (Soybean Bowman-Birk inhibitor), CEOM (Chicken Egg White Ovomucoid), TEI (Turkey Egg White Inhibitor), STI (Soybean Trypsin Inhibitor), LBI (Lima Bean Inhibitor), LBI/TEI (0.1% LBI + 0.1% TEI), and SBBI/STI/TEI (0.066% SBBI + 0.066% STI + 0.066% TEI).
*Significantly different from the Control (Dunnett two tailed test, $P \leq 0.05$).

al., 1995), the rice weevil, *Sitophilus oryzae* (Baker, 1982), and the maize weevil, *S. zeamais* (Baker, 1982). The majority of these curculionids have slightly acidic midguts and cysteine proteinases provide the major midgut endoproteolytic activity (Murdock et al., 1987; Michaud et al., 1995, 1996). Nevertheless, aspartic and/or serine proteinases are detected in some of these species (Purcell et al., 1992). Baker (1982) reported that protein digestion was provided by serine proteinases in some species of *Sitophilus*, but recent reports indicated the presence of cysteine proteinases (Liang et al., 1991; Irie et al., 1996; Matsumoto et al., 1997). Aminopeptidase and carboxypeptidase activities in curculionids have been reported by Baker (1982).

A peak of digestive azocaseinolytic activity at pH 5.0–7.0 was identified in *A. mariaefranciscae* larvae and adults, that was not inhibited by any of the serine, cysteine, aspartyl and metalloprotease specific inhibitors tested, nor enhanced by cysteine proteinase activators, indicating the presence of a protease activity that does not fit to any of the mechanistic classes established. The midgut extracts of *A. mariaefranciscae* adults were able to hydrolyze ZAA_2MNA and ArgNA, specific substrates for cathepsin B and H, respectively. According to these hydrolytic activities, it could be argued that the azocaseinolytic peak at pH 5.0–7.0 might be due to the action of cysteine proteinases. However, when ZAA_2MNA and ArgNA were used as substrates their optimum of activity was at pH 7.5–8.0, and their sensitivity to protease inhibitors did not correspond to cysteine proteinases. In fact, the hydrolysis of both substrates was not affected by the cysteine proteinase inhibitors E-64 and IAA or the cysteine proteinase activators DTT and L-cysteine. On the contrary, the hydrolysis of ZAA_2MNA and ArgNA respond to inhibitors and activators in the same way as the leucine aminopeptidase substrate LpNa, suggesting that all of them are hydrolyzed by aminopeptidases. An aminopeptidase isolated from larval midguts of the dermestiid *Attagenus megatoma* has been shown to hydrolyze several aminoacyl-β-naphthylamides, including ArgNA (Baker & Woo, 1981). Likewise, Novillo et al. (1997b) have shown that in larvae of the Colorado potato beetle, *Leptinotarsa decemlineata*, both aminopeptidases from the midgut epithelium and cathepsin H-like proteinases located in the endoperitrophic space are able to hydrolyze ArgNA. In addition, crude insect gut homogenates contain a number of different enzymes, such as di- and tripeptidases, as well as esterases, which might act on synthetic substrates such as ZAA_2MNA and ArgNA (Terra & Ferreira, 1994).

Effects of protease inhibitors in vitro *and* in vivo. Several authors have demonstrated that ingestion of proteinase inhibitors in natural or artificial diets can retard growth and development of several species of insect pests, suggesting that these inhibitors may serve as an effective means of controlling insects (review in Jongsma & Bolter, 1997). However, mortality was quite low in most studies, and protection not complete, even though high levels of inhibitors were used. There are some accounts of high levels of mortality with some curculionid species, but they were obtained with low-molecular-weight synthetic inhibitors and peptide proteinase inhibitors of microbial origin. Elden (1995) reported that larval survival of the alfalfa

weevil, *Hypera postica*, was significantly decreased by ingestion of the cysteine proteinase inhibitors E-64, p-hydroxymercuribenzoid acid and leupeptin, and the aspartic proteinase inhibitors antipain and pepstatin. Recently, Pittendrigh et al. (1997) found that E-64 increased mortality and delayed developmental time in larvae of the rice weevil.

According to the presence of digestive serine proteinases in larvae and adults of *A. mariaefranciscae*, several serine proteinase inhibitors were tested *in vitro* and *in vivo* to establish their potential as resistance factors. We have found that survival was strongly reduced and development significantly retarded with larvae fed an artificial diet containing 0.2% (w/w) of the proteinaceous proteinase inhibitors SBBI, TEI, STI and LBI. The mortality rates obtained with these proteinase inhibitors represent an increment of 35–45% with respect to the mortality of the controls, and are among the higher reported so far with plant proteinase inhibitors on curculionids. Purcell et al. (1992) found no mortality or stunting associated with feeding high levels of SBBI and STI to larvae of the boll weevil. However, Graham et al. (1997) have observed that the expression of cowpea trypsin inhibitor in strawberry is effective in protecting roots from vine weevil larval feeding.

From our data, no clear relationship can be established between the effect of proteinase inhibitors on the *in vitro* activity of digestive proteinases and the effect of these inhibitors on survival and development of *A. mariaefranciscae* in feeding trials. SBBI was the best inhibitor for chymotrypsin- and elastase-like activities and the second best for trypsin-like, but *in vivo* its activity was similar to that of STI, TEI and LBI. In addition, no effect was associated with feeding CEOM to larvae, despite the fact that this protein was the best inhibitor of trypsin-like activity. It has been suggested that the adaptation of insects to plant protease inhibitors can drastically influence their efficacy *in vivo* (Jongsma & Bolter, 1997). Indeed, some protease inhibitors are sensitive to proteolysis by non-target digestive proteases in insects (Michaud et al., 1995, 1996). Our results indicate that *in vitro* screening may be useful to select effective proteinase inhibitors for a particular insect, but validation in feeding trials before a crop plant is transformed is needed.

We have found in our feeding assays with proteinase inhibitors some individuals that presented disturbances at the time of molting and metamorphosis, mostly insects with intermediate morphological characteristics between larvae and pupae and between pupae and adults, that died within a few days. There were also some larvae that showed head-capsule slippage, a typical sign of apolysis, but the subsequent ecdysis step was inhibited resulting in incomplete removal of the old cuticle. Similar molting disruptions, at either the third or fourth instar, have been reported in some larvae of the budworm *Helicoverpa punctigera* feeding on diet containing trypsin and chymotrypsin inhibitors from neonates (Heath et al., 1997). Faktor & Raviv (1997) reported that injection of SBBI or STI into larvae of the Egyptian cottonworm, *Spodoptera littoralis*, disrupted larval molt, suggesting the presence of endogenous targets for protease inhibitors. Billings et al. (1992) have reported that following oral administration in mice, SBBI pass the gut barrier and can be found in almost every organ, the blood and the urine. However, no information about the possibility that proteinase inhibitors may get access through the insect gut to endogenous targets is available. Alternatively, the effects on the molting process indicated in our study and those reported by Heath et al. (1997) might also be due to physiological alterations derived from their action inside the midgut.

Interestingly, when dietary mixtures of two or three inhibitors were fed to *A. mariaefranciscae* larvae, a synergistic effect on mortality was observed that reduced the survival rate to 10–12.5%. The complementary action of these inhibitors may explain their synergistic effect when supplied together to the larvae. Burgess et al. (1994) reported a synergistic effect from the combination of protease inhibitors on the survival and growth of the black field cricket, *Teleogryllus commodus*. Oppert et al. (1993) found that dietary mixtures of cysteine and serine proteinase inhibitors exhibit synergistic toxicity towards larvae of the red flour beetle, *Tribolium castaneum*. Markwick et al. (1995) reported that when larvae of the codling moth, *Cydia pomonella*, were fed with protease inhibitors containing diets, the most significant reductions in growth rate occurred with combinations of protease inhibitors.

Transgenic cultivars of sugar beet have been proved agronomically useful in greenhouse and field trials (Mannerlof et al., 1997). This technology allows the possibility to obtain transgenic sugar beet plants that, expressing a suite of protease inhibitors that match to the digestive enzymes of *A. mariaefranciscae*, would enhance their resistance to this pest.

Acknowledgements

This work was supported by 'Comisión Interministerial de Ciencia y Tecnología (CICYT)' (grant AGF95-0065-CO2-01).

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