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Proteolytic Digestive Enzymes and Peritrophic Membranes during the Development of *Plodia interpunctella* (Lepidoptera: Piralidae): Targets for the action of Soybean Trypsin Inhibitor (SBTI) and Chitin-Binding Vicilin (EvV)

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The digestive system of *P. interpunctella* was characterized during its larval development to determine possible targets for the action of proteinaceous enzyme inhibitors and chitin-binding proteins. High proteolytic activities using azocasein at pH 9.5 as substrate were found. These specific enzymatic activities (AU/mg protein) showed an increase in the homogenate of third instar larvae, and when analyzed by individual larvae (AU/gut), the increase was in sixth instar larvae. Zymograms showed two bands corresponding to those enzymatic activities, which were inhibited by TLCK and SBTI, indicating that the larvae mainly used serine proteinases at pH 9.5 in their digestive process. The presence of a peritrophic membrane in the larvae was confirmed by chemical testing and light microscopy. In a bioassay, *P. interpunctella* was not susceptible to the soybean trypsin inhibitor, which did not affect larval mass and mortality, likely due to the weak association with its target digestive enzyme. EvV (*Erythrina velutina* vicilin), when added to the diet, affected mortality (LD_{50} 0.23%) and larval mass (ED_{50} 0.27%). This effect was associated with EvV-binding to the peritrophic membrane, as seen by immunolocalization. EvV was susceptible to gut enzymes and after the digestion process, released an immunoreactive fragment that was bound to the peritrophic matrix, which probably was responsible for the action of EvV.

KEYWORDS: Serine proteinases; peritrophic membrane; SBTI; chitin-binding vicilina; *Plodia interpunctella*; *Erythrina velutina*

1. INTRODUCTION

Plodia interpunctella (pyralid moth) is a generalist pest widely distributed in temperate and tropical areas of the world. In the larval stage, these insects have a significant negative economic impact when they attack storage products such as cereal and legume grains, dried fruits, and nuts (*1, 2*). Control of insect infestation in storage grains is done by treating the grain with methyl bromide, carbon disulfide, and several other chemicals considered environmentally harmful and very expensive for subsistence farmers (*3*). To increase the insect resistance of cultivated varieties, plant breeders are interested in understanding resistance mechanisms that operate in wild varieties. These are based on defense proteins such as proteinase and α-amylase inhibitors, lectins and chitin-binding proteins, which are usually expressed in response to herbivore insects, pathogens, and wounding (*4*−*7*). These defense proteins may affect the development of a wide range of potentially damaging organisms, such as bacteria, fungi, nematodes (*8*), and insects (*9*−*11*). The effects on insects can be observed when digestive enzymes are inhibited or when defense proteins bind to the gut structures of larvae and insects (*12*).

Currently, one of the most important aspects of alternative pest control is based upon the selection of target molecules, such as digestive enzymes and/or the chitin present in the peritrophic membrane (PM), a film-like structure that separates food from the gut tissue of many insect pests (*13*). This is because the digestive tube is the main interface region between

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insects and the environment, given that nutrition is a decisive factor in the evolutionary process of these organisms. These attempts seek to produce detrimental effects on larval and insect growth, retarding it and causing death by preventing the digestion and assimilation of nutrients.

In this study, we report on the classification of proteolytic digestive enzymes during *Plodia interpunctella* development and the detection of peritrophic membranes as possible targets for the action of the soybean trypsin inhibitor (SBTI) and the effect of EvV, a chitin-binding vicilin, on mass decrease and mortality of *P. interpunctella* in bioassays.

2. MATERIAL AND METHODS

2.1. Chemicals. All substrates, enzymes, synthetic inhibitors, SBTI, antirabbit IgG peroxidase conjugate, and the native protein deglycosylation kit were purchased from Sigma Chemical Co. (St. Louis, MO). Electrophoresis chambers and reagents were obtained from Bio-Rad Laboratories (Richmond, CA).

2.2. Insects. *P. interpunctella* larvae were reared on an artificial diet (10.4% finely ground sugar cane fibers, 3% wheat germ, 6.5% wheat flour, 12% crystal sugar, 9.9% yeast, 0.3% sodium benzoate, 0.9% HCl and 57% H_2O) (*14*), at a temperature of 25−30 °C and relative humidity of 70−80%. A laboratory colony was maintained with insects obtained from the Department of Genetics and Cell Biology, Bioscience Center, Federal University of Rio Grande do Norte, Natal, Brazil.

2.3. Preparation of Larval and Insect Gut Homogenates. Fifty larvae from the third to sixth instar were dissected in 0.15 M NaCl and the guts homogenized in 50 mM Tris-HCl buffer at pH 8.0 in a glass homogenizer. The homogenate was centrifuged at 10,000*g* for 30 min at 4 °C, and the clear supernatant was used in enzyme assays. All homogenates were freshly prepared. Fifty larvae from the first and second instar were totally homogenized in the same buffer; the homogenate was centrifuged at 10,000*g* for 30 min at 4 °C, and the clear supernatant was used in proteolytic enzyme assays.

2.4. Protein Determination. Protein concentration was estimated by Bradford's method (*15*), using bovine serum albumin as the standard.

2.5. Proteolytic Enzyme Assays. Azocaseinase activity: 50 μL aliquots of gut homogenates were incubated with 450 μL of appropriate buffer (50 mM Tris-HCl, 20 mM $CaCl_2$, pH 8.0) and 500 μL of 1.5% azocasein solution, at 37 °C for 30 min. The reaction was stopped by the addition of 150 μL of 20% TCA solution. Samples were centrifuged at 10,000*g* for 10 min and supernatants alkalinized with 0.2 N NaOH solution. The soluble peptides were measured by absorbance at 440 nm. One activity unit (AU) was defined as the amount of enzyme activity that increased absorbance by 0.01 at 440 nm. All the assays were performed in triplicate.

Hemoglobinase activity: 100 μL aliquots of gut homogenates were incubated with 50 μL of appropriate buffer (0.2 M sodium acetate buffer, pH 4.5) and 500 μL of 1.0% hemoglobin solution at 37 °C for 60 min. The reaction was stopped by the addition of 100 μL of 40% TCA solution. Samples were centrifuged at 10,000*g* for 10 min and supernatants alkalinized with 2.0 N NaOH solution. The supernatants were submitted to Folin's method, modified by Lowry et al. (*16*), and soluble peptides were measured by absorbance at 750 nm. One activity unit (AU) was defined as the amount of enzyme activity that increased absorbance by 0.01 at 740 nm. All the assays were performed in triplicate.

2.6. Determination of Azocaseinase and Hemoglobinase Activities at Different pH in Larval Gut on the Third and Sixth Instar Stage of Development. Acid proteinase activities were determined using hemoglobin as substrate and 50 mM acetate buffer (pH 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5). The basic proteinase activities were determined using azocasein as substrate and 50 mM acetate buffer (pH 4.5, 5.0, and 5.5), 50 mM phosphate buffer (pH 6.0, 6.5, and 7.0), 50 mM Tris-HCl buffer (pH 7.5, 8.0, 8.5, 9.0, and 9.5), and 50 mM glycine-NaOH buffer (pH 10.0, 10.5, 11.0, 11.5, and 12.0). The mixture was assayed for protease activity as previously described.

2.7. Proteinase Inhibitory Assays. Proteinases in the larval gut of the third and sixth instar were classified according to their sensitivity to specific inhibitors: serine proteinase, cysteine proteinase, metal proteinases, and aspartic proteinases. The inhibitors used and their final assay concentrations were 1 mM PMSF (phenylmethylsulfonyl fluoride, a serine proteinase inhibitor); 3 mM SBTI (soybean trypsin inhibitor, a serine proteinase inhibitor); 0.1 mM TLCK (*N*-*p*-tosyl-L-lysine chloromethyl ketone, a trypsin inhibitor); 0.1 mM TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone, a chymotrypsin inhibitor); 0.1 mM IA (iodoacetamide, a cysteine proteinase inhibitor); 0.01 mM E64 (*trans*-epoxysuccinyl-leucylamido-(4-guanidino) butane, a cysteine proteinase inhibitor); 0.01 mM PA (pepstatin A, an aspartic proteinase inhibitor); and 0.1 mM 1,10-phenanthroline (a metal proteinase inhibitor). These inhibitors were solubilized in appropriate solvents (*17*). Enzyme extracts (50 μL of gut extract from larval gut in the third instar and prepupal stage of development) were mixed with 450 μL of appropriate buffers, specific for each enzyme class, and preincubated for 10 min at 37 °C. The mixture was assayed for protease activity as previously described. The protease assay includes an internal control for inhibition caused by solvents. Average inhibition is expressed as a percentage, with 0% being the inhibition obtained in proteolytic assays from gut homogenates incubated without inhibitors.

2.8. Larval Gut Proteinase Activities in Polyacrylamide Gel. This analysis was performed in SDS−PAGE (*18*). Homogenate guts from third and sixth instar larvae were used in gel activity assays in the presence of serine proteinase inhibitors (0.1 mM TLCK and 3 mM SBTI). After the run, the gel was transferred to 2.5% Triton X-100 solution for 30 min at room temperature. The gel was then washed with 0.05 M Tris-HCl, pH 9.5, for 30 min and incubated with 2.5% azocasein for 30 min at 5 °C with gentle agitation. It was then transferred to 0.05 M Tris-HCl buffer at pH 9.5 for 30 min at 37 °C. After incubation, the reaction was stopped by transferring the gel to a staining solution (Coomassie brilliant blue R-250 in methanol/acetic acid/water, 3:1:6, v/v/v) and then to a destaining solution (methanol/acetic acid/water, 3:1:6, v/v/v).

2.9. EvV Purification and Antibody Production. *Erythrina velutina* seeds were obtained from the IBAMA (Brazilian Environmental Institute) seed bank of Natal, Brazil. Finely ground seed meal was extracted with 0.05 M borax buffer at pH 7.5 for 30 min at room temperature. After centrifugation for 30 min at 8,000*g* at 4 °C, the supernatant (crude extract) was precipitated with ammonium sulfate at a concentration of 70%−90%. This fraction, corresponding to the globulin fraction in legume seeds, was then dialyzed and freeze dried. Globulin (10 $mg \cdot mL^{-1}$) was applied to an affinity chromatograph on a chitin column (5 mL), equilibrated with 0.05 M borax buffer, pH 7.5. After protein adsorption, the matrix was washed with the equilibrium buffer, and adsorbed proteins were eluted with 100 mM glycine-HCl buffer, pH 2.0. Protein peaks were collected and recovered after dialysis and freeze-drying. Also, samples were tested to detect contaminants such as lectin activities, using ABO human erythrocytes, and trypsin and papain inhibitory activities. The sample (1 mg/mL) was then subjected to another gel filtration on Superose-6-10-300-GL using an AKTA purifier system, previously calibrated with protein markers: thyroglobulin (669 KDa); β-amylase (200 kDa); alcohol dehydrogenase (150 kDa); and carbonic anhydrase (29 kDa). All chromatographs were monitored at 280 nm. The peak obtained, denominated EvV, was pooled, freezer dried, and used in the bioassays. The purity of EvV was analyzed by SDS−PAGE and PAGE (*18*), and acid nature by PAGE.

Polyclonal antibodies against deglycosylated EvV (prepared following deglycosylation kit instructions) were performed by rabbit immunization according to Thorpe (*19*). IgG was obtained by affinity chromatography of the crude immune sera, using a protein A column (protein A bound to Sepharose CL-4B). Preimmune sera were collected before immunization and used as the control.

2.10. Insect Bioassay. To examine the effects of SBTI and EvV during the development of *P. interpunctella* larvae, an artificial diet system was assessed. Artificial diets (500 mg each) were prepared as described in section 2.2. Lyophilized SBTI and EvV at standard concentrations of 0.2, 0.5, 1.0, 2.0, and 4.0% w/w were added to the diet. The diets were presented to three neonate larvae per diet in a

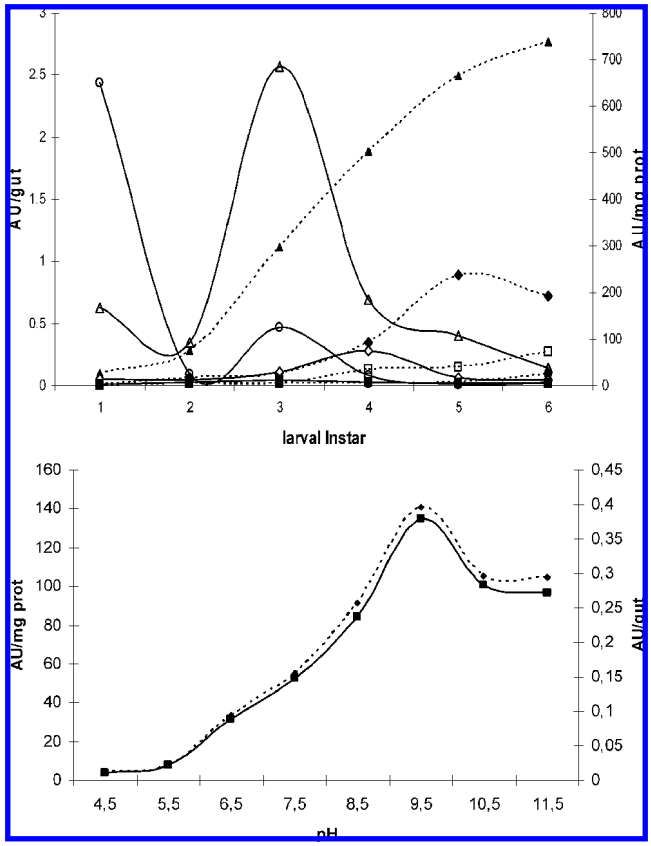

Figure 1. (**A**) Protease activities during *Plodia interpuncella* development. Black lines represent enzymatic activities per mg protein pH 5.5; (♦), pH 7.5 (■); hemoglobinase activity at pH 3.5 (●); hatched lines represent enzymatic activities per gut pH 5.5; (◊), pH 7.5 (□), pH 9.5 (Δ); hemoglobinase activity at pH 3.5 (○); Azocasein activities at. (**B**) Effect of pH on the specific activities of 3rd instar larvae and activities per gut of sixth instar larvae gut extract.

Table 1. Effect of Different Inhibitors on the Proteolytic Activity of *P. interpunctella* Gut Extracts[a]

inhibitor	concentration (mM)	proteinase class	inhibition (%)
SBTI	1	serine	96 ± 2.5
TPCK	0.1	serine (chymotrypsin-like)	8 ± 1.0
TLCK	0.1	serine (trypsin-like)	89 ± 3.1
iodoacetamide	0.1	cysteine	47 ± 2.5
E-64	0.01	cysteine	25 ± 2.0
EDTA	10	metalo-proteinase	28 ± 1.3
phenantroline	0.1	metalo-proteinase	5 ± 0.8

[a] Values are the mean of three determinations ± SD.

glass vial at a controlled temperature of 28 ± 1 °C and 60%−70% relative humidity in the growth chamber. After 15 days, the vials were opened, and the mass and number of larvae were recorded and compared to the mass and number of larvae found in the control. The experiments were carried out with six replicates, and the mean (±SEM) was calculated. Artificial control diets were prepared without SBTI and EvV. A completely random design was used, and the comparisons of mean larval weight was made by Tukey's test at a 5% level of probability.

2.11. Action Mechanism of EvV. *2.11.1. Detection of Chitin in the Gut of P. interpunctella Larvae.* Larvae were dissected under magnification in cold 0.15 M NaCl with the help of tweezers, and the guts were separated from the windpipes and Malpighian tubes. The guts were perforated, and the luminal contents were aspirated and reserved. The guts were washed to remove the remaining luminal contents. The presence of chitin in the larval guts was ascertained by the von Wisselingh color test (*20*). This qualitative test detects chitosan produced after treatment of the chitin-containing materials with saturated

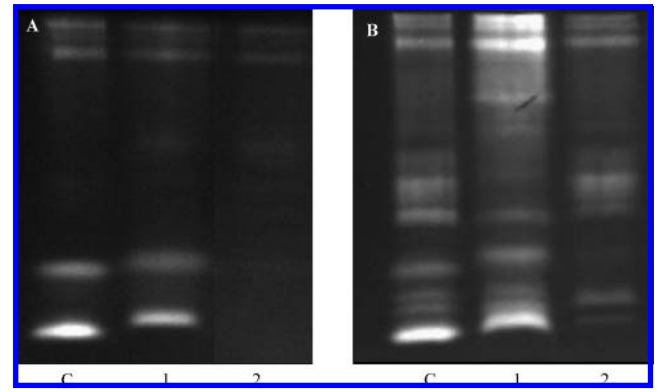

Figure 2. Substrate SDS−PAGE of gut extracts of 3rd instar larvae (**A**) and 6th instar larvae (**B**) treated with different specific inhibitors. The protein gut extract was incubated with 5 μL of different proteinase inhibitors. C = control without inhibitor; 1 = SBTI; and 2 = TLCK.

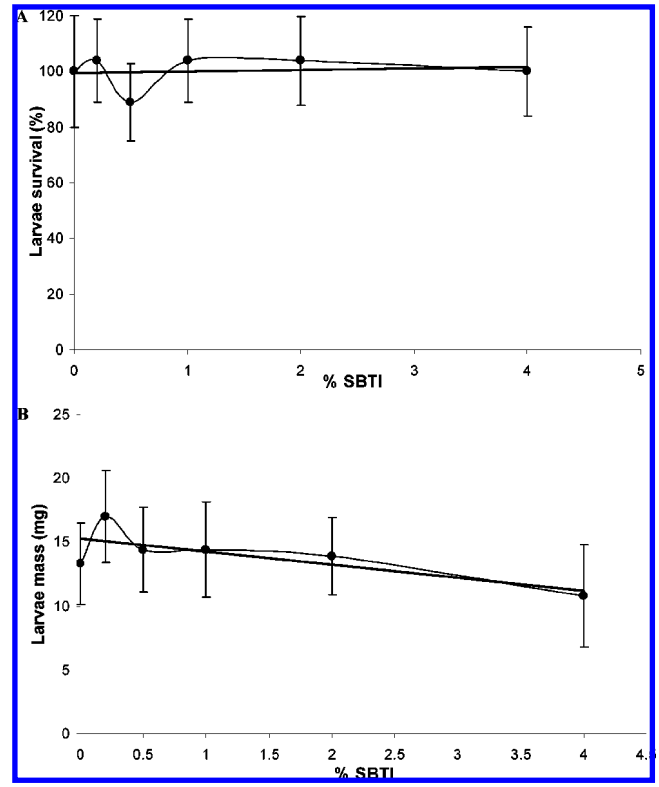

Figure 3. Effects of SBTI on *P. interpunctella* larval development in the artificial diet system. (**A**) % larval mass decrease; (**B**) % larval mortality. Mean values followed by the same letter were not statistically different ($P < 0.05$) in this test. Each mean represents six replicates (±SEM). The error bars represent the standard deviation.

KOH for 15 min at 160 °C. After the reaction, the presence of chitin was observed with a KI/ iodine solution. Controls used cellulose (−) and lobster chitin (+).

2.11.2. In Vivo Digestibility and EvV Binding to the Peritrophic Membrane. Sixth instar larvae were fed on artificial diet containing 80% insoluble starch, 20% sucrose, and 0.3% EvV (w/v). After 4 h, the larvae were transferred to the same diet without EvV for another 4 h, to dislocate the EvV contained in the gut. The larvae were removed and dissected, the peritrophic membranes were excised, and the excreta (feces) were collected. The excreta (feces) were extracted in 0.05 M borax buffer, pH 7.5, for 30 min at room temperature. The extracted feces were centrifuged at 10,000*g* for 10 min, and the supernatant was collected, dialyzed, and freeze-dried. The excised peritrophic membrane was washed with PBS to remove nonadsorbed membrane proteins. The concentration of nonadsorbed proteins was evaluated by Bradford's

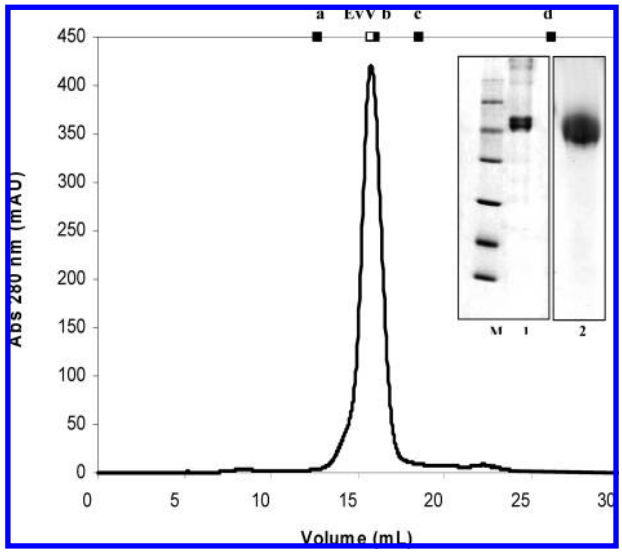

Figure 4. Elution profile on Superose 6-10-300-GL calibrated column of EvV peak eluted from the chitin column. Protein markers: (a) thyroglobulin (669 kDa); EvV (216 kDa); (b) β-amylase (200 kDa); (c) alcohol dehydrogenase (150 kDa); (d) carbonic anydrase (29 kDa). Inset: (1) SDS−PAGE (15%) analysis of EvV stained with Comassie Blue. (M) Molecular markers: β-galactosidase (119.0 kDa); bovine serum albumin (66.2 kDa); ovoalbumin (45.0 kDa); lactate dehydrogenase (35.0 kDa); restriction enzyme Bsp98 (25.0 kDa); β-lactoalbumin (18.4 kDa), lysozyme (14.4 kDa); (1) *E. veluntina* vicilin (10 μg). (2) PAGE (15%) analysis of purified EvV (10 μg) stained with Comassie Blue.

method (*15*). Adsorbed proteins were eluted with 0.1 M glycine-HCl buffer, pH 2.0. Extracted dried feces and eluted proteins from peritrophic membranes were subjected to SDS−PAGE (*18*) and Western blotting analysis (*21*). Control treatment with BSA was carried out under the same conditions.

2.11.3. Visualization of Peritrophic Membranes and Fluorescent Localization of the EvV-FITC Bond. Fluorescein-5-isothiocyanate was covalently coupled to EvV by the method developed by Johnston et al. (*22*) with modifications. FITC (0.2 mg/mL) was immediately added to 2 mg/mL of EvV (0.1:1, w/w) in the dark glass tube. The mixture was incubated and rotated at room temperature for 1 h. The FITC-labeled EvV was recovered by ammonium precipitation, dialyzed against distilled water, and freeze-dried.

Sixth instar larvae were fed on a diet containing 0.3% FITC-EvV (w/w) for 4 h at room temperature and then refed on a diet without EvV for an additional 4 h to purge FITC-vicilin contained in the gut before dissection. These larvae were removed and dissected, and the peritrophic membrane was washed with PBS. The peritrophic membranes were examined under a fluorescence microscope. Control diets containing 2.5% *N*-acetyl glycosamine (w/w) were administered under the same conditions described above.

3. RESULTS

3.1. Digestive Proteolytic Activities during *P. interpunctella* Larval Development. The values of total proteinase activity contained in the first to sixth instar larval gut homogenate are shown in **Figure 1A**. Proteolytic activities of azocasein as substrate showed a noticeable increase in the third instar larvae when expressed as mg/protein (specific activity) and in the sixth instar larvae when expressed as gut units. Homogenate gut from the third and sixth instar larvae were used to construct a pH curve (**Figure 1B**), where an optimum pH of 9.5 was observed. By inhibiting azocasein hydrolysis, using specific inhibitors of four enzyme classes (serine, cysteine, aspartic, and metalloproteinase), the relative contribution of the inhibited classes of proteinases to total proteinase activity in the gut could be observed (**Table 1**). Azocaseinase gut activity

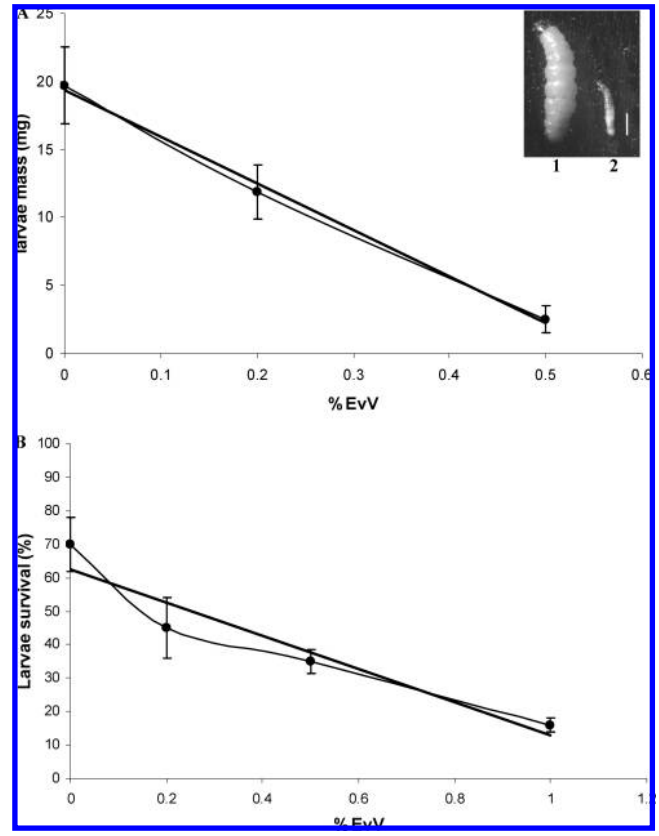

Figure 5. Effects of EvV (%, w/w) on larval development using an artificial diet system. (**A**) % larval mass decrease. (**B**) % larval mortality. Mean values followed by the same letter were not statistically different ($P <$ 0.05) in this test. Each mean represents six replicates (±SEM). The error bars represent the standard deviation. Inset: (1) Control; (2) larvae fed with EvV 0.5% (w/w).

at pH 9.5 was inhibited by serine proteinase inhibitors SBTI and TLCK, specific to trypsin-like enzymes, at 96% and 89%, respectively.

Digestive proteinases containing azocasein at pH 9.5 were observed in zymograms of the third and sixth instar larval gut homogenate, as clear zones in the gel against a dark blue background. Among these activities, two clear zones were totally inhibited by TLCK and partially inhibited by SBTI due to the clear retardation band, indicating the presence of two major trypsin-like activities in the larval guts (**Figure 2A** and **B**).

3.2. *In Vivo* Effect of SBTI on *P. interpunctella* Larvae. Enzymes from *P. interpunctella* guts were strongly susceptible to SBTI in the *in vitro* assays, producing 96% inhibition. On the basis of this *in vitro* enzyme inhibition study, a standard feeding trial was carried out to assess the potential effects of SBTI on *P. interpunctella* larvae. The influence of SBTI on the mass and number of *P. interpunctella* survivors was tested during larval development when the larvae were fed with a diet containing different SBTI concentrations. The diet with SBTI was not effective and did not reduce larval mass at the concentrations tested (**Figure 3A** and **B**).

3.3. Visualization of PM and Detection of Chitin in the Gut of *P. interpunctella* Larvae. PM was visualized in the digestive system of the larvae by light microscopy (see **Figure 7A**), and the presence of chitin in the gut was detected by the von Wisselingh color test (*20*). In this color test, KOH-treated larval guts were placed in a KI/iodine solution, and the appearance of a brown color indicated the presence of chitin in the larval guts. The presence of chitin was confirmed by the

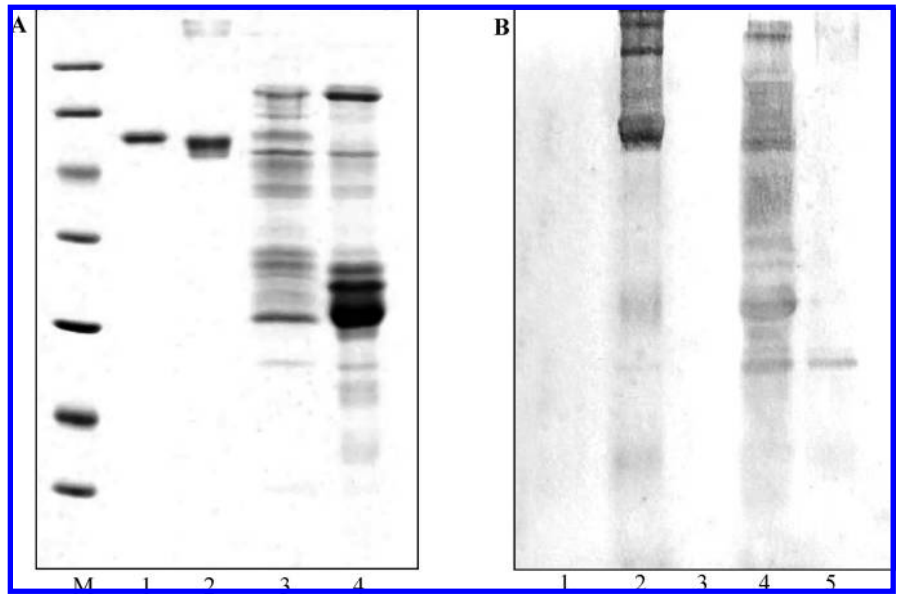

Figure 6. *In vivo* digestibility of EvV during the passage through the gut of *P. interpunctella* larvae. (**A**) SDS−PAGE. M = Molecular weight markers (β-galactosidase 116.0 kDa; bovine serum albumin 66.2 kDa; ovoalbumin 45.0 kDa; lactate dehydrogenase 35.0 kDa; restriction enzyme Bsp98 25.0 kDa; β-lactoalbumin 18.4 kDa, lysozyme 14.4 kDa); 1 = bovine serum albumin control; 2 = EvV; 3 = excreta from BSA treatment; 4 = excreta from EvV treatment. (**B**) Immunoblot. 2 = EvV; 4 = excreta from EvV treatment; 5 = elution from the PM of larvae fed an artificial diet containing EvV.

addition of 0.1% H_2SO_4 when the brown color turned violet. This molecule was then used to test the potential effect of a chitin-binding vicilin from *E. veluntina* seeds, called EvV, on *P. interpunctella* larvae.

3.4. Isolation, Purification, and Characterization of *E. velutina* Vicilin (EvV). A crude soluble protein extract obtained from the mature seeds of the *E. velutina* tree was initially precipitated at 0%−70% and 70%−90% saturation with ammonium sulfate. The F70−90 protein fraction (globulins) obtained was chromatographed on an affinity chitin column, and one peak was obtained. The retained chitin-matrix peak was assayed to detect contaminants, such as gut proteinase inhibitors, and lectin activities. This fraction showed no inhibitory and lectin activities (data not shown). After analysis, the retained peak was applied to Superose-6-10-300-GL gel filtration previously calibrated using an AKTA purifier system. The EvV peak had a molecular mass of 216.57 kDa (**Figure 4**) and when applied to SDS−PAGE, proved to be a multimeric protein with 2 major relative molecular mass subunits of 54.8 and 50.8 kDa (inset 1, **Figure 4**). EvV homogeneity was confirmed in a PAGE where it is a unique protein band with slow mobility in this native gel, similar to acid proteins (inset 2, **Figure 4**).

3.5. Insect Bioassay Using EvV. The effect of EvV on the development of *P. interpunctella* larvae was assessed by determining the number and mass of surviving larvae fed with a diet containing increasing amounts of EvV. The dose response of the effect of EvV on the growth (**Figure 5A**) and mortality (**Figure 5B**) of the insect larvae was indicative of its toxic effect. The chitin-binding vicilin was very effective, causing 50% mortality at a level of 0.23% (LD_{50}, lethal dose) and affected 50% of the larval mass with an ED_{50} (effective dose) of 0.27%. Regression analysis showed that for every 0.1% increase in EvV dose applied to *P. interpunctella*, there was a 20.0% increase in mortality. For each 0.1% increase in EvV dose, there was an 11% decrease in *P. interpunctella* larval mass.

3.6. Action Mechanism of EvV. *3.6.1. In Vivo Digestibility and Binding of EvV to PM of P. interpunctella Larvae.* The susceptibility of EvV to the action of digestive enzymes present in the gut of *P. interpunctella* larvae was assayed *in vivo* by adding vicilin to the larval diet; larval excreta and EvV-binding to PM were also analyzed. SDS−PAGE analysis of *P. interpunctella* larval excreta showed that EvV was susceptible to hydrolysis by digestive enzymes, where various protein fragments were still immunoreactive to IgG Anti-EvV (**Figure 6A**). Peritrophic membranes of larvae fed a diet containing EvV were excised; this was followed by extensive washing with PBS and treatment with 0.2 M glycine-HCl buffer to release adsorbed proteins. After this process, one immunoreactive protein fragment, obtained from vicilin hydrolysis, was eluted from PM larvae and visualized by Western Blotting (**Figure 6B**).

3.6.2. Fluorescent Location of the EvV-FITC Bond. The binding of EvV-FITC to the peritrophic membrane is shown in **Figure 7B**. This interaction could be occurring with the chitin component of the membrane. Control experiments were performed with EvV-FITC in the presence of 2.5% (w/v) *N*-acetyl glycosamine solution (**Figure 7C**). The lack of fluorescence in these controls demonstrated the specificity of EvV-binding to PM.

DISCUSSION

To effectively establish a novel insect control strategy based on biocompounds (enzyme inhibitors, lectins, chitinases, chitin binding proteins, and secondary metabolites) followed or not by transgenic plant methods, two initial steps are necessary: first, knowledge of the molecule targets present in the insect digestive or metabolite systems; and second, *in vitro* followed by *in vivo* assays using purified biocompounds. In this study, the biochemistry and physiology of the protein digestion of the Lepidoptera *P. interpunctella*, a phytophagous pest, was characterized first by detecting the class of proteolytic enzymes during larval development and second by the presence of binder molecules present in the structures of its digestive system. The knowledge gained about digestive enzyme classes was used to evaluate the response of this insect to proteinaceous inhibitors added to artificial diets, using serine proteinase classes as digestive enzyme targets. In addition, chitin (*N*-acetyl-glycosamine polymers), an important component of the larval peritrophic membrane, was evaluated as a target for chitin-binding proteins.

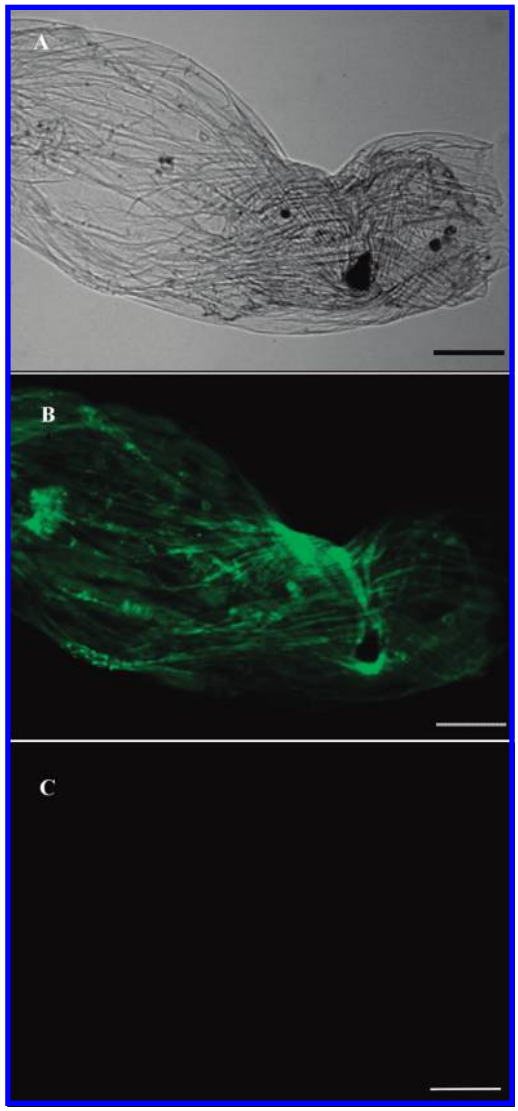

Figure 7. Fluorescence micrographs showing the location of FITC-labeled EvV in the peritrophic membrane (PM) of *P. interpunctella* larvae. (**A**) PM of larvae fed with EvV-FITC visualized by light microscopy. (**B**) PM of larvae fed with EvV-FITC. (**C**) PM of larvae fed a diet containing EvV-FITC and *N*-acetyl glycosamine.

Proteolytic enzymes are classified mainly as serine, cysteine, acid, and metalloproteinase, using specific inhibitors. Acid and cysteine proteinases are predominantly detected in Coleopterans of the Bruchidae family (*23*−*26*), but serine proteinases are also present (*27*). In general, serine proteinases are the major enzymes detected in the guts of the orders Lepidoptera, Hemiptera, Homoptera, and Diptera, but acid proteinases have also been isolated (*11*). To verify which enzyme classes were present in the guts of *P. interpunctella* larvae, azocasein and hemoglobin were used as substrates. Results showed that *P. interpunctella* larvae depend on complex proteolytic systems for protein digestion, with a small contribution from aspartic and cysteine digestive proteinase activities, whereas basic proteinase activities, especially serine proteinase, contribute with the major gut endoproteinases. During larval development, maximum specific azocaseinase activity at pH 9.5 was detected in the third larval instar, and absolute activity was displaced to the sixth larval instar. Zymograms of both homogenate larval instars showed the presence of two major trypsin-like proteinases with accentuated activity in the azocasein. Similar patterns of digestive proteinase activities were also reported throughout the larval development of the other Lepidoptera, such as *Manduca sexta* (L.) (*28*), *Heliothis zea* (Boddie) (*29*), *Spodoptera litura* (Boisd.) (*30*), tobacco budworm (*Heliothis virenscens*, Fab.) (*31*), and the western spruce budworm (*Choristoneura occidentalis*) (Valaitis, et al., 1999); as well as in the dipteran *Lucilia cuprina* (Wied.) (*32*)), and in coleopterans (*33*−*37*) such as the cotton boll weevil (*Anthonomus grandis*) (*38*). The use of serine proteinaceous inhibitors as candidates in insect control strategies has been suggested because insect digestive proteinases are promising targets in pest control.

When SBTI, a specific serine proteinaceous inhibitor, was assayed *in vitro* against *P. interpunctella* enzymes, an inhibition of 96% was detected, indicating the usefulness of this inhibitor in control strategies. However, when SBTI was incorporated into artificial diets and offered to *P. interpunctella* larvae, no effect was observed, in contrast with the results obtained *in vitro.*

This likely occurred because insect pests adapt to host plant proteinase inhibitors by synthesizing proteinases that are either insensitive to inhibitors (*39, 40*) or have the capacity to degrade them (*41, 42*). A zymogram using SBTI showed interactions between enzyme and inhibitor, with complex retardation during electrophoresis. This fact likely explains the instability of the enzyme−SBTI complex or the degradation of SBTI in the larval gut, promoting no SBTI activity *in vivo*.

Serine proteinases belong to multigene families that may have evolved to provide a more efficient mechanism for protein digestion as well as an adaptive advantage for phytophagous species feeding on plants or artificial diets that contain proteinase inhibitors (*43*). This has been proven by the presence of large amounts of inhibitors, including SBTI (*44*), in the diets of pests, which has obliged insects to adapt and produce proteinases that are activated by these serine proteinase genes and which are insensitive to the action of host plant inhibitors (*45*). As a result, pest control using proteinase inhibitors requires the isolation of inhibitors that are also active toward these insensitive proteinases (*46*). In this *P. interpunctella* model, the adaptive phenotypic plasticity of this insect likely provides increased production of insensitive proteinases due to inhibitor ingestion (*47*). This may be explained by the digestive system of *P. interpunctella* larvae, which is based on serine proteinases, with predominance of *in vivo* insensitive trypsin-like enzymes.

EvV was purified through a chitin matrix, and its effect tested against *P. interpunctella* larvae. EvV is a glycoprotein with a molecular mass of 216 kDa and two subunitis of 54.8 kDa and 50.8 kDa, in agreement with data reported by several authors for other legume vicilins (*48, 49*). The first study showing that chitin-binding vicilins could be involved in plant defense was reported by Sales et al. (*50*). These authors demonstrated that variant vicilins, isolated from resistant *C. maculatus* Nigerian cowpea seeds (*Vigna unguiculata*), strongly bound to the chitin matrix and had a significant effect on the bruchid (*48*). A later study showed that vicilins isolated from other legume seeds of distantly related species such as jack beans (*Canavalia ensiformis*), soybeans (*Glycine max*), and lima beans (*Phaseolus lunatus*) showed a highly detrimental effect on the larval development of *C. maculatus* (*49*). Bioassays showed that EvV, like other chitin-binding vicilins, had lethal and/or detrimental effects on organisms that contain chitin in the peritrophic membrane. EvV was lethal to *P. interpunctella* with ED_{50} (0.27%) and LD_{50} (0.23%), values lower than those observed in Nigerian resistant *V. unguiculata* vicilin (LD_{50} of 2.0% and ED_{50} of 1.07%) (*48*) and among other legume vicilins isolated from *Glycine max* (ED_{50} of 1.66%), *Phaseolus lunatus* (ED_{50} of 1.74%), and *Canavalia ensiformis* (ED_{50} of 2.15%) for *C.*

maculatus. The lethal EvV dose was comparable to the concentrations (0.1−1.0%) of WGA, rice lectins, nettle lectins, TEL (*Talisia esculenta* lectin), and *Enterolobium* vicilin tested for *C. maculatus* (*51*−*53*), which are not target hosts of this bruchid. The EvV action mechanism is probably associated with the release of one protein fragment from the digestion of EvV by digestive enzymes of *P. interpunctella* larvae and to the ability of this protein fragment to bind to the PM of this lepidopteran, as proven here by the immunoblotting and fluorescent binding of EvV-FITC. A similar pattern was found in the action of *Bacillus thuringiensis*, where the action of host digestive enzymes releases one protein fragment with insecticide action (*54*). These effects on the gut of *P. interpunctella* suggest that the binding of vicilins or fragments released by larvae enzyme digestion to the PM of larvae either causes interference with nutrient absorption (*55*), prevents or enhances movement between the endo- and exoperitrophic space, or prevents the formation of the membrane itself (*56*). The binding to epithelium cells could affect various functions of the cell membranes (*57*).

The strong negative effects of EvV on *P. interpunctella* larvae observed in this study suggest that EvV might be able to provide a viable alternative in designing insect-resistant transgenic crops or be used as a natural pesticide.

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