Toxicity of Hydrolyzed Vicilins toward *Callosobruchus maculatus* and Phytopathogenic Fungi

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Studies have shown that vicilins (7S storage proteins) from seeds were able to bind to the surface of the *Callosobruchus maculatus* larval midgut and to the peritrophic matrices of the midguts of *Diatraea saccharalis* and *Tenebrio molitor*, inhibiting larval development. Vicilins were also shown to inhibit yeast growth and bind to yeast cells through the association with chitin-containing structures. The present work studies the association of peptides from vicilins of genotypes of *Vigna unguiculata* (susceptible and resistant to bruchid) with acetylated chitin and the toxicity of vicilin fragments and chitin-binding vicilin fragments to *C. maculatus* and phytopathogenic fungi. Hydrolysis of vicilins with α-chymotrypsin results in a complex mixture of fragments that were separated by chitin-affinity chromatography. Chitin-binding peptides from both genotypes were toxic to *C. maculatus* larvae, and α-chymotrypsin-hydrolyzed vicilins were deleterious to the above insect and to *Fusarium oxysporum*, *Colletotrichum musae*, and *Saccharomyces cerevisiae* fungi.

KEYWORDS: Vicilins; chitin; *Vigna unguiculata*; *Callosobruchus maculatus*; chitin-binding proteins

INTRODUCTION

As the structure in which a plant embryo is dispersed, seeds are responsible for the initial development of a new plant and enable the embryo to survive the period between seed maturation and seedling establishment. Thus, the main metabolic changes occurring prior to and during germination include catabolic and anabolic reactions involving primary reserve metabolites. However, in addition to the first-order metabolic reactions, seeds also contain compounds that protect them by contributing to their defense against pathogens and predators (*1*).

Vicilins are 7S storage globulins that aggregate to form trimers with subunits of various molecular masses (45–53 kDa) and are mobilized during the germination of legume seeds (*2*). Studies have shown that vicilin-type storage proteins isolated from a variety of legumes including cowpea (*Vigna unguiculata* line IT81D-1045, a bruchid-resistant genotype), adzuki bean (*Vigna angularis*), common bean (*Phaseolus vulgaris*), lima bean (*Phaseolus lunatus*), soybean (*Glycine max*), and jack bean (*Canavalia ensiformis*) interfere with the development of the cowpea weevil (*Callosobruchus maculatus*) larvae (*3*, *4*). These proteins also interfere with the germination of spores or conidia of phytopathogenic fungi and inhibit both yeast growth and glucose-stimulated acidification of the medium by yeast cells (*5*, *6*).

Vicilins bind strongly to chitin [poly(β-1,4-*N*-acetyl-D-glucosamine)] (*4*, *7*), bind in vivo to the surface of the *C. maculatus* midgut (*8*), and bind in vitro to the peritrophic membrane of the midgut of *Diatraea saccharalis* larvae (*9*). Due to its specific binding to chitinous cell wall structures, vicilin strongly inhibits the growth of yeast cells, which was confirmed by demonstrating that yeast cell growth inhibition was reversed by incubating vicilin with *N*-acetyl-D-glucosamine, *N,N,N*-triacetylchitotriose, and other chitin-related oligosaccharides (*6*).

Chitin is a polysaccharide naturally found in the cell wall of fungi, in the exoskeleton of invertebrate animals, and in the lining the midgut epithelia of insects and other invertebrates, forming peritrophic matrices (PM) . The peritrophic matrices protect the absorptive epithelium from food particle abrasion and microorganism attack and facilitate recycling of digestive enzymes, among other functions (*10–12*). Therefore, chitin-binding proteins may interfere with the normal physiology of chitin-containing structures such as the peritrophic matrices by altering their architecture and permeability.

Vicilins from resistant cultivars show a stronger binding affinity for chitin of the *C. maculatus* larval midgut than vicilins

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Article

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from susceptible cultivars (*8*), and, despite their very high degree of sequence homology, Sales et al. (*13*) reported that vicilins from resistant cowpea seeds were also more refractory to digestion than vicilins from susceptible cowpea seeds. The latter authors suggested the antimetabolic effects of resistant or variant cowpea vicilins could be related to the low digestion rates through their association with chitin-containing structures of the insect's midgut, resulting in the impairment of nutrient uptake by midgut cells and a lower rate of development of *C. maculatus* larvae in seeds of *V. unguiculata* IT81D-1045.

The objective of this work was to investigate if hydrolyzed vicilin peptides from cowpea cv. EPACE-10 and IT81D-1045 were toxic to *C. maculatus* larvae, the fungi *Fusarium oxysporum* and *Colletotrichum musae*, and the yeast *Saccharomyces cerevisiae.*

MATERIALS AND METHODS

Seeds. Cowpea (*V. unguiculata* L.) seeds of the *C. maculatus*-susceptible cultivar EPACE-10 were supplied by the Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza, Brazil. *C. maculatus*-resistant cowpea seeds of the Nigerian line IT81D-1045 were obtained from IITA (International Institute of Tropical Agriculture, Ibadan, Nigeria) through Centro Nacional de Pesquisa do Arroz e Feijão CNPAF-EMBRAPA, Goiânia, Brazil.

Insect. *C. maculatus* (Coleoptera: Bruchidae) was obtained from a colony maintained in the Laboratório de Química e Função de Proteínas e Peptídeos from the Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil. The insects were reared on cowpea *V. unguiculata* (L.) Walp cv. EPACE-10 seeds at 25 °C, 60−80% relative humidity, and a photoperiod of 12/12 h light/dark.

Fungi. The fungi studied were *F. oxysporum* and *C. musae*, which were kindly supplied by CNPAF-EMBRAPA, Goiânia, Goiás, Brazil. *F. oxysporum* is an important phytopathogenic fungi and causal agent of diseases in several species of plants, including the severe *Fusarium* wilt (in cowpeas). *Colletotrichum* species have been considered to be excellent models for studies on pathology and fungal−plant interactions (*14*, *15*). Both fungi were maintained on Agar Sabouraud (1% peptone, 2% glucose, and 1.7% agar−agar). *S. cerevisiae* (access no. 1038), which is an excellent unicellular prokaryote model (easy cultivation and growth) for the study of the mechanisms of action of defensive proteins, was supplied by the Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil.

Vicilin Isolation and Hydrolysis. Vicilins were purified from cowpea seeds (cv. EPACE 10 and IT81D 1045) according to the described procedure, based on that of Macedo et al. (*3*). Ground meals were extracted by soaking in 0.05 M phosphate buffer, pH 7.6 (1:10 meal to buffer), for 30 min at room temperature, and centrifuged for 30 min at 8000*g* at 5 °C, and soluble proteins were fractionated by ammonium sulfate precipitation. The 70−90% saturation fraction was dialyzed against water, freeze-dried, and separated on a DEAE-Sepharose chromatography column (14 × 2.5 cm), equilibrated and eluted with 0.05 M Tris-HCl buffer, pH 8.0. After elution of the nonretained fraction, adsorbed proteins were desorbed by a gradient of 0−1 M NaCl. The vicilin fractions were dialyzed against water and freeze-dried.

Vicilin fractions (70 mg) from each cowpea genotype (EPACE 10 and IT81D 1045) were denatured with 1 mL of 8 M urea, pH 5.4, for 1 h at 45 °C. Denatured proteins were diluted with 0.1 M ammonium bicarbonate containing 0.1 M calcium chloride until the urea concentration reached 2 M. Then, an α-chymotrypsin solution (10 mg/mL) in 0.1 M HCl was added (*16*), and after 10 h of incubation at 37 °C, the hydrolysis was interrupted by freeze-drying the mixture. The resulting vicilin peptides were desalted using a Sephadex G-10 column (0.6 × 15 cm), equilibrated, and eluted with 0.2 M acetic acid. The hydrolyzed peptides (HP) were recovered and freeze-dried.

Gel Electrophoresis and Western Blotting. Native EPACE 10 and IT81D-1045 vicilins or HPs were analyzed by SDS−Tricine−gel electrophoresis performed according to the method of Schagger and Von Jagow (*17*). Samples of native vicilins or HPs (10 μg of proteins) were dissolved in 0.05 M Tris, 12% glycerol, and 0.01% bromophenol blue. The

gel was stained with 0.05% Coomassie blue and destained in acetic acid (10%).

After electrophoresis, separated vicilins were blotted onto a nitrocellulose membrane and subjected to Western blotting (*18*) using a 1:2000 dilution of an anti-*V. unguiculata* cv. EPACE 10 vicilin antibody produced in rabbit as the primary antibody and a 1:2000 dilution of a peroxidase-conjugated goat anti-rabbit IgG (Sigma) as the secondary antibody. Immune reactions were developed for 10 min using a tablet (Sigma) of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT) dissolved in 10 mL of distilled water. Antiserum against purified EPACE 10 vicilin was prepared according the method of Macedo et al. (*2*) by immunization of a white New Zealand rabbit, and preimmune serum was collected before immunization.

Protein Concentration Determination. The protein content of samples was determined according to the procedure of Bradford (*19*).

Acetylated Chitin Preparation. Chitin (5 g) was treated with 10% acetic acid (100 mL) under agitation for 24 h at room temperature. Then, ethanol (90 mL) was added, and the mixture was filtered. To the precipitate was added acetic anhydride (6 mL) under agitation, and the mixture was centrifuged for 30 min at 8000*g* (*20*). The obtained precipitate was dialyzed against water for 72 h, freeze-dried, and used as a matrix to prepare the acetylated chitin column for affinity chromatography.

Chitin Affinity Chromatography. Acetylated chitin (100 mg) was suspended in 50 mM sodium phosphate, pH 7.6 (2.5 mL), packed in a glass column, and washed with 10 mL of the same buffer. EPACE 10 or IT81D 1045 vicilin HPs (20 mg) were dissolved in 1 mL of 50 mM sodium phosphate, pH 7.6, and incubated on the acetylated chitin column for 30 min. After incubation, the column was eluted, and the nonretained vicilin fragments (NRF) were pooled. Adsorbed peptide fractions were desorbed from the chitin matrix with 100 mM *N*-acetylglucosamine, pH 7.6 (GlcNac), and designated AP1; the AP2 fraction was desorbed by 100 mM glycine, pH 3.0, and the AP3 fraction by 100 mM HCl. Each adsorbed fraction was dialyzed against water and freeze-dried, and its toxicity was tested against *C. maculatus.*

Insect Feeding Trials. To evaluate the potential deleterious effects of the hydrolyzed vicilin fragments on *C. maculatus* larval development, we employed an "artificial" seed system described by Macedo et al. (*3*). Artificial seeds (400 mg) were made of finely ground decorticated cowpea seed meal from the susceptible cultivar EPACE-10 pressed into a cylindrical brass mold with the help of a hand press. The vicilin fragments from EPACE 10 and IT81D 1045 seeds [α-chymotrypsin-hydrolyzed vicilin (HP fraction) and chitin-binding vicilin fragments (AP1, AP2, and AP3)] were incorporated into artificial seeds at a concentration of 2%, thoroughly mixed, and pressed as described above. Control artificial seeds consisted entirely of EPACE 10 meal (400 mg) and of EPACE 10 meal plus 0.04% α-chymotrypsin.

The seeds were infested with fertilized 3-day-old *C. maculatus* females, three insects per seed, in glass vials containing two seeds at 28 °C and 70% relative humidity. After 24 h, the excess eggs were removed, leaving three eggs per seed. After 20 days, the infested seeds were opened, and the weights of the larvae were measured and compared to those of control seeds. Experiments were run in triplicate, and the data show the average values.

Preparation of Fungal Inocula and Yeast Cell Culture. The fungal cultures were grown for 12 days on Petri dishes containing Agar Sabouraud. Fresh conidia suspensions were prepared by rinsing the surface of 12-day-old sporulated cultures with 0.15 M NaCl solution (10 mL). Conidia were quantified in a Neubauer chamber for appropriate dilutions.

S. cerevisae inocula were grown on Petri dishes containing Agar Sabouraud at 30 °C for 3 days. Plates were then rinsed with sterile 0.15 M NaCl solution (10 mL) and quantified in a Neubauer chamber to calculate the appropriate dilutions.

Antifungal Activity. To evaluate the toxic effect of HPs from EPACE 10 and IT81D 1045 vicilins, cells (20000/mL) in Sabouraud broth were incubated at 25 °C in 1.5 mL spectrophotometer plastic cuvettes with 1.0 mL of HP solution (0.8 mg/mL) in 0.1 M phosphate buffer, pH 7.6. Fungal growth was monitored by optical density at 660 nm from 0 to 62 h. A negative control containing bovine serum albumin at the same concentration was used, and a general control without the addition of protein was also performed. All experiments were run in triplicate.
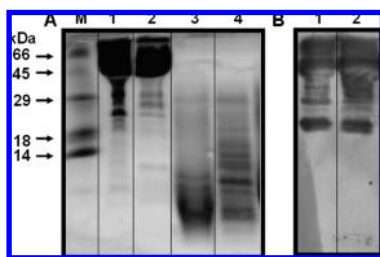
Figure 1. (**A**) SDS−Tricine−gel electrophoresis of proteins isolated by DEAE-Sepharose (native) and hydrolyzed vicilins. (**B**) Western blotting of native vicilins from *Vigna unguiculata* seeds. Lanes: M, molecular mass marker; 1, EPACE 10 native vicilin; 2, IT81D 1045 native vicilin; 3, EPACE 10 hydrolyzed vicilin; 4, IT81D 1045 hydrolyzed vicilin.
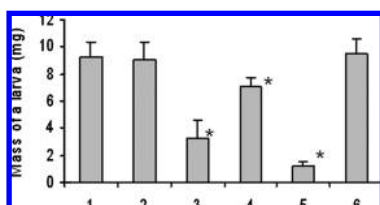

Figure 2. Toxicity of native and hydrolyzed vicilins to *Callosobruchus maculatus* larval development: 1, control; 2, EPACE 10 native vicilin (2%); 3, IT81D-1045 native vicilin (2%); 4, EPACE 10 hydrolyzed vicilin (2%); 5, IT81D-1045 hydrolyzed vicilin (2%); 6, α-chymotrypsin (0.04%). Experiments were run in triplicate, and the data shown are the average of these results. An asterisk (∗) indicates results statistically different from the control ($p < 0.05$ by Student's *t* test).

After a 62 h growth period, cells were separated from the growth medium by centrifugation for 2 min at 12000*g*, washed in 0.1 M Tris-HCl, pH 8.0, and plated for observation in an optical microscope at 1000× magnification.

After 62 h of incubation, fungal cultures were also evaluated for vicilin fragments bound to fungal chitinous structures. Cells were centrifuged for 2 min at 12000*g* and washed in 0.1 M Tris-HCl, pH 8.0. Pellets were then washed with 1 mL of 100 mM HCl and centrifuged for 5 min at 12000*g*, and the proteins in the supernatant were analyzed by gel electrophoresis and Western blotting.

Statistical Methods. Insect feeding trials and antifungal activity experiments were run in triplicate, and the values obtained were used to calculate averages and standard deviations. The results were analyzed using Student's *t* test, and significant differences were defined as $p < 0.05$ (*21*).

RESULTS

Vicilin Isolation and Hydrolysis Profile. Vicilins were isolated according to the protocol of Macedo et al. (*3*) by DEAE-Sepharose chromatography. A protein-rich adsorbed fraction that was desorbed with a NaCl gradient (data not shown) was visualized by SDS-PAGE and had a majority of the proteins that reacted with an anti-EPACE 10 vicilin antibody (**Figure 1**, lanes 1 and 2).

The hydrolysis of these vicilins with α-chymotrypsin creates fragments with varied molecular masses, mainly below 18 kDa (**Figure 1A**, lanes 3 and 4).

Toxicity of Native and Hydrolyzed Vicilin to *Callososbruchus maculatus*. Toxicity experiments with *C. maculatus* showed that EPACE 10 native vicilin and 0.04% α-chymotrypsin did not affect larval development significantly, whereas EPACE 10 hydrolyzed vicilin caused a 23% decrease of the larval mass (**Figure 2**). Native and hydrolyzed vicilin from the IT81D 1045 genotype were very toxic to larvae, reducing the larval masses 64.4 and 86%, respectively (**Figure 2**, bars 3 and 5).
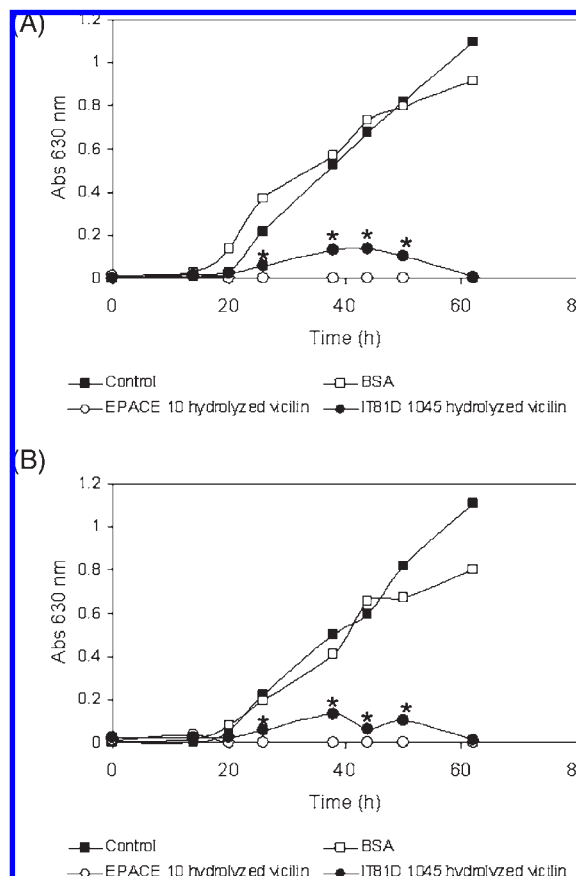

Figure 3. Effects of hydrolyzed vicilins from *Vigna unguiculata* EPACE 10 and IT81D 1045 genotypes on the growth of *Fusarium oxysporum* (**A**) and *Colletotrichum musae* (**B**). Experiments were run in triplicate, and the data shown are the average of these results. An asterisk (∗) indicates results statistically different from the controls ($p < 0.05$ by Student's *t* test).
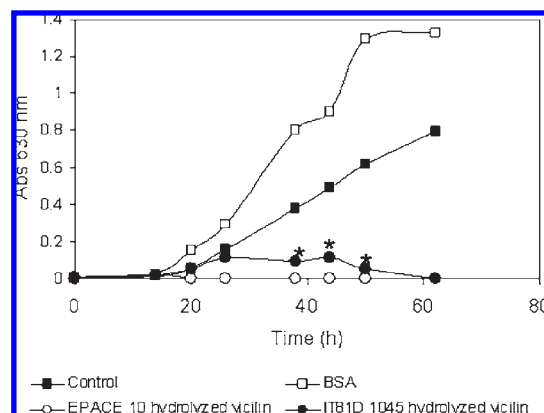

Figure 4. Effects of hydrolyzed vicilins from *Vigna unguiculata* EPACE 10 and IT81D 1045 genotypes on the growth of *Saccharomyces cerevisiae*. Experiments were run in triplicate, and the data shown are the average of these results. An asterisk (∗) indicates results statistically different from the controls ($p < 0.05$ by Student's *t* test).

Toxicity of Hydrolyzed Vicilin toward Fungi. Assays for fungal growth inhibition showed that HPs from vicilins of both genotypes inhibited the growth of *F. oxysporum*, *C. musae*, and *S. cerevisiae*. After 26 h of incubation, these vicilin fragments inhibited growth by 84% in *F. oxysporum* (**Figure 3A**), by 73% in *C. musae* (**Figure 3B**), and by 61% in *S. cerevisiae* (**Figure 4**). Both EPACE 10 and IT81D-1045 HPs were 100% toxic to fungal growth at 62 h.
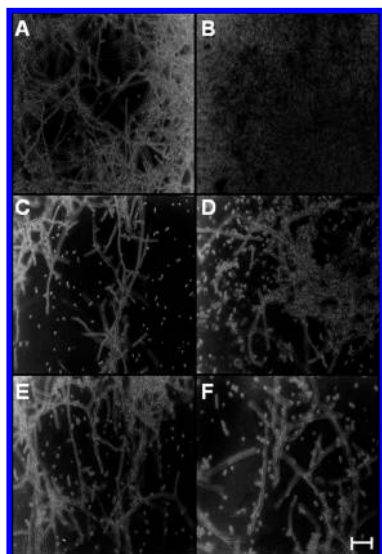
Article

J. Agric. Food Chem., Vol. 57, No. 17, 2009   **8059**

Figure 5. Light micrographs of *Fusarium oxysporum* fungi after 62 h of growth in the presence of control medium (**A**), BSA (**B**), EPACE-10 native vicilin (**C**), IT81D-1045 native vicilin (**D**), EPACE-10 hydrolyzed vicilin (**E**), and IT81D-1045 hydrolyzed vicilin (**F**). All samples are at a concentration of 60 μg mL^{-1}. Bar = 10 μm.
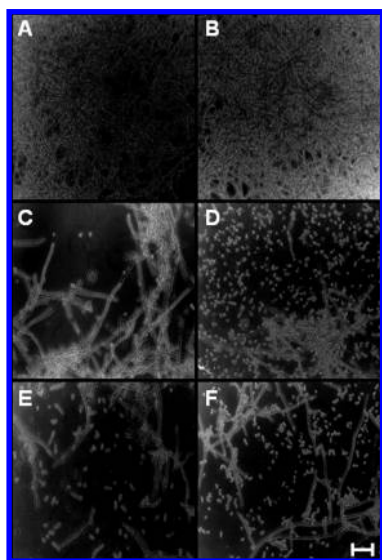

Figure 6. Light micrographs of *Colletotrichum musae* fungi after 62 h of growth in the presence of control medium (**A**), BSA (**B**), EPACE-10 native vicilin (**C**), IT81D-1045 native vicilin (**D**), EPACE-10 hydrolyzed vicilins (**E**), and IT81D-1045 hydrolyzed vicilin (**F**). All samples are at a concentration of 60 μg mL^{-1}. Bar = 10 μm.

Figure 7. (**A**) Affinity chromatography in an acetylated chitin column of EPACE 10 and IT81D 1045 hydrolyzed vicilins. NRF, nonretained fragments; AP1, adsorbed fragments desorbed by 100 mM GlcNAc; AP2, adsorbed fragments desorbed by 100 mM glycine, pH 3.0; AP3, adsorbed fragments desorbed by 100 mM HCl. (**B**) SDS—Tricine—gel electrophoresis of the AP1 fraction from both genotypes EPACE 10 (line 2) and IT81D 1045 (line 3). (**C**) Toxicity of the NRF and AP1 fractions on *Callosobruchus maculatus* larval development: a, control; b, EPACE 10 NRF (2%); c, IT81D 1045 NRF (2%); d, EPACE 10 AP1 fraction (2%); e, IT81D 1045 AP1 fraction (2%). An asterisk ($*$) indicates results statistically different from the control ($p < 0.05$ by Student's *t* tests).

We searched for possible alterations of *F. oxysporum* (**Figure 5**) and *C. musae* (**Figure 6**) morphology caused by vicilin HPs. Photomicrographs of the mycelia of the fungi were taken after 62 h of growth, and normal hyphal development was observed for both fungi in control cells (**Figures 5A** and **6A**) and in cells treated with BSA (**Figures 5B** and **6B**). However, cultures treated with native vicilins and hydrolyzed vicilins of both genotypes showed a notable inhibition of conidial germination and hyphal elongation as well as many morphological alterations of hyphae (differences in thickness) as compared with the control (**Figures 5** and **6**). The extraction of proteins bound to *F. oxysporum*, *C. musae*, and *S. cer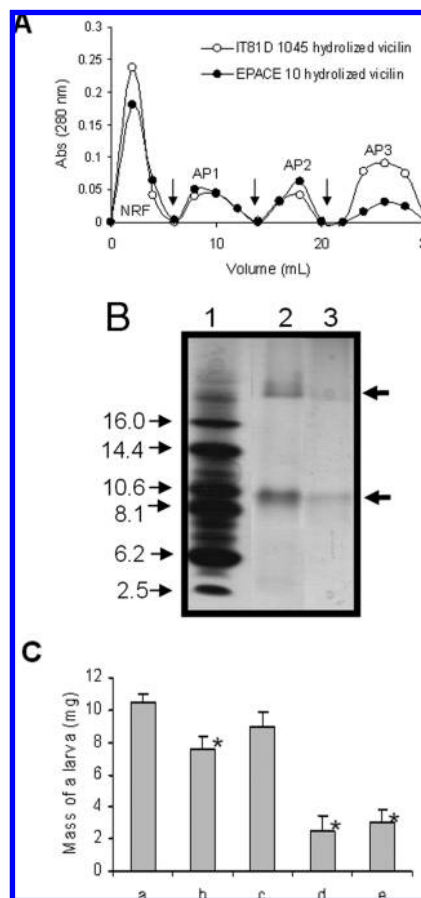evisiae* cell walls showed the presence of protein fragments that reacted with an anti-EPACE 10 vicilin antibody (data not shown).

Isolation of Chitin-Binding Vicilin Peptides and Toxicity to *C. maculatus*. Chitin-binding vicilin fragments were obtained using affinity chromatography on an acetylated chitin column. The chromatographic results showed the presence of three adsorbed fractions: AP1 desorbed by 100 mM *N*-acetyl-ᴅ-glucosamine (GlcNAc), AP2 desorbed by 100 mM glycine, pH 3.0, and AP3 desorbed by 100 mM HCl from both cowpea genotypes EPACE 10 and IT81D 1045 (**Figure 7A**). The AP1 fragments were analyzed by SDS—Tricine—gel electrophoresis, and the results showed a similar profile in the AP1 fractions from both the EPACE 10 and IT81D 1045 genotypes (**Figure 7B**). In both, the presence of two peptides was observed, one above 16 kDa and the other approximately 10 kDa (**Figure 7B**).

The chitin nonretained fractions (NRF) and AP1 fractions from both the EPACE 10 and IT81D 1045 genotypes were evaluated for effects on *C. maculatus* larval development. The results showed that AP1 fractions from the EPACE 10 and IT81D-1045 genotypes were toxic to *C. maculatus* (**Figure 7C**), inhibiting 75 and 71.6% of the larval growth compared with the

control, respectively (**Figure 7C**, bars d and e). The EPACE 10 NRF also decreased the larval mass, although to a much smaller extent than the AP1 fractions. Our results also showed that the AP2 and AP3 fractions consisted of fragments that bound even more strongly to chitin. However, these fractions were not tested against the insects because previous work showed that the acid solutions used to desorb chitin-bound proteins liberate chitin fragments that interfere with *C. maculatus* larvae development (*22*).

DISCUSSION

Previous work showed that IT81D-1045 native vicilins were toxic to *C. maculatus* at 2%, whereas EPACE-10 vicilins did not significantly affect insect larval development (*2, 3*). Both vicilins were also shown to be toxic to fungi (*5,6*). Further studies indicated that the toxicity of vicilin to fungi and insects was possibly due to its binding to chitin-containing structures present in the insect midgut (*4,7,9*) and in the fungal cell wall (*6*). In the present work, we studied the association of peptides from vicilin genotypes (susceptible and resistant to bruchid attack) of *V. unguiculata* with acetylated chitin and the toxicity of α-chymotrypsin-generated vicilin fragments and chitin-binding vicilin fragments to *C. maculatus* and phytopathogenic fungi.

Native and hydrolyzed vicilins from IT81D-1045 were very toxic to *C. maculatus* larvae, whereas the native EPACE 10 vicilin did not affect larval development and its derivative HP fraction mildly inhibited larval development. Meanwhile, chitin-binding vicilin fragments from both genotypes were toxic to *C. maculatus*. These results showed that although native EPACE 10 vicilins are not toxic to *C. maculatus*, the chitin-binding isolated fragments were highly deleterious to larval development, reinforcing the relationship between toxicity and chitin-binding properties suggested previously (*4−6,8*). Sales et al. (*8*) reported the presence of chitin in midgut structures of *C. maculatus* larvae and showed that vicilins from the IT81D-1045 and EPACE 10 *V. unguiculata* genotypes could bind to these structures, although EPACE 10 vicilins were less prone to do so than vicilins from the IT81D-1045 genotype.

The insect PM functions as a permeability barrier between the food bolus and the midgut epithelium, enhancing digestive processes and protecting the brush border from mechanical disruption and from attack by toxins and pathogens (*23*). Due to the importance of chitin, insect growth and fungal morphogenesis are strictly dependent on the ability to remodel chitin-containing structures (*11*).

Paes et al. (*24*) showed that vicilins from *V. unguiculata* seeds were toxic to *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae. These seed-storage proteins were capable of binding to the *T. molitor* larval PM that tested positively for the presence of chitin.

The toxicity of chitin-binding vicilin from *Enterolobium contortisiliquum* seeds was tested in *C. maculatus* and *Zabrotes subfasciatus* larvae, and the results showed that this protein was very effective against both bruchids, producing 50% mortality for *Z. subfasciatus* at 0.43% and for *C. maculatus* at 1.11% concentration (*25*).

Many proteins that bind to chitin are also related to plant defense mechanisms against organisms that contain this polysaccharide as a constituent of their peritrophic matrix. This group includes proteins that are generally toxic to insects and may affect the development of fungi, such as lectins, arcelins, and vicilins. The mechanisms of action of such chitin-binding proteins may differ, and suggestions have been made in the directions of increases on permeability of peritrophic matrices, interference with nutrient absorption, and rupture of gut membranes (*26*).

HPs from EPACE 10 and IT81D 1045 vicilins were toxic to fungi. When *F. oxysporum* and *C. musae* were treated with native vicilin and HP, there was an inhibition of conidial germination and hyphal elongation. The binding of vicilin fragments to *F. oxysporum*, *C. musae*, and *S. cerevisiae* was observed, suggesting that these vicilin fragments are potentially toxic due to their binding to chitin-containing structures of the fungal cell wall.

One Douglas-fir seedling protein (SSP 3279) with decreased levels after infection by *Phellinus sulphurascens* matched a vicilin-like storage protein precursor found in white spruce (*Picea glauca*) (*27*). The authors considered the possibility that the vicilin-like storage protein identified in the Douglas fir−*P. sulphurascens* pathosystem is used and eventually depleted in the production of an AMP in the seedlings. Interestingly, a new family of antimicrobial peptides (AMPs) identified in *Macadamia integrifolia* were seen to be formed by processing vicilin 7S globulin (*28*). The first example purified from nut kernels was a peptide of 45 amino acid residues (MiAMP2c) that inhibited various plant pathogenic fungi, in vitro. cDNA clones corresponding to MiAMP2c encoded a 666 amino acid precursor protein homologous to vicilin 7S globulin proteins. The deduced precursor protein sequence contained a putative hydrophobic N-terminal signal sequence (28 aa), an extremely hydrophilic N-proximal region (212 amino acids), and a C-terminal region of 426 amino acids, which is present in all vicilins (*28*). Ekramoddoullah et al. (*29*) reported an antimicrobial peptide (*Pm*AMP1) in western white pine, and later a homologue of *Pm*AMP1 was seen to be significantly upregulated in Douglas fir roots in response to *P. sulphurascens* infection (*30*).

Peptide fragments that exhibit in vitro antimicrobial activity have also been produced by cleavage from the hydrophilic region near the N-terminus of *Theobroma cacao* vicilin (*31*). Histidine-tagged versions of the putative antimicrobial vicilin-derived peptides from both *M. integrifolia* and *T. cacao* were expressed in *Escherichia coli*, purified, and demonstrated to conserve their in vitro antimicrobial activity (*31*).

Previous work showed that vicilin subunits are present in the HCl extracts of yeast cells pretreated with these proteins (*6*). Chitin-binding vicilin isolated from *E. contortisiliquum* was also toxic toward the phytopathogenic fungi *F. solani* and *C. lindemuntianum* (*25*).

In the future, we will be characterizing some of these defense-related cowpea vicilin-derived peptides, and detailed time course gene expression analysis of protein precursors will be carried out on some candidate genes using real-time PCR.

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Article

J. Agric. Food Chem., Vol. 57, No. 17, 2009   **8061**

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