Activity toward Bruchid Pest of a Kunitz-Type Inhibitor from Seeds of the Algaroba Tree (Prosopis juliflora D.C.)

Adeliana S. Oliveira,* Railene A. Pereira,* Liziane M. Lima,* Ana H. A. Morais,* Francislete R. Melo†‡,§, Octávio L. Franco†§, Carlos Bloch Jr.†‡³, Maria F. Grossi-de-Sá,† and Maurício P. Sales*¹

*Laboratório de Química e Função de Proteínas, Departamento de Bioquímica, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil; †Centro Nacional de Recursos Genéticos e Biotecnologia/EMBRAPA, Brasília, DF, Brazil; ‡Departamento de Biologia Celular and ¶Departamento de Química, Universidade de Brasília, Brasília, DF, Brazil; and §Universidade Católica de Brasília, Brasília, DF, Brazil

Received September 11, 2001; accepted December 6, 2001

A proteinaceous inhibitor with high activity against papain was found in seeds of the xerophytic algaroba tree (Prosopis juliflora). The proteinase inhibitor \( Pj \) was purified using Sephacryl S-200 gel filtration followed by reverse-phase high-performance liquid chromatography on a Vidad 18 TP. Inhibitor \( Pj \) showed a \( M_r \) of 20,000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and a \( M_r \) of 19.2K by mass spectrometry. The inhibition of papain by the \( Pj \) inhibitor was the noncompetitive type, with a \( K_i \) value of 0.59 \( \times \) 10\(^{-9}\) M. The gelatinase activity of papain was strongly inhibited by \( Pj \) too. The N-terminal amino acid sequence of the \( Pj \) inhibitor showed homology with the N-terminal amino acid sequence of the Kunitz-proteinase inhibitor family. \( Pj \) was strongly effective against digestive proteinases from bean weevil Acanthoscelides obtectus and cowpea weevil Callosobruchus maculatus and was moderately active toward midgut proteinases from pod weevil Mimosus mimusae and Mexican bean weevil Zabrottes subfasciatus. The data shown here suggest that the protein present in algaroba seeds is involved with defense responses to insects and may be an important tool to be used in engineering plants resistant to bean weevils.

Key Words: Prosopis juliflora; plant defense; bean weevils; phytocystatin; bruchids.

INTRODUCTION

Bruchids are the most serious pests attacking food legume seeds during storage. Substantial amounts of stored beans, such as common bean (Phaseolus vulgaris) and cowpea (Vigna unguiculata), are lost due to the damage caused by three main storage pests, the bean weevil Acanthoscelides obtectus, Mexican bean weevil Zabrottes subfasciatus, and cowpea weevil Callosobruchus maculatus. These bruchids use mainly cysteine proteinases to degrade proteins (1–3). The presence of cysteine proteinases in these insect guts has implicated enzyme inhibitors as proteins involved with exogenous defense mechanisms (4–7) and, also, various studies have shown cysteine proteinases isolated from guts of insect larvae to be inhibited by both nonproteinaceous and proteinaceous cysteine proteinase inhibitors or cystatins.

Plant proteinase inhibitors are important in a variety of ways, including acting as storage protein (8), as regulators of endogenous proteolytic activity (9), and as participants in mechanisms of programmed plant cell death (10). In addition, proteinase inhibitors are expressed in response to abiotic stress (11) and in plant defense processes against insect attacks (12, 13).

Plant cystatins, called phytocystatin, have been identified and studied in many plants such as rice (14, 15), maize (16), soybean (17), cowpea (18), potato (19), Chinese cabbage (20), and carrot (21). However, very limited efforts have been devoted to discover cystatins from other
plant sources such as the forest tree chestnut (22). Seeds from xerophytic trees, such as algaroba, seem particularly good candidates, because of their high moisture content at shedding and during the long periods that the seeds remain in the soil all through germination. Differences in regulation and mobilization of amino acids from protein storage tissues, during seed germination and plant development, and of plant defense components could be expected in such seeds when compared with those from cultivated species (22). Because of the inhibitory effects against insect exogenous proteinases from Hemipteran (23, 24) and from Coleopteran (25), insects, cystatins could be important in plant defense. Recently, studies with transgenic plants revealed that cystatins could provide effective protection against Coleopteran pests (26, 27).

In this report, we have identified and purified a papain inhibitor, Pj, from seeds of the xerophytic algaroba tree and we also showed a specific activity of this protein against two weevils that feed on stored grains.

MATERIALS AND METHODS

Inhibitor Isolation and Purification

Algaroba (Prosopis juliflora) seeds were obtained from the IBAMA (Environmental Brazilian Institute) seed bank at Natal, RN, Brazil. Finely ground seed meal was extracted with 0.05 M Tris-HCl buffer, pH 7.5, for 30 min at room temperature. After centrifugation for 30 min at 8000g at 4°C, the supernatant (crude extract) was precipitated with ammonium sulfate at a concentration of 0–30, 30–60, and 60–90. These fractions were then dialyzed against H2O and freeze-dried. The F30–60 fraction, which corresponds to a 30–60% saturation range, showed a high level of inhibitory activity against papain. This fraction was applied to a Sephacryl 200-SH size-exclusion column (84 cm × 2.5 cm column), equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. Fractions of 1.5 ml were collected at a flow rate of 30 ml h⁻¹. Three peaks with papain inhibitory activities were obtained (FI1, FI2, and FI3). The FI3 protein fraction was pooled, dialyzed, lyophilized, and then applied (1.0 mg/ml) to a reverse-phase high performance liquid chromatography (HPLC)² column (Vydac C-18 TP 1022), equilibrated with 0.1% trifluoroacetic acid (TFA) solution at flow rate of 1.0 ml min⁻¹. Samples were eluted with a linear gradient of 0–100% acetonitrile and the chromatography was monitored at 216 nm.

Protein Determination

A Total protein content was measured by the procedure of Bradford (28) with bovine serum albumin as protein standard.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted as described by Laemmli (29) at room temperature. Protein molecular weight markers were purchased from Pharmacia. Bromophenol blue was used as tracking dye. The molecular weight protein markers employed were α-lactoglobulin (14.3 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (19 kDa), ovalbumin (43 kDa), bovine serum albumin (68 kDa), and phosphorylase b (97 kDa).

Matrix-Assisted Laser Desorption-Time of Flight MALDI-TOF Analysis

Freeze-dried samples from peaks eluted with the imidazole-containing buffer were desalted and prepared for MALDI-TOF analysis on a Voyager-DE STR Bioworkstation (PerSeptive Biosystems, Framingham, MA). The samples were dissolved in 1.0% trifluoroacetic acid and the matrix sinapinic acid (a saturated solution dissolved in acetonitrile: 0.1% TFA, 1:1, v/v) from Sigma Chemicals Co. (St. Louis, MO) was added. The solution was then vortex mixed and 1 ml was applied onto the Voyager Bioworkstation sample plate. Samples were air-dried at room temperature. The spectrometer, equipped with a

² Abbreviations used: TCA, trichloroacetic acid; EDTA, ethylenediamine tetracetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; MCA, (4-methyl-7-coumaryl) amide; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide.
delayed-extraction system, was operated in linear mode. Sample ions were evaporated by irradiation with a N₂ laser at 337-nm wavelength and accelerated at 23 kV potential in the ion source with a delay of 150 ns. Samples were ionized with 100 to 200 shots of a 3-ns pulse width laser light. The signal was digitized at a rate of 500 MHz and averaged data were presented to a standard Voyager data system for manipulation.

**Preparation of the Proteinases**

*C. maculatus* and *Mimosestes mimosae* were supplied from Laboratório de Química e Função de Proteínas from Departamento de Bioquímica, UFRN, Natal, RN, Brazil. *Z. subfasciatus*, *A. oblectus*, and nematode *Meloidogyne javanica* were obtained from Centro Nacional de Recursos Genéticos e Biotecnologia (CENARGEM/EMBRAPA), Brasília, Distrito Federal, Brazil. Papain was purchased from Sigma Chemical Co.

Proteinases from 18 to 20-day larva weevils were obtained after dissection and extraction of the midguts according to Terra et al. (30). The midguts were surgically removed from the larvae and placed into an iso-osmotic saline (0.15 M NaCl) solution. Midgut tissue was stirred and centrifuged for 10 min at 10,000g at 4°C.

Homogenates of female nematodes were stirred and centrifuged for 10 min at 10,000g at 4°C. The supernatants were then removed and used for proteinase and proteinase inhibitor assays.

**Pj Inhibitory Assay against Proteinase Extracts from Bruchid Larvae and Nematodes**

The inhibitory activities were tested according to Solomon et al. (10), using 10 μM Phe-Arg-MCA (4-methyl-7-coumaryl)amide, 25 mM Tris–HCl, pH 6.5, and 20 mM dimethylsulfoxide (DMSO) solution as substrate. After 30 min, the reaction was stopped with 1.9 ml of 0.2 M Na₂CO₃. Inhibitory activities were measured using a spectrofluorimeter (DyNA Quand 500, Pharmacia-Biotech) with excitation and emission wavelengths of 365 and 460 nm, respectively. White proofs of the fluorescence were subtracted. The protein fractions were tested in one concentration standard of 50 μg ml⁻¹.

**Papain Inhibitory Activity Assays**

The inhibitory activity from protein fractions was analyzed using a 1% azocasein solution as substrate. Aliquots of 30 μl of papain (1 mg ml⁻¹ in 0.1 M phosphate buffer, pH 7.8, w/v) were preincubated with 40 μl of an activation solution containing 0.02 M ethylenediaminetetraacetic acid (EDTA) and 0.05 M cysteine, pH 8.0, and 40 μl 0.05 M Tris–HCl buffer, pH 7.5, for 10 min at 45°C. After this we added 60 μl of protein fractions (1.2 mg protein ml⁻¹ in 0.05 M Tris–HCl buffer, pH 7.5) and 330 μl of activation solution. The preincubation reaction was started by addition of 200 μl of 1% azocasein solution. After 10 min, the reaction was stopped by addition of 300 μl of 10% trichloroacetic acid (TCA) solution. Samples were centrifuged and supernatants were alkalinized with 0.25 N NaOH solution. The soluble peptides were measured by absorbance at 440 nm. One unit of inhibitory activity (UI) was defined as the amount of inhibitor that decreased absorbance by 0.01 at 440 nm.

**Assay of Pj Inhibitory Activity against Serine Proteinases**

Inhibitory activities of the *Pj* inhibitor toward two closely related serine proteinases were tested. Stock solutions of both enzymes were prepared: trypsin (0.3 mg ml⁻¹ in 0.0025 M HCl) and chymotrypsin (1 mg ml⁻¹ in 0.05 M Tris–HCl buffer, pH 7.5). *Pj* (1.2 mg ml⁻¹ in 0.05 M Tris–HCl buffer, pH 7.5) was used as inhibitor solution. The preincubation reaction with trypsin contained 50 μl of enzyme solution, 200 μl of 0.0025 M HCl solution, 50 μl of inhibitor solution, and 260 μl of 0.05 M Tris–HCl buffer, pH 7.5. The preincubation reaction with chymotrypsin contained 7 μl of enzyme solution, 50 μl of inhibitor solution, and 393 μl of 0.05 M Tris–HCl buffer, pH 7.5. These preincubation reactions were done in 15 min at 37°C and were started after addition of 500 μl
1% azocasein for 30 min. After 30 min at 37°C, the reaction was stopped by addition of 150 μl of 10% TCA solution. These samples were centrifuged and supernatants were alkalized with 0.25 N NaOH. The soluble peptides were measured by absorbance at 440 nm. One unit of inhibitory activity was defined as the amount of inhibitor that decreased absorbance by 0.01 at 440 nm.

Errors of Inhibitory Activity from Pj

Determination of the Kᵢ (app) value of the reaction between the Pj inhibitor and papain was conducted similarly to those assays described previously. The velocity rate of this reaction was expressed as 1/v(OD₄₄₀/h/ml) and the Kᵢ value was determined using a double-reciprocal plot of the data (31).

Papain Activity in Polyacrylamide Gel Containing 0.1% Gelatin

In order to determine papain inhibition in a polyacrylamide gel containing gelatin, three reaction mixtures were made: The control mixture contained 2.5 μl (1 μg μl⁻¹) papain, 3.8 μl activation solution (0.02 M EDTA and 0.05 M L-cysteine, pH 8.0), 26 μl of 0.05 M Tris–HCl buffer, pH 7.5. The T1 mixture contained 2.5 μl (1 μg μl⁻¹) papain, 3.8 μl activation solution (0.02 M EDTA and 0.05 M cysteine, pH 8.0). 6 μl of 0.05 M Tris–HCl buffer, pH 7.5, and 20 μl (2 μg μl⁻¹) of FI3. The T2 mixture contained the same amounts as T1 plus 26 μl (2 μg μl⁻¹) of FI3. T1 and T2 were preincubated for 10 min at 45°C. It was then supplemented with 3 μl of sample buffer and submitted to SDS–PAGE containing 0.1% gelatin (32). After being run at 5°C, the gels were washed with 2.5% Triton X-100 solution for 1–2 h to remove SDS and incubated with activation solution (0.001 M EDTA and 0.0025 M L-cysteine pH 8.0) for 60 min at 45°C. Gels were then stained with a Coomassie brilliant blue R-250 solution.

Assay of Inhibitory Activity in SDS–Polyacrylamide Gel

This analysis was made on SDS–PAGE, to which was applied (i) the protein molecular weight markers, (ii) 22 μg of FI3, and (iii) 45 μg of FI3. After being 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 7.5, for 30 min. After being washed, the gel was split in half as a control gel containing (i) and (ii) and a test gel containing (iii). The test gel was preincubated at 45°C for 10 min with 30 ml of 0.05 M Tris–HCl buffer, pH 7.5. To this reaction was then added 3 ml of a mixture containing 1.5 ml papain solution (1 mg papain ml⁻¹ in 0.05 M Tris–HCl, pH 7.5) and 1.5 ml activation solution (0.02 M EDTA and 0.05 M cysteine, pH 8.0). After incubation for 60 min, the reaction was stopped by transferring the test gel to a staining solution (Coomassie brilliant blue R-250) and then it was destained with methanol:acetic acid:water (3:1:6, v/v/v). The control gel was stained and destained in the same manner.

N-Terminal Sequencing

N-terminal amino acid sequence analysis was determined at the Centro Nacional de Recursos Genéticos (Cenargen-Embrapa, Brasília, Brazil) using an automated protein sequencer.

RESULTS

Purification of Algaroba Seed Papain Inhibitor

Crude soluble protein extract obtained from the mature seeds from an algaroba tree was initially precipitated at 30, 60, and 90% saturation with ammonium sulfate, and three protein fractions (F₀₀⁻₃₀, F₃₀⁻₆₀, and F₆₀⁻₉₀) were obtained. The F₃₀⁻₆₀ protein fraction obtained showed a strong inhibitory activity against papain, while the other fractions had weak inhibitory activities. The F₃₀⁻₆₀ was then applied on Sephacryl 200-SH and the peaks were assayed against papain. Three peaks (FI1, FI2, and FI3) showed the presence of an inhibitor group with 30, 43, and 68% papain inhibition, respectively (Fig. 1A). The FI3 fraction was then applied on reverse-phase HPLC (Fig. 1B). The elution of the FI3 fraction revealed several peaks (A to G). These peaks
FIG. 1. (A) Elution profile on Sephacryl 200-SH of cysteine proteinase inhibitor from *P. juliflora* seeds. Approximately 22 mg of protein was applied in the column equilibrated with 0.05 M Tris–HCl buffer, pH 7.5, and fractions were eluted and monitored at 280 nm. Fractions FI1, FI2, and FI3 were tested against papain. (B) Analytical reverse-phase HPLC of the FI3 peak. The separation was carried out on a Vydac 218TP analytical column using a flow rate of 1 ml/min. TFA (0.1%) was used as an ion-pairing agent and the dashed line indicates the acetonitrile gradient. The sample contained 1.0 mg of protein. (C) SDS–PAGE analysis of the G fraction, stained with Coomassie blue.
were assayed against papain and only peak G showed inhibitory activity toward papain. The purification procedure of this papain inhibitor from *P. juliflora* seeds resulted in high purification, 95fold with a 2.9% yield (Table 1).

**Electrophoretic and MALDI-TOF Analysis of Algaroba Seed Papain Inhibitor**

The crude extract and F30--60 fraction, when analyzed by SDS--PAGE, showed bands ranging from 68.0 to 15.0 kDa. In the FI3 protein fraction the presence of one strong band of approximately 20 kDa was also observed (data not shown). Electrophoretic analysis of peak G showed one protein of approximately 20 kDa (Fig. 1C). This G fraction or Pj inhibitor was applied to MALDI-TOF analysis showing a monomeric molecular mass of 19,278 Da and a dimeric molecular mass of 38,573 Da (Fig. 2).

**Kinetics of Inhibition from Algaroba Seed Papain Inhibitor**

In order to determine the inhibition mechanism of inhibitor Pj against papain, the inhibition kinetic data were analyzed by Lineweaver-Burk double reciprocal plots (Fig. 3). The analysis showed a noncompetitive type of inhibition, characterized for no changes of Km value and a decrease of Vmax, when compared to the reaction in the absence of the inhibitor. The $K_i$ value for the Pj inhibitor was determined using a double reciprocal plot of data (31), where the $K_i$ value was found to be $0.59 \times 10^{-9}$ M.

**Analysis of Papain Activity on Polyacrylamide Gel**

Polyacrylamide gel electrophoresis containing 0.1% gelatin was done according to

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total inhibitory activity (mg)</th>
<th>Total protein (mg)</th>
<th>Specific activity (UI/mg protein)</th>
<th>Purification (X)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>88,800.0</td>
<td>1634.40</td>
<td>54.33</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>F30--60</td>
<td>28,200.0</td>
<td>10.0</td>
<td>2820.00</td>
<td>52</td>
<td>31.7</td>
</tr>
<tr>
<td>FI3</td>
<td>12,427.0</td>
<td>3.23</td>
<td>3847.40</td>
<td>71</td>
<td>14.0</td>
</tr>
<tr>
<td>Inhibitor Pj</td>
<td>2,580.9</td>
<td>0.50</td>
<td>5161.72</td>
<td>95</td>
<td>2.9</td>
</tr>
</tbody>
</table>

*Note. One cysteine proteinase inhibitor unit (1 UI) was defined as the inhibitor amount that decreased the absorbance at 440 nm by 0.1 OD under the papain assay conditions.*
showed a high inhibition level against the cysteine proteolytic activity of *A. obtectus* (76.3%) and *C. maculatus* (72.0%). The results also showed moderate inhibitory activity toward digestive proteinases from *M. mimosaes*, with approximately 42% inhibition, and low inhibitory activity against digestive proteolytic activity from *Z. subfasciatus*, with 19.1% inhibition.

**N-Terminal Amino Acid Sequence Analysis**

The alignment of this N-terminal amino acid sequence of the *Pj* protein with other proteinase inhibitors had strong homology with the β-chain of the Kunitz proteinase inhibitor family (Table 3).

Michaud et al. (32) with minor modifications. By this method we visualized the proteolytic activity as a clear zone in the gel against a dark blue background. In Fig. 4A, the papain proteolytic activity was visualized in lane A and lanes B and C were papain treated with FI3, where a reduction of the activity was gradually observed. In another SDS-PAGE assay, FI3 linked to gel was tested against papain activity. The results showed one single band of approximately 20 kDa, probably a remainder from the proteolysis (Fig. 4B), corresponding to the *Pj* inhibitor that was also visualized in the SDS-PAGE and by MALDI-TOF analyses.

**In Vitro Pj Inhibitor Activity toward Several Proteinases**

Purified *Pj* inhibitor was assayed against different proteinases. In addition to the fact that several cystatins possess weak inhibitory activity against serine proteinases, we showed that inhibitor *Pj* also had weak activity toward trypsin and chymotrypsin (Table 2). Digestive proteinases from those bruchids were tested. Among the different proteinases tested, *Pj* inhibitor showed a high inhibition level against the cysteine proteolytic activity of *A. obtectus* (76.3%) and *C. maculatus* (72.0%). The results also showed moderate inhibitory activity toward digestive proteinases from *M. mimosaes*, with approximately 42%, inhibition, and low inhibitory activity against digestive proteolytic activity from *Z. subfasciatus*, with 19.1% inhibition.

**N-Terminal Amino Acid Sequence Analysis**

The alignment of this N-terminal amino acid sequence of the *Pj* protein with other proteinase inhibitors had strong homology with the β-chain of the Kunitz proteinase inhibitor family (Table 3).
KUNITZ-TYPE INHIBITOR AND ITS ACTIVITY TOWARD BRUCHID PEST

TABLE 2

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain</td>
<td>99.0 ± 0.70</td>
</tr>
<tr>
<td>Trypsin</td>
<td>4.2 ± 0.42</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>5.6 ± 1.76</td>
</tr>
<tr>
<td>AoP</td>
<td>76.2 ± 1.90</td>
</tr>
<tr>
<td>CmP</td>
<td>72.0 ± 4.24</td>
</tr>
<tr>
<td>MnP</td>
<td>42.0 ± 1.24</td>
</tr>
<tr>
<td>ZsP</td>
<td>19.1 ± 1.83</td>
</tr>
<tr>
<td>MjP</td>
<td>2.5 ± 0.28</td>
</tr>
</tbody>
</table>

* Assays against papain, trypsin, chymotrypsin, nematode M. javanica (MjP), and bruchid proteinases from A. obtectus (AoP), C. maculatus (CmP), M. mimosae (MnP), and Z. subfasciatus (ZsP).

* Values are means ± standard error.

3). The homology of the Pj inhibitor with Kunitz proteinase inhibitors from Glicine max (33), Albizia julibrissin (34), Acacia confusa (35), Adenanthera pavonina (36), and P. juliflora (37) was 57, 69, 86, 88, and 100%, respectively.

DISCUSSION

Crops can be attacked by a number of disease agents and several pests both in the field and during storage. One of the main pests of the legume seeds is bruchid weevils. The damage caused by these insects can reach up to 70% of the grains produced in a given region if necessary measures are not taken to check the attack (38). Many plants contain proteinaceous inhibitors that inhibit the digestive enzymes of insects. Proteinaceous inhibitors hold great promise for crop protection through biotechnology: their overexpression has been shown to make seeds resistant to those weevils whose digestive enzymes are sensitive to them (25, 26).

In previous studies, we have shown that protein fractions enriched with papain inhibitors from algaroba seeds have detrimental effects on development of the cowpea weevil C. maculatus larvae. The dose–response curve value was 1.7% and caused a reduction in 50% of the average mass of the 20-1 day-1 old larvae (39). In the present study we purified one papain inhibitor Pj from the same seeds. The inhibition of papain activity by Pj was noncompetitive, in opposition to Barrett (40), who suggested that cystatins, in general, are reversible and competitive inhibitors of papain. Other results have also shown that phytocystatins, such as corn cystatin I and oryza-proteinase inhibitors from Glicine max (33), cystatin-I (16), soyacystatins (41), and chestnut Albizzia julibrissin (34), Acacia confusa (35), cystatins (21), inhibit papain noncompetitively. The molecular mass of the Pj inhibitor is consistent with the molecular mass of other cystatins investigated. Phytocystatins demonstrate a range of molecular masses from 10 to 87 kDa such as Enterolobium contortisiliquum ~60 kDa (42); potato tubers, ~87 kDa (19); avocado, ~11 kDa (43); soybean seeds, ~26 kDa (17); rice, ~12 kDa (15); and chestnut, ~11.2 kDa (21).

Various proteinase inhibitors, such as trypsin and/or chymotrypsin inhibitors (44, 45), subtilisin inhibitor (45), cathepsin D inhibitor (46, 47), and papain and/or cathepsin L (48), have been purified from legumes, cereal seeds, and

TABLE 3

<table>
<thead>
<tr>
<th>Species*</th>
<th>N-terminal sequence</th>
<th>Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. juliflora</td>
<td>SDR-CKDLGISIDIEENN-RRL-V</td>
<td>100%</td>
</tr>
<tr>
<td>A. pavonina</td>
<td>LE-CKDLGISIDDDDN-RRLAV</td>
<td>88%</td>
</tr>
<tr>
<td>A. confusa</td>
<td>DDESCKDLGISIDDENN-RRLV</td>
<td>86%</td>
</tr>
<tr>
<td>A. julibrissin</td>
<td>KDDHCYDKL-SIIDDE</td>
<td>69%</td>
</tr>
<tr>
<td>G. max</td>
<td>DDK-CCDLGISIDDDGTTRRLV</td>
<td>57%</td>
</tr>
</tbody>
</table>

* Species.* The alignment was made using the program Fast 3. Totally conserved residues are in boldface type.

* P. juliflora, Accession No. A45588 (38); A. pavonina, Accession No. B24376 (37); A. confusa, Accession No. JH0607 (36); A. julibrissin, Accession No. P24927 (35); and G. max, Accession No. A01310 (34).
tubercles, (33, 49). These proteinaceous inhibitors have molecular masses (20 to 24 kDa) and primary structures similar to those of the β-chain of the soybean Kunitz-trypsin inhibitor family (7, 48) and are grouped in a Kunitz-type proteinase inhibitor superfamily. They play a significant role in defense-mechanisms of plants against insect and phytopathogen attacks (44, 45, 47, 50).

Several studies have shown that insects of the Bruchidae family have cysteine proteinases as their predominant digestive enzymes (9, 51). Among these cysteine proteinase-possessing bruchids are the cowpea weevil C. maculatus (3), the common bean weevil A. obtectus (51, 52), and the Mexican bean weevil Z. subfasciatus (2). By contrast, another bruchid, the pod weevil M. minosae (53; unpublished data), uses mainly serine proteinases as its major digestive enzymes. According to our results, Pj showed strong activity against those bean weevils which use primarily digestive cysteine proteinases, in particular, the common bean weevil A. obtectus and the cowpea weevil C. maculatus. These results indicate that Pj may be an effective bioinsecticide in the protection of bean seeds.

ACKNOWLEDGMENTS

This work was supported by the Brazilian agencies CAPES, PRONEX, and Departamento de Bioquímica da Universidade Federal do Rio Grande do Norte.

REFERENCES


3. C. P. Silva, and J. Xavier-Filho, Comparison between the levels of aspartic and cysteine proteinases of the larval midgut of Callosobruchus maculatus (F.) and Zabrotes subfasciatus (Boh.)(Coleoptera:Bruchidae), Comp. Biochem. Physiol. 99B, 529 (1991).


