Identification of an α-Amylase Inhibitor from *Pterodon pubescens* with Ability To Inhibit Cowpea Weevil Digestive Enzymes

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Cowpea seeds (*Vigna ungiculata*) are widely cultivated by poor farmers in Latin America and Africa and are often severely damaged by the cowpea weevil *Callosobruchus maculatus*. A proteinaceous inhibitor of cowpea weevil digestive enzymes, *PpAI*, was purified from white sucupira seeds (*Pterodon pubescens*) and biochemically characterized in this study. Proteins were extracted from seeds and precipitated with ammonium sulfate at 100% saturation. This fraction was applied onto a Red-sepharose CL-6B column, and the retained peak showed 70% inhibitory activity toward larval *C. maculatus* digestive α-amylases. The retained peak was then purified using an analytical reversed-phase HPLC column. Purified *PpAI* showed 65% inhibitory activity against larval *C. maculatus* enzymes. Enzymatic assays also showed that the purified *P. pubescens* inhibitor was unable to reduce the activity of mammalian α-amylases, suggesting specificity toward insect enzymes. Moreover, artificial seeds containing *PpAI* were able to reduce larval weight by 36% and cause 55% mortality. Mass spectrometry and SDS-PAGE analyses indicated that *PpAI* showed a molecular mass of approximately 5.0 kDa. This α-amylase inhibitor, coming from a native Cerrado plant, could be used to construct a genetically engineered cowpea with enhanced resistance against weevil pests.

KEYWORDS: *Callosobruchus maculatus*; *Pterodon pubescens*; plant defense; α-amylase inhibitor

INTRODUCTION

Insect pests and plant pathogens are responsible for severe worldwide crop losses, causing a reduction of approximately 40% on crop production (*1*). Nutritional compounds found in leguminous seeds, such as starch and proteins, are essential to human nutrition, especially to poor farmers from Latin America and Africa who grow cowpea as a subsistence crop. Unfortunately, the larvae of the bruchid pest *Callosobruchus maculatus* heavily damage cowpea seeds, especially during storage periods. *C. maculatus* and other insect pests synthesize α-amylolytic enzymes (α-1,4-glucanohydrolases; EC 3.2.1.1) in abundance (*2*, *3*, *30*), transforming starch into mono- and disaccharides, which are used as a source of metabolic energy.

To defend themselves from insect predators, seeds are able to produce several antifeedant compounds such as digestive enzyme inhibitors (*4*, *5*). α-Amylase inhibitors (α-AIs) are present in cereals and leguminous plants, and their role in plant resistance has been the object of several studies (*2*, *6*). These proteinaceus compounds prevent insect growth by reducing carbohydrate digestion (*7*). On the basis of their tertiary structure, α-amylase inhibitors have been classified into different families such as lectin-like, knottin-like, cereal-type, Kunitz-like, γ-purothionin-like, and thaumatin-like (*8*). All these inhibitor families are commonly found in seeds from different plant species and display a wide range of mechanisms of action (*8*). Moreover, each family has a different three-dimensional structure and characteristic specificity (*8*). Some well-studied examples of α-amylase inhibitors from bean (*Phaseoulus vulgaris*) are α-AI1 and α-AI2, which belong to the lectin-like family. α-AI1 and α-AI2 are two allelic variants with different α-amylase specificities. α-AI1 inhibits PPA (porcine pancreatic α-amylase), CMA (*C. maculatus* α-amylases), and CCA (*Callosobruchus chiensis* α-amylases) but shows no inhibitory activity against α-amylolytic enzymes from the Mexican bean weevil *Zabrotes subfasciatus* (ZSA). On the other hand, α-AI2 is able to inhibit ZSA but does not inhibit PPA, CMA, and CCA (*3*, *7*, *9*).

Although several α-amylase inhibitors have been isolated from crop plants such as wheat, bean, rye, rice, and maize (*10*, *11*), there are very few studies that were carried out with native

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plants (*8*, *12*). Particularly regarding tropical biomes, the potential of α-amylase inhibitors from native plants has not been thoroughly investigated and explored. One of the few examples in the literature is a study by Kotowaroo et al. (*12*), where traditional antidiabetic medicinal plants of Mauritius were screened for putative α-amylase inhibitory effects in vitro. However, no assay against insect α-amylases was performed in this study. Here, we investigated a novel α-amylase inhibitor from *P. pubescens* (*PpAI*) seeds, a native plant from Cerrado, a Brazilian savannah-like vegetation, which is a stressing environment to many plants. *P. pubscens* is known to be an important source of compounds with antiarthritic (*31*) and antinociceptive activities (*32*). Furthermore, sucupira is also known for its cercaricidal action (*33*). Because of its biological effect, this plant has the potential to be used in the prophylaxis of schistosomosis caused by *Schistosoma mansoni* (*33*). Commonly, plants that are able to grow in this region of Cerrado, characterized by two contrasting seasons, one with high rainfall and another of severely dry conditions, show enhanced protection against biotic and abiotic stresses. This fact suggests that Cerrado plants could be important sources of novel defense factors against pests and pathogens. Therefore, *PpAI* was also evaluated in both in vitro and in vivo against *C. maculatus* to demonstrate the feasibility of this approach.

MATERIALS AND METHODS

Purification of *P. pubescens* Inhibitor (*PpAI*). Sucupira (*P. pubescens*) seeds (100 g) were frozen in liquid nitrogen and macerated into a fine powder (90 g). Lipids were removed from the crude extract by treatment with 50% acetone (w/v). After being air-dried, the crude extract flour was resuspended in a solution containing 0.6 M NaCl and 0.1% HCl in a proportion of 1:3 (w/v) by stirring for 4 h at 4 °C. The crude extract was then centrifuged at 5000*g* for 40 min at 4 °C, and the supernatant was dialyzed overnight against distilled water. The crude extract obtained was precipitated with ammonium sulfate (100%), and then the supernatant obtained was centrifuged as previously described. The protein pellet was dissolved in distilled water and dialyzed overnight against distilled water and after lyophilization was named protein-rich fraction. This inhibitor fraction (3.0 mg) was applied onto a Red-sepharose Cl-6B affinity column with a diameter of 2 cm, length of 5 cm, and total volume of 6 mL, equilibrated with 0.1 M Tris-HCl buffer, pH 7.0, containing 5.0 mM $CaCl_2$. Non-retained proteins were eluted with equilibration buffer. The retained proteins were eluted with a single use of 0.1 M Tris-HCl buffer, pH 7.0, containing 3.0 M NaCl. Fractions of 3.0 mL were collected, and the optical density was measured at 280 nm. After dialysis and lyophilization, 1.0 mg of the *PpAI* fraction was dissolved in 250 μL of 0.1% trifluoroacetic acid and applied, by using a loop system of the same volume, onto an analytical reversed-phase HPLC column (Vydac C18-TP) in a Variant binary system at a flow rate of 1.0 mL min^{-1}. Proteins were eluted with a linear acetonitrile gradient (0−100%) for 40 min, generating several peaks. Optical densities were measured at 216 nm.

α-**Amylase Inhibitory Assays.** *C. maculatus* was reared in cowpea seeds in complete darkness at 28 °C and a relative humidity of 70%. Guts of 17−20 day-old larvae were surgically dissected into an ice-cold 0.15 M NaCl iso-osmotic solution. Guts were homogenized and centrifuged at 3000*g* for 15 min at 4 °C. The supernatant was designated as CMA and used for enzymatic assays. PPA was bought from Sigma Co. α-Amylases and α-amylase inhibitory activities were estimated as described by Bernfeld (*13*). Enzyme and inhibitors, buffered with 50 mM acetate buffer, pH 6.5, containing 5.0 mM $CaCl_2$ and 10.0 mM NaCl, were preincubated for 20 min at 37 °C. *PpAI* was assayed at standard concentrations of 25, 50, and 100 μg mL^{-1} against PPA and CMA to determine the IC$_{50}$. Both enzymes were assayed at a concentration of 25 μg mL^{-1}. Protein content was measured by the Bradford method (*35*). Starch 1.0% (w/v) was used as the substrate. After the addition of 3,5-dinitrosalicilic acid (DNS), the reaction was stopped by incubation at 100 °C for 10 min. Absorbance was measured at 530 nm. Each assay was carried out in triplicate. Distilled water was used as the negative control.

Molecular Mass Analyses. Fractions obtained from HPLC were analyzed in a 12% SDS-PAGE according to Laemmli (*14*) with minor modifications, using bromophenol blue as the tracking dye. Gel was run for 40 min at 200 V. Protein bands were detected by silver staining. Mass spectrometry analysis was carried out according to Nordhoff et al. (*34*). Freeze-dried samples were prepared for matrix-assisted laser desorption ionization-time-of-flight analysis (MALDI-ToF) on a Voyager-DE STR Bioworkstation (PerSeptive Biosystems, Framingham, MA). Samples were dissolved in 1.0% trifluoroacetic acid (TFA) and *a*-cyan (a saturated solution dissolved in acetonitrile/0.1% TFA 1:1, v/v) from Sigma. After homogenization, 1.0 mL aliquots were applied onto the Voyager Bioworkstation sample plate. Samples were air-dried at room temperature. The spectrometer, equipped with a delayed-extraction system, was operated in linear mode. Sample ions were evaporated by irradiation with a N_2 laser at a wavelength of 337 nm and accelerated by a 23 kV potential in the ion source with a delay of 150 ns. Samples were ionized with 100−200 shots of a 3 ns pulse width laser light. The signal was digitalized at a rate of 500 MHz, and the averaged data were presented to a standard Voyager data system for manipulation. The MALDI-ToF was calibrated using a Saquazyme calibration mixture (Applied Biosystems) consisting of bovine insulin (5734 Da), *Escherichia coli* thioredoxin (11 674 Da), and horse apomyoglobin (16 952 Da).

Feeding Tests. In vivo bioassays were performed with artificial seeds constructed with cowpea flour. Thus, after seed epicarp removal, cotyledons were ground in a flourmill. Flour was compacted using a hand compressor into an artificial seed (approximately 0.9 cm diameter × 0.6 cm height, weight 300 mg). For feeding tests, the *PpAI* protein-rich fraction was added to the flour to obtain the desired concentrations. Bioassays were performed using seeds containing three different concentrations of *PpAI* (2.0, 1.0, and 0.5% w/w). Seeds without *PpAI* were used as a negative control. Groups of five seeds were set in plastic containers, and 10−15 fertilized females were introduced for 24 h to allow oviposition. After this period, all seeds were observed under a stereoscopic microscope to confirm oviposition, leaving just two eggs per seed. This experimental design evaluated the influence of the *PpAI* inhibitor on both immature stages (mortality after 15 days of development in the seed) and adults (longevity and fecundity of females). Mortality of immature stages was evaluated considering the initial number of eggs and adults obtained in each plastic container (*n* = 5 for each treatment and control). Effects on adults were analyzed using insects obtained in each treatment; males and females were joined in couples and isolated in individual plastic containers with five cowpea seeds (*n* = 10 for each treatment and control). Seeds were observed every 24 h to evaluate insect longevity and also the number of eggs deposited by females. All evaluations were performed using a stereoscopic microscope and the total number of eggs counted, and 10 replicates were used for each treatment. Data were analyzed using ANOVA and Dunnett's test for median multiple comparisons ($p < 0.05$). The Kruskal−Wallis test was used when the data were not normally distributed.

RESULTS AND DISCUSSION

Purification and Biochemical Characterization of *PpAI*. To find novel α-amylase inhibitors in *P. pubescens*, a Brazilian native tree, seeds were extracted and precipitated with ammonium sulfate (100%). Apparently, leaves, stems, and roots do not synthesize α-amylase inhibitors since we did not observe any inhibitory activity when these tissues were used in enzyme assays (data not shown). After dialysis, the obtained ammonium sulfate-rich fraction was applied onto a Red-sepharose CL-6B affinity column to isolate cationic proteins, and only one retained peak was obtained (PpRP) (**Figure 1A**). Other studies have shown that Red-sepharose columns can be utilized for the purification of several plant defense proteins including α-amylase (*15*, *16*), proteinase inhibitors (*17*), γ-thionins (*18*), and 2S albumins (*19*). Enzymatic assays revealed that the PpRP

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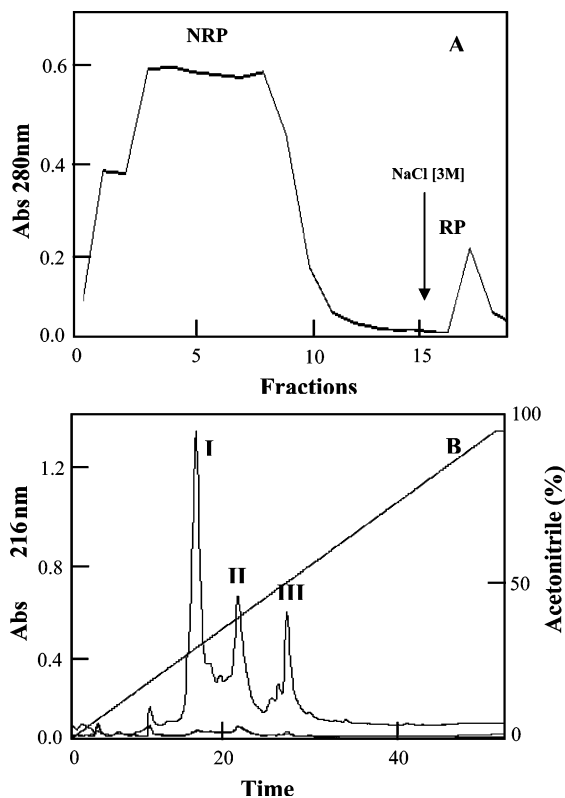
Figure 1. (**A**) Red-sepharose chromatography profile of *P. pubescens* seed crude extract. NRP corresponds to non-retained peak and RP to the retained peak. The black arrow indicates a single elution step with NaCl 3.0 M. (**B**) The retained peak was applied onto a reversed-phase HPLC (Vydac C-18TP) generating three peaks. The diagonal line represents the linear acetonitrile gradient (0–100%).

fraction was unable to inhibit PPA but inhibited CMA (36%) (**Figure 2** and **Table 1**). PpRP was then applied onto an analytical reversed-phase HPLC column (Vydac C_{18}–TP 522), yielding three major peaks (**Figure 1B**). Only fraction II demonstrated a clear inhibitory activity of approximately 65% against *C. maculatus* α-amylases (**Figure 2**). The purification improvement of *PpAI* is shown in **Table 1**. In addition, the IC_{50} of *PpAI* was calculated, and it was determined that 41.7 μg mL^{-1} was able to inhibit 50% of CMA activity. Earlier studies have shown that α-amylase inhibitors isolated from cereal kernels and leguminosae seeds showed inhibitory activity against bruchid enzymes (*2, 3, 11, 20, 21*). Recent studies demonstrated that an α-amylase inhibitor purified from papaya seeds was also effective toward CMA, showing a CL_{50} of approximately 49 μg mL^{-1}. This study is interesting because it shows that seeds from fruits are also part of the selective group of seeds capable of producing α-amylase inhibitors (*5*). Surprisingly, our data indicated that *PpAI* is probably specific to insect α-amylases. Nevertheless, further experiments with other enzyme sources will be performed to prove this hypothesis. Specificity toward insect α-amylases is a desirable characteristic because it may increase consumers' safety. Insect specificity has also been observed before. Lectin-like α-amylase inhibitors showed an unusual specificity pattern. While the α-AI1 variant showed activity toward PPA, CMA, and CCA, the α-AI2 variant showed activity against ZSA but no inhibition toward α-amylases affected by α-AI1 and vice versa (*22*). Additionally, some wheat inhibitors, such as 0.53, showed enhanced specificity toward bean bruchid enzymes (*10*). Finally, α-amylase inhibitors isolated from *Amaranthus hipocondriacus* seeds revealed a
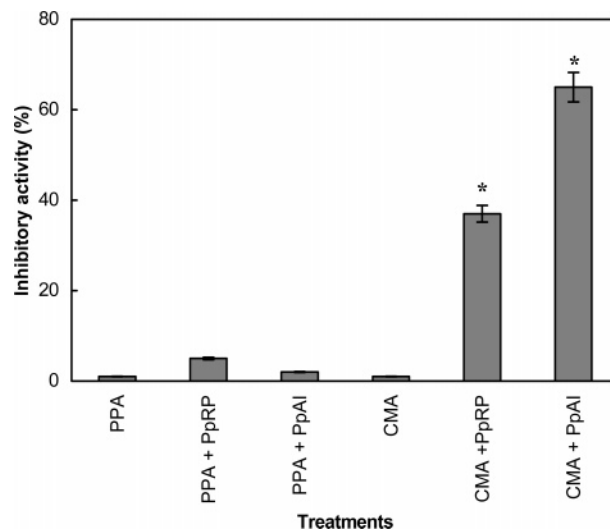
Figure 2. Inhibitory α-amylolitic (CMA and PPA) assays against *P. pubescens* retained fraction from Red-sepharose (PpRP) and purified *PpAI*. Enzyme and inhibitors, buffered with 50 mM acetate buffer, pH 6.5, containing 5.0 mM $CaCl_2$ and 10.0 mM NaCl, were preincubated for 20 min at 37 °C. *PpAI* was assayed at a standard concentration of 50 μg mL^{-1} against PPA and CMA. Both enzymes were assayed at a concentration of 25 μg mL^{-1}. Starch 1.0% (w/v) was utilized as a substrate. After the addition of 3.5 DNS, the reaction was stopped by incubation at 100 °C for 10 min. Vertical bars correspond to standard deviations. Each assay was carried out in triplicate. Asterisk indicates treatments statistically different from control (Dunnett's test $p < 0.05$).

**Table 1.** Purification Improvement of *PpAI* α-Amylase Inhibitor[a]

purification step	protein (μg)	activity (U)	yield (%)	specific activity (U μg^{-1})	fold purification
crude extract	3000	240	100	0.08	1
RED-sepharose Cl-6B	760	195	82	0.25	3.2
reversed-phase HPLC	118	112	46.7	0.94	11.7

[a] Activity was determined by the Bernfeld method (*13*) as described in the text, using starch as the substrate. One inhibitory enzyme unit is defined as the amount of inhibitor necessary to decrease the optical density by 0.1 at 530 nm in comparison to CMA with no inhibitor.

specific inhibitory activity against insect α-amylases being unable to inhibit mammalian α-amylases (*23*).

Molecular Mass Analyses. Purified *PpAI* was also analyzed by SDS-PAGE showing a single protein of about 5000 Da (data not shown). This protein was also analyzed by mass spectrometry (**Figure 3**), showing a monomer of 5230.60 Da. Similar results were observed by Melo et al. (*15*) and Bloch and Richardson (*16*), who found small α-amylase inhibitors with similar low molecular masses. Furthermore, a similar molecular weight was observed for CpAI, an α-amylase inhibitor purified from papaya seeds (*5*). These molecular masses are typical of the γ-purothionin family, found in legumes and cereal seeds, small and cationic proteins that are involved in different defense mechanisms of several plants, including inhibition of protein synthesis, membrane permeabilization, and enzymatic inhibition (*24*). So far, only the *Vigna radiata* plant defensin (VrD1) mechanism of action, which showed activity against insect amylases, was reported (*28*). VrD1 inhibits *Tenebrio molitor* α-amylase, and computational docking experiments were used to study the inhibitor–enzyme interaction. The results suggested that VrD1 inserts itself into the active site of TMA, thereby preventing the substrate from reaching the catalytic site (*28*).
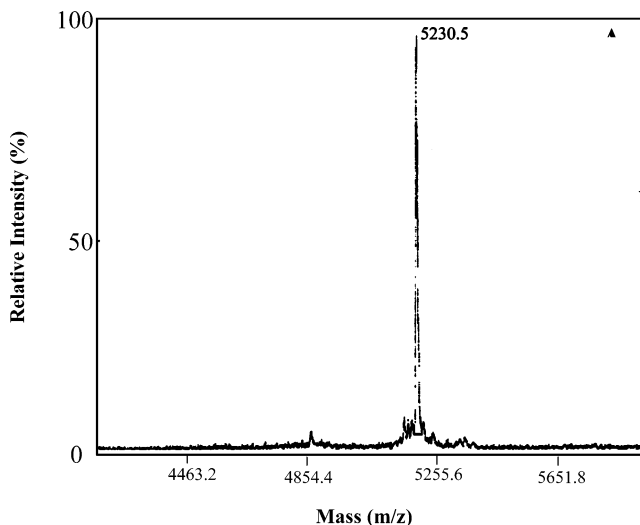
Figure 3. MALDI-TOF spectrum analysis of purified *PpAI*.

On the basis of its molecular mass and purification procedures, it is possible that *PpAI* belongs to the γ-purothionin family and could have a similar mechanism of action; however, further studies will be necessary to classify this proteinaceous compound.

In Vivo Bioassays. In vivo effects of *PpAI* toward *C. maculatus* were evaluated by incorporating the *P. pubenscens* inhibitor-rich fraction into cowpea artificial seeds. In this procedure, two parameters were analyzed: larval weight (**Figure 4A**) and insect mortality (**Figure 4B**). Results from feeding tests showed that inhibitor concentrations of 0.5 and 1.0% (w/w) caused a low larval weight reduction (~20%) (**Figure 4A**). Artificial seeds with an enhanced inhibitor concentration (2.0%) reduced in 36% larval weight. Furthermore, the *PpAI*-rich-fraction concentrations of 0.5 and 1.0% (w/w) caused 20% mortality. When the inhibitor concentration was increased to 2.0%, 55% mortality was obtained. LD_{50} was calculated, showing that a concentration of 1.6% was able cause 50% mortality. Similar results were observed in bioassays using two purified wheat inhibitors named 0.19 and 0.53. Both were able to inhibit *A. obtectus* development and decrease its survival (*10*). Moreover, an α-amylase inhibitor isolated from rye kernels was able, at low concentration (0.89% w/w), to cause an enhanced mortality (83%) to the cotton boll weevil *A. grandis* (*21*). Finally, results from bioassays using *C. papaya* α-amylase inhibitors showed that inhibitor concentrations of 0.5 and 1.0% (w/w) slightly reduced adult longevity (data not shown). Artificial seeds containing a high inhibitor concentration (2.0%) caused a larval mortality of 50% and a clear decrease in adult fecundity (*5*). Although expressive deleterious effects toward cowpea weevil development were observed only with a 2.0% (w/w) inhibitor concentration, *PpAI* presents the advantages of specificity and most likely can be easily expressed (in heterologous systems) due to its low molecular mass. Moreover, this inhibitor also could be combined with other proteinaceous factors to improve plant resistance.

A vast number of genes conferring pest resistance have been expressed in heterologous systems (*21*, *29*) or incorporated into different crops (*25*) including digestive enzyme inhibitors, which are thought to inhibit larvae growth by slowing down the digestion process, reducing carbohydrate assimilation. Transgenic peas and adzuki beans expressing *Phaseolus vulgaris* α-AI1 have enhanced resistance to certain species of Bruchidae, whose digestive α-amylases are affected by α-amylase inhibitors (*26*, *27*). No study where transgenic plants expressing α-amylase
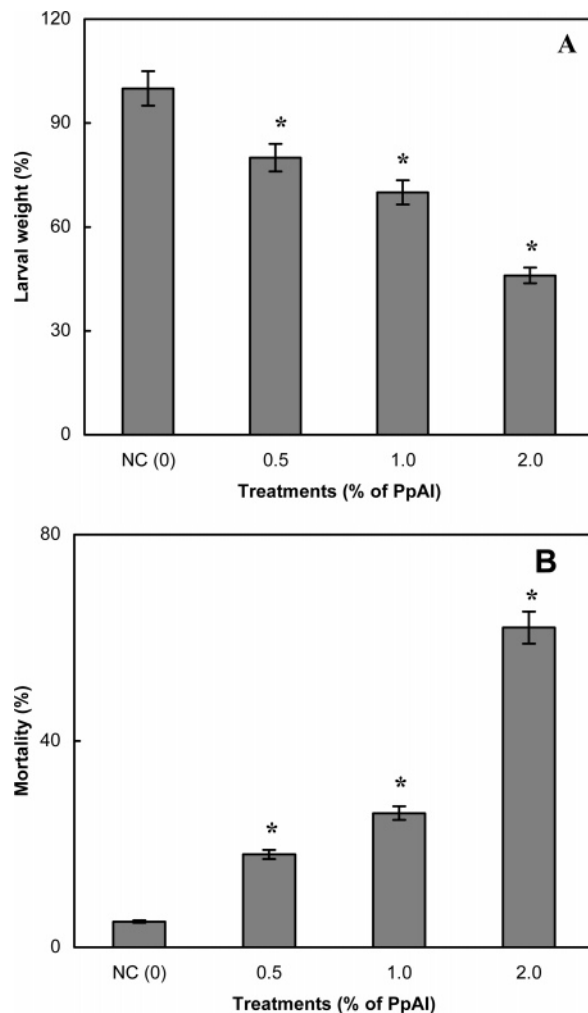
Figure 4. Feeding tests using the *P. pubescens*-rich fraction at standard concentrations of 0.5, 1.0, and 1.5% (w/w). Bioassays evaluate (**A**) larval weight and (**B**) adult mortality for 20 days. Each experiment was carried out in triplicate, and vertical bars represent the standard deviation. Asterisk indicates treatments statistically different from control (Dunnett's test $p < 0.05$).

inhibitors from native plants has been reported yet. For this reason, great efforts must be made to discover novel proteinaceous inhibitors from alternative sources. These inhibitors probably have different structures and biochemical properties and will help researchers to find new ways to reduce pest resistance. In summary, here we reported the isolation of a novel α-amylase inhibitor from the seeds of a Cerrado plant. This inhibitor, purified from sucupira seeds, showed a high specificity toward insect enzymes. This surprising finding suggests the implication of *PpAI* in the plant defense mechanism, indicating that this inhibitor probably could be utilized, by using genetic engineering techniques, as an alternative strategy to enhance cowpea crop resistance. The data reported here add a new activity to the already well-known list of uses for sucupira that include antiarthritic (*31*), antinociceptive (*32*), and cercaricidal (*33*) characteristics. More importantly, this work highlights the great opportunities presented by native plants for the discovery of novel useful compounds with biotechnological potential.

ABBREVIATIONS USED

α-AI1 and α-AI2, α-amylase inhibitors from *Phaseolus vulgaris*; CCA, *Callosobruchus chinensis* α-amylase; CMA,

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Callosobruchus maculatus α-amylase; HPLC, high-performance liquid chromatography; MALDI-ToF, matrix-assisted laser desorption ionization-time of flight; *PpAI, Pterodon pubescens* α-amylase inhibitor; PPA, porcine pancreatic α-amylase; PpRP, *Pterodon pubescens* retained peak; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TMA, *Tenebrio molitor* α-amylase; VrD1, *Vigna radiata* plant defensin; ZSA, *Zabrotes subfasciatus* α-amylase.

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