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Identification of a Kunitz-Type Proteinase Inhibitor from *Pithecellobium dumosum* Seeds with Insecticidal Properties and Double Activity

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A trypsin inhibitor, PdKI, was purified from *Pithecellobium dumosum* seeds by TCA precipitation, trypsin−sepharose chromatography, and reversed-phase-HPLC. PdKI was purified 217.6-fold and recovered 4.7%. SDS-PAGE showed that PdKI is a single polypeptide chain of 18.9 kDa and 19.7 kDa by MALDI-TOF. The inhibition on trypsin was stable in the pH range 2−10 and at a temperature of 50 °C. The K_i values were 3.56×10^{-8} and 7.61×10^{-7} M with competitive and noncompetitive inhibition mechanisms for trypsin and papain, respectively. The N-terminal sequence identified with members of Kunitz-type inhibitors from the Mimosoideae and Caesalpinoideae subfamilies. PdKI was effective against digestive proteinase from *Zabrotes subfasciatus*, *Ceratitis capitata*, *Plodia interpunctella*, *Alabama argillaceae*, and *Callosobruchus maculatus*, with 69, 66, 44, 38, and 29% inhibition, respectively. Results support that PdKI is a member of the Kunitz inhibitor family and its insecticidal properties indicate a potent insect antifeedant.

KEYWORDS: ***Pithecellobium dumosum*; plant defense; Kunitz type inhibitor; insect pests**

1. INTRODUCTION

Beginning in 1980, new strategies of insect pest control, such as integrated pest management (IPM) control and the use of transgenic crops, have been proposed and tested to avoid these crop production losses. These strategies have been employed because of worldwide crop loss that was estimated at 70% of production without the use of pesticides (*1*), and despite of the use of pesticides, preharvest losses had reached 15% of the total crop production (*2*). During the past decade, the uses of transgenic crops have been rapidly advancing with the discovery of effective plant genes that can be transferred and offer resistance to crop plants against pests and pathogens.

The inhibitors are known for their roles in response to abiotic (*3*, *4*) and biotic stresses, especially in plant defense processes against insect pest attack (*5*), where they are effective against insect digestive enzymes (*6*−*9*). Among the proteinaceous inhibitors, those belonging to the Kunitz family and Bowman−Birk members in particular are abundant in the Leguminosae, where they are presumed to serve in seed defense against insect predation, as well as simple seed storage (*5*). The Kunitz trypsin inhibitors in general are small, stable, and abundant proteins (*10*) that are found in plant storage tissues, such as seeds, tubers, leaves, and fruits (*11*, *12*). Most of these inhibitors bind to cognate enzymes according to a common substrate-like canonical mechanism (*6*, *13*). The use of Kunitz trypsin inhibitors as candidates in control strategies of insects has good potential, because insect digestive proteinases are promising targets in the control of lepidopteran, such as *Heliothis zea* (Boddie) (*14*), *Spodoptera litura* (Boisd.) (*15*), *Agrotis ipsilon* (Hufnagel), *Heliothis zea* and *Heliothis virescens* (Fab.), *Choristoneura occidentalis*, *Manduca sexta* (L.) (*16*); dipteran *Lucilia cuprina* (Wied.) (*17*); coleopteran such as the cotton boll weevil (*Anthonomus grandis*) (*18*) and others coleopterans (*5*, *18*−*21*).

Several plants have been screened to isolate and characterize such proteinase inhibitors, among them the species *Pithecellobium dumosum* (Jurema Branca), a member of the family Mimosoideae, which are tropical and subtropical trees and shrubs. In this study, we have reported on the purification, characterization, and amino acid sequence analysis of a related Kunitz inhibitor from Jurema Branca tree seeds. We have also tested its effect

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in vitro toward digestive enzymes from insect pests of different orders, which use as major digestive proteinases the cysteine and serine enzyme classes. The purified protein could help the search for indication of active inhibitors toward insect pests that may provide new potential bioinsecticides to pest control.

2. MATERIALS AND METHODS

2.1. Reagents. Trypsin (bovine pancreas), chymotrypsin (bovine pancreas), elastase (porcine pancreas), papain (papaya latex) and bromelain (pineapple steam), *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BApNA), *N*α-benzoyl-DL-Arg β-naphthylamide (BANA), trichloroacetic acid (TCA), and protein molecular weight markers were purchased from Fermentas Life Science.

2.2. Isolation and Purification of *P. dumosum* Trypsin Inhibitor (PdKI). *P. dumosum* seeds were obtained from the seed bank from IBAMA (Brazilian Environmental Institute of natural and renewable resources) in Natal-RN, Brazil. The crude protein extract was obtained from 100 g of seeds by continuous stirring with 50 mM sodium tetraborate buffer, pH 7.5 (1:10, w/v), at room temperature for about 3 h. After centrifugation for 30 min at 12000*g* at 4 °C, the proteins of the supernatant (crude extract) were precipitated by adding 20% TCA solution to a final concentration of 14%. After 30 min centrifugation at 12000*g* at 4 °C, the supernatant was dialyzed against 50 mM sodium tetraborate buffer, pH 7.5. This sample, with anti-tryptic activity, denoted JB14 (13 mg mL^{-1}) was applied to a trypsin−sepharose affinity column (10 cm × 1.5 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The retained proteins were eluted with 1 mM HCl solution at flow rate of 30 mL/h. The anti-tryptic peak, denoted JBAf, was pooled and submitted to a reverse-phase HPLC column (Vydac C-18), connected to LC-10A Shimadzu HPLC, and equilibrated with 0.1% trifluoroacetic acid (TFA) solution with a gradient of solvent B (60% acetonitrile/0.1% TFA/$H_2$0); the chromatography was monitored at 220 nm. The proteins were separated in semipreparative (2.2 × 25.0 cm, Vydac C-18 TP 1022) column at a flow rate of 9 mL/min with a gradient of 5−35% solution B for 10 min, followed by 35−60%, solution B for 25 min, 60−68% solution B for 5 min, 68−88% solution B for 20 min, 88−95% solution B for 10 min, 95−5% solution B for 2 min, and finally 5% solution B for 1 min. Four anti-tryptic peaks were obtained (JB1, JB2, JB3, and JB4), and JB1 was then again subjected to analytical reverse-phase (0.46 × 25.0 cm, Vydac C-18 TP 104) column at a flow rate of 1 mL/min with a gradient of 5% solution B by 5 min follwed 5−45% solution B for 5 min, 45−62 solution B for 17 min, 62−95% solution B for 2 min, and 5% solution for 1 min. The purified Kunitz-type trypsin inhibitor, PdKI, was subjected to further analysis.

2.3. Protein Concentration. Protein concentrations were determined by the dye-binding method of Bradford (*22*), with bovine serum albumin as the standard.

2.4. Characterization of the Kunitz Inhibitor from *P. dumosum* Seeds (PdKI). *2.4.1. Trypsin Inhibition Assay.* The inhibition of trypsin was determined by measuring the residual enzymatic activity toward the substrate BApNA at pH 7.5 as describe by Erlanger et al. (*23*). Ten microliters of trypsin (0.3 mg/mL in 50 mM Tris-HCl buffer, pH 7.5, containing 20 mM $CaCl_2$) solution was preincubated for 10 min at 37 °C with 100 μL of each retained fraction (inhibitor) obtained from trypsin−sepharose affinity column and 390 μL of 50 mM Tris-HCl buffer, pH 7.5. The reaction was started with the addition of 250 μL of 1.25 mM BApNA solution, prepared in 1% (v/v) DMSO and 50 mM Tris-HCl buffer, pH 7.5. After 15 min at 37 °C, the reaction was stopped by adding 60 μL of 30% acetic acid solution. Blanks were prepared in the same conditions as the tests, without addition of substrate, which was added after the addition of 30% acetic acid solution. The enzymatic hydrolysis of the substrate was evaluated by recording the absorbance at 410 nm. The constant of dissociation (K_i) was determined for bovine trypsin enzyme by preincubating the enzyme with increasing concentrations of purified inhibitor (2, 3, 4, and 5 μg) in 50 mM Tris-HCl, pH 7.5, 37 °C, followed by measurement of the residual activity using the synthetic substrate BApNA (0.5, 0.75, 1.0, 1.25, 2.0, and 2.5 mM). The velocity of the reaction was expressed as 1/V (OD_{440} h^{-1} mL^{-1}), and the K_i value was determined using a double-reciprocal plot of data.

2.4.2. Papain Inhibition Assay. The papain inhibitory assay was determined essentially as described by Zhao et al. (*24*) using BANA as substrate. Ten microliters of papain (0.1 mg/mL in 25 mM sodium phosphate buffer, pH 6.0) solution was incubated for 10 min at 37 °C with 20 μL of an activation solution containing 2 mM EDTA and 3 mM DTT in pH 6.0, 20 μL of PdKI (1 μg/μL), and 250 μL of 25 mM sodium phosphate buffer, pH 6.0. Reaction were started with the of 100 μL of 1 mM BANA solution, prepared in 1% (v/v) DMSO and 25 mM sodium phosphate buffer, pH 6.0. After 20 min at 37 °C, the reaction was stopped by adding 250 μL of 2% HCl in ethanol. Blanks were prepared in same conditions as the tests, without addition of substrate, which was added after the addition of 2% HCl in ethanol. The color product was developed by the addition of 250 μL of 0.06% *p*-dimethylaminocinnamaldehyde in ethanol and measured by absorbance at 540 nm. The constant of dissociation (K_i) was determined for papain by preincubating the enzyme with increasing concentrations of purified inhibitor (5, 10, 20, 30, and 40 μg) in 25 mM sodium phosphate buffer, pH 6.0 at 37 °C, followed by measurement of the residual activity using the synthetic substrate BANA (0.4, 0.8, 1.0, 1.5, 2.0, and 3.0 mM in 50 mM Tris-HCl, pH 7.5. The velocity rate of this reaction was expressed as 1/V ($O.D._{540}$ h^{-1} mL^{-1}) and the K_i value was determined using a double-reciprocal plot of the data.

2.4.3. Thermal and pH Stability of PdKI. The thermal stability of PdKI (1 μg/uL) was tested by incubation of protein at different temperatures (37, 40, 60, 70, 90, and 100 °C) for 30 min. After the samples were cooled at 4 °C for 10 min, the inhibitory assays against trypsin were performed. The stability in a broad range of pH was also checked. Samples of PdKI (1 μg/μL) were prepared with 100 mM glycine-HCl (pH 2−3), 100 mM sodium phosphate (pH 6−8), and 100 mM glycine-HCl (pH 11−12). After incubation in each buffer for 30 min at 37 °C, the samples was dialyzed against 50 mM Tris-HCl buffer, pH 7.5, and the inhibitory activity assays against trypsin were performed using BApNA as substrate. All assays were done in triplicate. The results of each series were expressed as the mean value ± SD.

2.4.4. Specificity of PdKI toward Serine and Cysteine Proteinases. The ability of PdKI to inhibit other serine (bovine chymotrypsin and porcine elastase) proteinases and bromelain, a cysteine proteinase, was assayed using azocasein as substrate, as described by Xavier-Filho et al. (*25*).

2.4.5. Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (15% SDS-PAGE) was conducted as described by Laemmli (*26*) at room temperature in the absence or presence of β-mercaptoethanol (0.1 M). For protein detection, gels were stained with 0.1% Coomassie brilliant blue R-250 followed by revelation with silver.

2.5. N-Terminal Sequencing. N-terminal amino acid sequence analysis of PdKI was determined at the Departamento de Bioquímica e Imunologia, ICB, UFMG, Brazil, using an automated protein sequencer from Shimadzu PSSQ-21A.

2.6. Mass Spectrometry. The molecular mass of PdKI was determined at the Departamento de Bioquímica e Imunologia, ICB, UFMG, Brazil using an Ultraflex II Matrix-assisted laser desorption-time-of-flight (MALDI-TOF/TOF) from Bruker Daltonics, Billerica, MA. The sample was dissolved in a solution containing 5.0 mg of α-ciano-4-hidroxi-cinâmico, 300 μL of Milli-Q water, 200 μL of acetonitrile, and 50 μL of 3% TFA. The solution was then vortex-mixed and 1 μL was applied onto the sample plate.

2.7. In vitro Effects of PdKI toward Digestive Enzymes from Insect Pests. *2.7.1. Preparation of Insect Gut Proteinases. C. maculatus, Z. subfasciatus*, and *P. interpunctella* were supplied by the Laboratório de Química e Função de Proteínas from Departamento de Bioquímica, UFRN, Brazil. *A. argillacea* was obtained of the Centro de Pesquisa do Algodão (CNPA/EMBRAPA), Campina Grande, Brazil, and *C. capitata* was obtained from the Laboratório de Mosca das Frutas of the Departamento de Biologia Celular e Genética, UFRN, Brazil. Insect colonies were maintained at 28 ± 2 °C, 60−80% RH, and photoperiod of 12 h. Insect larvae proteinases were obtained after dissection and extraction of the guts. The guts were surgically removed from the animal and placed into an iso-osmotic saline (0.15 M NaCl) solution. Gut tissue

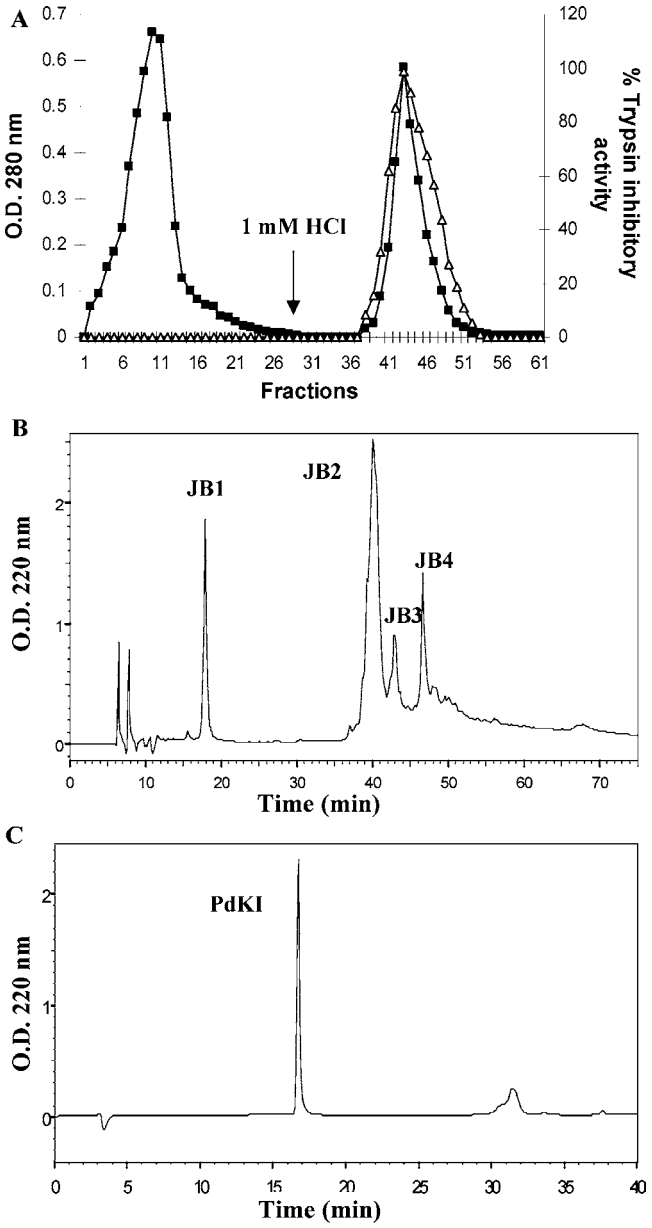

Figure 1. (**A**) Elution profile of JB14 on a trypsin–sepharose column. Column (10 cm × 1.5 cm) was equilibrated with 50 mM Tris-HCl buffer, pH 7.5, and the retained proteins were eluted with 1 mM HCl solution. The fractions of 2 mL were obtained and monitored at (■) 280 nm and (△) assayed against trypsin. (**B**) Elution profile of JBAf on HPLC (Vydac C-18) column. The fractions obtained from the trypsin–sepharose column were separated by a semipreparative reverse-phase HPLC column at a flow rate of 9 mL/min. The elution of the JBAf fraction revealed four peaks named JB1, JB2, JB3, and JB4. (**C**) The JB1 fraction was then subjected to another analytical reverse-phase HPLC column at a flow rate of 1 mL/min, and the single peak obtained was named of PdKI.

was homogenized in a potter at 4 °C for 10 min and centrifuged at 12000*g* at 4 °C for 10 min, and the supernatants were then recovered and used for in vitro assays.

2.7.2. PdKI Inhibitory Assay against Proteinases from Insect Pests. PdKI effects on the proteolytical activity of whole gut extracts were measured by using 1% azocasein as substrate. The assays were run in 50 mM Tris-HCl buffer, pH 7.5, for *C. maculatus*, *Z. subfasciatus*, and *A. argillaceae*, and 50 mM Tris-HCl buffer in pH 8.5 and 9.5 for *C. capitata* and *P. interpunctela*. Aliquots of 290 μL of the respective buffers were incubated with 50 μL gut extracts and 20 μL (1 μg/μL) of PdKI at 37 °C for 15 min. Reactions were started with the addition of 500 μL of 1% azocasein solution. After 30 min at 37 °C, the reaction

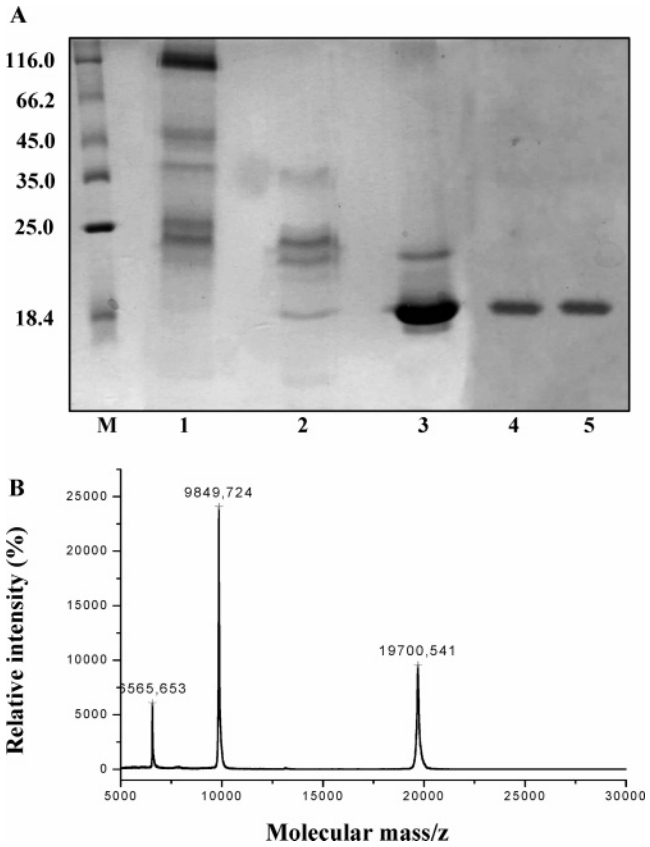

Figure 2. (**A**) SDS-PAGE (15%) of purified PdKI from *P. dumosum* seeds, stained with Coomassie followed by revelation with silver. (M) Protein molecular weight markers: β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), lactate degydrogenase (35 kDa), restriction endonuclease *Bsp*981 (25 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa). (1) Crude extract; (2) fraction treated with TCA (JB14); (3) trypsin–sepharose retained peak (JBAf); (4) PdKI; (5), PdKI treated with β-mercaptoethanol. (**B**) MALDI-TOF/TOF spectrum of PdKI.

was stopped by adding 150 μL of 20% TCA solution. The samples were centrifuged for 30 min at 12000*g* at room temperature, and the supernatants alkalinized with 2 N NaOH solution. The residual proteolytical activity was measured by absorbance at 440 nm. All assays were done in triplicate. The results of each series were expressed as the mean value ± SD.

3. RESULTS

3.1. Purification of PdKI. The soluble protein fraction obtained from 14% TCA precipitation showed strong inhibitory activity against trypsin and was applied to a trypsin−sepharose affinity column; the retained peak obtained had high antitryptic activity (**Figure 1A**). The anti-tryptic peak was then submitted to a reverse-phase high performance liquid chromatography (HPLC), and the elution profile (**Figure 1B**) showed separation of four protein peaks (named JB1, JB2, JB3, and JB4) with strong inhibitory activity against trypsin. The JB1 protein fraction was rechromatographed by reversed phase HPLC (**Figure 1C**), and a single peak with anti-tryptic activity (PdKI) was purified. SDS-PAGE (**Figure 2A**) in the absence and presence of β-mercaptoethanol showed that PdKI consisted of a single polypeptide chain with a molecular mass of 18.9 kDa and by MALDI-TOF analysis with molecular mass of 19.7 kDa (**Figure 2B**). The three peaks in the MALDI-TOF graphs represent different charged variants of the same protein, with charges of 1+, 2+, and 3+. The methodological procedure resulted in a high purification of 217.6-fold with a 4.7% yield (**Table 1**).

Table 1. Purification Steps of Kunitz Inhibitor from *P. dumosum*

steps	total inhibitory units (IU)	total protein (mg)	specific activity (UI mg $^{-1}$)[a]	purification (fold)	yield (%)
crude extract	118560	1041.20	113.87	1.0	100
JB14	80800	18.00	4488.88	39.4	68
JBAf	8190	1.76	4653.40	40.9	6.9
PdKI	5526	0.223	24780.26	217.6	4.7

[a] One trypsin protein inhibitor unit (1 UI) was defined as the inhibitor amount that decreased the absorbance at 410 nm by 0.1 O.D. in the trypsin assay conditions.

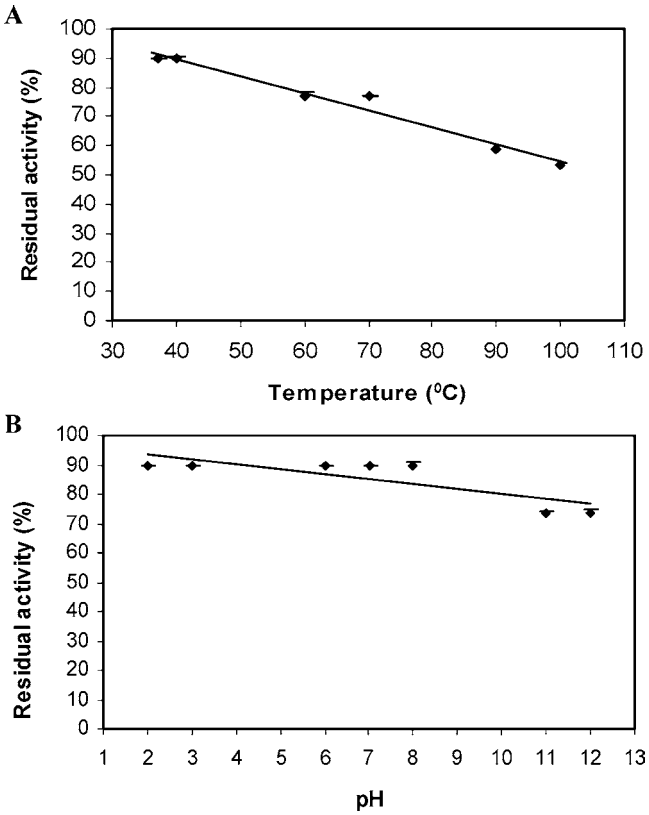

Figure 3. (**A**) Temperature stability of PdKI. (**B**) pH stability of PdKI. The residual trypsin inhibitory activity was measured using BApNA as substrate in 50 mM Tris-HCL buffer, pH 7.5, after incubation for 30 min at 37 °C. Each mean represent three replicates (±SE).

3.2. Characterization of PdKI. *3.2.1. Thermal and pH Stabilities.* The study of the temperature effect on PdKI showed that the inhibitory activity was stable at 50 °C, losing only 40% of activity at 100 °C. (**Figure 3A**). Preincubation of the inhibitor in the pH range (2.0−12.0) did not affect trypsin activity (**Figure 3B**).

3.2.2. Specificity of PdKI to Serine and Cysteine Proteinases. PdKI weakly inhibited elastase and chymotrypsin, two serine proteinases of the same class of trypsin that was strongly inhibited (88.69% of inhibition). Cysteine proteinases were also tested, and it was observed that 34.01% of inhibition to papain and inhibition to bromelain was weakly detected (**Table 2**).

3.2.3. Kinetics of PdKI. To determine the inhibition mechanism of PdKI against trypsin and papain, we analyzed the inhibition kinetic data by Lineweaver−Burk plots (panels **A** and **B** of **Figure 4**). The analysis showed noncompetitive type kinetic of inhibition to papain and competitive to trypsin. The K_i value of trypsin was 3.56×10^{-8} M and that of papain was 5.1×10^{-7} M.

3.2.4. N-Terminal Amino Acid Sequence Analysis. The alignment of the N-terminal amino acid sequence of the protein PdKI with other proteinase inhibitors showed similarity with N-terminal sequences of the Kunitz inhibitor families (**Figure 5**). The similarity of alignment of the PdKI with Kunitz inhibitors from *Leucaena leucocephala* (LlTKI), *Copaifera langsdorffii* (ClTKI), and *Psophocarpus tetragonolobus* (PtTKI) were 40, 60, and 40%, respectively.

Table 2. Inhibitory Effect of PdKI toward Serine and Cysteine Proteinases

enzymes[a]	inhibition (%)[b]
serine proteinases	
bovine trypsin	88.69 ± 0.86
porcine elastase	4.03 ± 0.04
bovine chymotrypsin	5.04 ± 0.11
cysteine proteinases	
bromelain	1.10 ± 0.05
papain	34.01 ± 1.44

[a] Assays against elastase, chymotrypsin and bromelain, measured using 1% azocasein as substrate; the inhibitory activity against trypsin and papain was measured using BApNA and BANA as substrate, respectively. [b] Values are mean ± standard error, and each mean represents three replicates.

3.3. In Vitro Effect of PdKI on Digestive Proteinases from Different Orders of Insect Pests. The digestive proteinases from Coleopteran (*Z. subfasciatus, C. maculatus*) Lepidopteran (*P. interpunctella,* A. *argillaceae*), and Dipteran (*C. capitata*) pests were tested (**Table 3**). Among the different gut proteinases tested, PdKI showed high in vitro inhibitory effect on *Z. subfasciatus* (68.87%) and *C. capitata* (65.53%). Moderate inhibitory activity was observed for gut proteinases of *P. interpunctella* (44.35%), and *A. argillaceae* (38.36%), and low inhibitory activity was observed against gut proteinase from *C. maculatus* (29.18%).

4. DISCUSSION

Proteinaceous inhibitors have been purified and characterized from a variety of seed plant sources (*27*−*33*). The role of these inhibitors as defensive compounds against predators was studied as early as 1947, when Mickel and Standish (*34*) observed that larvae of certain insects were unable to develop on soybean products. A later report showed that trypsin inhibitors were toxic to *Tribolium confusum* (flour beetle) larvae (*35*). Following these studies, several research groups have investigated these proteins as candidates for developing new pest control strategies. Various proteinase inhibitors such as trypsin and/or chymotrypsin inhibitors (*36*, *37*), subtilisin inhibitor (*38*), cathepsin D inhibitor (*39*, *40*), and papain and/or cathepsin L inhibitor (*41*) have been purified from legume, cereal seeds, and tubercles (*30*, *40*). These proteinaceous inhibitors have molecular masses (20−24 kDa) and primary structures similar to those of β-chain of soybean Kunitz-trypsin inhibitor family (*19*, *39*) and are reunited in a Kunitz-type proteinase inhibitors superfamily. They play a significant role in the defense mechanism of plant against insect and phytopathogen attacks (*35*, *36*, *38*, *41*).

In this study, a related Kunitz inhibitor from *P. domusum* seeds was purified and characterized and its effects on digestive proteinsases from insect pests were examined in vitro. The PdKI

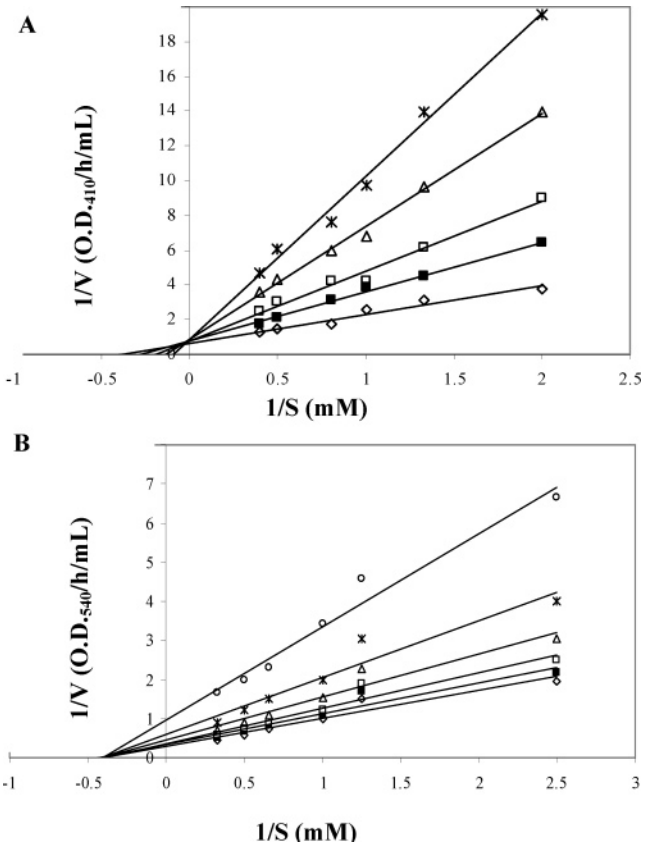

Figure 4. Kinetic analysis of trypsin and papain inhibition by PdKI activity. (**A**) Inhibition of trypsin activity by PdKI was competitive. Inhibition kinetic data are illustrated in Lineweaver–Burk double-reciprocal plots. PdKI activity was determined using various concentrations of BApNA as substrate. ◇, No inhibitor in the assay mixture; ■, 2 μg of PdKI; □, 3 μg of PdKI; △, 4 μg of PdKI; *, 5 μg of PdKI. (**B**) Inhibition of papain activity by PdKI was noncompetitive. Inhibition kinetic data are illustrated in Lineweaver–Burk double-reciprocal plots. PdKI activity was determined using various concentrations of BANA as substrate. ◇, No inhibitor in the assay mixture; ■, 5 μg of PdKI; □, 10 μg of PdKI; △, 20 μg of PdKI; *, 30 μg of PdKI; ○, 40 μg of PdKI.

purified is a protein with a unique polypeptide chain of molecular mass of 19.7 kDa, in agreement with the molecular mass of other trypsin inhibitors (*42*−*49*). Thermal inactivation of PdKI at different temperatures resulted in a progressive loss of trypsin-inhibiting activity at temperatures >40 °C and an ∼40% decrease at 100 °C. This property was similar to other trypsin inhibitors (*30*, *43*, *49*−*52*). The inhibitory activity of PdKI was not sensitive to pH in the range 2−12, as reported for ACTI (*53*), DMTI−II (*49*), PPTI (*47*), and PDTI (*43*). This relative stability of PdKI was possible due to intramolecular disulfide bridges, which are presumably responsible for the functional stability of Kunitz-type inhibitors in the presence of physical and chemical denaturants such as temperature and pH. PdKI, belonging to the Mimosoideae subfamily, weakly inhibited other serine proteinases, such as elastase and chymotrypsin, and was active against papain, a cysteine proteinase. Until now, only three related Kunitz-type inhibitors, all belonging to the Mimosoideae subfamily, were found to be active against papain-like enzymes, and were strongly active against enzymes from the midgut of bruchid larvae (*8*, *9*, *51*), suggesting that Kunitz-type inhibitors from this family are bifunctional inhibitors. The specificity of the Kunitz inhibitors varies among the subfamilies of the Fabaceae family. Some Kunitz-type inhibitors isolated from the most primitive species of the Caesalpinioideae subfamily have only activity for trypsin (*42*, *43*, *46*, *48*), and others from the Papilionoideae or Faboideae subfamily contain chymotrypsin inhibitors and are known as Bowman−Birk type inhibitors (*1*, *5*, *54*), but Kunitz-type inhibitors are also found (*33*, *51*, *52*). The K_i for trypsin and papain of PdKI showed patterns similar to those of trypsin−papain Kunitz inhibitors, with higher K_i for trypsin than for papain (*9*, *51*) and competitive to trypsin and noncompetitive to papain. Additional study using molecular modeling and docking experiments with PTPKI, another trypsin−papain Kunitz inhibitor, showed that the bifunctionality was due to the presence of two binding sites for the enzymes and that these sites were overlapping (*7*). This combination of activity against both serine and cysteine proteinases is highly attractive for the development of transgenic crops expressing these inhibitors.

Table 3. Inhibitory Effect of PdKI toward Gut Insect Proteinases

digestive enzymes[a]	inhibition (%)[b]
coleopteran	
C. maculatus	29.18 ± 1.10
Z. subfasciatus	68.87 ± 2.38
lepidopteran	
A. argillaceae	38.36 ± 1.22
P. interpunctella	44.35 ± 1.64
dipteran	
C. capitata	65.53 ± 0.58

[a] Assays against insect proteinases were measured using 1% Azocasein as substrate. [b] Values are mean ± standard error, and each mean represents three replicates.

The amino acid alignment analysis of the N-terminal sequence of PdKI with the Kunitz inhibitors from Mimosoideae *L. leucocephala* (*55*), Caesalpinioideae *C. langsdorffii*, (*56*) and Papilionoideae *P. tetragonolobus* (*57*) seeds showed similarities of 40, 60, and 40%, respectively. The relation between amino acid sequence similarities and enzyme specificities of Kunitz inhibitors could suggest that there is some relationship between this inhibitor family found in Fabaceae seeds and the evolution of these plants, but analysis of the complete amino acid sequence and comparative protein modeling could support this suggestion.

To effectively establish a novel insect control strategy, based on proteinaceous inhibitors, two initial steps are necessary, first

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                          10         20      % Similarity
                           |         |
LlTKI_m       QVLVDLDGDPLYN-GMSYYILL-          40
ClTKI_c       -RLVDTDGKPIENDGAEYYILL-          60
PtTKI_p       EPLLDSEGELVRN-GGTYYLLL-          40
PdKI_m        -GVVLYSVTQVQY-RARYILLG
                :: . : * :*
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Figure 5. Partial sequence of the trypsin inhibitor (PdKI) aligned with other Kunitz family inhibitors. *L. leucocephala*, LlTKI, gi: 18202442, NCBI (*55*). *C. langsdorffii*, ClTKI, gi: 49258682, NCBI (*56*). *P. tetragonolobus*, PtTKI, gi: 124938, NCBI (*57*).

purification of the inhibitor and second the knowledge of the digestive system of target insects. The digestive system of phytophagous pests is based mainly on serine and cysteine proteinase classes, where serine proteinases are the major enzymes found in Lepidoptera and Diptera orders and acid and cysteine proteinases are predominant in Coleopterans of the Bruchidae family (*58*−*61*). To verify the activity of purified PdKI, the digestive proteinases of Coleopteran, Lepdopteran, and Dipteran pests were extracted and assayed against PdKI. Results showed high in vitro inhibitory effect on colepterans *Z. subfasciatus* (68.87%) and dipteran *C. capitata* (65.53%). Moderate inhibitory activity was also observed for gut proteinases of lepidopteran *P. interpunctella* (44.35%) and A. *argillaceae* (38.36%); low inhibitory activity was observed against gut proteinase from coleopteran *C. maculatus* (29.18%). A number of Kunitz-related inhibitors from plants were evaluated in vitro for their potential of decreasing the activity of Lepidopteran/Coleopteran larvae digestive enzymes. SKTI showed a potent in vitro inhibitory activity against *A. grandis* gut proteinases (*21*). SKTI and CpTI were shown to be active against tomato moth larvae (*L. oleracea*). Serine proteinases (*62*) acted on *H. armigera*, *H. virescens*, and *L. cuprina*, in which SKTI was the most effective among the inhibitors tested (*63*−*68*). ApTI and DMTI−II inhibited 84% (*9*) and 80% (*69*) of the digestive trypsin-like enzymes of Coleopteran (Bruchidae) *C. maculatus*. Inhibitors of proteinases have been used successfully for protection of engineered plants. In many cases, transgenic plants containing genes encoding only serine proteinase inhibitors have shown enhanced resistance toward insect pests (*16*, *62*, *68*), but this effect is lost when insect pests develop escape mechanisms.

This work shows a trypsin−papain Kunitz inhibitor active in vitro toward insect pests. These results could indicate that transgenic plants expressing the PdKI gene could probably enhance resistance against potential predators that utilize serine proteinase and cysteine proteinases or might be unable to express cysteine proteinases after exposition at proteinase inhibitors.

ABBREVIATIONS USED

ApTI, *Adenanthera pavonina* trypsin inhibitor; ACTI, *Acacia confusa* trypsin inhibitor; ClTKI, *Copaifera langsdorffii* trypsin Kunitz inhibitor; CpTI, cowpea trypsin inhibitor; DMTI−II, *Dimorphandra mollis* trypsin inhibitor; LlTKI, *Leucaena leucocephala* trypsin Kunitz inhibitor; PDTI, *Peltophorum dubium* trypsin inhibitor; PPTI, *Poecilanthe parviflora* trypsin inhibitor; PTPKI, *Prosopis* trypsin-papain Kunitz inhibitor; PtTKI, *Psophocarpus tetragonolobus* trypsin Kunitz inhibitor; SKTI, soybean Kunitz trypsin inhibitor.

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