Two Kunitz-Type Inhibitors with Activity Against Trypsin and Papain from *Pithecellobium dumosum* Seeds: Purification, Characterization, and Activity Towards Pest Insect Digestive Enzyme

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Abstract: Two trypsin inhibitors (called PdKI-3.1 and PdKI-3.2) were purified from the seeds of the *Pithecellobium dumosum* tree. Inhibitors were obtained by TCA precipitation, affinity chromatography on Trypsin-Sepharose and reversed-phase-HPLC. SDS-PAGE analysis with or without reducing agent showed that they are a single polypeptide chain, and MALDI-TOF analysis determined molecular masses of 19696.96 and 19696.36 Da, respectively. The N-terminal sequence of both inhibitors showed strong identity to the Kunitz family trypsin inhibitors. They were stable over a wide pH (2-9) and temperature (37 to 100 ^{o}C) range. These inhibitors reduced over 84% of trypsin activity with inhibition constant (Ki) of 4.20 x 10^{-8} and 2.88 x 10^{-8} M, and also moderately inhibited papain activity, a cysteine proteinase. PdKI-3.1 and PdKI-3.2 mainly inhibited digestive enzymes from *Plodia interpunctella*, *Zabrotes subfasciatus* and *Ceratitis capitata* guts. Results show that both inhibitors are members of the Kunitz-inhibitor family and that they affect the digestive enzyme larvae of diverse orders, indicating a potential insect antifeedant.

Keywords: *Pithecellobium dumosum*, Leguminosae, Kunitz inhibitor, Pest proteases.

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1. INTRODUCTION

Proteinaceous inhibitors are small protein molecules that have the ability to inhibit the action of target proteolytic enzymes, blocking, altering or preventing access to the active enzyme site. They are present in multiple forms in numerous animal, microorganism and plant tissues [1]. In plants, the proteinase inhibitors are one of the most abundant protein classes in vegetative tissues, reproductive organs and storage organs of a wide range of both dicotyledonous and monocotyledonous plants [2,3]. Plant proteinase inhibitors are important in a variety of ways, including as storage proteins [4], as regulators of endogenous proteolytic activity [5], and as participants in mechanisms of programmed plant cell death [6]. In addition, proteinase inhibitors are expressed as a response to abiotic stress [7] and in plant defense processes against insect attacks [8,9].

Proteinaceous inhibitors are classified as serine, cysteine, aspartic and metalloprotease inhibitors. Legume seeds contain various proteinase inhibitors, classified into several families such as Kunitz, Bowman-Birk, potato I, potato II, squash, cereal super family and thaumatin-like among others [10]. The two best characterized families of plant serine proteinase inhibitors are the Kunitz and Bowman–Birk family inhibitors. Bowman–Birk family inhibitors have lower molecular mass (8–10 kDa), high cysteine content and two reactive sites. In contrast, Kunitz family inhibitors have a molecular mass of 18–32 kDa [11-13], one or two polypeptide chains [14-16], low cysteine content (usually with four Cys residues in two disulfide bridges) and one reactive site [17].

They are found in the Solanaceae family and in all Leguminosae subfamilies: Mimosoideae, Caesalpinoideae, and Papilionoideae. The Mimosoideae usually contain proteinase inhibitors formed by two polypeptide chains linked by a disulphide bridge [13,15], thus differing from the other Kunitz-type single-chain inhibitors from the Caesalpinoideae and Papilionoideae subfamilies [18].

Proteinase inhibitors have received an enormous amount of attention for their ability to inhibit insect digestive proteinases [11,12,19] and suppress growth and development when they are fed to insects [15,20]. A number of Kunitz trypsin inhibitors were capable of inhibiting the proteolytic activity of several lepidopterans, such as *Anagasta kuehniella* [19], *Alabama argillacea* [11,12], *Spodoptera litura* [21] and coleopterans such as *Callosobruchus maculatus* [22,20] and *Zabrotes subfasciatus* [11,12].

This study reports on the purification and characterization of two similar Kunitz inhibitors from *P. dumosum* seeds and the analysis of their *in vitro* effects on larval digestive

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enzymes from lepidopterans *A. argillacea* and *Plodia interpunctella*, coleopterans *C maculatus* and *Z. subfasciatus*, and the dipteran *Ceratitis capitata*.

2. MATERIAL AND METHODS

2.1. General Experimental Procedures

Seeds from *P. dumosum*, Bovine serum albumin (BSA), bovine pancreatic trypsin (E.C. 3.4.21.4), bovine pancreatic chymotrypsin (E.C. 3.4.21.1), pig pancreatic elastase (E.C. 3.4.21.36), papain (E.C. 3.4.22.2) from Papaya (*Carica papaya*) latex, bromelain (E.C. 3.4.22.32) from pineapples (*Ananas comosus*) stems, BApNA (Nα-benzoyl-DL-arginine-p-nitroanilide), BANA (Nα-Benzoyl-DL-arginine-2-naphthylamide), azocasein and trichloroacetic acid (TCA) were purchased from Sigma (St. Louis, MO, USA). Protein molecular weight markers were purchased from Fermentas Life Science. All other chemicals and reagents used were of analytical grade.

2.2. Purification Procedure for PdKI-3.1 and PdKI-3.2 from *P. dumosum* Seeds

P. dumosum seeds were obtained from the seed bank of IBAMA (Brazilian Environmental Institute of Natural and Renewable resources) in Natal, Brazil. Purification procedure for PdKI-3.1 and PdKI-3.2 followed methodology developed by Oliveira *et al.* [23]. The two purified Kunitz-type trypsin inhibitors, PdKI-3.1 and PdKI-3.2, were subjected to further analysis.

2.3. Protein Quantification

Protein contents were estimated by Bradford's dye-binding method [24] using bovine serum albumin (BSA) as the standard.

2.4. Assay of Inhibitory Activity

2.4.1. Trypsin Inhibitory Assay

Trypsin activity was determined using BApNA as substrate at pH 7.5 as described by Erlanger *et al.* [25]. The dissociation constant (*K*i) was determined for bovine trypsin as described by Cheng and Prusoff [26].

2.4.2. Papain Inhibition Assay

The papain inhibitory assay was determined as described by Zhao *et al.* [27] using BANA as substrate.

2.4.3. Specificity Toward Serine and Cysteine Proteinases

The ability of PdKI-3.1 and PdKI-3.2 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.* [28].

2.5. Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis (SDS–PAGE) in the absence and presence of β-mercaptoethanol (0.1 M) was carried out as described by Laemmli [29]. Proteins were detected by silver development [30].

2.6. N-Terminal Sequencing

N-terminal amino acid sequence analysis of PdKI-3.1 and PdKI-3.2 was performed at the Department of Biochemistry and Immunology, ICB, UFMG, Brazil, using an automated protein sequencer from Shimadzu PSSQ-21A. The amino acid sequences had been submitted to automatic alignment, which was performed using FASTA and the NCBI-Blast search system.

2.7. Mass Spectrometry

The molecular mass of PdKI-3.1 and PdKI-3.2 was determined at the Department of Biochemistry and Immunology, ICB, UFMG, Brazil using an Ultraflex II Matrix-assisted laser desorption time-of-flight spectrometer (MALDI-TOF/TOF) from Bruker Daltonics, Billerica, MA.

2.8. Thermal and pH Stability of PdKI-3.1 and PdKI-3.2 Against Bovine Trypsin

The thermal stability of PdKI-3.1 and PdKI-3.2 (1 ug/uL) was tested by incubating protein at different temperatures (37, 40, 60, 70, 90, and 100 °C) for 30 min as described by Gomes *et al.* [12].

2.9. *In Vitro* Effects on Digestive Enzymes from Insect Pests

2.9.1. Preparation of Insect Gut Proteinases

C. maculatus, *Z. subfasciatus*, and *P. interpunctella* were supplied by Department of Biochemistry, UFRN, Brazil. *A. argillacea* was obtained from the Center for Cotton Research (CNPA/EMBRAPA), Campina Grande, Brazil, and *C. capitata* was obtained from Department of Cellular Biology and Genetics, UFRN, Brazil. 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 was homogenized, centrifuged and the supernatants were used for *in vitro* assays.

2.9.2. Inhibitory Assay against Proteinases from Insect Pests

The effects of PdKI-3.1 and PdKI-3.2 on the proteolytical activity of whole gut extracts were measured using 1% azocasein as substrate, as described by Xavier-Filho *et al.* [28]. All assays were done in triplicate. The results of each series were expressed as the mean value ± SD.

3. RESULTS AND DISCUSSION

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 anti-tryptic activity (data not shown). The anti-tryptic peak was then submitted to a reverse-phase high performance liquid chromatography (HPLC), and the elution profile (Fig. **1A**) showed separation of four protein peaks (named JB1, JB2, JB3, and JB4) with strong inhibitory activity against trypsin. The JB3 protein fraction was rechromatographed by reversed phase HPLC column (Fig. **1B**), and two peaks with strong anti-tryptic

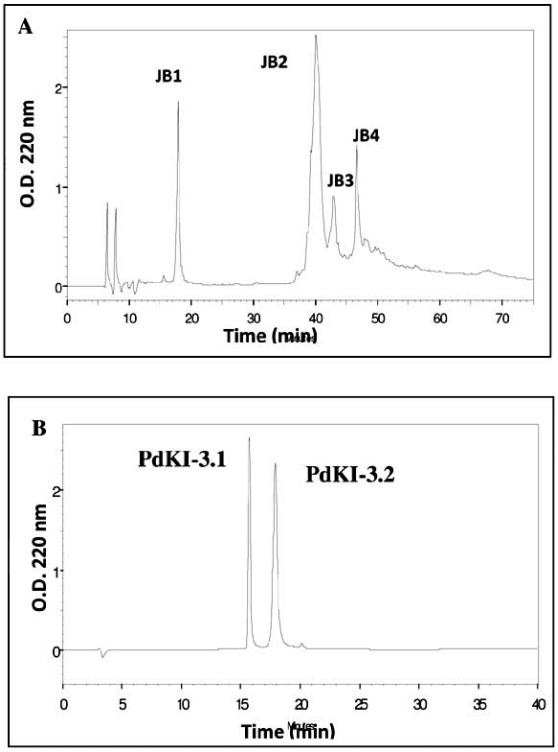

Figure 1. (A) Elution profile of JBAf on HPLC (Vydac C-18) column. The fractions obtained from the trypsin-sepharose column were separated by a semipreparative (2.2 x 25.0 cm) reverse-phase HPLC column at a flow rate of 9 mL/min. The elution of the JBAf fraction revealed four protein peaks named JB1, JB2, JB3, and JB4. **(B)** The JB3 fraction was then subjected to another analytical (0.46 x 25.0 cm) reverse-phase HPLC column at a flow rate of 1 mL/min, and two protein peaks eluted with 40.8 and 42% acetonitrile were purified and named of PdKI-3.1 and PdKI-3.2, respectively.

activities, named PdKI-3.1 and PdKI-3.2, eluted with 40.8 and 42.0% acetonitrile, respectively, were purified. SDS-PAGE (Fig. **2A**) in the absence and presence of β-mercaptoethanol showed a single protein band and molecular mass of 19697.96 and 19696.36 Da, respectively, was determined by MALDI-TOF analysis. The three peaks in the MALDI-TOF graphs represent different charged variants of the same protein, with charges of 1+, 2+, and 3+ (Fig. **2B** and **2C**). The molecular masses are in general agreement with those found for Kunitz-type trypsin inhibitors with one or two polypeptide chains [13,15,16, 31]. Inhibitors of the subfamily Mimosoideae belonging to the Leguminosae family generally have two polypeptide chains linked by disulphide bridges [15]. However, few inhibitors of this subfamily are formed by one polypeptide chain; among them are inhibitors purified from the seeds of *Dimorphandra mollis* [14,32] and *Inga laurina* [16], in agreement with the results observed for PdKI-3.1 and PdKI-3.2, which also have only one polypeptide chain confirmed by SDS-PAGE under reducing conditions. The methodological procedure resulted in 106.5-fold purification with a 1.84% yield for PdKI-3.1 and 156.1-fold purification with a yield of 1.79% for PdKI-3.2 (Table **1**).

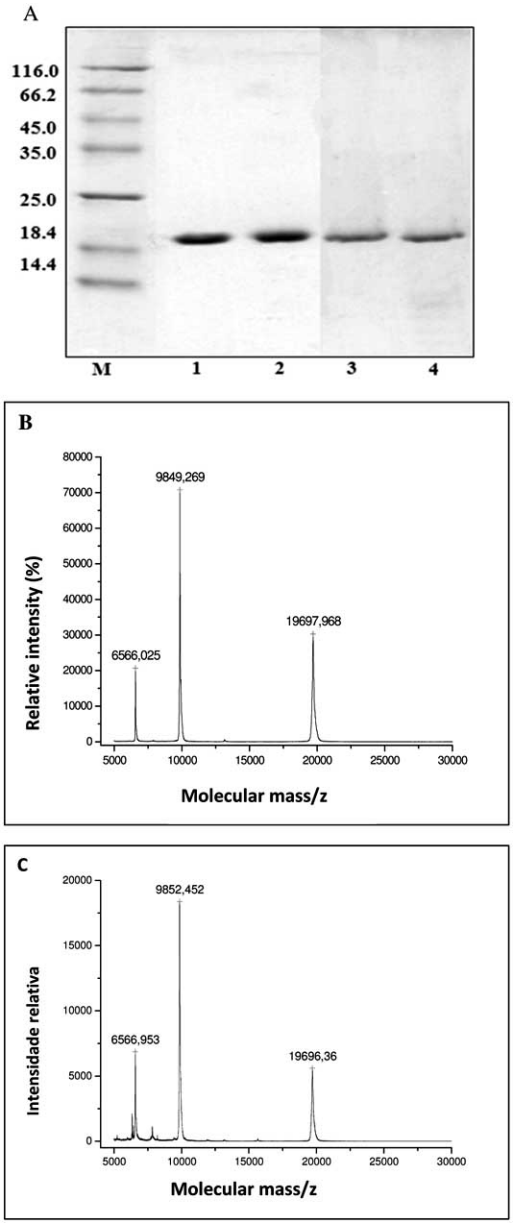

Figure 2. (A) SDS-PAGE (15%) of trypsin inhibitors purified from *P. dumosum* seeds, stained with Coomassie followed by silver revelation. (M) Standard proteins: *β*-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate degydrogenase (35.0 kDa), restriction endonuclease Bsp981 (25.0 kDa), *β*-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa). (1) PdKI-3.1 and (2) PdKI-3.2 obtained of reverse-phase HPLC column without reduction, (4) PdKI-3.1 and (5) PdKI-3.2 reduced with *β*-mercaptoethanol. MALDI-TOF/TOF spectrum of PdKI-3.1 **(B)** and PdKI-3.2 **(C)**.

The alignment of the N-terminal amino acid sequence of PdKI-3.1 and PdKI-3.2 with other Kunitz-type trypsin inhibitors from the subfamily Mimosoideae shows strong similarity (Fig. **3A** and **3B**). It shows over 73% sequence similarity with *Prosopis juliflora* trypsin-papain, PjTKI [22,33], *Adenanthera pavonina*, ApTKI [34], *Leucaena leucocephala*, LITKI [18], *Acacia confusa*, AcTKI [35] and *Enterolo-*

Table 1. Purification Steps of PdKI-3.1 and PdKI-3.2 from *P. dumosum* Seeds

Steps	Total Inhibitory Units (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	118560	1041.2	113.9	1.0	100
JB14	80800	18.0	4488.9	39.4	68
JBAf	8190	1.8	4653.4	40.9	6.9
PdKI-3.1	2183	0.18	12127.7	106.5	1.84
PdKI-3.2	2133	0.12	17775.0	156.1	1.79

One trypsin protein inhibitor unit (1 IU) was defined as the amount of inhibitor that decreased absorbance at 410 nm by 0.1 OD under the trypsin assay conditions.

bium contortisiliquum, EcTKI [13] inhibitors. In addition, 93% similarity was observed between the sequences of PdKI-3.1 and PdKI-3.2 (Fig. **3C**). These results indicate that the inhibitors purified here may be members of the Kunitz family.

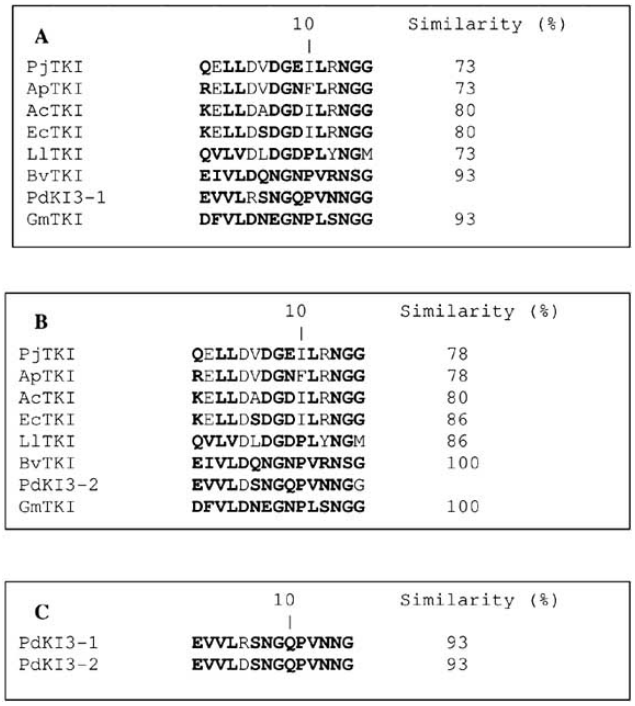

Figure 3. Partial sequence of PdKI-3.1 **(A)** and PdKI-3.2 **(B)** aligned with other Kunitz family inhibitors: *Prosopis juliflora* (PjTKI, gi: 243386, NCBI), *Adenanthera pavonina* (ApTKI, gi: 225058, NCBI), *Acacia confusa* (AcTKI, gi: 166234, NCBI), *Enterolobium contortisiliquum* (EcTKI, Batista *et al.*, 1996), *Leucaena leucocephala* (LlTKI, gi: 18202442, NCBI), *Bauhinia variegate* (BvTKI, gi: 5082208, NCBI) and *Glycine max* (GmTI, gi: 18770, NCBI). **(C)** Similarity between PdKI-3.1 and PdKI-3.2.

The functional stability of most Kunitz-type trypsin inhibitors in the presence of physical and chemical denaturants such as temperature and pH is generally associated with intramolecular disulfide bridges [36]. The study of the temperature effect on PdKI-3.1 and PdKI-3.2 showed a significant decrease in inhibitory activity when pre-incubated for 30 min at temperatures between 37-100 °C. However, the pre-incubation of PdKI3-2 at 100 °C led to a 30% decrease in its activity (Fig. **4A**). Pre-incubation of the inhibitors in the pH range (2.0–9.0) for 30 min did not affect trypsin inhibitory activity and over 65% of its activities were maintained after pre-incubation in pH 12 (Fig. **4B**). The high degree of thermal stability and pH amplitude observed for these inhibitors has also been observed for Kunitz-type inhibitors of seeds from *E. contortisiliquum* [13], *Archidendron ellipticum* [37], *Peltophorum dubium* [15], *Schizolobium parahyba* [38] and *Poecilanthe parviflora* [19].

The serine proteinases are differentially inhibited by Kunitz-type inhibitors. Among the few that inhibit only elastase is the glycosylated protein from *Bauhinia rufa*, named gBrEI [39]. Some inhibitors are specific for chymotrypsin and do not inhibit trypsin [40,41,], however, others are potent inhibitors of chymotrypsin but also inhibit trypsin to varying degrees [15]. In addition, other members of this family inhibit preferentially trypsin but also inhibit chymotrypsin to varying degrees [12,13]. The structural compatibility of the reactive site of the proteinase inhibitor with the target proteinase substrate-binding site reflects the efficacy of these inhibitors. Table **2** shows the *in vitro* inhibition of the inhibitors purified here against serine proteinases. PdKI-3.1 and PdKI-3.2 strongly inhibit bovine trypsin with dissociation constants of 4.20×10^{-8} and 2.88×10^{-8} M, respectively. The dissociation constant establishes a higher affinity between the trypsin and PdKI-3.2 than with PdKI-3.1 and is in agreement with Ki values reported for other trypsin inhibitors [18,42,43]**.** PdKI-3.2 and PdKI-3.1 also weakly affected the activity of the bovine chymotrypsin but showed no activity against elastase. These results reflect greater affinity of inhibitors with trypsin than with chymotrypsin and elastase. Interestingly, both inhibitors showed greater affinity for papain, a cysteine proteinase, than for serine proteinases such as chymotrypsin and elastase. The specificity assays for PdKI-3.1 and PdKI-3.2 showed that they had no effect on bromelain activity but a moderate affect on papain activity. The inhibition of trypsin and papain was also reported for a few members of the Mimosoideae subfamily, such as those purified from *P. juliflora* [22,33], *Crotalaria pallida* [12], *A. pavonina* [20] and others two trypsin-papain inhibitors recently purified from *P. dumosum* seeds [23,44].

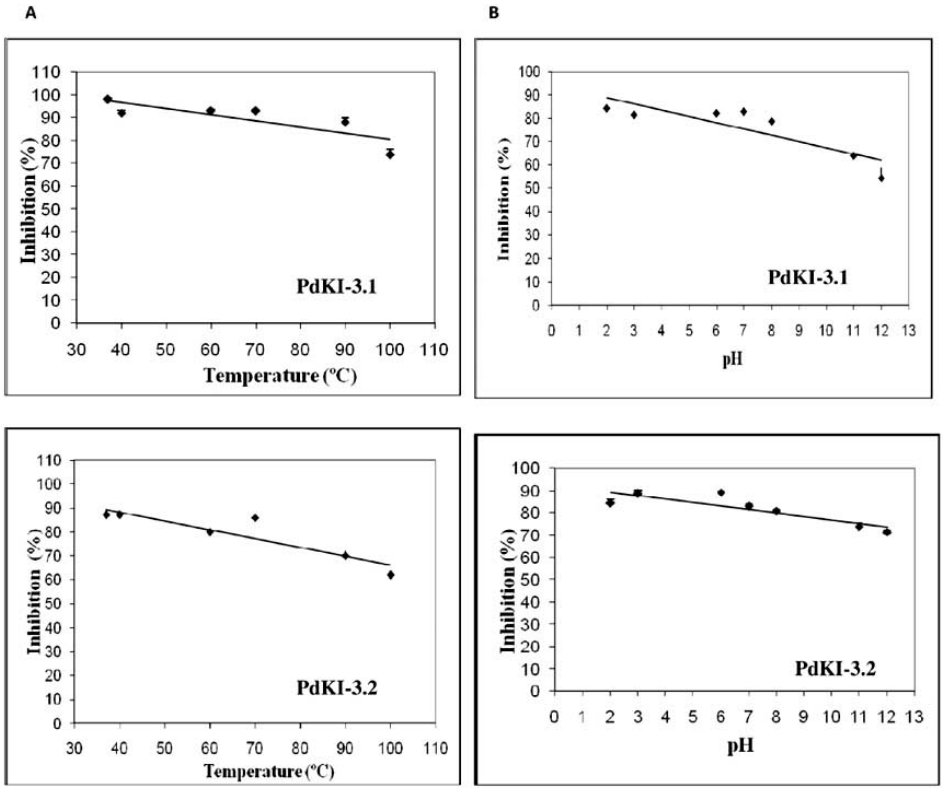

Figure 4. (A) Temperature stability of PdKI-3.1 and PdKI-3.2 after incubation for 30 min at the indicated temperatures. **(B)** pH stability of PdKI-3.1 and PdKI-3.2 after incubation for 30 min at 37 ^{0}C. Each mean represent three replicates (± S.E.).

Table 2. Specificities of PdKI-3.1 and PdKI-3.2 to Serine and Cysteine Proteinases from Diverse Sources

Enzymes [a]	PdKI-3.1 Inhibition (%) [b]	PdKI-3.2 Inhibition (%) [b]
Serine proteinases		
Bovine trypsin	84.30 ± 0.86	89.17 ± 1.89
Porcine elastase	1.53 ± 0.18	2.36 ± 0.28
Bovine chymotrypsin	8.38 ± 0.54	15.33 ± 0.62
Cysteine proteinases		
Bromelain	1.24 ± 0.29	1.16 ± 0.23
Papain	35.86 ± 0.24	32.93 ± 0.10

[a]Assays against elastase porcine, chymotrypsin bovine and bromelain were measured using 1% azocasein as substrate, the inhibitory assays against trypsin and papain were measured using BApNA and BANA as substrate, respectively. [b]Values are mean ± standard error, and each mean represent three replicates.

Phytophagous Lepidopteran and Dipteran larvae use serine proteinase, usually with high pH optima, as their major digestive enzymes [9,45,46]. On the other hand, most Coleopteran larvae of the Bruchidae family use cysteine proteinase, which exhibits optimum pH activity with acidic pHs [47,48], although serine proteinases are also present in this family of insects, contributing to a lesser extent to the digestion of diet proteins [49]. In addition, proteinaceous inhibitors are of interest as potential sources of resistance against pests and pathogens in transgenic plants. The defensive role of these inhibitors has been well established owing to their inhibitory activity against the digestive enzymes of pests and pathogens. These proteins also affect survival and/or larval development when incorporated into the artificial diet of many phytophagous insects [50,51]. To assess the hypothesis that the inhibitors purified here are involved in plant defense mechanisms and can protect plants against insect pests, we performed *in vitro* inhibitory assays on the trypsin-like digestive enzymes extracted from the larval midgut of Coleopterans (*Z. subfasciatus*, *C. maculatus*), Lepidopterans (*P. interpunctella*, *A. argillaceae*) and Dipterans (*C. capitata*). *C. maculatus* and *A. argillaceae* activity was relatively unaf-

Table 3. Specificities of PdKI-3.1 and PdKI-3.2 Towards Insect Digestive Enzymes

Enzymes [a]	PdKI-3.1 Inhibition (%) [b]	PdKI-3.2 Inhibition (%) [b]
Coleopteran		
C. maculatus	7.98 ± 1.93	5.17 ± 0.39
Z. subfasciatus	67.33 ± 1.51	56.93 ± 1.33
Lepidopteran		
A. argillaceae	15.52 ± 1.83	10.15 ± 1.53
P. interpunctella	49.00 ± 0.71	37.59 ± 1.33
Dipteran		
C. capitata	36.11 ± 1.35	29.44 ± 1.39

[a]Assays against insect trypsin-like digestive enzymes were measured using 1% azocasein as substrate. [b]Values are mean ± standard error, and each mean represent three replicates.

fected by the inhibitors, whereas the digestive enzymes from *C. capitata*, *P. interpunctella* and *Z. subfasciatus* were clearly inhibited, however, the *Z. subfasciatus* enzymes were more susceptible (Table **3**). Our results show compatibility between the reactive site of PdKI-3.1 and PdKI-3.2 and the active site of trypsin-like digestive enzymes from *C. capitata*, *P. interpunctella* and particularly Z. *subfasciatus*, suggesting that both PdKI-3.1 and PdKI-3.2 can exert harmful effects on these insects.

In conclusion, the potent inhibitory activity against trypsin, molecular mass and amino acid sequence similarity consistent with that of Kunitz-type trypsin inhibitors is a strong indication that PdKI-3.1 and PdKI-3.2 purified from *P. dumosum* seeds are the newest members of this inhibitor family. The *in vitro* insecticidal properties of these inhibitors suggest that they may affect the growth and/or survival of these insect pests when incorporated into their diet.

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