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Isolation and Properties of a Kunitz-Type Protein Inhibitor Obtained from *Pithecellobium dulce* Seeds

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We report for the first time the isolation and characterization of a protease inhibitor from the seeds of *Pithecellobium dulce*, which is a Leguminosae tree native to Mexico. The purification of the *P. dulce* trypsin inhibitor (PDTI) was a direct process. After its extraction (pH 8.0) and precipitation (80% $(NH_4)_2SO_4$), the pH was adjusted to 4.0, the supernatant was loaded onto a CM-Sepharose column, and a single peak of trypsin inhibitory activity was eluted (CM-TIA). The main component of CM-TIA was PDTI, a protein composed of two polypeptide chains joined by disulfide bridge(s), with a pI of 4.95 and a molecular weight determined by electrospray mass spectrometry of 19 614 Da. The N-terminal sequence of PDTI has the highest similarity with the seed inhibitor of *Acacia confusa.* PDTI lacks chymotrypsin inhibitory activity. A low rate of cytotoxicity of CM-TIA toward RINm5F cells contrasted with a high rate of the active fraction G75-TIA (gel filtration chromatography; LC_{50} of 0.04 mg/mL).

KEYWORDS: ***Pithecellobium dulce*; seed protein; cytotoxicity; Kunitz inhibitor; trypsin inhibitor**

INTRODUCTION

More than 60% of the Mexican territory suffers a severe edaphic degradation, and a large number of plant species are endangered (*1*, *2*). Mexico is homeland to many potential woody legumes; however, the lack of information about the properties of these legumes (e.g., agronomical, biological, nutritional) limits their use (*3*).

Pithecellobium dulce is a woody legume native to Sinaloa, in the NW of Mexico, where it is known as "guamuchil". Guamuchil tree has been used for fencing and tanning, fodder for feed, and pods for food. Infusions of different parts of guamuchil have been used traditionally to treat diseases, such as skin of the stem for dysentery, leaves for intestinal disorders, and seeds for ulcers, among others (*4*−*6*). Such ethnopharmacological properties have not been yet demonstrated. On the other hand, the powders and methanolic and aqueous extracts of *P. dulce* seeds have proved fungistatic and possess fungicidal effects against plant pathogens (*7*). Preparations of seeds of *Pithecellobium* spp. have shown interesting biological activities. A saponin contained in *P. racemosum* and other legume seeds has shown cytotoxic activity against cancer cell lines of ovaries (A2780) and lungs (M109) (*8*, *9*).

Despite seeds of *Pithecellobium* spp. having high contents of protein, dietary fiber, and unsaturated fatty acids, they are not commonly consumed by Mexicans (*10*−*13*).

Legume seeds also contain antinutritional compounds such as the protease inhibitors Bowman−Birk (MW ca. 8−9 kDa) and Kunitz (MW ca. 21 kDa) (*14*). Protein inhibitory activity has been registered for the seeds of several *Pithecellobium* spp. Trypsin inhibitory activity in *P. keyense* was higher than that described for soybean (*15*, *16*). The inhibitory activity identified in extracts of *P. dulce* seeds was against trypsin, chymotrypsin, and papain. However, there is a lack of information of the inhibitory proteins involved.

Protease inhibitors have shown biological effects ranging from antinutritional to beneficial (*12*, *17*−*19*). Pea seeds have protease inhibitors, with antifungal activity, which are secreted during germination, indicating a protective role of inhibitors (*20*). In evaluations with animal models and cell lines, the Bowman-Birk protease inhibitor of soybeans has showed anticarcinogenic properties; it has been suggested that an ca. 30 mg per food serving of chymotrypsin inhibitor is expected to protect against several forms of human cancer. The anticarcinogenic activity has been associated with a selective toxicity of Bowman-Birk inhibitors against premalignant (e.g., 10T1/2 myc and 308) or malignant cells (LNCaP), not being toxic to normal cells (*21*).

P. dulce seeds contain beneficial and antinutritional compounds that must be carefully evaluated before their introduction either as a meal or as a feed source. Despite its traditional use,

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P. dulce is underused in Mexico due to a lack of information about its beneficial properties. In this paper, we are reporting for the first time the isolation and characterization from *P. dulce* seeds of a protease inhibitor of the Kunitz family.

MATERIALS AND METHODS

Mature fruits of guamuchil (*P. dulce*) were collected from the NW of Sinaloa. The plant material was identified by Dr. Rito Vega-Aviña, and a voucher specimen (var 1455) was deposited in the herbarium of the Faculty of Agronomy, Autonomous University of Sinaloa. Pods were lyophilized without peel, the pericarp was manually removed, and the seeds were stored at 4 °C.

Sephadex G-75 and CM-Sepharose were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). All reagents for electrophoresis were obtained from BioRad (Mexico). Crude extracts of larval enzymes of *Prostephanus truncatus* were extracted as previously described (*22*). Bovine trypsin (EC 3.4.21.19) and bovine chymotrypsin (EC 3.4.21.1), as well as BAPNA, BAEE, SAAPLpNA, and SAAPPheNA, were supplied by Sigma (Mexico). RPMI-1640 medium, fetal bovine serum, trypsin-EDTA, penicillin, and streptomicin solutions were obtained from Gibco (Invitrogen Life Technologies, Carlsbad, CA), and a cell proliferation kit I (MTT) was obtained from Boehringer-Mannhein (Roche Diagnostic Corp.). All chemicals used were analytical grade.

Inhibitor Extraction. *Pithecellobium dulce* seeds were ground into a fine powder using a Terkman-A10 (Jankel & Kunkel, Willington, Germany) mill. The powder was sieved through a 0.875 μm screen and was stored at −20 °C.

The powder (5 g) was extracted by stirring overnight at 4 °C with 50 mL of 0.01 M Tris-HCl buffer pH 8.0, containing 0.01 M EDTA, and 1.5% PVP. The suspension was centrifuged at 16000*g* for 1 h. The supernatant was brought to 80% saturation with ammonium sulfate and centrifuged as above. The precipitate was dissolved in water and dialyzed with a Spectra/Por 3500 membrane.

Gel Filtration Chromatography. The dialyzate was fractionated on a 1.6 × 160 cm Sephadex G-75 chromatography column, equilibrated with water. Fractions containing trypsin inhibitor activity were pooled and lyophilized.

Cation-Exchange Chromatography. A fraction of dialyzed ammonium sulfate was adjusted to pH 4.0 with acetate buffer (0.02 M sodium acetate, pH 4.0) and clarified by centrifugation for 30 min at 15 000*g*. The supernatant was applied on a 1.7 × 24 cm CM-Sepharose column, equilibrated with 0.02 M sodium acetate buffer pH 4.0. The column was eluted with a linear gradient of NaCl (0.3−1 M NaCl in 0.02 M sodium acetate buffer pH 4.0) at a flow rate of 0.4 mL/min. Fractions containing trypsin inhibitor activity were pooled, dialyzed, and lyophilized.

Reverse-Phase HPLC. The lyophilized active fractions obtained from G-75 Sephadex and CM-Sepharose chromatography were separated by reverse-phase HPLC using a model 1100 Hewlett-Packard system and a Vydac C18 column (i.d. 4.6 mm, length 250 mm with 5-μm particle size). The two solvents used were 0.1% (v/v) TFA in water (solvent A) and 80% (v/v) acetronitrile in water (solvent B). During elution (flow rate 5.0 mL/min), we used a linear gradient, in which the solvent composition changed from 20% to 100% B (v/v) in 120 min. The eluent was monitored at 220 nm, and the active fractions were collected and lyophilized.

Enzyme Inhibition Assay. The crude extract of *P. truncatus* contains a mixture of enzymes. Hence, we used it to evaluate several enzyme activities. The purification of PDTI was followed using inhibitory activity against a crude extract. The inhibitory specificity of PDTI is reported only against purified commercial enzymes.

The inhibition of trypsin and trypsin-like activities was monitored according to the method of Schwert and Takenaka (*23*). We observed the rate of hydrolysis of BAEE at 253 nm.

Inhibitory activities of chymotrypsin and elastase-like enzymes were determined by a microplate assay using SAAPPhepNA (7.5 mg/mL) dissolved in DMSO and SAAPLpNA (7.09 mg/mL) dissolved in DMF as substrates, respectively. For the standard assay, 265 μL of 0.1 M Tris-HCl pH 8.0 and 10 μL of enzyme (100 μg/mL) were preincubated with an aliquot of the inhibitor preparation during 10 min at 30 °C. Afterward, 25 μL of substrate was added. This mixture was incubated for 10 min at 30 °C, and the plate was read at 405 nm with a Multiskan Plus microplate reader (BioRad). One proteinase unit was defined as the amount of enzyme that causes a change in absorbance of 0.01 units/min, under the assay conditions. The inhibitory activity was measured by the difference in enzyme activity with and without the presence of the inhibitor.

Electrophoresis. Samples from the different purification steps were analyzed by SDS-PAGE according to Shagger and Von Jagow (*24*) using a 13% acrylamide resolving gel. Proteins were detected by silver staining (*25*). In the experiment to demonstrate the presence of subunits joined by disulfide bridges, SDS-PAGE was carried out in the presence or absence of β-mecaptoethanol. Under reducing conditions, the sample buffer was added with 5% β-mercaptoethanol and the mixture was maintained in boiling water for 5 min.

Isoelectric Point. The isoelectric point of the purified trypsin inhibitor was performed on precast isoelectric focusing gels (pH 3−10). Sample preparation, isoelectrofocusing, and gel staining were carried out following instructions provided by Pharmacia using the Phast System Unit (Pharmacia, Piscataway, NJ).

Electrospray Mass Spectrometry. The molecular weight of the purified trypsin inhibitor was obtained using a Q/TOF micro electrospray mass spectrometer (Micromass). A solution (200 ng/μL) was prepared by dissolving the inhibitor in water/methanol/formic acid (49/49/2 v/v). Next, 50 μL was injected into the electrospray ion source by syringe infusion at a flow rate of 10 μL/min. The mass spectrometer was operated in the positive ion mode. The electrospray interface was operated using the following settings: capillary voltage, 3000 V; source temperature, 100 °C; sample cone voltage, 45 V. Nitrogen was used as the nebulizing and drying gas. Calibration was performed using myoglobin, 10 pmol/μL. The spectra from 120 scans were averaged, and the resultant spectra were used for analysis. The molecular mass was determined from the multiple charge ions using Maxentropy 1 software (MassLynx 3.5, Micromass).

Protein Assays. The protein concentration of the purified inhibitor fractions was determined by the BioRad microassay, using serum albumin as standard. The concentration of protein of all fractions eluted during the different chromatographic procedures was estimated by measuring the absorbance at 280 and 220 nm.

Cell Culture and Viability Assay. RINm5F cells were cultured as a monolayer in RPMI-1640 medium, supplemented with 5% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator with 95% O_2−5% CO_2 at 37 °C. Cells were plated at a density of 2×10^5 per mL in 96-well plates.

After 3 days, the fractions with trypsin inhibitor activity purified either by Sephadex G-75 (G75-TIA) or by CM-Sepharose chromatography (CM-TIA) were dissolved in distilled water, added to the culture, and incubated for 24 h. At the end of the incubation, the viability of the cells was determined using a cell proliferation kit (MTT based) according to the instructions of the manufacturer. The optical density of each well was measured at 595 nm using an automatic multiwell microplate spectrometer (BioRad). The viability of the control cells was considered to be 100%. For treated cells, the viability was expressed as the percentage of control cells. Each concentration for both fractions was tested in four replicates and was repeated three times in separate experiments.

All data are expressed as mean ± standard deviation (SD). Differences between control and treated cells were analyzed using the Student's t test. A $p < 0.05$ value was considered significant. The LC_{50} was obtained from the concentration-response through statistical software (Origin, Microcal Software Inc., Northampton, MA).

RESULTS

The inhibitory activity of the crude extract of *P. dulce* seeds was assayed against a crude preparation of enzymes from *P. truncatus*, showing the following results: trypsin-like, 3759 IU/mg; chymotrypsin-like, 517 IU/mg; and elastase-like, 1743 IU/mg. The crude extract of *P. dulce* also showed activity against the bovine enzymes: trypsin (6076 IU/mg) and chymotrypsin (846 IU/mg). The inhibitory activity against trypsin was higher,

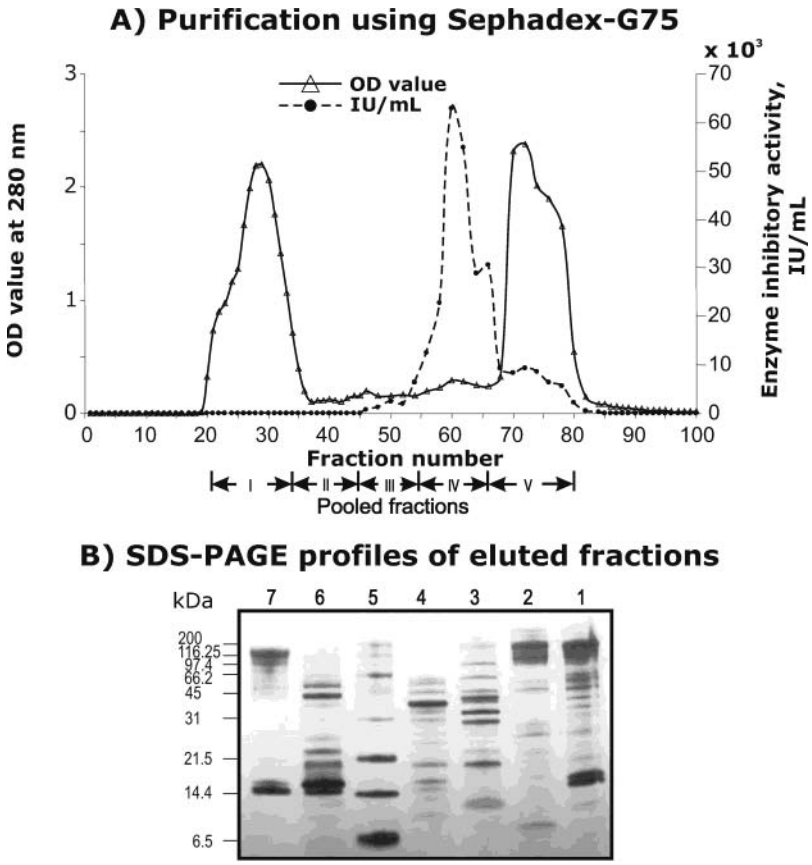

Figure 1. (**A**) Purification of an enzyme inhibitor from *Pithecellobium dulce* seeds using Sephadex G-75 chromatography. Fractions were pooled by considering the pattern of protein peaks (Roman numbers). The enzyme inhibitor was obtained from the eluted fractions from 56 to 66 (G75-TIA), as indicated by bar IV. (**B**) The profiles of SDS-PAGE of fractions obtained as indicated above: (1) raw fraction obtained by precipitation with 80% saturation $(NH_4)_2SO_4$; (2) pool I; (3) pool II; (4) pool III; (5) MW markers; (6) pool IV, active fraction; (7) pool V.

and it was used to follow the purification of the corresponding inhibitor. Most of the trypsin inhibitory activity from the crude extract was recovered by precipitation with $(NH_4)_2SO_4$ (80%, w/v). The desalted precipitate was then passed either through a Sephadex G-75 or through a CM-Sepharose column.

The purification of the *P. dulce* inhibitor (PDTI) involving gel filtration showed two major peaks of activity, pools IV and V (**Figure 1A**). Pool IV had the highest trypsin inhibitory activity, and it was named G75-TIA; this activity was associated with one of the lowest peaks of protein (OD_{280}). The active pool obtained from gel filtration showed several bands in SDS-PAGE. Such bands are in the MW range of proteins with inhibitory activity against trypsin (**Figure 1B**).

When CM-Sepharose chromatography was used, only one peak of trypsin inhibitory activity (CM-TIA) (**Figure 2A**) was eluted at 0.55 M NaCl, corresponding to a single band in SDS-PAGE (**Figure 2B**), and a MW of approximately 21 kDa. The CM-TIA fraction peak was collected.

The efficiency of the ion-exchange strategy was also evaluated by reverse-phase liquid chromatography (RP-HPLC). The CM-TIA active fraction was lyophilized and separated by RP-HPLC. We obtained a significant peak of protein, eluting at 51.6% (v/v) acetonitrile (**Figure 3A**). This peak remains significant in the G75-TIA fraction, but other compounds are notorious (data not shown). The RP chromatography showed that ion-exchange separation is the best strategy for the purification of this specific trypsin inhibitor. The active peak was collected and named PDTI.

PDTI shows a single band in SDS-PAGE, but two bands appeared after β-mercaptoethanol reduction, indicating that it has two polypeptide chains (**Figure 3B**). Through isolectric focusing determination, the PDTI obtained from CM-TIA showed a single band with a pI of 4.95 (**Figure 3C**, lane 3). The molecular weight of PDTI evaluated by electrospray mass spectrometry was 19 614 Da.

Table 1. Purification Scheme for the Kunitz Inhibitor of *P. dulce* Seeds

protein fraction	inhibitory specific activity[a] (IU/mg)	recovery number[b]
crude extract[c]	7962	1
precipitated with 80% $(NH_4)_2SO_4$	12 279	1.5
precipitated at pH 4.0	30 161	3.8
active fractions of CM-Sepharose	107 133	13.5
active fraction of RP-HPLC	114 200	14

[a] Inhibitory activity against trypsin of *P. truncatus* using BapNa. [b] Expressed as the increment in the inhibitory specific activity relative to the crude extract. [c] Obtained from 5 g of *P. dulce* seeds in dry weight basis.

The best purification scheme is shown in **Table 1**. First, we eliminated the precipitate formed by adjusting the pH at 4.0. We then applied CM-Sepharose chromatography. Both processes are major stages to eliminate a large quantity of impurities; the specific activity increased 3.8 and 14 times relative to the initial extract, respectively. The *P. dulce* seeds had large amounts of PDTI; our studies were carried out only with the inhibitor extracted from 5 g of milled seeds.

Purified PDTI was assayed against proteases from different sources. PDTI inhibits trypsin (54 333 IU/mg) but not chymotrypsin from bovine, even though chymotrypsin inhibitory activity was detected in the crude extract (846 IU/mg). The PDTI

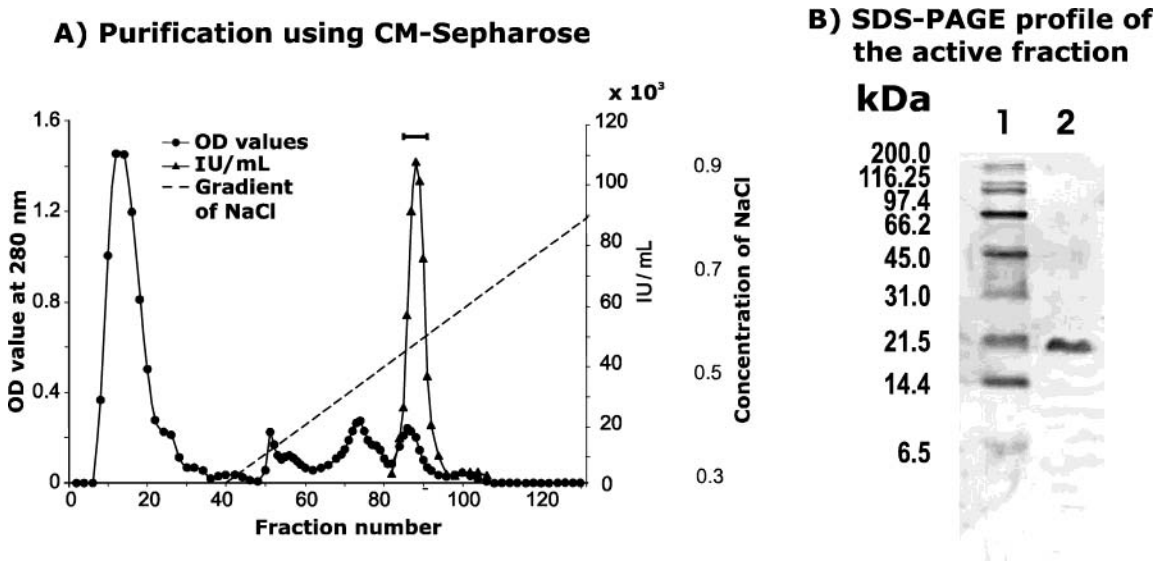

Figure 2. (**A**) Purification of an enzyme inhibitor from *Pithecellobium dulce* seeds using CM-Sepharose chromatography. The enzyme inhibitor was obtained from the eluted fractions from no. 85 to no. 91 (CM-TIA), as indicated by the bar. (**B**) SDS-PAGE profile of the active fraction: (1) MW markers and (2) active fraction.

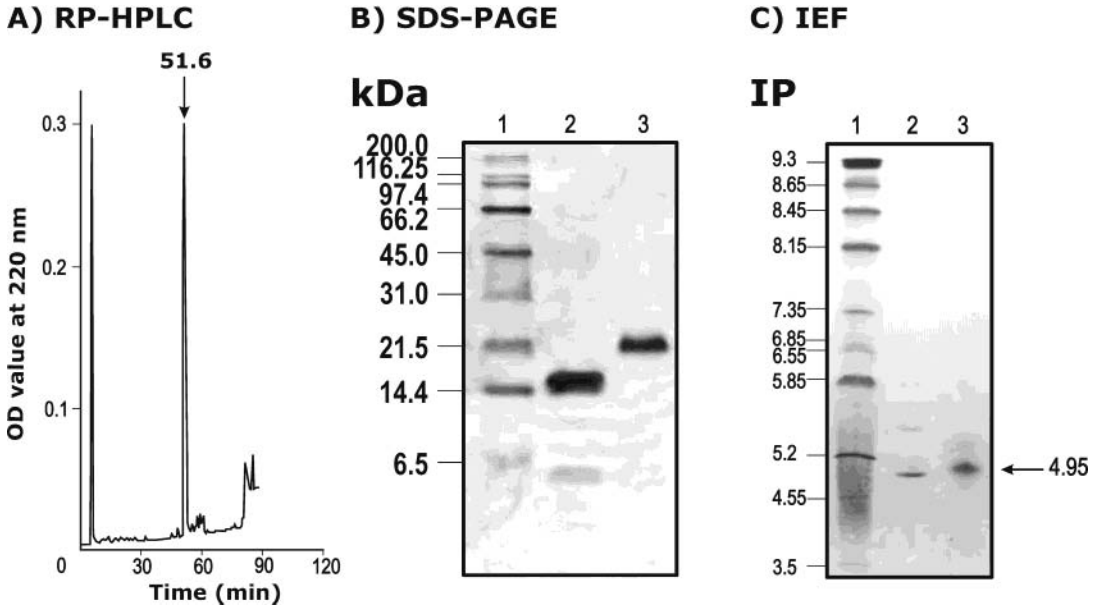

Figure 3. (**A**) RP-HPLC analyses of the active fraction obtained by using CM-Sepharose chromatography. The active peak is signaled by an arrow, and the corresponding value indicates the percentage of acetonitrile. (**B**) SDS-PAGE of the purified protein inhibitor: MW markers (1), and inhibitor in the presence (2) or the absence of reducing agents (3). (**C**) Polyacrilamide gel IEF: Isoelectric point markers (1), and inhibitor purified by using Sephadex G-75 (2) or CM-Sepharose (3); in both purification schemes, RP-HPLC was used as the last stage of purification.

inhibitory activities of crude extracts show chymotrypsin-like (3911 IU/mg) and elastase-like (5911 IU/mg) behavior.

The purified PDTI was subject to an N-terminal amino acid sequence analysis. PDTI shows significant homology with five sequences of the SwissProt Data Bank. All of them are protease inhibitors belonging to the Kunitz family. The N-terminal sequence of the trypsin inhibitor obtained from *Acacia confusa* seeds shows the highest homology, 68% identical residues (**Table 2**). According to the sequence homology, PDTI belongs to the Kunitz family of protease inhibitors.

The cytotoxic effects of G75-TIA and CM-TIA fractions were measured by the MTT assay on RINm5F cells. After 24 h of incubation, both fractions produced dose-dependent effects and significant toxicity at all concentrations (**Figure 4**). Because 100% inhibition was not obtained for the CM-TIA fraction, LC_{50} was obtained only for G75-TIA, at a value of 0.04 mg/mL. To compare their relative effects, we used the mean values obtained in the presence of the maximum concentration (1 mg/mL) of both fractions. The G75-TIA fraction was 49.86 times more potent than the CM-TIA fraction.

DISCUSSION

The PDTI consists of two disulfide-linked polypeptide chains: a large A-chain (MW 14.5 kDa) and a small B-chain (MW 5.6 kDa) (**Figure 3**). N-terminal analysis revealed only Asn, indicating that one N-terminal was blocked as in the *Leucaena leucocephala* Kunitz-type inhibitor (*26*). The PDTI sequence was used to search through the SwissProt Databank, and it had significant homology to several Kunitz-type inhibitors from different plant sources (**Table 2**). The N-terminal sequence of PDTI shows that residues Asp-5, Gly-8, and Ile-19, charac-

Table 2. Comparison of the Amino-Terminal Sequences (25 Amino Acids)[a]

Inhibitor in the seeds of (Accession number)	Sequence of amino acid																									Identity (%) /homology (%)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
P. dulce[b]	K	E	L	L	D	A	D	G	D	M	L	X	G	N	G	S	N	Y	I	L	P	V	K	R	G	
Acacia confusa (**P24924**)	**K**	**E**	**L**	**L**	**D**	**A**	**D**	**G**	**D**	I	**L**	R	N	G	**G**	A	Y	**Y**	**I**	**L**	**P**	A	L	**R**	**G**	68/76
Prosopis juliflora (**B45588**)	G	**E**	**L**	**L**	**D**	V	**D**	**G**	E	I	**L**	R	N	G	**G**	**S**	Y	**Y**	**I**	**L**	**P**	A	F	**R**	**G**	65/75
Albizzia julibrissin (**P24925**)	**K**	**E**	**L**	**L**	**D**	**A**	**D**	**G**	**D**	I	**L**	L	N	G	**G**	X	Y	**Y**	**I**	V						68/73
Leucaena leucocephala (**P83036**)	G	V	**L**	V	**D**	L	**D**	**G**	**D**	P	**L**	Y	N	G	M	**S**	Y	**Y**	**I**	**L**	**P**	**V**	A	**R**	**G**	66/70
Adenanthera pavonina (**P09941**)	R	**E**	**L**	**L**	**D**	V	**D**	**G**	N	F	**L**	R	N	G	**G**	**S**	Y	**Y**	**I**	V	**P**	A	F	**R**	**G**	56/64

[a] Residues identical to those of *P. dulce* inhibitor are shown in the sequence of the corresponding plant inhibitor as bold letters. [b] The amino-terminal sequence was determined in the sequencing laboratory of the Institute for Neurobiology, UNAM, Queretaro, Qro., Mexico.

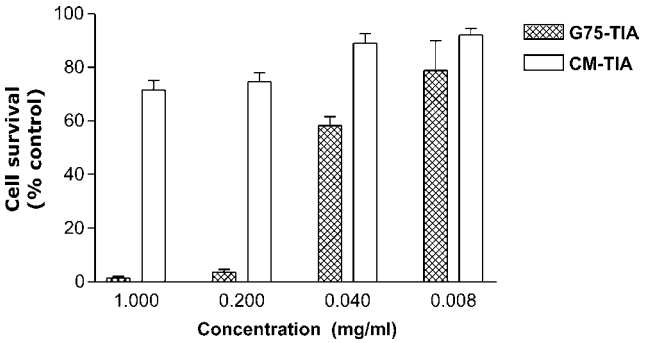

Figure 4. Cytotoxic activity of the fractions with trypsin inhibitory activity obtained by gel filtration (G75-TIA) and ion-exchange (CM-TIA) chromatographies. Cytotoxicity was assessed by MTT assay. Each bar represents the mean ± SD of 12 wells (4 wells/plate × 3 plates). The cytotoxic effect was significant at all concentrations tested ($p < 0.05$).

teristic of Kunitz-type inhibitors, are conserved. Another conserved residue from the Kunitz family is Tyr-17, but it is not present in PDTI, where it is changed for a nonpolar amino acid (Asn-17). The homologous sequences to PDTI (**Table 2**) correspond to seed proteins from legume plants; they belong to the subfamily Mimosoideae, whose Kunitz inhibitors have a MW ranging from 20 to 21 kDa comprising two polypeptide chains (*26*−*31*).

The purified PDTI is a single band (SDS-PAGE) with pI of 4.95. PDTI purity was corroborated by the mass spectrometry results. The results suggest the absence of isoforms. Some Kunitz inhibitors cited in **Table 2** showed various isoforms. The pI of PDTI is within the range of values for the inhibitor extract of *Adenanthera pavonina* (P09941) (8 isoforms, pI = 4.4−5.1), and it is lower than that reported for the *Prosopis juliflora* inhibitor (pI 8.8) (*28*, *30*).

PDTI does not inhibit bovine chymotrypsin, but it inhibits bovine trypsin. The inhibitors of the Mimosoideae subfamily show a variable range of activity; they inhibit trypsin, but the inhibitory activity against chymotrypsin is variable among them. The *L. leucocephala* inhibitor shows a similar activity against trypsin and chymotrypsin, whereas the inhibitor of *P. juliflora* shows a weak inhibition of chymotrypsin (*26*, *28*). The *P. juliflora* inhibitor also showed inhibitory activity against papain. The *P. juliflora* inhibitor has a high potential for pest control by its double inhibitory activity and considering that they could inhibit essential enzymes of some pest organisms such as cysteine proteases. Papain specificity has been associated with the residue Trp60, which makes hydrophobic interactions with papain side-chains. It was found that such residue is present in that position in the inhibitor of *A. confusa* (P24924). The *A. confusa* inhibitor has the highest homology with PDTI. Papain inhibitory activity in crude extracts of *P. dulce* seeds was previously registered (*15*, *32*); however, such activity was not detected in our crude extracts (data not shown). In this study, we observed that PDTI inhibits *P. truncatus* chymotrypsin but not bovine chymotrypsin; *P. truncatus* is one of the most damaging pests of maize. In addition, aqueous extracts of *P. dulce* seeds have fungistatic and fungicidal activities (*7*). The inhibitory activity of the *L. leucocephala* inhibitor (P83036) against other proteases such as plasmin and human plasma kallikrein has been registered. When administered intravenously, *L. leucocephala* inhibitor causes a decrease in paw edema induced by carragenin or heat in male Wistar rats (*26*); this inhibitor also showed inhibitory activity against papain (*33*).

The cytotoxic activity assay of natural products is helpful to discover new anticancer agents. The G75-TIA fraction showed the highest cytotoxic activity against the RINm5F cells, which is a cell line developed from a radiation-induced tumor in rat pancreas and maintained in tissue culture (*34*). PDTI is almost pure in the CM-TIA fraction, and it remains a major component in the G75-TIA fraction (**Figures 2** and **3**). Thus, the observed cytotoxic activity of the G75-TIA fraction must be mainly associated with its minor components.

The antitumor activity of PDTI cannot be discarded by considering their inhibitory and cytotoxic characteristics. It has been suggested that antinutritional properties of protein protease inhibitors are associated with trypsin inhibitory activity, considering that anticarcinogenesis relates to chymotrypsin inhibi-

tory activity (*21*). The low cytotoxicity of the CM-TIA fraction could be explained by the lack of chymotrypsin inhibitory activity of PDTI. Another factor may be the large size of PDTI (19 614 Da), which limits its opportunity to be internalized by cells (*35*). However, it has been demonstrated that inhibitory activity and antitumoral activity is not necessarily correlated (*36*) and, for Kunitz inhibitors, antitumorigenic effects are not necessarily associated with cytotoxic activity. It is suggested that proteins with Kunitz domains and Kunitz inhibitors may participate in establishing a balance between proteolysis and the inhibition of proteolysis on the surface of normal cells. Thus, such proteins (e.g., Kop, IαI, GsmK) may participate in the process leading to tumor invasion and metastasis (*37*−*40*).

PDTI is highly present in the seeds of *P. dulce*, and it shows a high specific activity. Thus, the use of *P. dulce* seeds as a human or animal food may be limited by its antinutritional properties. On the other hand, Kunitz-type inhibitors have a range of interesting biological activities, and the potential of PDTI as either an anticarcinogen or an insect deterrent needs further investigation. We have established an easy and efficient extraction and purification scheme for PDTI, and our study of the biological activities and properties of the G75-TIA fraction is still in progress.

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