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Research Article

Trypsin Isoinhibitors with Antiproliferative Activity toward Leukemia Cells from *Phaseolus vulgaris* cv "White Cloud Bean"

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A purification protocol that comprised ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, ion exchange chromatography on SP-Sepharose, and gel filtration by FPLC on Superdex 75 was complied to isolate two trypsin inhibitors from *Phaseolus vulgaris* cv "White Cloud Bean". Both trypsin inhibitors exhibited a molecular mass of 16 kDa and reduced the activity of trypsin with an IC_{50} value of about 0.6 μM. Dithiothreitol attenuated the trypsin inhibitory activity, signifying that an intact disulfide bond is indispensable to the activity. [Methyl-^{3}H] thymidine incorporation by leukemia L1210 cells was inhibited with an IC_{50} value of 28.8 μM and 21.5 μM, respectively. They were lacking in activity toward lymphoma MBL2 cells and inhibitory effect on HIV-1 reverse transcriptase and fungal growth when tested up to 100 μM.

1. Introduction

Protease inhibitors have been purified from an array of leguminous and nonleguminous species encompassing *Torresea cearensis* [1], *Erythrina caffra* [2], *Dolichos lablab* [3], *Crotalaria paulina* [4], *Medicago scutellata* [5, 6], *Canavalia gladiata* [7], *Pisum sativum* [8], *Dimorphandra mollis* [9], *Swartzia pickellii* [10], *Psophocarpus tetragonolobus* [11], *Delonix regina* [12], *Poecilanthe parviflora* [13], *Adenanthera pavonina* [14], *Cajanus cajan* [15], *Dolichos biflorus* [16], *Phaseolus acutifolius* [17], *Arachis hypogaea* [18], *Leucaena leucocephala* [19], *Bauhinia bauhinioides* [20], *Bauhinia variegata* [21], *Bauhinia ungulata* [22], *Vigna unguiculata* [23], *Lens culinaris* [24], *Glycine max* [25], *Peltophorum dubium* [26], *Pithecellobium dulce* [27], *Glycine soja* [28], and barley [29].

Protease inhibitors impair the activity of insect midgut proteases and thus adversely affect protein digestion and health in insects. They represent one of the multitude of entomotoxic proteins [30, 31]. In addition to insecticidal activity [32–35], protease inhibitors demonstrate antiproliferative and antitumor activities [36–45]. The objective of the present study was to isolate and characterize proteins with protease inhibitory activity from white cloud beans.

2. Material and Methods

2.1. Isolation of Trypsin Inhibitor. An aqueous extract of the beans (250 g) was produced by blending in distilled water (3 ml/g) followed by centrifugation (14000 g for 25 minutes at 4°C). The resulting supernatant was applied to a 5 × 20 cm column of DEAE-cellulose (Sigma) in 10 mM Tris-HCl buffer (pH 7.4). After elution of unadsorbed proteins (fraction D1), the column was eluted successively with 0.2 M NaCl and 1 M NaCl in the Tris-HCl buffer. Fraction D2 eluted with 0.2 M NaCl was dialyzed to remove NaCl and then subjected to affinity chromatography on a 5 × 15 cm of Affi-gel blue gel (Bio-Rad) in 10 mM Tris HCl buffer (pH 7.4). The unadsorbed fraction (B1) was dialyzed against 10 mM NH_4OAc buffer (pH 5) and then applied to a 2.5 × 20 cm column of SP-Sepharose (GE Healthcare). After elution of unadsorbed proteins (fraction S1), the column was eluted with a 0-1 M NaCl concentration gradient in the NH_4OAc buffer. The first and second adsorbed fractions (SP2 and SP3) were then further purified by gel filtration on a Superdex 75 HR 10/30 column (GE Healthcare) in 0.2 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier (GE Healthcare). The second absorbance peak represented purified trypsin inhibitor.

2.2. Electrophoresis, Molecular Mass Determination, and N-Terminal Sequence Analysis. The molecular mass of the isolated proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the procedure of Laemmli and Favre [46]. Gel filtration on an FPLC-Superdex 75 column, previously calibrated with molecular mass marker proteins (GE Healthcare), was employed to determine the molecular mass of the protein. The N-terminal sequence of the protein was analyzed by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System [47].

2.3. Assay for Trypsin Inhibitory Activity. The test sample (20 μl) was added to 160 μl of a 1% casein solution in 0.1 M Tris-HCl buffer (pH 7.4). Trypsin (20 μl of a 0.5 mg/ml solution) was then added and the mixture was incubated at 37°C for 15 minutes followed by addition of Trichloroacetic acid (0.4 ml, 5%) that was added to bring the reaction to an end. After centrifugation the absorbance of the resulting supernatant, which indicates the amount of casein fragments produced by trypsin, was read at 280 nm. The % inhibition of trypsin activity is equal to the % decrease in absorbance of the supernatant [48].

2.4. Effect of Dithiothreitol (DTT) on Trypsin Inhibitory Activity. The isolated trypsin inhibitor (2.5 μM) was treated with dithiothreitol (DTT) at the final concentration 2.5, 10 and 40 mM for 5, 20, and 80 minutes at 37°C. Soybean trypsin inhibitor from Sigma (2.5 μM) was similarly treated and used as a positive control. The reaction was terminated by adding iodoacetamide at twice the amount of thiol functions at each DTT concentration. Residual trypsin inhibitor activity was measured at pH 7.4 as described above in assay for trypsin inhibitory activity. The highest iodoacetamide concentration used in the test was devoid of any effect on trypsin activity and the trypsin inhibitory activity of isolated trypsin inhibitor and soybean trypsin inhibitor [47].

2.5. Assay for Antiproliferative Activity Toward Tumor Cells. The antiproliferative activity of the purified trypsin inhibitor was assayed as described below. The cell lines L1210 (human leukemia) and MBL2 (murine lymphoma) were obtained from American Type Culture Collection. The cell line was maintained in Dulbecco Modified Eagles' Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/l streptomycin, and 100 IU/ml penicillin, at 37°C in a humidified atmosphere of 5% CO_2. Cells (1×10^4) in their exponential growth phase were seeded into each well of a 96-well culture plate (Nunc, Denmark) and incubated for 3 hours prior to addition of the trypsin inhibitor. Incubation was performed for an additional 48 hours. Radioactive precursor, 1 μCi ([^{3}H-methyl]-thymidine, from GE Healthcare), was then introduced to each well and the incubation continued for 6 hours. The cultures were then harvested using a cell harvester. The radioactivity incorporated was measured in a liquid scintillation counter [49].

2.6. Assay for HIV-1 Reverse Transcriptase Inhibitory Activity. The assay for HIV reverse transcriptase inhibitory activity was carried out in view of the report that trypsin inhibitors manifest this activity [50, 51]. It was conducted according to instructions supplied with the assay kit from Boehringer Mannheim (Germany). The assay makes following use of the ability of reverse transcriptase to synthesize DNA, commencing from the template/primer hybrid poly (A) oligo (dT) 15. The digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one of the same DNA molecule, which is freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity are based on a sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with strepatavidin. In the following step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the last step, the peroxidase substrate is added. The peroxidase enzyme affects the cleavage of the substrate, yielding a colored reaction product. The absorbance of the sample at 405 nm which is directly correlated to the level of RT activity can be measured using a microtiter plate (ELISA) reader. A fixed amount (4–6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of the trypsin inhibitor was calculated as percent inhibition compared to a control without the trypsin inhibitor [49].

2.7. Assay of Ability to Inhibit HIV-1 Integrase

2.7.1. Expression and Purification of Recombinant HiV-1 Integrase. The plasmid that expressed His-tagged wild-type HIV-1 integrase, pT7-7-His (Y | TX)-HIV-1-IN, was a generous gift from Professor S.A. Chow (School of Medicine, UCLA). To express the enzyme, a 1-liter culture of *E. coli* BL21 (DE3) cells containing the expressing plasmid was grown at 37°C until it reached OD600 0.7-0.8. Cells were induced by addition of 0.8 mM IPTG (isopropyl-β-d-thiogalactopyranoside) and harvested, after 4 hours of incubation, by centrifugation at 6000 g for 10 minutes at 4°C. Cells were suspended at a concentration of 10 ml/g wet cell paste in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA, 2 mM β-mercaptoethanol, 0.5 M NaCl, and 5 mM imidazole. Lysozyme was added to a concentration of 0.2 mg/ml. After incubation at 4°C for 1 hour, the lysate was sonicated and centrifuged at 40 000 g at 4°C for 20 minutes. The pellet was homogenized in 50 ml buffer A (20 mM Tris-HCl, pH 8.0, 2 M NaCl, 2 mM β-mercaptoethanol) which contained 5 mM imidazole. The suspension was rotated at 4°C for 1 hour and cleared by centrifugation at 40 000 g at 4°C for 20 minutes. The supernatant was applied to a 1 ml chelating Sepharose (GE Healthcare) column charged with 50 mM imidazole. The column was eluted with five column volumes of buffer A containing 5 mM imidazole, and the protein was eluted with three column volumes of buffer A containing 200 and 400 mM imidazole, respectively. Protein containing fractions were pooled, and EDTA was added to a final concentration of 5 mM, followed by dialysis against buffer B (20 mM HEPES, pH 7.5, 1 mM EDTA, 1 M NaCl, 20% glycerol) containing 2 mM β-mercaptoethanol and then against buffer B containing 1 mM

Table 1: Yields and trypsin inhibitory activities of various chromatographic fractions (from 250 g dry white cloud beans).

Fraction	Yield (mg)	IC_{50} (μg/ml)	Fraction	Yield (mg)	IC_{50} (μg/ml)
Extract	**4150**	**236.8**	**SP2**	**82.1**	**17.3**
D1	1079	—	**SP3**	**102.6**	**23.5**
D2	**904**	**72.4**	SP4	109.4	—
D3	958	—	SP2SU1	33.2	—
B1	**469.8**	**45.1**	**SP2SU2**	**39.9**	**9.6**
B2	179.1	—	SP3SU1	41.8	—
SP1	47.9	—	**SP3SU2**	**31.7**	**8.8**

Trypsin inhibitor-enriched fractions are highlighted in boldface.

dithiothreitol. Aliquots of the protein were stored at −70°C [49].

2.7.2. HIV-1 Integrase Assay. A nonradioactive ELISA-based HIV-1 integrase assay was carried out according to the DNA-coated plate method. In this study, 1 μg of SmaI-linearized pBluescript SK was coated onto each well in the presence of 2 M NaCl as target DNA. The donor DNA was prepared by annealing VU5BR (5′-biotin-GTGTGG-AAAATCTCTA- GCAGT-3′) and VU5 (5′-ACTGCTAGA-GATTTTCCACAC-3′) in 10 mM Tris-HC1, pH 8.0, 1 mM EDTA, and 0.1 M NaCl at 80°C, followed by 30 minutes at room temperature. Integrase reaction was conducted in 20 mM HEPES (pH 7.5) containing 10 mM $MnCl_2$, 30 mM NaCl, 10 mM dithiothreitol, and 0.05% Nonidet-P40 (Sigma). After the reaction, biotinylated DNA immobilized on the wells was incubated with streptavidin-conjugated alkaline phosphatase (Boehringer-Mannheim, Mannheim, Germany), followed by colorimetric detection with 1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$. The absorbance was read at 415 nm. The ribosome inactivating protein trichosanthin was employed as a positive control [49].

2.8. Screening for Inhibitory Effect on SARS Coronavirus Protease. The activity of SARS coronavirus (CoV) protease was reflected by a cleavage of designed substrate which is composed of two proteins linked by a cleavage site for SARS CoV protease. The reaction was carried out in a mixture containing 5 μM SARS CoV protease, 5 μM sample, and 20 μM substrate and buffer [20 mM Tris-HCl (pH 7.5), 20 mM NaCl and 10 mM beta-mercaptoethanol] for 40 minutes at 37°C. The reaction was then terminated by heating at 100°C for 2 minutes. Then the reaction mixture was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). If SARS CoV protease is inhibited by the test sample, there is only one band, which is the intact substrate, shown in SDS-PAGE [52].

2.9. Assay for Antifungal Activity. This assay was performed since some trypsin inhibitors demonstrate antifungal activity [8]. The assay for antifungal activity toward *Botrytis cinerea* and *Fuserium oxysporum* was executed in 100 mM × 15 mM

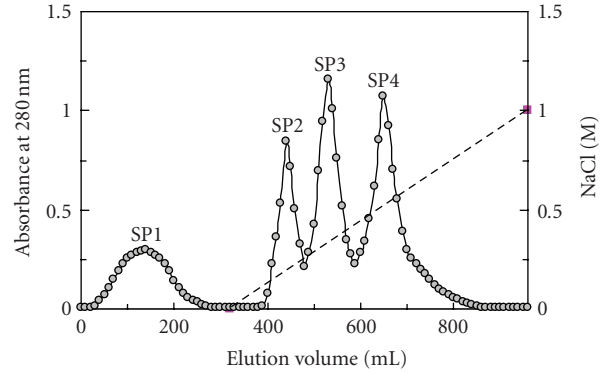


Figure 1: Results of ion exchange chromatography on SP-Sepharose column (2.5 × 20 cm). The sample is fraction of white cloud bean extract previously adsorbed on DEAE-cellulose and eluted with 0.2 M NaCl added to the buffer, and subsequently unadsorbed on Affi-gel blue gel in the Tris-HCl buffer. Starting buffer for SP-Sepharose chromatography is 10 mM NH_4OAc buffer (pH 5). Broken line across the right half of the chromatography represents the linear 0-1 M NaCl gradient in 10 mM NH_4OAc buffer (pH 5) employed to elute adsorbed proteins. Trypsin inhibitory activity was found in both fractions SP2 and SP3.

petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had grown to a sufficiently large size, sterile blank paper disks (0.625 cm in diameter) were laid at a distance of 0.5 cm away from the edge of the mycelial colony. An aliquot (15 μl) of the trypsin inhibitor was added to a disk. The plates were exposed at 23°C for 72 hours until mycelial growth had enveloped the disks containing the control and had produced crescents of inhibition around disks containing samples with antifungal activity [49].

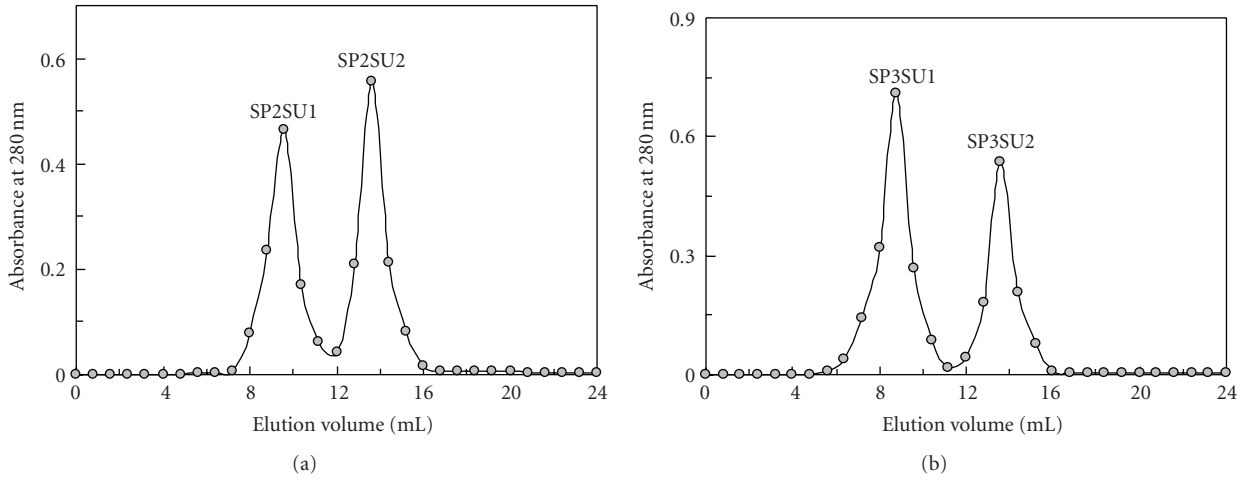
3. Results

3.1. Isolation of Trypsin Inhibitor. Anion exchange chromatography of the bean extract on DEAE-cellulose resolved it into three fractions, an unadsorbed fraction D1 together with two adsorbed fractions D2 and D3. Trypsin inhibitory activity was confined to fraction D2. After affinity chromatography of fraction D2 on Affi-gel blue gel, the activity appeared in the unadsorbed fraction B1 (data not shown). Ion exchange chromatography of fraction B1 on SP-Sepharose resolved it into a small unadsorbed fraction SP1 and three large adsorbed fractions (SP2, SP3, and SP4) of about the same size (Figure 1). Trypsin inhibitory activity was detected in fractions SP2 and SP3. Final purification of SP2 on Superdex 75 produced two fractions, SP2SU1 and SP2SU2 (Figure 2(a)). Gel filtration of SP3 on Superdex 75 yielded two fractions SP3SU1 and SP3SU2 (Figure 2(b)). Fractions SP2SU2 and SP3SU2, both with a molecular mass of 16 kDa as determined by gel filtration on Superdex 75, were the only fractions with trypsin inhibitory activity. The remaining fractions SP2SU1 and SP3SU1 were inactive. The yields of the various chromatographic fractions are presented in Table 1. Both SP2SU2 and SP3SU2 displayed a molecular mass of 16 kDa in SDS-PAGE (Figure 3) and gel filtration (Figure 2). Their N-terminal sequences are shown in Table 2.

Table 2: N-terminal sequence comparison of white cloud bean trypsin inhibitors SP3SU2 and SP2SU2 with other leguminous trypsin inhibitors (Results of BLAST search).

Trypsin inhibitor	Amino acid sequences
SP3SU2	GSGHRHESTDEPSSSKAACCD
SP2SU2	GH -HRHESTDEPSESKKACCDHCACTKKIPPQCRRDLLLL
PVTI (34–62)	–HRHESTDEPSESSKACCDHCACTKSIPPQ
VUTI (80–118)	–NHHDSDSSDEPSESSEPCCDSCICSKSIPPQCHHTDIRL
VMTI (32–51)	–SGRHHESTDEPSESSKPCCD
PLTI (32–51)	–SGHHHESTDEPSESSKPCCD

Identical corresponding residues are underlined.
VUTI: *Vigna unguiculata* trypsin inhibitor.
PVTI: double-headed trypsin inhibitor from *Phaseolus vulgaris.*
PLTI: double-headed trypsin inhibitor from *Phaseolus lunatus.*
VMTI: double-headed trypsin inhibitor from *Vugna mungo.*



(a) (b)

Figure 2: (a) Fast protein liquid chromatography gel filtration of fraction SP2 from SP-Sepharose column on a Superdex 75 HR10/30 column in 0.2 M NH_4HCO_3 buffer (pH 8.5) at a flow rate of 0.4 ml/min. Fraction size is 0.8 ml. Trypsin inhibitory activity was restricted to the second fraction (SP2SU2). (b) Fast protein liquid chromatography gel filtration of fraction SP3 from SP-Sepharose column on a Superdex 75 HR10/30 column in 0.2 M NH_4HCO_3 buffer (pH 8.5) at a flow rate of 0.4 ml/min. Fraction size is 0.8 ml. Trypsin inhibitory activity was restricted to the second fraction (SP3SU2).

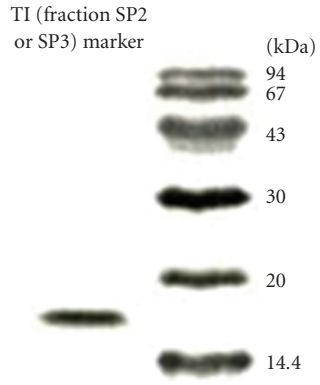


Figure 3: Results of SDS-PAGE of the two white cloud bean trypsin inhibitors (TI, fractions SU2 and SU3) and molecular mass marker. Molecular mass of both trypsin inhibitors was calculated to be 16 kDa.

These two white cloud bean trypsin inhibitors inhibited trypsin with an IC_{50} of about 0.6 μM (Figure 4).

3.2. Characterization of Isolated Trypsin Inhibitor. DTT treatment curtailed the trypsin inhibiting activity in a dose- and time-dependent manner (Table 3). The two trypsin inhibitors did not inhibit HIV-1 reverse transcriptase when tested at various concentrations up to 100 μM (Table 4). They lacked antifungal activity when tested up to 24 μg/disk (100 μM, 15 μl) (data not shown). The IC_{50} values of the inhibitory effects of the trypsin inhibitors on L1210 cells were, respectively, 21.5 μM and 28.8 μM (Table 4). However, there was no activity toward MBL2 cells when tested up to 100 μM. They showed marked homology to partial sequences of other leguminous trypsin inhibitors.

4. Discussion and Conclusions

The present study disclosed the production of two trypsin inhibitors with closely related N-terminal sequences chromatographic behavior and bioactivities by the white cloud bean variety of *Phaseolus vulgaris*. The trypsin inhibitors demonstrate the same molecular mass; both are adsorbed on DEAE-cellulose and unadsorbed on Affi-gel blue gel

Table 3: Inhibition rate (%) of dithiothreitol (DTT) on the trypsin inhibitory activity of white cloud bean trypsin inhibitor and soybean trypsin inhibitor after incubation at 37°C for different durations.

Incubation time (min)	Isolated trypsin inhibitor (SP3SU2)			Isolated trypsin inhibitor (SP2SU2)			Soybean trypsin inhibitor
	2.5 mM DTT	10 mM DTT	40 mM DTT	2.5 mM DTT	10 mM DTT	40 mM DTT	2.5 mM DTT
5	9.7 ± 1.1^{a}	24.7 ± 2.5^{b}	28.8 ± 2.4^{c}	9.0 ± 1.0^{a}	22.8 ± 1.9^{b}	27.0 ± 2.2^{c}	78.2 ± 5.7^{a}
20	30.6 ± 2.9^{d}	55.0 ± 4.1^{e}	67.4 ± 4.1^{f}	28.3 ± 3.0^{d}	54.1 ± 3.6^{e}	65.5 ± 4.3^{f}	93.7 ± 5.0^{b}
80	61.7 ± 6.5^{g}	83.2 ± 5.9^{h}	94.5 ± 5.6^{i}	59.6 ± 4.7^{g}	80.6 ± 6.5^{h}	91.9 ± 6.6^{i}	96.4 ± 4.3^{c}

Results are presented as mean ± SD ($n = 3$). Different alphabets (e.g., a, b, and c) indicate statistically significant difference ($P < .05$) when (I) data at same time point and different DTT concentrations or (II) data at same DTT concentration but different time point were analyzed by analysis of variance followed by Duncan's multiple range test.

Table 4: Inhibition rate (%) of white cloud bean trypsin inhibitors on L1210 cells, MBL2 cells, and HIV-1 reverse transcriptase (RT). Results are presented as mean ± SD ($n = 3$).

Dose (μM)	Trypsin inhibitor (SP3SU2)			trypsin inhibitor (SP2SU2)		
	L1210 cells	MBL2 cells	HIV-1 RT	L1210 cells	MBL2 cells	HIV-1 RT
100	93.2 ± 5.7^{a}	4.8 ± 1.5	3.8 ± 0.6	88.1 ± 7.2^{a}	5.0 ± 1.4	5.0 ± 0.8
50	79.3 ± 5.0^{b}	2.7 ± 0.7	5.3 ± 1.1	68.6 ± 5.3^{b}	4.1 ± 0.8	4.7 ± 1.0
25	56.0 ± 3.8^{c}	3.4 ± 1.2	3.4 ± 1.3	44.9 ± 3.4^{c}	3.5 ± 1.5	4.5 ± 1.3
12.5	24.7 ± 3.1^{d}	4.2 ± 0.9	4.0 ± 1.8	21.3 ± 2.2^{d}	4.3 ± 1.0	3.1 ± 0.6

Different alphabets (e.g., a, b, c, and d) indicate statistically significant difference ($P < .05$) when data were analyzed by analysis of variance followed by Duncan's multiple range test.

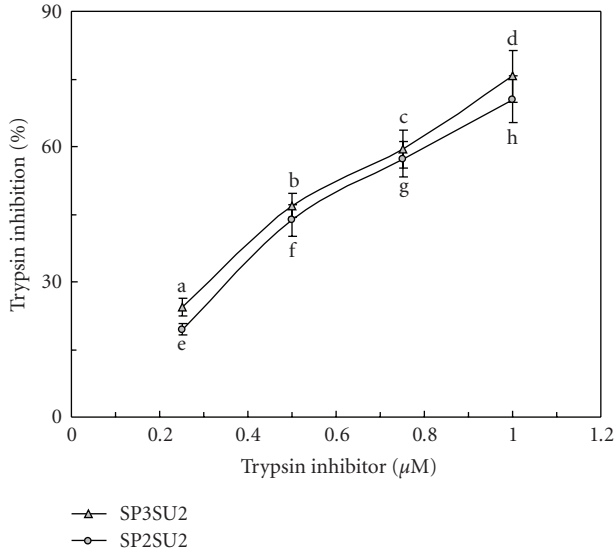


Figure 4: Inhibitory effects by the two white cloud bean trypsin inhibitors on trypsin. IC_{50} = circa 0.6 μM. IC_{50} for soybean trypsin inhibitor = 1.5 μM. Results are presented as mean ± SD ($n = 3$). Results are presented as mean ± SD ($n = 3$). IC_{50} = 0.25 μM. Different letters (a, b, c, d) next to the data points indicate statistically significant difference ($P < .05$) when the data are analyzed by analysis of variance followed by Duncan's multiple range test.

but can be separated by ion exchange chromatography on SP-Sepharose. They have approximately the same trypsin inhibitory potency, and neither of them inhibits HIV-1 reverse transcriptase inhibitory activity, HIV-1 integrase inhibitory activity, SARS coronavirus proteinase inhibitory activity, or fungal growth. Both inhibitors exhibit an antiproliferative activity toward L1210 cells albeit with a small difference in potency, while there is little activity toward MBL2 cells. The difference in yields of the two trypsin inhibitors from the white cloud beans is only slight.

The two trypsin inhibitors exhibit N-terminal sequence homology with those of other leguminous trypsin inhibitors such as inhibitors mungbean (*Vigna mungo*), cowpea (*Vigna unguiculata*), and lima bean (*Phaseolus lunatus*). Whereas leguminous antifungal proteins [48, 49], like nonleguminous antifungal proteins [53], are unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel, white cloud bean trypsin inhibitors are adsorbed on DEAE-cellulose and unadsorbed on Affi-gel blue gel. Thus the purification procedure adopted in the present investigation can be conveniently used to separate trypsin inhibitors from antifungal proteins. The antiproliferative activity of white cloud bean trypsin inhibitors is consistent with similar observations on field bean trypsin inhibitor [39, 40, 42, 43, 54]. It is noteworthy that white cloud bean trypsin inhibitors do not exert a similar action on lymphoma MBL2 cells. Thus the action of white cloud bean trypsin inhibitors is tumour cell-specific. The ribosome inactivating protein trichosanthin exerts different antiproliferative potencies toward different tumor cells [55]. In contrast to broad bean trypsin inhibitor [48, 56], those from white cloud bean do not inhibit HIV-1 reverse transcriptase. A variety of trypsin inhibitors exhibit antifungal activity [48, 56]. However, white cloud bean trypsin isoinhibitors lack such activity. This is reminiscent of the finding that lentil and Vigna mungo inhibitors have little or no HIV-1 reverse transcriptase inhibitory and antifungal activities [57, 58].

In summary, the isolation of two trypsin inhibitors with very similar biochemical and biological characteristics from white cloud beans was achieved in the present investigation. The presence of multiple trypsin inhibitors has previously been reported in *Momordica cochinchinensis* seeds [59]. White cloud bean trypsin inhibitors demonstrate antiproliferative activity against tumor cells but do no inhibit mycelial growth or HIV-1 reverse transcriptase. Previously isolated *P. vulgaris* trypsin inhibitors have not been so tested [60–64]. They reportedly have a molecular mass of about 9 kDa [63, 65] or 13 kDa [61], smaller than the value of 16 kDa obtained for white cloud bean trypsin inhibitors

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