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A stable trypsin inhibitor from Chinese dull black soybeans with potentially exploitable activities

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ABSTRACT

A dimeric 40-kDa Kunitz-type trypsin inhibitor was isolated from seeds of the Chinese black soybean *Glycine max* cv. 'Dull Black'. The purification protocol comprised ion exchange chromatography on Q-Sepharose, SP-Sepharose, and Mono Q, and gel filtration on Superdex 75. The trypsin inhibitor inhibited chymotrypsin, albeit to a lesser extent than it inhibited trypsin. Its trypsin-inhibitory activity was unaffected after exposure to pH 1–14, or to temperatures up to 80 °C. The trypsin inhibitor was inhibited by dithiothreitol in a dose-dependent (from 2.5 to 50 mM) and a time-dependent (from 5 to 120 min) manner. Besides inhibiting serine proteases, the trypsin inhibitor demonstrated additional biological activities including stimulation of nitric oxide production by macrophages. It inhibited HIV-1 reverse transcriptase, cell-free translation and proliferation of liver cancer cells and breast cancer cells, with an IC_{50} value 9.4, 14, 39 and 70 μM, respectively. However, it did not exhibit antifungal, antibacterial or mitogenic activity.

1. Introduction

Protease inhibitors have drawn the attention of many investigators due to their potential value. For instance, HIV protease inhibitors and SARS coronavirus proteinase inhibitors may be used to combat HIV and SARS virus, respectively. Plant protease inhibitors may have anti-insect and antifungal activities. They may be involved in the regulation of programmed cell death in plants. They can cause inhalant allergies and also food allergies [1]. One of the common types of protease inhibitors is trypsin inhibitors which have been isolated from animal tissues and also from plant tissues [1,2]. There are several types of plant trypsin inhibitors. Kunitz-type trypsin inhibitors have a molecular mass of about 20 kDa, a low cysteine content and a single reactive site while Bowman–Birk trypsin inhibitors are approximately 8 kDa in size and possess a high cysteine content and two reactive sites [3–8]. The conformation in Kunitz-type trypsin inhibitors is mainly β-sheet with a small amount of regular sheet. The insecticidal activity of Kunitz-type trypsin inhibitors has been demonstrated using transgenic plants [2]. Kunitz-type trypsin inhibitors have been ***isolated from the seeds of *Erythrina* species [9], *Psophocarpus tetragonolebus* [10], *Prosopis juliflora* [11], *Acacia confusa* [12], *Enterolobium contortisiliquum* [13], *Bauhinia variegata* [14], *Delonix regia* [15], *Crotalaria paulina* [16], *Mucuna pruriens* [17], *Dimorphandra mollis* [18], *Copaifera langsdorfii* [19], *Phaseolus vulgaris* [20], *Pithecelobium dumosum* [21], *Veronica hederifolia* [22] and *Mirabilis jalapa* [23]. Kunitz-type trypsin inhibitors also inhibit other enzymes such as chymotrypsin, α-amylase and human plasmin, and block the conversion of prothrombin to thrombin [1]. The formation, degradation and gene expression of Kunitz-type trypsin inhibitor in the soybean have been reported [1]. Some seeds, e.g., those of *Glycine max* produce both Bowman–Birk and Kunitz-type trypsin inhibitors [24]. The seeds of bitter gourds, sponge gourds, wax gourds and *Momordica cochinchinensis* produce squash-type trypsin inhibitors with a molecular mass of about 3 kDa [25].

Chinese dull black soybean is a special cultivar of *G. max*. It has a different usage in traditional Chinese medicine from the yellow soybean. The intent of the present study was to purify and characterize a trypsin inhibitor from Chinese dull black soybean.

2. Materials and methods

2.1. Isolation of trypsin inhibitor

Chinese dull black soybeans (*G. max* cv. 'Dull Black Soybean') from China (100 g) were extracted with distilled water (10 ml/g) at room temperature in a blender for 10 min followed by centrifugation at 13,000 rpm and 4 °C for 30 min. Tris–HCl buffer (1 M, pH 7.4) was added to the resulting supernatant until the final concentration of Tris attained 10 mM. The supernatant was then loaded on a 5 cm × 20 cm column of Q-Sepharose (GE Healthcare) in 10 mM Tris–HCl buffer (pH 7.4). After removal of unadsorbed proteins, the column was eluted successively with 0.1 M, 0.4 M and 1 M NaCl added to the Tris–HCL buffer. The fraction eluted

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with 0.1 M NaCl was dialyzed against distilled water and then its concentration was adjusted to 10 mM Tris–HCl (pH 7.4) before ion exchange chromatography on a 2.5 cm × 20 cm column of SP-Sepharose (GE Healthcare) in the same buffer. After removal of unadsorbed proteins, the column was eluted sequentially with 0.2 M NaCl and 1 M NaCl added to the Tris–HCl buffer. The fraction desorbed with 0.2 M NaCl was dialyzed prior to FPLC-ion exchange chromatography on Mono Q (GE Healthcare). After unadsorbed proteins had been eluted, the column was eluted, first with a linear gradient of 0–0.3 M NaCl in the starting buffer, and then with a linear gradient of 0.3–1 M NaCl. Fraction S3, the adsorbed fraction desorbed with 0.2 M NaCl, was finally purified on an FPLC-gel filtration Superdex 75 HR10/30 column (GE Healthcare) using an AKTA Purifier (GE Healthcare). The single peak obtained represented black soybean trypsin inhibitor (BSKTI).

2.2. Electrophoresis, molecular mass determination, and N-terminal sequence analysis

The purified protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular mass determination in accordance with the method of Nielsen and Reynolds [26]. After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular mass of the isolated protein was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from GE Healthcare. Gel filtration on an FPLC-Superdex 75 column, which had been calibrated with molecular mass markers (GE Healthcare), was conducted to determine the molecular mass of the protein. The N-terminal sequence of the protein was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System.

2.3. Assays for trypsin-inhibitory and chymotrypsin-inhibitory activities

Trypsin activity was determined by using *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) from Sigma as substrate [25]. Ten microliters of a bovine pancreatic trypsin (USB Corporation, OH, USA) solution (250 μg/ml) in assay buffer (50 mM Tris–HCl, pH 8, containing 20 mM $CaCl_2$) were added to 980 μl of assay buffer, and then 10 μl of BAEE in assay buffer was added to give a final concentration of 0.6 mM. The reaction rate was determined by monitoring the absorbance change at 253 nm for 1 min.

To assay for trypsin-inhibitory activity, 10 μl test sample in assay buffer was added to trypsin, and incubated at 25 °C for 15 min before addition of substrate (BAEE) to initiate the reaction. Trypsin-inhibitory activity was calculated as follows:

$$\text{Trypsin-inhibitory activity (U)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control} \times \text{trypsin (mg)}}$$

where Abs control is absorbance change in absence of sample, Abs sample is absorbance change in presence of sample, trypsin (mg) is the amount of trypsin in assay mixture. One unit of trypsin-inhibitory activity refers to the activity capable of inhibiting 1 mg trypsin.

Chymotrypsin activity was determined by using *N*-α-benzoyl-L-tyrosyl ethyl ester hydrochloride (BTEE) as substrate and bovine pancreatic chymotrypsin (USB, Corporation, OH, USA) [27]. Chymotrypsin-inhibitory activity was assayed as described above for trypsin-inhibitory activity but with chymotrypsin and BTEE replacing trypsin and BAEE, respectively.

To investigate thermal stability and pH stability, the isolated trypsin inhibitor was exposed to 0–100 °C (0, 10 °C, etc., at 10 °C intervals) or pH 1–14 (pH 1, 2, 3, etc.) and the assay of trypsin-inhibitory activity was then conducted as mentioned above.

2.4. Effect of dithiothreitol (DTT) on trypsin-inhibitory activity

The isolated trypsin inhibitor (2.41 μM) was incubated with dithiothreitol (DTT) at the final concentrations of 2.5, 20 and 50 mM for 5, 20, 60 and 120 min at 37 °C. For comparison, the soybean trypsin inhibitor (Sigma) (2.88 μM) was similarly treated. The reaction was terminated by adding iodoacetamide at twice the amount of thiol functions contained in each DTT concentration. The remaining trypsin-inhibitory activity was measured at pH 8 as described above. The highest iodoacetamide concentration used in the test was devoid of any effect on the activity of trypsin and the trypsin-inhibitory activity on isolated trypsin inhibitor and soybean trypsin inhibitor [27].

2.5. Assay of antibacterial activity

Bacteria (*Streptococcus aureus*) were incubated in 10 ml of nutrient broth in a thermal shaker for 12 h at 37 °C, and then 5 ml of this bacterial suspension was transferred to 50 ml of nutrient broth and incubated for another 3–6 h in order to shift bacterial growth to the mid-logarithmic phase. The bacterial suspension was then centrifuged at 2000 × *g* for 10 min, and the bacterial pellet was washed and resuspended in normal saline. A total of 10^5 or 10^7 bacteria per ml were obtained by dilution guided by the optical density at 595 nm. In the experiment, every condition was prepared in triplicate; one aliquot of bacterial suspension was mixed with the isolated trypsin inhibitor at 0.5, 0.25 and 0.125 mg/ml and one aliquot was mixed with only bacteria in saline as a control. The samples were then incubated in a shaker and aliquots were obtained at four time points [0, 3, 6 and 12 h], serially diluted with nutrient broth, and spread on agar plates. After incubation at 37 °C for 24 h, the colonies were counted. The number of bacteria for each condition and dilution was determined from the average colony counts for three plates. The leguminous defensin-like peptide sesquin [28] was used as a positive control emperor banana lectin [30] and bovine serum albumin was used as a negative control.

2.6. Assay of mitogenic activity

Four C57BL/6 mice [20–25 g] were killed by cervical dislocation, and the spleens were aseptically removed. Spleen cells were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve and resuspended to 5×10^6 cells/ml in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units penicillin/ml, and 100 μg streptomycin/ml. The cells [7×10^5 cells/100 μl/well] were seeded into a 96-well culture plate and serial dilutions of a solution of the isolated trypsin inhibitor in 100 μl medium were added. After incubation of the cells at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h, 10 μl [methyl-^{3}H]-thymidine [0.25 μCi, GE Healthcare] was added, and the cells were incubated for a further 6 h under the same conditions. The cells were then harvested with an automated cell harvester onto a glass fiber filter, and the radioactivity was measured with a Beckman model LS 6000SC scintillation counter. All reported values are the means of triplicate samples. Con A was used as positive control and bovine serum albumin as a negative control [29].

2.7. Assay of nitric oxide production by murine peritoneal macrophages

The assay was conducted as described by Wong and Ng [30]. Macrophages were collected from the peritoneal cavity of mice after an intraperitoneal injection of a 3% thioglycolate solution. The cells were washed and resuspended in RPMI medium containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Cells [2×10^5 cells/well] were seeded in a 96-well culture plate for 1 h before incubation with the isolated trypsin inhibitor (23.76 μM) or soybean trypsin inhibitor (25 μM) for 24 h. The amount of nitric oxide in the culture medium was determined by the colorimetric method using sodium nitrite as a standard. In the assay, a 100 μl aliquot of cell-free culture medium from each culture well was allowed to react with 50 μl of Griess reagent [1% sulfanilamide in 5% H_3PO_4–0.1% naphthalene-ethylenediamine dihydrochloride] for 10 min before absorbance was read at 540 nm using a microplate reader. Lipopolysaccharide was used as a positive control and knife bean lectin as a negative control in this assay [29].

2.8. Assay of anti-proliferative activity on tumor cell lines

Breast cancer MCF-7 cells and hepatoma HepG2 cells were suspended in RPMI medium and adjusted to a cell density of 2×10^4 cells/ml. A 100 μl aliquot of this cell suspension was seeded to a well of a 96-well plate, followed by incubation for 24 h. Different concentrations of the trypsin inhibitor in 100 μl complete RPMI medium were then added to the wells and incubated for 72 h at 37 °C. After 72 h, 20 μl of a 5 mg/ml solution of [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] [MTT] in phosphate buffered saline was spiked into each well and the plates were incubated for 4 h. The plates were then centrifuged at 324 × *g* for 5 min. The supernatant was carefully removed, and 150 μl of dimethyl sulfoxide was added in each well to dissolve the MTT-formazan at the bottom of the wells. After 10 min, the absorbance at 590 nm was measured by using a microplate reader [28] and green lentil trypsin inhibitor [31] was used as a negative control in the assay.

2.9. Assay for HIV-1 reverse transcriptase-inhibitory activity

The assay for HIV reverse transcriptase-inhibitory activity was carried out according to instructions supplied with the assay kit from Boehringer Mannhein (Germany). The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting 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 follows a sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with strepatavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the sample at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly correlated to the level of RT activity. 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 as compared to a control without the protein [28]. The leguminous defensin-like protein sesquin was used as a positive control [28] and the antifungal protein mungin [32] as a negative control.