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Bowman-Birk proteinase inhibitor from *tepary* bean (*Phaseolus acutifolius*) seeds: purification and biochemical properties

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Abstract

The present work describes the purification and characterisation of a Bowman-Birk-like trypsin and chymotrypsin inhibitor from the seeds of *tepary* bean (*Phaseolus acutifolius*). Simple purification steps of ion exchange, hydrophobic interaction, and size exclusion chromatography were applied to purify a 17.8 kDa *tepary* bean protease inhibitor (TBPI). Analysis of the amino acid profile of the TBPI revealed that aspartic acid, glutamic acid and serine as the dominant amino acids. Stoichiometry of the reactions of trypsin and chymotrypsin with TBPI showed a molar ratio of 1:1 between the inhibitor and these enzymes. A 50% reduction in antitrypsin activity was observed when the free amino groups of TBPI were chemically modified with 2,4,6-trinitrobenzene; however, antichymotrypsin activity was not altered by this modification. These results show that purified TBPI reacts distinctly with trypsin and chymotrypsin, indicating that it might have two different reactive sites.

Introduction

Protease inhibitors (PIs) are antimicrobial proteins that exist in all kingdoms of life (Birk, 2003). In plant seeds, PIs usually act as storage proteins (nitrogen sources) and as defence mechanisms against pathogens and insects (Mosolov and Valueva, 2005). Plant PIs are being examined for their critical roles in a wide range of biological activities. Their high selectivity for inhibition of specific-enzymes has led to increased interest in these substances for the treatment of many health-threatening diseases such as cancer, inflammation, blood-clotting, heart and neurodegenerative diseases. Recently, several PIs have been isolated from various plant sources and their inhibitory; physiological and chemical attributes have been studied (Prasad *et al*., 2010). The richest sources of PIs are leguminous seeds (Benjakul *et al*., 2000). PIs from legumes are grouped into the Bowman-Birk family according to their molecular weight, cysteine content and enzyme specificity (Guillamón *et al*., 2008; Prasad *et al*., 2010). Bowman-Birk inhibitors are of relatively small size and have several disulphide bonds and two active sites; consequently, they inhibit both trypsin and chymotrypsin (Chen *et al*., 1992).

Tepary bean (*Phaseolus acutifolius*) is a drought-, heat-, and salt-tolerant legume grown in arid and semi-arid environments of North America, where Native Americans used it in their diets for centuries (Osman *et al*., 2003). Currently, the seeds of this plant are rarely consumed, and their high protein (21 - 31.9%) and carbohydrate (65.3 - 69.1%) content are underutilised due to the presence of substantial amounts of antinutritional factors, such as trypsin inhibitor, phytic acid and lectin (Osman *et al*., 2003). Despite the large volume of reports on legume PIs, information regarding PIs in *tepary* beans is scarce (Campos *et al*., 1997; Gonzales-Garza *et al*., 1982). The apparent molecular weight of *tepary* bean protease inhibitor (TBPI) in denatured form is 7.1 kDa, whereas in native form is 17.2 kDa (Campos *et al*., 1997). In a previous study, the initial isolation of a 17.6 kDa trypsin inhibitor from the seeds of *tepary* beans was reported (Gonzales-Garza *et al*., 1982). However, to date, no detailed study has been conducted

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on the amino acid profile, inhibitory potentials, and N-terminal sequence of TBPI. Therefore, complete purification and characterisation of the TBPI is needed and will contribute to comparative studies with known PIs. The present work thus describes the studies designed to provide further information on the PIs of *tepary* bean.

Materials and methods

Materials

Tepary beans were purchased from the market (AZ, USA), manually cleaned, ground to a fine powder, sieved using a 40-mesh sieve, and stored at -20°C until isolation of the protease inhibitor. Bovine pancreas trypsin (EC 3.4.21.4), bovine pancreas chymotrypsin (EC 3.4.21.1), pepsin (EC 3.4.23.1), synthetic substrates [α-N-benzoyl-DL-arginine p-nitroanilide (BAPNA) and N-glutaryl-L-phenylalanine p-nitroanilide (GPNA)], CM-cellulose resin, and size exclusion and SDS-PAGE molecular weight markers were procured from Sigma (St. Louis, MO, USA). Phenyl-Sepharose, DEAE A25 Sephadex, and G-75 superfine Sephadex resins were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Extraction and purification of TBPI

A sample of 200 g *tepary* bean powder was dissolved in 2,000 mL Tris-HCl buffer (50 mM, pH 7.5, containing 1 mM EDTA, 1 mM NaN_3, and 500 mM NaCl). After overnight stirring, the suspension was centrifuged for 15 min at 11,000 *g* and the supernatant was collected. Thereafter, crude extract was obtained by filtering the supernatant through glass wool, heating for 10 min at 80°C in water bath, and then cooling in an ice bath. Next, the heat-coagulated protein was removed from the extract by centrifugation at 11,000 *g* for 10 min, and the supernatant was assayed for anti-trypsin activity. Then, the supernatant was subjected to a salting-out process using ammonium sulphate at 70% saturation, and the contents were stirred for 2 h before centrifugation at 11,000 *g* for 20 min. The precipitate was dissolved in 100 mL 50 mM Tris-HCl (pH 7.5) and then dialysed against excess volume (2 L) of deionised distilled water for 2 d at 4°C. The dialysate was centrifuged (11,000 *g*, 30 min) and assayed for trypsin inhibitory activity. After that, the clarified dialysate was mixed (1:1, v/v) with 50 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA and 1 mM NaN_3 and then loaded onto a DEAE-Sephadex A25 column (2.6 × 25 cm) equilibrated with the same buffer. The protein was eluted with a linear gradient (0 - 0.4 M) of NaCl in equilibration buffer at a flow rate of 1.1 mL/min. The trypsin inhibitory activity was assessed, and active fractions were combined. The active fraction was dialysed against double distilled water (ddH_2O) at 4°C overnight and again analysed for protein concentration and trypsin inhibition activity. Thereafter, solid ammonium sulphate was added to the dialysed fraction to reach 16% saturation (1.2 M ammonium sulphate) and then loaded onto a Phenyl-Sepharose column (2.6 × 20 cm) equilibrated with 50 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA, 1 mM NaN_3, and 1.2 M ammonium sulphate. The column was washed with the same buffer and the protein was eluted with a linear gradient of ammonium sulphate (1.2 - 0.0 M) at a flow rate of 0.75 mL/min. The anti-trypsin active fractions were combined, dialysed overnight at 4°C against ddH_2O, and then concentrated to 5 mL using Amicon YM-10 membrane (EMP Millipore, Darmstadt, Germany). The concentrated sample was mixed (1:1, v/v) with a buffer (5 mM sodium acetate (pH 4.0), 1 mM EDTA, 1 mM NaN_3) and applied to a CM-cellulose column (2.6 × 18 cm) equilibrated with the same buffer. The active trypsin inhibitor was eluted from the column using a linear gradient of NaCl (0 - 1.0 M) at 0.67 mL/min flow rate. The active fractions were collected and dialysed against ddH_2O and then concentrated using Amicon® membrane (EMP Millipore). Following concentration, the sample was loaded onto a Sephadex G-75 column (1.6 × 95 cm) equilibrated with a buffer [50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM NaN_3, and 100 mM NaCl]. The protein was eluted with the same buffer at a flow rate of 0.16 mL/min. The active fraction was pooled, dialysed, and lyophilised, and then used for biochemical characterisation of the purified inhibitor.

Determination of inhibitory activity

Trypsin and chymotrypsin inhibitor activities were assessed using synthetic substrates BAPNA and GPNA, respectively (Klomklao *et al*., 2010). Briefly, inhibitor diluted in Tris-HCl buffer (50 mM, pH 8.0; 20 mM $CaC1_2$) was added to an aliquot containing 10 µg protease and the mixture was incubated at 37°C for 15 min. After that, the remaining trypsin or chymotrypsin activity was measured spectrophotometrically using BAPNA (for trypsin) and GPNA (for chymotrypsin). Inhibitor activity was expressed as percent of protease activity relative to activity in the control assay using Equation 1:

$$\%\ Inhibition = \frac{Activity\ without\ inhibitor - Activity\ with\ inhibitor}{Activity\ with\ inhibitor} \times 100 \quad \text{(Eq. 1)}$$

where one unit of inhibitor was defined as the amount that caused 50% inhibition of substrate hydrolysis by 10 µg of trypsin or chymotrypsin.

Determination of protein content

The bicinchoninic acid (BCA) method of Smith *et al*. (1985) was adopted for the determination of protein concentrations in the extracts using bovine serum albumin as the standard.

Electrophoretic analysis

The homogeneity of the purified protein was verified under non-reducing (15% native-PAGE) and reducing (15% SDS-PAGE) conditions as described previously (Laemmli, 1970). For non-reducing conditions, polyacrylamide gels were prepared without a reducing agent (SDS), and the samples were not treated with denaturing buffers or heated. SDS-PAGE was performed using a 15% acrylamide separating gel and a 4% acrylamide stacking gel. For electrophoresis, protein samples were mixed with SDS-PAGE buffer (50 mM Tris-HCl, pH 6.8, containing 30% glycerol, 1% SDS, 2% 2-mercaptoethanol, and 0.01% bromophenol blue). The mixtures were heated in a boiling water bath for 5 min, cooled, and then loaded into the gel holes and electrophoresed at 30 mA/gel for 2 h. Following electrophoresis, staining of the gels was carried out for 1 h at room temperature using Coomassie Brilliant Blue R-250:acetic acid:methanol (0.25:10:50%) solution. Removal of the background dye was achieved by immersing the gels in de-staining solution (7.5% acetic acid and 5% methanol).

Amino acid analysis and N-terminal sequencing

For amino acid determination, purified TBPI was initially hydrolysed with 6 N HC1 for 20 h at 110°C. Then, the hydrolysate was analysed using a Beckman Model 121 M amino acid analyser (Beckman Coulter, Brea, CA, USA). The determination of cystine and cysteine was carried out using the performic acid oxidation method (Hirs, 1967). N-terminal sequencing of TBPI was performed using a gas phase protein sequencer (Applied BioSystems 470A, Thermo Fisher Scientific, Waltham, MA, USA).

Determination of molecular weight of TBPI

A high-performance size exclusion (Superose 12) column was used to assess the molecular weight of TBPI (GE Healthcare Lifesciences, Pittsburgh, PA, USA). The column (1.5 × 60 cm) was equilibrated in Tris-HCl buffer (20 mM, pH 7.5, 1 mM EDTA, 1 mM NaN_3, and 100 mM NaCl). Prior to analysis, the column was calibrated using standard proteins, namely, bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12 kDa), and aprotinin (0.5 kDa). Molecular weight was also estimated using 15% SDS-PAGE with bovine serum albumin (66 kDa), egg albumin (45 kDa), pepsin (34 kDa), trypsinogen (24 kDa), lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa) as markers.

Effect of varying substrate concentration

Increasing concentrations (0 to 5 mM) of the substrate, BAPNA, or GPNA in 50 mM Tris-HCl buffer (pH 8.0) containing 20 mM $CaCl_2$ were used in assays containing aliquots of enzymes (10 µg) and inhibitor (0, 5, 10, or 15 µg). Lineweaver-Birk plots were used to determine the type of inhibition exhibited by TBPI.

Determination of dissociation constant (Kd)

The determination of the dissociation constant (Kd) of TBPI-enzyme complex was carried out using varied amounts (0 - 5 µg) of TBPI and concentrations (1.5 and 3 mM) of the substrate (Dixon, 1953).

Chemical modifications

Arginine residues in TBPI were modified using 1,2-cyclohexanedione (CHD) (Patthy and Smith, 1975) and ninhydrin (Chaplin, 1976). Thereafter, about 2 mg TBPI was dissolved in 4.5 mL 200 mM borate buffer (pH 9.0). Then, 0.5 mL CHD (5%, w/v) in ddH_2O or 0.7 mL ninhydrin (1.8%) in ddH_2O was added, and the mixtures were incubated in the dark at 37°C for various periods. Thereafter, 50 µL aliquots of the reaction mixture were taken and examined for residual trypsin- and chymotrypsin-inhibitory activities.

Free amino residues of TBPI were modified using 2,4,6-trinitrobenzene sulfonate (TNBS) (Plapp *et al*., 1971). Briefly, 1.5 mg TBPI was dissolved in 3 mL sodium borate buffer (50 mM, pH 9.5) to which 0.3 mL 0.8% TNBS in dH_2O was added, and the reaction mixture was incubated for 1 to 4 h at 25°C. Thereafter, 50 µL aliquots were taken from the reaction mixture and analysed for residual inhibition activity against trypsin and chymotrypsin activities.

The disulphide bonds in TBPI were reduced using dithiothreitol (DTT) (Cleland, 1964). TBPI (0.78 mg) in 3 mL 50 mM Tris-HC1 (pH 7.5) was incubated with 100 mM DTT under N2 at 25°C in the dark for various times. Thereafter, aliquots were withdrawn from the reaction mixture and assessed for residual trypsin- and chymotrypsin-inhibitory activities. TBPI disulphide bonds were also modified using 8 M urea and 2-mercaptoethanol (Anfinsen and Haber, 1961).

Results and discussion

Extraction and purification

Tepary bean protease inhibitor (TBPI) was purified to homogeneity by overnight extraction in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM NaN_3, and 500 mM NaCl followed by heating, ammonium sulphate precipitation, and a combination of different chromatographic approaches. Heat treatment at 80°C was performed for 10 min as the first purification step to quickly eliminate undesired heat-sensitive proteins, which resulted in a 1.1-fold increase in the purity of TBPI (Table 1). The thermal treatment of plant extracts can encourage thermal coagulation of bulk protein and consequently improves the specific activity of protease inhibitors (Benjakul *et al*., 2000; Chaijan, 2011). Salting-out using 70% ammonium sulphate resulted in a significant increase in the specific inhibitor activity (131.4 units/mg protein) and purity (3.9-fold) of TBPI. This salting-out procedure not only enables the efficient removal of coloured materials from the extract, but also concentrates the protein to a workable volume that can be used in subsequent purification steps (Ahmed *et al*., 2009). In agreement with our findings, Klomklao *et al*. (2011) reported that using heat treatment at 90°C followed by the 30 - 65% ammonium sulphate precipitation step increased the purity of Thai mung bean trypsin inhibitor by 2.84- and 3.98-fold, respectively. In the present work, the ammonium sulphate fraction that showed inhibitor activity was subjected to a further purification step by anion exchange chromatography on a DEAE-Sephadex column. The proteins were eluted into three major peaks, and the active TBPI was recovered in the second peak at 125 - 250 mM NaCl (data not shown). This purification step resulted in a significant increase in specific activity (288.1 units/mg protein) and purity (8.7-fold) of TBPI (Table 1). However, only 22.1% of the total TBPI was recovered from the DEAE-Sephadex A25 column. It is possible that a small, undetectable amount did not bind to the DEAE-Sephadex and was eluted with a significant amount of protein that passed directly through the column at pH 7.5. The active fractions of the DEAE-Sephadex column were combined and exposed to sequential purification using Phenyl-Sepharose, CM-cellulose, and Sephadex G75 columns. Most of the TBPI was eluted from the Phenyl-Sepharose column at an ammonium sulphate concentration between 0.2 and 0.0 M, while a minor quantity of TBPI was eluted during the flushing of the column with ddH_2O (data not shown). This purification step increased the purity and specific activity of TBPI 10.8-fold and 360.5 units/mg, respectively (Table 1). The major peak of the CM-cellulose column at 100 - 250 mM NaCl (Figure 1a) contained most of TBPI activity, resulting in improved specific activity (401.8 units/mg) and purity (12.1-fold). Gel filtration chromatography was used as a final step to purify the inhibitor. Only one symmetrical peak having constant specific activity was obtained when the dialysed concentrated fraction was loaded onto a Sephadex G75 column equilibrated with 0.1 M NaCl in 50 mM Tris-HCl (pH 7.5) (Figure 1b). This purification increased the specific activity and purity of TBPI to 565.7 units/mg protein and 17.0-fold (Table 1), respectively. Similarly, Dantzger *et al*. (2015) reported that using a Superdex G75 gel filtration column as a final purification step improved the purity of *Clitoria fairchildiana* proteinase inhibitor (CFPI) to 17.0-fold. In addition, Klomklao *et al*. (2011) observed that the application of gel filtration on Sephadex G50 as the last purification step for trypsin inhibitor from Thai mung bean seed led to a 13.51-fold increase in the inhibitor activity. Overall, the purification procedure developed in the present work yielded 47.6 mg of pure TBPI per 200 g of *tepary* bean flour.

Structural properties

Polyacrylamide gel electrophoresis under both reducing and non-reducing conditions showed that the TBPI was highly purified, as it migrated as only one band on SDS-PAGE, suggesting the existence of a

Table 1: Summary of the purification of the proteinase inhibitor from tepary beans.

Fraction	Total protein (mg × 10^3)	Total activity (units × 10^3)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
Crude extract	19.7	656.0	33.3	100.0	1.0
Heat treatment	16.9	598.0	35.4	91.2	1.1
$(NH_4)SO_4$ fraction	3.22	423.2	131.4	64.5	3.9
DEAE Sephadex	0.504	145.2	288.1	22.1	8.7
Phenyl-Sepharose	0.152	54.8	360.5	8.40	10.8
CM cellulose	0.112	45.0	401.8	6.90	12.1
G-75 Sephadex	0.047	28.0	565.7	4.30	17.0

Inhibitory activity was determined by trypsin inhibition assay, where one trypsin inhibitory unit was defined as the amount that caused 50% inhibition of substrate hydrolysis by 10 µg of trypsin.

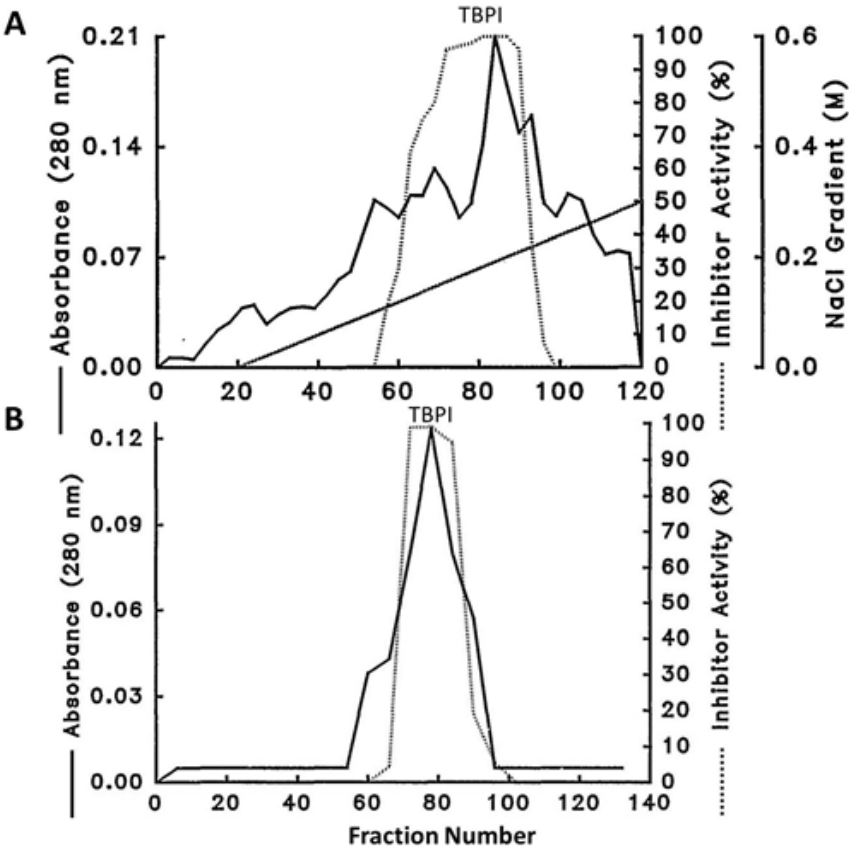

Figure 1. Elution profiles of TBPI on cation-exchange and gel filtration columns. (A) Elution profile of TBPI on a CM-cellulose column equilibrated with 5 mM sodium acetate (pH 4.0), 1 mM EDTA, and 1 mM NaN_3 and eluted with a 0 - 300 mM NaCI gradient in the same buffer. (B) Elution profile of TBPI on a 1.6 × 95 cm Sephadex G-75 gel filtration column. The protein was eluted with 0.05 M Tris-HC1 (pH 7.5), 0.001 M EDTA, 0.001 M NaN_3, and 0.1 M NaCl.

single polypeptide chain having a 17.8 kDa molecular weight (Figure 2a). Additionally, the native molecular mass of TBPI was assessed by using a size exclusion chromatography column and comparing the retention time of the TBPI on the column with that of standard proteins. Molecular weight of the native TBPI was estimated to be 17.8 kDa (Figure 2b), which is similar to that obtained with SDS-PAGE. Interestingly, molecular weight of TBPI in the present work was apparently different from the previously isolated inhibitors from *tepary* bean (Campos *et al*., 1997; 2004), suggesting that it could be a new isoinhibitor. The existence of manifold isoinhibitors in legumes appears to be a rule rather than an exception, as multiple isoforms have been reported from various legumes including *tepary* bean (Campos *et al*., 2004). The molecular weight of purified TBPI is well known to be within the range of 8 - 20 kDa reported for most Bowman-Birk-like protease inhibitors (Losso, 2008). Similarly, a monomeric protease inhibitor of the Bowman-Birk family having a molecular weight of 17.0 kDa was recently isolated from seeds of brown kidney bean (*Phaseolus vulgaris*), a closely related leguminous species (Chan *et al*., 2013). Moreover, Dantzger *et al*. (2015) isolated a dimeric Bowman-Birk protease inhibitor from *C. fairchildiana* seeds having an apparent molecular mass of 15 kDa under non-reducing conditions and two identical masses of 7.973 kDa when analysed by MALDI-TOF analysis. Furthermore, several Bowman-Birk protease inhibitors isolated from leguminous plants such as *P. acutifolius* (Campos *et al*., 1997), *P. coccineus* (de Azevedo Pereira *et al*., 2007), and *Lens culinaris* (Ragg *et al*., 2006) have multiple isoforms. These results suggest that some Bowman-Birk protease inhibitors could be of monomeric nature despite the well-known polymeric nature of known protease inhibitors in this family.

The amino acid analysis revealed that glutamic acid, aspartic acid, and serine were the dominant amino acids in TBPI. These three amino acids accounted for more than 40% of the total amino acid content in TBPI (data not shown). Another important observation was that TBPI contained 14 half cysteines; the half-cysteine residues are more likely to form seven disulphide bonds, as reported

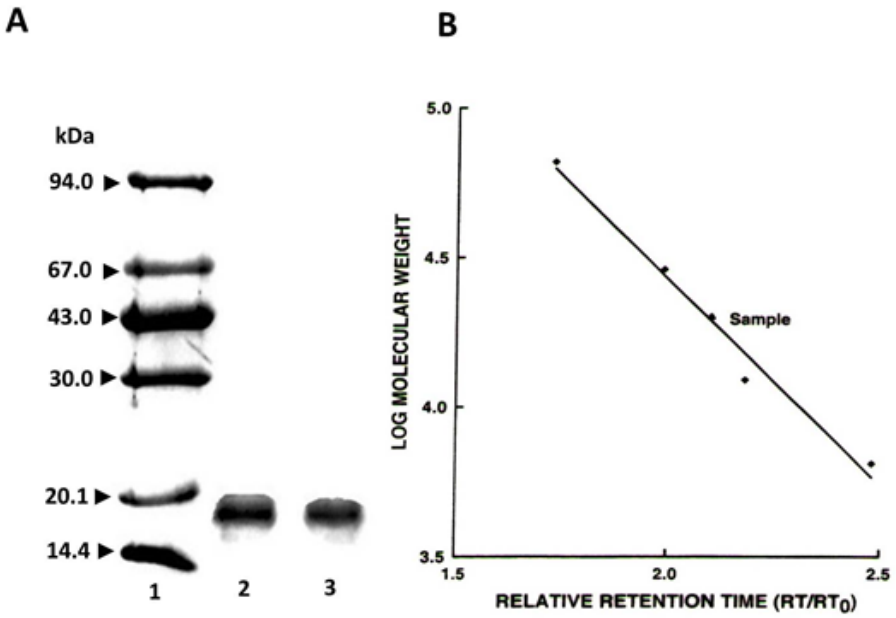

Figure 2. Electrophoretic analysis and molecular weight determination of TBPI under denaturing and non-denaturing conditions. (A) SDS-PAGE of purified TBPI. Lane 1 molecular-weight markers, lane 2 and 3, purified TBPI under non-reducing and reducing conditions, respectively. (B) Molecular weight measurement of TBPI by gel permeation on a Superose G12 column. Standard proteins: 1, bovine serum albumin (M.W. 66 kDa); 2, carbonic anhydrase (M.W. 29 kDa); 3, cytochrome c (M.W. 12.4 kDa); 4, aprotinin 6.5 kDa).

for other Bowman-Birk inhibitors (Campos *et al*., 2004). The amino acid profile of TBPI was deficient of methionine residue. Purified TBPI had an amino acid profile comparable to those of cysteine-rich low molecular weight inhibitors of the Bowman-Birk family (Campos *et al*., 2004). Molecular weight of TBPI as calculated from the amino acid contents was approximately 10.6 kDa, which was within the molecular weight range of 0.8 - 13 kDa reported for most Bowman-Birk trypsin inhibitors isolated from *Phaseolus* species (Campos *et al*., 2004; de Azevedo Pereira *et al*., 2007), suggesting that TBPI belongs to the Bowman-Birk family. Previous studies have indicated that protease inhibitors of Bowman-Birk family isolated from black gram (Prasad *et al*., 2010), *camaratus* bean (Paiva *et al*., 2006), and kidney bean (Kumar *et al*., 2004) have a tendency of self-association that forms dimers or tetramer polypeptides. Self-association is likely the cause of the higher molecular weight of TBPI.

The sequence of 16 amino acids at the N-terminus of TBPI was determined and compared with other leguminous plant protease inhibitors in the NCBI database (Table 2). The N-terminal sequence of TBPI exhibited high similarity with that of protease inhibitors of the Bowman-Birk family, demonstrating that the TBPI belongs to this family. The highest similarities were 94% with TBPI-B (Campos *et al*., 2004), 92% with MBTI (Wilson and Chen, 1983), 85% with IIC (Ishikawa *et al*., 1985), and 75% with CPPI (Rao and Suresh, 2007), with a lesser but still significant similarity of 63% with LBPI (Debreczeni *et al*., 2003).

Table 2: N-terminal sequence alignment of TBPI with other Bowman–Birk proteinase inhibitors. Identical residues are shaded in grey.

Inhibitor	Source	Amino terminal sequence (first 16 residues)	Identity (%)
TBPI	*Phaseolus acutifolius*	S G H H H H D S S D E P S E C S	100
TBPI-B	*Phaseolus acutifolius*	S G H H H H D S S D E P S E S S	94
MBTI	*Vigna radiata*	S S H H H – D S S D E P S E S S	92
IIC	*Vigna angularis*	S V H H H – D S S D Q P S E S S	85
CPI	*Vigna unguiculata*	S G H H E – D S T D E P S E S S	75
LBPI	*Phaseolus lunatus*	S G H H E H – S T D Z P S Z S S	63

TBPI = *tepary* bean protease inhibitor; TBPI-B = *tepary* bean protease inhibitor isoform B (Campos *et al*., 2004); MBPI = mung bean protease inhibitor (Wilson and Chen, 1983); IIC = *azuki* bean protease inhibitor (Ishikawa *et al*., 1985); CPI = cowpea protease inhibitor (Rao and Suresh, 2007); LBPI = lima bean protease inhibitor (Debreczeni *et al*., 2003).

Inhibitory properties and dissociation constant

The inhibitory potential of TBPI was measured against both trypsin and chymotrypsin, a typical feature of Bowman-Birk family protease inhibitors.

The results showed that TBPI inhibited both enzymes at a similar molar ratio, as 1 mg TBPI inhibited 1.4 mg of trypsin (Figure 3a), specifying that 1.0 mole of TBPI inactivates 1.0 mole of trypsin (molecular weight of bovine trypsin is 24 kDa and that of TBPI is 17.8 kDa). Similarly, 1.0 mole of TBPI inactivated 1.0 mole chymotrypsin (Figure 3b). Several Bowman-Birk family protease inhibitors have been reported to inhibit both trypsin and chymotrypsin at a 1:1 (inhibitor:enzyme) molar ratio, including CFPI from *C. fairchildiana* seeds (Dantzger *et al*., 2015), DBPI from *Dolichos biflorus* seeds (Ramasarma *et al*., 1994), and BTCI from *Vigna unguiculata* (Barbosa *et al*., 2007).

The Dixon plot analysis revealed a competitive inhibition of trypsin and chymotrypsin by TBPI (data not shown). The apparent K_i values of TBPI for trypsin and chymotrypsin were calculated from plots as 2.8×10^{-7} and 6.8×10^{-8} M, respectively. These values agreed with those reported for CFPI from *C. fairchildiana* seeds (Dantzger *et al*., 2015).

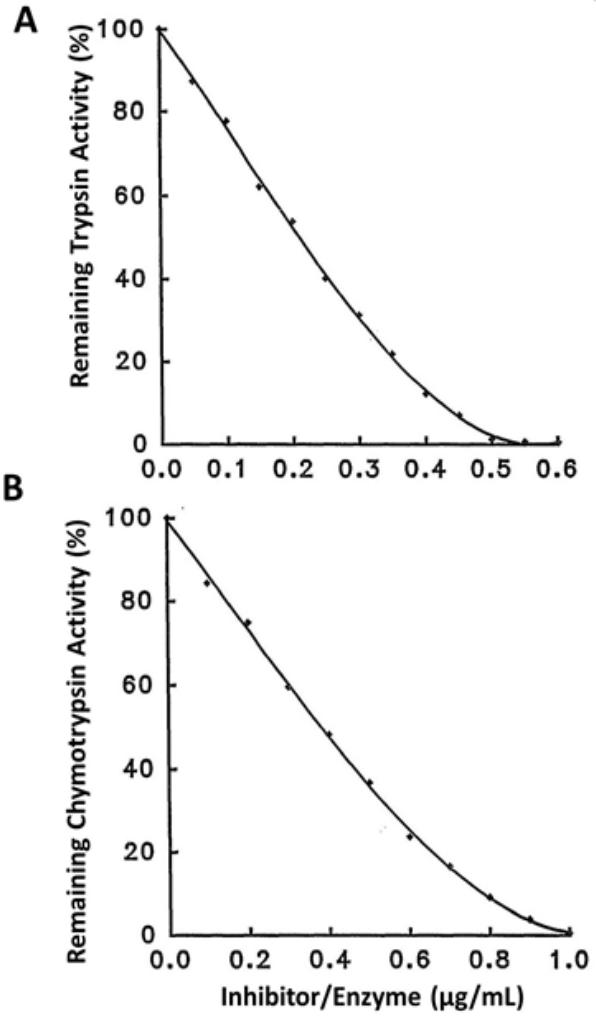

Figure 3. Trypsin (A) and chymotrypsin (B) inhibition by the purified TBPI.

Chemical modification

The results of chemical modifications of arginine residues of TBPI with CHD and ninhydrin are not shown. Modifying arginine residues with both compounds did not alter the activity of TBPI against both enzymes. Treatment of TBPI with TNBS caused a 58.8% loss in antitrypsin activity, but this treatment had no effect on the anti-chymotrypsin activity of TBPI, indicating that lysine residues are critical for the inhibition of TBPI against trypsin. The TNBS study also established that TBPI is a double-headed inhibitor that has two different binding sites: one for trypsin and the other for chymotrypsin. In contrast to this observation, Kunitz-type inhibitors which are high molecular weight inhibitors, have only one reactive site for trypsin. However, several low molecular weight inhibitors of the Bowman-Birk family purified from legume seeds are in fact double-headed inhibitors having two reactive sites (Campos *et al*., 2004; Kumar *et al*., 2004; Paiva *et al*., 2006; Prasad *et al*., 2010; Dantzger *et al*., 2015). In the present work, treatment of TBPI with urea, DTT, and mercaptoethanol denatured the protein and caused great loss of antitrypsin and antichymotrypsin activities (data not shown), indicating that disulphide bridges play crucial roles in the conformation of TBPI. Many investigators have recently reported similar observations on the loss of inhibitory activity of Bowman-Birk family protease inhibitors isolated from the seeds of various leguminous plants (Prasad *et al*., 2010; Dantzger *et al*., 2015).

Conclusion

The present work demonstrated the purification and characterisation of TBPI from the seeds of *tepary* beans. TBPI was extremely stable over a wide pH range and at high temperatures. However, it was sensitive to reduction with DTT, urea, and 2-mercaptoethanol. Based on the N-terminus residues, amino acid profile, inhibitory behaviour, and chemical modification responses, we propose that the TBPI be considered a Bowman-Birk type inhibitor.

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