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Purification and Characterization of Bowman-Birk Trypsin Inhibitor from Soybean

Chunmei Gu[1], Xinxiu Song[1], Linlin Zhao[1], Shu Pan[1], Guixin Qin[2,*]

[1]Institute of Food Science and Engineering, Jilin Agricultural University, Changchun, China
[2]Institute of Animal Science and Technology, Jilin Agricultural University, Changchun, China
*Corresponding author: qgx@jlau.edu.cn

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Abstract In this paper, crude extract of a Bowman-Birk trypsin inhibitor from soybean meal was firstly isolated by a combination of alcohol precipitation, thermal denaturation, isoelectric precipitation and acetone precipitation. Then this extract was purified by DE-52 ion exchange and affinity chromatography. The results showed that soybean Bowman-Birk trypsin inhibitor (SBBI) was purified to 50.07-fold with trypsin activity of 822.31 U·mg-1. The purified SBBI gave a single protein band in SDS-PAGE electrophoresis. The accurate molecular mass of this inhibitor was determined to be 8837.46Da by MALDI-TOF. N-terminal sequence showed high homology with other serine proteinase inhibitors belonging to the Leguminosae family.

Keywords: *soybean Bowman-Birk trypsin inhibitor, purification, characterization*

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1. Introduction

Bowman-Birk inhibitor (BBI) is a family of serine protease inhibitors with similar structure features consisting of many forms and isoforms commonly found in a variety of legume seeds, such as soybean [1], pigeon pea [2], tepary bean [3], cowpea, bambara ground-nuts [4], mung bean seed (Phaseolus mungo) [5], Australian wattle seed (Acacia victoriae Bentham) [6], dry bean [7], velvet bean and jack bean [8], broad beans [9].

BBI was first identified by Bowman in the 1940s [10] and purified by Birk in 1960s [11]. The primary structure of many representatives BBI shows high homology on the conserved cysteine positions [12]. The protein is small peptide (Mr8~10 kDa) containing 71 amino acids and seven disulfide bridges. Disulfide bridges and (or) extensive hydrogen bond networks help to form a symmetrical structure of two tricycle domains, each containing an independent serine protease binding loop joined via a disulphide bond between flanking cysteine residues. BBI has two separate functional inhibitory domains: one domain inhibits trypsin-like serine proteases, and the other inhibits chymotrypsin-like serine proteases. BBI can form a complex with two separate proteases through the interaction between the binding loop of inhibitor and enzyme reactive center, typically allowing simultaneous inhibition of chymotrypsin and trypsin. The three-dimensional structures are now known in Molecular Modeling Database (PDB code 1BBI) [13] and the complete Bowman-Birk type inhibitor in ternary complex with trypsin has also been reported [14].

The inhibitory mechanism of BBI is well established, and the identity of the P1 residue in the binding loop is considered to define the enzyme-inhibitor specificity. The interaction of BBI with serine proteases is similar to that of enzyme-substrate. The inhibitory loop of BBI inserts deeply into the active center of the protease, taking up the reorganization sites and combination sites and forming hydrophobic bond contact between the active site residue and the P1 residue to block off the active center of protease. The protease-inhibitor complex is so tight as to result in low rates of hydrolysis [1].

Many reports are available on the isolation and purification of protease inhibitor from plants [15,16,17]. For example, a trypsin inhibitor from mung bean seeds was extracted and characterised by Sappasith et al. through determining the optimal extraction medium and extract time, heat-treatment, ammonium sulphate precipitation and gel filtration on Sephadex G-50 and purified to 13.51-fold with a yield of 30.25% [18]. In addition, an Apios americana trypsin inhibitor (AATI) from Apios tubers purified by chromatography on DEAE Cellulofine A-500 and Sephadex G-50 was reported by Zhang et al. [19], and protein was purified by up to 27.2 times with trypsin inhibitory activity of 847.6 U.mg-1. However, these purification procedures were rather complicated and poorly efficient. So we will study a simple purification method with higher purification fold, and this work will be beneficial to further research of SBBI.

2. Materials and Methods

2.1. Materials

Soybean was provided by Jilin Academy of Agricultural Sciences. DEAE-cellulose DE-52, trypsin-sepharose 4B were purchased from Beijing Ruida Henghui Science and Technology Development Co. (Beijing, China). Trypsin and BAPNA (N-benzoyl-DL-arginine-p-nitroaniline hydrochloride) were purchased from Shanghai Sanjie Biotechnology Co. (Shanghai, China). Marker (low) was purchased from Beijing Dingguo Changsheng Biotechnology Co. (Beijing, China).

2.2. Extraction and Purification of BBI

Soybean seeds, free of integument, were finely ground to a fine powder, which was depigmented and defatted with several washes of hexane. The air–dried seed powder (100 g) was extracted with 50% ethanol (700 g) and incubated in water at 65°C for 60 min. The extract was placed at room temperature for 12 h and centrifuged at 10,000 r·min-1 for 30 min. The pH of the supernatant was adjusted to 4.2±0.05 with 0.1 M HCl and then the hexane was added (quality of hexane g/quality of the adjusted aqueous g was 2:1) for precipitating the trypsin inhibitors in the liquor. The precipitate obtained by centrifugation at 10000 r·min-1 was re-dissolved in a small amount of deionized water and then dialyzed against 0.05 M Tris-HCl buffer (pH 8.2) (buffer A) for 24 h and finally freeze-dried for further studies. 5 mL of this crude inhibitor preparation was applied onto a DEAE cellulose column previously equilibrated with 0.05 M Tris-HCl, pH 8.2. The proteins were eluted using a linear gradient of 0-1.0 M NaCl in the same buffer at a flow rate of 0.5 ml/min and absorbance was recorded at 280 nm. Fractions corresponding to trypsin inhibitory activity were pooled, dialyzed against 0.05 mol/L Tris-HCl, pH8.5 and loaded onto a trypsin-Sepharose 4B column (1.3×15 cm), pre-equilibrated with 0.05 mol/L Tris-HCl, pH 8.5, containing 0.5 mol/L NaCI. The bound proteins were eluted with Glycine-HCI, pH 2.0 at a flow rate of 0.4 ml/min. The fractions with TI activity were pooled, and concentrated using PEG 20,000.

2.3. Assay of Inhibitory Activity

Trypsin inhibitory activity assay was carried out by estimating the remaining esterolytic activity of trypsin towards the substrate BAPNA. Trypsin was pre-incubated with BBI for 5 min at 37°C in 0.05 M Tris-HCl buffer containing 0.02 M CaCl2, pH 8.0, prior to the reaction [20]. One unit of trypsin enzyme activity (TU) is defined as the increase in the absorbance of 0.01 at 410 nm under assay conditions.

2.4. Estimation of proteins

Protein contents were estimated by Coomassie blue staining (dye-binding method) [21]. BSA (1 mg/ ml) was used as the standard protein. The protein content of individual fractions collected from different columns was determined by UV absorbance at 280 nm.

2.5. Urea-SDS-PAGE electrophoresis

The content of various solutions in Urea-SDS-PAGE electrophoresis was described as Table 1:

Table 1. Various solutions of Urea-SDS-PAGE electrophoresis

	stacking gel (pH 6.8) 100 ml	separation gel (pH 8.8) 100 ml	running buffer (pH 8.3) 1 L	loading buffer (pH 6.8) 40 ml
Sucrose(g)		1		
Acr(g)	4.8625	13.384		
Bis(g)	0.1375	0.366		
SDS(g)	0.1	0.1	10	0.04
urea(g)	36.0375	36.03		14.42
Tris(g)	1.5125	4.54	30	0.61
TEMED(μl)	187.5	60		
20%AP(μl)	500	200		
Gly(g)			144	
β-mercaptoethanol(ml)				4

2.6. MALDI-TOF Analysis

Molecular mass of BBI was determined by MALDI-TOF. BBI was mixed with an equal volume of matrix and 2 μl of this mixture was applied on a MALDI sample plate and allowed to crystallize at room temperature. External standards (Bruker's protein calibration standard: Bradykinin, 757.399 Da; Angiotensin-II, 1046.541 Da and ACTH (18–39), 2465.198 Da) were used for calibration. The BBI spots separated in SDS-electrophoresis were excised, and destained with acetonitrile: 25 mM NH4HCO3 (1:1). After destaining, the excised spots were incubated for 5 min in 100% acetonitrile to completely dry them. After discarding the acetonitrile, the gel spots were incubated with 10 mM DTT for 1 h at 56°C to reduce the protein and subsequently alkylated by incubating with 55 mM iodoacetamide at room temperature in dark for 45 min. After washing with 25 mM NH_4HCO_3, the excised spots were incubated in 100% acetonitrile for drying. Sufficient quantity of acetonitrile: trifluoro acetic acid:

water (50:1:49) was added to the tubes and vortexed well. The supernatant was concentrated using Speed Vac and analyzed using MALDI-TOF.

2.7. Amino Acid Sequence Analysis

The purified peptide was subjected to urua SDS-PAGE and then transferred to PVDF membrane. The partial N-terminal amino acid sequence of BBI was determined by automated Edman degradation, performed with an automatic amino acid sequencer [22].

3. Results

3.1. Extraction and Purification of BBI

Through alcohol precipitation, heat treatment, isoelectric precipitation and acetone precipitation, crude total protein (3250 mg) with a specific activity of 17.62U·mg^{-1} was extracted (as shown in Table 2) and subsequently loaded on DEAE-cellulose column. An amount of protein was absorbed to the column and the proteins were eluted with a linear gradient of NaCl from 0 to 0.1M in equilibrating buffer, but strong trypsin inhibitory activity was detected in the flow through fractions(Figure 1, fraction II). Hence the fraction was polled, and then the proteins were applied onto a trypsin-sepharose 4B column. As shown in Figure 2, affinity chromatography resolved the proteins into two peaks, and only the second peak showed a strong trypsin inhibitory activity of 882.31 TIU/mg. Urea-SDS-PAGE analysis of the fraction gave a single band (Figure 3).

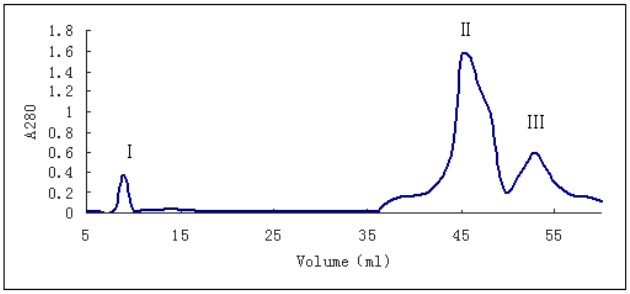

Figure 1. Ion-exchange chromatography on DEAE-52

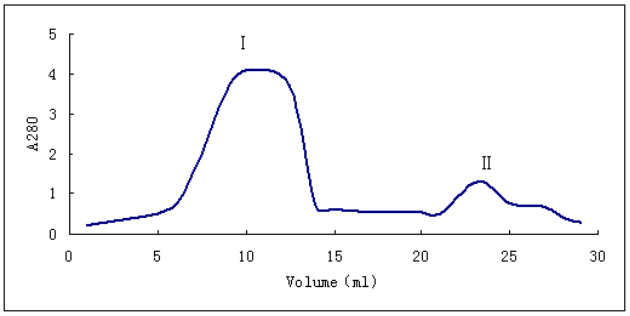

Figure 2. Affinity chromatography on trypsin-sepharose 4B column

The purification of SBBI was summarized in Table 2. The total trypsin inhibitory activity of the crude extract from soybean flour was 57258TIU. Samples with trypsin inhibitory activity were subjected to DEAE-Cellulose-52 chromatographic column where the inhibitor was 3.34-fold purified with a yield of 80.12% (Table 2). When the inhibitor was purified using trypsin affinity chromatography, the purified protein was up to -50.07fold with a yield of 69.34%.

Table 2. Result of parameters detection

Purification step	protein/mg	activity/U	Specific activity /U·mg-1	Purification fold	Yield/%
Crud extract of BBI	3250	57258	17.62	1	100
DEAE-Cellulose column52	780	45876	58.82	3.34	80.12
Trypsin Sepharose 4B column	45	39704	882.31	50.07	69.34

3.2. Electrophoretic Analysis of SBBI

From Figure 3, we observed that SBBI consisted of a single polypeptide with Mr of approximately 8 kDa.

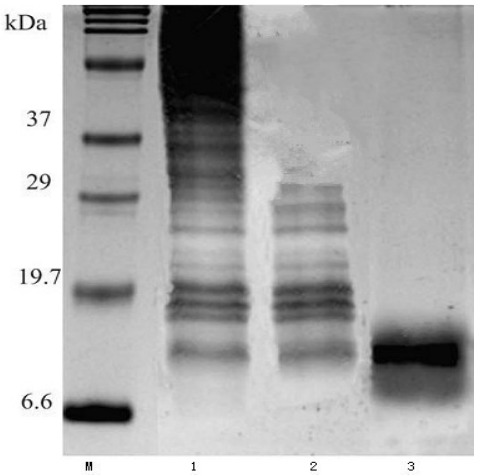

Figure 3. SDS-PAGE electrophoresis

Note: M:molecular mass standards; 1:crude extract with being diluted 10 times; 2:active frcaction after DEAE-52; 3:active frcaction after Trypsin-Sepharose-4B.

3.3. MALDI-TOF and Amino Acid Analysis of SBBI

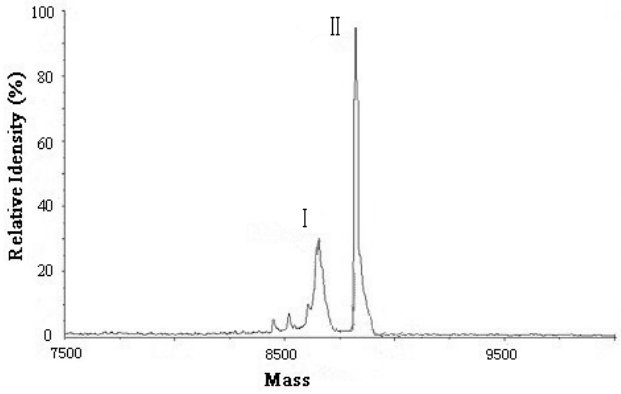

Figure 4. MALDI-TOF analysis of the BBI

MALDI-TOF mass spectrometry analysis of SBBI showed a double-signal peak at 8501.14 Da (Figure 4, peak I) and a single-signal peak at m/z of 8837.46Da (Figure 4, peak II), which indicated that accurate molecular mass of SBBI was 8837.46Da. This result was similar to protease inhibitors of the Bowman-Birk family.

Accession NO.: AAW84292.1,Lens culinaris; JC1066, mung bean; ABD97867.1, Vigna marina; CAQ52823.1, Phaseolus filiformis; AAD09816.1, Glycine max; BAB86783.1, Glycine soja; Asterisks indicate identical residues in all sequences. Colons indicate completely conserved residues and periods indicate incompletely conserved residues. Gaps (-) have been inserted to maximize the global alignment.

Table 3. Partial sequences of soybean BBI aligned with different known Bowman-Birk inhibitors

Species	N-terminal sequence	Identity(%)
BBI	PCCDSCACTGSNKPQCI-CSDMRCKSCHSAC	100
Glycine max	PCCDQCACTKSNPPQCRCSDMRLNSCHSAC	80
Glycine soja	PCCDQCACTKSNPPQCRCSDMRLNSCHSAC	80
Vigna marina	PCCDKCACTRSI-PPQCRCSD-LRLNSCHSAC	73
Phaseolus grayanus	--CCDQCACTKSI-PPQCRCSD-LRLNSCHSAC	72
Lens culinaris	PCCDS-CI-CTKSI-PPQCHCTD-IRLNSCHSAC	70
mung bean	PCCDS-CDCTKSKPPQCHCAN-IRLNSCHSAC	60
	.* * *. * ** * ** * *:. :* : ******	

The N-terminal amino acid sequence of SBBI was identified with automated Edman degradations (Table 3). Homology searches used the NCBI-BLAST and ClustalW 2. Multiple sequence alignment of SBBI and Bowman-Birk inhibitors from Leguminosae family revealed variable levels of sequence similarity and many conserved residues (Table 3). Among these Bowman-Birk inhibitors, SBBI showed the highest homology with AAD09816.1 (Glycine max Kunitz inhibitor, Leguminosae, 90%) with many conserved residues.

4. Discussion

4.1. Separation and Purification of BBI

Peroxidase is the major water soluble protein component of soy seed coats [23]. To eliminate the coextraction of peroxidase with BBI, which would occur with buffer or water extraction of the seed coats, we used 50% ethanol extraction method followed by acetone precipitation of the proteins in the alcohol extractables as applied to soybean cotyledons [24]. This alcohol extraction and acetone precipitation technique may also coprecipitate any phospholipid materials extracted with the protein. To eliminate that possibility, we defatted the seed with hexane, which is hydrogen-bond breaking solvents that are known to remove phospholipids [25].

DE-52 separated the crude extract into three peaks, where the fraction represented by peak II showed anti-trypsin activity (Figure 1). This step was important for elimination of pigmentation excess present in crude extract, eliminated basically in the first peak. As shown in Figure 2, affinity-chromatography resolved the proteins into peaks, but only the second peak showed a specific activity of 882.31TIU/mg and a yield of 69.34% (as shown in Table 2). Purity and high yield are desirable features for proteins. Affinity chromatography is proved to be a very convenient way of isolating PIs, although the possibility of limited digestion of PIs by immobilized trypsin during purification cannot be excluded [26].

4.2. Electrophoretic and MALDI-TOF Analysis of BBI

Electrophoretic separation of BBI showed a single band with an approximate molecular mass of 8 kDa in SDS-PAGE. In agreement, MALDI-TOF mass spectrum of BBI also showed the presence of a peak at 7837.46 Da. These observations corroborated with the previous reports on BBI type PIs isolated from Glycine soja [27] and Phaseolus coccineus [28].

However, under reducing condition BBI showed the presence of many sharp bands in SDS–PAGE, which may be due to incomplete reduction and alkylation of the disulfide bridges, resulting in the different three-dimensional shapes of the PIs. The appearance of purified PIs in such several sharp bands under reducing conditions was suggested to be very common among BBI type inhibitors [29]. In general, BBI type PIs have the tendency of self-association, which results in the formation of homodimers or trimers or more complex oligomers in solution [30,31]. However, such self-association tendency might be negligible in BBI, as evident through the appearance of a single band in SDS-PAGE (Figure 3). Based on the analysis of molecular mass, SBBI belonged to the Bowman-Birk family.

4.3. N-terminal Amino Acid Analysis

After multiple sequence alignment with different known Bowman-Birk inhibitors using CLUSTAL W 2.0, we found that the purified protein has sequence similarity to the Bowman-Birk type protein inhibitors containing 9 cysteine residues. As we all known, the conserved cysteines are indispensable for formation of disulfide linkages that are responsible for the functional stability of Bowman-Birk inhibitors in the presence of physical and chemical denaturants. Furthermore, it is reported that the BBI in plants has a peptide consisting of 9 amino acids, i.e. ‘CTP1SXPPQC’ (P1 representing residues of Arg, Lys or Leu and X representing any amino acids) [32], while in this study, the similar sequence was ‘CTGSNKPQC’. Due to the different source of the soybean as well as the experimental conditionals such as precision of the instrument and completeness of the database, multiple sequence alignment can not reach 100%.

5. Conclusions

Through preparation for crude extraction, DE-52 ion exchange and affinity chromatography, we obtained the purified SBBI with a 50.07 purification fold and a specific activity of 822.31. Urea SDS-PAGE electrophoresis gave a single protein band. The accurate molecular mass of this inhibitor was determined as 8837.46Da by MALDI-TOF, which was homogenous with various members of the Bowman-Birk inhibitor family.

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