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Heterologous Expression and Biochemical Characterization of a Peanut Bowman-Birk Type Inhibitor

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Abstract:

Objectives To heterologously express peanut (*Arachis ipaensis*) Bowman-Birk inhibitor (BBI) and characterize its properties.

Results A putative BBI gene from peanut was overexpressed in *Pichia pastoris* with the maximal yield of 11.1 mg/L in a 250-mL shaking flask fermentation. The recombinant peanut BBI (rPBBI) was purified and its molecular weight was estimated to be 9 kDa. Purified rPBBI showed 5223.6 TIA/mg inhibitory activity toward trypsin. It retained more than 95% of its inhibitory activity over wide range of temperatures (40 to 90 ^{o}C) and pH (2.0 to 10.0) after incubation for 60 min. When presence of 100 mM dithiothreitol, rPBBI lost more than 80% inhibitory activity in 30 min.

Conclusion The current investigation expressed a peanut BBI with stable inhibitory activity against trypsin and exposed the potential of heterologous overexpression approach in large scale production of BBI.

Key words: Bowman-Birk inhibitor; *Arachis ipaensis*; Overexpression; Biochemical characterization

Plant protease inhibitors (PIs) are abundant in nature and widely utilized for regulating proteolytic activity in food, feed, biotechnological and pharmaceutical industries (Clemente et al. 2020). Based on secondary and tertiary structure similarities as well as inhibitor functions, plant PIs are classified into more than 12 families, in which Bowman-Birk inhibitor (BBI) has been extensively studied (Hellinger and Gruber 2019).

BBIs were originally classified as antinutritional factors because they reduce digestive efficiency of the main GIT proteases (Losso 2008). They have a broad activity toward proteolytic enzymes, and unique resistance to extreme pH and temperature (Müller et al. 2017). BBIs have also been exploited for therapeutic purposes. For instance, soybean BBI exhibited significant inhibition activity toward dengue virus protease, and was able to block HIV entrance and replication in macrophages (Tomlinson et al. 2009; Ma et al. 2016; Ma et al. 2018). Soybean BBI also inhibited the activation of pro-matrix metalloproteinase-1, thus possessed potential *in vivo* health enhancing abilities in degenerative angiogenic diseases onset and/or progression (Losso et al. 2004). The black-eyed pea BBI (BTCI) and related peptides were demonstrated to have antihypertensive and vasodilator effects, which highlight the therapeutic potentials in the treatment of hypertension (de Freitas et al. 2020).

However, preparation of purified BBI from plants is complicated with low yield. BBI from peanut seed was purified through multi-procedures including a set of chromatographic separations with the yield of 3.25 mg from 1 kg peanuts (Norioka et al. 1982). As alternative, heterologous expression of BBI is eco-friendly and economical, and thousand important proteins have been prepared in large scale (Ahmad et al. 2014). BBIs from soybean, rice bean, and *Rhynchosia sublobata* were successfully

expressed in *Escherichia coli* with the yields of 0.5 mg/L, 12 mg/L and 2.5 mg/L, respectively (Flecker 1987; Katoch et al. 2014; Mohanraj et al. 2018). BBI genes from mustard and *Glycine max* were expressed and prepared in *Pichia pastoris* with the yields of 40-160 mg/L and 50-56 mg/L, respectively (Volpicella et al. 2000; Yakoby and Raskin 2004).

Currently, the purified peanut-originated BBIs can only be obtained by complex isolation method from the nature samples and its homologs are often coexisted (Norioka et al. 1982). This study aimed at expressing a putative open reading frame encoding for a peanut BBI in *P. pastoris* and characterizing the properties of the recombinant BBI. The heterologous overexpression of peanut BBI provided an alternative for its large-scale preparation and encouraged its further application in various fields.

Materials and methods

Strains and cultivation conditions

E. coli JM109 and *P. pastoris* GS115 (Invitrogen) were used as hosts for plasmid amplification and gene expression, respectively. Plasmid pPIC9k (Invitrogen) was used for gene cloning and expression. *E. coli* JM109 was grown in Luria-Bertani (LB) medium, and *P. pastoris* GS115 and its recombinants were cultured in yeast extract peptone dextrose (YPD) medium (Niu et al. 2017). Minimal dextrose medium (MD), buffered minimal glycerol-complex medium (BMGY), and buffered minimal methanol-complex medium (BMMY) were prepared according to the Multi-Copy Pichia Expression Kit Instructions (Invitrogen).

Expression of peanut BBI

The coding nucleotide sequence of peanut BBI gene (*pbbi*) (GenBank ID: **XM_016319515**) was identified by similarity alignment from NCBI GenBank database. The codon optimization was carried out using JCat (http://www.jcat.de/). The optimized sequence was chemically synthesized and cloned

into pUC57 by Sangon Biotech (Shanghai) Co., Ltd. It was then used as template to amplify *pbbi* by PCR with a pair of primers HS-F (5'-GTTAGATTGGATCCTAGTTTGATGCTGT-3') and HS-R (5'-TGCTCTAGATCAAGATCTACATTCAGTGACTGGACATCTTC-3'; underlined sequences represented *Xba*I restriction site). The PCR amplification was carried out in a 50 μL reaction mixture mediated by LA *Taq* DNA polymerase (Takara). The amplified fragment was digested with *Xba*I and cloned into pPIC9k to yield recombinant plasmid and then linearized with *Sac*I and transformed into *P. pastoris* GS115 by electroporation (Micropulser Electroporator, Bio-Rad) at 1.5 kV, 200 Ω, and 25 mF. High expressing yeast colonies were selected according to the manufacturer's protocol.

Preparation and purification of the recombinant PBBI

The recombinant PBBI (rPBBI) was prepared according to Pichia Expression Kit manual (Invitrogen). The secreted peptide was purified to homogeneity using ammonium sulfate precipitation, desalted with DP-10 Spin Adopter (GE Healthcare), and purified with G75 gel chromatography. Elution fraction containing target protein was lyophilized and used for further experiments. The purified rPBBI was confirmed by Tricine-SDS-PAGE according to the method described by Schägger (2006).

Protein concentration was determined by Bradford method with bovine serum albumin Fractionation V (Sigma-Aldrich) as standard. Concentration of trypsin and chymotrypsin was calculated from their absorbance. The following factors were used: $A^{1\%}_{280}$=15.0 for bovine trypsin, and $A^{1\%}_{282}$ = 20.0 for bovine chymotrypsin (Norioka et al. 1982).

Biochemical characterization of rPBBI

Protease inhibitory activity assay

The inhibitory activity of rPBBI toward trypsin, chymotrypsin, pepsin, papain, and subtilisin

89 (Yuanye, Shanghai, China) was assayed according to a colorimetric trypsin inhibitor assay by titrating
90 the substrates benzoyl-L-arginine *p*-nitroanilide monohydrochloride (L-BAPA, Sigma-Aldrich) with
91 rPBBI (Zhang et al. 2008). Briefly, appropriately diluted inhibitor and bovine L-trypsin (Solarbio,
92 Beijing, China) were mixed and incubated for 10 min at 37 °C and pH 8.0, followed by addition of
93 L-BAPA solution (0.6 mg/mL). The reaction was terminated by adding acetic acid to the reaction
94 mixture and absorbance of the hydrolysis product *p*-nitroaniline was measured at 410 nm. The
95 inhibitory activity toward chymotrypsin was measured in a similar way using 0.05 M Tris-HCl buffer
96 (pH 7.0) containing 0.02 M $CaCl_2$, and 24 mM acetyl-L-tyrosine ethyl ester (ATEE, Sigma-Aldrich)
97 solution, and the absorbance was measured at 237 nm (Yakoby and Raskin 2004). The inhibitory effect
98 of rPBBI against pepsin, papain or subtilisin was assayed using casein as substrate (Liu et al. 2020).
99 One unit of the inhibitory activity is defined as the amount of inhibitor which inactivates 1 μg of active
100 enzyme. Specific inhibitory activity is defined as the inhibitory units per mg of inhibitor.
101 The inhibitory activity of rPBBI toward trypsin and chymotrypsin was also assayed through
102 inhibitory activity staining (Felicioli et al. 1997). Briefly, 10 g/L gelatin was added to the
103 polyacrylamide in Tricine-PAGE. After electrophoresis, the slab was washed with distilled water and
104 incubated at 37 °C in a solution containing 25 μg/mL trypsin (in 100 mM Tris-HCl buffer, pH 8) and
105 containing 100 μg/mL chymotrypsin (in 50 mM Tris-HCl buffer, pH 7). After washing with distilled
106 water, the slab was stained with Coomassie blue (Bio-Safe™ Coomassie Stain, Bio-Rad), and then
107 destained with water.
108 *Effects of pH, temperature, metal ions and chemicals on the inhibitory activity of rPBBI*
109 Purified rPBBI was incubated in different buffers from pH 2.0 to 11.0 at room temperature. After
110 incubation for 1 h, the pH was adjusted to 8.0 and the residual trypsin inhibitory activity was

111 determined as described above. The buffers used were 50 mM dibasic sodium phosphate-citric acid
112 buffer (pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) and 50 mM glycine-sodium hydroxide buffer (pH 9.0,
113 10.0, and 11.0).

114 For thermostability assay, rPBBI was prepared in 0.05 M Tris-HCl buffer (pH 8.2, containing 0.02
115 M $CaCl_2$), and then incubated at temperatures of 40, 50, 60, 70, 80, and 90 °C, respectively, for 1 h and
116 then cooled to room temperature. Residual trypsin inhibitory activity was determined as described
117 above.

118 To assess the effect of chemicals on rPBBI, metal ions and EDTA were added individually to the
119 reaction system at 37 °C and pH 8.0 at a final concentration of 1 or 5 mM. The mixture was incubated
120 with rPBBI for 30 min, followed by the addition of trypsin and L-BAPA. The inhibitory activity of
121 rPBBI was determined as mentioned above. To illustrate the sensitivity to dithiothreitol (DTT), rPBBI
122 was incubated with 1, 10 or 100 mM DTT for 30-120 min at 37 °C. The reaction was terminated by
123 adding iodoacetamide twice the amount of DTT followed by a 15-min incubation (Azarkan et al. 2006).
124 The residual trypsin inhibitory activity was measured as mentioned earlier.

125 *Inhibitory kinetic parameters of rPBBI*

126 To determine the inhibitory kinetic parameters of rPBBI, trypsin activity assays were conducted
127 with different concentrations (0.4, 0.6, 0.8, and 1.0 mM) of L-BAPA at 37 °C and pH 8.0 for 5 min. The
128 kinetic parameters K_m and V_{max} of trypsin can be obtained by plotting with double reciprocal method
129 (Lineweaver-Burk plotting). The inhibition constant (K_i) was estimated by the nonlinear regression
130 analysis of Graphpad Prism 8.0 (GraphPad Software, Inc.).

131 **Bioinformatic analysis**

132 The alignment of different genes were performed using NCBI BLAST

(https://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid sequence analysis and classification was carried out with InterPro (http://www.ebi.ac.uk/interpro). Signal peptide was predicted using SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP). Cysteines disulfide bonding state and connectivity were predicted by DISULFIND (http://disulfind.dsi.unifi.it). Phylogenetic and molecular evolutionary analysis were conducted using MEGA version X (https://www.megasoftware.net). The three-dimensional models of BBIs were generated by the online Swiss-Model server (http://www.swissmodel.expasy.org).

Results and discussion

Sequence analysis, cloning, and expression of rPBBI

By using BLAST and alignment tools, a 324 bp open reading frame (**XM_016319515**) composed of 107 amino acid residues in the *A. ipaensis* genome (**GCF_000816755.2**) was predicted to be a putative serine protease inhibitor and renamed as PBBI. It contained a typical eukaryotic signal peptide (20 amino acid residues from N-terminal) and its matured peptide contained 87 amino acid residues with the molecular weight of 9,467 Da and predicted pI of 4.93. Further homolog alignment analysis revealed that it was strongly similar to the Bowman-Birk type protease inhibitor A-II from other species of *Arachis* (Fig. 1). PBBI had the same amino acid sequence as that of *A. hypogaea* A-II except for 17 additional amino acids in the N-terminal regions and the 61st amino acid residue [PBBI is phenylalanine (F), others are tyrosine (Y)] (Norioka and Ikenaka 1983) (Fig. 1). The amino acids responsible for the reactive loop with reactive site of R36-R37 (R56-R57 in the whole sequence) and R64-S65 (R84-S85 in the whole sequence), the reactive bonds and the protease binding sites in A-II are all conserved in the BBI (Suzuki et al. 1993). All 14 cysteine residues in the PBBI were deduced to

155 form seven disulfide bridges, the disulfide bonded residues in the matured PBBI were predicted
156 Cys48-Cys105, Cys49-Cys66, Cys52-Cys100, Cys54-Cys64, Cys73-Cys80, Cys77-Cys92, and
157 Cys82-Cys90, according to the crystal structure of A-II (Suzuki et al. 1993). Three-dimensional
158 structure of PBBI created by SWISS-MODEL was similar to that of A-II (Fig. 2). The reported amino
159 acids responsible for the reactive loop with reactive site of R36-R37 (R56-R57 in the whole sequence)
160 and R64-S65 (R84-S85 in the whole sequence) were highlighted. The two typical domains of BBIs
161 with corresponding reactive loops were well-conserved in the tertiary structures of both inhibitors
162 (Norioka and Ikenaka 1983). Compared with A-II, the major structure difference observed was a
163 extended loop at N-terminal of PBBI. These results indicated a high similarity of PBBI in functional
164 characteristics with aforementioned peanut BBIs, while minor variations may exist.

165 **Fig. 1.**

166 **Fig. 2.**

167 Subsequently, the nucleotide sequence encoding the mature coding region of PBBI was codon
168 optimized, chemically synthesized, and cloned into pPIC9k, resulting a recombinant plasmid
169 pPIC9k-PBBI. It was then linearized and electroporated into *P. pastoris* GS115. The transformant
170 GS-PBBI was selected on MD medium and verified by PCR. After shake flask fermentation, the
171 recombinant PBBI (rPBBI) was produced by GS-PBBI with the yield of 11.1 mg/L. The molecular
172 mass of purified rPBBI was estimated to be 9 kDa based on Tricine-SDS-PAGE (Fig. 3a), which
173 corresponded to the predicted size. Its inhibitory activity toward trypsin was significantly revealed by
174 inhibitory activity staining (Fig. 3b), while only slight inhibitory activity was identified toward
175 chymotrypsin (Fig. 3c). These results clearly indicated that PBBI was functionally expressed in *P.*
176 *pastoris* GS115. Norioka et al. (1982) demonstrated that as low as 3.25 mg individual BBI was

obtained from 1 kg peanuts using chromatographic separation method. In comparation, the heterologous expression of BBI in *P. pastoris* was a more convenient and efficient method.

Fig. 3

Purification of rPBBI

The rPBBI was purified to homogeneity through precipitation, desalting and gel filtration. The results were showed in Fig. 3a and Table 1. Its specific inhibitory activity was 5223.6 TIA/mg (Table 1), which was slightly higher than that of the purified peanut BBIs (4790 TIA/mg) (Norioka et al. 1982).

Inhibitory patterns of rPBBI

The inhibitory activities of rPBBI against trypsin, chymotrypsin, pepsin, papain or subtilisin were studied (Table 2). The rPBBI exhibited strong inhibitory activity against trypsin, and slight inhibitory activity against chymotrypsin. No detectable inhibitory activity against other proteases was observed. The inhibitory activity of rPBBI against trypsin was similar to previously identified peanut BBIs A-I, A-II, B-I, B-II or B-III (Norioka et al. 1982). However, the inhibitory activity of rPBBI against chymotrypsin was much lower than peanut BBIs A-I, A-II, B-I, B-II or B-III (Norioka et al. 1982).

The inhibition parameters of rPBBI against trypsin was further examined and calculated by Lineweaver-Burk plots (Fig. 4). The inhibition constant (K_i) toward trypsin was estimated to be 3.83×10^{-7} M. The rPBBI was a competitive inhibitor ,which is consistent with Norioka et al.(1982).

Fig. 4

Effect of temperature and pH on the inhibitory activity of rPBBI were examined. The results revealed that the inhibitory activity was existed in a wide range of temperatures and pH (Fig. 5a, b). Almost 95% relative inhibitory activity was maintained by rPBBI at 30-90 ^{o}C and pH 2.0 to 10.0 (Fig. 5a, b). Effect of metal ions and EDTA on rPBBI were studied (Fig. 5c). No obvious activation or

inhibitory effect was observed. Losso et al. (2004) reported soybean BBI contained magnesium, calcium and zinc, and demineralized BBI at 30 nM inhibited matrix metalloproteinase-1 activity whereas mineralized BBI was inhibitory at 115 nM. However, no available data were reported for effect of metal ions on peanut BBI. A typical characteristic of BBIs is the tolerance to heat, which is to a large extent dependence upon the existence of the disulfide bonds (Norioka and Ikenaka 1983). The sensitivity of rPBBI to the presence of the reducing agent was assessed. Nearly half of the inhibitory activity was lost after treatment with 10 mM DTT for 60 min and about 90% of the inhibitory activity was lost after treatment with 100 mM DTT for 60 min (Fig. 5d). The results confirmed the importance of disulfide bonds on the inhibitory activity of rPBBI, as was demonstrated for other BBIs from soybean and peanut (Norioka et al. 1987).

Fig. 5

Conclusion

To the best of our knowledge, the peanut BBI gene was overexpressed in the *P. pastoris* system for the first time. The amino acid sequence of the rPBBI showed high similarity with five reported peanut BBIs. The purified rPBBI exhibited stable inhibitory activity toward trypsin in a wide range of temperatures and pH. But the activities were lost on the reduction of rPBBI with DTT. The present study provided a base for the large-scale preparation of PBBI, and encouraged its further application in food, feed, and pharmaceutical industries.

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Conflict of Interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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301 **Table 1** Purification of rPBBI

Step	Total protein (mg)[b]	Total inhibitory activity (TIA)[c]	Specific inhibitory activity (TIA/mg)	Purification fold	Yield (%)
Crude extract[a]	1.57	1001.7	637.6	1	100
Ammonium sulfate 30-40% cut[d]	0.103	388.5	3768	5.91	38.8
Superdex G75 column (pooled peak)	0.0556	292.3	5223.6	8.19	29.2

302 [a] From 5 mL of supernatant, obtained by centrifuging at 3000 × *g* for 5 minutes.

303 [b] Protein concentration determined by Bradford assay using BSA as a standard protein.

304 [c] Trypsin inhibitory activity measured as described in the methods section.

305 [d] Crude extract material that is soluble at 30% but precipitates at 40% saturated ammonium sulfate.

306 **Table 2** Inhibitory activity of rPBBI against different proteases

Ratio (M/M)	Inhibitory rate against different proteases (%)				
	Trypsin	Chymotrypsin	Pepsin	Papain	Subtilisin
I/E=0.1	5.3	0	0	0	0
=1	46.7	0	0	0	0
=2	92.6	0	0	0	0
=10	97.4	32.6	0	0	0

307

Fig. 1 Amino acid sequence alignment of PBBI with five reported peanut BBIs. The sequence of signal peptides is colored in gray. The amino acids composed the reactive loop are boxed. The reactive bonds for trypsin are marked with ▲. The protease binding sites are marked with ○, while the disulfide bond related amino acids with ●. The GenBank accessory numbers for PBBI is XP_016175001, A-II, A-I, B-I and B-III is P01066 and B-II is P01067.

Fig. 2 Predicted 3D structures of PBBI and A-II created by SWISS-MODEL. a: Predicted 3D structures of PBBI aligned with crystal structure of barley BBI, PDB ID: 1c2a.1.A; b: Predicted 3D structures of A-II aligned with crystal structure of chickpea BBI, PDB ID: 6lh6.1.B. The amino acid residues of the reactive sites were labeled.

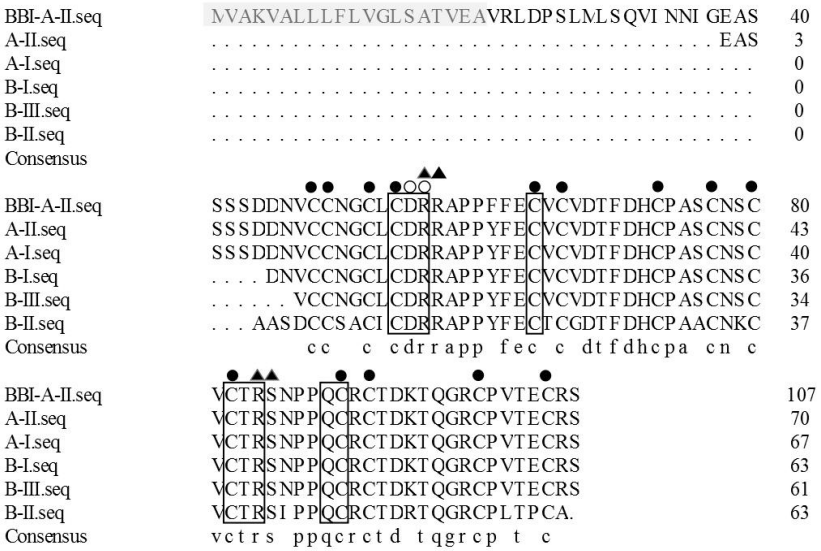
Fig. 3 Profiles of purified rPBBI and activity assay. a: Tricine-SDS-PAGE chromatogram of purified rPBBI. Lanes M: Protein molecular weight standard; 1: Supernatant of GS-PBBI; 2: Purified rPBBI; 3: supernatant of *P. pastoris* GS115-pPIC9k as control. b: Inhibitory activity staining of rPBBI against trypsin. c: Inhibitory activity staining of rPBBI against chymotrypsin.

Fig. 4 Lineweaver-Burk plots of trypsin activity-rPBBI kinetics. Trypsin activity assays were conducted with 0.4, 0.6, 0.8, and 1.0 mM of L-BAPA at 37 ^{o}C and pH 8.0, with 0 (●), 5 (▲), and 10 (■) μg/mL of rPBBI, respectively.

Fig. 5 Effect of temperature, pH, and chemicals on the trypsin inhibitory activity of rPBBI. a: Temperature. The thermostability of rPBBI was determined by incubating rPBBI at pH 8.2 and 40, 50, 60, 70, 80 or 90 ^{o}C for 1 h, the residual inhibitory activities were then determined according to the method part; b: pH. The rPBBI was incubated in buffers ranged from pH 2.0 to 11.0 at room temperature for 1 h, the pH was adjusted to 8.0 and the residual inhibitory activity was determined; c: DTT. The rPBBI was incubated with 1mM (▲), 10 mM (●), and 100 mM (■) of DTT for 120 min

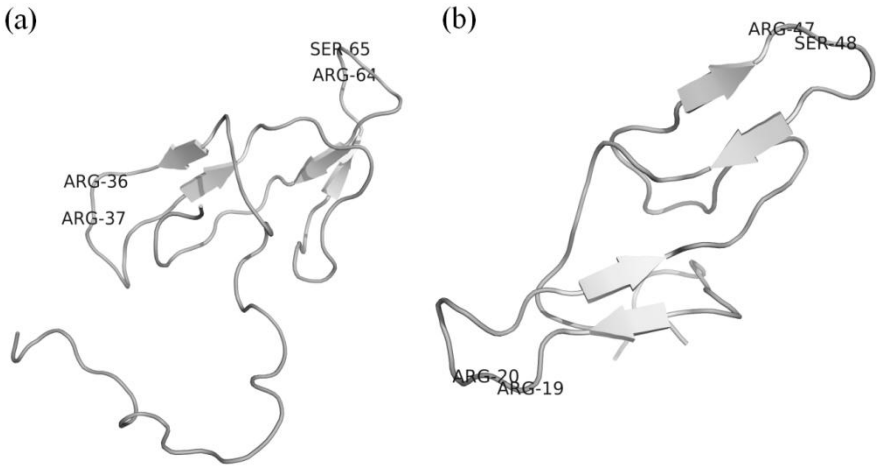
330 and the residue activity of rPBBI was assayed according to the method part.

331 **Fig. 1**

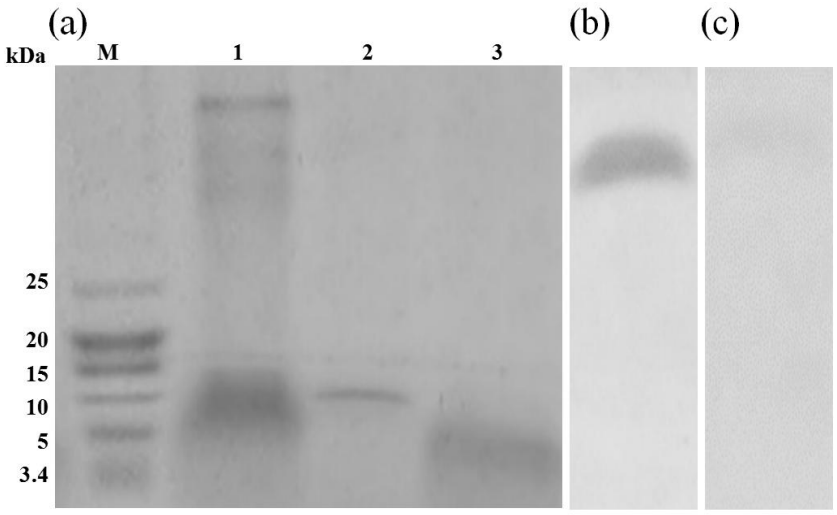

BBI-A-II.seq MVAKVALLLFLVGLSATVEAVRLDPSLMLSQVINNIGEAS 40
A-II.seq .....EAS 3
A-I.seq 0
B-I.seq 0
B-III.seq 0
B-II.seq 0
Consensus
BBI-A-II.seq SSSDDNVCCNGCLCDRRAPPFFECVCVDTFDHCPASCNSC 80
A-II.seq SSSDDNVCCNGCLCDRRAPPYFECVCVDTFDHCPASCNSC 43
A-I.seq SSSDDNVCCNGCLCDRRAPPYFECVCVDTFDHCPASCNSC 40
B-I.seq ....DNVCCNGCLCDRRAPPYFECVCVDTFDHCPASCNSC 36
B-III.seq ......VCCNGCLCDRRAPPYFECVCVDTFDHCPASCNSC 34
B-II.seq ...AASDCCSACICDRRAPPYFECTCGDTFDHCPAACNKC 37
Consensus cc c cdrrapp fec c dtfdhcpa cn c
BBI-A-II.seq VCTRSNPPQCRCTDKTQGRCPVTECRS 107
A-II.seq VCTRSNPPQCRCTDKTQGRCPVTECRS 70
A-I.seq VCTRSNPPQCRCTDKTQGRCPVTECRS 67
B-I.seq VCTRSNPPQCRCTDKTQGRCPVTECRS 63
B-III.seq VCTRSNPPQCRCTDKTQGRCPVTECRS 61
B-II.seq VCTRSIPPQCRCTDRTQGRCPLTPCA. 63
Consensus vctrs ppqcrctd tqgrcp t c

332

Fig. 2

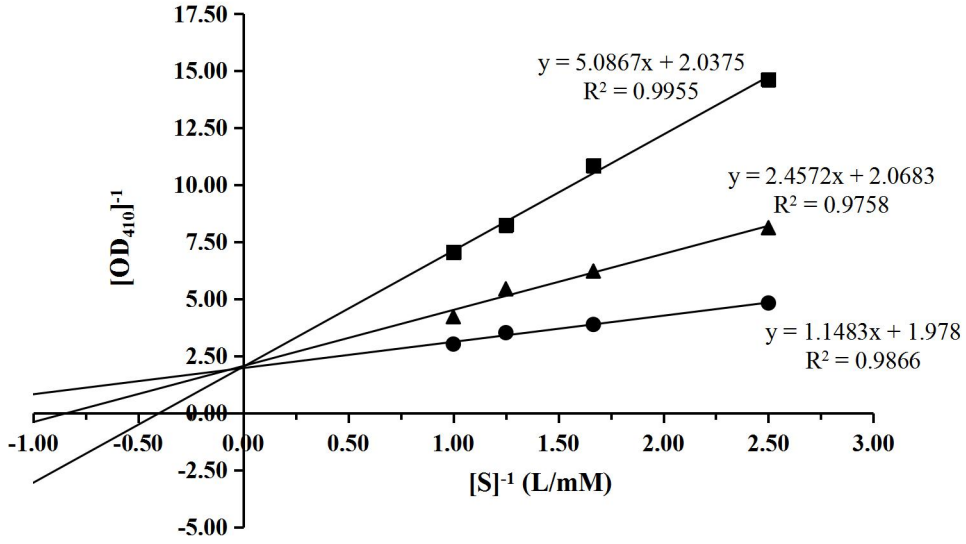


335 **Fig. 3**



336

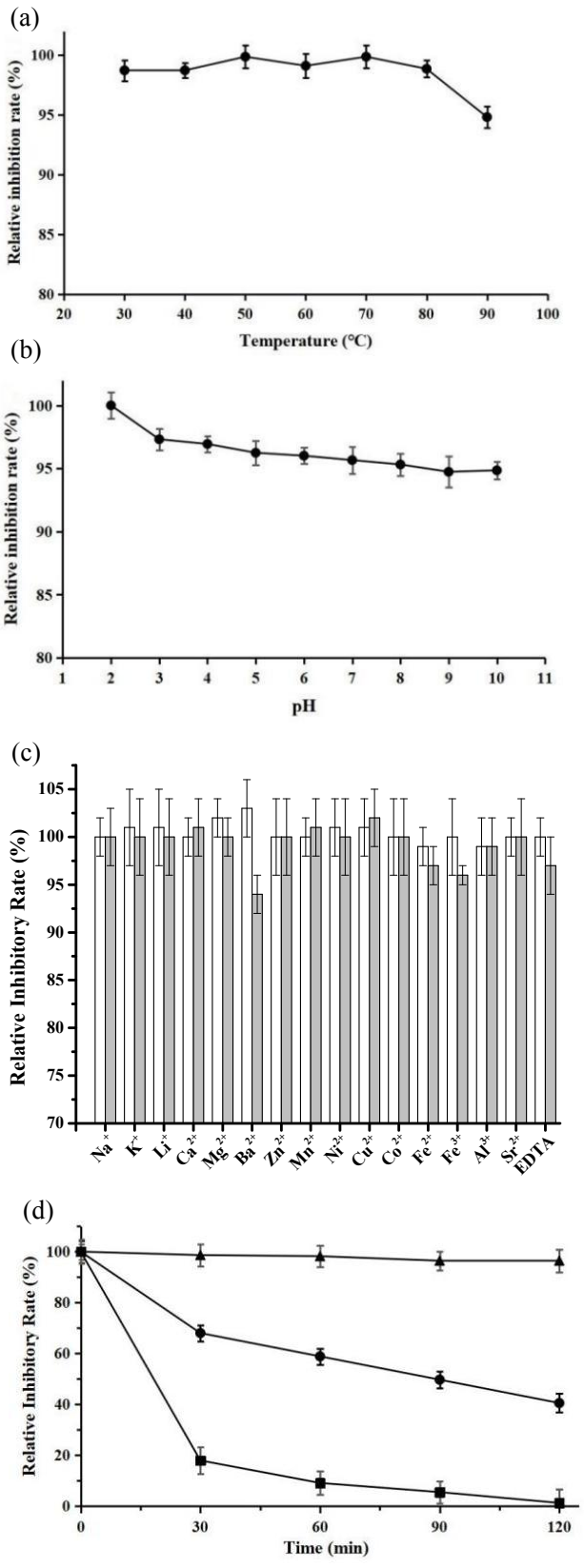
337 **Fig. 4**


y = 5.0867x + 2.0375
R² = 0.9955
y = 2.4572x + 2.0683
R² = 0.9758
y = 1.1483x + 1.978
R² = 0.9866
[OD410]-1
[S]-1 (L/mM)
17.50
15.00
12.50
10.00
7.50
5.00
2.50
0.00
-2.50
-5.00
-1.00
-0.50
0.00
0.50
1.00
1.50
2.00
2.50
3.00

338

339 **Fig. 5**

340


(a)
Relative inhibition rate (%)
Temperature (°C)
(b)
Relative inhibition rate (%)
pH
(c)
Relative Inhibitory Rate (%)
Na+ K+ Li+ Ca2+ Mg2+ Ba2+ Zn2+ Mn2+ Ni2+ Cu2+ Co2+ Fe2+ Fe3+ Al3+ Sr2+ EDTA
(d)
Relative Inhibitory Rate (%)
Time (min)

Figures

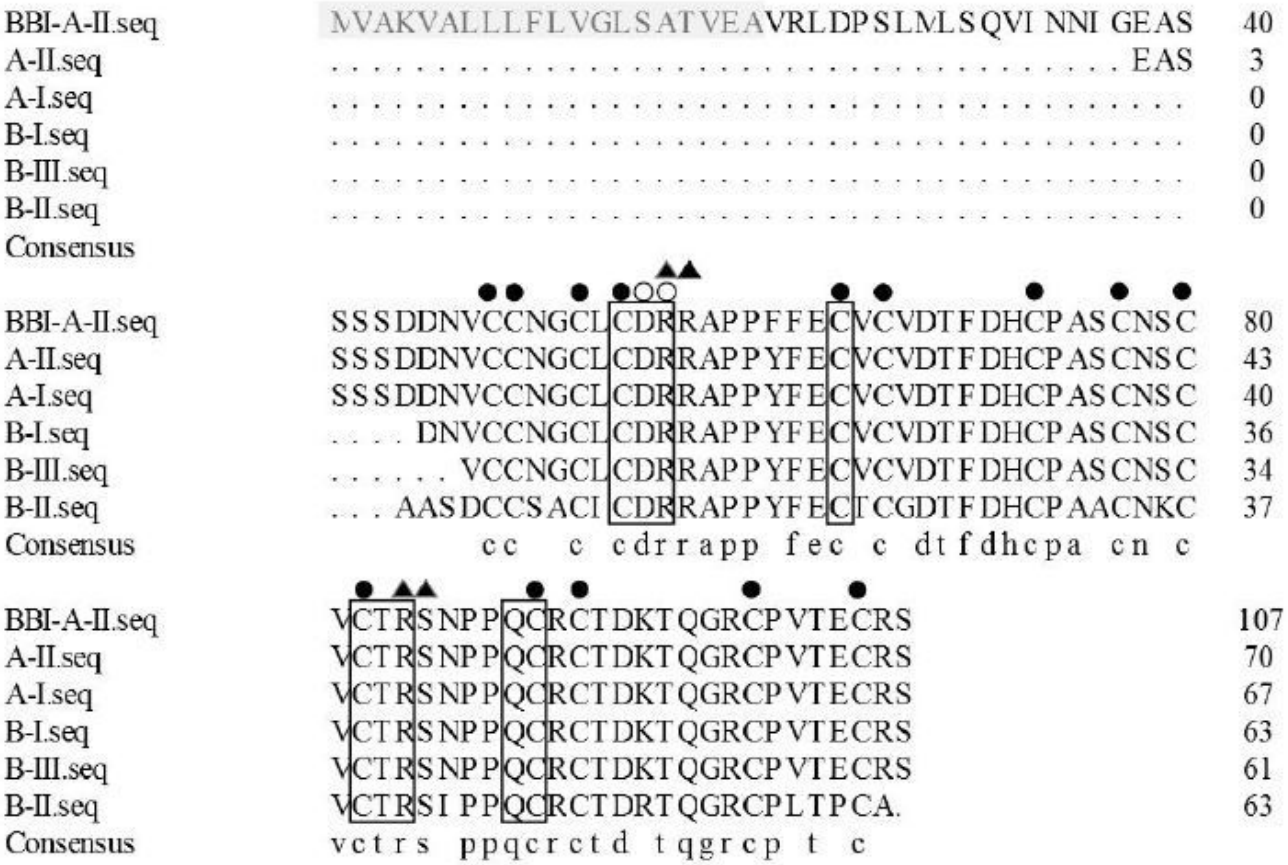


Figure 1

Amino acid sequence alignment of PBBI with five reported peanut BBIs. The sequence of signal peptides is colored in gray. The amino acids composed the reactive loop are boxed. The reactive bonds for trypsin are marked with ▲. The protease binding sites are marked with ●, while the disulfide bond related amino acids with ○. The GenBank accessory numbers for PBBI is XP_016175001, A-II, A-I, B-I and B-III is P01066 and B-II is P01067.

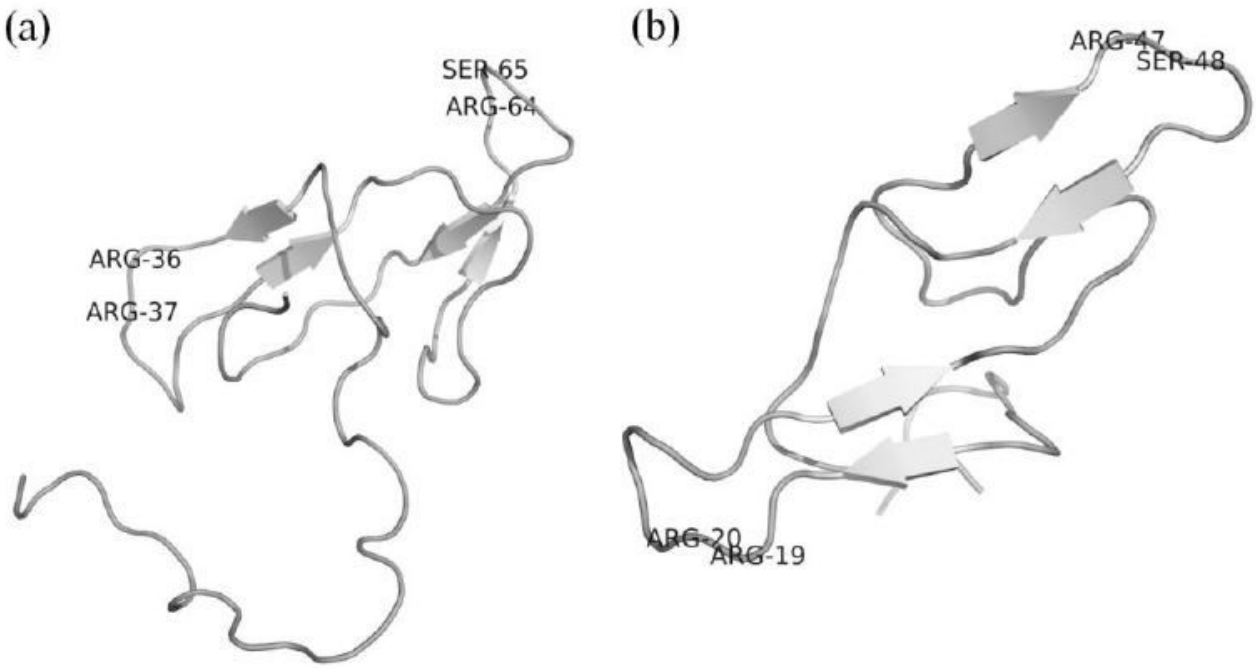


Figure 2

Predicted 3D structures of PBBI and A-II created by SWISS-MODEL. a: Predicted 3D structures of PBBI aligned with crystal structure of barley BBI, PDB ID: 1c2a.1.A; b: Predicted 3D structures of A-II aligned with crystal structure of chickpea BBI, PDB ID: 6lh6.1.B. The amino acid residues of the reactive sites were labeled.

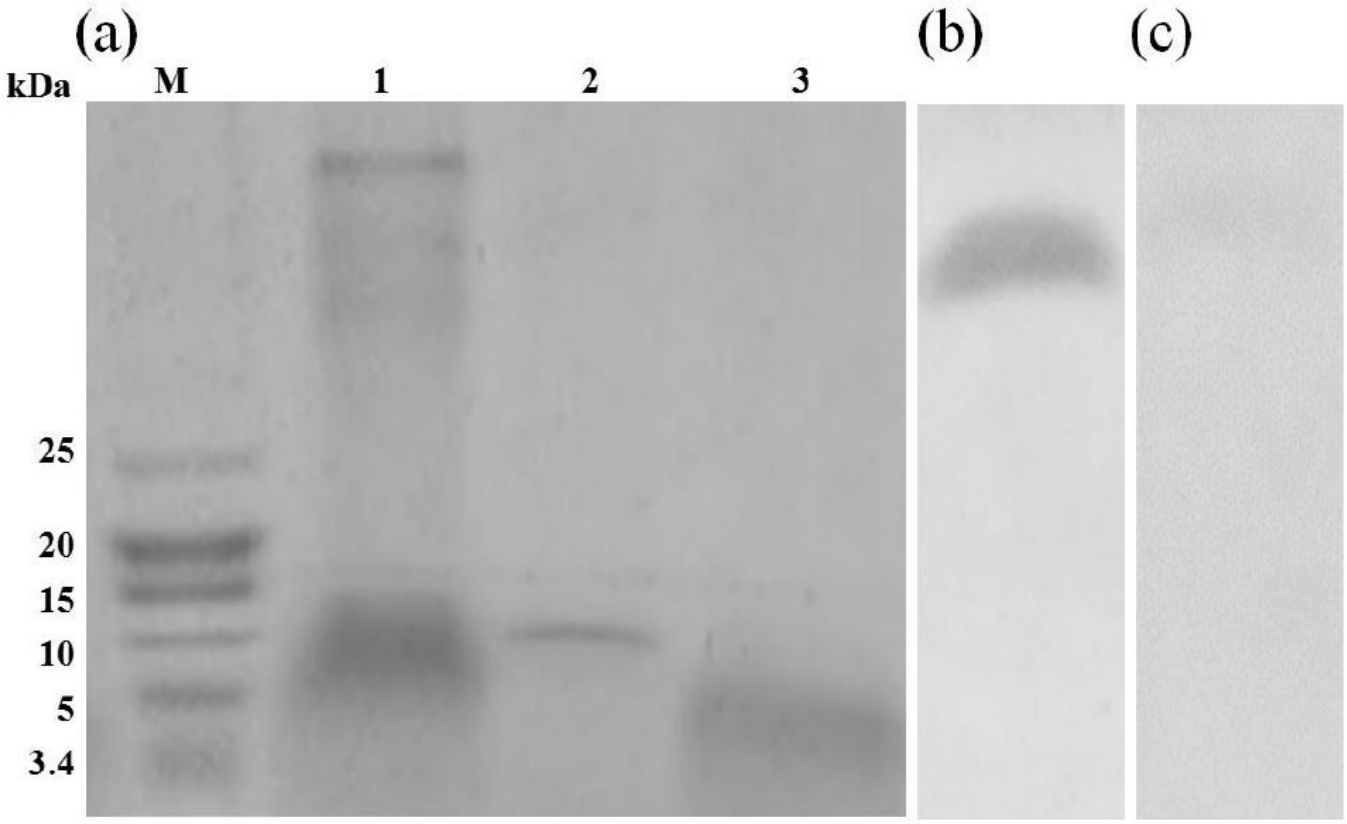


Figure 3

Profiles of purified rPBBI and activity assay. a: Tricine-SDS-PAGE chromatogram of purified rPBBI. Lanes M: Protein molecular weight standard; 1: Supernatant of GS-PBBI; 2: Purified rPBBI; 3: supernatant of P. pastoris GS115-pPIC9k as control. b: Inhibitory activity staining of rPBBI against trypsin. c: Inhibitory activity staining of rPBBI against chymotrypsin.

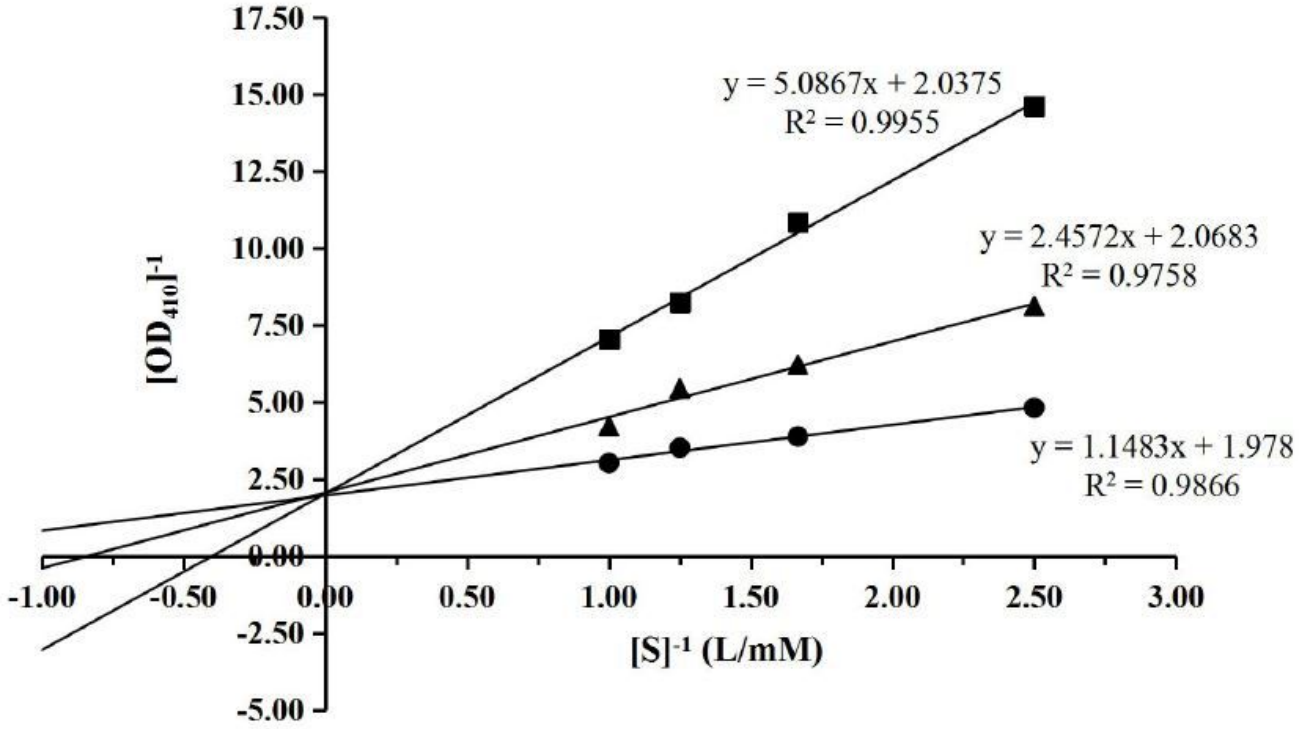


Figure 4

Lineweaver-Burk plots of trypsin activity-rPBBI kinetics. Trypsin activity assays were conducted with 0.4, 0.6, 0.8, and 1.0 mM of L-BAPA at 37 oC and pH 8.0, with 0 (▲), 5 (■), and 10 (●) µg/mL of rPBBI, respectively.

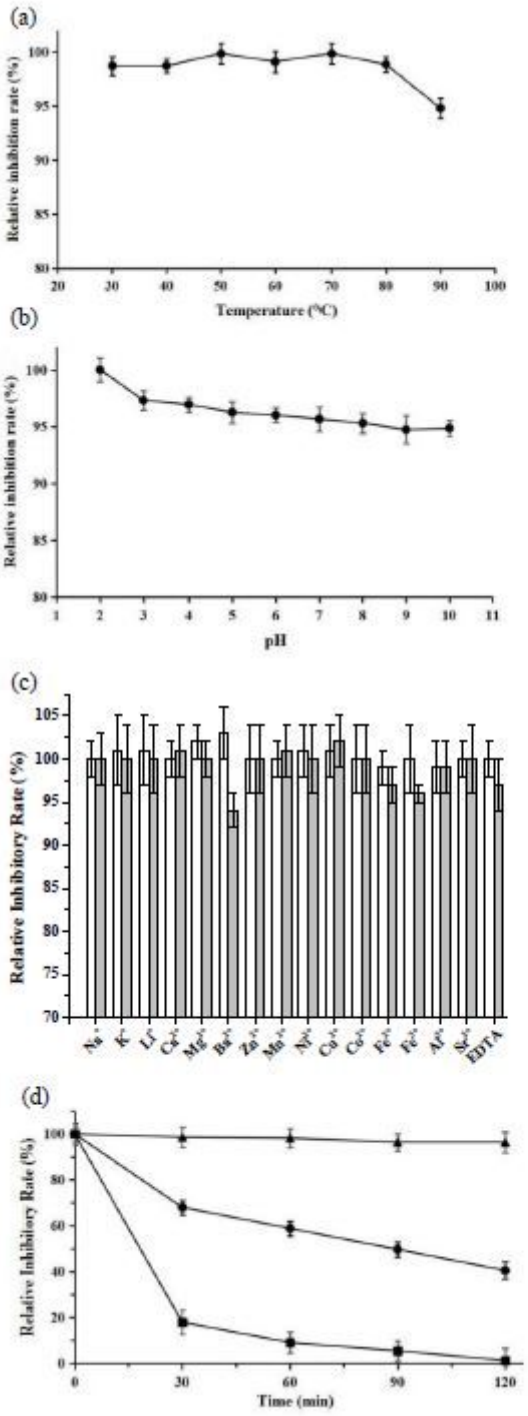


Figure 5

Effect of temperature, pH, and chemicals on the trypsin inhibitory activity of rPBBI. a: Temperature. The thermostability of rPBBI was determined by incubating rPBBI at pH 8.2 and 40, 50, 60, 70, 80 or 90 oC for 1 h, the residual inhibitory activities were then determined according to the method part; b: pH. The rPBBI was incubated in buffers ranged from pH 2.0 to 11.0 at room temperature for 1 h, the pH was adjusted to 8.0 and the residual inhibitory activity was determined; c: DTT. The rPBBI was incubated with 1mM (□), 10

mM (□), and 100 mM (□) of DTT for 120 min and the residue activity of rPBBI was assayed according 330 to the method part.