

Original article

A comparison study of the impact of boiling and high pressure steaming on the stability of soybean trypsin inhibitor

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Summary The aim of the study is to compare the effect of boiling and high pressure steaming (HPS) on the degradation, inhibitory activity reduction and gastric digestibility of soybean trypsin inhibitor (STI). Thermal stability analysis showed that HPS treatment was effective in eliminating the inhibitory activity of STI than boiling. SDS-PAGE and Western blot analysis indicated that boiling has less impact on the gastric digestibility of STI than HPS. More importantly, boiling-pretreated STI revealed high inhibitory activity against trypsin even after digestion by pepsin in simulated gastric fluid (SGF), while HPS treatment was more effective. SDS-PAGE analysis further verified that after boiling, STI still revealed strong binding ability to trypsin, while STI could be completely degraded by trypsin after HPS treatment for 30 min.

Keywords Boiling, gastric digestibility, high pressure steaming, inhibitory activity, soybean trypsin inhibitor.

Introduction

Soybean is an economical crop, served as proteins, energy, vitamins and minerals for human and animal consumption. However, there are many antinutritional factors (ANFs) in soybeans, especially the soybean trypsin inhibitor (STI), which was first isolated from soybean seeds by Kunitz in 1945 (Van den Hout *et al.*, 1998; Kumar Dixit *et al.*, 2011). STI consists of a single polypeptide chain with molecular weight of approximately 21.5 kDa containing 181 amino acid residues with two disulphide bonds (Cys 39-Cys 86 and Cys 138-Cys 145) (Krauchenco *et al.*, 2004; Tettenbaum & Miller, 2001). STI exhibits inhibitory activity on serine proteinases, such as trypsin and chymotrypsin. Hence, over existence of active STI in foods would impair protein digestion, leading to nutrition losing, and even cause growth inhibition, pancreatic hypertrophy and hyperplasia (DiBella & Liener, 1969).

So far, a series of processing methods including chemical and physical treatments were used to inactivate STI (Huang *et al.*, 2008). Chemical reagents such as the NADP/thioredoxin systems were used to reduce the disulphide bonds, thereby inactivate STI by changing its conformation (Jiao *et al.*, 1992). However, these processes would probably bring the chemical reagents into

final food products, which would be harmful for human beings. Several physical processing studies have also been carried out to reduce the inhibitory activity of STI. Among which, high temperature heating (Kwok *et al.*, 1993; Osman *et al.*, 2002), ultrasound (Huang *et al.*, 2008), pressure and temperature (Guerrero-Beltrán *et al.*, 2009) and gamma irradiation (Hafez *et al.*, 1985; Kumar Dixit *et al.*, 2011) were shown to be effective.

As an allergen in soybean, STI is more thermal stable than nonallergenic proteins and stable in digestion by simulated gastric fluid (SGF) (Astwood *et al.*, 1996). Preheating at 100 °C or 90 °C for 5 min was a general processing to inactivate STI, while no obvious degradation of STI could be found in SGF after such short-time treatment (Roychaudhuri *et al.*, 2004; Takagi *et al.*, 2003). Because little information was available about the effect of physical processing on the digestibility of STI, the major purpose of our present work was to compare the extent of destruction and *in vitro* digestibility of STI under different heating-processing conditions.

To investigate the potential mechanism of an effective and well-controlled technology for STI inactivation, the effects of boiling and HPS treatment were compared, in terms of inhibitory activity against trypsin, heat-induced conformational change and immunoreactivity. In addition, *in vitro* SGF was adopted to investigate the digestive stability of STI after boiling and HPS treatments.

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Materials and methods

Materials

Dried soybean (*Glycine max*) was purchased from Jimei market, Xiamen. DEAE-Sephacel, SP-Sephacel and Protein A Sepharose were purchased from GE Healthcare (Piscataway, NJ, USA). Protein markers for tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE) were from Fermentas (Vilnius, Lithuania). Prestained protein standards for Western blot were purchased from New England BioLabs (Beverly, Mass, USA). Fluorogenic substrate t-Butyloxy-carbonyl-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) was obtained from Peptide Institute (Osaka, Japan). Horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG and porcine trypsin were purchased from Sigma (St. Louis, MO, USA). Porcine pepsin was purified according to the methods as previously described (Zhou *et al.*, 2007). All other reagents were of analytical grade.

Purification of soybean trypsin inhibitor and preparation of polyclonal antibody

Dried soybean was powdered in a grinder and soaked in 3-fold acetone for 2 h at room temperature and centrifuged at 5000 *g* for 15 min. The precipitate was washed with 80% ethanol, and the procedures were repeated four times. The resulting precipitate was dissolved in 0.08–0.125 M H₂SO₄ and centrifuged at 5000 *g* for 15 min. Then, the supernatant was adjusted to pH 4.7 and centrifuged at 12000 *g* for 20 min, and ammonium sulphate was added to the supernatant to 70% saturation. After centrifugation at 8000 *g* for 20 min, the resulting pellet was dissolved in a minimum volume of 20 mM Tris-HCl, pH 8.0 (buffer A), and dialysed against the same buffer extensively. The dialysed solution was subsequently applied to a DEAE-Sephacel column (2.5 × 27 cm) equilibrated with buffer A. The adsorbed proteins were eluted at flow rate 1.0 mL min⁻¹ with a linear gradient of NaCl from 0 to 0.5 M in buffer A. Trypsin inhibitory fractions were pooled and dialysed with 20 mM HAc-NaAc, pH 3.5 (buffer B), overnight and then applied to an SP-Sephacel column (2.5 × 27 cm) equilibrated with buffer B. Bound proteins were eluted with 20 mM HAc-NaAc from pH 3.5 to 6.0, and fractions with trypsin inhibitory activity were collected as purified STI and dialysed with 20 mM Tris-HCl (pH 8.0). For antibody preparation, purified STI was inactivated by heat treatment and used to immunise rabbit, and the serum was thus applied to purify IgG by passing through a Protein A Sepharose affinity column.

Protein concentration assay

Protein concentrations were determined by the method of Bradford using the Bio-Rad protein assay kit (Hercules,

CA, USA) or by measuring the absorbance at 280 nm of the sample solution on column chromatography.

SDS-PAGE, tricine-SDS-PAGE, native PAGE and reverse zymography

For electrophoresis, samples were separated by 12% SDS-PAGE according to the method of Laemmli (1970). Tricine-SDS-PAGE was based on a glycine-Tris and tricine-Tris buffer system (Schagger, 2006). Native PAGE was performed in a similar manner as SDS-PAGE, without heating of samples and addition of SDS to both samples and gel. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (CBB). Reverse zymography was performed using tricine-SDS-PAGE containing 1 mg mL⁻¹ gelatin in the gel according to the method of Kleiner and Steler-Stevenson (1994). Briefly, samples without heating were mixed with one-quarter of SDS sample buffer. The gels were electrophoresed at 4 °C. After electrophoresis, gels were washed twice (30 min each time) to remove SDS with 50 mM Tris-HCl (pH 8.0) containing 2.5% (v/v) Triton X-100 by gently shaking. After rinsing with deionised water at room temperature, gels were soaked in 50 mM Tris-HCl (pH 8.0) containing appropriate amount of porcine trypsin and incubated at 37 °C for 12 h followed by staining with CBB. The band zone in the gel was the inhibitor that could not hydrolysed by trypsin.

Inhibitory activity assay of soybean trypsin inhibitor

The inhibitory activity assay of STI against trypsin was performed in duplicate using Boc-Phe-Ser-Arg-MCA as substrate according to the method as described by Jiang *et al.* (2006). Appropriately diluted porcine trypsin (50 µL) and STI (50 µL) solutions were added to 900 µL buffer A and preincubated at room temperature for 10 min. The reaction was initiated by adding 50 µL of 10 µM substrate and reacted at 37 °C for 10 min and quenched by the addition of 1.5 mL of stopping agent (methyl alcohol/isopropyl alcohol/distilled water = 35:30:35, v/v/v). Enzymatic activity was detected by measuring the fluorescence intensity of the liberated 7-amino-4-methylcoumarin (AMC) at an excitation wavelength of 380 nm and an emission wavelength of 450 nm using a fluorescence spectrophotometer (FP-6200; Jasco, Japan). Control tests were performed under same conditions except that STI solution was replaced by buffer A. One unit of trypsin activity was expressed as the amount of the enzyme to release 1 nmol of AMC min⁻¹. Inhibitory activity was expressed as a percentage of inhibition (I) of a control assay using the following equation: $I (\%) = ((T - Ti) / T) \times 100$, where T and Ti are proteinase activities without and with STI, respectively. One unit

of inhibitory activity was defined as the inhibition (50%) of 1 unit of porcine trypsin activity.

Thermal stability of soybean trypsin inhibitor

To investigate the thermal stability of STI, 90 μL of purified STI (1 mg mL^{-1}) was added to different sealed tubes (0.5 mL) and treated with boiling (100°C) or high pressure steaming (HPS) (0.14 MPa, at 121°C) in an autoclave (KT-30 L, ALP, Tokyo, Japan). The heating treatments were stopped at different time intervals of 0, 0.5, 1, 1.5, 2, 3 and 4 h. Then, the tubes were immediately chilled on ice and samples were analysed by SDS-PAGE.

The residual inhibitory activity of STI after heat treatment was examined. Purified STI ($0.5\text{--}10 \mu\text{g mL}^{-1}$) was treated with boiling or HPS in sealed tubes for 0–2 h. The solutions were chilled on ice, and the residual inhibitory activity against porcine trypsin was determined. In control tests, STI was not treated by heating.

Digestive stability and residual inhibitory activity of soybean trypsin inhibitor

The digestibility of STI in SGF was examined according to the method as described by Thomas *et al.* (2004) with some modifications. Three different treatment groups of STI (untreated, boiled for 30 min and HPS treated for 30 min) were prepared. SGF was prepared according to the United States Pharmacopoeia (1995) and consisted of porcine pepsin (160 U mg^{-1}) at pH 1.2. For the final digestion conditions, a ratio of 0.33 U of pepsin activity $\mu\text{g protein}^{-1}$ was selected for all tests (pepsin to STI was 1:200 (w/w)), and the final concentration of STI was 0.2 mg mL^{-1} . Digestion was performed at 37°C with continuous rocking in an eppendorf tube with total volume of 1 mL. At each scheduled time point (1, 2, 5, 10, 15, 30, 60 min), 100 μL of the reaction mixture was removed and added to a separate sampling tube containing 30 μL of 200 mM Na_2CO_3 followed by addition of 26 μL of $4 \times$ Laemmli buffer. The tube was then heated at 95°C for 10 min, and the contents were subjected to SDS-PAGE or Western blot. For the 0 min sample, protein samples were added to SGF that was previously neutralised with Na_2CO_3 . In the control experiments, each protein sample was mixed with the reaction solution without pepsin and then reacted at 37°C for 60 min. All the experiments were repeated in triplicate.

To better reflect the residual inhibitory activity after SGF, inhibitory activity was examined with different ratio of pepsin to STI. The ratio of pepsin to STI was selected as 1:640, 1:320, 1:160, 1:80, 1:40, 1:20, 1:10 (w/w). Digestion was performed at 37°C with continuous rocking for 1 h and residual inhibitory activity

was determined. Other procedures were carried out as described above.

Western blot

Western blot was carried out as described by Towbin *et al.* (1979). Briefly, protein samples were subjected to tricine-SDS-PAGE followed by electrophoretic transfer to nitrocellulose membrane. The membrane was blocked with 5% nonfat milk for 1 h, followed by washing with Tris-buffered saline-Tween-20 (TBST) containing 0.145 M NaCl and 0.05% Tween-20. Then, the membrane was incubated with rabbit anti-STI polyclonal antibody (1:2000 dilution) at room temperature for 2 h and washed with TBST for five times. After incubation for 1 h with HRP-labelled goat anti-rabbit IgG secondary antibody (1:20000 dilution), the membrane was washed extensively with TBST. Specific protein bands were visualised by chemiluminescent detection reagents (Amersham Biosciences, Uppsala, Sweden).

The interaction between soybean trypsin inhibitor and trypsin

To investigate the interaction of STI with trypsin, 1 mg mL^{-1} of trypsin was mixed with different ratios of STI (1:3, 1:2.5, 1:2, 1:1.5, 1:1, 1:0.5, 1:0.25, STI/trypsin, n/n (mol/mol)). Protein solutions (100 μL) were incubated at room temperature for 1 h, and 33 μL of Laemmli buffer was immediately added and separated by SDS-PAGE at 4°C . In control tests, trypsin or STI was replaced by buffer A. To study the effect of heating temperature on the mobility of STI on SDS-PAGE, STI (0.2 mg mL^{-1}) was heated at 40, 45, 50, 60, 70, 80°C in SDS solution and separated by 12% gel.

Statistical analysis

The statistical analysis was performed using the Statistical Package for Social Science (SPSS, software version 17.0) (SPSS Inc., Chicago, IL, USA). Least significance difference test was used to determine difference between means. A *P* value of <0.05 was taken as the level of statistical significance.

Results and discussion

Purification of soybean trypsin inhibitor

In the present study, STI was purified from soybean by a series of pretreatments followed by ammonium sulphate fractionation and sequential column chromatographies of DEAE-Sephacel and SP-Sepharose. As shown in Fig. S1a, STI with the molecular mass of 21.5 kDa was obtained, which was consistent with our previous report

(Jiang *et al.*, 2006). The result of native PAGE (Fig. S1b) and reverse zymography (Fig. S1c) also revealed a single band, suggested STI was highly purified.

Effects of boiling and high pressure steaming on the thermal stability of soybean trypsin inhibitor

Boiling and HPS treatments were performed on purified STI at pH 8.0 and analysed by SDS-PAGE. As shown in Fig. 2a, STI revealed as a monomer form before boiling, suggesting STI could not form dimer in natural state. With the increasing of boiling time, a decrease in the band density of monomer form was observed, while the dimer and polymer bands (higher than 116 kDa) progressively formed. After boiling for 4 h, the polymer bands increased slowly and the monomer still existed. On the other hand, degradation fragments appeared slowly as time went on. It was of interest to notice that the formation of STI polymer was more easily under HPS treatment (Fig. 2b). With time extending, the original band of STI decreased and even disappeared after 3 h, replaced by the increasing formation of polymer band. However, no dimer band was observed. All these results suggested that STI was highly thermal stable and the structure of STI was variable during thermal processing.

It has been reported that food allergenic proteins would form new intermolecular interactions and rearrangements of disulphide bonds at 90–100 °C, and aggregation and chemical modifications on side chains happened at 100–125 °C (Davis & Williams, 1998). As a major allergen in soybean, STI contains two disulphide bonds (cys-39-cys-68, cys-138-cys-145), which play important roles in stabilising the protein structure and sustaining the inhibitory activity (Tetenbaum & Miller, 2001). Heat treatment might break the disulphide bridges of STI and therefore form dimer and polymer through reconstructing disulphide bridges. Furthermore, HPS treatment facilitated the formation of polymer more easily than boiling treatment.

Effects of boiling and high pressure steaming on the inhibitory activity of soybean trypsin inhibitor

In consideration of the digestibility for human beings, inactivation of STI was necessary during soybean food processing. As shown in Fig. S3a, the inhibitory activity of STI decreased progressively with time extending. When the concentration was 0.5 $\mu\text{g mL}^{-1}$, the inhibitory activity of STI was completely lost after boiling for 1 h. With the increasing of STI concentration, the decreasing of inhibitory activity was slowed down. When the concentration of STI was below 2.5 $\mu\text{g mL}^{-1}$, the residual inhibitory activity was lower than 20% after boiling for 1 h, while approximately 40% residual inhibitory activity ($P < 0.05$) remained when the

concentration of STI was 10 $\mu\text{g mL}^{-1}$ under the same condition. After boiling for 2 h, the inhibitory activity remained in all the four groups was below 10%. This result was not the same with the report by Osman *et al.* (2002) that about 60% inhibitory activity remained after boiling for 2 h when the concentration of STI was 200 $\mu\text{g mL}^{-1}$. We propose that it is mainly due to the different inhibitory activity evaluating systems and the concentrations of STI used.

As the inactivation of STI by boiling treatment was not effective when its concentration was 10 $\mu\text{g mL}^{-1}$, high pressure steaming (HPS) at 0.14 MPa, 121 °C, was then employed. As shown in Fig. S3b, the loss of inhibitory activity of STI was significant with HPS treatment. At 10 $\mu\text{g mL}^{-1}$, after boiling for 10 min, the residual inhibitory activity of STI was 21.2% ($P < 0.05$) (Fig. S3b). The inhibitory activity further decreased nearly to 5.0% with HPS treatment for 30 min. These results demonstrated that HPS was more effective in breaking the structure and reducing the inhibitory activity of STI than boiling.

Heating temperature, pH and the concentration of STI were three important factors affecting the elimination of inhibitory activity of STI. Wallace *et al.* (1971) showed that complete destruction of the inhibitory activity of soymilks at 98 °C was from 76 min to 11 min by increasing pH from 6.8 to 9.9. At pH 6.5, inactivating 90% of the inhibitory activity of soymilk was reached for 60 min, 56 and 23 s when the heating temperatures were 93 °C, 143 °C and 154 °C, respectively (Kwok *et al.*, 1993). However, it was not clear about the concentration of STI in these samples. In the present study, we found that under same pH condition (20 mM Tris-HCl, pH 8.0), the time consumed for inhibitory activity losing is proportionally related to the concentration of STI, and 121 °C, 30 min treatment could completely eliminate the inhibitory activity of STI when the concentration is lower than 10 $\mu\text{g mL}^{-1}$. The effect of HPS treatment on soybean products was also evaluated, and the results showed that the residual inhibitory activity of three soybean products (soybean milk, soy flour and defatted soybean flour) was 0.82%, 0.47% and 0.32%, respectively, after HPS treatment for 30 min, indicating that HPS treatment would be an effective method to inactivate STI in soybean products.

Effects of boiling and high pressure steaming on the gastric digestibility of soybean trypsin inhibitor

The digestion pattern of STI in SGF system was then examined by SDS-PAGE and Western blot. In these experiments, the pepsin-containing SGF system was chosen, and the ratio of pepsin to STI was 1:200 (w/w). As shown in Fig. S4a, native STI was hardly digested by SGF, and only a bit of digested fragment (5 kDa)

could be detected after 30 min. These results indicated that STI was quite resistant to proteolysis in SGF, which was in agreement with previous reports (Astwood *et al.*, 1996). Compared with untreated STI, the original STI band with boiling and HPS treatments gradually decreased with the formation of digested fragments (Fig. S4b and c). This result, however, was not consistent with previous reports (Roychaudhuri *et al.*, 2004; Takagi *et al.*, 2003), which indicated that extending heating had no effect on the digestion of STI in SGF. Interestingly, for HPS treatment, the original STI band almost completely disappeared in SGF after 30 min (Fig. S4b) and it was hard to identify the degradation products after 1 h (Fig. S4c). Thus, compared with boiling, HPS treatment could greatly promote the degradation of STI in SGF. Yu *et al.* (2011) reported that HPS treatment can enhance the digestibility of the major allergen tropomyosin (TM) in shellfish in simulated gastrointestinal digestion, as compared to boiling. Similar to TM, STI is also an allergen. The significant enhancement of digestibility of STI by HPS should be owing to the protein structure change under that condition, which exposed the internal sites and subsequently promoted the interaction between pepsin and denatured STI.

It has been reported that recombinant antibody against Bowman–Birk inhibitor (BBI) was effective to target soybean BBI and was applicable for probing it during soybean processing (Muzard *et al.*, 2012). To clarify the residual immunoreactivity of STI after digestion, rabbit anti-STI polyclonal antibody was used to identify the original band of STI. The results showed that untreated STI was resistant to SGF digestion, and the original STI band was only partially decreased, and very strong immunoreactivity revealed after digestion for 60 min (Fig. S5a). As shown in Fig. S5b, the decreasing of STI band was not obvious when time went on. Untreated STI and boiled STI for 30 min still revealed strong immunoreactivity after digestion for 60 min in SGF when the pepsin to STI ratio was 1:200. For HPS treatment, the original STI band disappeared within 30 min in SGF (Fig. S5c). Therefore, comparing with boiling treatment, HPS was more effective in promoting the degradation of STI.

On the other hand, residual inhibitory activity was another important factor reflecting the digestibility of STI. Thus, the effect of the ratio of pepsin to its substrate used in an SGF assay on the residual inhibitory activity was evaluated. Untreated STI was used as control in each group. As illustrated in Fig. S6, with the increasing of pepsin concentration, the inhibitory activity decreased. For control (untreated STI), the inhibitory activity was seen as 100% after digestion in SGF when the ratio of pepsin/STI (w/w) was 1:640 (Fig. 6). As high as 60% inhibitory activity still retained even the ratio of pepsin to STI (w/w) was up

to 1:40, and the inhibitory activity was not significantly different ($P < 0.05$) with the increasing of pepsin concentration. Compared with control, the inhibitory activity of STI (pretreatment at 100 °C for 30 min) decreased 70% when the ratio of pepsin to STI was 1:640. These results further confirmed that the digestibility of STI in SGF was greatly influenced by the ratio of pepsin and its substrate present in the assay (Fu *et al.*, 2002). When the ratio was higher than 1:80, the inhibitory activity did not change any more and was not significantly different ($P < 0.05$). On the other hand, the inhibitory activity of STI almost completely lost when the ratio of pepsin/STI was lower than 1:320 after HPS treatment (Fig. S6). These results strongly suggested that HPS treatment for 30 min would be an ideal processing method to accelerate the degradation of STI in SGF, eliminating the inhibitory activity of STI by effective denaturation.

Effects of boiling and high pressure steaming on the interaction between soybean trypsin inhibitor and trypsin

It was reported that the inhibitory active site of STI is between Arg-63 and Ile-64, which could bind with trypsin (Sweet *et al.*, 1974). The changing of interaction between STI and trypsin was therefore carried out in the present study. STI treated with boiling and HPS was incubated with different concentrations of trypsin respectively at room temperature for 1 h. Laemmli buffer was immediately added without heating and then analysed by SDS-PAGE at 4 °C. As shown in Fig. S7a, a new band with the molecular mass of about 50 kDa was observed, which should be the complex of native STI and trypsin. Because the band of STI (21.5 kDa) was close to that of trypsin (23 kDa), it was difficult to distinguish them on SDS-PAGE. The concentration of this 50-kDa band gradually increased when the ratio of STI to trypsin was below 1:2 (STI/trypsin, n/n). When STI was pretreated with boiling, the band density of the complex formed did not increase as the ratio was above 1:1.5 (STI: trypsin, n/n) (Fig. S7b), which was not much the same as the situation of native STI, suggesting some trypsin-binding sites in STI were destroyed by boiling. Figure S7c shows the interaction between trypsin and HPS-treated STI. The original band of STI disappeared gradually, and no new band indicating the formation of STI and trypsin complex could be found, suggesting the structure and the trypsin-binding sites in STI were effectively destroyed by HPS treatment, leading to its degradation by trypsin.

As shown in Fig. S7a and Fig. S7b, it was of interest to notice that purified STI revealed two bands of 25 and 21.5 kDa, which was inconsistent with the pattern as shown in Fig. S1a. This may be due to different heating temperatures during SDS treatment, which

finally altered the electrophoretic mobility of the protein sample (Nakamura & Mizushima, 1976). Therefore, the effect of heating temperature on the mobility of STI in SDS gels was observed. As shown in Fig. S8a, when heating temperature was below 60 °C, purified STI revealed as two bands (25 and 21.5 kDa). With the increasing of incubation temperature, the 25 kDa band gradually disappeared, and only the 21.5 kDa band could be observed. It was reported that the conformation and electrophoretic mobilities of the major outer membrane proteins (O-8, O-9, O-10) of *Escherichia coli* K-12 changed when they were heated in SDS solution, and a conformational change from β -structure to α -helix was identified by far-UV circular dichroism (CD) spectra (Nakamura & Mizushima, 1976). However, STI is composed of β -sheet (22.5%), β -turn (16.2%) and random coil (61.3%) and without α -helix (Huang *et al.*, 2008). It has been reported that in nonhelical proteins, the bends, loops and other irregular segments of the polypeptide chains would chiefly transform to α -helix conformation with SDS treatment (Jirgensons, 1973, 1982). Therefore, we proposed that in STI, the β -structure might turn to α -helix through SDS treatment in heating treatment, then leading to the change in the structure of STI and affecting the mobility of STI on SDS-PAGE.

Conclusions

The inhibitory activity of STI was reduced after both boiling and HPS treatments in a time- and concentration-dependent manner. Compared with boiling treatment, HPS affected the structure of STI more dramatically, leading to its inhibitory activity elimination and increasing its digestibility in SGF. HPS treatment for 30 min could effectively eliminate the inhibitory activity of STI. Furthermore, HPS-treated STI could be degraded by trypsin, and its interaction with trypsin was completely destroyed, indicating that HPS treatment is an effective way to inactivate STI in soybean products.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) Tricine-SDS-PAGE, (b) native PAGE and (c) reverse zymography of purified soybean trypsin inhibitor (STI). M, protein marker; 1, purified STI.

Figure S2. Detection of the effect of thermal stability. Soybean trypsin inhibitor (STI) was incubated with boiling and high pressure steaming (HPS) for different time intervals. After incubation, samples were applied to SDS-PAGE. STI treated with boiling (a) and HPS (b). M, protein marker. Incubation time was indicated above each lane.

Figure S3. Residual inhibitory activity of soybean trypsin inhibitor (STI) with boiling (a) and high pressure steaming (HPS) (b) treatments. The remaining inhibitory activity was determined in buffer A. Differ-

ent concentrations of STI were preincubated at different temperatures [$(\square)$ 10 $\mu\text{g mL}^{-1}$; $(\blacktriangle)$ 2.5 $\mu\text{g mL}^{-1}$; $(\bullet)$ 1.0 $\mu\text{g mL}^{-1}$; (M) 0.5 $\mu\text{g mL}^{-1}$]. Vertical bars represented $\pm$ standard error.

Figure S4. Effect of simulated gastric fluid (SGF) digestion on soybean trypsin inhibitor (STI). STI was untreated (a), boiling (b) and high pressure steaming (HPS) (c). Enzymatic digestion was performed, followed by tricine-SDS-PAGE. After electrophoresis, proteins were visualised by staining with Coomassie Brilliant Blue R-250 (CBB). In the control experiments (con), proteinase was replaced with buffer A, M, protein marker.

Figure S5. Western blot detection of the degradation of soybean trypsin inhibitor (STI) in simulated gastric fluid (SGF). M, protein marker; STI was untreated (a); treated with boiling (b); treated with high pressure steaming (HPS) (c). The proteins were separated by tricine-SDS-PAGE and transferred to nitrocellulose, followed by an immunological detection with rabbit anti-STI polyclonal antibody to detect the IgG binding of digested samples.

Figure S6. Residual inhibitory activity of soybean trypsin inhibitor (STI) after boiling and high pressure steaming (HPS) treatments. The concentration of STI was 0.25 mg mL^{-1} . Control was untreated; boiling was at 100 °C for 30 min; HPS was at 121 °C for 30 min. STI was incubated with different ratio of porcine pepsin for 1 h, and then, its residual inhibitory activity was determined. Vertical bars represented $\pm$ standard error. Columns with the same letter was not significantly different ($P < 0.05$).

Figure S7. Effect of the interaction between soybean trypsin inhibitor (STI) and trypsin. STI was untreated (a); STI treated with boiling (b); STI treated with high pressure steaming (HPS) (c). Soybean trypsin inhibitor (STI) was incubated with trypsin at room temperature for 1 h and then 4× Laemmli buffer was added and incubated for 30 min at room temperature followed by checking with SDS-PAGE at 4 °C. In control experiment (con), proteinase was replaced with 20 mM Tris–HCl, pH 8.0; M, protein marker; T, trypsin.

Figure S8. Mobility of soybean trypsin inhibitor (STI) heated at different temperatures. M, protein marker.

Figure Legends

Fig. S1. (A) Tricine-SDS-PAGE, (B) Native PAGE and (C) Reverse Zymography of purified STI. M, protein marker; 1, Purified STI.

Fig. S2. Detection of the effect of thermal stability. STI was incubated with boiling and HPS for different time intervals. After incubation, samples were applied to SDS-PAGE. STI treated with boiling (A) and HPS (B). M, protein marker. Incubation time was indicated above each lane.

Fig. S3. Residual inhibitory activity of STI with boiling (A) and HPS (B) treatments. The remaining inhibitory activity was determined in buffer A. Different concentrations of STI was preincubated at different temperatures ((A) ($\square$) 10 $\mu\text{g/mL}$; ($\blacksquare$) 5 $\mu\text{g/mL}$; ($\blacktriangle$) 2.5 $\mu\text{g/mL}$; ($\bullet$) 1.25 $\mu\text{g/mL}$; ($\triangle$) 0.5 $\mu\text{g/mL}$. Vertical bars represented $\pm$ standard error.

Fig. S4. Effect of SGF digestion on STI. STI was untreated (A), boiling (B) and HPS (C). Enzymatic digestion was performed, followed by Tricine-SDS-PAGE. After electrophoresis, proteins were visualized by staining with CBB. In the control experiments (con), proteinase was replaced with buffer A, M, protein marker.

Fig. S5. Western blot detection of the degradation of STI in SGF. M, protein marker, STI was untreated (A); treated with boiling (B); treated with HPS (C). The proteins were separated by Tricine-SDS-PAGE and transferred to nitrocellulose, followed by an immunological detection

with rabbit anti-STI polyclonal antibody to detect the IgG-binding of digested samples.

Fig. S6. Residual inhibitory activity of STI after boiling and HPS treatments. The concentration of STI was 0.25 mg/mL. Control was untreated; boiling was at 100 °C for 30 min; HPS was at 121 °C for 30 min. STI was incubated with different ratio of porcine pepsin for 1 h, then its residual inhibitory activity was determined. Vertical bars represented $\pm$ standard error. Columns with the same letter was not significantly different ($P < 0.05$).

Fig. S7. Effect of the interaction between STI and trypsin. STI was untreated (A); STI treated with boiling (B); STI treated with HPS (C). STI was incubated with trypsin at room temperature for 1 h, then 4 $\times$ Laemmli buffer was added and incubated for 30 min at room temperature followed by checking with SDS-PAGE at 4 °C. In control experiment (con), proteinase was replaced with 20 mM Tris-HCl, **PH-pH** 8.0; M, protein marker; T, trypsin.

Fig. S8. Mobility of STI heated at different temperatures. M, protein marker

Figure S1

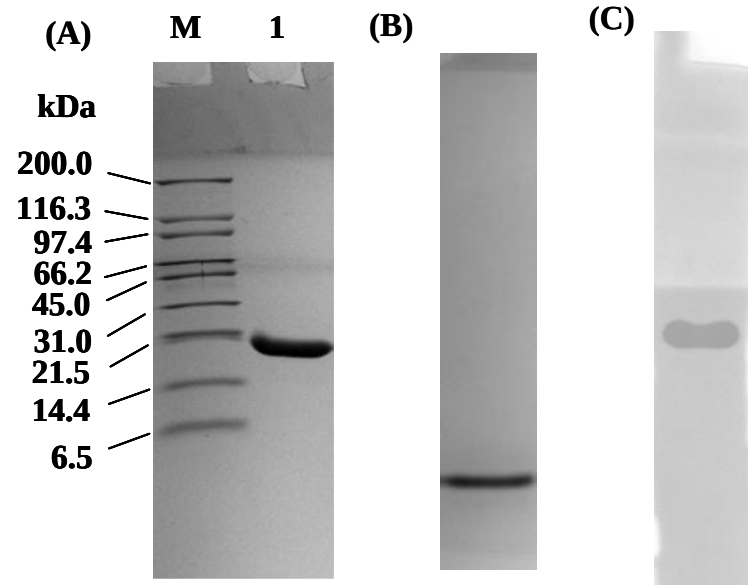


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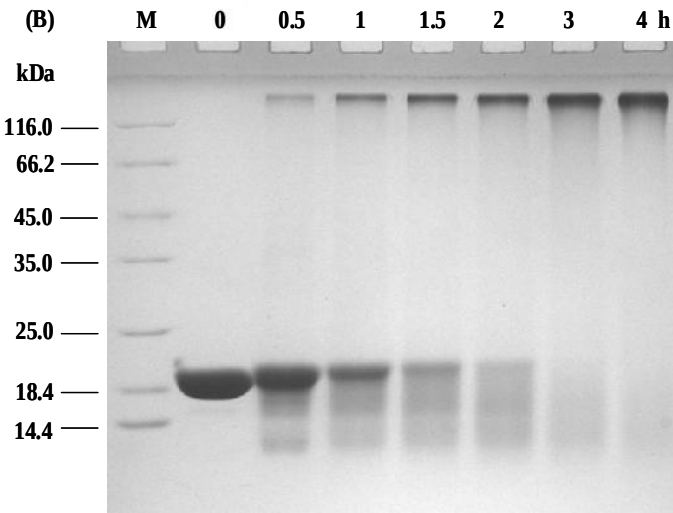
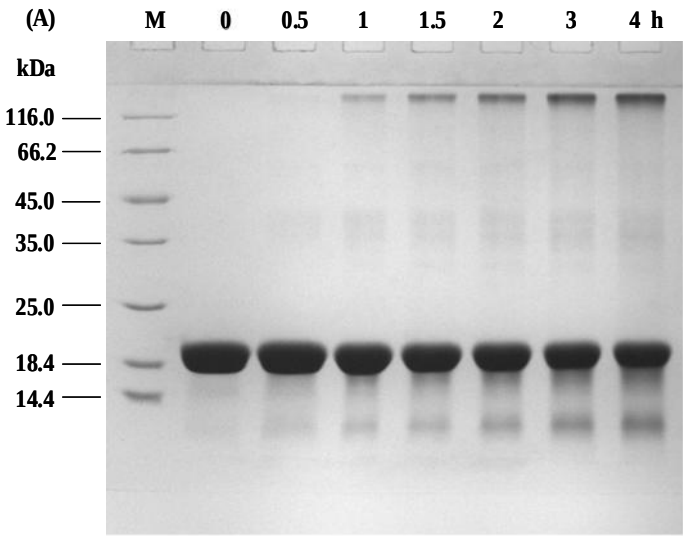
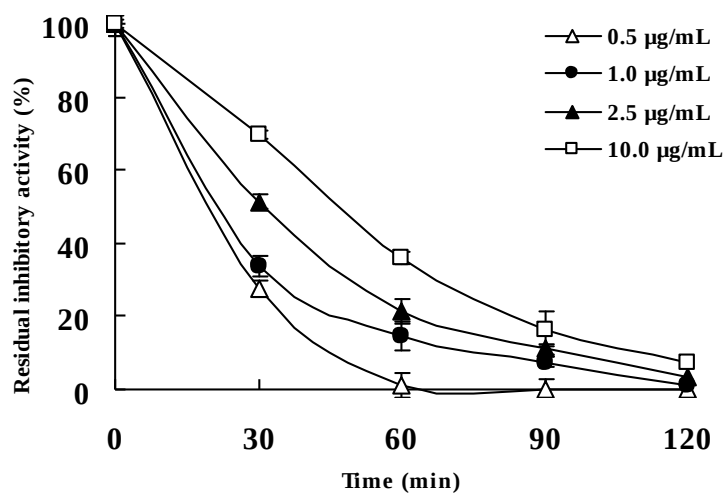
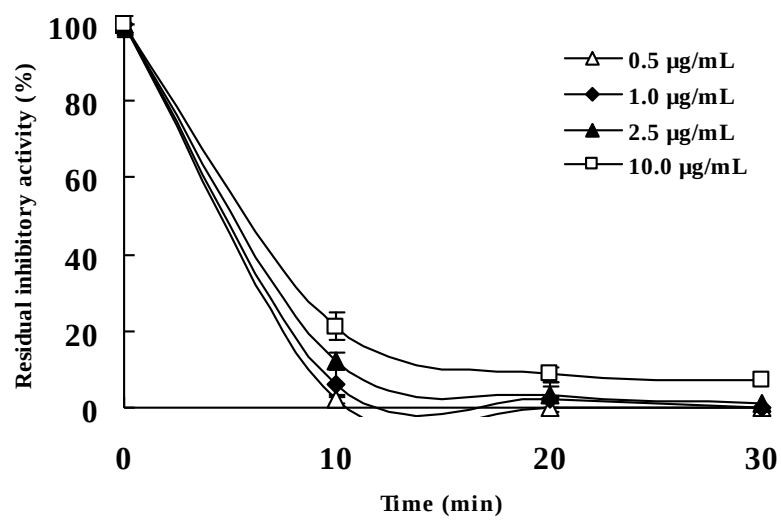


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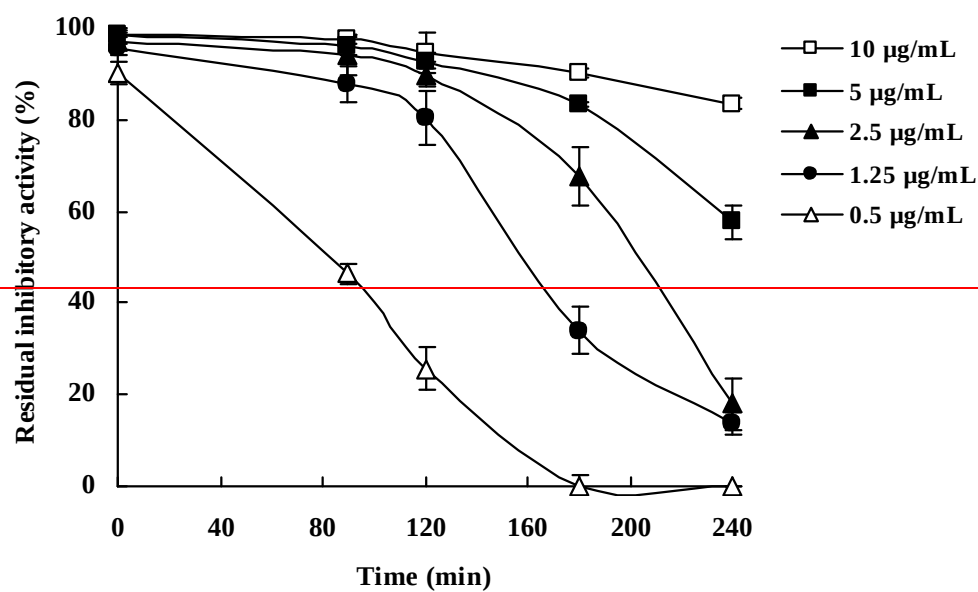
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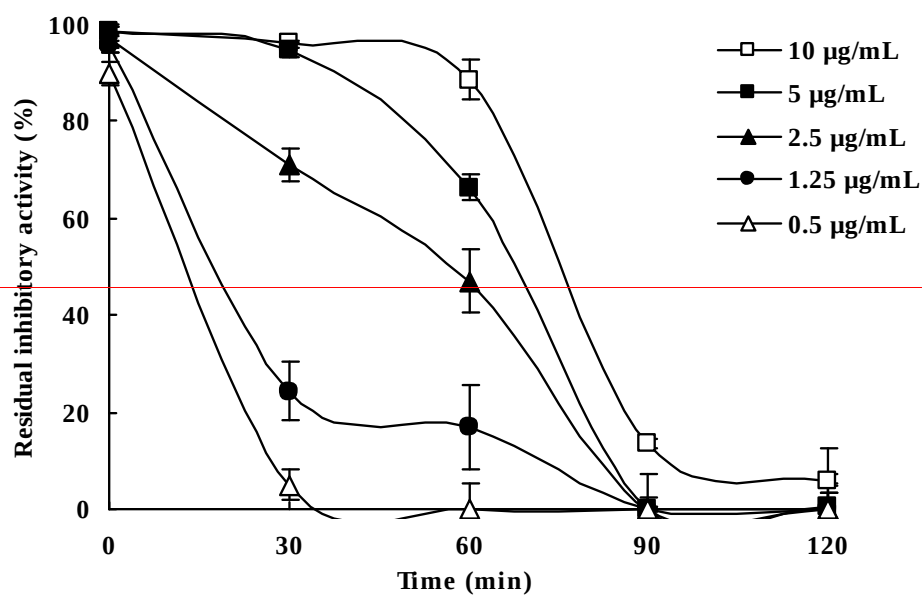
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(A)



(B)



82 **Figure S4**

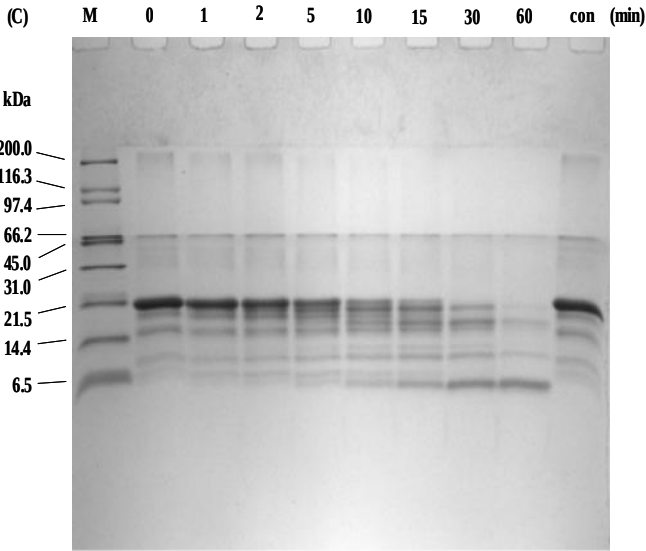
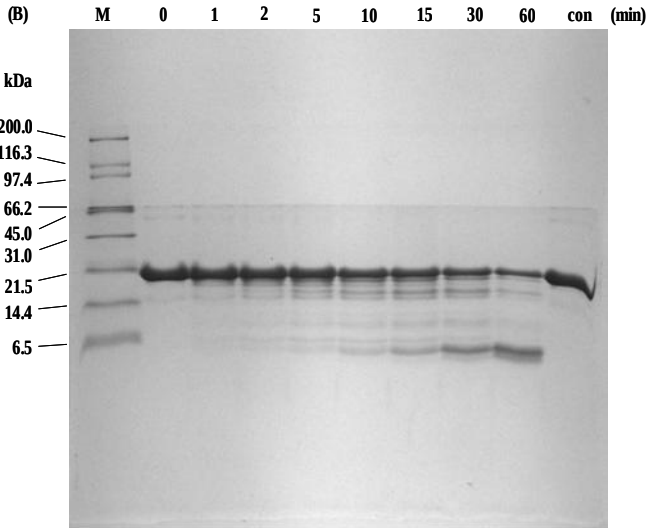
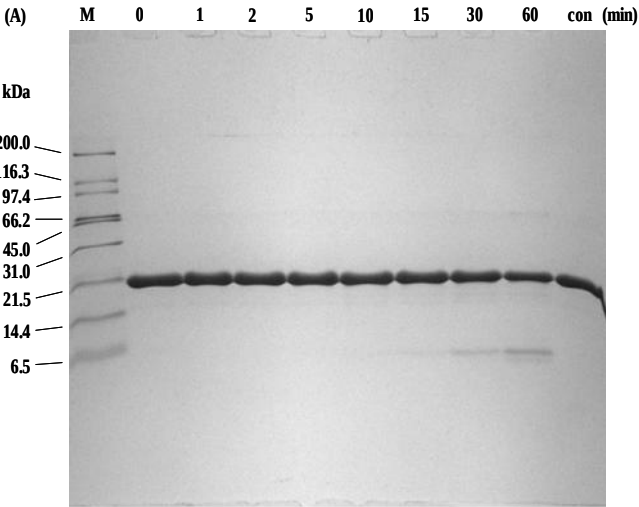


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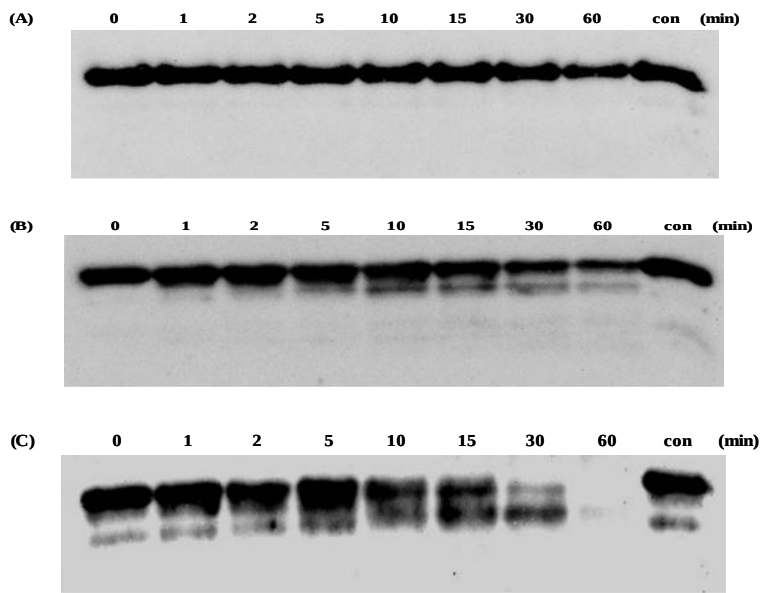


Figure S6

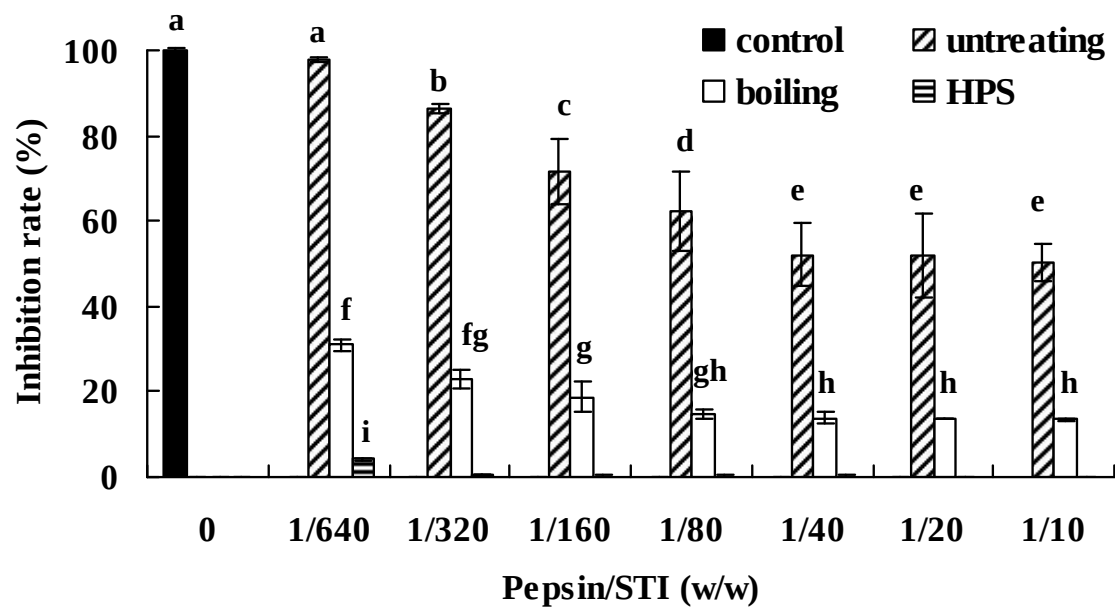
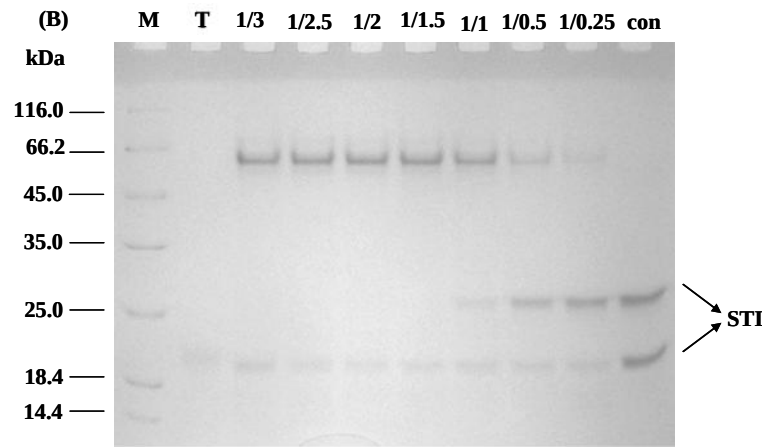
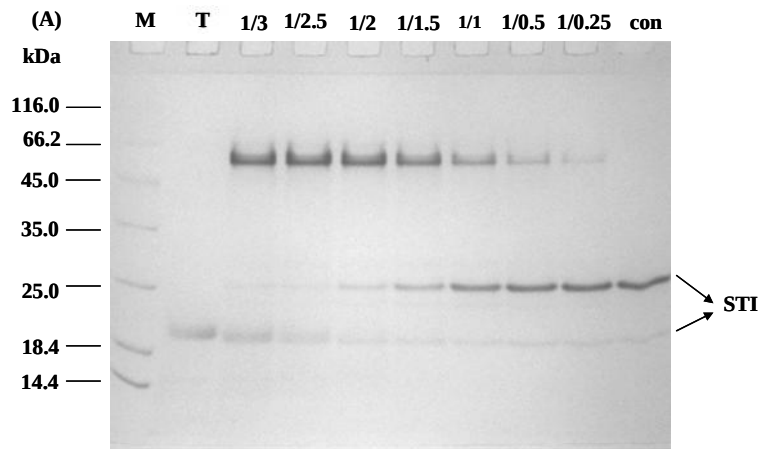
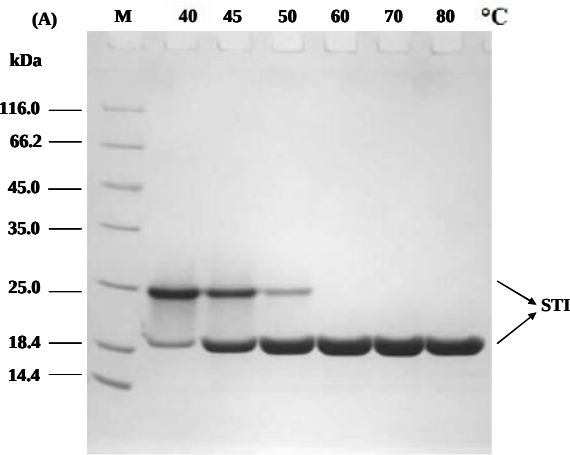


Figure S7



127 **Figure S8**



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