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The Heat-Induced Protein Aggregate Correlated with Trypsin Inhibitor Inactivation in Soymilk Processing

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ABSTRACT: Kunitz trypsin inhibitor (KTI) and Bowman–Birk inhibitor (BBI) have trypsin inhibitor activities (TIA), which could cause pancreatic disease if at a high level. It is not clear why some KTI and BBI lose TIA and some does not in the soymilk processing. This would be examined in this study. TIA assay showed residual TIA was decreased with elevated temperature and TIA was decreased quickly in the beginning and then slowly in boiling water bath. Interestingly, ultracentrifugation showed low residual TIA soymilk had more precipitate than high residual TIA soymilk and soymilk TIA loss had a high correlation coefficient ($R^2 > 0.9$) with precipitate amount. In addition, the TIAs of floating, supernatant, and precipitate obtained by ultracentrifugation were assayed and >80% residual TIA was concentrated in the supernatant. Tricine-SDS-PAGE showed KTI in supernatant was mainly a noncovalent bound form which might exist as itself and/or incorporated into a small protein aggregate, while KTI in precipitate was incorporated into a protein aggregate by disulfide and/or noncovalent bonds. Chymotrypsin inhibitor activity (CIA) assay showed about 89% of the original CIA remained after 100 °C for 15 min. Ultracentrifugation showed that >90% residual CIA was concentrated in supernatant. Tricine-SDS-PAGE showed soymilk (100 °C, 15 min) BBI mainly existed in supernatant but not in precipitate. It was considered that BBI tended to exist as itself with its natural conformation. Thus, it was suggested residual TIA was mainly from the free BBI and TIA inactivation was mainly from KTI incorporation into protein aggregate. This study is meaningful for a new strategy for low TIA soymilk manufacture based on the consideration of promoting protein aggregate formation.

KEYWORDS: *soymilk, protein aggregate, Kunitz trypsin inhibitor (KTI), trypsin inhibitor activity (TIA), Bowman–Birk inhibitor (BBI), chymotrypsin inhibitor activity (CIA), sulfhydryl group, disulfide*

INTRODUCTION

Soymilk is a very popular beverage in the East Asia. Nowadays, it is more and more consumed in the Western countries. This should be due to the positive health aspects of soy products.[1] However, there also exist some antinutritional factors in soy products, which include the well-known Kunitz trypsin inhibitor (KTI) and Bowman–Birk inhibitor (BBI).[2,3] They could bind to trypsin to decrease the protein digestibility and cause pancreatic hypertrophy.

KTI has a molecular weight of 20 kDa and two intrachain disulfides, while BBI has a molecular weight of 8 kDa and seven intrachain disulfides.[4,5] Both of them do not have free sulfhydryl group (SH). KTI just has the trypsin inhibitor activity (TIA). BBI is not only a trypsin inhibitor but also a chymotrypsin inhibitor.[6] Generally, KTI is described as "heat-labile" protein, while BBI is referred as "heat-stable" protein. BBI has a stable conformation even after disulfide bonds (SS) are broken.[7] When pure KTI and BBI solutions were heated at 100 °C, it was found KTI lost its TIA after 180 min while BBI retained over 75% of its TIA even after 360 min.[8] This is well in agreement with the reports above. However, KTI and BBI in the aqueous extract of defatted soy flour lost their activities more quickly than their pure solutions. Unexpectedly, BBI was inactivated more quickly than KTI in defatted soy flour matrix.[8] These revealed KTI and BBI showed different inactivation ways in different systems.

Yuan et al. reported soymilk TIA could be decreased to 13% of TIA in original raw soymilk by traditional treatment (100 °C, 20 min).[9] Kwok et al. reported heating at 143 °C for about 60s can deactivate TIA to 10%.[10] Johnson et al. reported the kinetics of TIA inactivation followed behavior exemplified by the summation of two first-order reactions: the heat-labile reaction was attributed to KTI and the heat-stable reaction to BBI.[11,12] The former accounted for about 85% of original TIA. Rouhana et al. reported KTI could be deactivated very easily (60% of soymilk TIA was from KTI), and TIA inactivation was not protein unfolding.[13]

All the researches above examined the TIA inactivation from the kinetics and suggested some conclusions, but they could not explain why >80% TIA could be deactivated and <20% TIA was still remaining in the soymilk. Generally, SH/SS interchange reaction was considered as one reason for TIA inactivation.[14] It was believed that there were some other reasons for the TIA inactivation. Ono et al. examined the protein behavior in soymilk processing. They found that soybean protein, mainly glycinin and β-conglycinin, dissociated into their subunits/polypeptides and reassociated into protein aggregates and protein monomers by heating. The protein aggregates contained small ones (<40 nm) and large ones (about 100 nm).[15] Although they did not mention KTI and BBI in their studies, KTI and BBI (two proteins) should also be

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involved in this process. Then it is well-known that protein biological activity is closely correlated with its natural conformation. If KTI and BBI were involved in this process and incorporated into protein aggregates, they might lose their biological activities owing to their conformation change induced by their incorporation into protein aggregates regardless of their locations on the protein aggregate surface or in the protein aggregate core. Thus, this study would examine the soymilk TIA inactivation on the viewpoint of colloidal science.

In China, the traditional cooking method is still widely used because of widespread soymilk manufacture by soymilk machine. Therefore, soymilk TIA inactivation mechanism under the treatment ($\leq$100 °C) would be examined in this study. This might supply some points for making low TIA soymilk with less heat time or low heat temperature, which is meaningful for soymilk nutrition and the lifestyle of modern people. The soymilk could be separated into floating, supernatant, and precipitate fractions by ultracentrifugation. The TIAs in the three fractions obtained from soymilks by different heat temperatures or heat time were assayed, respectively. The existing states of KTI and BBI in the supernatant and precipitate fractions were examined by the nonreducing and reducing Tricine-SDS-PAGE.

MATERIAL AND METHODS

Materials. Soybean Heinong 54, harvested in 2011, was purchased from the Northeast Soybean Research Institute and stored at 4 °C until use. All Reagents were purchased from Sigma-Aldrich Trading Co., Ltd. or of analytical reagent grade.

Soymilk Preparation. First, 10 g of soybean was soaked in deionized (DI) water for 18 h at 4 °C. DI water was added to make the total weight of 200 g and was ground in a Waring blender (18000 rpm, MJ-60BE01B, Midea) for 3 min. The homogenate was filtered through four layers of gauze and then 200-mesh sieve. The filtrate was designated as raw soymilk. Then 20 mL of raw soymilk was added into five 50 mL beakers and heated in water bath at 60, 70, 80, 90, and 100 °C for 15 min, respectively,[13] or 20 mL of raw soymilk was added into six 50 mL beakers and heated in boiling water bath for 1, 2, 5, 15, 30 and 60 min, respectively. The free sulfhydryl in raw soymilk was determined. Then 20 mL of raw soymilk was added into three 50 mL beakers. *N*-Ethylmaleimide (NEM) was added to make NEM/sulfhydryl (μmol/μmol) = 1:1, 2:1 and 20:1, respectively. They were cultivated for an hour and heated in boiling water bath for 15 min. All beakers were covered with preservation film before heating to suppress water evaporation. Raw soymilk was used as control.

Preparation of Soymilk Floating, Supernantant and Precipitate Fractions by Ultracentrifugation. Soymilk was treated by using a Beckman Optima L-XP ultra centrifuge (197000*g*, 1 h) and separated into three fractions: floating (mainly oil body), supernatant, and precipitate fractions. According to the method by Guo et al.,[16] protein aggregates larger than 34 nm were precipitated. Protein in supernatant was small protein aggregates (<34 nm) and monomer protein. The three fractions were collected carefully and sampled. The floating fraction was carefully collected and put into 25 mL beaker. DI water was added to make the volume of original soymilk. The supernatant was collected by syringe. Precipitate was collected and put into a beaker. DI water was added to make the volume of original soymilk and kept overnight. Then it was mixed well by magnetic stirrer and precipitate solution was obtained.

Trypsin Inhibitor Activity (TIA) Assay. Trypsin inhibitory activities (TIA) in soymilk, floating fraction, supernatant, and precipitate were measured by the method of Liu and Markakis with slight modifications.[17]

TIA standard curve: 0−1.0 mL trypsin inhibitor standard solution (0.01%, w/v) was pipetted into a set of test tubes, and the volume was made up to 2 mL with DI water. The test tubes were placed and warmed in a 37 °C water bath. Then 5 mL of 0.04% (w/v) N_α-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPA) in pH 8.2 Tris-HCl buffer (1% dimethylsulfoxide, v/v; 0.02 M $CaCl_2$), which should be prepared freshly and prewarmed in 37 °C water bath, was pipetted into test tubes followed by 2 mL of 0.01% (w/v) trypsin in 0.04 mM HCl solution. Exactly 10 min later, the reaction was terminated by adding 1 mL of 30% acetic acid (v/v) into each tube. After mixing, the content of each tube was filtered with membrane (0.45um, φ50 LBK), and the absorbance of the filtrate was measured at 410 nm by using UV-2100 spectrophotometer (UNICO, WFZ UV-2100) against a reagent blank. The reagent blank was prepared by adding 1 mL of 30% acetic acid (v/v) to a test tube containing trypsin and water before 5 mL of BAPA solution was added. The linearity of the calibration curve was r = 1.

Trypsin inhibitory activity of sample (soymilk, floating fraction, supernatant, or precipitate): The samples should be diluted to the point where 1 mL produces trypsin inhibition of 40−60%. Then 1 mL of diluted sample was added to tubes, and it was done just as above. One trypsin inhibitory activity (TIA) unit is arbitrarily defined as an increase of 0.01 absorbance units at 410 nm per 10 mL of the reaction mixture under the conditions used.

Chymotrypsin Inhibitor Activity (CIA) Assay. Chymotrypsin inhibitor activities (CIA) in soymilk and supernatant were determined by using a method modified from Tan et al.[18]

The samples were diluted to the point where 0.5 mL produced 30−40% chymotrypsin inhibition. Then 0.5 mL of diluted samples were pipetted into test tubes and adjusted to 1.0 mL with DI water. Then 1.0 mL of α-chymotrypsin solution (0.02 mg/mL dimethylformamide) was added, followed by 0.4 mL of *N*-benzoyl-L-tyrosine *p*-nitroanilide (BTpNA) solution (0.4 mg/mL dimethylformamide) and thorough mixing. Exactly 10 min later, the reaction was terminated by adding 0.5 mL of 30% (w/v) acetic acid. The mixture was then centrifuged at l0000g for 10 min, and absorbance of the supernatant was measured at 410 nm against a reagent blank by using a UV-2100 spectrophotometer (UNICO, WFZ UV-2100). The reagent blank was prepared by adding 0.5 mL of 30% (w/v) acetic acid to a test tube containing chymotrypsin and water (1 mL each) before 0.4 mL of BTpNA solution was added. A sample blank is prepared by adding 0.4 mL of BTpNA solution to the diluted sample and DI water (0.5 mL each), incubating the mixture at 37 °C for 10 min, and then adding 0.5 mL of acetic acid followed by the addition of 1 mL of α-chymotrypsin.

Chymotrypsin inhibitor activity (CIA) is estimated from the residual chymotryptic activity of the mixture of diluted sample and α-chymotrypsin. One unit of α-chymotrypsin activity was arbitrarily defined as an increase of 0.01 absorbance unit (at 410 nm) of the reaction mixture under the conditions used.

Sulfhydryl (SH) and Disulfide (SS) Determination. The method for SH and SS determination was that of Ou et al. with some modifications.[19] First, 1 mL of soymilk and 9 mL of anhydrous acetone were mixed and held for 10 min at 25 °C before being centrifuged at 3000*g* for 15 min. Then the precipitate was washed twice (5 mL acetone; 3000*g*, 15 min). Finally, the acetone was removed by evaporating the solvent with streams of cold air.

Total Free SH Determination. The dry precipitate above was dissolved in 5 mL of buffer 1 [pH 8.0, 1.04% Tris (w/v), 0.69% glycine (w/v), 0.12% EDTA (w/v), 8 M urea]. This was deemed as pretreated soymilk. Then 1 mL of pretreated soymilk, 2.0 mL of buffer 1, and 0.02 mL of 0.4% Ellman's reagent (w/v) were mixed and reacted at 25 °C for 5 min. The absorbance at 412 nm was measured by a UV-2100 spectrophotometer (UNICO, WFZ UV-2100). The reacted solution without pretreated soymilk was a blank, while the reacted solution without Ellman's reagent was used for the turbidity determination.

Total Cysteine Content Determination. First, 0.2 mL of pretreated soymilk, 1 mL of buffer 2 [pH 8.0, 1.04% Tris (w/v), 0.69% glycine (w/v), 0.12% EDTA (w/v), 10 M urea], and 0.02 mL of 2-mercaptoethanol (2-ME) (14.4 mol/L, purity >99%) were mixed and held at 25 °C for 1 h, and then 10 mL of 12% TCA (w/v) was added and again held at 25 °C for 1 h and then centrifuged at 5000*g* for 15 min. The precipitate was twice resuspended in 5 mL of 12% (w/v) TCA and centrifuged at 5000*g* for 10 min. At last, the precipitate was

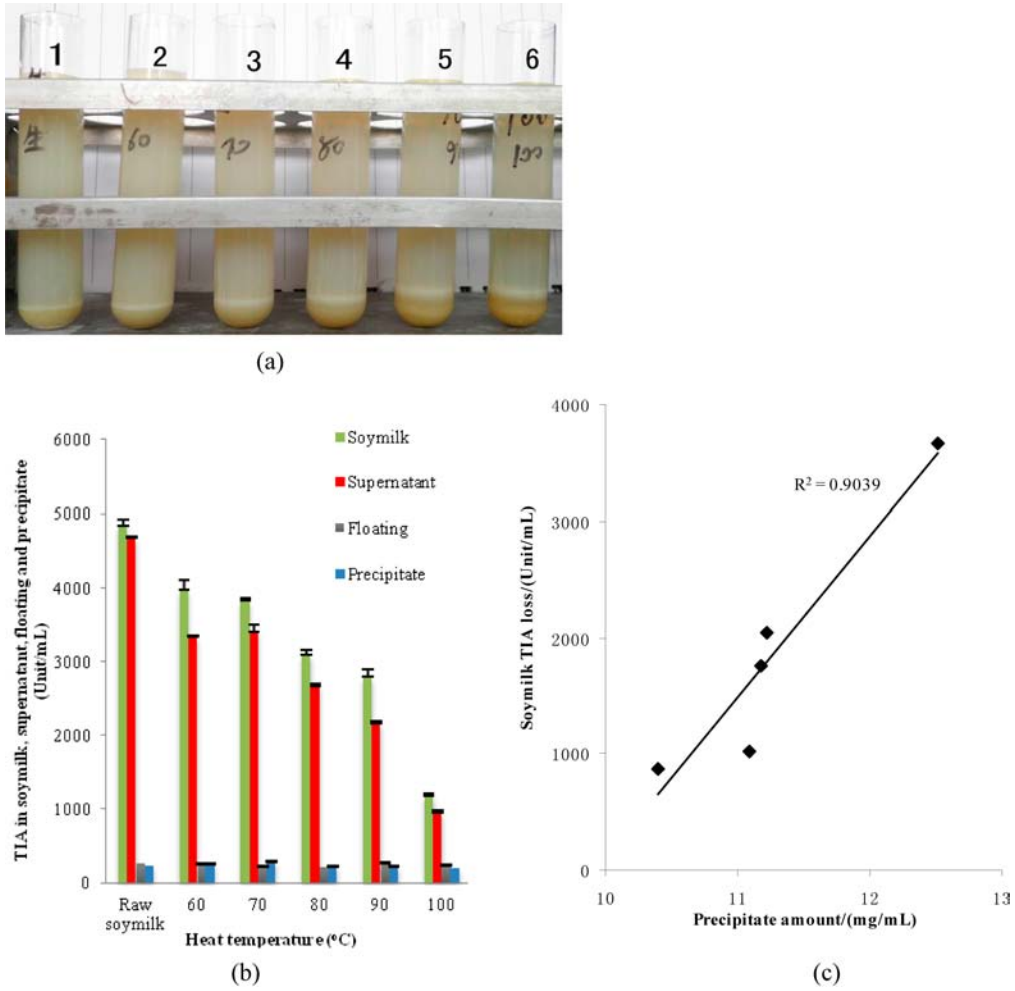

Figure 1. The temperature effect on soymilk trypsin inhibitor activity (TIA). (a) Soymilks after ultracentrifugation (197000*g*, 60 min); centrifuge tube 1 was raw soymilk, 2−6 were soymilks heated by 60, 70, 80, 90, and 100 °C for 15 min, respectively; (b) TIAs in soymilk, soymilk supernatant, precipitate, and floating fractions obtained by ultracentrifugation (197000*g*, 60 min); (c) the correlation between the precipitate amount and the TIA loss.

dissolved in 3.0 mL of buffer 3 [pH 8.0, 1.04% Tris (w/v), 0.69% glycine (w/v), 0.12% EDTA (w/v)], the color was developed with 0.05 mL of 0.4% (w/v) Ellman's reagent, and the absorbance was read at 412 nm. The reacted solution without pretreated soymilk was as blank soymilk without Ellman's reagent for the turbidity determination.

The contents of total free SH and total cysteine contents were calculated according to the following formula of Beveridge et al. with slight modifications:[20]

$$\mu\text{mol SH/mL} = (73.53 \times A_{412} \times D)/1000$$

A_{412} = difference of absorbance at 412 nm between with and without DTNB in color developing solutions. D is the dilution factor, for total free SH, $D = 3.02 \times 5 = 15.1$, for total cysteine, $D = (3.05/2) \times 5 = 7.625$.

Tricine-SDS-PAGE. Tricine-SDS-PAGE was usually applied to efficiently separate protein with small molecular weights. The molecular weights of KTI and BBI are 20 and 8 kDa, respectively, so we attempted to separate them with this method. Tricine-SDS-PAGE was carried out according to the procedure of Schagger with some modifications.[21] The separating gel, spacer gel, and stacking gel were well prepared and solidified.

For supernatant, the highest protein concentration sample (obtained from raw soymilk by ultracentrifugation) was diluted to 2 mg/mL. The other supernatants were diluted with the same dilution ratio. This was different from the normal method, but it could show the amount of changes of KTI and BBI. Precipitate samples were treated with the same method as supernatant. Then 0.5 mL of diluted sample was mixed with 0.5 mL of sample buffer [0.05 M Tris-HCl at pH 6.8, 4% (w/v) SDS, 12% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and 2% (v/v) 2-ME (or no for nonreducing)] effectively and heated for 3 min in a boiling water bath, then centrifuged at 10000 rpm for 3 min. Then 10 μL or 20 μL of each sample was loaded into a well. Tricine-SDS-PAGE was performed at 30 V for 1 h, and then up to 100 V. After electrophoresis, the gels were stationed for 2 h in stationary liquid [45.4% (v/v) methanol, 9.2% (v/v) glacial acetic acid] and then stained for 2 h using 0.1% (w/v) Coomassie Brilliant Blue R-250. After staining, the gels were destained using a 10% (v/v) acetic acid solution until a clear background was obtained.

RESULTS AND DISCUSSION

It was considered there were two necessary factors for protein biological activity. One fundamental factor was the protein conformation, the other was the contact of protein with its target. Soymilk was obtained by heating raw soymilk. Ono et al. reported soymilk protein could be divided into two parts, soluble protein (<40 nm, containing small protein aggregate and protein monomer) and large protein aggregate (also called protein particle, 100 nm).[15] Thus, it is possible some KTI and BBI might be incorporated into protein aggregate (including

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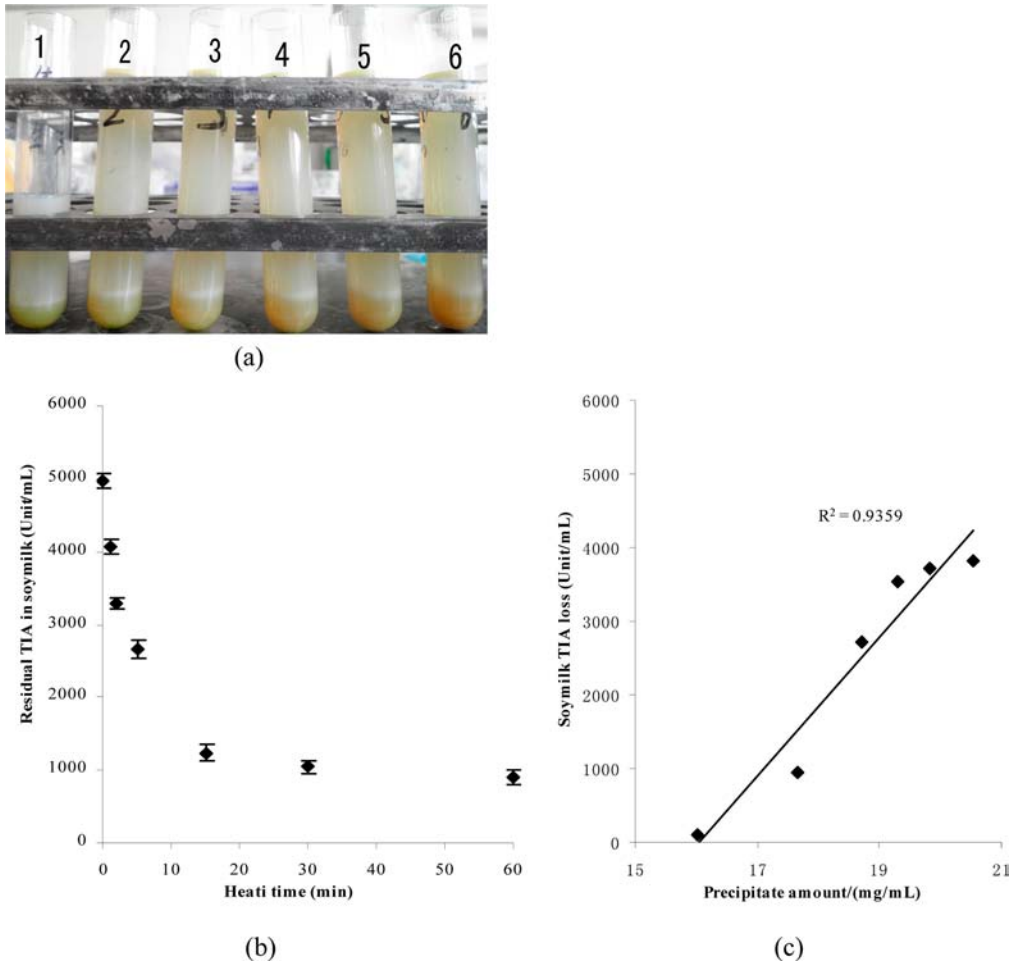

Figure 2. The heat time effect on soymilk trypsin inhibitor activity (TIA). (a) Soymilks after ultracentrifugation (197000*g*, 60 min); Centrifuge tubes 1−6 were treated by 100 °C for 1, 2, 5, 15, 30 and 60 min, respectively; (b) the residual TIA of soymilk treated by 100 °C for 1, 2, 5, 15, 30 and 60 min, respectively; (c) the correlation between the precipitate amount and the TIA loss.

the small protein aggregate and large protein aggregate), which could induce the soymilk TIA loss. The other KTI and BBI, which still exist as themselves after heating, might show different TIA. KTI might lose most of its TIA owing to its conformation change,[8,13] while BBI might keep most of its TIA because of its heat stable conformation.[7,8]

TIA in Different Fractions of Soymilk. Soymilk protein, mainly β-conglycinin and glycinin, have different denaturation temperatures. Therefore, the heat-induced protein aggregate formation would be different at different temperatures. Figure 1a shows soymilk is separated into floating (mainly oil body), supernatant (<34 nm small protein aggregate, protein monomer, carbohydrate, and minerals), and precipitate (mainly large protein aggregate) fractions by ultracentrifugation (197000*g*, 60 min). It was obvious that precipitate was increased with heat temperature. The floating, supernatant, and precipitate fractions were collected carefully, and their TIAs were assayed. Figure 1b shows soymilk TIA is decreased with temperature, and the residual TIA by 100 °C 15 min is about 20% of raw soymilk. It was interesting that >80% soymilk residual TIA was in the supernatant and the rest of the residual TIA was in the floating and precipitate fractions. In addition, it is found the precipitate amount is highly correlated (R^2 = 0.9039) with the TIA loss (Figure 1c). These revealed that more and more KTI/BBI might be incorporated into protein aggregate and lost their TIA with increasing heat temperature and a few of KTI/BBI still existed as themselves in the supernatant owing to their small sizes (should be <10 nm).

Figure 2a shows precipitate amount is increased with heat time (100 °C). Figure 2b shows a two-phase TIA inactivation behavior, TIA is decreased sharply within 0−15 min, then slowly after 15 min. Figure 2c also shows soymilk TIA loss is highly correlated with the precipitate amount (R^2 = 0.9359).

The heat time effect on soymilk TIA was similar to the heat temperature effect on soymilk TIA. Both of them revealed precipitate amount was highly (R^2 > 0.9) correlated with the TIA loss, and >80% of soymilk residual TIA was in the supernatant obtained from soymilk by ultracentrifugation (197000*g*, 60 min). Thus, it was considered that soymilk KTI/BBI might be divided into two parts, incorporated into protein aggregate, and existed as themselves (free forms). The TIA loss should be mainly due to the incorporation of KTI and BBI into the protein aggregate (including small and large protein aggregate), and the residual TIA should be mainly due to the free KTI and BBI.

Sulfhydryl (SH) and Disulfide (SS) in Different Fractions of Soymilk. Many researches reported SH/SS interchange reaction and SH oxidation might be related to KTI and BBI inactivation.[14] Therefore, this section would examine the total free SH and SS in soymilk. As stated above, soymilk was separated into floating, supernatant, and precipitate fractions by ultracentrifugation. The total cysteine content

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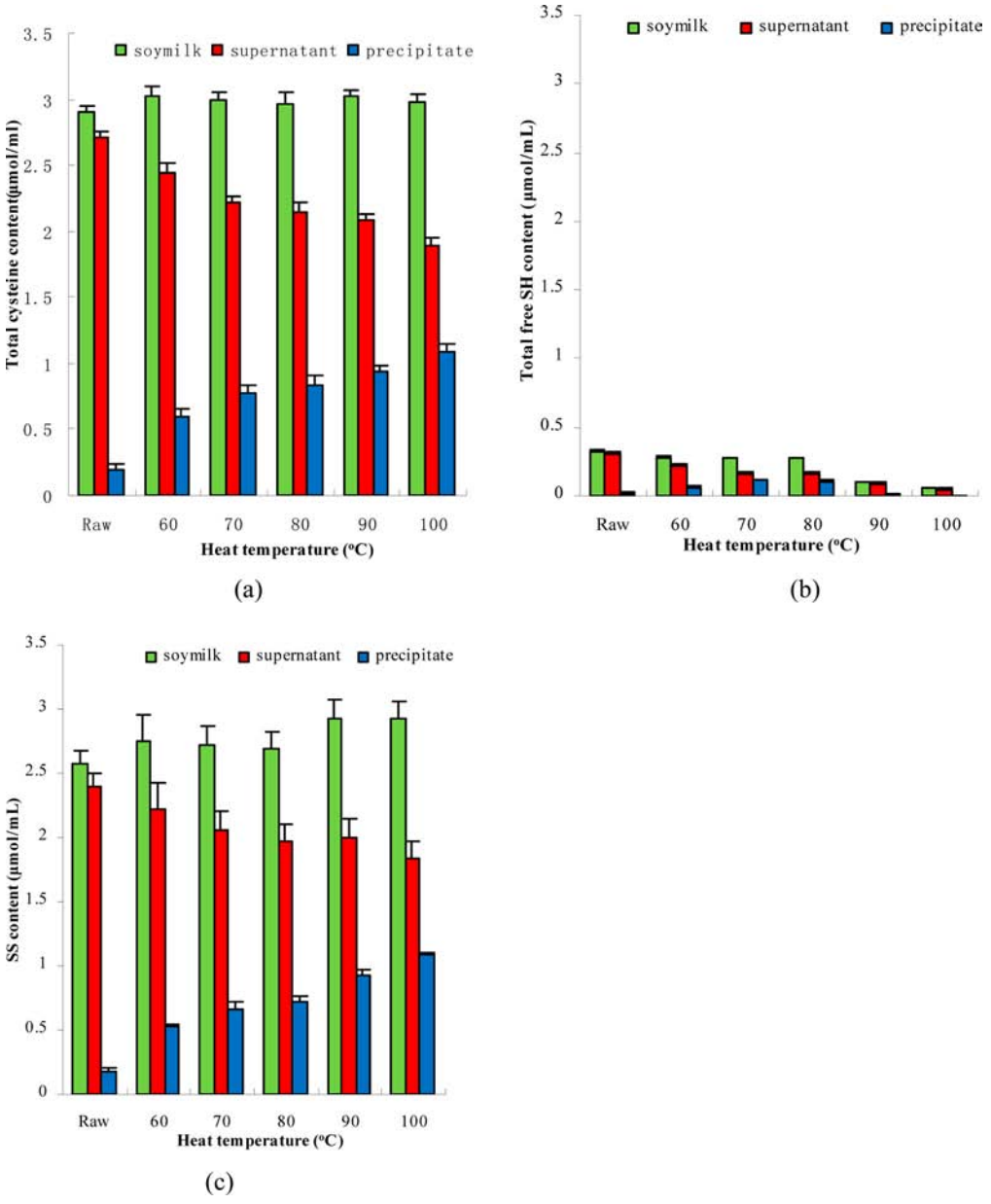

Figure 3. Heat temperature effect on sulfhydryl (SH) and disulfide (SS) in soymilk. (a) Heat temperature effect on total cysteine in soymilk, supernatant, and precipitate; (b) heat temperature effect on free SH in soymilk, supernatant, and precipitate; (c) heat temperature effect on SS in soymilk, supernatant, and precipitate.

and total free SH content were determined in soymilk and supernatant. The total SS contents in soymilk and supernatant could be calculated. Because the protein in the floating fraction was very small, the total cysteine, total free SH, and total SS contents in precipitate could be calculated from the results above. Figure 3a shows the total cysteine content (about 3 μmol/mL) is constant in soymilk. Total cysteine content in precipitate is increased, while that in supernatant is decreased with heat temperature. Supernatant total cysteine is composed of about 93% (63%) soymilk total cysteine for raw soymilk (100 °C heated soymilk). This revealed more and more cysteines were incorporated into precipitate (37%, 100 °C heated soymilk) with heat temperature. Figure 3b shows there is 0.325 μmol/mL total free SH in raw soymilk and is decreased to 0.052 μmol/mL by heat treatment (100 °C, 15 min). Raw soymilk supernatant total free SH was 0.307 μmol/mL and decreased to 0.051 μmol/mL. It is interesting that total free SH in precipitate was increased from raw soymilk to 80 °C heated soymilk and almost decreased to zero for 100 °C heated soymilk. This revealed protein aggregates of 70−80 °C heated soymilk contained some free SH in addition to SS, while those of 100 °C heated soymilk just contained SS. Figure 3c shows soymilk SS is increased, supernatant SS is decreased, and precipitate SS is increased with temperature. These results revealed soymilk free SH was decreased by heating. Because SH/SS interchange reaction did not change the total free SH amount, free SH decrease should be caused by the SH oxidation. Supernatant total free SH was decreased. This was caused by the protein aggregate formation and SH oxidation (Figure 3b). Precipitate total free SH was increased in ≤80 °C treated soymilk and almost decreased to zero for 100 °C heated soymilk. This would be explained as below: When heat

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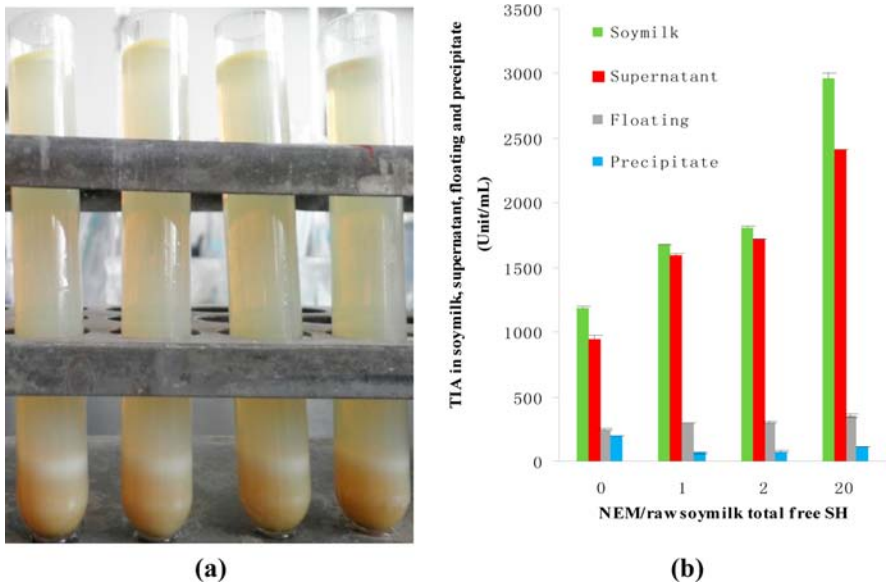

Figure 4. (a) Soymilks without NEM and with NEM preaddition (NEM/SH = 1/1, 2/1, 20/1) treated after ultracentrifugation (197000*g*, 60 min); SH is the total free SH of raw soymilk. (b) The NEM preaddition effects on TIAs of soymilk, floating, supernatant, and precipitate fractions obtained by ultracentrifugation.

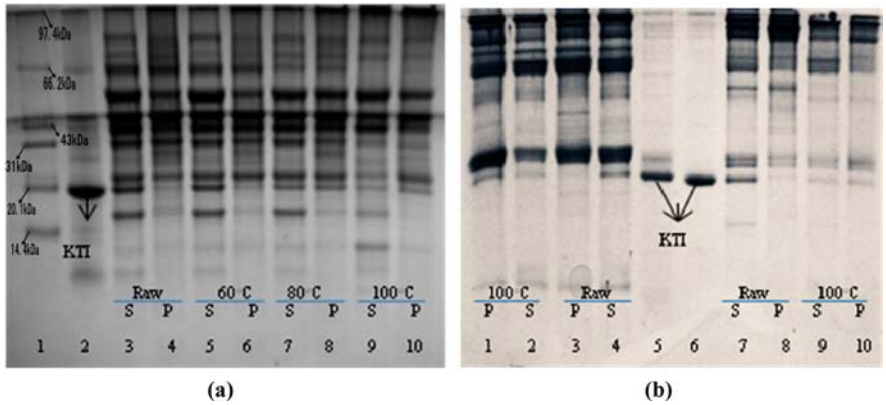

Figure 5. Tricine-SDS-PAGE for supernatant (S) and precipitate (P) protein. (a) Nonreducing Tricine-SDS-PAGE for supernatant protein of raw soymilk, 60, 80, and 100 °C treated soymilk. Lane 1 is marker, rabbit phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), rabbit action ovabumin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20.1 kDa), hen egg white lysozyme (14.4 kDa); lane 2 is KTI; lanes 3, 5, 7, and 9 are the supernatants of raw soymilk, 60, 80, and 100 °C treated soymilks; lanes 4, 6, 8, and 10 are the precipitates of raw soymilk, 60, 80, and 100 °C treated soymilks; (b) reducing and nonreducing Tricine-SDS-PAGE of supernatant and precipitate of raw and 100 °C treated soymilk; lanes 1 and 2 are reducing 100 °C precipitate and supernatant, lanes 3 and 4 are reducing raw soymilk precipitate and supernatant, lanes 5 and 6 are reducing and nonreducing KTI, lanes 7 and 8 are nonreducing raw supernatant and precipitate, lanes 9 and 10 are nonreducing 100 °C supernatant and precipitate.

temperature was ≤80 °C, soy protein would be unfolded although the extents of different proteins were different. This would cause the exposure of protein hydrophobic sites, which was beneficial for the protein aggregate formation. At the same time, SH oxidation also happened on these proteins. But the protein aggregate formation rate was quicker than the SH oxidation rate owing to the low temperature. This caused the precipitate total free SH to increase. When the heat temperature was >80 °C, the protein aggregate formation rate was slower than the SH oxidation rate. This caused the precipitate total free SH decreased.

The results above showed SH/SS interchange reaction and SH oxidation should be happening in the soymilk processing, and the key components were the free SH and SS. The raw soymilk used in this study contained 13 μmol free SH/g protein, composed of 11% total cysteine. Then the free SH was decreased to 2% by 100 °C heat treatment. The free SH could be supplied by glycinin, β-conglycinin, and some other proteins while the SS could be supplied by glycinin, KTI, BBI, and some other proteins.[22,23] It was considered that the KTI and BBI might be deactivated by the SH/SS interchange and SH oxidation as below:

$$\text{protein}-\text{SH}+\text{KTI (BBI)}\rightarrow\text{protein}-\text{SS}-\text{KTI (BBI)}-\text{SH}$$

$$\text{protein}-\text{SS}-\text{KTI (BBI)}-\text{SH}+\text{protein}-\text{SH} + 1/2\text{O}_2 \rightarrow \text{protein}-\text{SS}-\text{KTI (BBI)}-\text{SS}-\text{protein} + \text{H}_2\text{O}$$

Protein mainly represents the major soybean proteins, glycinin and β-conglycinin.

***N*-Ethylmaleimide (NEM) Preaddition Effect on TIA.** The results above suggested SH/SS interchange reaction and

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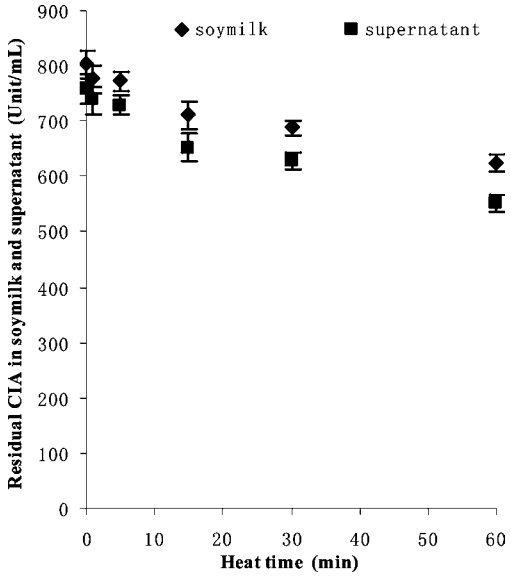

Figure 6. The heat time effect (100 °C, 0–60 min) on soymilk and supernatant chymotrypsin inhibitor activity (CIA).

SH oxidation might be correlated with the TIA loss. In this section, the effect of SH/SS interchange reaction and SH oxidation on TIA was examined. NEM, a well used SH blocking reagent, was added into raw soymilk to make NEM/SH (mol/mol) of 1:1, 2:1 and 20:1, respectively. These were cultivated for an hour and heated at 100 °C for 15 min and cooled with tap water. Soymilk without NEM preaddition was used as control. They were treated with ultracentrifugation (197000*g*, 60 min). Figure 4a shows the precipitates of four soymilks are almost the same. This revealed NEM preaddition did not have effect on protein aggregate formation. TIAs of soymilk, supernatant, floating, and precipitate were determined. Figure 4b shows residual TIA in soymilk is increased with NEM preaddition and >80% residual TIA is in the supernatant. When NEM/SH was 20:1, soymilk residual TIA was 60% of the original raw soymilk TIA. However, soymilk residual TIA without NEM preaddition was 20% of the original raw soymilk TIA. This showed that about 40% of raw soymilk TIA inactivation was correlated with the SH/SS interchange reaction and SH oxidation in the condition of heat treatment (100 °C, 15 min). The other 40% should be caused by other reasons, which were considered as the heat-induced protein aggregate formation (Figures 1a, 2a, and 4a). van den Hout et al. suggested that KTI and BBI in soy flour were deactivated in two ways: SH/SS interchange and heat.[24] This would be examined for soymilk with Tricine-SDS-PAGE below.

KTI Distribution in Soymilk Precipitate and Supernatant. Nonreducing and reducing Tricine-SDS-PAGE were conducted. The supernatant and precipitate of soymilks heated at 60, 80, and 100 °C were treated by the nonreducing Tricine-SDS-PAGE (Figure 5a). It was found the KTI band of supernatant became light, while that of precipitate became dense with heat temperature. This revealed KTI could be incorporated into protein aggregate by non-SS bonds, which might be another reason for TIA inactivation. This was in agreement with the results of Figure 4a,b.

Figure 4 shows SH/SS interchange reaction and SH oxidation does correlate with the TIA inactivation. Nonreducing and reducing Tricine-SDS-PAGE were conducted to confirm it. The supernatant and precipitate of 100 °C treated soymilk were examined (Figure 5b). The raw soymilk was used as control. It was found reducing KTI band was obviously denser than the nonreducing KTI band for 100 °C treated soymilk precipitate, while the reducing KTI band was almost the same (maybe a little denser) as the nonreducing KTI band for 100 °C treated soymilk supernatant. This clearly revealed some KTI was incorporated into protein aggregate by SS and the other by noncovalent bonds. Thus, the results above (Figures 2b, 5a,b) showed KTI was tended to be incorporated into protein aggregate by SS and/or noncovalent bonds. The KTI in supernatant might have low TIA because of its possible heat-induced conformation change, which was affected by the heat time.[8,13] The longer the heat time is, the more conformation change is.[8] The results above showed the TIA originated from KTI was inactivated easily and might be the reason for the quick phase of TIA inactivation.

BBI Distribution in Soymilk Supernatant and Precipitate. TIA was not only from KTI but also from BBI. BBI also had chymotrypsin inhibitor activity (CIA). Thus, it was considered that the heat effect on CIA could be used to analyze the BBI denaturation and possibly its TIA. Figure 6

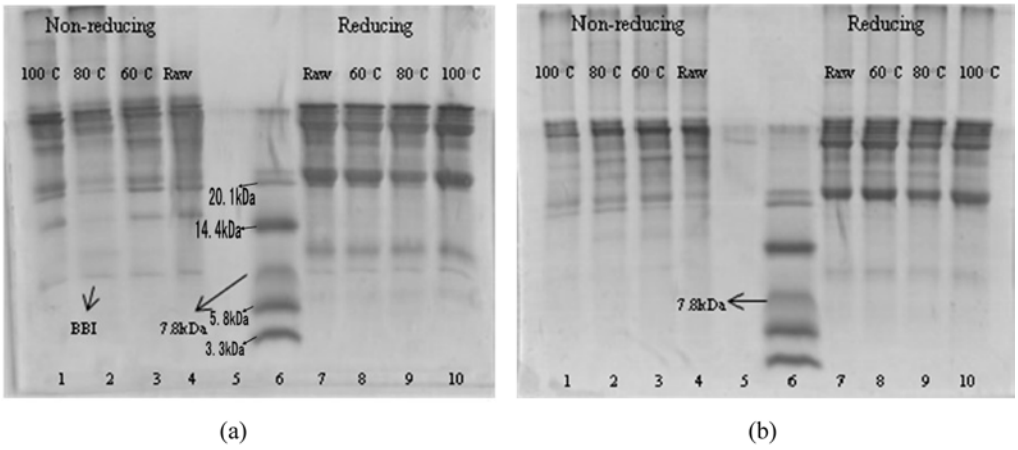

Figure 7. The protein profiles (nonreducing and reducing Tricine-SDS-PAGE) in supernatant (a) and precipitate (b) obtained from raw soymilk and soymilks (60, 80, 100 °C, 15 min). Lane 6 is marker: trypsin inhibitor (20.1 kDa), lysozyme (14.4 kDa), ABI-80 (7.8 kDa), ABI-81(5.8 kDa), ABI-95 (3.3 kDa), ABI-80, ABI-81, and ABI-95 are three polypeptides.

shows soymilk residual CIA is about 89% and 78% of original CIA after heating for 15 and 60 min at 100 °C, and about >90% residual CIA is concentrated in supernatant. This revealed BBI was difficult to be deactivated and might be the reason for the slow phase of TIA inactivation.

Figure 7a,b shows there is BBI in supernatant but few BBI in precipitate for soymilks (60−100 °C, 15 min), and the densities of BBI bands are almost the same for the supernatants obtained from raw soymilk and soymilks. This revealed BBI was tended to remain in supernatant as themselves. It was considered that this was closely correlated with the BBI structure.[14] BBI contained many hydrophilic amino acid residuals and should be a hydrophilic protein. This might be the reason why it was difficult to be incorporated into protein aggregate and was tended to exist as itself. Figure 6 shows CIA was difficult to be deactivated, so it was considered that BBI tended to exist as itself with its natural conformation. Thus, soymilk BBI should still possess most of its TIA.

According to the results above, the KTI and BBI behavior in soymilk processing would be described as below: By heating, KTI was easily incorporated into protein aggregate (including small and large protein aggregate) by SS and/or noncovalent bonds to lose its TIA (Figure 2b) while the free KTI was changed in conformation and lost its TIA, and the quick phase of TIA inactivation should result from KTI. BBI tended to exist as itself with its natural conformation (Figures 6 and 7), and the slow phase of TIA inactivation and the residual TIA should result from BBI. In all, this study might supply a new strategy for low TIA soymilk manufacture on the basis of the consideration of promoting the protein aggregate formation.

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Notes

The authors declare no competing financial interest.

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