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**Heat-induced inactivation mechanisms of Kunitz trypsin inhibitor
and Bowman-Birk inhibitor in soymilk processing**

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20 **ABSTRACT**

21 Trypsin inhibitor activity (TIA) is an important antinutritional factor in soymilk. In this study, the
22 effects of NaCl preaddition on TIA and the heat-induced TIA inactivation mechanisms were
23 examined. The results showed that Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor
24 (BBI) contributed 74% and 26% to raw soymilk TIA, respectively. The heat-induced quick KTI
25 incorporation into protein aggregates was the reason for its quick TIA inactivation. The
26 heat-induced slow cleavage of one BBI peptide bond was the reason for its slow TIA inactivation.
27 Heat-induced protein aggregate formation had little effect on BBI inactivation owing to the fact
28 that BBI and its degradation product tended to remain in the supernatant (197,000 g, 1 h) in all
29 conditions used in this study. NaCl could accelerate the KTI incorporation into protein aggregates
30 and the cleavage of one BBI peptide bond, which supplied a simple and quick method for low TIA
31 soymilk processing.

32 *Keywords:* soymilk, NaCl, Kunitz trypsin inhibitor (KTI), trypsin inhibitor activity (TIA),
33 Bowman-Birk inhibitor (BBI), chymotrypsin inhibitor activity (CIA), disulfide bond, protein
34 aggregate; peptide bond cleavage.

35

36 **1. Introduction**

37 Soymilk is a very popular protein beverage in East Asia and is gradually becoming accepted by
38 Western consumers owing to the positive health aspects of soy products. But there exist some
39 antinutritional factors in soymilk, such as the well known Kunitz trypsin inhibitor (KTI) and
40 Bowman-Birk inhibitor (BBI) (Liener, 1976). KTI mainly possesses trypsin inhibitor activity (TIA)
41 and a little chymotrypsin inhibitor activity (CIA) (De Vonis Bidlingmeyer, Leary, & Laskowski,
42 1972). BBI contains two independent inhibitory binding sites, one for trypsin and the other for
43 chymotrypsin, and it binds each protease to form a 1:1 complex (Kay, 1979). Since the inhibition
44 is non-competitive, BBI has the ability to form a ternary complex with both enzymes (Birk, 1985).
45 High levels of TIA could decrease protein digestibility (Hackler, van Buran, Streinkraus, Rawi, &
46 Hand, 1965) and might cause pancreatic disease (Holm, Reseland, Thorsen, Flatmark, & Hanssen,
47 1992). Therefore, many technologies, such as high pressure (Guerrero-Beltran, Estrada-Giron,
48 Swanson, & Barbosa-Canovas, 2009), ultrasound (Huang, Kwok, & Liang, 2008), microwave
49 (Wing & Alexander, 1971), fermentation (Song, Frias, Martinez-Villaluenga, Vidal-Valverde, &
50 de Mejia, 2008) and irradiation (Siddhuraju, Makkar, & Becker, 2002), were examined for
51 inactivating TIA, and showed some good aspects for soymilk processing. However, the method
52 widely used is still heat treatment, including traditional processing (boiling), steam injection batch
53 processing and ultrahigh-temperature processing (UHT) (Yuan, Chang, Liu, & Xu, 2008). The
54 former method is still widely used in China because of the popularity of one kind of machine for
55 home-made soymilk, while the latter two are used for large-scale soymilk processing in industry.
56 Hackler et al. (1965) reported that the extent of destruction of soymilk TIA for the highest

nutritive value or protein efficiency ratio was 90%. Kwok et al. (2002) reported that the heating time needed for destroying 90% of soymilk TIA was temperature-dependent (100 °C, 40 min; 125 °C, 5 min; 143 °C, 60 s). For the home-made soymilk, boiling for 40 min is too long, which is not convenient in a modern lifestyle. Therefore, a quick method is desirable for home-made soymilk (low TIA), which is also beneficial for the nutritive values and sensory attributes of soymilk (Hackler, van Buran, Streinkraus, Rawi, & Hand, 1965; Luttrell, Wei, Nelson, & Steinberg, 1981; Song, An, & Kim, 2003). For large-scale soymilk processing, shortening the heating time is also meaningful for saving energy (environment-friendly), decreasing cost and should be also good for increasing the qualities of soymilk. Identification of the heat-induced inactivation mechanisms is key for designing a quick method for low TIA soymilk.

As stated above, TIA is mainly resulted from two proteins of KTI and BBI. KTI, with molecular weight (MW) of 20 kDa, has two intrachain disulfide bonds, while BBI, with MW of 8 kDa, has seven intrachain disulfide bonds. In the early researches, purified KTI and BBI were examined and it was found that they were heat-stable and BBI was more heat-stable than KTI (Birk, 1961; Obara & Watanabe, 1971). DiPietro and Liener (1989) reported that the water extract of defatted soy flour lost its TIA and CIA faster than the purified KTI and BBI solutions, assumed to be a result of the interaction of KTI and BBI with other materials in the extract. Johnson et al. (1980) reported the kinetics of soymilk TIA inactivation could be expressed as the summation of two first-order reactions: the heat-labile reaction (quick phase of TIA inactivation) was attributed to KTI and the heat-stable reaction (slow phase of TIA inactivation) to BBI. Rouhana et al. (1996) reported that soymilk KTI could be easily inactivated, and the residual TIA could be ascribed to BBI exclusively.

79 Xu et al. (2012) reported that the amount of heat-induced protein aggregates was positively and
80 highly ($R^2 > 0.9$) correlated with soymilk TIA inactivation, which supplied a hypothesis for a
81 quick method for making low TIA soymilk through promoting protein aggregate formation. They
82 also demonstrated that KTI was incorporated into protein aggregates through disulfide and/or
83 non-covalent molecular interactions, which was suggested to be the mechanism of KTI's TIA
84 inactivation, but BBI tended to exist as itself with high residual CIA (residual CIA was 78% by
85 heating at 100 °C for 60 min). It was known that a high ionic strength could decrease the
86 ζ -potential of a colloidal system and was beneficial for the heat-induced protein aggregation
87 owing to the electrostatic screening effects. NaCl, a well used condiment, was used to confirm the
88 above hypothesis in this study. The difficulty of BBI inactivation should be correlated with the
89 fact that BBI tended to exist as itself and not likely to be incorporated into protein aggregates,
90 which was different from KTI (Xu, Chen, Kong, Zhang, & Hua, 2012). It was reported (Wu &
91 Sessa, 1994) that BBI still had a stable conformation after heated at 80 °C for 1 h, and even after
92 its disulfide bonds were broken. However, BBI did slowly lose biological activities by heating and
93 the reason for this was still not clear until now. Thus, this study aimed to confirm whether NaCl
94 could accelerate TIA inactivation and examine the inactivation mechanism of BBI.

95 **2. Materials and methods**

96 *2.1. Materials*

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

100 2.2. Soymilk preparation

101 Soybeans (20 g) were soaked in de-ionized (DI) water for 18 h at 4 °C. The soaked soybeans
 102 were washed with fresh DI water three times and then fresh DI water was added to make a total
 103 weight of 200 g. This was ground in a Waring blender (18000 rpm, MJ-60BE01B, Midea) for 3
 104 min. The homogenate was filtered through four layers of gauze and then a 200-mesh sieve. The
 105 filtrate was deemed as raw soymilk. NaCl was added into the raw soymilk to make concentrations
 106 of 0, 10 mM, 20 mM and 30 mM NaCl, sealed and mixed well. They were heated in a boiling
 107 water bath for 15 min and immediately cooled in tap water.

108 NaCl was added into raw soymilk to make a NaCl concentration of 30 mM and the solution
 109 mixed well. The NaCl preadded raw soymilk was divided into seven parts (sealed) and heated at
 110 75 °C, 85 °C, 95 °C or in a boiling water bath for 0, 1, 2, 5, 15, 30, 60 min, respectively. Soymilk
 111 without NaCl was used as a control.

112 2.3. Soymilk supernatant fraction preparation by ultracentrifugation

113 Soymilk was treated by using BECKMAN OptimaTM L-XP ultra centrifuge (197,000 g, 1 h) and
 114 separated into three fractions: floating, supernatant and precipitate. Protein particles (large protein
 115 aggregates) larger than 34 nm were precipitated. The supernatants, containing protein with particle
 116 size of < 34 nm, were collected carefully by syringe and sampled.

117 2.4. Trypsin inhibitor activity (TIA) assay

118 Soymilk and supernatant TIAs were measured by the method of Liu and Markakis (1989) with

119 slight modifications.

120 TIA standard curve: 0 to 1.0 ml trypsin inhibitor standard solution (0.01%, w/v) was pipetted
121 into a set of test tubes and the volume was made up to 2 ml with DI water. The test tubes were
122 warmed in a 37 °C water bath. 5 ml of 0.04% (w/v) N_α -Benzoyl-L-arginine 4-nitroanilide
123 hydrochloride (BAPA) in pH 8.2 Tris-HCl buffer (1% dimethylsulfoxide, v/v; 0.02 M CaCl_2),
124 which should be prepared freshly and pre-warmed in a 37 °C water bath, was pipetted into test
125 tubes followed by 2 ml of 0.01% (w/v) trypsin in a 0.04 mM HCl solution. Exactly 10 min later
126 the reaction was terminated by adding 1 ml of 30% acetic acid (v/v) into each tube. After mixing,
127 the contents of each tube was membrane filtered (0.45 μm , ϕ 50 LBK), and the absorbance of the
128 filtrate was measured at 410 nm by using a UV-2100 spectrophotometer (UNICO, WFZ UV-2100)
129 against a reagent blank. The reagent blank was prepared by adding 1 ml of 30% acetic acid (v/v)
130 to a test tube containing trypsin and DI water before the 5 ml of BAPA solution was added. The
131 linearity of the calibration curve was $r = 1$.

132 Trypsin inhibitory activity of the sample (soymilk or supernatant): the sample should be diluted
133 to the point where 1 ml produces trypsin inhibition of 40–60%. Then 1 ml of diluted sample was
134 added to tubes, as described above. One trypsin inhibitory activity (TIA) unit was arbitrarily
135 defined as an increase of 0.01 absorbance units at 410 nm per 10 ml of the reaction mixture under
136 the conditions used.

137 2.5. Chymotrypsin inhibitor activity (CIA) assay

138 Soymilk and supernatant CIA was determined by using a method from Tan et al. (1984) with

139 some modifications.

140 The samples were diluted to the point where 0.5 ml produced 30–40% chymotrypsin inhibition.

141 An aliquot of 0.5 ml of diluted samples were pipetted into test tubes and adjusted to 1.0 ml with

142 DI water. 1.0 ml of α -chymotrypsin solution (0.02 mg/ml dimethylformamide) was added,

143 followed by 0.4 ml of N-Benzoyl-L-tyrosine p-nitroanilide (BTpNA) solution (0.4 mg/ml

144 dimethylformamide) and thoroughly mixing. Exactly 10 min later, the reaction was terminated by

145 adding 0.5 ml of 30% (w/v) acetic acid. The mixture was then centrifuged at 10000 g for 10 min,

146 and the absorbance of the supernatant was measured at 410 nm against a reagent blank by using

147 the UV-2100 spectrophotometer. The reagent blank was prepared by adding 0.5 ml of 30% (w/v)

148 acetic acid to a test tube containing chymotrypsin and DI water (1 ml each) before 0.4 ml of

149 BTpNA solution was added. A sample blank was prepared by adding 0.4 ml of BTpNA solution to

150 the diluted sample and DI water (0.5 ml each), incubating the mixture at 37 °C for 10 min, and

151 then adding 0.5 ml of acetic acid followed by the addition of 1 ml of α -chymotrypsin. CIA was

152 estimated from the residual chymotryptic activity of the mixture of diluted sample and

153 α -chymotrypsin. One unit of α -chymotrypsin activity was arbitrarily defined as an increase of 0.01

154 absorbance unit (at 410 nm) of the reaction mixture under the conditions used.

155 2.6. SDS-PAGE and Tricine-SDS-PAGE

156 SDS-PAGE was performed to examine KTI according to the method proposed by Laemmli

157 (1970), with the concentrations of stacking gel and separating gel being 5% and 12.5%,

158 respectively. Tricine-SDS-PAGE was carried out to examine BBI according to the procedure of

159 Schagger (2006) with some minor modifications, with the concentrations of stacking gel and

separating gel being 4% and 16%, respectively.

For soymilks, all were diluted to 2 mg/ml with the same dilution ratio because they had the same protein concentrations. For supernatants, they were diluted with the dilution ratio above. This was different from the normal method, but it could show the amount changes of KTI and BBI on the gel, which could be analyzed by the software of Quantity One (Bio-Rad, USA). Then, 0.5 ml of diluted sample was mixed with 0.5 ml of SDS-PAGE sample buffer or Tricine-SDS-PAGE sample buffer, 10 μ l was loaded into the sample well and electrophoresed. The gels were stained with Coomassie Brilliant Blue G-250.

2.7. MALDI-TOF/TOF-MS

The protein spots of interest were excised from the Coomassie Brilliant Blue G-250 stained gel, washed in distilled water and destained completely in a 30% acetonitrile (v/v) solution containing 100 mM NH_4HCO_3 . After a 100% acetonitrile washing, the destained gel piece was placed into a reducing solution (10 μ l of 100 mM DTT, 90 μ l of 100 mM NH_4HCO_3) for 30 min at 56 °C. After a 100% acetonitrile washing, 70 μ l of 100 mM NH_4HCO_3 and 30 μ l of 200 mM iodoacetic acid were added and incubated for 20 min in the dark. After a 100% acetonitrile washing again, 5 μ l of 10 μ g/ml trypsin (Promega, V5111) was added and refrigerated at 4 °C for 30 min. Then, 25 mM NH_4HCO_3 solution was added. The peptides were generated after 20 h incubation at 37 °C. The solution containing peptides was collected and concentrated at 30 °C to get a dry powder. 3 μ l of 0.1% trifluoroacetic acid (v/v) was used to dissolve the dry powder. In the final step before MALDI-TOF/TOF analysis, the sample was prepared by mixing 0.7 μ l of the sample and 0.7 μ l of 4-hydroxy- α -cyanocinnamic acid (matrix) on a MALDI target and air-dried. All mass spectra were

obtained with an ultrafleXtreme MALDI-TOF/TOF (Bruker Daltonics, Germany) and were analyzed by flexAnalysis software provided by Bruker Daltonics Corp. Protein identification was performed by searching plant proteins in the latest version of the NCBI nr database.

3. Results and discussion

3.1. The effect of NaCl preaddition on the soymilk protein aggregate formation

Fig. 1a shows the soymilks heated for 0–60 min without NaCl and Fig. 1b shows the soymilks heated for 0–60 min with 30 mM NaCl after ultracentrifugation (197,000 g, 1 h), revealing that the precipitate amount of soymilk without NaCl is obviously less than that of the corresponding soymilk with NaCl. Fig. 1c reveals that protein in the supernatant shows a two-phase behaviour with heating time regardless of NaCl: decreases quickly from 0 to 15 min and slowly after 15 min, and the supernatant protein concentrations of soymilks with NaCl were less than the corresponding ones without NaCl, which clearly reveals that NaCl could accelerate protein aggregate formation. Xu et al. (2012) reported that protein aggregate formation had positive and high correlation with TIA inactivation. Thus, it was considered that NaCl preaddition might be a simple and quick method to make low TIA soymilk.

3.2. The effects of NaCl on soymilk TIA and CIA

Fig. 2a shows that TIA is also decreased in a two-phase behaviour: quickly from 0 to 15 min and slowly after 15 min without NaCl, and TIA inactivation is accelerated by NaCl, which is promoted by the increasing amount of NaCl. The residual TIA of soymilk with NaCl was significantly lower than that of the corresponding soymilk without NaCl. It was interesting that the residual TIA of soymilk (100 °C, 5 min) with NaCl was almost the same as that of soymilk (100 °C, 15 min) without NaCl. This revealed that the NaCl preaddition into raw soymilk before

heating was a quick method for making low TIA soymilk.

Fig. 2b shows the residual CIA decreases gradually with heating time and CIA inactivation is also accelerated by NaCl, which is promoted by the increasing amount of NaCl. The residual CIA of soymilk with 30 mM NaCl was significantly lower than that of soymilk without NaCl except within a heating time of 1–5 min. However, the CIA inactivation was much slower than the TIA inactivation. It is known that TIA originated from KTI and BBI, while CIA mainly originated from BBI. These results clearly show that BBI was heat-stable while KTI was heat-labile in the soymilk processing (Johnson, Deyoe, Hoover, & Schwenke, 1980; Johnson et al., 1980; Rouhana, Adler-Nissen, Cogan, & Frokier, 1996).

Van den Hout et al. (1998) gave an inactivation model for the total TIA from KTI and BBI, which is shown as equation 1. TIA_t was the residual TIA of soymilk which was heated for time of t . TIA_0 was the original TIA of the raw soymilk. The kinetic parameters λ_1 , λ_2 and A were estimated by fitting equation 1 to the experimental data (Fig. 2a) using the Software Origin with $R^2 > 0.99$. Also, the CIA inactivation model is shown as equation 2. The kinetics parameters λ and B were estimated by fitting equation 2 to the experimental data (Fig. 2b) using the Software Origin with $R^2 > 0.99$. These parameters are shown in Table 1.

$$TIA_t/TIA_0 = A e^{-\lambda_1 t} + (1-A) e^{-\lambda_2 t} \quad (1)$$

$$CIA_t/CIA_0 = B e^{-\lambda t} \quad (2)$$

λ is the inactivation rate constant of CIA originating from BBI. It was found that λ was exactly the same as λ_2 (Table 1). It is known that BBI has one bioactive site for TIA and one bioactive site for CIA (Kay, 1979). Therefore, it was considered that $A e^{-\lambda_1 t}$ should represent the residual TIA originated from KTI, while $(1-A) e^{-\lambda_2 t}$ should represent the residual TIA originated from BBI;

λ_1 was the inactivation rate constant for TIA originated from KTI and λ_2 was the inactivation rate constant for TIA originated from BBI. λ_1 was about 40 times larger than λ_2 , which clearly revealed KTI was inactivated more quickly than BBI. The parameter A was near 0.74, this showed KTI contributed 74% and BBI contributed 26% to the total TIA of raw soymilk. This was in good agreement with another three reports (DiPietro & Liener, 1989; Friedman, Brandon, Bates, & Hymowitz, 1991; van den Hout, Pouw, & Gruppen, 1998), in which KTI contributed 79%, 70% and 78% to the total TIA, respectively.

According to the results above, KTI and BBI inactivation could be expressed as equations (3) and (4).

$$KTI_t/KTI_0 = e^{-\lambda_1 t} \quad (3)$$

$$BBI_t/BBI_0 = e^{-\lambda_2 t} \quad (4)$$

KTI_0 (BBI_0), the bioactive KTI (BBI) in raw soymilk; KTI_t (BBI_t), the bioactive KTI (BBI) in soymilk heated for time of t.

KTI and BBI inactivation behaviours as a function of heating time are expressed in Fig 2c and 2d, revealing a big difference, in that KTI inactivation rates (the slopes of the curves in Fig. 2c) are decreased with heating time, while BBI inactivation rates (the slopes of the curves in Fig. 2d) are not obviously affected by heating time. Fig 2c shows that KTI is quickly inactivated from 0 to 15 min and slowly after 15 min; KTI inactivation is accelerated by NaCl, and it is enhanced by the increasing amount of NaCl, which shows the same trend as protein aggregate formation (Fig. 1c).

A time of 15 min was needed for the total KTI inactivation of soymilk with NaCl, but 30 min was needed for the soymilk without NaCl. Fig. 2d shows that BBI is slowly inactivated with heating time, and its inactivation is also accelerated by NaCl, which is enhanced by the increasing amount

247 of NaCl. Xu et al. (2012) reported that KTI could be incorporated into protein aggregates while
248 BBI tended to exist as itself and not likely to be incorporated into protein aggregates by heating.
249 Therefore, it was considered that the different relationships between heat-induced protein
250 aggregate and KTI/BBI might be the reason for the different inactivation behaviours of KTI and
251 BBI. But it was still not clear why KTI/BBI inactivation could be accelerated by NaCl. Through
252 the incorporation into protein aggregates induced by heating (Xu, Chen, Kong, Zhang, & Hua,
253 2012), the conformation of KTI would be changed and subsequently lose its activity. It was
254 considered that the effect of NaCl on accelerating KTI inactivation should be that the KTI
255 incorporation into protein aggregates was accelerated by NaCl. Differently, BBI, possessing a very
256 stable conformation (Wu & Sessa, 1994), was not likely to be incorporated into protein aggregates.
257 As a result, it was considered that BBI in soymilk processing should not be inactivated as KTI, but
258 in another way.

259 3.3. The kinetic and thermodynamic studies of TIA and CIA inactivation with 30 mM NaCl

260 Fig. 3a shows the effect of temperature on the TIA inactivation of soymilk with 30 mM NaCl.
261 TIA is quickly decreased at first and then declines slowly, and TIA inactivation is enhanced by
262 high temperature. Fig. 3b shows CIA is gradually decreased by heating and the decrease is also
263 enhanced by high temperature. Table 1 shows the kinetic parameters of TIA and CIA inactivation
264 at different heat temperatures. The parameter A was about 0.74, and λ_2 was exactly the same as λ_1 .
265 These were in agreement with the results above. λ_1 and λ_2 should be the inactivation rate
266 constants of KTI and BBI, respectively.

267 Inactivation rate constants (λ_1 and λ_2) are assumed to be dependent on temperature following
268 the Arrhenius equation (5) or (6).

$$\lambda = k_0 \exp (-E_a/RT) \quad (5)$$

$$\ln(\lambda) = \ln(k_0) - (E_a/R) * (1/T) \quad (6)$$

λ = inactivation rate constant (λ_1 or λ_2), E_a = activation energy, R = universal gas constant, k_0 = frequency factor and T = absolute temperature.

Fig. 3c shows the $1/T$ has a good linear relationship with $\ln(\lambda)$. The E_a for KTI and BBI inactivation with 30 mM NaCl were calculated as 20 kJ/mol and 85 kJ/mol, respectively. Rouhana et al. (1996) reported that E_a values for KTI and BBI inactivation without NaCl were 25 kJ/mol and 102 kJ/mol, respectively. These showed that NaCl could decrease the E_a for KTI and BBI inactivation, which supplied the thermodynamic evidence for the quicker TIA and CIA inactivation induced by NaCl.

3.4. KTI inactivation

Soymilk could be separated into floating, supernatant and precipitate fractions by ultracentrifugation (197,000 g, 1 h). The percentage of supernatant residual TIA/CIA in the corresponding soymilk residual TIA/CIA was calculated (data not shown). It was found that the supernatant contributed > 80% TIA and > 90% CIA to the soymilk residual TIA and CIA, regardless of NaCl. This revealed that bioactive KTI (if any) and BBI of heated soymilk remained mainly in the supernatant (Xu, Chen, Kong, Zhang, & Hua, 2012). The protein in supernatant had a particle size smaller than 34 nm, which revealed that the proteins in the supernatant should be mainly composed of protein monomers and small protein aggregates. Generally, bioactive proteins must keep their natural conformations to maintain bioactivity, and must contact its substrate to express bioactivity. Therefore, it was considered that soymilk residual TIA and CIA should mainly come from the free forms of KTI (if any) and BBI in the supernatant.

Fig. 4a shows non-reducing SDS-PAGE profiles for soymilks with and without 30 mM NaCl. The band, possessing the same MW as the KTI standard, was confirmed as KTI by MALDI-TOF/TOF-MS. The KTI band of soymilk with and without 30 mM NaCl became weak, clearly revealing that more and more KTI was bound to other proteins by disulfide bond (SS) with increased heating time. Fig. 4b shows non-reducing SDS-PAGE profiles for supernatants with and without 30 mM NaCl. It clearly revealed that the supernatant KTI band with NaCl became weak more quickly than that without NaCl, and the KTI band (with NaCl) had almost disappeared after 15 min of heating, revealing that almost all of KTI was incorporated into protein aggregates through interacting with other proteins by SS and/or non-covalent molecular interactions. This was in good agreement with KTI inactivation behaviour (Fig. 2c). Thus, it was concluded that KTI inactivation was a result of the loss of its free form through KTI incorporation into protein aggregates in soymilk processing (Fig. 4b); NaCl could accelerate the KTI incorporation into protein aggregates (Fig. 4b), which was considered as the reason for the NaCl-accelerated KTI inactivation.

3.5. BBI inactivation

In contrast to KTI, BBI was inactivated slowly at a considerably constant rate, regardless of NaCl, although NaCl did accelerate BBI inactivation (Fig. 2d). Fig. 5a and 5b show the non-reducing Tricine-SDS-PAGE profiles for soymilk and supernatant as a function of heating time. There were three bands (bands 1–3) with molecular weights (MWs) of around 10 kDa. These were examined by MALDI-TOF/TOF-MS. Band 1 was not matched to any protein. The peptide of DDESSKPCCDQCACTKSNPPQCR was detected for both bands 2 and 3, and matched to two kinds of BBI, named BBI₁ (NCBI: gil4191566) and BBI₂ (NCBI: gil157830209) in this study. It

313 was found that the primary structure of BBI₁ was the same as the N-terminal (from D1 to A42) of
 314 BBI₂. BBI₁ held 42 residues with MW of 5097 Da and BBI₂ held 71 residues with MW of 8665
 315 Da. Fig. 5a and 5b show that BBI₁ (band 3) appears on Tricine-SDS-PAGE gel after ≥ 15 min
 316 heating, meaning that band 2 should be contributed by BBI₂ and band 3 should be contributed by
 317 BBI₁, and that BBI₁ should be originated from BBI₂ through the heat-induced cleavage of one
 318 peptide bond of BBI between A42 and L43. The intensities of bands 2 and 3 were examined by
 319 Software Quantity One. With the same heating time, the intensity of band 2 from soymilk (or
 320 supernatant) with 30 mM NaCl was weaker than that from soymilk (or supernatant) without NaCl,
 321 and the intensity of band 3 for the former was larger than the latter one, especially after heating
 322 time (≥ 15 min). These results are perfectly in agreement with the results in Fig. 2b, which shows
 323 that $> 95\%$ of raw soymilk CIA remained within 5 min of heating and the residual CIA
 324 considerably decreased after heating time (≥ 15 min). These results mean that the heat-induced
 325 BBI₁ formation from BBI₂ should be the reason for BBI inactivation, and NaCl-accelerated BBI₁
 326 formation should be the reason for the accelerated BBI inactivation. It was reported that the E_a of
 327 BBI inactivation was about 102 kJ/mol for soymilk without NaCl (Rouhana, Adler-Nissen, Cogan,
 328 & Frokier, 1996) and generally the bond energy of the peptide bond was about 84 kJ/mol
 329 (Jorgensen & Gao, 1988), which showed that the heat-induced cleavage of the BBI peptide bond
 330 between A42 and L43 was possible. Low E_a (85 kJ/mol) was needed for BBI inactivation when
 331 soymilk had 30 mM NaCl preadded, which meant that NaCl preaddition was beneficial for the
 332 heat-induced cleavage of the BBI peptide bond between A42 and L43.

333 In order to clarify the relationship between heat-induced protein aggregate and BBI inactivation,
 334 the precipitates obtained (197,000 g, 60 min) from soymilks (100 °C, 15 min) preadded with 0

335 mM, 10 mM, 20 mM and 30 mM NaCl were treated by non-reducing and reducing
336 Tricine-SDS-PAGE on the same gel. It was found that there are no bands around 10 kDa in the
337 non-reducing conditions, while there is band 4 around 10 kDa in the reducing condition (Fig. 5c).
338 Band 4 was examined by MALDI-TOF/TOF-MS and matched to the A5 polypeptide of glycinin.
339 These results clearly showed that there was no BBI incorporated into protein aggregates by
340 heating, even after NaCl preaddition. This should be closely correlated with the high
341 hydrophilicity of BBI, which is composed of approximately 73% hydrophilic amino acid residues
342 (NCBI database).

343 *3.6. Heat-induced TIA inactivation mechanism*

344 In the kinetics study, it was suggested that 74% of raw soymilk TIA was originated from KTI
345 and about 26% of raw soymilk TIA was originated from BBI (Table 1). Almost all of KTI could be
346 inactivated by 15 min of heating, while BBI was inactivated slowly. It was also found that the TIA
347 of BBI was inactivated at the same rate constant as the CIA of BBI (Table 1), revealing that BBI
348 inactivation could cause the simultaneous inactivation of its TIA and CIA.

349 KTI was quickly inactivated owing to its incorporation into protein aggregates through disulfide
350 bonds and/or non-covalent molecular interactions (Fig. 4a and 4b), which could change the natural
351 conformation of KTI and inhibit KTI interact with its substrate. The disulfide bonds among KTI
352 and other proteins was increased with heating time (Fig. 4a), and it was found that the KTI
353 inactivation rate decreased with heating time (Fig. 2c), which corresponded perfectly to the
354 protein aggregate formation rate with heating time (Fig. 1c). This further confirmed that KTI
355 incorporation into protein aggregates was the reason for KTI inactivation.

356 In contrast to KTI, BBI was inactivated slowly at a considerably constant rate by heating. This

should be closely correlated with the properties of BBI, which was a highly hydrophilic protein with a stable structure resulting from seven intrachain disulfide bonds. This was unbeneficial for BBI unfolding and the interactions among BBI and other proteins. As a result, BBI was difficult to be incorporated into protein aggregates and bound to other proteins by SH/SS exchange reaction (Fig. 5c). It was found that the heat-induced cleavage of one BBI peptide bond was the reason for BBI inactivation (Fig. 5a and 5b).

NaCl could promote the KTI incorporation into protein aggregates (Fig. 4b) and the cleavage of one BBI peptide bond (Fig. 5a and 5b), which should be the reason for the NaCl-accelerated TIA inactivation. This also could be supported by the thermodynamic study of KTI and BBI inactivation.

4. Conclusions

This study supplies a simple and quick method (NaCl preaddition) for making low TIA soymilk. This is very useful for soymilk nutritional value and for those who enjoy soymilk. In addition, this study respectively clarified the KTI and BBI inactivation mechanisms, which were directly correlated with TIA inactivation. The heat-induced, rapid KTI incorporation into protein aggregates was the reason for its quick inactivation, and 100% of KTI inactivation could be obtained by short heating times. The heat-induced cleavage of one BBI peptide bond, needing high energy, could simultaneously inactivate the TIA and CIA of BBI, and protein aggregate formation had little effect on BBI inactivation.

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452

Figure captions

Fig.1. (a) Image of soymilks (without NaCl) treated by ultracentrifugation (197,000 g, 1 h). Soymilks in tubes 1–7 were heated in a boiling water bath for 0, 1, 2, 5, 15, 30 and 60 min. (b) Image of soymilks (with 30 mM NaCl) treated by ultracentrifugation (197,000 g, 1 h). Soymilks in tubes 1–7 were heated in a boiling water bath for 0, 1, 2, 5, 15, 30 and 60 min. (c) The protein concentrations of supernatants obtained from soymilks (without NaCl or with 30 mM NaCl) by ultracentrifugation (197,000 g, 1 h).

Fig. 2. The effects of NaCl preaddition (0 mM, 10 mM, 20 mM and 30 mM) on residual TIA (a) and CIA (b) of soymilk. Soymilk is heated in a boiling water bath for 0, 1, 2, 5, 15, 30 and 60 min. Original TIA/CIA is the raw soymilk TIA/CIA. (c) The KTI_t/KTI_0 as a function of heating time, KTI_t is the bioactive KTI in soymilk for heating time of t, and KTI_0 is the bioactive KTI in raw soymilk. (d) The BBI_t/BBI_0 as a function of heating time, BBI_t is the bioactive BBI in soymilk for heating time of t, and BBI_0 is the bioactive BBI in raw soymilk.

Fig. 3. (a) The effects of temperature (75 °C, 85 °C, 95 °C and 100 °C) on the soymilk residual TIA. (b) The effects of temperature (75 °C, 85 °C, 95 °C and 100 °C) on the soymilk residual CIA. (c) The Arrhenius plots for the inactivation of KTI and BBI in the temperature range of 75–100 °C.

Fig. 4. The non-reducing SDS-PAGE protein profiles of soymilk and supernatant. (a) Soymilks without NaCl or with 30 mM NaCl are obtained by heating raw soymilk in a boiling water bath for 0, 1, 2, 5, 15 and 30 min. (b) Supernatants obtained from soymilks above by ultracentrifugation (197,000 g, 1 h). N means soymilk without NaCl and P means soymilk with 30 mM NaCl. Lanes 1

474 and 14, KTI standard.

475 **Fig. 5.** The non-reducing Tricine-SDS-PAGE protein profiles of soymilk and supernatant. (a)

476 Soymilks without NaCl or with 30 mM NaCl are obtained by heating raw soymilk in a boiling

477 water bath for 0, 1, 2, 5, 15, 30 and 60 min. (b) Supernatants are obtained from soymilks above by

478 ultracentrifugation (197,000g, 1 h). N means soymilk without NaCl and P means soymilk with 30

479 mM NaCl. (c) The reducing and non-reducing Tricine-SDS-PAGE protein profiles for precipitates

480 obtained from soymilks (100 °C, 15 min) by ultracentrifugation (197,000 g, 1 h). Lanes 1–4

481 (reducing), precipitates obtained from soymilks with 30 mM, 20 mM, 10 mM and 0 mM NaCl;

482 lane 5, marker; lanes 6–9 (non-reducing), precipitates obtained from soymilks with 0 mM, 10 mM,

483 20 mM and 30 mM NaCl.

484

485 **Table 1.** Inactivation rate constants of TIA and CIA of soymilk in different conditions.

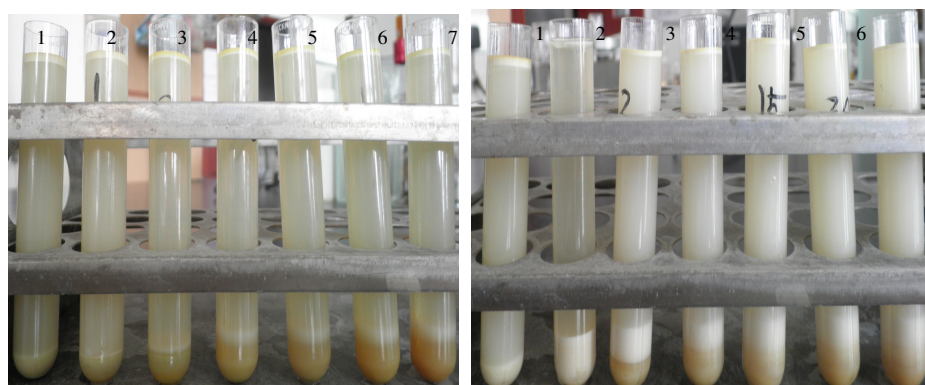
		$TIA_t/TIA_0 = A e^{-\lambda_1 t} + (1-A) e^{-\lambda_2 t}$			$CIA_t/CIA_0 = B e^{-\lambda t}$	
NaCl preaddition (mM)		A	λ_1	λ_2	B	λ
100 °C	0	0.65	0.1995	0.0043	0.9914	0.0043
	10	0.73	0.3322	0.0095	1.0046	0.0095
	20	0.73	0.4573	0.0116	1.0102	0.0116
	30	0.75	0.5383	0.0118	0.9978	0.0118
Temperature (°C)						
30 mM NaCl preaddition	75	0.74	0.3378	0.0015	1.0005	0.0015
	85	0.74	0.4031	0.0035	1.001	0.0035
	95	0.75	0.4778	0.0064	1.0009	0.0064
	100	0.75	0.5383	0.0118	0.9978	0.0118

486

487 **Fig. 1.**

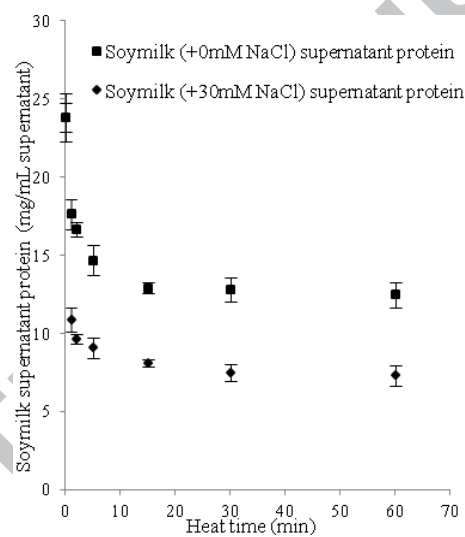
488

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(a)

(b)



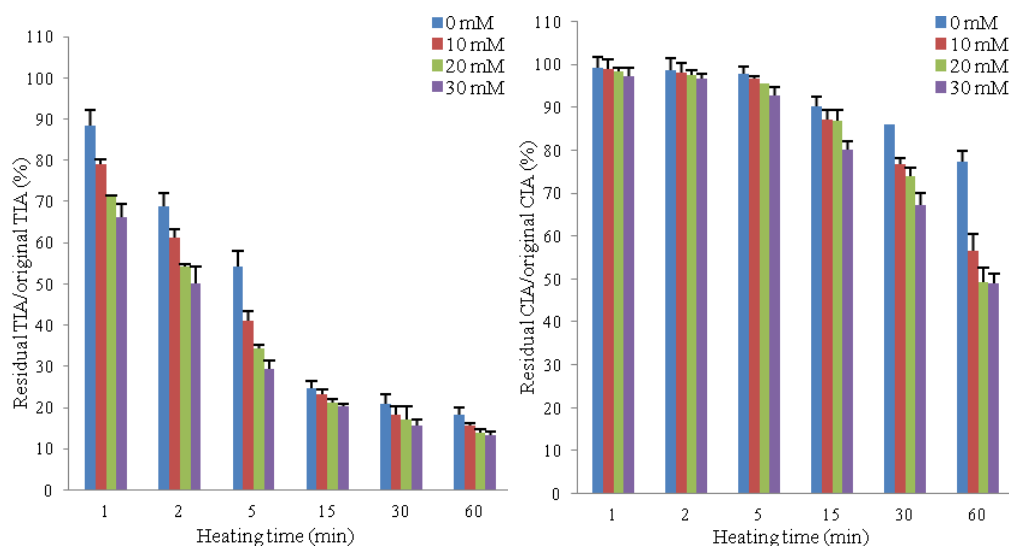
(c)

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491

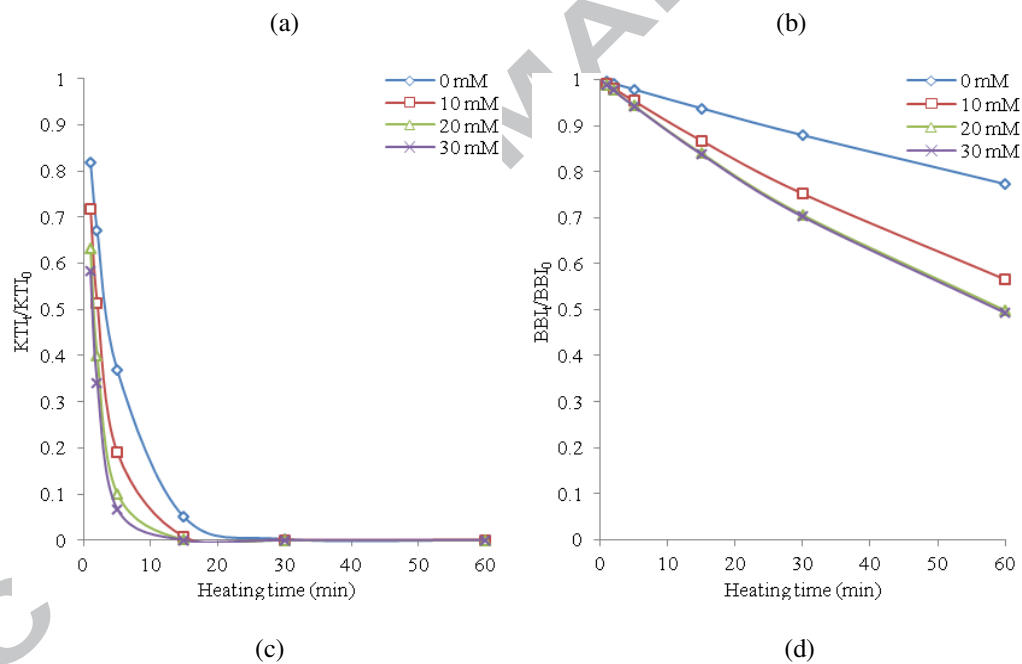
492

493 **Fig. 2.**



494

495

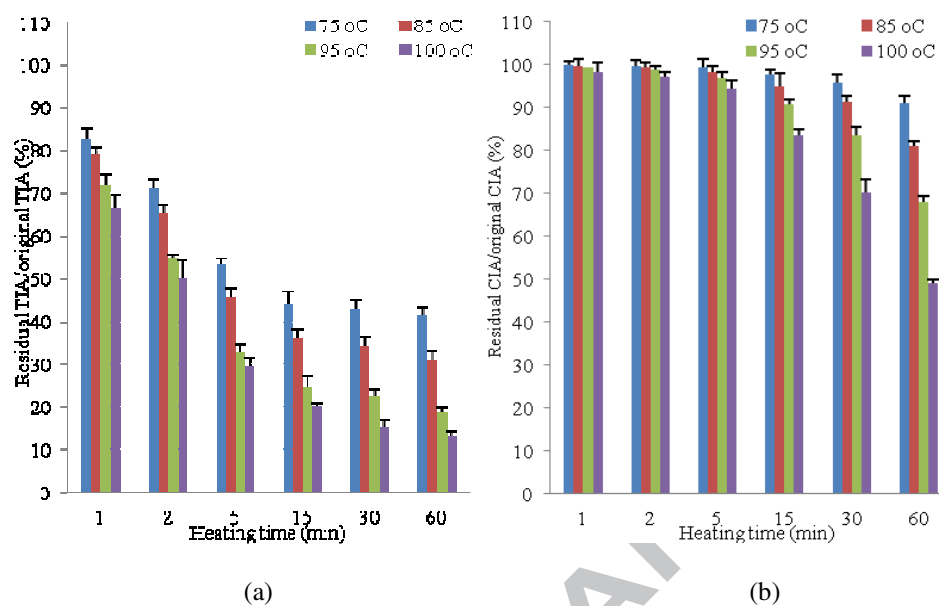


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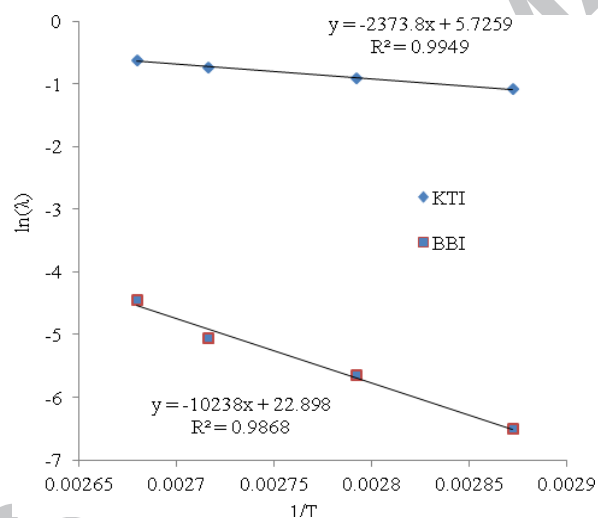
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499 **Fig. 3.**



500

501



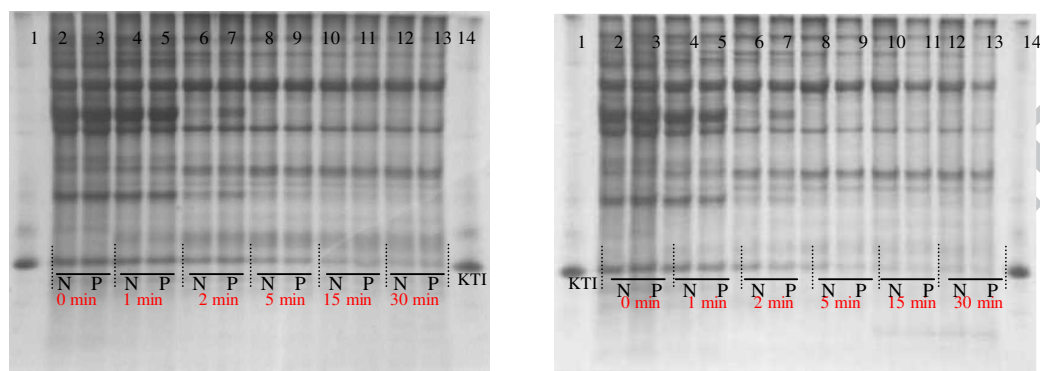
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503

(c)

504

505 **Fig. 4.**



506

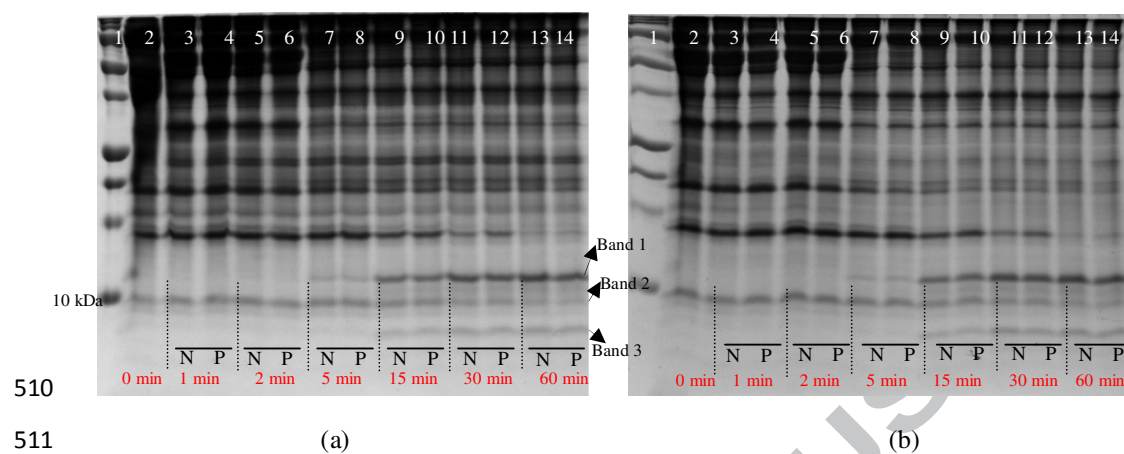
507

(a)

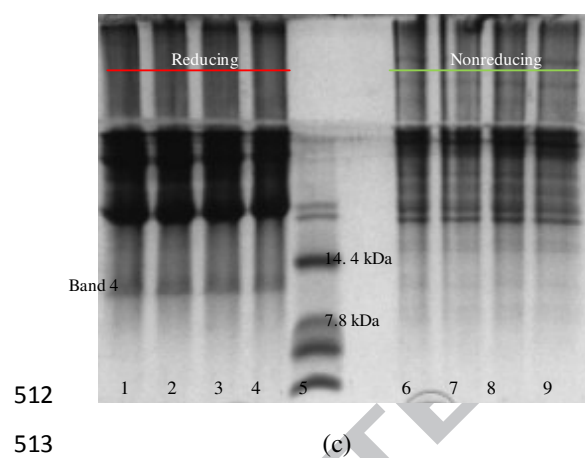
(b)

508

509 **Fig. 5.**



511



513

514 **Highlights**

- 515 1) A simple and quick method for low TIA soymilk is supplied
- 516 2) Heat-induced KTI incorporation into protein aggregates causes KTI's TIA loss
- 517 3) Heat-induced peptide bond cleavage of BBI causes BBI's TIA/CIA loss
- 518 4) NaCl accelerates protein aggregate formation and peptide bond cleavage of BBI

519

520