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Pressure and temperature combination for inactivation of soymilk trypsin inhibitors

José A. Guerrero-Beltrán [a], Yokiushirdhilgilmara Estrada-Girón [b], Barry G. Swanson [c], Gustavo V. Barbosa-Cánovas [d,*]

[a] Depto. Ing. Química y Alimentos, Universidad de las Américas-Puebla, Sta. Catarina Mártir, Cholula, Puebla 72820, Mexico
[b] Departamento de Ingeniería Química, Universidad de Guadalajara, Boulevard Marcelino García Barragán 1421, Colonia Olímpica, Guadalajara, Jalisco 44430, Mexico
[c] Food Science and Human Nutrition Department, Washington State University, Pullman, WA 99164-6376, USA
[d] Biological Systems Engineering Department, Washington State University, Pullman, WA 99164-6120, USA

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ABSTRACT

High hydrostatic pressure (HHP) processing, an emerging technology for food preservation, in combination with thermal treatment (250/50, 550/19, 550/65, and 550/80 MPa/°C) was applied to soymilk made from previously soaked soybeans (in distilled water or 0.5% sodium bicarbonate solution). First order kinetics constants ranging from 0.081 to 0.217 min^{-1}, for residual trypsin, were estimated in soymilk from soaked soybeans at selected pressure–temperature combinations. Residual trypsin, at 550 MPa and 80 °C, was high at higher HHP holding times. The highest percentage of residual trypsin (76%) was estimated after a 15 min holding time. The use of sodium bicarbonate for soaking of soybeans synergistically decreased the trypsin inhibitor activity in soymilk in comparison with residual trypsin using distilled water alone.

1. Introduction

Protease inhibitors (PIs) are generally considered the main antinutritional factors in soybeans. Soybean PIs belong to a broad class of proteins that inhibit proteolytic enzymes, such as trypsin and chymotrypsin. Both compounds are important animal digestive enzymes for splitting proteins to render dipeptides and tripeptides (Scheider, 1983). However, the specificity of these inhibitors is not necessarily restricted to trypsin and chymotrypsin but also to elastase and serine proteases for which serine constitutes the active site. Nevertheless, the literature reports two main types of soybean PIs, specifically called trypsin inhibitors (TIs). The Kunitz soybean inhibitor, with a molecular weight of 20,000 and two disulfide bridges, exhibits specificity to inhibit trypsin. The Bowman–Birk inhibitor, on the other hand, with a molecular weight ranging from 6000 to 10,000 and seven disulfide bonds, exhibits specificity to inhibit chymotrypsin (Liener, 1994).

The many different methods by which soybeans are processed usually reduce TIs content by up to 90% (Hackler, Van Buren, Steinkraus, El-Rawi, & Hand, 1965; Van Buren, Steinkraus, Hackler, El-Rawi, & Hand, 1964). Inactivation of TIs in whole soybeans can be achieved by atmospheric steaming (15 min) if the initial moisture content is about 20%. If beans are soaked in water overnight to reach 60% of moisture, 5 min in boiling water is sufficient to inactivate trypsin inhibitors (Salunkhe, Chavan, Adsule, & Kadam, 1992). The extrusion/steaming method at high pressure and temperature is the only one reported to destroy up to 100% of TIs (Harper, 1981). However, thermal destruction of these compounds depends on temperature, heating time, particle size, and physical condition of the product. The inactivation can be accelerated or delayed by the addition of basic or acid compounds (Baker & Mustakas, 1973; Rackis, 1974).

Other established technologies, such as the ultra-high temperature (UHT) process, have also been reported to inactivate such TIs. In this process, the holding times required to inactivate 90% of trypsin inhibitors in soymilk (pH 6.5) were 60 min, 56 s, and 23 s at 93, 143, and 154 °C, respectively (Kwok, Qin, & Tsang, 1993). Moreover, there are still questions concerning the ideal UHT processing conditions, which would produce a commercially sterile soymilk with minimum nutrient degradation. Kwok et al. (1993) also recommended heat-inactivation of trypsin inhibitors in soymilk at temperatures below 100 °C prior to the UHT process because prolonged heating at higher temperatures may destroy lysine, sulfur amino acids and vitamins.

Because of the increasing use of soybean products in human nutrition, it is important to keep in mind the risk to human health that could be associated with the consumption of soybean products in which the TIs have not been completely inactivated. It has been reported that thermal treatment destroys 90% of TIs. However, most commercially available soybean products are found to have less than this expected percentage reduction. Soymilk,

* Corresponding author. Tel.: +1 509 335 6188; fax: +1 509 335 2722.
E-mail addresses: barbosa@mail.wsu.edu, angel.guerrero@udlap.mx (G.V. Barbosa-Cánovas).

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soy-based infant formula, tofu, soy protein isolates and concentrates, and textured meat analogues receive sufficient thermal treatment to eliminate approximately 80% of total TIs in raw soybeans.

Soybean trypsin inhibitors can alter the normal human pancreatic functions through an enlargement of the pancreas or hypertrophy. Consequently, hyperplasia or an increase in size of the acinar cells of the pancreas (Cheftel, Cuq, & Lorient, 1985) may occur. Therefore, alternative technologies, besides thermal treatments, should be used to process raw soy products to reduce the soybean TIs content to a minimum.

Two soybean TIs are usually found in soybeans: the Kunitz soybean trypsin inhibitor (KSTI), commonly described as the heat-labile inhibitor, and the Bowman–Birk inhibitor (BBI), which is often referred to as the heat-stable inhibitor (Rouhana, Adler-Nissen, Cogan, & FrØkiÆr, 1996). When using thermal treatment, researchers usually relate their experiments to both KSTI and BBI TIs due to their difference in thermal stability (Van den Hout, Pouw, Gruppen & Van't Riet, 1998; Rouhana et al., 1996; DiPietro & Liener, 1989).

According to Rouhana et al. (1996), the activity contribution of KSTI can be neglected, because after 1 min of boiling the relative concentration of KSTI drops to 3% from its initial concentration in soymilk. Therefore, the decrease in TIs activity, after processing at boiling temperature for 1 min, is a consequence of the inactivation of BBI. In traditional soymilk processing, soybeans are soaked in water; however, it is reported that sodium bicarbonate positively contributes to the inactivation of TIs during thermal treatment (Nelson, Steinberg, & Wei, 1976).

It has been demonstrated that HHP can inactivate microorganisms and enzymes. Studies that combine HHP with thermal treatment must be conducted to develop food products with better nutritious quality. Hence, the purpose of this work was to evaluate the effect of HHP-thermal treatment combination, at selected holding times, on the TIs of soymilk from soybeans soaked in distilled water and sodium bicarbonate solution.

2. Materials and methods

2.1. Materials

Soybeans (*Glycine max*, L.) of the Chapman cultivar were purchased from Schlessman Seeds Company (Milan, OH). N-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA), Tris–HCl, dimethylsulfoxide, and bovine tryspin were purchased from Sigma (St. Louis, MO).

2.2. Soymilk preparation

Soybeans were weighed and soaked in distilled water or 0.5% $NaHCO_3$ solution in a ratio of 1:2 (soybeans/water or solution) for 18 h at room temperature. After the soaking period, seeds were rinsed with distilled water. Soymilk was prepared according to the accepted standard water/dry beans ratio of 10:1 (Beddows & Wong, 1987). The mixture was ground for 4 min using a commercial laboratory blender, model 31 BL91 (Waring, Torrington, CT). The slurry obtained was filtered with a double layer of cheesecloth. Raw soymilk obtained in this manner had a solids content of about 7.8% and pH of 6.5.

2.3. Sample preparation

Fifty ml of soymilk were placed in a sterile plastic pouche (Whirl-Pak, 4 oz. Cole-Parmer Instrument, Co., Vernon Hills, IL), tabs folded, and held in ice water prior to HHP treatment. Samples containing soymilk were wrapped in polyethylene bags (Power Plastics Inc., Paterson, NJ) and heat-sealed using a TISH-300 heat-sealer (E-Z Audit Bankpak Inc., Baltimore). Samples were equilibrated prior to HHP processing in a warm water bath, corresponding to the working temperature, to ensure uniform temperature during the HHP treatment.

2.4. HHP treatment

Raw soymilk was HHP-processed at selected pressures and temperatures (250/50, 550/19, 550/65, and 580/80 MPa/°C) for times ranging from 0 to15 min. An isostatic pressing system (Engineering Pressure System Inc., Haverhill, Mass., USA), connected to a computer to control pressure, was used in this study. To transmit pressure, the pressure chamber was filled with 5% Houghton Hydrolubic 123B-soluble oil/water solution (Houghton International of Valley Forge, PA). The heating system to heat the HHP-chamber was turned on the night before pressurization. The come-up time (CUT: time necessary to reach the working pressure) was measured using a chronometer. Wrapped samples were placed in the HHP processing chamber and pressurized for selected times (0–15 min). After pressure treatment, samples were kept at 4 °C and subsequently analyzed. Each test was performed in triplicate.

2.5. Trypsin inhibitors extraction

Soymilk was diluted with an equal volume of 0.02 N NaOH and mixed for 2 min in a high shear mixer. After mixing, the whole was centrifuged at 10,000 × *g* for 30 min. Before TIs analysis, the extract was filtered through Whatman paper No. 42.

2.6. Trypsin inhibitor assay

In this study, total trypsin inhibitor activity was assessed, using the enzymatic method instead of assessing KSTI and BBI separately. The enzymatic assay was performed, based on the inhibition of trypsin method, in which N-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA) is the substrate that renders a yellow colour due to liberation of nitroanilide if trypsin is still active. The Rouhana et al. (1996) method was used with modifications. Soymilk samples were diluted using Tris buffer (50 mM Tris and 20 mM $CaCl_2$, pH 8.2) to reach 40–60% trypsin inhibitor activity. A mixture of 100 µl of soymilk and 50 µl of 1 mM HCl containing 10 µg of bovine trypsin was incubated at 37 °C for 10 min. The reaction was started by adding 1 ml of substrate (25 mg of BAPNA in 1 ml of dimethylsulfoxide, adjusted to 100 ml with prewarmed Tris buffer) to the trypsin-soymilk mixture to determine the reaction rate. The increase of absorbance at 37 °C was measured at 410 nm as a function of time for a minimum of 10 minutes, using an 8452A diode array spectrophotometer (Hewlett–Packard, Palo Alto, CA). The slope (reaction rate) was computed from the linear part of the absorbance vs. time representation. The absorbance of trypsin alone, using the same assay as for soymilk samples, was evaluated, substituting soymilk for water. The trypsin inhibitor activity was expressed as residual trypsin. Residual trypsin was calculated based on the trypsin absorbance and the absorbance of the soymilk–trypsin mixture. One unit of residual trypsin was defined as 0.001 $\Delta A_{420}\ min^{-1}\ ml^{-1}$. The trypsin inhibitor activity was determined in triplicate.

2.7. Data analysis

The residual trypsin was analyzed as a first-order kinetic model:

$$\ln[TA_t] = \ln[TA_i] - k't \quad (1)$$

where $[TA_t]$ is the residual trypsin (%) remaining after treatment time t, $[TA_i]$ is the initial trypsin (720 enzyme activity units, theoretically 100%), k' is the slope (min^{-1}). The inactivation rate constant (min^{-1}) is obtained as $2.303 * k'$.

2.8. Statistical analysis

Linear regression and standard deviation were determined using a Microsoft Excel programme. The analysis of variance (ANOVA) was calculated with the SAS (1999) programme. An α of 0.05 was used for making decisions about significant differences.

3. Results and discussion

Figs. 1 and 2 illustrate the residual trypsin after HHP processing (550 MPa), at selected temperatures (19, 65, and 85 °C), for soymilk made from soybeans soaked in distilled water or sodium bicarbonate solution (0.5%), respectively. First order kinetics were observed for residual trypsin in soymilk treated at 19 and 65 °C, in both cases, since correlation coefficients were above 0.970 (Table 1). It was also observed that the higher the holding time, the lower was the residual trypsin in both types of soymilk. About 5 and 8% of residual trypsin in soymilk from soybeans soaked in water and sodium bicarbonate, respectively, were observed after 15 min of HHP treatment at 250 MPa (Fig. 3). This low amount of residual trypsin was because HHP-processing and holding time were not enough to inactivate TIs in soymilk. However, with processing at 550 MPa, at high holding times and temperatures, more residual trypsin remained in the soymilk. This was because of the increased inactivation of TIs as pressure and processing time increased. It was observed that, at 550 MPa, in combination with 19, 65 or 85 °C, there was a slight difference in the residual trypsin between soymilk from soybeans soaked in water (Fig. 1) and sodium bicarbonate (Fig. 2); lower residual trypsin values in soymilk from soybeans soaked in water were observed. The same tendency holds for residual trypsin data at 250 MPa in combination with 50 °C in soymilk from different soaking sources (Fig. 3). At 550 MPa and 65 °C, and all selected holding times, the percentage of residual trypsin was higher, due to the high TIs inactivation. Therefore, inactivated TIs did not inhibit trypsin completely. Trypsin inhibitors are proteins that can be affected by pressure at the molecular level. Pressure may rupture the protein noncovalent (ionic, hydrogen, hydrophobic) and covalent (disulfide) bonds (Michel & Autio, 2001). Thus, TIs inactivation increases as pressure, temperature and processing time increase. These molecular changes lead to protein denaturation, aggregation, or gel formation (Rovere, 2001).

Results observed using sodium bicarbonate in the soaking water were significantly different ($p < 0.05$) from those results observed when soaking in distilled water, for all treatment combinations. Sodium bicarbonate may act synergistically to inactivate TIs under HHP. For those treatments, using sodium bicarbonate at 250 MPa and 50 °C, the residual trypsin was approximately 8% after 15 min of holding time.

The number of disulfide bonds in the TIs molecules confers major stability of these compounds during the inactivation treatment. The Kunitz soybean trypsin inhibitor, with two disulfide bonds, could be less stable during thermal or pressurization treatments. The Bowman–Birk inhibitor, on the other hand, with a molecular weight of 8000, is strongly stabilized by its seven disulfide bonds. The Bowman–Birk inhibitor is a polypeptide consisting of 71 amino acid residues; 20% of these aminoacids is cysteine (Rackis, Wolf, & Baker, 1986). Even though disulfide interactions present high stability to pressure, these hydrophobic interactions in proteins can be either disrupted or stabilized according to the magnitude of the applied pressure (Johnson, Austin, & Murphy, 1992). The fact that the proportion of residual trypsin increases as pressure and temperature processing increases, suggests that TIs (proteins) lose

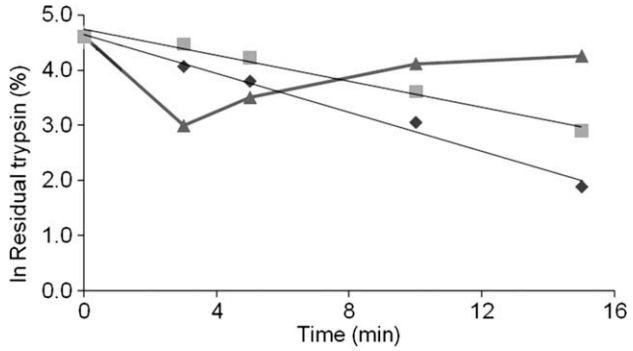

Fig. 1. Residual trypsin in HHP-processed (550 MPa) soymilk from soybeans previously soaked in water. Temperatures: 19 (◇), 65 (□), and 80 °C (△).

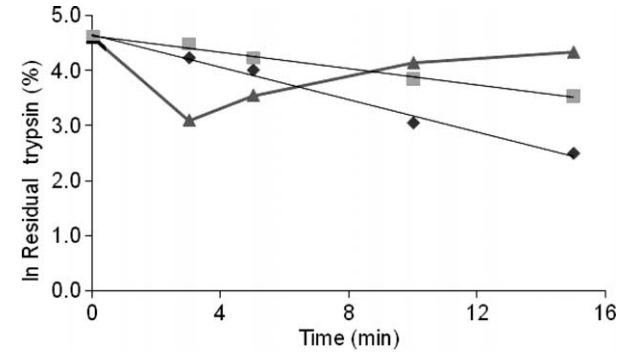

Fig. 2. Residual trypsin in HHP-processed (550 MPa) soymilk from soybeans previously soaked in sodium bicarbonate solution (0.5%). Temperatures: 19 (◇), 65 (□), and 80 °C (△).

Table 1
First order kinetics constants for residual trypsin in soymilk after selected treatments.

Treatment	Slope (min^{-1})	ln $[TA_i]$	R^2	k (min^{-1})
Soymilk from soybeans soaked in distilled water				
550 MPa, 19 °C	−0.186	4.70	0.976	0.428
550 MPa, 65 °C	−0.125	4.62	0.986	0.288
250 MPa, 50 °C	−0.217	4.59	0.981	0.499
Soymilk from soybeans soaked in $NaHCO_3$ solution				
550 MPa, 19 °C	−0.154	4.66	0.970	0.355
550 MPa, 65 °C	−0.081	4.64	0.985	0.186
250 MPa, 50 °C	−0.164	4.52	0.990	0.378

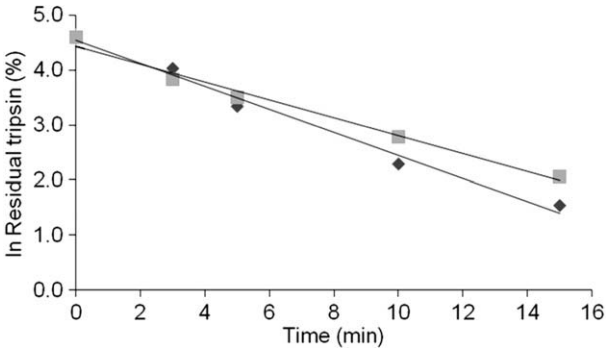

Fig. 3. Residual trypsin in HHP-processed (250/50 MPa/°C) soymilk from soybeans soaked in distilled water (◇) or sodium bicarbonate solution (0.5%) (□).

their activity because HHP and temperature may cause denaturation (Heremans, 2001).

The kinetic parameters of the residual trypsin in soymilk after inactivation of TIs are presented in Table 1. The rates of inhibition for trypsin activity in treatments using sodium bicarbonate were relatively higher than in treatments using distilled water during soaking of soybeans. The rate constant of the remaining residual trypsin in soymilk treated at 250 MPa and 50 °C, using sodium bicarbonate in the soaking water, was 0.378 min^{-1} in comparison with 0.499 min^{-1} when soybeans were soaked in distilled water alone. Increasing pressure treatment to 550 MPa resulted in smaller rate constants of the residual trypsin in soymilk. At 550 MPa and 19 °C the rate constants were 0.428 and 0.355 min^{-1} for soymilk from soybeans soaked in distilled water and sodium bicarbonate, respectively. Upon increasing temperature to 65 °C and 550 MPa, the rate constants were 0.288 and 0.186 min^{-1} for soymilk when soaking in distilled water and sodium bicarbonate, respectively. These data may suggest that HHP processing, combined with temperature, can alter the structural integrity of TIs, as pointed out above. This may also suggest that after increasing pressure, temperature and treatment time, the rate constant of the inactivation of the TIs increased leading to more residual trypsin.

In the case of treatments at 550 MPa and 80 °C (Figs. 1 and 2), data did not fit a first order kinetics model, since the percentages of residual trypsin increased as treatment time increased. Therefore, higher quantities of residual trypsin were observed. There was no significant difference ($p > 0.05$) of the residual trypsin averages, at the same treatment times, after soaking soybeans in distilled water or sodium bicarbonate solution. However, treatment time had a significant effect ($p < 0.05$) on the quantity of residual trypsin. After 15 min of treatment, 76% of the residual trypsin remained in soymilk, while only 32% remained after 5 min of holding time.

In products such as cow's milk and eggs, which contain disulfide bonds, researchers have reported that the formation of disulfide bonds was enhanced by pressure treatment. Keim and Hinrichs (2004) reported that disulfide bonds (which stabilize molecular interaction) predominated in gels from whey protein isolate treated at 600 MPa (30 °C). They pointed out that the content of native whey protein decreased while the amount of intermolecular disulfide bonds increased in pressurized whey protein isolate at prolonged holding time.

Ninety percent inactivation of TIs in soymilk, for maximum nutritive value or protein efficiency ratio, has been reported when thermal treatment was used alone in soy products. Attempts to inactivate TIs through thermal processing below 100 °C have shown that a long time is required to reduce TIs to 10% which is the desired level for safe consumption of soy products. In this research, the use of HHP, combined with temperature, resulted in 76% residual trypsin after 15 min of HHP processing at 550 MPa and 80 °C (considering, as 100%, the initial trypsin activity), indicating that important inactivation of TIs was achieved in soymilk. As explained above, HHP, in combination with thermal treatment, may target disulphide bonds to destabilize TI molecules. However, the exact mechanism through which high pressure acts on these molecules is unknown, since no research is reported using HHP as an alternative process for soymilk; therefore, further research is needed.

4. Conclusions

Residual trypsin was measured in soymilk subjected to selected pressures, temperatures and holding times. Treatment combination at higher pressures and temperatures, for selected holding times resulted in an increased inhibition rate of trypsin inhibitors in soymilk. It was not possible to obtain inactivation rate parameters for treatments at 550 MPa and 80 °C because the data did not fit a first order kinetics model. However, a clear increase of residual trypsin was observed as treatment times increased.

Soaking of soybeans in sodium bicarbonate solution, prior to preparation of soymilk, resulted in smaller inhibition rates of trypsin at the working selected pressures, combined with thermal treatment and holding times, than in soybeans soaked in distilled water. The use of sodium bicarbonate, as soaking medium of soybeans, did not result in a significant increase in the percentage of residual trypsin in soymilk treated at 550 MPa and 80 °C for the selected holding times.

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