

THE INFLUENCE OF SOYBEAN GENOTYPES AND HTC PROCESSING METHOD ON TRYPSIN INHIBITOR ACTIVITY OF SOYMILK

Sladana P. Stanojević*, **Miroljub B. Barać**,
Mirjana B. Pešić and **Biljana V. Vucelić-Radović**

University of Belgrade, Faculty of Agriculture,
Nemanjina 6, 11081 Belgrade-Zemun, Serbia

Abstract: Kunitz inhibitor (KTI) and Bowman-Birk trypsin inhibitor (BBI) are inhibitors of digestive enzymes in raw soybeans. Due to their antinutritive properties in the active state, their inactivation by heat treatment is commonly used. Soymilk is a turbid and stable colloidal solution, obtained by thermal treatment of soybean. In this study soymilk was made on a pilot-plant scale from six soybean cultivars using hydrothermal cooking (HTC) as the production method. This procedure is significantly different from the traditional one. The aim of this investigation was to evaluate the impact of the HTC processing for soymilk production and different soybean genotypes on trypsin inhibitor content and activity. Obtained soymilk contained BBI in trace amounts, in the BBI-polymeric forms. The BBI monomeric forms were not detected. The soymilk of the investigated soybean genotypes had very similar KTI levels (2.34–2.99%). Results have suggested that the soybean genotype does not have substantial effects on the levels of KTI, as well as on the value of residual trypsin inhibitor activity (rTIA). The total content of TI and rTIA showed a strong dependence ($r=0.91$; $p<0.05$). HTC-soymilk rTIA was $<20\%$ (7.15–19.89%). These results have indicated that HTC processed soymilk is applicable for human consumption.

Key words: soymilk, hydrothermal cooking (HTC), soybean genotype, trypsin inhibitors.

Introduction

Soy foods and beverages have become popular among consumers because of their potential health benefits and low prices. Soymilk is a traditional Asian beverage derived from soybeans. In Western countries, soymilk is mainly intended for populations that cannot consume cow's milk because of lactose intolerance, allergies to milk protein, or they do not consume animal milk out of conviction.

*Corresponding author: e-mail: sladjas@agrif.bg.ac.rs

Soymilk is a turbid and stable colloidal solution that primarily consists of carbohydrates, proteins and oil bodies (Ono, 2008). Chen and Ono (2014) classified soymilk proteins (by scanning electron microscopy) into three categories: 1) large protein particles (40–258 nm) that incorporate lipoxygenase, γ -conglycinin, lectin, Kunitz trypsin inhibitor as well as β -conglycinin and glycinin; 2) small stable protein particles (20–40 nm) which are correlated with the β subunits of β -conglycinin; and 3) soluble proteins (<20 nm) comprised of monomers and oligomers of α' , α and acidic glycinin polypeptides (disulfide-linked or not). Together, the large protein particles and soluble proteins <20 nm contribute to 90% of total soymilk protein.

In addition to glycinin and β -conglycinin, which together comprise about 70% of total soymilk protein, biologically active proteins such as lipoxygenase, β -amylase, lectin, Kunitz and Bowman-Birk trypsin inhibitors are also important soymilk components. KTI and BBI are inhibitors of digestive enzymes in raw soybeans. However, they play an important role in prevention of cancer, after application of thermal treatment. Namely, trypsin inhibitor (TIA) contains a high level of sulfur-containing amino acids, desirable from a nutritional point of view, but their activity must be reduced commonly by thermal treatment.

Depending upon conditions of processing, soymilk nutritional, sensory and techno-functional quality may be affected. Conventional soymilk manufacturing procedures include heating freshly prepared soymilk or slurry to boiling in an open vessel for about 30 minutes. This partly destroys the antinutritional factors, but traditionally prepared soymilk has painty and beany flavors. In our societies, this flavor is unacceptable to most consumers. Hydrothermal cooking comprises continuous dispersion of ground soy seeds in hot water under pressure. HTC processing (high pressure/high temperature/short time) has been developed to produce soymilk with better nutritional and techno-functional characteristics and that has less beany flavor (Wang et al., 2003). The HTC process, because of the short time action of lipoxygenase, produces soymilk with much less present beany flavor. Moreover, undesirable volatile components were removed with water vapor at high pressure. Furthermore, this process increases the yield of dry matter and proteins in HTC milk (Johnson et al., 1981). In addition, HTC milk is suitable for preparing tofu with good characteristics (Stanojevic et al., 2011).

It is known that protein composition and quality of soy protein products vary among genotypes (Pesic et al., 2005, Stanojevic et al., 2011), as well as the levels of TI (Pesic et al., 2007; Stanojevic et al., 2013). Trypsin inhibitor activity is also affected by the genotype (Vollmann et al., 2003; Pesic et al., 2007; Stanojevic et al., 2013). However, little information is available about the content of TI and TIA in soymilk. Therefore, this investigation was performed to evaluate a possible impact of high-pressure hydrothermal process production of soymilk on the trypsin inhibitor content and activity from six different soybean genotypes.

Material and Methods

For soymilk preparation, six commercial soybean genotypes grown in field conditions were used: ZPS-015, Nena, Lana (obtained from Maize Research Institute “Zemun Polje”, Belgrade, Serbia) Krajina, Novosadjanka and Balkan (supplied by the Institute of Field and Vegetable Crops, Novi Sad, Serbia). The genotype Lana was selected without the Kunitz type of trypsin inhibitor. The pilot-plant scale with hydrothermal cooking (HTC) was used for soymilk production (Stanojevic et al., 2012). Briefly, after soaking of soybeans for 14 h in water at 5–7°C and grounding, the suspension was cooked by a steam injection system at 110°C/1.8 bar/8 min (SoyaCow VS 30/40, model SM-30, Russia). To separate soymilk from okara, the obtained slurry was filtered. Soymilk proteins were extracted at room temperature with 0.03 M Tris-HCl buffer, pH 8.00 (tris-hydroxymethyl-aminomethane), which contained 0.01 M β -mercaptoethanol, for 120 min (the sample to buffer ratio was 1:10), then, the mixture was centrifuged at 7558g for 15 min at room temperature. The soymilk protein extracts were further analyzed by SDS- and native-PAGE electrophoresis.

The procedure proposed by Fling and Gregerson (1986) was used to perform electrophoresis in dissociating conditions (SDS-PAGE) detailed by Stanojevic et al. (2011, 2012). The stacking gel was 5% (m/v) (pH 6.80) and the separating gel (pH 8.85) was 12.5% (m/v). Briefly, the protein extracts were adjusted to a concentration of 2 mg/mL by sample buffer (pH 6.80) and were subjected to 90°C for 5 min, and then cooled to room temperature. After that, 25 μ L of sample was loaded into the gel wells and electrophoresis was run for 6 h at 160 mA per two gels in a buffer solution (pH 8.30). After gel fixation, staining with 0.23% (m/v) Coomassie brilliant blue R-250 and destaining with 8% (v/v) acetic acid and 18% (v/v) ethanol, the gels were analyzed.

The procedure proposed by Davis (1964) was used to perform electrophoresis in native conditions (native-PAGE), detailed by Stanojevic et al. (2013). The stacking gel was 5% (m/v) (pH 6.70) and the separating gel was 7% (m/v) (pH 8.90). Briefly, the protein extracts were adjusted to a concentration of 2 mg/mL by sample buffer (pH 8.00) and 25 μ L were loaded into the gel wells. The electrophoresis was run for 4.30 h at 90 mA in buffer solution (pH 8.30). Gels were stained and destained as previously described. The analysis was performed with two replications. All gels were analyzed by SigmaGel software version 1.1 (JandelScientific, San Rafael, CA, USA). The trypsin inhibitor content was calculated as the percentage of the respective area with respect to the total area of the densitogram.

The method of Liu and Markakis (1989) was used to determinate the trypsin inhibitor activity (TIA) in soymilk using a crystalline bovine trypsin and α -N-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA) as substrate. These

substances were supplied by Sigma, USA. Briefly, the soymilk samples were extracted for 30 min with distilled water at a sample to water ratio 1:100 in a mechanical shaker. After extraction, filtration was performed using Whatman no. 4 paper. An aliquot of 10 mL of the filtrate was diluted with 10 mL 0.05 M Tris-HCl buffer (pH 8.2) and then filtered. Further dilution of obtained filtrates with distilled water was made until 1 mL of filtrate inhibited 30–70% of the enzyme. An aliquot of 1 mL of the diluted sample, 1 mL of 0.92 mM BAPA and enzyme solution (16 µg/mL in 0.001 M HCl) were heated at 37°C for 10 min. The addition of 30% (v/v) acetic acid was used to stop the reaction. The determination of absorbance was performed at 410 nm. The trypsin units inhibited (TUI) per milligram of the dry sample were used to express the trypsin inhibitor activity. The TIA was also expressed as residual activity in relative percent to defatted soybean flour.

Statistica software of version 6.0 (StatSoft Co., Tulsa, OK, USA) was applied for data analysis. Student's *t*-test procedure for independent samples (at $p < 0.05$) was used to evaluate significant differences between means. The results are presented as mean values. The correlation analyses were also performed using a Pearson two-tailed significance correlation test (at $p < 0.05$).

Results and Discussion

Electrophoretic analysis

TI was registered on SDS and PAGE gels of soymilk extractable proteins (Figures 1 and 2) and the content was analyzed (Table 1).

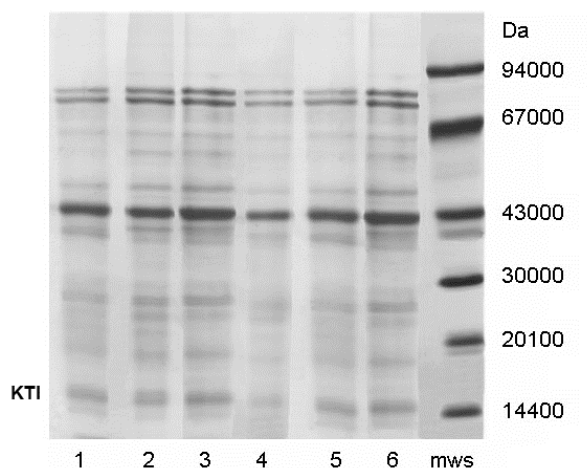


Figure 1. SDS-PAGE analysis of soymilk. Lanes: 1 – Lana, 2 – Novosadjanka, 3 – Krajina, 4 – Balkan, 5 – Nena, 6 – ZPS-015, mws – molecular weight standards.

According to our previous results of soybean and okara PAGE analysis, the BBI type of trypsin inhibitors was observed in two zones (zone of polymeric forms – BBIp and zone of monomeric forms – BBIm), whereas the KTI was detected as one band (Pesic et al., 2007; Stanojevic et al., 2013). The zone with lower electrophoretic mobility represents the polymeric forms of BBI, whereas the other represents monomeric forms of BBI (Barac and Stanojevic, 2005). The polymeric forms of BBI are the result of their self-aggregation under non-dissociating conditions (Sessa and Wolf, 2001). In investigated samples of soymilk BBIm was not detected, but BBIp was detected in trace amounts (0.01–0.04%, Table 1). Xu et al. (2012) reported that in soymilk, heated for a long time, KTI was incorporated into protein particles while BBI, a very hydrophilic protein, existed as a monomer. HTC process applied in these studies included the high pressure/high temperature/short time treatment, not a long time treatment.

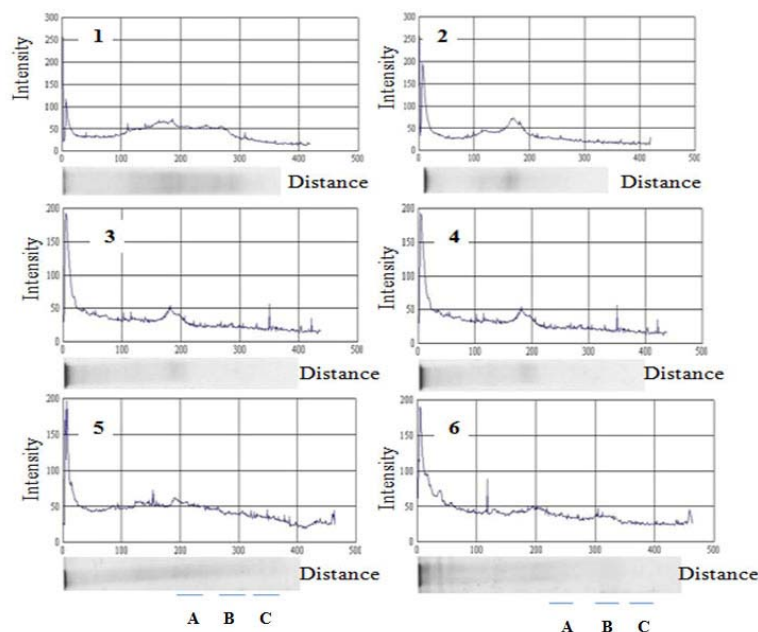


Figure 2. PAGE analysis of soymilk proteins from investigated genotypes; 1 – Nena, 2 – Krajina, 3 – Novosadjanka, 4 – ZPS-015, 5 – Balkan, 6 – Lana. A – zone of polymeric forms of BBI, B – zone of KTI, C – zone of monomeric forms of BBI.

We could assume that conditions of applied HTC process did not lead to hydrophobic protein interaction of BBI molecules due to a very low level of self-aggregation of BBI molecules. This might be expectable because the self-

association of BBI was observed even in the presence of SDS, mercaptoethanol and urea (Sessa and Wolf, 2001). Furthermore, the results have indicated that the polymeric forms of BBI molecules exist in trace amounts in soymilk, after the applied HTC process. Namely, PAGE analysis of the results has suggested that the conditions of applied HTC process lead to the occurrence of different products of protein hydrolysis, which are close to the electrophoretic mobility of this type of inhibitor, so it could not be detected with certainty. However, a soluble protein state (i.e., as small protein particles or monomers) in soymilk has remained unclear (Chen and Ono, 2014).

As it could be observed, the soymilk of the investigated soybean genotypes had very similar KTI levels (2.99–2.34% of total extractable protein, Table 1). A very strong positive correlation was found between the concentrations of KTI and total TI ($r = 0.98$, $p < 0.05$). These results strongly suggested that levels of total TI in HTC soymilk mostly existed, under the applied experimental conditions, in form of KTI. Namely, contents of total TI ranged from 2.35% to 3.02% of total extractable protein. The exception was Lana soymilk (level of total TI was 0.02%, Table 1). Lana was a cultivar lacking the Kunitz type of trypsin inhibitor, so because of that Lana soymilk was not included in statistical correlation analysis.

Table 1. Trypsin inhibitor composition (% extractable protein) and activity of soymilk from investigated genotypes¹.

Genotype	KTI ²	BBIp ³	TI ⁴	TUI ⁵ /mg	rTIA ⁶ (%)
Nena	2.44±0.03 ^b	0.04±0.001 ^a	2.48 ^b	46.24±0.27 ^b	13.00±0.24 ^{c d}
Krajina	2.99±0.02 ^a	0.02±0.001 ^b	3.01 ^a	49.23±0.29 ^a	16.95±0.19 ^b
Novosadjanka	2.98±0.07 ^a	0.04±0.001 ^a	3.02 ^a	39.29±0.33 ^c	19.89±0.22 ^a
Balkan	2.34±0.03 ^c	0.01±0.000 ^b	2.35 ^c	46.88±0.17 ^b	14.00±0.09 ^{b c}
ZPS - 015	2.51±0.04 ^b	0.01±0.001 ^b	2.52 ^b	40.01±0.13 ^c	11.76±0.12 ^d
Lana	/	0.02±0.001 ^b	0.02 ^d	10.71±0.09 ^d	7.15±0.07 ^e

¹Means in the same column with different superscript roman letters are significantly different ($p < 0.05$); ²KTI – content of Kunitz-type soybean trypsin inhibitor; ³BBIp – content of polymeric forms of Bowman-Birk trypsin inhibitor; ⁴TI – total content of trypsin inhibitor; ⁵TUI/mg – activity of trypsin inhibitor; ⁶rTIA – residual activity of trypsin inhibitor.

Activity of trypsin inhibitor

TI has high nutritive value due to the presence of cystein amino acids (Liener, 1994), but the activity of TI showed antinutritive properties at high level. To

reduce their activity to an acceptable level, heat treatment is commonly applied. The presented results indicated that HTC treatment was effective to reduce trypsin inhibitor activity in soymilk. The residual trypsin inhibitor activity of soymilk from 7.15% to 19.89% was registered (Table 1). The lowest value of rTIA was determined in Lana soymilk, genotype without KTI. These results have indicated that HTC processed soymilk is applicable in human nutrition, due to TIA lower than 20%. The most commercially heated soybean meals retain up to 20% of the activity of Bowman-Birk's chymotrypsin and trypsin inhibitors BBI and KTI (Friedman and Brandon, 2001).

Our previous studies have suggested that TIA of soybeans and okara varies among genotypes (Pestic et al., 2007; Stanojevic et al., 2013). The obtained results suggested that soybean genotype did not have substantial effects on the value of rTIA in HTC soymilk. In fact, these values were similar according to the statistical analysis (Table 1). Furthermore, a strong correlation between the rTIA and total TI contents in soymilk ($r = 0.91$; $p < 0.05$) was found, which was similar to soy okara, but different to soy grain. Namely, Pestic et al. (2007) did not report any correlation between TIA and TI contents ($r = 0.34$; $p < 0.05$) in 12 soybean genotypes. Contrary to that, Stanojevic et al. (2013) found a strong correlation between rTIA and TIs contents ($r = 0.89$; $p < 0.05$) in soy okara. These results showed that correlation between rTIA and TI contents was well balanced after heat treatment.

Conclusion

According to our results, it could be concluded that the applied HTC process (thermal/pressure treatment/short time) conditions had an impact on the production of soymilk in which BBI_m was not detected, but BBI_p was detected in trace amounts. The soymilk of the investigated soybean genotypes had very similar KTI levels. The soybean genotype did not have substantial effects on the value of rTIA in HTC soymilk. The total content of TI and value of rTIA showed a strong dependence. The total TI in HTC soymilk, under the applied experimental conditions, was mostly in form of KTI. HTC soymilk rTIA was <20%. These results suggested that soymilk obtained by HTC processing could be taken by consumers from the food safety aspect.

Acknowledgements

This investigation was supported by the Serbian Ministry of Education, Science and Technological Development (Project No. TR 31022) and the EU FP7 project Grant Agreement No. 316004 (REGPOT-AREA). Also, we are grateful to Maize Research Institute, "Zemun Polje", Serbia and Institute of Field and Vegetable Crops, Novi Sad, Serbia, for providing soybean genotypes.

References

- Barac, M. & Stanojevic, S. (2005). The effect of microwave roasting on soybean protein composition and components with trypsin inhibitor activity. *Acta Alimentarija*, 34, 23-31.
- Chen, Y. & Ono, T. (2014). Protein particle and soluble protein structure in prepared soymilk. *Food Hydrocolloids* 39,120-126.
- Davis, J. (1964). Disc electrophoresis–II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences*, 121, 404-427.
- Fling, S.P. & Gregerson, D.S. (1986). Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris-buffer system without urea. *Analytical Biochemistry* 155, 83-88.
- Friedman, M. & Brandon, L.D. (2001). Nutritional and health benefits of soy protein. *Journal of Agricultural and Food Chemistry*, 49(3), 1069-1086.
- Liener, I.E. (1994). Implications of anti-nutritional components in soybean foods. *CRC Critical Review of Food Science and Nutrition* 34, 31-67.
- Liu, F. & Markakis, P. (1989). An improved colorimetric method for determining antitryptic activity in soybean products. *Cereal Chemistry* 66, 415-422.
- Ono, T. (2008). The mechanism of soymilk and tofu formation from soybean, and the factors affecting the formation. *Nippon Shokuhin Kagaku Kaishi*, 55(2), 39-48.
- Pesic, M.B., Vucelic-Radovic, B.V., Barac, M.B. & Stanojevic, S.P. (2005). The influence of genotypic variation in protein composition on emulsifying properties of soy proteins. *Journal of American Oil Chemistry Society*, 82, 667-672.
- Pesic, B.M., Vucelic-Radovic, V.B., Barac, B.M., Stanojevic, P.S. & Nedovic, A.V. (2007). Influence of different genotypes on trypsin inhibitor levels and activity in soybeans. *Sensors*, 7, 67-74.
- Sessa, D.J. & Wolf, W.J. (2001). Bowman-Birk inhibitors in soybean seed coats. *Journal of American Oil Chemistry Society* 14, 73-83.
- Stanojevic, P.S., Barac, B.M., Pesic, B.M. & Vucelic-Radovic, V.B. (2011). Assessment of soy genotype and processing method on quality of soybean tofu. *Journal of American Oil Chemistry Society*, 59(13), 7368-7376.
- Stanojevic, P.S., Barac, B.M., Pesic, B.M. & Vucelic-Radovic, V.B. (2012). Composition of proteins in okara as a byproduct in hydrothermal processing of soymilk. *Journal of American Oil Chemistry Society*, 60, 922-9228.
- Stanojevic, P.S., Barac, B.M., Pesic, B.M., Jankovic, S.V. & Vucelic-Radovic, V.B. (2013). Bioactive proteins and energy value of okara as a byproduct in hydrothermal processing of soy milk. *Journal of American Oil Chemistry Society*, 61, 9210-9219.
- Vollmann, J., Grausgruber, H., Wagentristl, H., Wohleser, H. & Michele, P. (2003). Trypsin inhibitor activity of soybean as affected by genotype and fertilisation. *Journal of Agricultural and Food Chemistry*, 83, 1581-1586.
- Wang, C., Johnson, A.L. & Wilson, A.L. (2003). Calcium coagulation properties of hydrothermally processed soymilk. *Journal of American Oil Chemistry Society*, 80 (12), 1225-1229.
- Xu, Z., Chen, Y., Zhang, C., Kong, X. & Hua, Y. (2012). The heat-induced protein aggregate correlated with trypsin inhibitor inactivation in soymilk processing. *Journal of Agricultural and Food Chemistry*, 60, 8012-8019.

Received: May 19, 2016

Accepted: June 30, 2016

UTICAJ GENOTIPA SOJINOG ZRNA I HTC PROIZVODNOG POSTUPKA
NA AKTIVNOST TRIPSIN INHIBITORA SOJINOG MLEKA

**Sladana P. Stanojević*, Miroljub B. Barać,
Mirjana B. Pešić i Biljana V. Vucelić-Radović**

Univerzitet u Beogradu, Poljoprivredni fakultet,
Nemanjina 6, 11080, Beograd-Zemun, Srbija

R e z i m e

U sirovoj soji Kunitz-ov (KTI) i Bowman-Birk-ov inhibitor tripsina (BBI) su inhibitori digestivnih enzima. Da bi se poboljšao nutritivni kvalitet hrane od soje, tripsin inhibitori (TI) se inaktiviraju uglavnom termičkom obradom. Sojino mleko je mutni i koloidni rastvor, dobijen nakon termičkog tretmana sojinog zrna. U ovoj studiji sojino mleko je napravljeno od šest sorti soje u pilot postrojenju metodom koja uključuje hidrotermičko kuvanje (engl. *hydrothermal cooking* – HTC). Ovaj postupak se znatno razlikuje od tradicionalnog. Cilj ovog rada je bio da se proceni uticaj HTC postupka za proizvodnju sojinog mleka i različitih genotipova sojinog zrna na sadržaj i aktivnost tripsin inhibitora. Dobijeno sojino mleko je sadržalo BBI u tragovima, u formi BBI-polimera. BBI-monomerne forme nisu detektovane. Sojina mleka od ispitivanih genotipova soje imaju veoma slične sadržaje KTI (2,34–2,99%). Rezultati su pokazali da genotip sojinog zrna nema značajne efekte na sadržaj KTI, kao i na vrednost rezidualne tripsin inhibitorske aktivnosti (rTIA). Ukupan sadržaj TI i vrednost rTIA je u snažnoj korelaciji ($r=0,91$; $p<0,05$). HTC sojina mleka imala su vrednosti za rTIA <20% (7,15–19,89%). Ovi rezultati su pokazali da sojina mleka dobijena nakon HTC obrade mogu biti namenjena za ishranu ljudi.

Ključne reči: sojino mleko, hidrotermičko kuvanje (HTC), genotip soje, tripsin inhibitori.

Primljeno: 19. maja 2016.

Odobreno: 30. juna 2016.

*Autor za kontakt: e-mail: sladjas@agrif.bg.ac.rs