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Bioactive Proteins and Energy Value of Okara as a Byproduct in Hydrothermal Processing of Soy Milk

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ABSTRACT: The nutritional properties of raw okara obtained as a byproduct from six soybean varieties during hydrothermal cooking (HTC) of soy milk were assessed. The composition and residual activity (rTIA) of trypsin inhibitors (TIs), contents of lectin, proteins, fats, and carbohydrates, and energy values (EV) were correlated with the respective physicochemical properties of soybean and okara. Kunitz (KTI) and Bowman−Birk (BBI) TIs both comprised okara rTIA. TIs content was higher in okara (5.19−14.40%) than in soybean (3.10−12.17%), which additionally enriched okara by cysteine. Contents of KTI ($r = 1.00; p < 0.05$) and BBI ($r = 0.89; p < 0.05$) as well as BBI monomeric ($r = 0.89; p < 0.05$) and polymeric forms ($r = 0.95; p < 0.05$) in okara and in soybean were strongly correlated. Low urease index activity indicated that okara was heated adequately to inactivate antinutritional factors. The proximate composition of raw okara, advantageous rTIA, and a very low EV (2.74−3.78 kJ/g) qualify this byproduct for potential application in food preparation as a functional ingredient in dietary products.

KEYWORDS: *okara, HTC processing, soybean genotype, proteins, carbohydrates, trypsin inhibitors, urease activity, lectin, energy values*

INTRODUCTION

In recent years soybean has gained much attention because of its health-promoting properties. Many studies have reported on the beneficial components of soybean such as protein, dietary fiber, fatty acids, isoflavones, and other phytochemicals.[1−4] Soy foods are becoming more popular as low-cost substitutes for traditional dairy products and an ideal nutritional supplement for lactose-intolerant subjects. Raw okara, also called "soy pulp", is a byproduct of the soy milk industry. It is a white-yellowish material consisting of the insoluble parts of the soybean seeds, which remain in the filter sack when pureed soybeans are filtered for the production of soy milk.[1,5−10] The high protein content of about 30% (31.81−40.36% on dry basis)[11] makes okara a potential source of low-cost plant protein for human nutrition. Moreover, the ratio of essential amino acids to total amino acids in okara is similar to that in soy milk and tofu.[12] The main components of okara are protein[11,13] and dietary fiber (52.8−58.6%).[6]

Food intake rich in dietary fiber in the daily diet is important to reduce or regulate plasma cholesterol and triacylglycerol levels and to promote health.[14] The hypocholesterolemic effect of dietary fiber has been reported in different animal models[15−17] and in humans.[18] It was shown that higher supplementation of okara fiber significantly reduced total cholesterol. It has also been found that not only fiber but other components of okara, such as protein, could be involved in the reduction of serum lipids. Other studies have shown that soy protein consumption has an inhibitory effect on the development of atherosclerosis in mice.[19] Several studies have assessed okara for potential hypolipidemic ingredients and for other health-promoting properties in animals.[15,17] Okara also contains isoflavones (0.14 g/100 g dry basis[20]). Due to this specific composition, okara might have a potential use in the food industry.

Legumes play an important role in the dietary pattern intended to reduce cancer risk. Of the plants in the Leguminosae family, the soybean has undoubtedly received the most research attention for its role in cancer chemoprevention.[21] Among other soybean phytochemicals, biologically active proteins such as inhibitors of digestive enzymes Kunitz (KTI) and Bowman−Birk (BBI) protease inhibitors (TIs), and lectins are responsible for this activity. Thus, soy protein is not an ideal protein because adverse nutritional effects following consumption of raw soybean meal have been attributed to the presence of trypsin inhibitors and lectins and to their poor digestibility.[3,21] The activity of these inhibitors must be reduced, but because they are high in sulfur-containing amino acids, their presence is desirable. To improve the nutritional quality of soy foods, inhibitors and lectins are generally inactivated by heat treatment. A lot of research has been done on employing different high-pressure/high-temperature treatments for improvement of the nutritional quality of soybean[22,23] and okara.[8] Besides different conditions used during processing (pH, humidity, duration, temperature, pressure), the effect of inactivation treatments also depends on soybean genotype. Pesic et al.[24] have found, by examining 12 different varieties of soybeans, a significant difference in content and TIs activity among genotypes. The content of KTI ranged from 4.28 to 6.85% and BBI varied from 0.6 to 6.32% of

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total extractable proteins, and the TIs activity among genotypes varied from 60.36 to 100.95 TUI/mg. Therefore, the aim of this study was to assess the influence of a pilot plant method that uses high-pressure hydrothermal processing for soy milk production on content, activity, and energy value of proteins and other main nutritional components in okara prepared from six different soybean genotypes. Also, the aim was to examine correlation relationships between soybean grain and okara nutrients.

MATERIALS AND METHODS

Materials. For soy milk and okara preparation six commercial soybean genotypes grown in field conditions were used: ZPS-015 (0 maturity group), Krajina (00 maturity group), Novosađanka and Balkan (I maturity group), and Nena and Lana (II maturity group). Three genotypes (ZPS-015, Lana, and Nena) were selected by the Maize Research Institute Zemun Polje (Belgrade, Serbia) and the others (Novosadjanka, Krajina, and Balkan) by the Institute of Field and Vegetable Crops (Novi Sad, Serbia). The genotype Lana lacked the Kunitz type of trypsin inhibitor. Although Novosadjanka was selected as a high seed-protein cultivar, all of the genotypes were of food grade and were characterized by the high protein content in grain.

Soy Milk and Okara Processing. Okara was made on the pilot plant scale using a production method that includes hydrothermal cooking (HTC)[23] for soy milk preparation, modified by Stanojevic et al.[11] Briefly, soybeans were soaked in water at 5−7 °C for 14 h. Soaked beans were ground and cooked by a steam injection system at 110 °C/1.8 bar/8 min (SoyaCow VS 30/40, model SM-30, Russia). The slurry was filtered to separate soy milk from okara.

Preparation of Samples for Chemical Analyses. The seeds were ground in a Micro-Mill grinder (Fisher, Germany). The soybean powder and raw okara were then defatted using hexane (okara or soybean powder/hexane = 1:20). Samples were dried at room temperature. From all samples (soy flour, soy milk, and defatted okara), 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. For defatted soy flour, the sample to buffer ratio was 1:20; for soy milk and okara, the sample to buffer ratio was 1:10. Then, the mixture was centrifuged at 7558*g* for 15 min at room temperature. The protein extract was used for SDS- and native-PAGE analyses.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Dissociating electrophoresis for all samples was performed according to the Fling and Gregerson[25] procedure, detailed by Stanojevic et al.,[11,26] using 5% (m/v) (pH 6.80) stacking and 12.5% (m/v) separating gels (pH 8.85). Briefly, the protein extracts were diluted with sample buffer (pH 6.80) to a concentration of 2 mg/mL, then heated at 90 °C for 5 min, and cooled to room temperature. A 25 μL sample was loaded onto each well, and then the gels were run at 80 mA per gel for 6 h in a buffer solution (pH 8.30). The gels were fixed, stained with 0.23% (m/v) Coomassie brilliant blue R-250 for 50 min, and destained with 8% (v/v) acetic acid and 18% (v/v) ethanol. Molecular weight markers included phosphorylase B (94 kDa), bovine albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (Pharmacia, Sweden).

Native-Polyacrylamide Gel Electrophoresis (PAGE). Native-PAGE was performed according to the Davis[27] procedure using 5% (m/v) (pH 6.70) stacking and 7% (m/v) separating gels (pH 8.90). Briefly, aliquots of 25 μL of okara protein extracts diluted with sample buffer (pH 8.00) up to 2 mg protein/mL were loaded per well. The gels were run at 12 °C in a buffer solution (pH 8.30) for 30 min (40 mA) and then for 4 h (90 mA) to completion. Gels were stained in the same way as in SDS-PAGE. PAGE and SDS electrophoresis were performed in electrophoresis unit LKB-2001-100 using power supply LKB-Macrodrive 5 and LKB-Multitemp as a cooling unit (Pharmacia, Sweden). Two gels were run simultaneously in the same electrophoretic cell, and two aliquots of the same sample were analyzed at the same time.

Densitometric Analysis. All destained gels were scanned and subsequently analyzed by SigmaGel software version 1.1 (Jandel Scientific, San Rafael, CA, USA). Quantitative analysis of each identified subunit was calculated as the percentage of the respective area with respect to total area of the densitogram.

Trypsin Inhibitor Activity (TIA). TIA in soybean and okara was estimated according to the method of Liu and Markakis[28] using a crystalline bovine trypsin (Sigma, USA) and α-*N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA; Sigma, USA) as substrate. Briefly, the samples were extracted with distilled water (sample/water = 1:100) for 30 min in a mechanical shaker, and the extract was filtered through no. 4 Whatman paper. An aliquot of the filtrate (10 mL) was diluted with 0.05 M Tris-HCl buffer (pH 8.2) (extract/buffer = 1:1) and filtered. The filtrates were then further diluted with distilled water so that 1 mL of filtrate inhibited 30−70% of the enzyme. A 1 mL sample of the diluted filtrate was incubated at 37 °C for 10 min with 0.92 mM BAPA (1 mL) and enzyme solution (16 μg/mL in 0.001 M HCl). After 10 min, the reaction was stopped by the addition of 30% (v/v) acetic acid. The absorbance was measured at 410 nm. The inhibitor activity was expressed in trypsin units inhibited (TUI) per milligram of the dry sample (defining a trypsin unit as an A_{410} increase of 0.01 under the conditions of the assay). It was also expressed as residual activity (rTIA in percent) relative to defatted soybean flour.

Urease Activity (ΔpH; UA). UA was determined according to the method of Caskey and Knapp[29] and defined as the amount of urease required to release nitrogen from 1 g of urea per minute. Samples were incubated with a buffered solution of urea (pH 7.00; samples/urea = 1:50) for 30 min, at 30 °C, and were intensively shaken every 5 min. Exactly 5 min after preparation of test samples, the incubation of control samples was started. Control samples were prepared from soybean flour (or okara) with phosphate buffer (pH 7.00; samples/buffer = 1:50). Then, exactly 5 min after incubation, the pH value of all samples was measured. Ammonia release rate was found to increase pH values as compared to the control. The difference between the pH values of test samples and control samples was the index of activity of urease (ΔpH). Urease activity was also expressed in percent, relative to the urease activity index of defatted soybean flour (UA).

HPLC. Quantification of monosaccharides and disaccharides was done on a Carbohydrate Analysis column (Waters Chromatography Div., Millipore, Bedford, MA, USA) in the device consisting of the solvent and sample delivery system (Waters-600E System Controller) and the detector (Waters-410 differential refractometer). A GuardPak module containing Resolve Silica (the same manufacturer) was used as a precolumn. As a mobile phase acetonitrile/water = 83:17 was used. The column was calibrated by mobile phase, with gradual flow increase of 0.5−2 mL/min for 5 h. Individual sugars were quantified by comparison with external standards. The standard solution contained xylose, arabinose, glucose, fructose, sucrose, and mannose (Sigma, USA). The standards were dissolved in redistilled water (1%) and filtered through a 0.25 μm membrane (Nucleopore Corp., Pleasanton, CA, USA). Ten microliters of standard solution was injected into the column and eluted for 25 min at 30 °C. Software Maxima 820 (Waters Chromatography Div., USA) was used to calculate sugars content. Samples were extracted with 95% ethanol in a water bath at 50 °C, filtered (Whatman 42), and evaporated under vacuum to dryness (Devarot, type-D-3, Slovenia). The samples were diluted with redistilled water (initial sample/water = 1:4) in an ultrasonic bath (Sonic, Italy), filtered through a 0.25 μm membrane (Nucleopore Corp.), and injected into the column in an amount of 20 μL. The conditions of chromatography were a flow rate of 2 mL/min during 25 min at 30 °C.

Other Analyses. Total nitrogen content in samples was determined by using the micro-Kjeldahl method,[30] and total protein content was calculated (N × 6.25). Total fat content in samples was determined by extraction with diethyl ether in a Soxhlet system.[31] Ash content was calculated from the weight of sample after burning at 550 °C for 2 h in a muffle furnace.[32] Insoluble fiber was determined by the standard ISO method[33] using Fibertec 2010 and M6-Gold Extractor

Table 1. Some Characteristics of Raw Okara[a] (Percent Fresh Matter)

genotype	total fat	total protein	carbohydrate[b]	insoluble fiber	ash	moisture
Nena	1.30 d	8.82 c	8.01 b	2.38 b	0.53 abc	78.94 e
Krajina	1.62 c	7.87 e	7.82 c	2.11 c	0.51 abc	80.06 b
Novosadjanka	1.65 c	9.71 a	7.05 e	1.89 d	0.40 bc	79.28 d
Balkan	2.05 b	8.54 d	7.02 e	1.94 d	0.55 a	79.88 c
ZPS-015	2.09 b	9.39 b	7.51 d	2.56 a	0.45 c	77.99 f
Lana	2.30 a	6.25 f	8.21 a	1.75 e	0.49 abc	80.89 a

[a]Means in the same column with different letters are significantly different ($p < 0.05$). [b]Carbohydrate by difference.

Table 2. Composition of Monosaccharides and Disaccharides and Content of Dietary and Crude Fiber Registered in Raw Okara[a] (Percent Fresh Matter)

	genotype					
components (t_R in min)[b]	Nena	Krajina	Novosadjanka	Balkan	ZPS-015	Lana
mannose (4.11)	4.11 a	nd	nd	3.12 b	nd	nd
fructose (9.08)	nd	0.51 b	0.72 b	nd	1.05 a	0.17 c
sucrose (17.13)	1.78 c	1.61 d	1.84 c	2.21 a	2.07 b	2.11 b
maltose (20.63)	nd	nd	nd	0.31 b	nd	2.24 a
monosacharides	4.11 a	0.51 e	0.72 d	3.12 b	1.05 c	0.17 f
disaccharides	1.78 d	1.61 e	1.82 d	2.52 b	2.07 c	4.35 a
available carb[c]	5.89 a	2.12 e	2.56 d	5.64 a	3.12 c	4.52 b
dietery fiber[d]	2.12 d	5.70 a	4.49 b	1.38 e	4.39 b	3.69 c
total fiber[e]	4.50 e	7.81 a	6.38 c	3.32 f	6.95 b	5.44 d

[a]Means in the same row with different letters are significantly different ($p < 0.05$); not detected carbohydrates were not included in statistical analysis; nd, not detected. [b]t_R, retention time. [c]Available carbohydrate = sum of monosaccharides and disaccharides. [d]Dietary fiber = % of carbohydrates − % of available carbohydrate. [e]Total fiber = % of dietary fiber + % of insoluble fiber.

1021 (FOSS, Sweden). Moisture and volatiles were determined by using standard AACC procedure.[34] Carbohydrate content was calculated by subtracting moisture, protein, fat, ash, and insoluble fiber values from 100%. Dietary fiber content was calculated by subtracting mono- and disaccharide values from carbohydrate content. Available carbohydrate was calculated as the sum of monosaccharides and disaccharides on a raw okara dry matter basis. The total energy content was calculated from the proximate composition.[35] The quantities measured per gram of okara of the various fractions (protein, lipid, and carbohydrate) were multiplied by the known mean combustion equivalents (in the body) of these compounds (17 kJ g^{-1} for protein, 37 kJ g^{-1} for lipid, 17 kJ g^{-1} for available carbohydrate, and 8 kJ g^{-1} for dietary fiber).[29] If otherwise not stated, the results were expressed on a dry weight basis.

Statistical Analysis. The data were analyzed using Statistica software version 6.0 (StatSoft Co., Tulsa, OK, USA). The significance of differences between means was determined by Student's t test procedure for independent samples at $p < 0.05$. The results are presented as mean values. Regression analyses were also carried out. The different parameters were correlated with each other by Person two-tailed significance correlation at $p < 0.05$ level. If not otherwise stated, the experiments were performed in triplicate.

RESULTS AND DISCUSSION

Proximate Composition. The proximate composition of raw okara is presented in Table 1. The okara fresh matter was nutritious and contained mainly carbohydrates (7.02−8.21%) and protein (7.87−9.71%). However, the proximate composition of okara in the literature is very different.[5,6,36,37] O'Toole[5] reported that the proximate composition of okara will depend on the amount of water phase extracted from the ground soybeans and whether further water is added to extract residual extractable components. On a wet weight basis Tadano et al.[36] found the proximate composition of raw okara to be 84.50% moisture, 1.50% fat, 4.73% protein, 7.00% sugars, 1.50% fiber, and 0.40% ash, which is similar to our results.

Content of okara total fat in the literature is very different, too. Our results showed only 1.30−2.30% of total fat in okara (Table 1), which is in agreement with results by Tadano et al.,[36] who registered only 1.5% fat in raw okara. On the contrary, Khare et al.[37] reported a wide range of fat content of 3−9% on a dry matter basis. Other authors reported, depending on the genotype of soybean, that okara may contain even 8−10% of total fat.[6,7,18,38] Because total fat in grain of investigated soy genotypes ranged from 15.25 to 20.48%, it may be assumed that, due to applied pressure/thermal processing, a significant portion of fat was retained in soy milk. The nutritional value of okara would be higher if it contained higher levels of fat, because okara fat is rich in linolenic and oleic acids.[9,38] However, the result of our soy milk production process (HTC) was okara with a lower fat content. From the technological aspect, the low fat content may be an advantage in terms of storability and product shelf life as well.

The high content of protein in okara improves the nutritional quality because the okara proteins are high in essential amino acids.[10,12,39] Our group has previously examined the effects of HTC treatment on okara protein composition, finding the basic 7S globulin (Bg7S) as the main one in okara.[11] Such a high content of Bg7S in okara is desirable because of its nutritional value. Bg7S is a cysteine-rich glycoprotein, and it is of special interest because of its ability to bind both insulin and insulin-like growth factors.[40] Total protein content and carbohydrate content in okara from the examined soybean genotypes were negatively but not significantly correlated ($r = -0.76$; Table 7).

The dry weight of a typical soybean contains around 11% of soluble carbohydrates, which are heat stable and may be partly present in okara.[41] Of these carbohydrates only monosaccharides and disaccharides are positive for metabolizable energy.[42] Both raffinose and stachyose, belonging to the family of oligosaccharides, due to the absence of enzymes capable of

breaking the α-galactosidic linkages, are unavailable for human metabolism. Monosaccharide and disaccharide contents of okara are presented in Table 2. Our results show that raw okara prepared from investigated genotypes after the applied heat/pressure treatment contained small amounts of monosaccharides (0.51−4.11%), disaccharides (1.61−4.35%), and insoluble fiber (1.75−2.56%) on a fresh matter basis. However, our data were higher than those of Van der Reit et al.,[6] who reported monosaccharide (0.60−0.70%) and sucrose (1.30−2.30%) contents on a dry matter basis of okara from three cultivars of soybeans. Meteos-Aparicio et al.[38] reported only 0.6% of sucrose and 0.1% of fructose in okara on a dry matter basis. The same authors[8] concluded that the ratio of soluble to insoluble dietary fiber in okara increased with increasing hydrostatic pressure and temperature. These facts indicate that carbohydrate contents in okara depend on okara heat/pressure treatment during production. In addition, our results confirmed that carbohydrate content in okara depends on the soybean cultivar, too. Due to a high content of total fiber (3.32−7.81%) comprising almost half of the total carbohydrates in the majority of samples, okara might be recognized as an important food fiber, desirable for use in human nutrition.

Bioactive Proteins with Antinutritional Activity in Okara. The quality of soybean proteins is limited by soybean's high content of antinutritional factors including lectins and trypsin inhibitors (TIs). By SDS and PAGE analysis of soybean and okara extractable proteins (Figure 1−4) we registered the following bioactive components: lectin and TIs (Tables 3 and 4). Imbalanced activity of bioactive components with antinutritional effect can cause a disorder of amino acid and vitamin metabolism, the development of pancreatic hypertrophy, adverse changes in the digestive tract, and disturbance of mineral composition in the body. However, contemporary research has shown that if the content of these physiologically active compounds is balanced, they have a favorable effect on human health. They show preventive and therapeutic effects in many diseases, such as diabetes, cardiovascular, bone and kidney diseases, and cancer.[1−3] To improve the nutritional quality of soy foods, inhibitors and lectins are generally inactivated by heat treatment.[43]

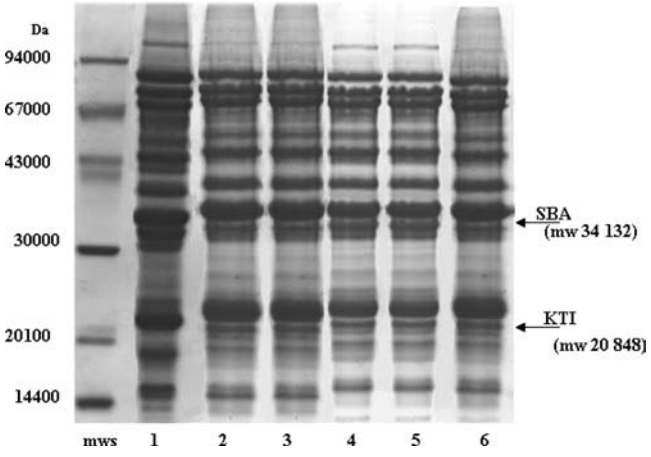

Figure 1. SDS-PAGE analysis of protein composition in soybean from investigated genotypes. Lanes: 1, Lana; 2, Krajina; 3, Novosadjanka; 4, Balkan; 5, Nena; 6, ZPS-15; mws, molecular weight standards.

Trypsin Inhibitor Activity (TIA). Because the TIs are cysteine-rich proteins,[44] heat treatment should aim to preserve their content and to reduce their activity to make them nutritionally valuable. Our results showed that applied thermal/pressure treatment to soybeans was sufficient to get such a result in okara. When compared to soybean (95.61−197.68 TUI/mg), the applied treatment significantly reduced TIA in okara (4.61−14.93 TUI/mg; Table 5). At the same time, TIs content was higher in okara (5.19−14.40%) than in soybean (3.10−12.17%; Tables 3 and 4). Total content of TIs in okara was in significant correlation with total TIs content in soybean (r = 0.97; Table 9). Also, TIA in okara was in significant correlation with TIA in soybean (r = 0.83; Table 9). This suggests that TIA and TIs total contents in okara depend on their initial values in the grain. Furthermore, we have found a strong correlation between the TIA and total TIs content in okara (r = 0.89; Table 8), but this relationship was not significant in soy grain (r = 0.80; Table 10). These results showed that correlation between TIA and TIs contents was set up after heat treatment. These results were in accordance with Pesic et al.,[24] who did not find correlation between TIA and TIs content (r = 0.34; $p < 0.05$) in 12 soybean genotypes. This high content of TIs additionally enriched the investigated okara by cysteine, because our previous results showed the cysteine-rich

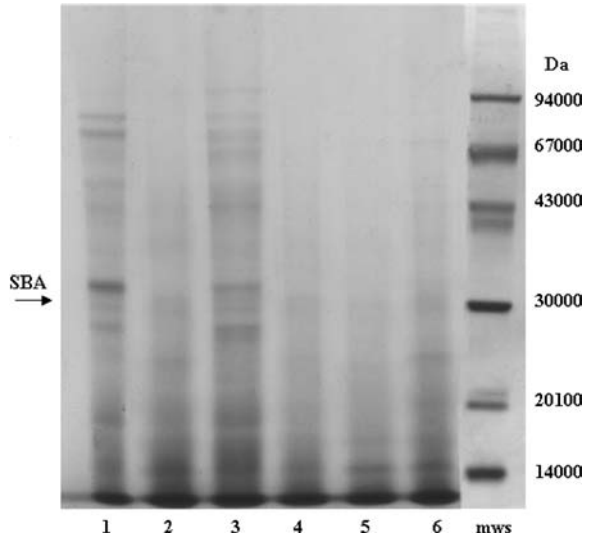

Figure 2. SDS-PAGE analysis of protein composition in okara from investigated genotypes. Lanes: 1, Nena; 2, Krajina; 3, Novosadjanka; 4, Balkan; 5, Lana; 6, ZPS-15; mws, molecular weight standards.

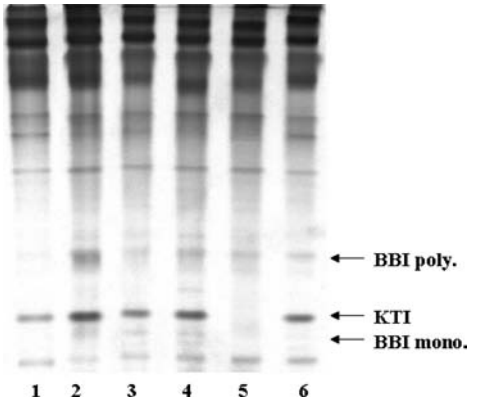

Figure 3. PAGE analysis of protein extracts of soybean from investigated genotypes. Lanes: 1, Balkan; 2, Krajina; 3, Nena; 4, ZPS-15; 5, Lana; 6, Novosadjanka; mws, molecular weight standards.

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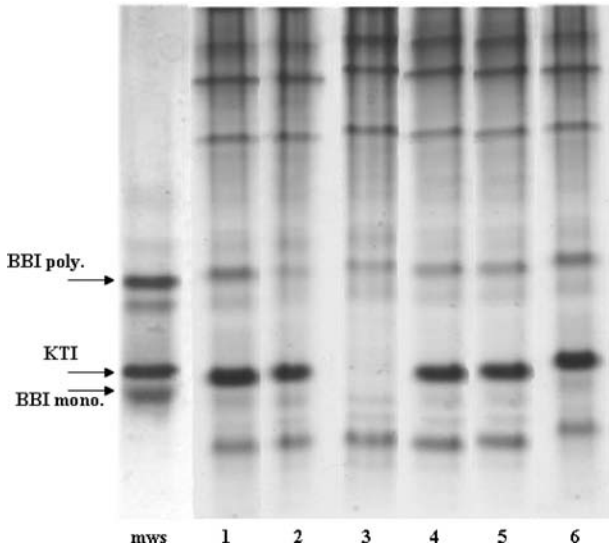

Figure 4. PAGE analysis of protein extracts of okara from investigated genotypes. Lanes: 1, Nena; 2, Balkan; 3, Lana; 4, Krajina; 5, Novosadjanka; 6, ZPS-15; mws, molecular weight standards.

Bg7S globulin as the okara major protein.[11] This is certainly desirable, as it is known that protein products based on soybean are characterized by limited amounts of amino acids containing sulfur. On the other hand, a high content of Bg7S that enriches cysteine in okara may be the cause of this low TIA in okara, in view of the fact that Friedman and Brandon[3] showed that the soy trypsin inhibitors are inactivated more readily by heat in the presence of cysteine or *N*-acetylcysteine. The fact that our results for TIA in okara were much lower than some literature data might also be the consequence of the applied HTC processing. For example, Vishwanathan and others[45] reported significantly higher levels of TIA in okara produced using membrane technology (33.75 TUI/mg).

Such low TIA in okara corresponded (r = 0.87; Table 8) to a very low residual TIA (4.82−7.99% of defatted soy flour activity; Table 5), which suggested that okara obtained by hydrothermal cooking could be applied for human consumption, because it featured <20% of soybean TIA. Friedman and Brandon[3] reported that the most commercially heated meals retain up to 20% of the activity of Bowman−Birk's chymotrypsin and trypsin inhibitor (BBI) and the Kunitz trypsin inhibitor (KTI). The molecule of BBI consists of a single polypeptide chain containing 71 amino acid residues, being one of the smallest proteins found in soybeans with a molecular weight of only 7861.[44] However, the seven disulfide cross-links make the molecule fairly rigid and, consequently, remarkably stable to heat or acid proteolytic digestion. On the other hand, KTI with 181 amino acid residues has more than twice the molecular mass of BBI, but the extent of cross-linking is much smaller because there are only two disulfide bonds in the molecule.[44] Therefore, BBI might be contributing to the residual TIA remaining in heated soybean protein products such as okara. Furthermore, anionic polysaccharides are known to inactivate the activity of soy Kunitz trypsin inhibitor.[46] It is well-known that okara contains a high amount of carbohydrates and even Bg7S is glycosylated.[47] Therefore, on the basis of its chemical structure, we might assume that BBI was responsible for TIA in okara. Looking at the relationship between okara TIA and total content of KTI in okara and in soybean, as well as the relationship between okara TIA and values for KTI/BBI in okara and soybean, we registered the same correlation factor (r = −0.88) for all four relationships (Tables 8 and 9). On the contrary, okara TIA had no significant relationship with BBI content in okara (r = −0.14) and soybean (r = −0.05; Tables 8 and 9). These results suggested that the KTI may be responsible for the TIA remaining in heated soybean protein products such as okara. The reason for these relationships might be that although KTI is more heat labile than BBI, a much higher content of KTI than BBI was found in protein extract of okara (Table 4). We assumed that such a small molecule like BBI had a higher solubility and that therefore a greater quantity of BBI was released in soy milk than retained in okara. However, because of its rigid molecule, stable to heat/pressure, we concluded that BBI and KTI both comprised okara residual TIA.

From the nutritional point of view, in soy protein products it would be desirable that BBI is the carrier of the residual TIA, because it is believed that one of the reasons for BBI's cancer preventive activity is the presence of cysteine in the molecule.[3] We might assume that the high sulfur amino acid content of okara from BBI and Bg7S may act synergistically as cancer preventive agents, which certainly requires more research. Regardless of the results that showed the BBI content in okara to be smaller than KTI content, it should be sufficient to exert anticancerogenic activity, because the animal studies have indicated that dietary BBI concentrations as low as 0.01% suppress carcinogenesis.[48]

Total BBI and Bg7S contents in okara of investigated soybean genotypes were in strong significant correlation (r = 0.96; Table 8), so we could assume that because of the presence of multiple disulfide links in molecules, they reacted in a similar

Table 3. Trypsin Inhibitor Composition of Soybean from Investigated Genotypes[a]

	soybean (% extractable protein)					
		BBI				
genotype	KTI[b]	BBIp[c]	BBIm[d]	BBI[e]	TI[f]	KTI/BBI
Nena	4.32 d	2.27 d	0.91 a	3.18 c	7.50 c	1.36
Krajina	5.77 a	5.77 a	0.63 b	6.40 a	12.17 a	0.90
Novosadjanka	4.56 c	2.09 e	0.11 c	2.20 e	6.76 e	2.07
Balkan	4.24 d	0.54 f	0.53 b	3.07 d	7.31 d	1.38
ZPS-015	5.51 b	3.39 b	0.58 b	3.97 b	9.48 b	1.39
Lana		2.91 c	0.19 c	3.10 cd	3.10 f	

[a]Means in the same column with different letters are significantly different (p < 0.05). [b]KTI, total content of Kunitz-type soybean trypsin inhibitor. [c]BBIp, polymeric forms of BBI. [d]BBIm, monomeric forms of BBI. [e]BBI, total content of Bowman−Birk-type soybean trypsin inhibitor. [f]TI, total content of trypsin inhibitors.

Table 4. Trypsin Inhibitor Composition of Raw Okara from Investigated Genotypes[a]

	okara (% extractable protein)					
		BBI				
genotype	KTI[b]	BBIp[c]	BBIm[d]	BBI[e]	TI[f]	KTI/BBI
Nena	8.78 a	3.81 c	1.81 a	5.62 b	14.40 a	1.56
Krajina	7.33 c	3.51 d	1.65 b	5.16 d	12.49 c	1.42
Novosadjanka	7.73 b	3.07 e	1.23 c	4.30 e	12.03 d	1.80
Balkan	6.85 d	3.58 d	1.83 a	5.41 c	12.26 cd	1.27
ZPS-015	7.81 b	4.21 a	1.63 b	5.84 a	13.65 b	1.34
Lana		3.99 b	1.20 c	5.19 d	5.19 e	

[a]Means in the same column with different letters are significantly different ($p < 0.05$). [b]KTI, total content of Kunitz-type soybean trypsin inhibitor. [c]BBIp, polymeric forms of BBI. [d]BBIm, monomeric form of BBI. [e]BBI, total content of Bowman–Birk-type soybean trypsin inhibitor. [f]TI, total content oftrypsin inhibitors.

Table 5. Trypsin Inhibitor and Urease Activity of Raw Soybean and Raw Okara from Investigated Genotypes[a]

	soybean		okara			
genotype	TUI[b]/mg	ΔpH[c]	TUI[b]/mg	rTIA[d] (%)	ΔpH[c]	UA[e] (%)
Nena	197.68 a	2.28 a	10.69 d	5.41 e	0.04 c	1.75 c
Krajina	181.69 d	2.10 b	14.93 a	7.99 a	0.08 a	3.81 a
Novosadjanka	197.50 a	2.26 a	12.08 c	6.12 c	0.04 c	1.77 c
Balkan	195.36 b	2.22 ab	12.51 b	6.38 b	0.06 b	2.70 b
ZPS-015	184.86 c	2.15 ab	10.23 e	5.53 d	0.03 cd	1.39 d
Lana	95.61 e	1.95 c	4.61 f	4.82 f	0.02 d	1.03 e

[a]Means in the same column with different letters are significantly different ($p < 0.05$). [b]TUI/mg, trypsin inhibitor activity. [c]ΔpH, index of urease activity. [d]rTIA, residual trypsin inhibitor activity. [e]UA, urease activity.

Table 6. Energy Values of Raw Okara[a] (Kilojoules per Gram Fresh Matter)

genotype	total energy[b]	P-energy[c]	F-energy[d]	AC-energy[e]	DF-energy[f]
Nena	3.14 b	1.49 c	0.48 d	1.00 a	0.17 d
Kraijna	2.74 d	1.32 d	0.60 c	0.36 e	0.46 a
Novosadjanka	3.05 c	1.65 a	0.61 c	0.43 d	0.36 b
Balkan	3.28 a	1.45 c	0.76 b	0.96 a	0.11 e
ZPS-015	3.24 a	1.59 b	0.77 b	0.53 c	0.35 b
Lana	2.98 c	1.06 e	0.85 a	0.77 b	0.30 c

[a]Means in the same column with different letters are significantly different ($p < 0.05$). [b]Total energy = sum of energy from proteins, fats, and carbohydrates. [c]P-energy = energy value from proteins. [d]F-energy = energy value from fats. [e]AC-energy = energy value from available carbohydrates. [f]DF energy = energy value from dietary fiber.

Table 7. Correlation Coefficients between Soybeans Characteristics and Okara Properties[a]

relationship	r
okara carb[b] content – okara protein content	−0.76
okara SBA[c] content – okara protein content	0.88[+]
okara SBA content – okara ESP[d]	0.57
okara SBA content – okara ash	−0.96[+]
okara SBA content – soybean ash	−0.95[+]
okara P-energy[e] – protein content in okara	1.00[+]
okara P-energy – okara ESP	0.94[+]
okara F-energy[f] – fat content in okara	1.00[+]
okara AC-energy[g] – AC[h] content in okara	1.00[+]
okara AC-energy – dietary fiber content in okara	−0.95[+]
okara AC-energy – total fiber content in okara	−0.93[+]
okara AC-energy – okara DF energy[i]	−0.95[+]
okara DF-energy – DF content in okara	1.00[+]
okara DF-energy – AC content in okara	−0.95[+]
okara DF-energy – total fiber content in okara	0.98[+]
okara DF-energy – monosaccharide content in okara	−0.85[+]

[a]+, significant at $p < 0.05$. [b]carb, carbohydrate. [c]SBA, lectin. [d]ESP, extractable soluble protein content. [e]Okara P-energy, energy derived from proteins. [f]Okara F-energy, energy derived from fats. [g]Okara AC-energy, energy derived from available carbohydrate. [h]AC, available carbohydrate in okara. [i]Okara DF-energy, energy derived from dietary fiber.

manner to the applied heat/pressure treatment. This strong dependence of BBI and Bg7S contents in okara may be the consequence of the correlations between content of BBI–okara polymeric form with okara Bg7S ($r = 0.86$), whereas BBI monomeric form and Bg7S were in the middle but showed no significant dependence ($r = 0.61$; Table 8). There was no interdependence of these two forms of BBI in okara or in soybeans (Tables 8 and 9), but contents of polymeric ($r = 0.95$) and monomeric forms ($r = 0.89$; Table 9) among soybean and okara were correlated. This indicated that the content of BBI forms in okara depended on soybean genotype. Our results showed significantly higher participation of BBI polymeric than of BBI monomeric form both in okara extractable protein and in soybean for all investigated genotypes (Tables 3 and 4). Unlike BBI monomeric form in okara, polymeric forms of BBI in soybean and in okara were in significant correlation with total content of BBI in soybean and in okara (Tables 8 and 10). On that basis, we could assume that the polymeric forms of BBI in okara might be responsible for the residual activity of this inhibitor.

There was no interdependence between two types of TI in okara (Table 8), but when we compared these results with our previously published data,[3] we found that certain okara protein properties were related to KTI and BBI contents in okara. Okara extractable soluble protein content (ESP = 27.83–32.53%)[11] was in significant dependence with okara total TIs

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Table 8. Correlation Coefficients between Okara Properties[a]

pkara property	TI[b] content	rTIA (%)[c]	KTI[d] content	BBI[e] content	KTI/BBI	BBI poly[f]	BBI mono[g]
TIA (TUI/mg)[h]	0.89[+]	0.87[+]	−0.88[+]	−0.14	0.88[+]		
rTIA (%)[c]			0.86[+]	−0.22			
KTI content				−0.05			
BBIpoly[f]				0.86[+]			
BBImono[g]				0.61		0.21	
Bg7S[i]				0.96[+]		0.86[+]	0.96[+]
UA[j] (ΔpH)[k]		0.96[+]		−0.09	−0.57		

[a+], significant at $p < 0.05$. [b]TI, total trypsin ihhibitor content. [c]rTIA, residual trypsin inhibitor activity. [d]KTI, Kunitz trypsin inhibitor. [e]BBI, Bowman−Birk trypsin inhibitors. [f]BBI poly, polymeric form of Bowman−Birk trypsin inhibitors. [g]BBI mono, monomeric form of Bowman−Birk trypsin inhibitors. [h]TIA (TUI/mg), trypsin inhibitor activity in trypsin units inhibited (TUI) per milligram of the dry sample. [i]Bg7S, basic 7S globulin content. [j]UA, urease activity (%). [k]ΔpH, urease index activity.

Table 9. Correlation Coefficients among Soybean Characteristics with Okara Properties[a]

	okara							
soybean	TI[b] content	rTIA (%)[c]	TIA (TUI/mg)[d]	KTI[e] content	KTI/BBI	BBI[f] content	BBI poly[g]	BBI mono[h]
TI content	0.97[+]		0.83[+]	−0.88[+]		0.33		
TIA (TUI/mg)			0.83[+]					
KTI content			−0.88[+]	1.00[+]				
KTI/BBI			−0.88[+]	1.00[+]				
total protein content			0.78	−0.89[+]	−0.89[+]	−0.33		
Bg7S[i]				−0.82[+]	−0.82[+]	−0.31		
Bg7S-$L_{I,II}$				−0.92[+]	−0.92[+]	−0.15		
BBI content			−0.05			0.89[+]	0.84[+]	0.50
BBI poly							0.95[+]	
BBI mono			−0.05					0.89[+]
UA[j] (ΔpH)[k]		0.14		−0.83[+]	−0.83[+]	−0.09		

[a+], significant at $p < 0.05$. [b]TI, total tripsin ihhibitors content. [c]rTIA, residual trypsin inhibitor activity. [d]TIA (TUI/mg), trypsin inhibitor activity in trypsin units inhibited (TUI) per milligram of the dry sample. [e]KTI, Kunitz trypsin inhibitor. [f]BBI, Bowman−Birk trypsin inhibitors. [g]BBI poly, polymeric form of Bowman−Birk trypsin inhibitors. [h]BBI mono, monomeric form of Bowman−Birk trypsin inhibitors. [i]Bg7S, basic 7S globulin content. [j]UA, urease activity (%). [k]ΔpH, urease index activity.

Table 10. Correlation Coefficients between Soybean Characteristics[a]

soybean parameter	TI[b] content	BBI mono[c]	BBI poly[d]	UA[e] (ΔpH)[f]
TIA (TUI/mg)[g]	0.80			
BBImono			0.41	
rTIA (%)[h]				0.92[+]
KTI[i] content				−0.84[+]
BBI content		0.58	0.95	−0.31
KTI/BBI				−0.84[+]

[a+], significant at $p < 0.05$. [b]TI, total trypsin ihhibitor content. [c]BBI mono, monomeric form of Bowman−Birk trypsin inhibitors. [d]BBI poly, polymeric form of Bowman−Birk trypsin inhibitors. [e]UA, urease activity (%). [f]ΔpH, urease index activity. [g]TIA (TUI/mg), trypsin inhibitor activity in trypsin units inhibited (TUI) per milligram of the dry sample. [h]rTIA, residual trypsin inhibitor activity. [i]KTI, Kunitz trypsin inhibitor.

content ($r = 0.92$), as well as with content of okara KTI ($r = −0.95$, unlike BBI), leading to the same dependence of KTI/BBI ratio in okara ($r = −0.95$; Figure 5). Unlike KTI, BBI content in okara was in strong correlation ($r = 0.85$; Figure 5) with okara protein extractability (protein extractability ranging from 79.29 to 90.45%).[11] This may be a result of the different chemical structures of BBI and KTI molecules.

Okara extractable soluble protein (ESP) content was in strong correlation with content of okara proteinase inhibitors ($r = 0.92$; Figure 5), indicating a significant contribution of TIs to okara ESP. Okara ESP content and soybean KTI content were negatively correlated ($r = −0.95$). Also, okara ESP content was correlated with total soybean TI content ($r = 0.87$), which led to the strong significant dependence with soybean TIA ($r = 0.94$; Figure 5). This might be important for studying the functional characteristics of okara proteins, as it is known that they mainly depend on relationships between nutritional and technological functional properties of soluble proteins.

Furthermore, we noted that unlike okara BBI content, the content of KTI in okara significantly depended on the soybean genotype. We registered strong correlations between KTI content in okara with the following: content of KTI ($r = 1.00$) and TIs in soybean ($r = −0.88$), soybean total proteins ($r = −0.89$), content of Bg7S in soybean ($r = −0.82$), and soybean $L_{I,II}$ subunit of Bg7S ($r = −0.92$; Table 9). None of these relationships were found for okara BBI content (Table 9) with the exception of soybean total BBI content ($r = 0.89$; Table 9). This indicated that the content of KTI in okara significantly depended on genotype characteristics.

Urease Activity (UA and Urease Index Activity, ΔpH). This is an indirect test for the level of trypsin inhibitor activity,[29] and in an adequate heat treatment of soy products ranges of urease index activity (ΔpH) should be between 0.05 and 0.20.[49,50] Table 5 shows the urease index activity, measured as pH difference, and the percentage of urease activity in soybeans and respective samples of okara (UA). Raw soybeans had ΔpH approximately 1.95−2.28, which indicated that raw soybeans are not adequate for human consumption. The ΔpH of okara was around 0.02−0.08, which indicated that okara was

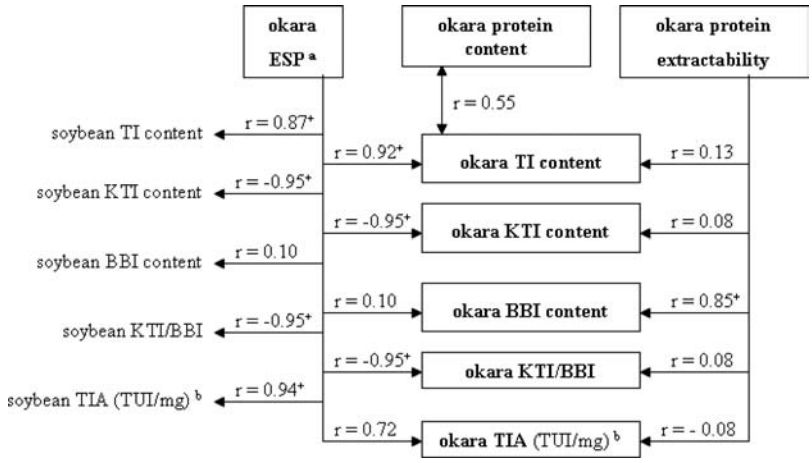

Figure 5. Correlation relationships among okara proteins properties with okara and soybean Bowman–Birk (BBI) and Kunitz (KTI) trypsin inhibitor (TI) characteristics: + significant at $p < 0.05$. [a] ESP, extractable soluble protein content. [b] TIA(TUI/mg), trypsin inhibitor activity in trypsin units inhibited (TUI) per milligram of the dry sample.

heated adequately to inactivate antinutritional factors. Statistical analysis showed the same strong correlation between okara residual TIA and okara ΔpH and UA (r = 0.96; Table 8). Similar relationships for these values were registered for soybeans, too (r = 0.92; Table 10). These results confirmed that heat treatment in an identical way affected the activity of urease and trypsin inhibitors. We found the same relationship between soybean UA (ΔpH) and okara KTI/BBI, as well as with okara KTI content (r = −0.83; Table 9), which indicated significant dependence of these okara parameters on grain characteristics. The lowest urease index found was 0.02 in Lana okara, which also had the lowest okara TIA (4.61 TUI/mg). Lana genotype had the lowest urease index (1.95; Table 5).

Unlike soybean KTI, content of soybean BBI was not correlated with soybean UA (and soybean ΔpH) (Table 10). Also, no correlation was registered in okara after thermal treatment (Table 8). These results indicated that KTI but not BBI was responsible for relationship between TIA and UA in soybean, but neither inhibitor in okara showed such correlation.

Lectin (Hemagglutinin, SBA) Content. We detected very low lectin content in okara ranging from 0.07 to 1.73%. Because we recorded the distribution of lectin from soybean (5.48–7.74%) into soy milk (5.96–8.84%) and finally to okara (0.07–1.73%, Figure 6), we could assume that after the applied thermal process, lectin was mainly transferred and retained in the milk. Such low lectin content in okara increased its nutritional value. It is known that the soy hemagglutinin among

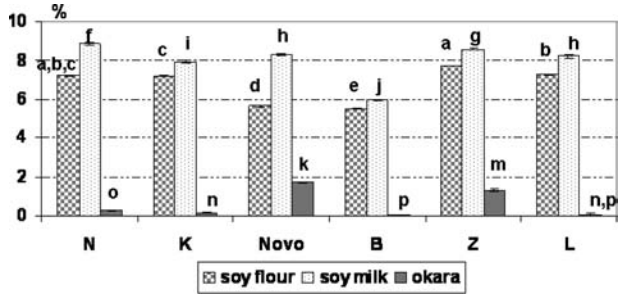

Figure 6. Distribution of lectin content in soy protein products (percent extractable protein). Bars with different letters are significantly different ($p < 0.05$).

other adverse effects interferes with intestinal absorption of nutrients in experimental animals, thus exhibiting growth inhibition.[3,21] For this reason, soybean-derived lectin has traditionally been recognized as having antinutritional properties. We could assume that the okara, under the conditions studied, having such a low lectin content, was characterized by a low level of residual lectin activity. Because the inhibitors are more heat-stable than the lectins, it is believed that achieving a satisfactory level of residual TI activity sufficiently decreases the activity of lectins.[3,43] By statistical analysis of data we got the information that okara with higher content of soluble proteins would have a higher content of inhibitors, whereas okara with higher content of total protein would have a higher content of lectins (Figure 5; Table 7). This could be important for growers and processors to obtain a protein product with a balanced TI or lectin activity.

Energy Values. Soy protein products make a significant contribution to weight reduction, mainly by providing essential high-quality protein in a concentrated form for specially designed, low-calorie/high-nutrient meals. The energy available for metabolism from soy protein products can be estimated by calculating the contributions from the carbohydrate, fat, and protein contents, taking into account the digestibility of each and their heat of combustion. In the literature, there are no data on the energy value of raw (fresh) okara. Data on the energy value of dry okara are available.[18,51,52] Given the fact that the fresh okara can be applied as food without previous drying, we calculated the energy value that comes from each of the components of raw okara, as well as total energy. Samples of okara, obtained by the applied method of production, had a very low energy values, from 2.74 to 3.28 kJ/g (Table 6). A major portion of energy came from protein. Energy derived from fat, from proteins, from available carbohydrates, and from dietary fiber significantly depended on the total content of each of these components in raw okara (r = 1.00; Table 7). Also, we have registered a strong correlation between okara energy values derived from proteins and okara extractable soluble protein content (r = 0.94; Table 7).

In summary, the investigated soybean genotypes all produced okara containing mainly protein and carbohydrates, having favorable contents and activity of bioactive components, such as trypsin inhibitors and lectin. The applied heat treatment (HTC) significantly reduced TIA in okara compared to

soybean, whereas the TI contents were higher in okara than in soybean, thus greatly improving the nutritional value of okara, because the TIs are cysteine-rich proteins. Furthermore, the content of KTI and BBI in okara extractable protein significantly depended on genotypic characteristics of soy seeds. Our present study pointed out a favorable proximate composition of okara, which can be utilized for human consumption, because it features <20% of residual TIA. Under the applied production method, the urease index activity of okara was low and indicated that okara was heated adequately to inactivate antinutritional factors. Okara, obtained by the applied method of production, had low energy values with the major portion of energy from protein. Finally, we summarize that HTC treatment opens the way to a new perspective, especially a more effective use of okara as a plant protein source that could be used in the development of low-energy protein- and fiber-rich foods.

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