CHARACTERIZATION OF ALKALI-MODIFIED SOY PROTEIN CONCENTRATE

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To study the influence of the preparation mode, including mild alkali modification, of soy protein concentrate on soluble protein content and composition, some of its nutritive and functional properties were investigated. Soy protein concentrate prepared by aqueous alcohol leaching was modified in mild alkaline solutions (pH 8.0) at 40, 50 and 60° C for 60 minutes and compared with two principal types of commercial soy protein concentrate. Soluble protein content, composition and properties of soy protein concentrate, as well as their potential use are essentially determined by the preparation mode. Limited mild alkali hydrolysis increased protein solubility by 40-71%, while emulsion stability was increased by 18-56%. Major storage soybean proteins exhibited different stability to alcohol denaturation and mild alkali modification. The most susceptible were acidic -A_3- and -A_5- subunits of glycinin.

KEYWORDS: Hydrolysis; soy protein concentrate; solubility; subunits composition

INTRODUCTION

Soybeans are an abundant source of proteins that have long been recognized for high nutritional value and excellent functional properties in food systems (1). Defated soy flour, soy protein concentrate and soy protein isolate are three major soy protein products. Soy protein concentrate is manufactured by selectively removing the soluble carbohydrates from soy protein flour by either aqueous alcohol (referred to as traditional concentrate) or isoelectric leaching, and it contains more than 65% proteins. Nutritionally, the soy protein concentrate maintains the same excellent nutritional profile of soy flour (2). However, the most widely used methods based on aqueous alcohol washing denature and insolubilize

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proteins, rendering them poorly functional in food (3). To improve their solubility as well as other properties, traditional concentrate can be modified.

The physicochemical and functional properties of soy proteins could be changed by physical, chemical and enzymatic treatment. These treatments include heating, pH adjustment, hydrolysis and covalent attachment of other constituents (4). Thermal treatment has been recognized as a way of soy protein modification. Nakai and Li-Chen (5) reviewed the effect of heating on soy protein functional properties. For this purpose, Wang and Johnson (6) suggested the use of steam-infusion treatment known as steam-infusion method.

In the recent, literature numerous authors have discussed the influence of different enzymatic methods on soybean proteins. Most of these studies were conducted on pure major soybean proteins or on soy protein isolates. Kamata et al. (7) and Shutov et al. (8) investigated the influence of limited proteolysis on pure β-conglycinin and glycinin. Petrucelli and Anon (9), Wu et al. (10) and Anon and Ortiz (11) analyzed products, possible mechanisms and solubility of protease modified soy protein isolates.

Traditional concentrates have high nutritive value and low costs. Better solubility will make them very useful as a replacement ingredient for more expensive isolates and dairy proteins in many food systems. The method based on solubilizing at slightly alkaline pH and increased temperature is one of the possible ways that is used for this purpose. However, the effects of mild alkali modification on dominant concentrate proteins have not been reported yet. The main aim of this work was to study the effect of preparing mode, including mild alkali modification of traditional concentrate on soluble protein content and composition and some nutritive and functional properties.

EXPERIMENTAL

Concentrate preparation. Soybean flour (Glycine max var. Hodgson) was prepared from dehulled meal by defating with *n*-hexane, desolventizing by drying at room temperature. To prepare traditional concentrate nonprotein components were extracted by stirring the flour with 65% aqueous alcohol solution (flour: aqueous alcohol ratio wt/V, 1:10) for 90 minutes at 40°C. Concentrate was separated on glass filter at reduced pressure, washed with the same aqueous alcohol solution (1:5 wt/volume), dried over night at 40°C and ground. Functional concentrate was prepared from defeated flour by extracting with dilute acid (HCl) at pH 4.5 and 30°C for 90 minutes (flour: dilute acid ratio 1:10 wt/V). Concentrate was separated on glass filter at reduced pressure, washed with the same acid solution. Resulting slurry was neutralized with 1M NaOH and lyophilized.

Modified concentrate preparation. Amounts of 5.00g of traditional concentrate were dispersed in deionized water (concentrate: water ratio 1:15 wt/V) and stirred for 10 min to obtain uniform dispersions. Dispersions were then adjusted to pH 8.0 with 1M NaOH and stirred by magnetic stirrer for 60 minutes at 40, 50 and 60°C. Each portion was adjusted to pH 7.0, freeze-dried, ground, sealed in glass bottles and stored at 4°C until analyzed. Alkali modified samples were prepared in triplicate.

To determine protein solubility, samples were extracted according to Than and Shibasaki (12). Soy flour and concentrates were extracted for 120 min at room temperature and centrifuged at 13500 x g for 15 min. Soluble protein content was determined by the method of Lowry et al. (13), using bovine serum albumin (Sigma, USA) as a standard. Total protein content was determined in 0.1 g samples by the micro-Kjeldahl method (14).

Protein solubility was calculated as:

$$\text{Solubility (\%)} = \text{soluble protein content/total protein content} \times 100$$

The change of soluble protein composition was determined by polyacrylamide gel electrophoresis (PAGE), while the change of soluble subunits composition was determined by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric analysis of destained gels. Soluble proteins extracted with 0.03 M Tris-HCl buffer containing 0.01M 2-mercaptoethanol (pH 8.0) were diluted to 2 mg/ml with sample buffer pH 8.0. PAGE was performed according to Davis (15) on 14.5 x 15.5 x 0.15-cm slab gel. Stacking gel was 5% and running gel was 7%. Samples (0.025 ml) were run at 30 mA per gel for 7 h. The gel was stained with 0.1 % Coomassie Blue R-25 for 45 min, and destained in 7 % methanol-acetic acid. SDS-PAGE was performed according to Fling and Gregerson (16) on the same size slab gel. Stacking gel was 5%, and running gel was 12.5 % acrylamide. Prior to electrophoresis samples were diluted to 2 mg/ml with sample buffer (pH 6.8). Samples (0.025 ml) were run at 30 mA per gel for 6 h, stained with 0.23 % Coomassie Blue R-250 solution which contained 3.9 % TCA, for 45 min and destained in ethanol - acetic acid solution (18% and 8% respectively). For both methods, electrophoresis unit LKB-2001-100 was used in conjunction with power supply LKB-Macrodrive 5 and LKB-Multitemp as a cooling unit (LKB, Sweden). Destained gels were scanned using Scanexpress 12000SP (Mustek, Germany). Densitometric analysis of the scanned gels was performed using SigmaGel for Windows software (Jandal Sci. Co, USA). Protein subunits were identified by the use of 7S and 11S fraction separated according to Than and Shibasaki (12), and low molecular weight calibration kit (Pharmacia, Sweden). Molecular weight markers included: phosphorylase b (94 kDa), albumin (64kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-laktalbumin (14.4 kDa).

Emulsion stability index (ESI) was measured by the method of Pearce and Kinsella (17). Pure corn oil (15 ml) and 45 ml of 0.1% protein solution (pH 7.0) were homogenized in a mechanical homogenizer at a highest setting for 1 min. Fifty-microliter portions of the emulsion were pipetted from the bottom of the container at 0 and 10 min after homogenization. Each portion was diluted with 5 ml of 0.1 % SDS solution. Absorbances of these diluted emulsions were measured at 500 nm (Unicam, Great Britain). Absorbances measured immediately (A_0) and 10-min (A_{10}) after emulsion formation were used to calculate emulsion stability index:

$$\text{ESI (min)} = A_0 \times \Delta t / \Delta A$$

where $\Delta t = 10$ min and $\Delta A = A_0 - A_{10}$.

Trypsin inhibitor (TI) activity was assayed and quantified as described by Liu and Markakis (18) using α-N-benzoyl-DL-arginine-p-nitroanilide hydrochloride as a substrate for bovine trypsin (Sigma, USA). Samples were extracted with deionized water for 30 min (sample/water ratio 1:100 wt/vol.). Ten cm^3 of suspension were diluted with 0.05 mol/dm^3 Tris-HCl buffer pH 8.0 (suspension/buffer ratio 1:1vol/vol) and filtered. Diluted extracts were incubated with substrate and trypsin solution for 10 min at 37°C and then stopped with 30% acetic acid. Absorbance was measured at 410 nm (Unicam, Great Britain) from three replicates. Residual activity was expressed in trypsin units inhibited (TUI) per g of dry sample. Determinations were conducted in triplicate.

Phytic acid content was measured according to supernatant difference method of Erdman and Thompson (19), modified by Velickovic et al. (20). The content of phytic acid was calculated by multiplying the amount of phytic acid phosphorus with the corresponding conversion factor (3.55).

RESULTS AND DISCUSSION

Protein solubility was measured in Tris-HCl (pH 8.0) extract of concentrate and hydrolysates. The buffer was selected to identify the components that may be participating as the soluble fraction. The way of nonprotein components extracting determines essentially soy protein concentrate solubility. The aqueous-alcohol leaching reduced protein solubility to 33 % (Figure 1A). Protein insolubilization during isoelectric leaching was minor. Therefore, no significant difference (p<0.05) between soy flour and isoelectric functional concentrate were observed. Mild alkali hydrolysis significantly improved protein solubi-
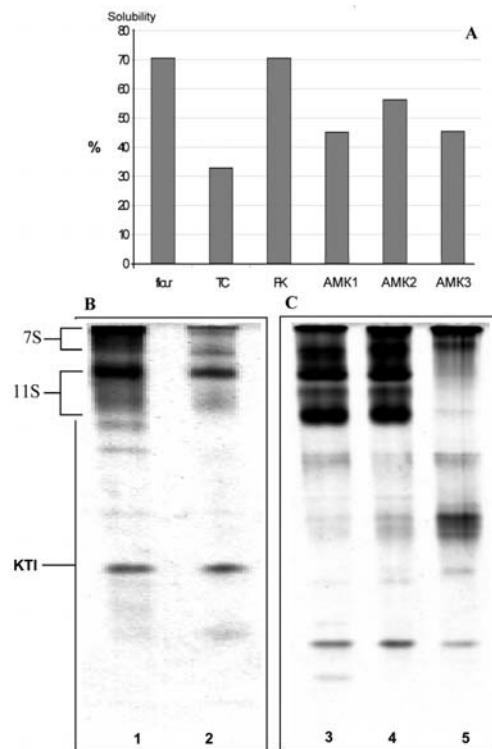

Fig. 1. The effect of mild alkali modification on traditional concentrate solubility and soluble protein composition. **A**. The change of traditional concentrate solubility. TC-traditional concentrate, AMK1, AMK2 and AMK3- samples modified at 40, 50 and 60 ˚C, respectively. **B**. PAGE patterns of soluble flour proteins (1), traditional concentrate (2). **C**. PAGE patterns of samples modified at 40°C (3), 50°C (4) and 60°C (5)

lity of traditional concentrate. Due to the hydrolysis, the solubility has increased to 45-56%.

The soluble protein composition was analyzed by PAGE (Figure 1B). Soluble soybean flour proteins were separated into multiple components. The pattern was similar to that obtained by Than and Shibasaki (12). Band with highest relative mobility corresponded to Kunitz inhibitor, while glycinin existed in two bands (monomer and dimer form) and 7S fraction as a diffuse band. According to PAGE results, the low solubility of traditional concentrate is the result of high insolulubilization of 11 S and 7S fractions. Mild alkali hydrolysis at lower temperatures (40, 50°C) promoted degradation of insoluble protein aggregates and their reassociation into soluble products. Thus, the zones of 7S and 11 S fractions were characterized with two and three protein bands, respectively (Figure 1C, line 3 and 4). Higher temperature (60°C) caused further denaturation, especially of 11S fraction. As a result, three dominant fractions with higher relative mobility were observed (Figure 1C, line 5).

The SDS patterns of soy flour proteins (Figure 2A, line 3) contained five major bands, three of which (mw 81 kDa, 74 kDa, 49 kDa) were identified as subunits of β-conglycinin, while the other two (mw. 36.3 kDa, 25.5 kDa) were identified as glycinin subunits. The minor bands of 39.7, 36.3, 32.125.5 kDa were identified as glycinin subunits, and the 93.9 kDa-band as lipoxygenase. Soy flour protein subunits exhibited different susceptibility to alcohol denaturation.

After treatment with alcohol (Figure 2B, line 6), the band of lipoxygenase in traditional concentrate sample disappeared, completely. New polypeptide species of 41.5 kDa (indicated in Figure 2B with arrow) as end product of alcohol denaturation was detected. Also, due to partial degradation of high molecular weight polypeptides such as polypeptides of lipoxygenase, β- and γ- conglycinin as well as β-amilase polypeptides, more intensive band of 57.1 kDa was registered. Subunits of glycinin were more susceptible than those of β-conglycinin, as evidenced by the diffuse SDS-patterns, and reduced relative contents. Furthermore, acidic subunits were denatured to a greater extent, especially $-A_3$- and $-A_5$- acidic subunits. Their content was reduced to 1.95 and 4.17% respectively (Figure 3B). Soluble basic subunits were observed as diffuse bands, which represented 19 % of soluble subunits (Figure 3A). These results suggest that alcohol denaturation proceeds initially on the acidic subunits and later on basic subunits of glycinin. This is in agreement with the localization of the basic subunits in the interior of the molecule, suggested by Marcone et al. (21).

The subunits content of β-conglycinin was at the same level as in the soy flour extracts (Figure 3A)., This was partially due to the loss of minor polypeptides, but these results also indicated their higher resistance to the alcohol denaturation.

The SDS-pattern of alkali modified concentrates (Figure 2 B, line 4, 5, 7) showed that the method used increased the solubility of the both major protein subunits. The temperature of hydrolysis determined polypeptide composition of soluble protein extracts. At lower temperature (40, 50°C), the increase of all dominant subunits has been observed, particularly of those registered as alcohol susceptible, such as acidic $-A_3$-, and $-A_5$-and basic $-B_{1.2.3.4}$- subunits. The contents of $-A_3$- and $-A_5$- subunits were 6.1-7.4% and 5.6- 6.9%. Also, in the extracts of the samples treated at 40°C (Figure 2B, line 5) the band of 92.7 KDa was detected, which may be characterized as partially degraded polypeptides of lipoxygenase. At higher temperatures, this band disappeared. These results, which are in agreement with PAGE results, suggest that lower temperatures promote dissociation of insoluble aggregates, without significant denaturation of the major subunits.
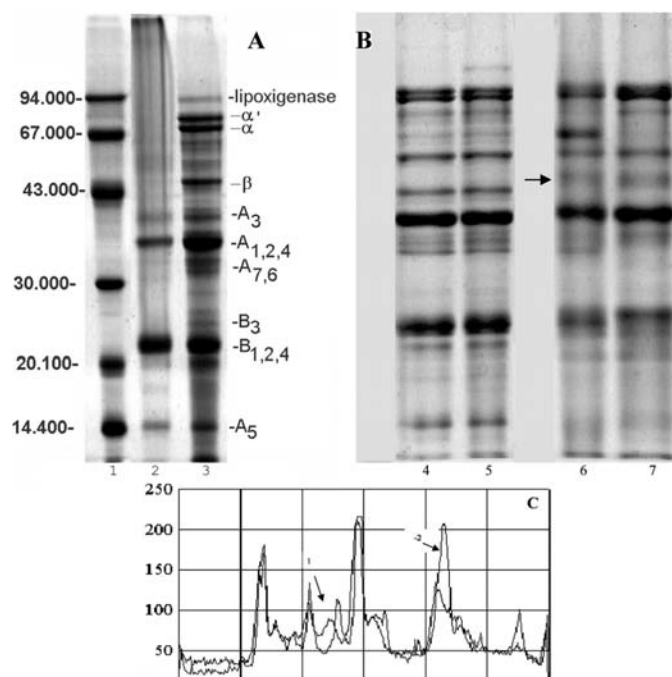
Fig. 2. SDS-PAGE analysis of soy flour, traditional and modified concentrates. **A**. Analysis of soy flour, line 1- molecular weight (Da) markers, line 2-11S fraction, line 3 – soy flour. **B**. Analysis of traditional protein concentrate (line 6), and alkali-modified samples at 40°C (line 5), 50°C (line 4) and 60°C (line 7). **C**. Comparative densitometric analysis of samplesmodified at 40°C (2) and 60°C (1)

At higher temperature (60°C) subunits of glycinin were more denatured. A_3- and A_7- subunits disappeared completely, while the other acidic subunits content decreased. At the some time, basic subunits were registered as diffuse zones in the range of 26.0 - 22.0 KDa that contained three bands. Due to the decrease of the acid subunits content, the ratio of glycinin and β-conglycinin subunits changed from 2.95: 1 (samples treated at 50°C) to 1.78 : 1.

Mild alkali modifications yielded significant (p<0.05) improvement of emulsion stability. Depending on temperature, ESI of traditional concentrate increased for 17.8-56.1% (Figure 4). The ESI of samples treated at 60°C and functional concentrates prepared by isoelectric leaching were at the same level.

The level of inhibitor activity is determined by the way of nonprotein components extraction (Table 1). Residual inhibitor activity registered in traditional concentrate samples (17.3%) has no antinutritive effect. Therefore, intensive band of soluble KTI registered on PAGE pattern (Figure 1B, line 2) and low residual activity suggest that most of these components exist in partially disrupted native conformation and inactive form. The presence of inactive forms of inhibitors is important because of their amino acids composition and potential health benefits. Further decrease of inhibitor activity during mild alkali modification (15.5-14.8%) has no practical significance.
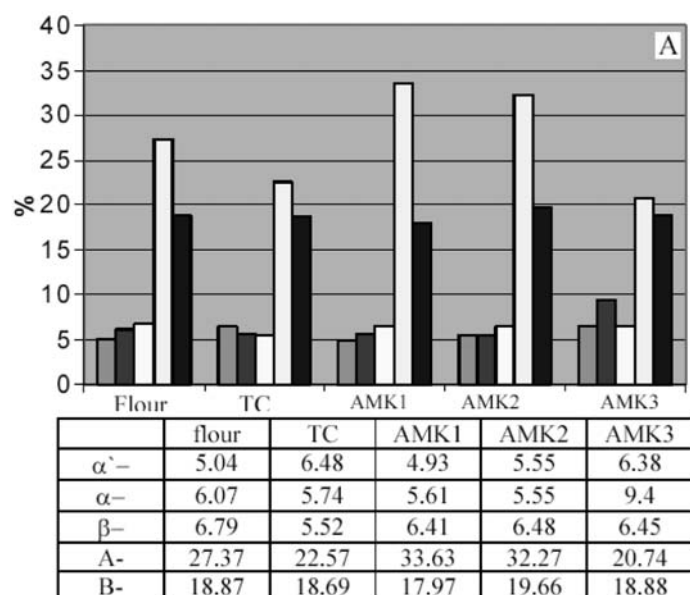
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	flour	TC	AMK1	AMK2	AMK3
α`−	5.04	6.48	4.93	5.55	6.38
α−	6.07	5.74	5.61	5.55	9.4
β−	6.79	5.52	6.41	6.48	6.45
A-	27.37	22.57	33.63	32.27	20.74
B-	18.87	18.69	17.97	19.66	18.88

Fig. 3 A. The change of polypeptide composition (%) of dominant concentrate proteins*.
A. subunits content of β-conglycinin (α`-,α-, β-) and glycinin (A-acid and B-basic) subunits
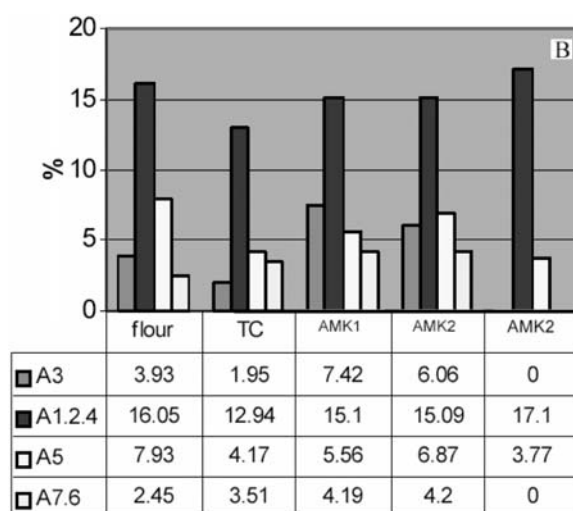*Means of two densitometric measurements of soluble protein extracts by SDS-PAGE

	flour	TC	AMK1	AMK2	AMK2
■ A3	3.93	1.95	7.42	6.06	0
■ A1.2.4	16.05	12.94	15.1	15.09	17.1
□ A5	7.93	4.17	5.56	6.87	3.77
□ A7.6	2.45	3.51	4.19	4.2	0

Fig. 3 B. The change of polypeptide composition (%) of dominant concentrate proteins*
B. Soluble acid subunits content of glycinin. TC-traditional concentrate. AMK1, AMK2,
AMK3 – alkali modified samples treated at 40, 50, and 60°C, respectively
*Means of two densitometric measurements of soluble protein extracts by SDS-PAGE

The methods of nonprotein components leaching reduced also phytic acid content. No significant differences between traditional and functional concentrate were observed (Table 1). Mild alkali modification at lower temperatures (40, 50°C) reduced phytic acid content to 3.89±0.03% – 3.90±0.05%. Samples treated at higher temperature (60°C) contained 4.61 ±0.20% phytic acids. No significant differences between traditional concen-

Table 1. Trypsin inhibitor (TI) acivity and phytic acid content of traditional (TC), functional (FC) and alkali-modified (AMK1, AMK2, AMK3) traditional concentrates*

Sample	Residual TI-activity		Phytic acid** (%)
	TUI/g**	%	
Row flour	187±0.71	-	4.10±0.07
TC	31.57±0.80	17.34	4.95±0.03
FC	135.62±0.08	76.01	4.81±0.05
AMC1	29.61±0.86	15.51	3.89±0.03
AMC2	27.23±0.77	15.34	3.90±0.05
AMC3	25.78±0.52	14.78	4.61±0.02

TC-traditional concentrate, FC- functional concentrate, AMK1, AMK2, AMK3-alkali modified concentrates at 40°C, 50°C and 60°C, respectively
* The present value are means ± standard deviation from three replicates except for the % of residual activity. These values represent the % of initial inhibitor activity of soy flour.
** The letter a denoting significant difference at 5% vs. traditional concentrates.
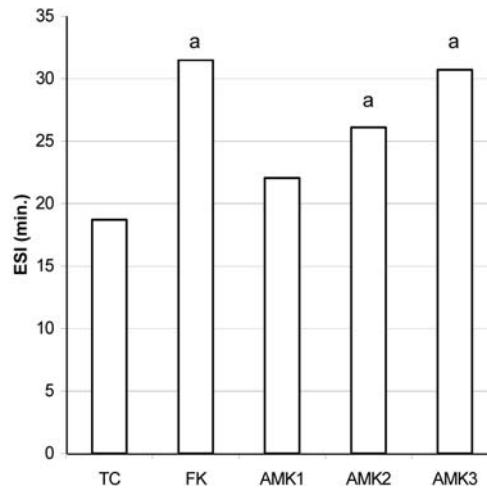

Fig. 4. The effect of mild alkali modification on emulsion stability of traditional concentrates*
*Means of three meassurements from three replicates, the letter a denoting significant difference at 5% vs. traditional concentrates. TC- traditional concentrate, FC- functional concentrate. AMK1, AMK2, AMK3 - alkali modified concentrates at 40°C, 50°C and 60°C, respectively

trates and these samples were observed. These results for the samples treated at 60°C indicate that the higher degree of denaturation probably promotes interactions between phytic acid and denatured proteins. In traditional nutritional theory, phytic acid has been considered as a nutritient. The later investigations also showed that phytic acid might have beneficial nutritional effects (22,23). Thus, the higher content of phytic acid in soy protein products is desirable.

CONCLUSION

In the conclusion, modification at slightly alkaline pH and increased temperature is a simple method to improve solubility of traditional soy protein concentrate. Major storage soy protein fraction showed different stability towards alcohol denaturation, as well as to the proposed method of modification. Because of higher solubility and emulsion stability, alkali-modified concentrates could find their use in products that require these properties.

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КАРАКТЕРИЗАЦИЈА АЛКАЛНО-МОДИФИКОВАНИХ СОЈИНИХ ПРОТЕИНСКИХ КОНЦЕНТРАТА

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У оквиру овог рада испитиван је ефекат начина припреме, укључујући и ефекат алкалног модификовања на садржај и састав растворљивих протеина и неке функционалне карактеристике традиционалног концентрата протеина соје. Традиционални

протеински концентрат (добијен испирањем непротеинских компоненти 65%-ним етанолом) модификован је у алкалној средини (pH 8,0) при температури од 40, 50 и 60°C у току 60 минута. Садржај, састав и функционалне особине, а тиме и могућа примена сојиног протеинског концентрата одређени су начином његове припреме. Алкалним модификовањем садржај растворљивих протеина традиционалног концентрата се повећава за 40-70%, док је стабилност емулзије образоване овим узорцима повећана за 17,8-56,1%. Доминантни протеини соје испољили су различиту осетљивост при алкохолној екстракцији и алкалном модификовању.

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