Changes in the isoflavone profile and in the chemical composition of tempeh during processing and refrigeration

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Abstract – The objective of this work was to analyze changes in the isoflavone profile, determined by high performance liquid chromatography, at different processing stages and after refrigeration of tempeh. For tempeh production, clean soybean grains from cultivars BR 36 (low isoflavone content) and IAS 5 (high) were dehulled, and the separated cotyledons were hydrated and then cooked in boiling water for 30 min. Spores of the fungus *Rhizopus microsporus* var. *oligosporus* were inoculated in the cooked and cooled cotyledons, and incubated at 32ºC for 6, 12, 18, and 24 hours in perforated polypropylene bags, for fermentation. The resulting tempeh was stored at 4ºC for 6, 12, 18, and 24 hours. After 24-hour fermentation, isoflavone glucosides were 50% reduced, and the aglycone forms in the tempeh from both cultivars was increased. The malonyl forms reduced 83% after cooking. Less than 24 hours of refrigeration did not affect the isoflavone profile of tempeh from either cultivar, which is a good indicator of its quality. The tempeh maintains the high and low isoflavone content of the cultivars, which indicates that cultivar differences in this trait should be considered when processing tempeh.

Index terms: *Rhizopus microsporus* var. *oligosporus*, aglycone, fermentation, functional food, high performance liquid chromatography, malonyl.

Mudanças no perfil de isoflavonas e na composição química de 'tempeh' durante o processamento e a refrigeração

Resumo – O objetivo deste trabalho foi analisar mudanças no perfil de isoflavonas, determinado por meio de cromatografia líquida de alta eficiência, em diferentes estágios do processamento e após refrigeração de 'tempeh'. Para a produção do 'tempeh', grãos de soja limpos das cultivares BR 36 (baixo teor de isoflavonas) e IAS 5 (alto teor) foram descascados, e os cotilédones foram hidratados e, em seguida, cozidos por 30 min em água fervente. Em seguida, esporos do fungo *Rhizopus microsporus* var. *oligosporus* foram inoculados nos cotilédones cozidos e resfriados, e incubados a 32ºC por 6, 12, 18 e 24 horas em sacos perfurados de polipropileno, para fermentação. O 'tempeh' resultante foi refrigerado por 6, 12, 18 e 24 horas. Após 24 horas de fermentação, os teores de glicosídeos de isoflavonas reduziram-se em 50%, e os das formas agliconas aumentaram, em ambas as cultivares. Após o cozimento, as formas malonil reduziram-se em 83%. A refrigeração por menos de 24 horas não afetou o perfil de isoflavonas no 'tempeh' das duas cultivares, o que é um bom indicador de sua qualidade. O 'tempeh' mantém os altos e baixos conteúdos de isoflavonas das cultivares, o que indica que as diferenças entre cultivares, para esta característica, devem ser consideradas durante o processamento de 'tempeh'.

Termos para indexação: *Rhizopus microsporus* var. *oligosporus*, aglicona, fermentação, alimento funcional, cromatografia líquida de alta eficiência, malonil.

Introduction

The chemical composition of soybean cultivars varies depending on genetics and environmental conditions (Liu, 1997), but the average composition is generally 40% proteins, 21% lipids and 34% carbohydrates. Silva et al. (2009) worked with grain-type and food-type soybean cultivars, which ranged from 40.1 to 44.5% of protein, from 18.1 to 20% of lipids and from 30.6 to 34.4% of carbohydrates. Moraes et al. (2006) observed greater variations for protein content (40.7 to 47.8%), and that an increase in protein was accompanied by a corresponding reduction in oil (16.7 to 20.8%); these authors also reported a carbohydrate content between 30 and 33.6%. The availability of soybean cultivars with high protein content should improve the nutritional value of soybean products (Liu, 1997).

Isoflavones are very important biologically active substances contained in soybeans. They are diphenolic compounds with antioxidant and antifungal activity (Liggins et al., 2000; Brouns, 2002), and recognized for their benefits to human health, reducing menopause symptoms and lowering the risk of chronic diseases, such as cardiovascular ones, osteoporosis, and breast, prostate and colon cancer (Setchell et al., 2001; Kris-Etherton et al., 2002; Jefferson, 2003; Delmonte et al., 2006). Among the different forms of isoflavones, aglycones are especially important because they are readily bioavailable to humans.

To be converted into aglycone forms – daidzein, genistein and glycitein – the isoflavone glycosides require an initial hydrolysis by ß-glycosidase enzymes produced by intestinal microflora of the human body (Liggins et al., 1998; Setchell et al., 2001). Fermentation is a processing technique in which the hydrolysis of glycosides can occur, as a result of microbial activities, forming aglycones (Nakajima et al., 2005; Haron et al., 2009).

Tempeh is a traditional fermented soybean food product from Indonesia. It is normally consumed fried, boiled, steamed or roasted. Processing soybeans into tempeh by fermenting with the fungus *Rhizopus microsporus* var. *oligosporus* (Saito) (Schipper & Stalpers, 1984) improves the texture, flavor and aroma of the product. Tempeh has a soft texture due to breaking down, by the fungus, of the intercellular matrix between plant cells. A mild mushroom-like aroma is evident in tempeh as a result of the action of the fungal mycelium on protein and lipids of the soybeans (Hermana & Karyadi, 2001). The fermentation process improves the nutritional value of tempeh by increasing the availability of isoflavone aglycones. These aglycones exist in smaller amounts in other nonfermented soy products such as tofu and soymilk (Wang & Murphy, 1994; Astuti & Dalais, 2000).

Carrão-Panizzi et al. (1998) studied the influence of the environment on the growth of Brazilian soybean cultivars and concluded that differences in the isoflavone content, in different sowing locations, allows the selection of locations for the production of soybean with low or high isoflavone contents.

The objective of this work was to analyze changes in the isoflavone profile, determined by high performance liquid chromatography, at different processing stages and after refrigeration of tempeh.

Materials and Methods

The soybean cultivars BR 36 and IAS 5 have low (53.7 mg per 100 g) and high (136.4 mg per 100 g) isoflavone contents, respectively (Carrão-Panizzi et al., 1998). Grains of these cultivars were supplied by Embrapa Soja, located in Londrina, PR, Brazil. The chemical composition and the isoflavone profile analyses were performed on dehulled soybean grains (cotyledons) and on fresh tempeh from both cultivars.

The tempeh was prepared according to traditional methods (Wei, 1991). Clean soybean grains were dehulled, and the separated cotyledons were cooked in boiling water for 10 min. After that, the water was drained and the cotyledons were soaked in tap water for 17 hours at room temperature. The water was discarded, and the hydrated cotyledons were cooked in boiling water for 30 min. After cooking, the water was drained, and the cooked cotyledons were cooled at room temperature. Then, spores of the fungus *Rhizopus microsporus* var. *oligosporus* were inoculated in the cooked and cooled cotyledons, which were packed in perforated polypropylene bags and incubated at 32ºC for 24 hours. The resulting tempeh was stored at 4°C for 24 hours.

Freeze-dried *Rhizopus microsporus* var. *oligosporus* inoculum was acquired from Intsoy (International Soybean Program, University of Illinois, USA). To multiply spores of *R. microsporus*, the inoculum was suspended in a saline solution (0.85%), inoculated in Petri dishes containing potato dextrose agar (BDA), and incubated at 32°C for 48 hours. The resulting spores were inoculated in Petri dishes containing 60 g of raw ground rice, previously autoclaved for 15 min at 121°C, and 40 mL of distilled water, and incubated at 32°C for 72 hours. After the development of spores, the Petri dishes were opened and dried in a ventilated oven (50°C for 24 hours). After drying, the contents of the Petri dishes were ground in a DCG-20 grinder (Cuisinart Coffee Grinder, East Windsor, NJ, USA) and stored in glass bottles.

The moisture, protein, lipid, ash and carbohydrate contents were determined as described by Normas Analíticas do Instituto Adolfo Lutz (2008), with three replicates, and the results were expressed in percentage of dry basis.

Assessments of isoflavone profiles were made in cotyledons and in tempeh. As for tempeh, the assessments were made in the following processing

stages: after the 10-min cooking, in the hydration phase; after the 30-min cooking; after 6, 12, 18, and 24 hours of fermentation; and after 6, 12, 18, and 24 hours of refrigeration.

A high performance liquid chromatography system (HPLC) model 2690 (Waters, Milford, MA, USA) was used to analyze the isoflavones (Kitamura et al., 1991; Kudou et al., 1991). The separation was carried out in an ODS C18 reverse column – YMC-Pack ODS-AM, S-5 mm, 120 A, 250 mm long x 4.6 mm diameter (YMC America Inc., Allentown, PA, USA) –, and a photodiode array detector model 996 (Waters, Milford, MA, USA) was used to detect the isoflavones at a wavelength of 260 nm.

In order to extract the isoflavones, 100 mg of ground samples were transferred to test tubes with lids. Four milliliters of 70% ethanol containing 0.1% of acetic acid were added to each test tube. The tubes were agitated every 15 min during the 4-hour extraction period. In an Eppendorf microcentrifuge, model 5417 R (Eppendorf, Hamburg, Germany), 1.5 mL of the obtained extract was centrifuged for 10 min at 20,000 *g*. Then, 80 μL of the supernatant was pipetted into the tubes of the chromatograph's automatic injector. These tubes were covered and sealed. For separation and quantification of the isoflavones, 10 μL aliquots were injected (with automatic injectors) into the chromatograph.

The separation was carried out in the before mentioned ODS C18 type reverse column, and the also mentioned photodiode array detector was used to detect the isoflavones at a 260 nm wavelength. A linear gradient system was used for separation. The mobile phase consisted of 0.1% acetic acid and acetonitrile. Initially, the system had 20% acetonitrile and 80% acetic solution. At the end of the first 20 min, the mobile phase composition in the system was 50% of acetonitrile and 50% of acetic acid, reaching 100% of acetonitrile shortly thereafter. This concentration was maintained for 5 min and reduced to the initial concentration (20% acetonitrile and 80% acetic acid) for the final 15 min. This completed the 40 min required for the separation of the isoflavones. The mobile phase flow was 1.0 mL per minute.

The standard solutions were a mixture of daidzin, genistin, daidzein and genistein prepared with a solution of 70% ethanol containing 0.1% acetic acid at different concentrations: 0.00625, 0.0125, 0.0250, 0.0500, and 0.1000 mg mL^{-1}, for each component of the mixture. The isoflavones were separated, quantified according to standard references, and expressed in mg per 100 g for each component, on a dry basis. The concentrations of malonyl-glycosides and aglycones were calculated from the standard curves of their corresponding β-glycosides, using the similarity of the molar extinction coefficients of malonyl-isoflavones and their β-glycosides (Coward et al., 1998). The values for isoflavones can be presented in aglycone equivalents (Góes-Favoni et al., 2010) or as the sum of the different forms (Yue et al., 2010). In the present work, data are shown as the sum of each compound within each group of isoflavones (glycosides, malonyls and aglycones).

A randomized complete block experimental design was used with factorial arrangement between soybean cultivars (BR 36 and IAS 5) and processing treatments (cotyledons and tempeh), with seven replicates. The data were subjected to the analysis of variance (ANOVA). Following the evaluation, the assumptions of ANOVA were verified and the means compared by Tukey's test, at 5% probability, using the SAS scientific package (SAS Institute, 2002).

Results and Discussion

Soybean moisture content for both cultivars was 10.23% on average (Figure 1). In tempeh produced

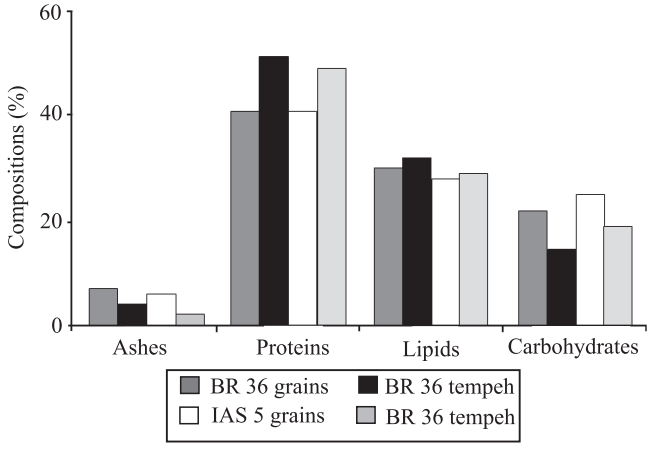

Figure 1. Approximate chemical composition, on dry matter basis, of cotyledons and tempeh obtained from cultivars BR 36 and IAS 5. Coefficient of variation (CV) in grains of 'BR 36' and 'IAS 5' for: ash, 1.36%; protein, 0.86%; lipids, 1.93%; carbohydrates, 3.14%. CV in tempeh of 'BR 36' and 'IAS 5' for: ash, 2.21%; protein, 1.26%; lipids, 1.35%; and carbohydrates, 4.80%.

from both cultivars, the moisture content was 64.61% on average. Both cultivars had 5% average ash content. In tempeh, however, a 50% decrease in ash content was observed due to the processing procedures. Tempeh had 21% higher protein content than the cotyledons. The soaking and cooking during the tempeh processing resulted in the loss of soluble substances, such as sugars and minerals, which may explain the higher protein and the lower ash contents.

Grains from cultivar BR 36 have high (39%) protein content (Mandarino et al., 1992). Tempeh maintained this characteristic and showed an even higher protein content in both products: cotyledons and tempeh. Lipid content was approximately the same for cotyledons of 'BR 36' and 'IAS 5' (29.18% average), while a small increase was observed in tempeh. The carbohydrate content in cotyledons ranged from 21.29% for 'BR 36' to 25.13% for 'IAS 5'. Carbohydrate content decreased 15.21% and 19.53%, respectively, for tempeh made from 'BR 36' and 'IAS 5'. This result was due to the solubilization of sugars during the tempeh processing. Vaidehi et al. (1985) found similar results for the chemical composition of soy tempeh in combination with other oilseeds.

The chemical composition of tempeh in this study was higher than that reported by Haron et al. (2009), who observed, for raw tempeh, 65% average moisture, 17.5% protein, 9.2% lipid, 7.6% carbohydrate and 0.6% ash. The USDA database (United States Department of Agriculture, 2010) reports 59.6% moisture, 18.5% protein, 10.8% lipid, 9.4% carbohydrate and 1.6% ash. Differences observed in the present study may be due to the different methods of analysis or to the expression of experimental data on a dry basis, which concentrates the components.

The total isoflavone content in the cotyledons of 'BR 36' and 'IAS 5' were, on average, 87.17 and 281.85 mg per 100 g, respectively (Table 1). 'BR 36' is a genetically low-isoflavone content genotype, which was also observed in other studies (Carrão-Panizzi et al., 1998). Isoflavone content in different soybean cultivars is affected by genetics, crop year and growth location (Wang & Murphy, 1994; Carrão-Panizzi et al., 2009). The total glycoside forms (daidzin and genistin) in soybean cotyledons decreased in processed tempeh for both cultivars. Malonyl forms, which react unsteadily to heat treatments, were greatly reduced during the preparation of tempeh as a result of cooking. Carrão-Panizzi et al. (2004) showed that malonyl compounds were reduced at higher temperatures and in the processing of functional soybean foods. These authors also reported that hydrothermal treatments of soybean grains and high-isoflavone content soybean cultivars can enhance the development of aglycone forms. The aglycone forms (daidzein and genistein) were found in very small amounts in cotyledons of 'IAS 5', and they were not even detected in 'BR 36'. In general, the aglycone forms are not present

Table 1. Total glycoside, malonyl and aglycone isoflavones (mg per 100 g dry basis) during different steps of tempeh processing[(1)].

Processing stage	'BR 36'	'IAS 5'	Means
		Glycoside isoflavones	
Cotyledons	13.61±0.24	45.79±3.17	29.70±7.33AB
10 min cooking	13.87±0.69	54.03±4.16	33.95±9.17A
Soaking	8.34±0.45	44.23±7.41	26.28±8.68AB
30 min cooking	11.68±0.09	53.05±5.42	32.36±9.56A
6 hour fermentation	9.54±0.29	40.20±3.16	24.87±7.00AB
12 hour fermentation	7.20±0.32	30.21±5.99	18.71±5.80B
18 hour fermentation	4.65±0.15	12.93±0.57	8.79±1.87B
24 hour fermentation	3.65±0.29	14.79±1.50	9.22±2.58B
6 hour refrigeration	6.51±0.29	16.36±0.69	11.43±2.23A
12 hour refrigeration	7.33±0.26	18.5±0.80	12.93±2.53A
18 hour refrigeration	7.85±0.63	16.40±0.22	12.12±1.93A
24 hour refrigeration	7.42±0.36	17.43±0.83	12.43±2.27A
		Malonyl isoflavones	
Cotyledons	73.55±2.41bA	229.21±14.85aA	151.38±A
10 min cooking	53.92±1.22bB	162.37±9.08aB	108.14±B
Soaking	41.06±0.95bB	115.24±8.62aC	78.15±17.03C
30 min cooking	12.46±0.71	37.03±1.61	24.74±5.55AB
6 hour fermentation	9.81±0.10	30.06±1.87	19.94±4.60AB
12 hour fermentation	9.98±0.10	34.28±3.46	22.13±5.65AB
18 hour fermentation	10.51±0.18	33.96±1.87	22.23±5.31AB
24 hour fermentation	10.07±0.20	37.70±1.65	23.88±6.22AB
6 hour refrigeration	11.74±0.61	37.87±2.47	24.01±5.95AB
12 hour refrigeration	10.78±0.92	39.80±2.53	25.29±6.60A
18 hour refrigeration	12.25±0.58	37.26±1.93	24.75±5.66AB
24 hour refrigeration	11.78±0.13	38.45±0.73	25.11±5.97A
		Aglycone isoflavones	
Cotyledons	0.0±0.0	6.83±0.51	3.41±1.54B
10 min cooking	2.28±0.11	8.84±0.28	5.56±1.47A
Soaking	3.57±0.21bB	12.27±1.38aC	7.92±2.04C
30 min cooking	2.46±0.16	8.87±0.19	5.67±1.43A
6 hour fermentation	3.76±5.1	12.43±0.80aB	8.10±2.38A
12 hour fermentation	5.19±0.05bAB	24.19±3.61B	14.69±4.55B
18 hour fermentation	9.29±1.29bAB	30.75±1.89aAB	20.02±4.90AB
24 hour fermentation	11.52±0.81bAB	34.78±1.97AA	23.15±5.29A
6 hour refrigeration	12.71±0.55bA	35.35±2.38aA	24.03±5.18A
12 hour refrigeration	11.98±1.39BA	36.32±2.28aA	24.15±5.63A
18 hour refrigeration	13.16±0.38bA	36.77±2.90aA	24.96±5.44A
24 hour refrigeration	12.70±0.34bA	35.44±1.48aA	24.07±5.13A

[(1)]Means followed by equal letters, uppercase in the columns, and lowercase in the rows, within each group of variables, do not differ by Tukey's test, at 5% probability.

in soybeans which were not exposed to hydrolysis or to fermentation. These processes are necessary to activate the ß-glycosidase enzyme that hydrolyzes the glycosides to form the aglycones. In tempeh, the aglycone content increased from 0 to 11.52 mg per 100 g for 'BR 36', and from 6.83 to 34.78 mg per 100 g for 'IAS 5', as a result of the 24-hour fermentation process.

Aglycones are important compounds due to their bioavailability, which is greater than that of other isoflavone forms. Therefore, fermented tempeh can be a functional food with health benefits for humans. Nakajima et al. (2005) also reported that the amount of aglycones increased with fermentation time, and it effectively doubled after 24-hour fermentation. Haron et al. (2009) reported higher values of aglycone forms in raw tempeh.

Isoflavone glucosides did not change markedly during the first stages of tempeh processing. Even after a 6-hour fermentation, only a small reduction of these compounds was observed. Fermentation time, however, had a significant effect on these compounds, and, in the 12-hour fermentation, there was a glucoside reduction of about 25%, which continued to decrease (50%) in the late stages of fermentation (18 and 24 hours). While refrigerated, the content of glucosides remained constant, and was not affected by different storage times.

Malonyl forms, which are heat labile, decreased continuously during each step of the tempeh processing, mainly due to cooking. Total malonyl in cotyledons of 'BR 36' were 73.55 mg per 100 g, and for 'IAS 5' 229.21 mg per 100 g. After 10-min cooking, the malonyl forms in 'BR 36' were reduced by 73%, and in 'IAS 5' by 70%. An average reduction of 72% was observed after soaking. After 30 min of cooking, malonyl forms had decreased by 83% relatively to the amount in cotyledons. During the fermentation process, malonyl forms did not change and remained stable during refrigeration. Tempeh contains substantially reduced levels of malonyl isoflavones, in contrast to soybeans, of which malonyl is the main isoflavone compound (Kudou et al., 1991).

The aglycone forms, however, strongly changed as a result of the hydrolysis of the isoflavone glucoside by ß-glycosidase enzyme during the fermentation process (Nakajima et al., 2005; Carrão-Panizzi et al., 1999).

No aglycone was observed in the cotyledons of the BR 36 cultivar. In cultivar IAS 5, which has a high content of total isoflavones, a small amount of aglycone compounds was observed (6.83 mg per 100 g), which is normally the case in freshly harvested soybeans. To form aglycones, soybeans must be exposed to certain levels of moisture or fermentation, in order to activate the β-glycosidase enzyme (Matsuura & Obata, 1993; Carrão-Panizzi & Bordingnon, 2000; Carrão-Panizzi et al., 2004).

During the cooking processes, no significant changes were observed in the level of aglycones. However, changes occurred during fermentation, confirming that special conditions are needed to form aglycones. The amount of aglycones increased significantly with fermentation time. During the first stage of fermentation (6 hours), aglycone content increased by 58% in comparison to cotyledons, and after 24-hour fermentation, both cultivars contained more aglycones in tempeh than in cotyledons. Other authors also observed that aglycone increased with fermentation time, and that after 24-hour fermentation, the amount of isoflavone aglycone doubles (Nakajima et al., 2005; Haron et al., 2009). Silva et al. (2011) reported that the autoclaved whole soybean flour fermented with *Aspergillus oryzae* contained 36.25% of aglycones in comparison to the autoclaved whole soybean flour (6.94%), after 24 hours. The amount of aglycones in tempeh of either cultivar did not change while refrigerated. Results indicate that tempeh refrigerated for 24 hours maintains its isoflavone composition, which is a good characteristic for tempeh.

Cultivar BR 36, which has less total isoflavones than IAS 5, had smaller amounts of aglycone. Tempeh from 'IAS 5' provided about 35 mg per 100 g (Table 1) of aglycone isoflavones. According to Messina et al. (2006), if median Asian isoflavone intake is used as a guide, approximately 8 or 9 g of soy protein, with concentrations of about 30 to 35 mg per 100 g of aglycones, could be recommended. In order to facilitate soybean consumption and to reach the recommended intake of isoflavones, it is important to consider cultivar differences for isoflavone content. Tempeh is a highly nutritional product and should be considered a functional food. As a form of vegetable protein, it can be used in different dishes in the occidental diet. This work showed the change of different forms of isoflavones during tempeh processing. The

concentration of aglycones in tempeh is high. However, this amount could be even higher if soybean cultivars with high isoflavone levels, like IAS 5, were used as raw material.

Conclusions

1. Tempeh prepared with soybean cultivars with high-isoflavone content can be considered a functional food with positive effects on human health.
2. The fermentation process changes the isoflavone profile increasing the amount of aglycone forms.
3. Malonyl forms decrease continuously during tempeh processing, mainly due to cooking.

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