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# Interactions between isoflavones and soybean proteins: Applications in soybean-protein–isolate production

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ABSTRACT

The effects of several modifications to soybean protein isolate (SPI) production on genistein series composition and content were analyzed. Combined variations of protein extraction pH and protein precipitation pH revealed that the maximum content of total genistein series was obtained with protein extraction at pH 8.0 and protein precipitation at pH 3.5. When protein extraction was carried out at pH 11.0, the saponification of malonylgenistin, resulting in genistin production, led to a decrease in isoflavone content. This fact was attributed to the low affinity of genistin for β-conglycinin. Malonylgenistin exhibited a high level of association with both glycinin and β-conglycinin at precipitation conditions. When temperature was reduced from 20 to 0 °C during protein precipitation, the genistein series content increased if the β-glucosidase activity was inhibited. This increase was at the expense of glucosilated forms. The nature of the interaction between soybean proteins and malonylgenistein and genistin seemed to be – at least in part – enthalpic in nature. In particular, malonylgenistin at pH 3.5 establishes another kind of interaction with protein, besides enthalpic ones. Glycinin exhibited higher affinities for genistein series components than β-conglycinin, the interactions being more stable at acidic than at alkaline pHs.



## 1. Introduction

Soybean-protein isolates (SPIs) are ingredients in a number of food products and contain small amounts of isoflavones in association with the main storage globulins: β-conglycinin and glycinin. Soybean proteins and isoflavones have been postulated as responsible of at least part of the beneficial health effects of soybean consumption (Crouse et al., 1999; Erdmann, Cheung, & Schröder, 2008; Speroni et al., 2009). SPIs are obtained by alkaline extraction from soybean flour and subsequent isoelectric precipitation of storage proteins.

The isoflavones genistein, daidzein, and glycitein, which belong to the phytoestrogens, may be found as four molecular species: an aglucon, a glucoside, and glucoside esters (malonyl-glucoside and acetyl-glucoside). Chemical and enzymatic reactions occurring during soybean processing (cooking, soaking, fermentation) can interconvert these molecules, by hydrolysis of the ester or the β-glucoside (hemiketal) bond (Matsuura, Obata, & Fukushima, 1989; Otieno, Ashton, & Shah, 2005; Wang & Murphy, 1996).

The various isoflavones may establish different kinds of interactions with proteins because of their diverse polarity and hydrophobicity as well as their ability to form hydrogen bonds. Moreover, malonyl-glucosides dissociate to produce negatively charged species at pHs near neutrality, since they have a pKa of ca. 5.7 (Rickert, Meyer, Hu, & Murphy, 2004). A study on the interactions between polyphenols and proteins from barley, suggested that the main contribution would be from hydrogen bonding and/or hydrophobic interactions (Asano, Shinagawa, & Hashimoto, 1982). An important role in this molecular linkage would be the degree of stacking between the polyphenol rings and the aliphatic moieties of the proline residues of proteins. In this regard, the phenolic hydroxyl group is an excellent hydrogen donor for the formation of hydrogen bonds with the amide carbonyl of the peptide backbone of proteins (Siebert, 1999). Nevertheless, Asano et al. (1982) postulated that ionic bonding was not involved in such protein–polyphenol interactions.

The final isoflavone content in a given soybean-derived product depends on the association/release of isoflavones and proteins during each step of production. For SPI, pHs of protein extraction and protein precipitation are important variables (Speroni, Milesi, & Añón, 2007).

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Knowledge concerning the nature of the interactions between isoflavones and soybean proteins would allow an optimization of the conditions of commercial processing (e. g., pH, temperature, time) in order to improve, and ultimately maximize, the isoflavone content of soybean-containing products. The acquisition of this information was thus the objective of this investigation.

## 2. Materials and methods

### 2.1. Materials

Soybean-protein isolates, glycinin, and β-conglycinin were prepared from different samples of flash-desolventized, defatted soybean flour manufactured by Solae S.A. (Porto Alegre, Brazil). The standard samples of genistin, acetylgenistin, and malonylgenistin were purchased from Nakalai-Teske (Kyoto, Japan); the genistein was from Sigma Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile and ethanol were used, and the other reagents were of analytical grade.

### 2.2. Standard production of SPIs

Soybean flour was suspended in distilled water (1:10, w/v). The suspension was adjusted to pH 8.0 with 2 mol/L NaOH, stirred at room temperature for 90 min, and centrifuged at 10,000 × g for 30 min at 4 °C. The alkaline supernatant was adjusted to pH 4.5 with 2 mol/L HCl and centrifuged at 3750 × g for 15 min at 4 °C. The pellet was washed with water at pH 4.5 and centrifuged. The final pellet was suspended in water and the pH was adjusted to 7.4 with 2 mol/L NaOH. This suspension was frozen at −80 °C and subsequently freeze-dried to obtain the SPI.

Several modifications of this standard technique were evaluated as follows:

(i) *Variation of pH during protein extraction and protein precipitation.* The initial aqueous suspension of flour, adjusted to pH 8.0 or 11.0, was stirred at room temperature for 90 min and centrifuged as above. The pH of the alkaline supernatant was then acidified to pH 3.5 or 4.5 and the rest of the protocol carried out as described above for the standard SPI-production procedure.

(ii) *Variation of pH, time, and temperature during protein precipitation.* The alkaline pH of the supernatant was adjusted to 3.5, 4.5, or 5.6 before stirring for 5 h at 0 °C or 20 °C. To prevent microbial growth, 0.002 g/L $NaN_3$ was added to the suspension.

(iii) *Variation of pH, time, and temperature during protein precipitation with inhibition of ß-glucosidase.* The initial flour suspension was prepared in an aqueous solution of 0.012 mol/L $AgNO_3$. The alkaline supernatant of the initial centrifugation step was adjusted to pH 3.5, 4.5, or 5.6, and the resulting suspension was stirred for 5 h at 0 °C or 20 °C. $NaN_3$ (0.002 g/L) was added to prevent microbial growth, while 0.012 mol/L $AgNO_3$ was included as a β-glucosidase inhibitor (Hsieh & Graham, 2001). The goal was to prevent the formation of different amounts of aglucone from the β-glucoside at the two temperatures assayed since the activity of this enzyme is temperature-sensitive. In the presence of the inhibitor the total amount of each molecule remains constant and thus available for interaction with proteins.

### 2.3. Preparation of fractions enriched in specific protein subunits

Preparations enriched in β-conglycinin (the 7S fraction) and glycinin (the 11S fraction) were obtained from defatted flour following the protocol described by Nagano, Hirotsuka, Mori, Kohiyama, and Nishimari (1992).

### 2.4. Ultrafiltration of glycinin and β-conglycinin suspensions

Suspensions of fractions enriched in β-conglycinin and glycinin were prepared at 6.25 g/L, at pHs 3.5, 4.5, or 8.0. The suspensions were stirred for 30 min at room temperature and then filtered through a membrane with a cutoff of 3000 Da using an AMICON 8010 device (Beverly, USA) with $N_2$ pressure (40 psi) The resulting eluate was analyzed by online HPLC to determine isoflavone concentration in the water-soluble fraction.

### 2.5. Extraction of isoflavones from SPI and from the fractions enriched in globulins

Isoflavone extraction was carried out on 0.050–0.057 g of freeze-dried samples with 1.0 mL of 0.6 L/L aqueous ethanol at room temperature for 2 h. After centrifugation (14,000 × g, 20 min, 4 °C), the supernatant was separated and centrifuged under the same conditions; the second supernatant was then analyzed by HPLC.

### 2.6. HPLC analysis of isoflavones

Analysis of isoflavones was performed by a modification of the method described by Barnes, Kirk, and Coward (1994). A nonlinear HPLC gradient (combining convex and concave curves), generated with 0.001 L/L acetic acid in water (solvent A) and 0.001 L/L acetic acid in aqueous acetonitrile (0.5 L/L, solvent B), was used. Following injection of 30 μl of sample, the percentage of solvent B was increased from 0 to 75% over a 40-min interval. The solvent flow rate was 1 mL/min. The HPLC system consisted in a pump and controller (the Waters 600 E Multisolvent Delivery System) along with a Waters 717-Plus Autosampler plus a Waters 996a Photodiode Array Detector. The column was Sephasil Peptide C8 12 μ ST4.6/250 (Pharmacia Biotech, Uppsala, Sweden).

The contribution of each of the four forms of genistein was estimated by summing the genistein equivalent computed through the use of coefficients derived from the molecular weights of each genistein conjugate, as follows:

Total genistein plus conjugates (TG) = genistein + genistin × 270/432 + malonylgenistin × 270/518 + acetylgenistin × 270/474.

### 2.7. Statistical analysis

All data were the average of three independent assays. Results are reported as mean ± standard error. Statistical analysis was carried out by the Sigmastat software. Analyses of variance were conducted. Differences between the sample means were analyzed by Tukey's test at an alpha = 0.05.

## 3. Results and discussion

The three flour samples analyzed contained no acetylgenistin, nor did this molecule appear in the SPI (it was apparently not formed from malonylgenistin during the different experimental procedures). Therefore, the values for TG reported throughout this report represent the sum of genistein, genistin, and malonylgenistin contents.

### 3.1. Variation in the pHs of extraction and precipitation

In a previous study, we observed that the pH during protein extraction and subsequent precipitation strongly affected the isoflavone content in the resulting SPI: at a protein extraction pH (EX-pH) of 8.0, the optimum yield of isoflavones occurred at a protein precipitation pH (PR-pH) of 3.5; whereas if PR-pH was fixed to 4.5,