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ORIGINAL PAPER

Effect of Extraction and Precipitation Conditions During Soybean Protein Isolate Production on the Genistein Series Content

F. J. Speroni Aguirre · V. Milesi · M. C. Añón

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Abstract The influence of the conditions for isolation of soy protein on the content of genistein and its conjugated forms was studied. The major components of the genistein series isolated from soybean flour were malonyl genistin (54.3%), genistin (36.9%), and equal amounts (4.4%) of genistein and acetyl genistin. A modification in the conjugation profile of genistein between pH 4.5 and 8.0 and above pH 10 was attributed to the action of β-glucosidase and the saponification reaction, respectively. A decrease in the content of total genistein in the insoluble flour residue and in the soy protein isolate (SPI) with increasing extraction pH was detected, while in the whey, the total genistein content was not affected by pH. The effect of pH variation during acid precipitation on the content of genistein and its conjugated forms, at a constant extraction pH (8.0), was also studied. Under these conditions, the highest absolute content of total genistein in the SPI was obtained at pH 3.5 and the lowest was obtained at pH 5.6. The total genistein content in the whey followed an inverse trend compared with that of the protein yield. A temperature increase did not substantially affect the distribution of the different genistein forms or their total contents. The content of total genistein was higher in the glycinin than in the β-conglycinin protein fraction. The effect of the pH during the alcohol/water extraction of the isoflavones was also analyzed. The efficiency of the extraction was lower at pH values between 3.25 and 3.5 than at other pH values.

Keywords Genistein · Protein isolates · Soybean extraction and precipitation · Glycinin · β-Conglycinin

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Introduction

Soybean has a high content of isoflavones, the concentration of genistein being much higher than that of daidzein and glycitein. In soy products, isoflavones can be found in four forms: 6″-*O*-malonyl-β-glucosides (the more stable, soluble and abundant form in the plant), 6″-*O*-acetyl-β-glucosides, β-glucosides, and aglycones, the last three being derived from the first type (Fig. 1 shows the series corresponding to genistein). These species are related by enzymatic and nonenzymatic pH-sensitive reactions. β-Glucosidase, which can transform β-glucosides into aglycones, is present in soybean and has optimal activity at pH values between 6.0–6.5. Desterification of 6″-*O*-malonyl-β-glucosides and 6″-*O*-acetyl-β-glucosides to form β-glucosides, a process called saponification, is favored by alkaline pH values. These reactions can occur during protein isolation. Therefore, the proportion of each isoflavone form depends on the soy variety, growing conditions and postharvest processing and storage conditions.

The distribution of genistein in its four possible forms in soy foods is important for genistein absorption.

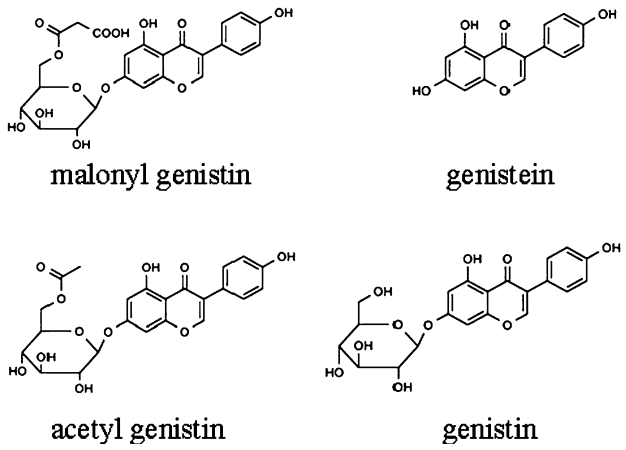

Fig. 1 Genistein series

Aglycones are absorbed directly from the stomach [1], whereas enzymes present in the human gut are unable to digest malonyl genistin [2]. The antioxidant activity of aglycones is also greater than that of the conjugated forms owing to the presence of one OH group [3]. In addition, aglycones may be responsible for the bitter and astringent taste of certain soy products, such as soymilk or tofu [4, 5].

Among soy products, protein isolates are very important for their functional and nutritional properties. Because of their wide range of functional properties, isolates are commonly included in the formulation of different foods (health drinks, meat extenders, and dairy products), in which they can replace milk proteins, especially caseinates. Soy isolates are mainly composed of the storage globulins β-conglycinin and glycinin.

The isoflavone content of protein isolates varies depending on the different suppliers. Information regarding the retention of isoflavones during protein isolate processing is usually scarce. Wang and Murphy [6] and Wang et al. [7] showed that after extraction at pH 8.0 and precipitation at pH 4.5 only 26–40% of the total isoflavone content in the soy flour was retained in the isolate. The highest losses occurred during alkaline extraction [6] and during washing of the isoelectric precipitate [7]. These studies also showed that the aglycone content is much higher in the isolates than in the original soy flour.

Extraction conditions for preparing soy protein isolate (SPI) could affect the isoflavone distribution. Thus, the aim of the current study was to assess the effect of different soy protein isolation conditions on the quantity of each genistein form in the final product. This knowledge will be useful for controlling the conjugation profile of genistein.

Experimental Procedures

Materials

SPIs were prepared from flash-desolventized defatted flour manufactured in different years by Solae (Porto Alegre, Brazil). Standard samples of genistin, daidzin, acetyl genistin, acetyl daidzin, malonyl genistin, and malonyl daidzin were purchased from Nakalai-Teske (Kyoto, Japan) and daidzein, glycitein, and genistein from Sigma Aldrich (St Louis, MO, USA). HIgh-performance liquid chromatography (HPLC) grade acetonitrile and ethanol were used. Other reagents were ACS grade.

Soaking of Flour at Different pHs

Defatted flour was dispersed in water (flour-to-water ratio 1:10) and adjusted to acid (1.5, 4.5, 5.0, 5.5, 6.0, and 6.5), neutral, or alkaline (7.0, 8.0, 9.0, 10.0, and 11.0) pH with either 2 N HCl or 2 N NaOH. The dispersions obtained were stirred for 90 min at room temperature; all dispersions were then adjusted to pH 7.4 (Fig. 2). The extraction of isoflavones was carried out on 0.5 mL of each dispersion using 0.75 mL of absolute ethanol at room temperature over 10 h. After centrifugation (14,000*g*, 20 min, 4 °C), the supernatant was retained and centrifuged again under the same

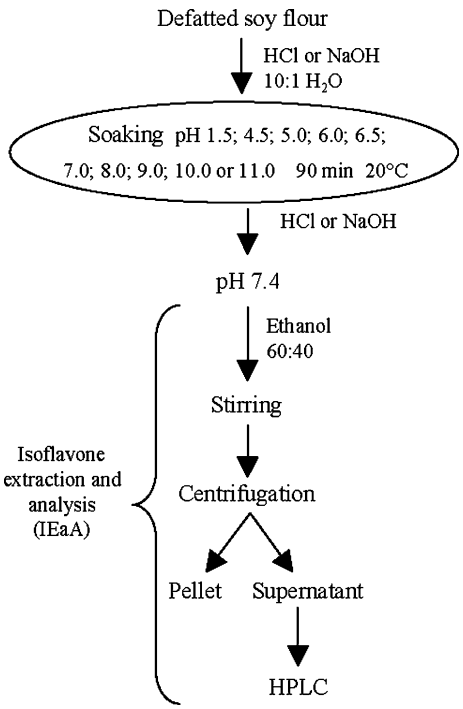

Fig. 2 The soaking of soy flour at different pHs

conditions. The second supernatant was directly injected into a HPLC apparatus.

Standard SPI Production

Soy flour was dispersed in distilled water (1:10 w/w). The dispersion was adjusted to pH 8.0 with 2 N NaOH, stirred at room temperature for 90 min, and centrifuged at 10,000*g* for 30 min at 4 °C. The supernatant was adjusted to pH 4.5 with 2 N HCl and centrifuged at 3,750*g* for 15 min at 4 °C. The pellet was washed with distilled water at pH 4.5 and centrifuged as above. The final pellet was suspended in distilled water and the pH was adjusted 7.4. Finally, the alkaline precipitate constituted by the insoluble flour residue (IFR), the whey, and the SPI was frozen at –80 °C and freeze-dried [8].

Several modifications of this standard method are described:

1. Extraction with pH variation at 20 °C. Initial water dispersions were adjusted to pH 7.0, 8.0, 9.0, 10.0, or 11.0 and were stirred at room temperature for 90 min. The rest of the protocol was identical to that described for the standard SPI production (Fig. 3a).
2. Extraction with pH variation at 50 °C. The protocol described for room temperature was followed, but the alkaline extraction was performed at 50 °C (Fig. 3a).
3. Precipitation with pH variation. The initial dispersion was performed at pH 8.0 for 90 min at 20 °C. Proteins in the alkaline supernatant were precipitated at pH 3.0, 3.5, 3.75, 4.0, 4.5, 5.0, 5.6, or 6.0. The rest of the procedure was as described above (Fig. 3b).
4. Variable pH of the final suspension. Proteins were extracted at pH 8.0 as described above and were precipitated at pH 3.5. After washing and a second centrifugation at 3,750*g*, the precipitate was suspended at the following pHs: 1.9, 2.75, 3.0, 3.25, 3.5, 3.75, 4.5, 5.6, 6.2, 7.4, and 8.43. Suspended samples were frozen and lyophilized (Fig. 4).

Preparation of Purified Protein Subunits

β-Conglycinin (7S fraction) and glycinin (11S fraction) were obtained from defatted flour following the protocol described by Nagano et al. [9].

To verify the influence of the extraction time and the water volume to solid matter ratio, glycinin and *β*-conglycinin were prepared following the procedure described in the preceding section until the precipitation step. From that point onwards, the following modifications were introduced: In every precipitation or dialysis step, the water volume to solid matter ratio

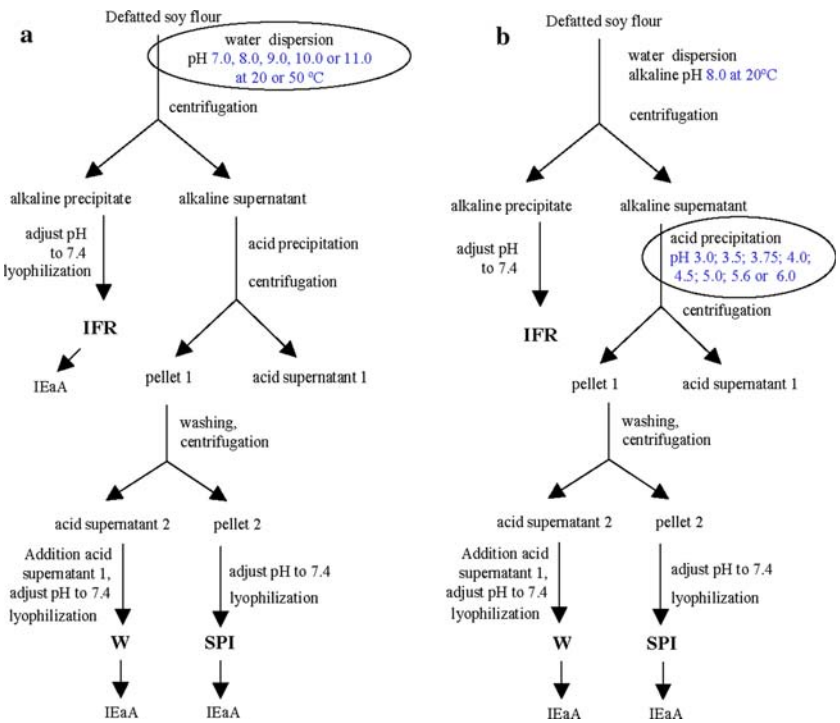

Fig. 3 The standard soy protein isolate (*SPI*) production including the modifications introduced in the extraction step (**a**) (experiments 1 and 2) and in the precipitation step (**b**) (experiment 3). These modifications are indicated by an *ellipse*. *IFR* insoluble flour residue, *W* whey

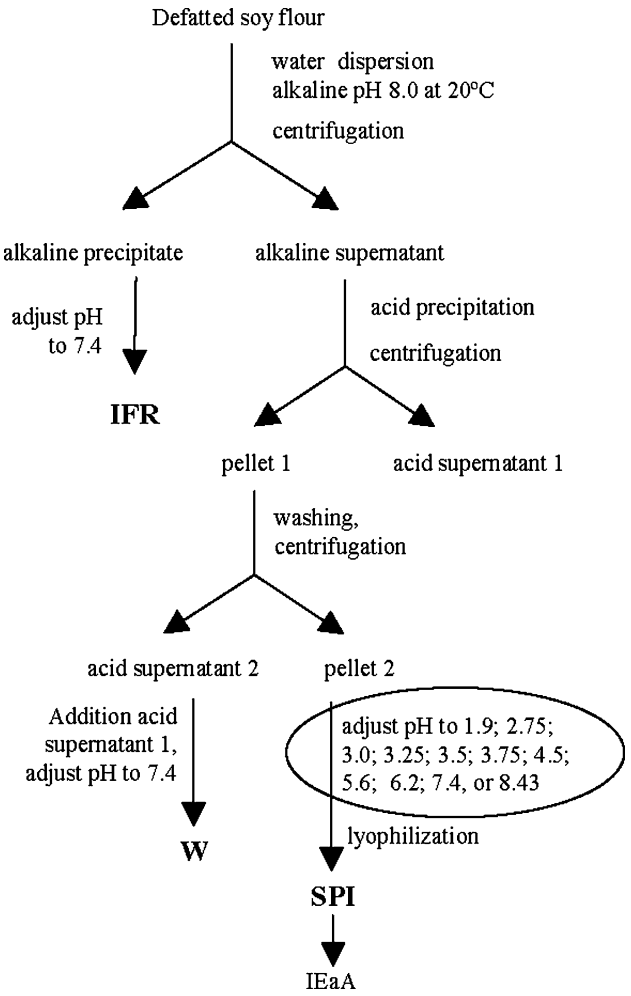

Fig. 4 The SPI production including the modification introduced in the final suspension step. This modification is indicated by an *ellipse*

and the contact time were maintained constant in all preparations. Both subunits were obtained after the same total processing time.

Glucosidase Activity Measurement

Genistein β-Glucoside Hydrolysis

A dispersion of soy flour was prepared in water at pH 6.5 (1:10 w/v), thermally treated (90 °C, 15 min), and incubated at room temperature for 12 h (control sample). After adding ethanol to reach a 60:40 (v/v) ratio of ethanol to water, the dispersion was centrifuged and the genistein content of the supernatant was measured. An enzyme-active sample was obtained using a similar procedure without thermal treatment.

O-Nitrophenyl-β-D-galactopyranoside hydrolysis

A flour dispersion was prepared in water at pH 6.5 (0.5:10 w/v) and then 0.66 mM *O*-nitrophenyl-β-D-galactopyranoside was added. The dispersion was thermally treated at 90 °C for 15 min. Different aliquots were incubated for various times and then were centrifuged (10,000*g*, 15 min, 10 °C); the supernatants were diluted 1:10 with 0.2 M Na_2CO_3 before measurement of the absorbance at 400 nm. An enzyme-active sample was obtained using a similar procedure without thermal treatment.

Extraction of Isoflavones from SPI and Flour

The extractions of isoflavones were carried out from 50 to 57 mg of freeze-dried samples with 1.0 mL of 60% aqueous ethanol, at room temperature over 10 h. After centrifugation (14,000*g*, 20 min, 4 °C) the supernatant was separated and centrifuged again under the same conditions. The second supernatant was directly injected into a HPLC apparatus.

HPLC Analysis of Isoflavones

Analysis of isoflavones was performed by a modification of a method described by Barnes et al. [10]. A nonlinear HPLC gradient generated with 0.1% glacial acetic acid in H_2O (solvent A) and 0.1% glacial acetic acid in acetonitrile/H_2O (50:50 v/v) (solvent B) was used. Following injection of 30 μL of sample, solvent B was increased from 0 to 75% over 40 min. The solvent flow rate was 1 mL/min. The HPLC system included the Waters 600 E multisolvent delivery system with a Waters 717 plus autosampler and a Waters 996 photodiode array detector. A Sephasil Peptide C8 (12 μm, 4.6 mm × 250 mm) (Pharmacia Biotech, Uppsala, Sweden) column was used for the separation.

The contribution of each of the four genistein forms was estimated by adding up the genistein equivalent (which was converted using factors derived from molecular weights):

$$\text{Total genistein} = \text{genistein} + \text{genistin} \times 0.625 + \text{malonyl genistin} \times 0.521 + \text{acetyl genistin} \times 0.569.$$

Statistical Analysis

Experiments and sample analyses were done in at least triplicate. Statistical analysis was completed using the Sigmastat software (Systat Software, USA). Analyses

of variance were conducted. Differences between the sample means were analyzed by Tukey's test at $\alpha = 0.05$.

Results and Discussion

Isoflavone Content of Soy Flour

The content of the combined isoflavones varied between 1,371.6 and 1,939.4 μg/g of flours obtained in different years. Various forms of genistein account for 58.4–60.7% of total isoflavones, followed by daidzein (37.4–38.3%) and glycitein (1.9–3.3%). Considering the differences in composition, these values lie within the wide range of values obtained previously for soybean, which depend on the cultivar and the cultivation conditions [7, 11, 12].

Genistein Content of Protein Isolates and Intermediate Products

Effect of pH During the Flour Soaking Step

The first step in the SPI processing is the dispersion of soy flour in water. In the present work, a wider range of pH (1.5–11.0) than that used in the standard method was assayed to analyze the effect of pH on genistein conjugation. In order to maximize protein extraction, a 10:1 water-to-flour ratio was used. A study by Kao et al. [12] showed that, in the case of soymilk, the maximum extraction of proteins and isoflavones was achieved with a water-to-seed ratio between 9:1 and 11:1. Figure 5 shows the content of genistein, genistin, acetyl genistin, and malonyl genistin, expressed as a percentage, for soaking water pHs between 1.5 and 11.0. The first point corresponds to the content of each genistein form in the original dry flour. The major fraction in the original flour corresponded to malonyl genistin (54.3%), followed by genistin (36.9%), and equivalent amounts of genistein and acetyl genistin (which together accounted for the remaining 9%).

According to Wang et al. [7], the predominant form within the genistein series in soy flour is malonyl genistin (66.8%), followed by genistin (30.7%). Since these authors did not detect the presence of acetyl genistin, the remaining 2.5% was represented by genistein. Considering the content of each genistein form in the original flour, the results of the present study show a decrease of the genistin fraction in the pH range between 4.5 and 8.0, with maximum conversion at pH 6.5 (36.9–29.1%), and an increase in the genistein fraction (4.6–13.3%). From pH 10.0 onwards, there was a noticeable increase in the genistin content (95% of the total genistein at pH 11.0) and a decrease of the malonyl genistein and acetyl genistein fractions from 54.3 to 1% and from 4.2 to 0%, respectively, at pH 11.0.

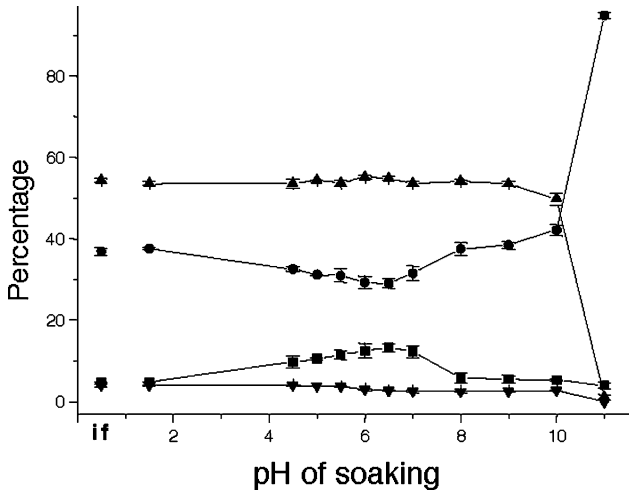

Fig. 5 Conjugation profile of isoflavones corresponding to aqueous defatted soy flour dispersions adjusted to different pHs (1.5–11) after soaking for 90 min. Percentage of genistein (*squares*), genistin (*circles*), acetyl genistin (*inverted triangles*) and malonyl genistin (*up triangles*). The initial value in the flour is represented by *If*

The variations in the content of genistein and its conjugated forms detected at alkaline pH can be attributed to hydrolysis of the ester bond under alkaline conditions [13], while those found at acid pH may be due to hydrolysis of glucoside isoflavones through the action of β-glucosidase (optimum pH 6.0–6.5) present in soy seed. Similar variations have been detected as a consequence of thermal treatment [6, 14, 15] and during the preparation of soymilk [12].

To verify the existence of enzymatic activity, the amounts of the different forms of genistein were analyzed in thermally treated and untreated flour. By comparing the percentage of each genistein form in untreated flour dispersions with that in original flour, a substantial conversion of genistin (5%) into genistein (41%) without a reduction in the content of malonyl genistin (54%) was evident (Fig. 6a). Thermally treated samples showed only a reduction in the malonyl genistin fraction (genistin 48%, genistein 6%, malonyl genistin 41%, and acetyl genistin: 4%), possibly due to higher levels of decarboxylation favored by the temperature increase [6].

The substantial conversion of genistin into genistein suggests the presence of active glucosidases in the original flour; thus, the enzymatic activity was measured. As in the previous experiment, glucosidase

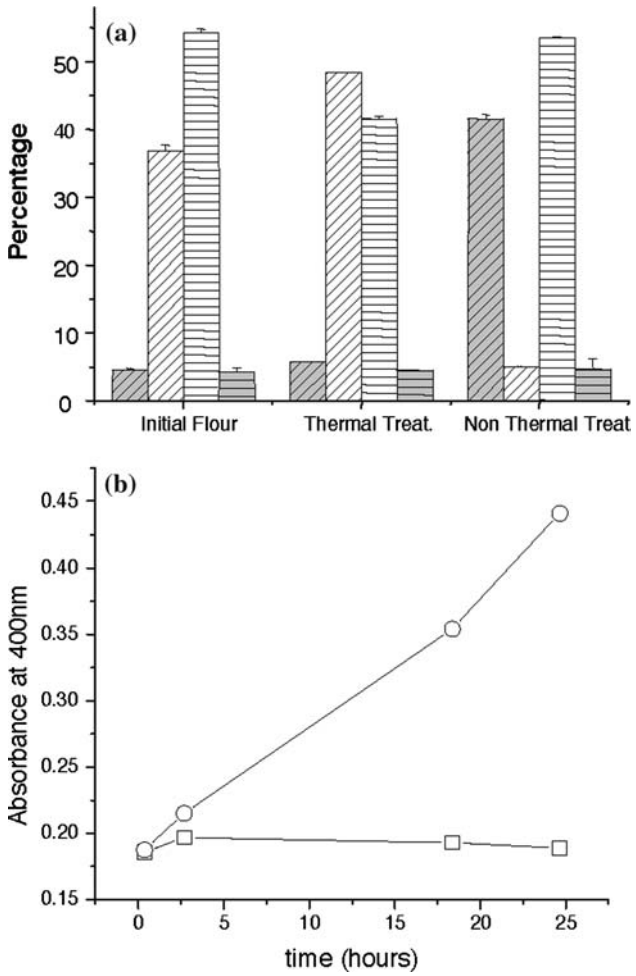

Fig. 6 **a** Percentage of genistein (*dark bars with diagonal hatching*), genistin (*light bars with diagonal hatching*), malonyl genistin (*light bars with horizontal hatching*), and acetyl genistin (*dark bars with horizontal hatching*) expressed as fractional numbers in the initial flour and after 12 h of soaking with or without thermal treatment . **b** Absorbance increase due to formation of *O*-nitrophenol (enzyme activity). Non-thermally treated sample (*circles*) and thermally treated sample (*squares*)

activity was detected in supernatants from flour dispersions not subjected to thermal treatment (Fig. 6b). These results indicate that the thermal treatment used to eliminate residual oil from flour was insufficient to inactivate the β-glucosidase.

Effect of Protein Extraction Conditions

Figure 7a–c shows the variation in the content of genistein and its conjugated forms in the IFR (pH 7.0–11.0), the whey, and the SPI obtained from isoelectric precipitation. Considering the yield of each product, the results were expressed as micrograms of each genistein form and total genistein per gram of initial flour. These products retained approximately 90% of the total genistein present in the original flour, without significant differences between the different pH values assayed (7–11). A decrease in the content of total genistein in the IFR was observed with increasing extraction pH (Fig. 7a). The SPI was enriched in total genistein with increasing extraction pH (Fig. 7c), while in the whey, the total genistein was not affected by pH (Fig. 7b).

The distribution of total genistein in the IFR, whey, and SPI may also be expressed as the percentage of the initial total genistein remaining in each product: the SPI was enriched in total genistein with increasing extraction pH (40, 50, and 54% at pH 7.0, 9.0, and 11.0, respectively) ($P < 0.05$), while the total genistein percentage in the IFR diminished (38.5, 26.5, and 20.3%, at pH 7.0, 9.0, and 11.0, respectively) ($P < 0.05$), and that of the whey remained approximately constant (21–26%). Since isoflavones are weak acids, with pK_a values ranging from alkaline (9.74–11 for the glycosylated forms and aglycones) to acid (5.7 for malonyl daidzein) [14], their water solubilities should increase at higher pHs owing to an increase in their dissociation. These molecules would become insoluble again during isoelectric precipitation and would coprecipitate with proteins, thus enriching the isolate.

The changes in the distribution of the genistein forms at 20 °C (Fig. 7a–c) were consistent with those discussed above (Fig. 5). It is interesting to note, however, that at pH 7.0 the IFR had a much higher content of genistein and a lower content of genistin. This difference could be attributed to a higher enzymatic activity because the dispersion was maintained at pH 7.0 for a longer period before freezing, which favored the action of β-glucosidase.

Wang et al. [7] analyzed the distribution of isoflavones in the alkaline precipitate, acid whey, isolectric precipitate washes, and acid precipitate, after soy flour extraction at pH 8.0 and precipitation at pH 4.5. Their results agree with ours in terms of the relative percentages of each fraction in the whey. Wang et al. [7] found, as in the present study, that malonyl genistin was the predominant species in the IFR and acid precipitates (SPI), which was followed, at variance with our results, by genistein, genistin, and acetyl genistin.

The temperature increase (20–50 °C) during the extraction of proteins at neutral and alkaline pH did not significantly affect the results discussed previously. The main difference was a lower variability in the content of total genistein as a function of pH (Fig. 7d–f). A recent study by Ungar et al. [16] showed that, at temperatures between 70 and 90 °C, the apparent velocity constant for the degradation of genistein is approximately 4 times higher at pH 9.0 than at pH 7.0.

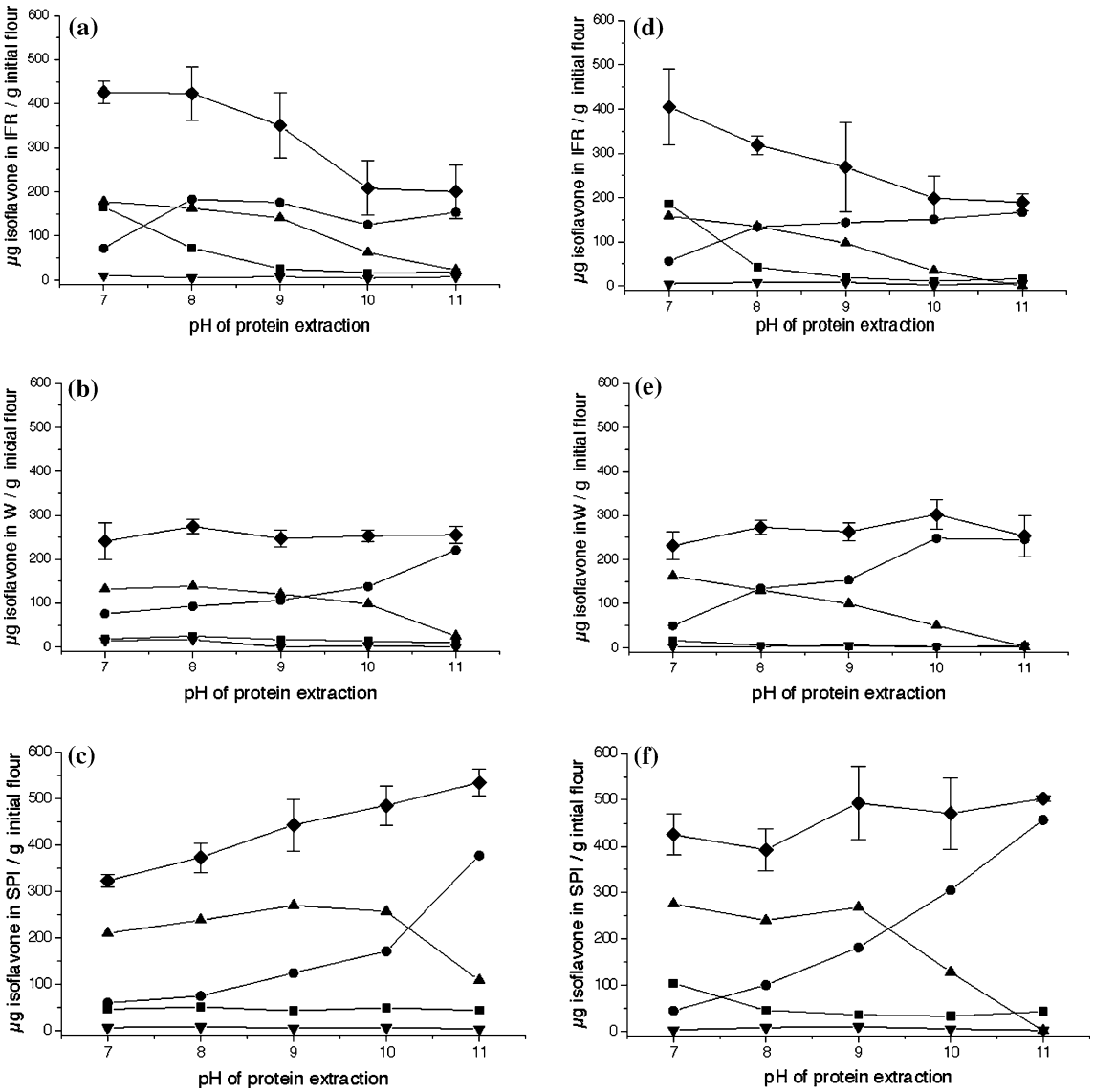

Fig. 7 Content of genistein (*squares*), genistin (*circles*), malonyl genistin (*up triangles*), acetyl genistin (*inverted triangles*), and total genistein (*diamonds*) in the insoluble flour residue (**a**, **d**), whey (**b**, **e**), and SPI (**c**, **f**) obtained after neutral or alkaline extraction (pH 7–11) of defatted flour at 20 °C (**a**–**c**) and 50 °C (**d**–**f**), corresponding to the isoelectric protein precipitation at pH 4.5 during SPI production

Wang and Chen [17] also showed that isoflavones were more affected by the thermal treatment in the presence of other components than in the pure form (genistin being less stable than genistein).

Effect of Protein Precipitation Conditions

The effect of pH, during acid precipitation of the protein extracted at pH 8, on the contents of genistein and its conjugated forms was analyzed. Several pHs between 3.0 and 6.0, which includes the average pI of storage globulins from soybeans, were assayed. In this pH range, protein solubility exhibited marked changes, especially at pHs 3.0 and 6.0, where the protein was more soluble. The higher solubility resulted in a dramatic change in the yield of SPI (Fig. 8a), which ranged from 27.8 to 5.4 g of isolate per 100 g of flour at pH 4.0 and pH 3.0, respectively. SPI obtained from pHs 3.5 to 5.6 contained an equivalent percentage of protein (pH 3.5, 77.72 ± 3.64%; pH 5.6, 80.49 ± 1.80%; water content, 11.5 ± 0.5%) and an equal proportion of glycinin and β-conglycinin (not shown). In these assays, 94% of the original content of total genistein in the flour was recovered. The highest content of total genistein in the SPI was obtained at pH 3.5 (Fig. 8b), and the lowest was obtained at pH 5.6 ($P < 0.05$). The increase in the

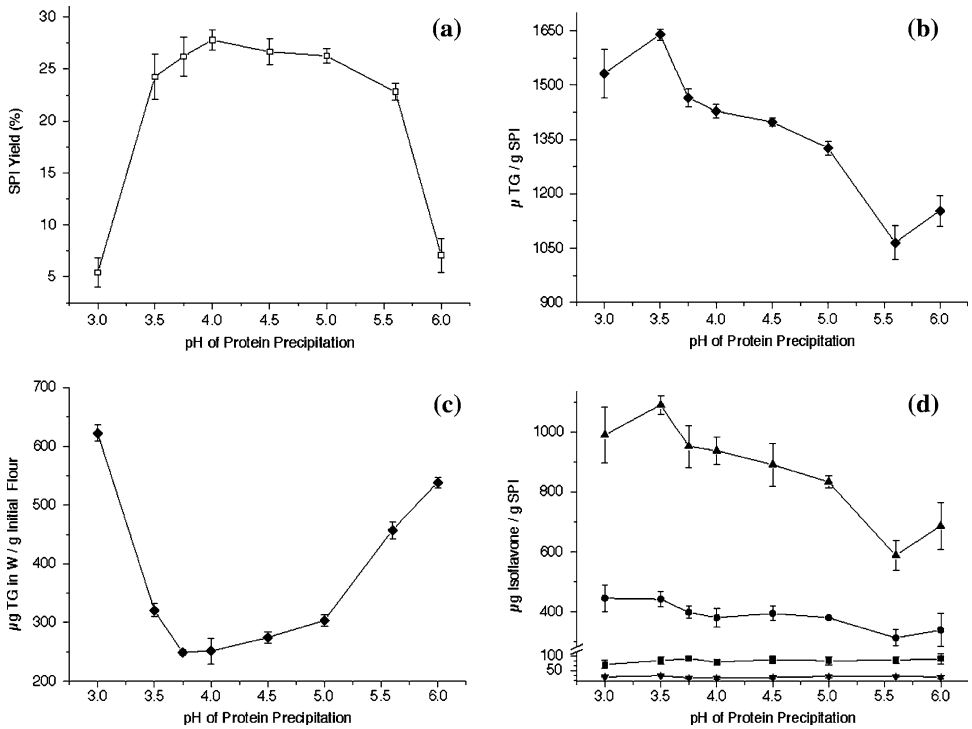

Fig. 8 Effects of pH during protein precipitation in SPI production. Protein isolate yield (**a**), content of total genistein in SPI (**b**) and in whey (**c**), and the contents of genistein (*squares*), genistin (*circles*), malonyl genistin (*up triangles*), acetyl genistin (*inverted triangles*) in SPI, expressed as their aglycone form equivalent (**d**). Protein extraction from the defatted flour was carried out at pH 8.0, with protein precipitation beginning conducted between pH 3 and 6. *TG* total genistein

total genistein detected at the more acid pH was due to an increase in the malonyl genistin and genistin contents, while the genistein content remained constant in the pH range assayed (Fig. 8d). The total genistein content in the whey (Fig. 8c) followed an inverse trend compared with that of the protein yield. From the total genistein extracted at pH 8.0 (approximately 650 μg/g of flour), 60% was precipitated at pH 3.5. However, when the yield difference was taken into account, the highest recovery (40%) was achieved at pH 4.0, where 21% of the isoflavone content was found in the whey and the remainder was found in the IFR. Thus, the distribution of conjugated forms of genistein can be affected by the pH during protein precipitation, suggesting a more intense interaction with the protein at pH 3.5, in addition to its effect on the isolate yield.

Taking these findings into account, SPIs were obtained under similar conditions (extraction at pH 8.0 and precipitation at pH 3.5) and were suspended at different pHs, frozen, and later subjected to isoflavone extraction with alcohol/water (60:40), so that the pH conditions during isoflavone extraction were similar to those during freezing. The amounts of malonyl genistin and genistin extracted were a function of the pH of the alcohol/water extraction medium, exhibiting minimum values between pH 3.25 and 3.5 ($P < 0.05$) (Fig. 9). These results reinforce the hypothesis that the electrostatic interactions between soy proteins and malonyl genistin and genistin depend on the pH in the sample, with increasing interaction at acid pH (3.25–3.5). However, protein conformation and, therefore, exposed hydrophobic regions and hydrogen bonding vary with pH. Below pH 3.5, glycinin and *β*-conglycinin denature, dissociate, and undergo an increase in hydrophobicity, which could lead to increased hydrophobic interactions between the aglycon moiety and soy proteins [18]. Owing to the changes in hydrophobicity, the extraction of isoflavone components from acid foods should be performed at pH greater than 5.0; otherwise an underevaluation of these bioactive compounds, due to the decrease in their extraction efficiency, will occur.

Genistein Content Variation in 7S and 11S Fractions

The content of total genistein was higher in glycinin (11S fraction) (1,162.0 ± 41 μg total genistein per gram) than in *β*-conglycinin (7S fraction) (339.8 ± 15.8 μg total genistein per gram; $P < 0.05$). The original working protocol, described by Nagano et al. [9] showed that *β*-conglycinin remained in water for 18 h longer than glycinin. Since isoflavones may leach out during the wet steps of the isolation process, a preparation was made where the time periods and the water-to-globulin ratios were equivalent for both fractions. The resulting protein fractions occurred in similar proportions as before. Thus, the differences in the isoflavone contents are not due to leaching of

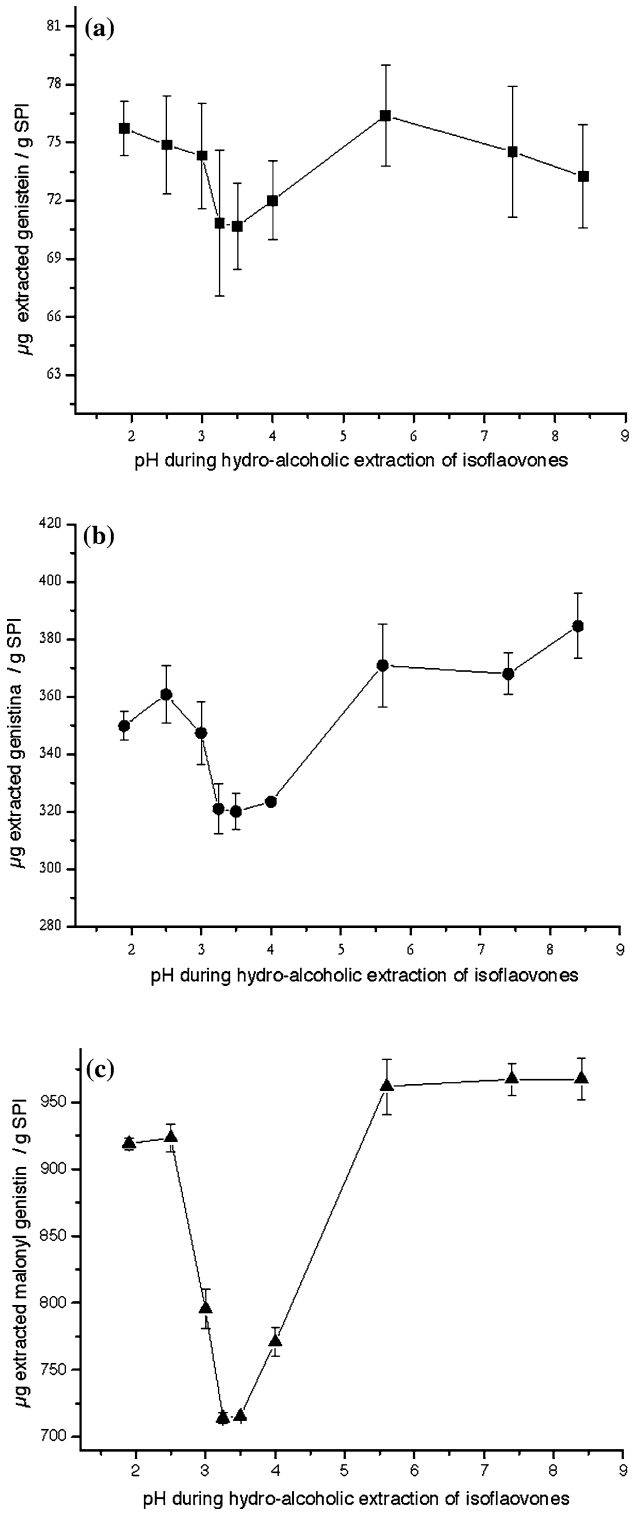

Fig. 9 Alcohol/water extraction of genistein (**a**), genistin (**b**), and malonyl genistin (**c**). The pH during the extraction of the isoflavones from SPI was fixed at different values

isoflavones, but are probably due to the purification method and to a differential affinity between the two globulins and the isoflavones.

The difference in the content of total genistein between glycinin and β-conglycinin could be due to structural differences in the proteins, which would give a higher affinity of isoflavones from the genistein family for glycinin. This difference could stem from differences in the surface hydrophobicity of the globulins.

Conclusion

On the basis of the results obtained in this work, we conclude that soaking or protein-extraction pHs between 4.5 and 8.0 favor the enzymatic hydrolysis of β-glucosylated forms, whereas extreme alkaline pHs favor the hydrolysis of the ester union of malonyl and acetyl derivatives. In terms of a higher content of total genistein in SPI, the optimal preparation conditions would be to precipitate the proteins at pH 3.5. The protein precipitation pH affects the SPI yield and the amount of total genistein in the whey and the SPI. The malonyl genistin–protein and genistin–protein association would be favored at acid pHs (3.25–4.0), whereas the association would diminish at higher pH values [5, 6].

The effect of pH on the interaction of the conjugated forms of genistein and the soybean proteins observed in this work is of great importance for both the production of foods based on soybean as well as for the quantification of isoflavones in acid soybean foods.

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