Chemical modification of isoflavones in soyfoods during cooking and processing

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ABSTRACT The principal chemical forms of isoflavones in soybean are their 6′′-O-malonyl-β-glucoside (6OMalGlc) conjugates. Experiments were carried out to determine the best conditions for extraction of isoflavones from soyfoods and the effects of commercial processing procedures and of cooking on isoflavone concentrations and composition. Hot alcohol extraction of ground soybeans deesterified 6OMalGlc conjugates. Although room temperature extraction slowed the conversion, extraction at 4°C for 2–4 h led to the highest yield of 6OMalGlc conjugates and the lowest proportion of β-glucoside conjugates. Analysis of soyfood products by reversed-phase HPLC–mass spectrometry revealed that defatted soy flour that had not been heat treated consisted mostly of 6OMalGlc conjugates; in contrast, toasted soy flour contained large amounts of 6′′-O-acetyl-β-glucoside conjugates, formed by heat-induced decarboxylation of the malonate group to acetate. Soymilk and tofu consisted almost entirely of β-glucoside conjugates; low-fat versions of these products were markedly depleted in isoflavones. Alcohol-washed soy-protein concentrates contained few isoflavones. Isolated soy protein and textured vegetable protein consisted of a mixture of all 3 types of isoflavone conjugates. Baking or frying of textured vegetable protein at 190°C and baking of soy flour in cookies did not alter total isoflavone content, but there was a steady increase in β-glucoside conjugates at the expense of 6OMalGlc conjugates. The chemical form of isoflavones in foods should be taken into consideration when evaluating their availability for absorption from the diet. Am J Clin Nutr 1998;68(suppl):1486S–91S.

KEY WORDS Genistein, daidzein, glycitein, glucosides, solvent extraction, isoflavones, food processing, malonylg glucoside conjugate, acetylglucoside conjugate, soyfoods

INTRODUCTION There is an apparent association between increased soy consumption and reduced cancer risk (1). In addition, a meta-analysis confirmed that soy protein lowers blood cholesterol concentrations (2), a finding observed almost 20 y before in rabbits (3) and in patients with hypercholesterolemia (4). The number of investigators carrying out both animal studies (5–10) and human clinical trials (11–13) with soy products has increased and, as a result, there is considerable interest in the soybean isoflavones and their potential chemopreventive effects.

Before 1990, the composition of isoflavones in soyfoods was thought to be largely determined by whether the foods were fermented (14, 15). Fermented soyfoods such as miso and tempeh contain the unconjugated isoflavone aglucones, whereas nonfermented soyfoods [soymilk, tofu, soy flour, soy-protein concentrate, and isolated soy protein (ISP)] contain their β-glucoside conjugates (Figure 1). Isoflavones at that time were recovered for analysis by extraction into hot, aqueous organic solvents such as acetonitrile, ethanol, or methanol. However, in 1991 it was found that extraction of soybeans with these solvents without the use of heat led to a different set of isoflavone glucosides, identified as the 6′′-O-malonyl-β-glucoside (6OMalGlc) conjugates (Figure 1) (16). Careful investigation of soyfoods with reversed-phase HPLC–mass spectrometry (HPLC-MS) and revised extraction protocols showed that most soyfoods contained mixtures of the β-glucoside, 6OMalGlc, and 6′′-O-acetyl-β-glucoside (6OAcGlc) conjugates (Figure 1) (17, 18). The 6OAcGlc conjugates had been previously identified in toasted soy flour (19).

Because the chemistry of the isoflavone conjugates is altered during the commercial processing of soy into food products, we set out to determine 1) whether the revised extraction procedures in use permit quantitatively reliable measurement of the different isoflavone conjugate types, 2) to what extent the consumer’s cooking the soyfood further alters the isoflavone glucoside conjugate composition, and 3) whether the isoflavone moiety is stable under these conditions. To properly interpret data, it is crucial that we know exactly what forms of isoflavones are consumed by the subjects in a clinical trial. The proportion of each chemical type of isoflavone absorbed into the blood remains poorly understood, although it was proposed that the aglucones are absorbed readily from the upper small
FIGURE 1. Chemical structures of the glucoside conjugates of genistein found in soyfoods. A, 6′-O-malonyl-β-glucoside; B, β-glucoside; C, 6′-O-acetyl-β-glucoside.

intestine, that the β-glucoside conjugates are absorbed from the distal small intestine after hydrolysis to the aglucone, and that the 6OMalGlc and 6AcGlc conjugates are absorbed from the large intestine after hydrolysis (20).

MATERIALS AND METHODS

Materials

HPLC-grade methanol and acetonitrile and ammonium acetate were purchased from Fisher Scientific (Norcross, GA). Trifluoroacetic acid was purchased from Sigma Chemical Company (St Louis). The disodium salt of fluorescein was purchased from JT Baker (Phillipburg, NJ). Genistin was isolated from a soy-protein extract prepared by Protein Technologies International (St Louis); genistin was made by acid hydrolysis of genistin (21). Daidzin was isolated from soy molasses (21) and daidzein was purchased from LC Labs (Woburn, MA). Tofu, soy flour, and textured vegetable protein (TVP) were purchased at a local health food store (Decatur, IL). Tofu, soy flour, and textured vegetable protein were provided by Archer Daniels Midland Co (Phillipburg, NJ). Toasted soy flour and soy molasses were provided by Archer Daniels Midland Co (Decatur, IL). Tofu, soy flour, and textured vegetable protein (TVP) were purchased at a local health food store.

Extraction conditions

Soyfoods (0.5 g) were mixed with 5 mL 80% aqueous methanol with 1 mg fluorescein as an internal standard (17). Milk samples (1 mL) were mixed with 4 mL 80% methanol containing 1 mg fluorescein. Extraction was carried out by tumbling the mixture for 2–24 h at room temperature (22–23°C) or 4°C or shaking in a water bath for 2–4 h at 80°C. Samples of each were centrifuged at 16000 × g for 5 min at 4°C before HPLC analysis.

Cooking conditions

Soy flour was either mixed with water to make a dough or mixed with wheat flour, sugar, butter, baking soda, and eggs to make a cookie. Both preparations were baked in a conventional oven at 190°C for 0–30 min. TVP was hydrated in boiling water and either fried over medium heat for 0–20 min or baked at 170°C for <60 min. Samples were stored frozen at −20°C until analyzed.

HPLC analysis

Reversed-phase HPLC analysis of isoflavones and their glucoside conjugates was carried out on a 22 cm × 4.6 mm internal diameter Brownlee Aquapore C8 column with a 1.5 cm × 3.2 mm internal diameter RP-C8 NewGuard precolumn (Rainin, Woburn, MA). Samples were eluted at a flow rate of 1.5 mL/min by using a gradient of 0–30% eluant B (95% acetonitrile with 0.1% trifluoroacetic acid) over 30 min. The column was washed with 100% eluant B for 5 min and equilibrated with eluant A (5% acetonitrile with 0.1% trifluoroacetic acid) for 5 min between runs. The eluted isoflavonoids were detected at 262 nm (Figure 2). Quantitative data for daidzein, daidzin, genistein, and genistin were obtained from comparison with known standards. Kudou et al (16) showed that the molar extinction coefficients of the 6OMalGlc conjugates approximate those of the β-glucoside conjugates, so the 6OMalGlc and 6AcGlc conjugates were calculated from the β-glucoside standards. Glycitin (7,4′-dihydroxy-6-methoxyisoflavone) and its conjugates were calculated by using daidzein and its conjugates because standards were not available. Data are expressed as means ± SDs.

Mass spectrometry analysis

The identity of each isoflavone conjugate was confirmed by HPLC-MS. Analyses were performed on an API III triple-quadrupole mass spectrometer (PE-Sciex, Concord, Canada) equipped with 2 Macintosh Quadra 950 computers (Apple Computer Inc, Cupertino, CA) for data analysis. The isoflavonoids were separated by reversed-phase HPLC on a 10-cm × 4.6-mm internal diameter Aquapore C8 column at a flow rate of 1.5 mL/min by using a linear 0–50% gradient of acetonitrile in the presence of 10 mmol ammonium acetate/L. Positive ions were introduced into the mass spectrometer after their generation by atmospheric pressure chemical ionization caused by a corona discharge needle in the heated nebulizer interface of the instrument (17). Each conjugate produced a [M+H]+ ion and the aglucone fragment.

FIGURE 2. Reversed-phase HPLC results of the isoflavone conjugates in textured vegetable protein. A, daidzin; B, glycitin; C, genistin; D, 6′-O-malonyl/daidzin; E, 6′-O-malonylglycitin; F, 6′-O-acetyl/daidzin; G, 6′-O-malonylgenistin; H, 6′-O-acetylgenistin; I, genistin; J, fluorescein (internal standard); AU, absorbance unit.
RESULTS

Extraction conditions

Isoflavone β-glucoside conjugates were extracted from soybeans at 80°C, at room temperature, and at 4°C for 2–72 h. Quantitative and reproducible recovery of the isoflavone glucosides was achieved after 2 h (data not shown). Extraction at 4°C gave the highest concentration of 6OMalGlc conjugates and the lowest concentration of β-glucoside conjugates (Table 1). At room temperature, a slight conversion of the 6OMalGlc conjugates to the β-glucoside conjugates was observed. Extraction at 80°C caused extensive conversion of the 6OMalGlc conjugates to the β-glucoside conjugates but not to the 6OAcrGlc conjugates or aglucones. Although the composition of the individual β-glucoside conjugates was drastically altered by temperature, the total amount of isoflavones extracted was constant. The 6OMalGlc conjugates were stable at 4°C for 24 h but prolonged storage also caused conversion to the β-glucoside conjugates (Figure 3).

Soyfoods

Soy flour, toasted soy flour, ISP, tofu, low-fat tofu, soymilk, low-fat soymilk, nonfat soymilk, and TVP were extracted at 4°C. Soy flour, which is prepared by grinding soybeans into a powder, contained predominantly the 6OMalGlc conjugates (Table 2). Toasting the flour increased the content of both the β-glucoside and 6OAcrGlc conjugates. ISP is prepared by first solubilizing the proteins at an alkaline pH to separate them from the insoluble carbohydrates and then precipitating the proteins at their isoelectric point (pH 5). After the mixture is neutralized, the protein is spray-dried to produce the commercial product. Isoflavones were found in ISP as a mixture of the 6OMalGlc and β-glucoside conjugates, with a small amount of the 6OAcrGlc conjugates and aglucones. TVP, prepared by extrusion technologies on soy flour, soy-protein concentrates, or ISP, contained a combination of all 3 conjugates, 30% occurring as the 6OAcrGlc conjugates. Tofu and soymilk, both of which are processed by hot aqueous extractions, contained predominantly the β-glucoside conjugates (Table 3). Low-fat and nonfat soymilk also contained predominantly β-glucoside conjugates but were relatively depleted of isoflavones, by 57% and 88%, respectively. Low-fat tofu was depleted of β-glucoside conjugates by 40%, but a slight increase was seen in the 6OAcrGlc conjugates and aglucones.

Cooked foods

When TVP, which contains each of the glucoside conjugates, was baked, the amounts of 6OAcrGlc conjugates and aglucones present were unchanged, but the 6OMalGlc conjugates slowly degraded to the β-glucoside conjugates. When TVP was fried, however, increases in the 6OAcrGlc conjugates and aglucones, as well as the β-glucoside conjugates, were observed (Figure 4). Under normal cooking times (60 min for baking and 7.5 min for frying), the total isoflavones recovered did not decrease once an increase due to the loss of water during cooking was taken into account. When soy flour was baked as a dough, the water in the dough regulated the internal temperature of the dough and 6OMalGlc conjugates decreased only slightly over the 30 min of cooking. When ingredients such as sugar and butter were added, the 6OMalGlc conjugates were degraded much more rapidly (Figure 5).

FIGURE 3. Mean (±SD) total concentrations of isoflavone glucoside conjugates from ground soybeans extracted with 80% aqueous methanol at room temperature (temp) for 2 h or at 4°C (cold) for 2 and 24 h, or after storage at 4°C for 24 or 72 h. For each set of bars, from left to right, the bars represent β-glucosides, 6′′-O-malonyl-β-glucosides, 6′′-O-acetyl-β-glucosides, and aglucones. n = 2.
of β-glucoside conjugates were more than doubled. If the cookie was dry and slightly burnt (after 15 min), the 6-O-MalGlc conjugates and aglucones were increased. As seen for the TVP, under normal cooking conditions total isoflavone concentrations were constant. If the cookie was charred, the isoflavone concentrations were decreased (L. Coward et al., unpublished observations, 1996).

**DISCUSSION**

This study showed that the isoflavone glucoside conjugates were easily altered during extraction, processing, and cooking. The 6-O-MalGlc conjugates were unstable when exposed to heat. Aqueous heating, as seen during extraction with aqueous methanol, caused conversion to the β-glucoside conjugates. Room temperature extraction also led to a loss of the 6OMalGlc

<table>
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<tr>
<th>Food and isoflavone</th>
<th>6OMalGlc</th>
<th>βGlc</th>
<th>6OAcGlc</th>
<th>Aglucone</th>
<th>Total</th>
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<tr>
<td><strong>TSF</strong></td>
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<td>Daidzein</td>
<td>402.0 ± 5.7</td>
<td>609.6 ± 6.4</td>
<td>331.8 ± 4.8</td>
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<td>525.9 ± 6.7</td>
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<td>296.4 ± 1.1</td>
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<td>Glycitein</td>
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<td>ND</td>
<td>242.5</td>
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<td>Soy flour</td>
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<tr>
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<td>Daidzein</td>
<td>366.2 ± 0.1</td>
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<td>ND</td>
<td>114.2</td>
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<td><strong>TVP</strong></td>
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<td>Daidzein</td>
<td>195.4 ± 8.3</td>
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<td>64.9 ± 0.8</td>
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<td>98.4</td>
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</table>

DISCUSSION

This study showed that the isoflavone glucoside conjugates were easily altered during extraction, processing, and cooking. The 6OMalGlc conjugates were unstable when exposed to heat. Aqueous heating, as seen during extraction with aqueous methanol, caused conversion to the β-glucoside conjugates. Room temperature extraction also led to a loss of the 6OMalGlc

![FIGURE 4](image1.png)

![FIGURE 5](image2.png)
conjugates, but at a much slower rate. The 6OMalGlc conjugates were stable for up to 24 h at 4°C, but storage for several days in the cold also led to breakdown (Figure 3).

Isoflavones are found in soybeans as their 6OMalGlc conjugates. The glucoside composition of soyfoods is determined by the processing conditions. Grinding to produce flour and hexane extraction to remove fats does not alter glucoside conjugation. Dry heat, as in toasting of soy flour or extrusion used to produce TVP, caused a loss of carbon dioxide and led to the formation of 6OAcGlc conjugates instead of the 6OMalGlc. Fermentation to produce miso and tempeh, causes a loss of the glucosides. The 6OMalGlc conjugates were most affected, being converted to 6OAcGlc conjugates. The present study showed that the composition of the isoflavone glucoside conjugates can vary from one food to another. Furthermore, cooking procedures may influence which glucoside conjugates are actually consumed by trial subjects. Because the composition of the isoflavone glucoside conjugates will alter the rate of absorption and possibly the degree of further metabolism, these alterations in the chemistry of isoflavones need to be taken into account when interpreting data from clinical trials.

REFERENCES

CHEMICAL MODIFICATION OF ISOFLAVONES 1491S