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# Enhancement of Tofu Isoflavone Recovery by Pretreatment of Soy Milk with Koji Enzyme Extract

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Isoflavones are novel nutraceutical constituents of soybeans, but considerable amounts are lost in the whey during conventional tofu manufacturing. In this study, in a small-scale process, 2 mL of koji enzyme extract (soybean koji/deionized water, 1/3, w/v) was combined with 600 mL of soy milk, and 30 mL aliquots were incubated at 35 °C for 0, 30, 60, 120, and 300 min, for enzyme pretreatment. After each treatment time, soy milk was heated to 85 °C, $CaSO_4$ was added to aggregate protein, and the mixture was centrifuged to separate the solids (tofu) from the whey. The tofu yield and moisture contents from soy milk treated for 30 or 60 min were higher than those from soy milk treated for 0 (control), 120, or 300 min. The protein content of freeze-dried tofu varied in a limited range, and native PAGE and SDS−PAGE patterns revealed slight quantitative and qualitative variations among products. Soy milk daidzein and genistein contents increased while daidzin and genistin contents decreased as the time of enzyme pretreatment of the soy milk increased. After 30 min of pretreatment, daidzin, genistin, daidzein, and genistein contents recovered in tofu products were higher than those of the control. In a pilot-scale process, aliquots (3 L) of soy milk were enzyme-treated for 30 min, aggregated with $CaSO_4$, and hydraulically pressed to remove the whey. As in pretreatments, soy milk daidzein and genistein contents increased while daidzin and genistin contents decreased. In a comparison of the control and enzyme-treated tofu products, the total recoveries of daidzin, genistin, daidzein, and genistein in the tofu products increased from 54.9% to 64.2%. When the tofu products were subjected to a sensory panel test, both products were judged acceptable.

**KEYWORDS: Tofu; isoflavone; soy milk; koji; β-glucosidase; enzyme pretreatment**

## INTRODUCTION

Isoflavones in human diets have been shown to have chemopreventive effects on cardiovascular diseases, osteoporosis, menopausal symptoms, and cancers (*1*−*4*). Soybean is one of the isoflavone-rich crops consumed worldwide. Isoflavone contents of soybeans vary considerably, depending upon variety, location, growing season, climate, cultivation practice, and storage (*5*). Genistin and daidzin constitute 99% of the total isoflavones in soybeans (*6*, *7*). In Asian countries, tofu is a widely consumed soybean product. Considerable amounts of isoflavones are lost in the whey during hydraulic pressing of soybeans to produce tofu (*8*, *9*). Attributes causing loss of isoflavones include differences in chemical polarity, e.g., the glucosidic isoflavones daidzin and genistein are more hydrophilic than their aglycons daidzein and genistein, and other chemical characteristics. Among the isoflavones, genistein has been reported to be more effective than genistin and other test compounds in inhibiting cell growth of human prostate cancer cells (*10*). Therefore, enzymatic transformation of the glucosidic isoflavones to their corresponding aglycons for the purpose of enhancing their functional and nutraceutical effects as well as increasing recovery during tofu manufacturing is worthy of further investigation.

In addition to its popularity in Asian countries, tofu is gaining wide acceptance in the United States and other western countries. According to Wang and Murphy (*11*), each gram of tofu contains 0.532 mg of isoflavones. In another study, the total isoflavone content in raw tofu was 0.297 mg/g, whereas cooked tofu contained 0.258 mg/g (*12*). Coward et al. (*2*) analyzed tofu products of two different brands and reported them to contain 0.031 and 0.015 mg/g genistein and 0.249 and 0.269 mg/g genistin. Variation of isoflavone contents in tofu products was governed by the original content in soybeans and extent of loss in whey during recovery of soybean curd. Most studies have addressed optimization of yield, physicochemical properties, and sensory quality of tofu products as affected by variety, coagulant, and processing parameters (*13*−*19*). Transformation and recovery of isoflavones during tofu preparation as affected

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by enzyme pretreatment of the soy milk for the purpose of enhancing the functional quality of tofu has been given meager attention.

Enrichment of genistein in soy protein concentrate has been achieved using hydrocolloids and β-glucosidase (*20*). Koji is a rich source of β-glucosidase and proteolytic enzymes (*21*, *22*). In this study, on the basis of the fact that daidzein and genistein are less hydrophilic than daidzin and genistin and have more affinity to partition into tofu, recovery of isoflavones in tofu by transformation of the glycosides to their aglycons by koji enzyme pretreatment was investigated. Tofu products were prepared by small-scale and pilot-scale processes. Tofu yields, moisture contents, changes of daidzin, genistin, daidzein, and genistein contents, and proportional recoveries of isoflavones in tofu products were investigated.

## MATERIALS AND METHODS

**Koji Preparation, Koji Enzyme Extraction, and β-Glucosidase Activity Determination.** The procedure of Chiou et al. (*23*) was followed. Briefly, soybeans were soaked in tap water (24−28 °C) for 4 h, drained, and then steam-cooked at 121 °C for 30 min. After being cooled to 40 °C in a bamboo tray, the cooked soybeans were mixed with the conidia of *Aspergillus oryzae* CCRC 33705, a soysauce koji mold (0.1%, mass of conidial powder/mass of soybean prior to soaking). The inoculum was at a population of $10^7$ CFUs/g of cooked soybean, estimated by subjecting the inoculated soybeans to homogenization, serially diluting, surface spreading the samples (0.1 mL) on a yeast malt agar (YMA) plate, and incubating them at 28 °C for 3 days before the colonies were counted. The inoculated soybeans were spread on the tray, covered with two layers of wet cheesecloth, and incubated at ambient temperature (24−28 °C) for 3 days. The koji was then pulverized with a cyclone mill, sealed in polyethylene (PE) plastic bags, and held at −25 °C until use.

For determination of β-glucosidase activity, the procedure described by Nagayami and Saito (*24*) was followed. The substrate solution consisted of 0.1 mM *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) (Sigma Chemical Co., St. Louis, MO) dissolved in 0.1 M sodium phosphate buffer (pH 6.0). For each determination, 2.0 mL of *p*NPG in a 10-mL test tube was adjusted to 30 °C in a water bath for 5 min prior to addition of 0.5 mL of koji enzyme solution. After mixing by vortexing, the tube was further incubated at 30 °C for 30 min, and 2.5 mL of a 0.5 M $Na_2CO_3$ aqueous solution was added to stop further enzyme reaction. The absorbance at 420 nm was measured (Spectrophotometer 2001, Hitachi Co. Ltd., Tokyo, Japan). Enzyme activity was expressed as absorbance units per milliliter of koji enzyme extract.

In a preliminary experiment to optimize the procedure for koji enzyme extraction, aliquots (3 g) of koji powder were homogenized with 3 mL (1:1, w/v), 9 mL (1:3), 15 mL (1:5), 21 mL (1:7), and 27 mL (1:9) of deionized water using a Polytron (PT3000, Kinematica AG, Littau, Switzerland) at 12000 rpm for 1 min. The homogenate was centrifuged (20000*g* at 4 °C) for 15 min (SCR 20B, Hitachi). The supernatant was analyzed for β-glucosidase activity. The β-glucosidase activity decreased with an increase of the water:powder ratio used for enzyme extraction. The higher the water:powder ratio, the larger the supernatant volume after centrifuging, and the lower the β-glucosidase activity per unit supernatant volume was achieved. From a practical viewpoint, with an attempt to maintain high enzyme activity and volume recovered, the koji enzyme extract prepared using a ratio of 1:3 (w/v) was used in the following experiments.

**Preparation of Soy Milk and Tofu by a Small-Scale Process.** Soy milk and tofu were prepared following a procedure modified from that reported by Mullin et al. (*18*). Soybeans (200 g) were soaked in 800 mL of tap water (24−28 °C) for 8 h. The drained soybeans were combined with a sufficient amount of deionized water to reach 2 kg of total mass, and the mixture was homogenized with a juice blender (YS-9828MX, Yen-Sun Industrial Co., Kaohsiung, Taiwan) set at the highest speed for 5 min. The °Brix of soy milk was measured with a refractometer (Hand Refractometer, Atago Co., Ltd., Tokyo, Japan), and sufficient deionized water was added to adjust the °Brix between 6 and 7. The homogenate was heated to 95 °C with constant stirring and held with occasional stirring for 10 min. Then the homogenate was filtered through a cloth, and the filtrate was cooled with occasional stirring to 35 °C at ambient temperature.

Soy milk (600 mL) was taken and mixed thoroughly with 2 mL of koji enzyme extract (0.33%, v/v). After vigorous stirring, aliquots (30 mL) were dispensed into a series of 100 mL bottles and incubated at 35 °C for 0, 30, 60, 120, and 300 min. After each treatment time, the soy milk was heated to 85 °C in a water bath set at 95 °C and held for 10 min prior to being transferred to 50 mL centrifuge tubes. A sample of treated soy milk (1 mL) was deposited into a 10 mL centrifuge tube containing 4 mL of methanol to analyze the isoflavone contents, 0.075 g of $CaSO_4 \cdot 2H_2O$ in 1 mL of warm deionized water was added (equivalent to 0.25% soy milk), and the mixture was agitated rapidly for 10 s with a stirring bar. After 15 min, the tubes were centrifuged (11000*g*, 15 min at 20 °C) (SCR 20B, Hitachi) to separate the whey (supernatant) and tofu (pellet).

The volume of whey was measured before storing it at 4 °C for determination of the isoflavone and soluble protein contents. The tofu was removed from the tube, placed in a preweighed flask, frozen at −30 °C, and freeze-dried. Subsequent mass determination enabled the moisture contents of tofu to be determined. The freeze-dried tofu was pulverized and stored at −25 °C for further analyses.

**Determination of Daidzin, Genistin, Daidzein, and Genistein Contents of Soy Milk, Whey, and Tofu Products.** The procedure of Chiou and Cheng (*21*) was followed. Using a 10 mL Teflon centrifuge tube, 100 mg of lyophilized, pulverized tofu powder was deposited and mixed with 4 mL of 80% methanol (v/v, with deionized water). After homogenization with a Polytron homogenizer equipped with an aggregate probe (PT-DA 3007/2) operated at 15000 rpm for 1 min, an additional 1 mL of 80% methanol used to clean the probe was added. The tubes were screw-capped and heated in a water bath at 70 °C for 30 min. During heating, the tubes were inverted by hand for agitation at 5 min intervals. The tubes were centrifuged at 20 °C at 15000 rpm for 30 min. A 1 mL sample was withdrawn from the middle layer and membrane-filtered (0.45 μM) for HPLC analysis. For extraction of isoflavones from the soy milk and whey samples, 1 mL of sample was deposited in a 10 mL centrifuge tube, 4 mL of methanol (100%) was added, and the tube was screw-capped. After vortexing, the tube was heated at 70 °C for 30 min following the procedure described above to extract isoflavones for quantification.

The filtrate was diluted 5-fold with 80% methanol, and 20 μL of the solution was injected into an HPLC system (Hitachi L-7100 pump, L-7420 UV−vis detector, and L-7455 diode array detector) for analysis. A reversed-phase $C_{18}$ column (250 mm × 4 mm, Thermal Hypersil ODS) was used. A gradient solvent system started with 20% solvent A (methanol) and 80% solvent B (water) and progressed to 80% A and 20% B within 16 min followed by holding for an additional 2 min. The flow rate was 1.0 mL/min. Standard daidzin, genistin, daidzein, and genistein (Sigma Chemical Co.) were run under identical conditions for quantitative and qualitative analysis. Isoflavone contents were expressed as micromoles per 100 mL of soy milk or per gram of the freeze-dried tofu powder, and recovery was estimated.

**Determination of Crude Protein Content, Extraction of Tofu Protein, and Gel Electrophoresis.** The soluble protein content in the whey was determined by the method of Bradford (*25*), following the product manual, and estimated using bovine serum albumin (BCA Protein Assay Reagent, Pierce, Rockford, IL) as a standard. The total nitrogen content of the freeze-dried tofu was determined with the Kjeldahl method, and the crude protein content was determined by multiplying the nitrogen content by 6.25.

For extraction of proteins from the freeze-dried tofu, 150 mg of tofu powder was weighed and combined with 4 mL of a buffer solution containing 5 mM $MgCl_2$, 5 mM EDTA, and 1% Triton X-100 (all dissolved in 10 mM Tris buffer, pH 7.5) in a screw-capped 10 mL centrifuge tube. After the reaction mixture was homogenized with a Polytron at 12000 rpm for 1 min, the tube was centrifuged (11000*g* at 20 °C for 15 min). The supernatant was withdrawn and subjected to native polyacrylamide gel electrophoresis (PAGE) and SDS−PAGE following published procedures (*26*, *27*). For native PAGE, 20 μL of the protein extract was mixed with 10 μL of tracking dye, and 20 μL

**Table 1.** Yields and Moisture Contents of Tofu Products Prepared by a Small-Scale and a Pilot-Scale Process and Volumes and Protein Contents of Whey after Pretreatment of the Soy Milk with Koji Enzyme Extract for Various Times[a,b]

| | enzyme pretreatment of | | | | |
|---|---|---|---|---|---|
| | 0 min | 30 min | 60 min | 120 mi | 300 min |
| yield of tofu prepared by a small-scale process, g of tofu/100 mL of soy milk | 29.4 ± 0.8b | 31.2 ± 0.1a | 31.1 ± 0.1a | 29.5 ± 0.4b | 24.6 ± 0.5c |
| moisture content of tofu prepared by a small-scale process, % | 80.1 ± 0.3b | 82.3 ± 0.6a | 81.5 ± 0.1a,b | 81.0 ± 0.3b | 78.3 ± 0.3c |
| protein content of the freeze-dried tofu prepared by a small-scale process, % | 48.0 ± 0.5a | 47.4 ± 0.2a | 47.9 ± 0.2a | 48.0 ± 0.1a | 48.1 ± 0.4a |
| volume of whey prepared by a small-scale process, mL/100 mL of soy milk | 68.4 ± 0.4c | 67.9 ± 0.9c | 68.8 ± 0.1c | 70.2 ± 1.4b | 73.2 ± 1.2a |
| protein content of whey prepared by a small-scale process, mg/mL | 6.00 ± 0.05e | 6.13 ± 0.02d | 6.31 ± 0.09c | 6.59 ± 0.04b | 7.55 ± 0.01a |
| yield of tofu prepared by a pilot-scale process, g of tofu/100 mL of soy milk | 28.5 ± 1.3a | 27.9 ± 1.0a | | | |
| moisture content of tofu prepared by a pilot-scale process, % | 80.5 ± 1.0a | 79.5 ± 0.5a | | | |

[a] Means ± SD of four determinations of two replicate experiments. [b] Values in the same row with different letters are significantly different ($p < 0.05$).

of the mixture was loaded into each well of a 12% polyacrylamide gel for electrophoresis. For SDS−PAGE, 20 μL of the protein extract was mixed with 10 μL of Laemmli tracking dye supplemented with 10% (v/v) mercaptoethanol and heated in a water bath at 95 °C for 5 min. After the mixture was cooled to ambient temperature, 20 μL was loaded into each well of a 12% polyacrylamide gel. Electrophoresis was conducted at 200 V using a vertical electrophoresis system (Hoefer miniEV, Hoefer Pharmacia Biotech Inc., San Franscisco, CA). After electrophoresis, the gels were stained with colloidal Coomassie Blue (containing 0.1% Coomassie Brilliant G-250, 0.2% phosphoric acid, 10% ammonium sulfate, and 20% methanol) overnight and destained with a solution containing 1% acetic acid and 1% glycerol. A low molecular weight marker (LMW, Amersham Biosciences Co., Piscataway, NJ) was run concurrently as a reference of molecular weight.

**Tofu Preparation by a Pilot-Scale Process.** For preparing tofu using a simulated conventional process, 2 kg of soybeans were soaked in 8 L of tap water at ambient temperature (24−28 °C) for 8 h, drained, and combined with a sufficient amount of deionized water to reach 20 kg. After the soybeans and water were homogenized with a juice blender (YS-9828MX, Yen-Sun) set at the highest speed for 5 min, the homogenate was cooked on a stove with constant stirring to raise the temperature to 95 °C in 20−30 min and held for an additional 15 min. The cooked homogenate was pressed through a filtration cloth. After the °Brix of the soy milk was measured, an appropriate amount of deionized water was added to the filtrate to adjust the °Brix to between 6 and 7, followed by cooling to 35 °C before koji enzyme extract was added.

Aliquots (3 L) of the soy milk at 35 °C were supplemented with 10 mL of koji enzyme extract and rapidly stirred. Right after enzyme supplementation, samples (1.0 mL) were deposited in 10 mL centrifuge tubes containing 4 mL of methanol to extract isoflavone. The soy milk aliquots (3 L) were incubated at 35 °C with manual stirring for 30 min. After further sampling of 1 mL of the soy milk into a 10 mL centrifuge tube containing 4 mL of methanol to extract isoflavone for analysis, the soy milk was heated to 85 °C and combined with 7.5 g of $CaSO_4 \cdot 2H_2O$ (the fine powder was suspended in 100 mL of warm deionized water, equivalent to 0.25% soy milk) as a protein coagulant, followed by vigorous stirring for 10 s. Protein coagulation was allowed to proceed for 15 min before the mixture was poured into a wooden tofu mold (21 cm × 21 cm × 11 cm) lined with a filtration cloth. A lid weighted with a bucket containing 15 kg of water was placed on top of the coagulated soy milk for 40 min to expel the whey. The curd was weighed to determine the tofu yield and cut into cubes (0.5 × 0.5 × 0.5 $cm^3$), half of which was subjected to an organoleptic acceptance test and the other half of which was freeze-dried to determine the moisture content. The dried tofu was weighed for estimation of the moisture content and pulverized for isoflavone analysis.

For preparation of control products (no enzyme pretreatment), soy milk (3 L) was cooked on a stove to 85 °C, after which 10 mL of the koji enzyme extract was added. After vigorous stirring, 1 mL of the tested soy milk was deposited in a 10 mL centrifuge tube containing 4 mL of methanol (100%) for isoflavone determination. The soy milk held at 85 °C was combined with $CaSO_4 \cdot 2H_2O$ to prepare tofu following the procedure described above.

**Organoleptic Acceptance Test.** The freshly prepared enzyme-treated (30 min of enzyme pretreatment) and control (0 min) tofu products prepared by the pilot-scale process were cut into cubes (0.5 × 0.5 × 0.5 $cm^3$) and subjected to organoleptic acceptance tests. A drop of soysauce was deposited onto each cube prior to evaluation, which was acclimating to a cold serving dish. Each of 18 panelists who were familiar with tofu products was invited to taste three cubes of each tofu product and subjectively evaluate the overall acceptance according to three categories, i.e., fully accepted, accepted, and poorly accepted. Prior to the organoleptic acceptance test, a commercial tofu bought from a local supermarket was used as a reference of a fully acceptable product to train the panelists.

**Statistics.** At least duplicate experiments and duplicate determinations for each experiment were conducted. Means of values with standard deviation are reported. Duncan's multiple range tests (SAS) were applied to analysis of variance (ANOVA) among the treatments, and a significance of 95% ($p = 0.05$) is reported.

## RESULTS AND DISCUSSION

The yields and moisture contents of tofu prepared by a small-scale process as affected by pretreatment of the soy milk with koji enzyme extract are shown in **Table 1**. The yields of the 30 and 60 min pretreated products were significantly higher than that of the control (0 min of enzyme pretreatment) ($p < 0.05$). Generally, the higher the tofu yield, the higher the moisture content was observed. Since all products were subjected to identical centrifugation conditions to separate whey and tofu, it is apparent that the water-holding capacity was enhanced by subjecting soy milk to pretreatment with koji enzymes for an appropriate time. Results from replicate determinations and duplicate experiments for each product deviated in a limited range; centrifuging to pellet protein aggregates to form tofu and separate whey in a laboratory gave consistent and reproducible products. The yield and moisture content as affected by a wide spectrum of variables have been extensively investigated (*13*−*19*). In those studies, whey was separated from aggregates by hydraulically pressing, a process similar to a conventional practice.

Changes in protein contents of tofu products were limited (**Table 1**). Native PAGE analysis (**Figure 1**) of proteins showed that large molecular proteins (higher than 97.0 kDa) were dominant for all products. A quantitative and qualitative comparison of samples as affected by enzyme pretreatment was

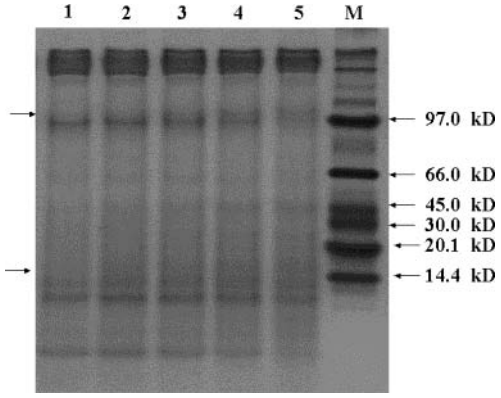


**Figure 1.** Native polyacrylamide gel electrophoretic patterns of the extracted tofu proteins as affected by pretreatment of the soy milks with koji enzyme extract for various times: 1, control; 2, 30 min; 3, 60 min; 4, 120 min; 5, 300 min; M, protein marker.

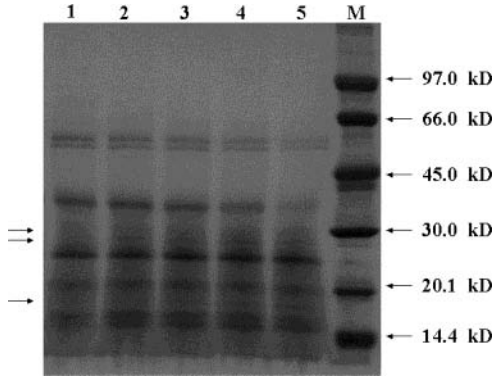


**Figure 2.** SDS–polyacrylamide gel electrophoretic patterns of the extracted tofu proteins as affected by pretreatment of the soy milks with koji enzyme extract for various times: 1, control; 2, 30 min; 3, 60 min; 4, 120 min; 5, 300 min; M, protein marker.

made. The native protein patterns of control and 30 min treated tofu products varied in a limited range, except that a band with a molecular mass of ca. 100 kDa and slightly more proteins with molecular masses around 14.4 kDa (both indicated by arrows on the left of **Figure 1**) were visualized in 30 min treated products (bands 1 and 2). For the 60−300 min treated products, in comparison to the control products, proteins with molecular masses in the range of 14.4−45.0 kDa increased, indicating that soy milk proteins were hydrolyzed by pretreatment with enzymes.

The protein extracts were subjected to SDS−PAGE analysis. Profiles are shown in **Figure 2**. In comparison to control products, as affected by enzyme pretreatment (lanes 1 and 2), two bands of protein with molecular masses of ca. 28 and 30 kDa appeared after 30 min of pretreatment (indicated by arrows on the left of **Figure 2**). After 60 min of pretreatment, an additional band with a molecular mass of ca. 17 kDa appeared. After 300 min of enzyme pretreatment, proteins with molecular masses higher than 30 kDa were extensively hydrolyzed. Since the soybean koji also contained potential proteolytic enzymes, it was inevitable that the soy milk proteins were hydrolyzed. This was further evidenced by the fact that longer enzyme pretreatment times resulted in higher whey protein content and lower tofu yield (**Table 1**).

Daidzin, genistin, daidzein, and genistiein contents in soy milk prepared by a small-scale process as affected by pretreatment of the soy milk with koji enzyme extract are shown in **Table 2**. Initially (0 min of pretreatment), daidzin, genistin, daidzein, and genistein contents were 28.1, 36.1, 15.7, and 16.3 $\mu$mol/100 mL of soy milk, respectively. In raw soybean seeds, the glucosidic conjugates of isoflavone are more predominant than their aglycons (*21*, *28*). Genistin and daidzin constitute 99% of the total isoflavones in dry soybeans (*6*). After pretreatment of soy milk with koji enzyme extract for 120 min, daidzin and genistin contents decreased, and consequently, levels of their hydrolyzed counterparts daidzein and genistein increased significantly ($p < 0.05$). It is obvious that increases of daidzein and genistein contents in the soy milk were enhanced by the action of $\beta$-glucosidase originating from *A. oryzae* during koji preparation (*29*). This was in agreement with the observation that daidzein and genistein contents increased while daidzin and genistin contents decreased during fermentation of miso containing soybean koji (*21*). On a molecular basis, the total isoflavone content of the soy milk decreased slightly as a result of pretreatment with koji enzyme. This may be due to further chemical alteration of the isoflavones by koji enzyme extract.

**Table 2.** Daidzin, Genistin, Daidzein, and Genistein Contents ($\mu$mol/100 mL of Soy Milk) of Soy Milks Prepared by a Small-Scale Process for Tofu Production As Affected by Pretreatment of the Soy Milk with Koji Enzyme Extract for Various Times[a,b]

| | enzyme pretreatment of | | | | |
|---|---|---|---|---|---|
| isoflavone | 0 min | 30 min | 60 min | 120 min | 300 min |
| daidzin | 28.1 ± 1.7a | 17.0 ± 0.5b | 13.1 ± 0c | 9.1 ± 0.4d | 9.3 ± 0.4d |
| genistin | 36.1 ± 1.8a | 24.3 ± 0.6b | 18.7 ± 0.1c | 13.6 ± 0.2d | 10.7 ± 1.2e |
| daidzein | 15.7 ± 0.4e | 23.8 ± 0.7d | 28.2 ± 0c | 32.1 ± 1.0b | 34.6 ± 0.5a |
| genistein | 16.3 ± 1.5e | 25.1 ± 0.1d | 29.7 ± 0.30c | 34.1 ± 0.8b | 36.0 ± 0.8a |
| total | 96.2 | 90.2 | 89.7 | 88.9 | 90.6 |

[a] Means ± SD of four determinations of two replicate experiments. [b] Values in the same row with different letters are significantly different ($p < 0.05$).

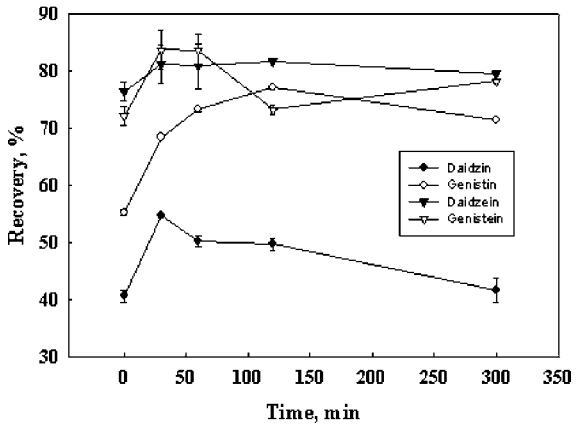


**Figure 3.** Molecular recoveries of daidzin, genistin, daidzein, and genistein from soy milks to tofu products prepared by a small-scale process as affected by the time of pretreatment of the soy milks with koji enzyme extract.

Alteration of soy milk proteins as well as other constituents caused by pretreatment with koji enzymes might further influence isoflavone recovery from soy milk to tofu. Recovery of daidzin, genistin, daidzein, and genistein as affected by the time of koji enzyme pretreatment were therefore investigated. Recovery was estimated by dividing the micromoles of each isoflavone in tofu products by the micromoles in the soy milks after enzyme pretreatment and heating to 85 °C but prior to the addition of $CaSO_4$ for protein coagulation. In general, except that of genistein after 120 min of pretreatment, the molecular recoveries of daidzein and genistein were higher than those of daidzin and genistin (**Figure 3**). This indicates that daidzein

**Table 3.** Recoveries of Daidzin, Genistin, Daidzein, and Genistein (on the Basis of 100 mL of Soy Milk) from Soy Milks to the Tofu Products Prepared by a Pilot-Scale Process As Affected by Pretreatment of the Soy Milk with Koji Enzyme Extract for 30 min[a]

| | daidzin | genistin | daidzein | genistein | total |
|---|---|---|---|---|---|
| soy milk (0 min), $\mu$mol | 27.6 ± 2.2 | 34.8 ± 2.3 | 16.9 ± 1.2 | 15.2 ± 1.8 | 94.5 |
| soy milk (30 min), $\mu$mol | 13.2 ± 2.1 | 26.7 ± 2.0 | 26.0 ± 1.6 | 21.7 ± 1.0 | 87.6 |
| tofu (0 min), $\mu$mol | 9.3 ± 1.8 | 20.1 ± 1.2 | 12.3 ± 0.4 | 10.2 ± 0.3 | 51.9 |
| tofu (30 min), $\mu$mol | 4.8 ± 0.2 | 14.1 ± 0.2 | 19.9 ± 0.7 | 17.4 ± 0.6 | 56.2 |
| tofu isoflavone recovery (0 min), % | 33.7 | 57.8 | 72.8 | 67.1 | 54.9 |
| tofu isoflavone recovery (30 min), % | 36.4 | 52.8 | 76.5 | 80.2 | 64.2 |

[a] Means ± SD of four determinations of two replicate experiments.

and genistein partitioned into tofu rather than whey. Interestingly, after 30 min of enzyme pretreatment, recovery of daidzin, genistin, daidzein, and genistein increased from 40.6% to 54.7%, from 55.3% to 68.5%, from 76.5% to 81.2%, and from 72.2% to 83.8%, respectively. However, after prolonged times of enzyme pretreatment, the recoveries varied. It is apparent that recovery of isoflavones from soy milk to tofu varied according to the nature of individual isoflavones and the physicochemical status of soy milk after enzyme pretreatment.

The tofu yield, moisture content, and daidzin, genistin, daidzein, and genistein recoveries from soy milk to tofu products prepared by a pilot-scale process, as affected by pretreatment of the soy milk with koji enzyme extract, are shown in **Tables 1** and **3**. The tofu yields and moisture contents varied insignificantly ($p > 0.05$) with 30 min of enzyme pretreatment, which is not in agreement with results obtained using a small-scale process. Apparently, tofu products prepared by the conventional pilot-scale process, i.e., hydraulic pressing, to remove whey were less consistent than products prepared by the small-scale process. Using the pilot-scale process, after pretreatment of soy milk with koji enzyme for 30 min, daidzin and genistin contents decreased and daidzein and genistein contents increased (**Table 2**). When estimated on a molecular basis, recovery from soy milk as affected by 30 min of enzyme pretreatment changed from 33.7% to 36.4%, from 57.8% to 52.8%, from 72.8% to 76.5%, and from 54.9% to 64.2% for daidzin, genistin, daidzein, and genistein, respectively. This is in agreement with observations using the small-scale process (**Table 2**). It is of merit to note that, after enzyme pretreatment of the soy milk caused isoflavone transformation, the overall molecular recovery from soy milk to tofu increased from 54.9% to 64.2%. On a molecular basis, glycosidic isoflavones are more hydrophilic than their aglycons, and thus, the aglycons have slightly more affinity for tofu (protein aggregates) than whey (aqueous fractions) during tofu processing. Isoflavone aglycons, e.g., genistein and daidzein, are generally believed more physiologically active than genistin and daidzin (*30*, *31*). Genistein has been reported to be much more effective than genistin in inhibiting cell growth of human prostate cancer (*10*).

When the control and enzyme-treated tofu products were subjected to an organoleptic evaluation, both products were fully accepted in comparison to a commercial tofu (data not shown). Even though soybean proteins were altered slightly after 30 min of enzyme treatment as evidenced by a slight increasing soluble protein in whey (**Table 1**) and the fact that tofu proteins varied slightly, as visualized in SDS−PAGE and native PAGE profiles (**Figures 1** and **2**), it appears that minor protein alterations did not influence organoleptic acceptance of the resultant tofu products. From the viewpoint of functional food development, it is important to note that enhancement of isoflavone transformation and recovery as well as retaining the tofu yield and sensory acceptance can be achieved by pretreatment of the soy milk with koji enzyme for 30 min. Since the strain of *A. oryzae* used in this study was one used to prepare soysauce koji and has been phenotypically and genotypically determined to be a nonaflatoxigenic producer (*32*), the use of the koji enzyme extract in tofu manufacturing is not a food safety concern.

In conclusion, minimizing isoflavone loss in whey during tofu manufacturing is important to processors and consumers. Soybean koji is a potent and economic source of $\beta$-glucosidase for pretreatment of soy milk destined for tofu preparation to enhance tofu isoflavone recovery. However, in addition to $\beta$-glucosidase, koji also contains many other enzymes, such as proteases that may hydrolyze soy milk proteins simultaneously and result in protein loss. When transformation of glucosidic isoflavones to their aglycons is desired, a purified $\beta$-glucosidase would be more specific than the crude koji enzyme extract. Nevertheless, tofu is a very common, popular, traditional food, and consumers have the impression that tofu products are generally affordable. Thus, from a practical and economic viewpoint, pretreatment of soy milk with koji enzyme extract for 30 min is recommended as an effective means to enhance isoflavone recovery and retain the tofu yield and sensory quality.

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