# Isoflavone Transformation during Soybean Koji Preparation and Subsequent Miso Fermentation Supplemented with Ethanol and NaCl

Robin Y.-Y. Chiou*,† and S.-L. Cheng‡

Graduate Institute of Biotechnology and Department of Food Science, National Chiayi University, Chiayi, Taiwan, Republic of China

Soybeans were soaked with water for 4 h, steam-cooked, inoculated with the conidia of *Aspergillus oryzae*, and incubated for 3 days for koji preparation. The koji was then mixed with water-soaked and steam-cooked soybeans (1:2, w/w), ground into paste, and supplemented with 15% ethanol and 12.5% NaCl or 3% ethanol and 6% NaCl for miso fermentation at 30 °C. Daidzin, genistin, daidzein, and genistein contents were extracted from the lyophilized and pulverized soybean powder or from the miso homogenate by a developed one-tube procedure and analyzed with an HPLC. After water soaking, daidzein and genistein contents increased markedly, whereas daidzin and genistin contents decreased. Further increases of daidzein and genistein contents and decreases of daidzin and genistin contents were observed after koji mold growth. During fermentation, fungal and lactic acid bacterial (LAB) growth in the miso products was inhibited, whereas soluble protein contents increased much more rapidly in the low-salt miso products supplemented with 3% ethanol and 6% NaCl than the other products. When the 4- and 8-week-fermented miso products were cooked with tofu for sensory evaluation, flavor ratings of the low-salt products were higher than that of a popular commercial product. In both products, the most daidzins and genistins were hydrolyzed after 4 weeks of fermentation. The hydrolytic enzymes contributing to isoflavone transformation originated from soybeans after water soaking and from koji with mold growth. It was of merit that the low-salt fermented products were fairly acceptable in flavor rating and rich in daidzein and genistein contents after 4 weeks of fermentation.

**Keywords:** *Isoflavone; miso; low-salt fermentation; koji; HPLC*

## INTRODUCTION

Soybean-based foods contain isoflavones that can confer significant health benefits. Their presence in the soybean-based foods and effectiveness in anti-carcinogenesis have attracted worldwide concerns. The isoflavones in soybean have been suggested as the compounds that delay the onset of certain types of cancer (*1*). In Asian countries, soybeans are largely consumed as tofu, soy sauce, miso, natto, soy milk, and tempeh. Genistin and daidzin constitute 99% of total isoflavones in the dry soybeans (*2*). Among the isoflavones, genistein has been reported to be the most effective in inhibiting cell growth of human prostate cancer cells compared to genistin (*3*). In a comparison of the commercial soybean products, higher genistein contents are observed in the fermented foods (soy sauce is not included) than in the unfermented foods (*4*, *5*). It is of merit to enhance genistein formation through fermentation. The higher genistein contents in the fermented foods are assumed to be the contribution of microbial enzymes for hydrolysis of genistin to form genistein during fermentation. During normal fermentation, enzymes could be contributed by the koji enzymes and from the enzymes produced by the halophilic yeasts and lactic acid bacteria (LAB). In the literature, transformation of isoflavones of soybeans during koji preparation and subsequent fermentation has been meagerly studied.

Ethanol supplemented at an appropriate level has a dual function in preserving against spoilage yet allowing enzymes originating from koji to contribute to the development of miso fermentation (*6*). The supplemented ethanol was also effective in inhibiting growth of the halophilic yeasts and LAB. Thus, the involvement of halophilic yeasts and LAB could be minimized by supplementation with ethanol and NaCl. In this study, changes of the microbial (fungal and LAB) populations and daidzin, genistin, daidzein, and genistein contents in the miso products supplemented with 15% ethanol and 12.5% NaCl or 3% ethanol and 6% NaCl during fermentation were periodically determined. For tracing the $\beta$-glucosidase-like enzyme sources prior to fermentation, changes of the daidzin, genistin, daidzein, and genistein contents in the soybeans after soaking with water, subsequent steam-cooking, artificial inoculation, and incubation with *Aspergillus oryzae* for koji preparation were investigated. In addition to the enhancement of genistein formation, soluble protein contents, pH values, and sensory flavor quality of the fermented products were also determined.

## MATERIALS AND METHODS

**Koji Preparation.** Soybean koji was prepared following the previous procedure (*7*). Soybeans were soaked with tap water (25−28 °C) for 4 h, drained, and then steam-cooked at 121 °C

* Author to whom correspondence should be addressed (telephone 8865-2857976; fax 8865-2775524; e-mail rychiou@mail.ncyu.edu.tw).

† Graduate Institute of Biotechnology.

‡ Department of Food Science.

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**Table 1. Changes of Daidzin, Genistin, Daidzein, and Genistein Contents during Soybean Koji Preparation**

| | mg/g of dry matter[a] | | | |
|---|---|---|---|---|
| treatment | daidzin | genistin | daidzein | genistein |
| soybeans prior to treatment | 0.270 ± 0.007 | 0.200 ± 0.003 | 0.059 ± 0.003 | 0.143 ± 0.005 |
| soybeans after 4 h of soaking in water | 0.181 ± 0.016 | 0.181 ± 0.023 | 0.118 ± 0.012 | 0.246 ± 0.017 |
| soybeans after steam-cooking at 121 °C for 30 min | 0.221 ± 0.019 | 0.195 ± 0.004 | 0.099 ± 0.002 | 0.196 ± 0.004 |
| soybeans after inoculation with *A. oryzae* | 0.213 ± 0.012 | 0.177 ± 0.005 | 0.087 ± 0.002 | 0.186 ± 0.003 |
| soybeans after 1 day of incubation | 0.132 ± 0.005 | 0.134 ± 0.007 | 0.101 ± 0.005 | 0.195 ± 0.009 |
| soybeans after 2 days of incubation | 0.114 ± 0.001 | 0.122 ± 0.001 | 0.119 ± 0.002 | 0.238 ± 0.003 |
| soybeans after 3 days of incubation | 0.104 ± 0.004 | 0.122 ± 0.001 | 0.110 ± 0.001 | 0.236 ± 0.001 |

[a] Mean of determinations with standard deviation ($n = 3$).

for 30 min. After cooling on a bamboo tray, the cooked soybeans were mixed with the conidia of *A. oryzae* CCRC 33705, a soy sauce koji mold, spread on the tray, covered with two layers of wet cheesecloth, and incubated at the ambient temperature (23−29 °C) for 3 days.

During incubation, triplicate samples were taken and lyophilized with a freeze-dryer. The dried soybeans were pulverized into powder with a coffee mill. The residual moisture contents of the pulverized powders were further determined by heating subsamples at 85 °C until constant weight was reached. Then the dry matter contents of the powders were determined.

**Miso Preparation.** Steam-cooked and cooled soybeans (~40 °C) were mixed with the prepared soybean koji (w/w, 2:1), ground into paste with a KitchenAid mixer, and used as the base substrate for miso preparation. Aliquots (60 g) of the base miso paste deposited into a series of glass jars were combined with 80 mL of 30% ethanol and 20 g of NaCl, resulting in 15% ethanol and 12.5% NaCl (w/w), or combined with 2.02 mL of 95% ethanol and 3.96 g of NaCl, resulting in 3% ethanol and 6% NaCl. Each sample was manually mixed and pressed, screw-capped, and incubated at 30 °C for fermentation.

**Characterization of Soybean Miso Supplemented with Ethanol and NaCl.** During fermentation, at least two jars from each treatment were periodically taken for characterization. For the jar fermented with 3% ethanol and 6% NaCl, an additional 77.98 mL of deionized and membrane-filtered (0.2 μM) water was refilled to make up 80 mL of the initial liquid volume. Then each jar was homogenized with a Polytron (Kinematica AG, Luzern, Switzerland) equipped with an aggregate probe (PT-DA 3012/2S) operated at 12000 rpm for 1 min. From the homogenates, enumeration of fungal and LAB populations and determinations of the soluble protein content and pH values were conducted following the previously reported procedures (*8*).

**Isoflavone Analysis.** According to an extensive trial conducted in the laboratory as a preliminary study, a one-tube procedure for extraction of the isoflavones including daidzin, genistin, daidzein, and genistein from the lyophilized soybean powders and from the miso homogenates was developed by modifying the procedure of Lu et al. (*9*). Using a 10-mL Teflon centrifuge tube (Nalgene 3110), 100 mg of the lyophilized and pulverized soybean powder was deposited and mixed with 4 mL of 80% methanol. After homogenization with a Polytron homogenizer equipped with an aggregate probe (PT-DA 3007/2) operated at 15000 rpm for 1 min, 1 mL of 80% methanol was applied for cleaning the aggregate probe and pooled. 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 every 5 min. Then the tubes were centrifuged at 20 °C at 15000 rpm for 30 min. One milliliter was withdrawn from the middle layer and membrane-filtered (0.45 μM) for HPLC analysis.

For extraction from the miso products, 1 mL of the homogenate was deposited in a 10-mL centrifuge tube and replenished with 3 mL of methanol (100%). After homogenization with a Polytron homogenizer equipped with an aggregate probe (PT-DA 3007/2) operated at 15000 rpm for 1 min, 1 mL of methanol was applied for cleaning the aggregate probe and pooled, resulting in 80% methanol of the final solution. The following extraction procedure was the same as that described above.

After 5-fold dilution of the filtrate with 80% methanol, 20 μL of the solution was injected into an HPLC system for analysis. A reversed phase $C_{18}$ column (LiChroCART 250-4, E. Merck, Darmstadt, Germany) was run with a gradient solvent system initiated with 95% of solvent A (0.1 M ammonium acetate/methanol, 60:40, v/v) and 5% of solvent B(methanol) to 70% A and 30% B in 20 min (Hitachi L-7100 pump, Tokyo, Japan). The flow rate was set at 1.2 mL/min. Standard daidzin, genistin, daidzein, and genistein (Sigma Chemical Co., St. Louis, MO) were run simultaneously for quantitative and qualitative analysis.

**Sensory Flavor Evaluation.** The miso products after 4 and 8 weeks of fermentation were homogenized as above and subjected to sensory flavor evaluation. Freshly prepared tofu was cut into fine cubes (~0.2 $cm^3$). Tap water (200 mL) and 20 g of the tofu cubes were combined and heated on a gas stove to near boiling. When the boiling point was reached, 20 mL of the homogenized miso was added and mixed and the heat was removed. The tofu−miso soups were immediately evaluated by seven panelists who were familiar with miso characteristics. Panelists evaluated the products according to the intensity of the unique pleasant volatiles and rated on a five-point scale in which 5, 4, 3, 2, and 1 indicate very strong and pleasant, strong and pleasant, fair, poor, and very poor, respectively. A popular commercial product obtained from the local market was prepared by homogenizing 60 g of miso with 80 mL of deionized water and used as a reference for evaluation and rated as 3.

**Statistics.** At least duplicate experiments were conducted. Means of determinations with standard deviation are reported.

## RESULTS AND DISCUSSION

Changes of daidzin, genistin, daidzein, and genistein contents during soybean koji preparation are shown in Table 1. When the soybeans were soaked with water for 4 h, daidzein contents increased from 0.059 to 0.118 mg/g and genistein contents increased from 0.143 to 0.246 mg/g. Meanwhile, daidzin and genistin contents in the soybeans decreased. Increases of daidzein and genistein contents in the soy protein concentrates can be enhanced by the incorporation of β-glucosidase during soy protein isolation (*10*). On the basis of the fact that daidzein and genistein contents increased in the soybeans after soaking with water for 4 h, β-glucosidase-like enzymes must be present in the soybeans and

**Table 2. Changes of Fungal and Lactic Acid Bacterial (LAB) Populations during 8 Weeks of Fermentation of the Soybean Miso Supplemented with Various Levels of Ethanol and NaCl**

| | microbial population[a] (CFU/g) | | | | |
|---|---|---|---|---|---|
| soybean miso | 0 weeks | 1 week | 2 weeks | 4 weeks | 8 weeks |
| | | Fungal Population | | | |
| with E15N12.5[b] | $1.0 \times 10^5$ | nd | nd | nd | nd |
| with E3N6[c] | $(2.9 \pm 1.2) \times 10^7$ | $(1.8 \pm 0.6) \times 10^6$ | $(2.0 \pm 0.1) \times 10^4$ | nd | nd |
| | | LAB Population | | | |
| with E15N12.5[b] | $1.0 \times 10^4$ | nd | nd | nd | nd |
| with E3N6[c] | $(1.7 \pm 0.3) \times 10^5$ | $(3.6 \pm 3.4) \times 10^4$ | nd | nd | nd |

[a] Mean of determinations with standard deviation ($n = 3$); the detection limits were $10^3$ CFU/g for the fungal population and $10^4$ CFU/g for LAB; nd, not detectable. [b] E15N12.5 = miso supplemented with 15% ethanol and 12.5% NaCl. [c] E3N6 = miso supplemented with 3% ethanol and 6% NaCl.

activated by the soaking with water. To confirm this hypothesis, the soybeans were cooked in boiling water for 10 min and subsequently soaked with water at the ambient temperature for 4 h. As a result, daidzein and genistein contents did not increase (data not shown). This was further support for the likely presence of $\beta$-glucosidase-like enzymes in the soybeans and also in agreement with the results of Matsuura and Obata (*11*), who have partially purified three isoforms of $\beta$-glucosidase from soybean cotyledons. When the rehydrated soybeans were steam-cooked at 121 °C for 30 min, daidzein and genistein contents slightly decreased, indicating that they were fairly resistant to heat treatment.

When the steam-cooked and cooled soybeans were artificially inoculated with the conidia of *A. oryzae* and incubated at 28 °C for 3 days, daidzein and genistein contents increased from 0.087 to 0.110 mg/g and from 0.186 to 0.236 mg/g, whereas daidzin and genistin contents decreased from 0.213 to 0.104 mg/g and from 0.177 to 0.122 mg/g, respectively. Because the $\beta$-glucosidase-like enzymes from soybeans should have been destroyed during steam-cooking, it was apparent that growth of *A. oryzae* had produced another $\beta$-glucosidase-like enzyme in the soybeans to hydrolyze daidzin and genistin and release daidzein and genistein. Riou et al. (*12*) have purified a glucose-tolerant $\beta$-glucosidase produced by *A. oryzae* and shown to be of potential in wine and juice processing.

Changes of the fungal and LAB populations during fermentation of the miso products supplemented with ethanol and NaCl are shown in Table 2. After 2 weeks of fermentation, the fungal and LAB growth was completely inhibited. This was in agreement with the previous observation that growth of the fungal (including koji molds and yeasts) and LAB populations in the rice miso products was effectively inhibited by the supplementation of ethanol and NaCl (*6*). In general, the enumerated fungal, originating mostly from the koji molds, and LAB populations decreased with time of fermentation and varied sensitively as affected by ethanol and NaCl concentrations.

During fermentation, changes of the soluble protein contents and pH values of the miso products supplemented with ethanol and NaCl are shown in Figure 1. In general, soluble protein content increased and pH value decreased during fermentation. In comparison, soluble protein contents increased much more rapidly in the miso products supplemented with 3% ethanol and 6% NaCl than in the products supplemented with 15% ethanol and 12.5% NaCl. Apparently, the proteolytic enzymes in the miso products were effectively inhibited by 15% ethanol and 12.5% NaCl.

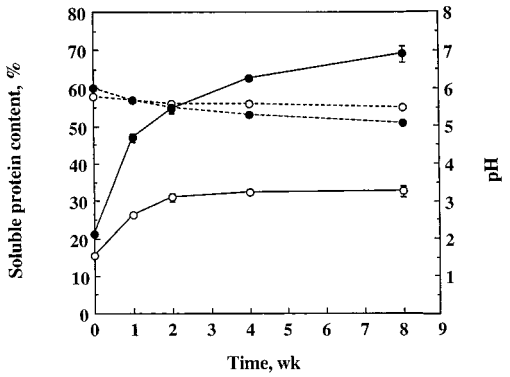


**Figure 1.** Changes in soluble protein content and pH value during fermentation of soybean miso supplemented with 15% ethanol and 12.5% NaCl or 3% ethanol and 6% NaCl: (○) E15N12.5 = 15% ethanol and 12.5% NaCl; (●) E3N6 = 3% ethanol and 6% NaCl; (—) soluble protein content; (- - -) pH.

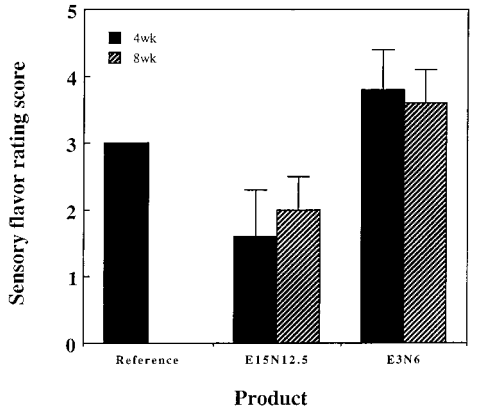


**Figure 2.** Sensory flavor evaluation of soybean miso supplemented with ethanol and NaCl after 4 and 8 weeks of fermentation: Reference, a commercial miso product; E15N12.5 = 15% ethanol and 3% NaCl; E3N6 = 3% ethanol and 6% NaCl.

Ratings for the 4- and 8-week-fermented miso products cooked with cubed tofu for sensory flavor evaluation are shown in Figure 2. The ratings of both 4- and 8-week-fermented products supplemented with 3% ethanol and 6% NaCl were higher than that of a commercial product used for reference. In comparison, the unique flavor rating was slightly higher for the 4-week-fermented products than for the 8-week-fermented products. This was in agreement with the previous study reporting that the flavor ratings of the low-salt miso products are comparable to or even better than the commercial products (*6*). When the ethanol-supplemented low-salt miso products (7.5% ethanol and 5% NaCl) were further subjected to GC-MS characterization (*13*),

**Table 3. Changes of Daidzin, Genistin, Daidzein, and Genistein Contents during 8 Weeks of Fermentation of Soybean Miso Supplemented with Various Levels of Ethanol and NaCl**

| soybean miso | fermentation time (weeks) | mg/g of dry matter[a] | | | |
|---|---|---|---|---|---|
| | | daidzin | genistin | daidzein | genistein |
| unfermented | 0 | 0.206 ± 0.038 | 0.178 ± 0.004 | 0.067 ± 0.008 | 0.166 ± 0.039 |
| with E15N12.5[b] | 4 | 0.019 ± 0.001 | 0.026 ± 0 | 0.152 ± 0.006 | 0.319 ± 0.018 |
| with E3N6[c] | 4 | 0.004 ± 0 | 0.008 ± 0.001 | 0.212 ± 0.017 | 0.495 ± 0.037 |
| with E15N12.5[b] | 8 | 0.024 ± 0.004 | 0.031 ± 0.003 | 0.162 ± 0.016 | 0.318 ± 0.030 |
| with E3N6[c] | 8 | 0.008 ± 0.002 | 0.010 ± 0.004 | 0.160 ± 0.033 | 0.375 ± 0.057 |

[a] Means of determinations with standard deviation ($n = 4$). [b] E15N12.5 = miso supplemented with 15% ethanol and 12.5% NaCl. [c] E3N6 = miso supplemented with 3% ethanol and 6% NaCl.

most volatiles except alcohols and acetals of the low-salt products were higher than those in the control products fermented with 9% NaCl and 0% ethanol. In this study, the products supplemented with 15% ethanol and 12.5% NaCl were rated much lower than the product of reference. This reveals that an appropriate supplementation of ethanol in miso not only enabled low-salt miso fermentation but also enhanced flavor formation.

Changes of daidzin, genistin, daidzein, and genistein contents in the miso products supplemented with ethanol and NaCl are shown in Table 3. Before fermentation, their contents were 0.206, 0.178, 0.067, and 0.166 mg/g, respectively. After 4 weeks of fermentation, most daidzins and genistins in the products supplemented with 15% ethanol and 12.5% NaCl and almost all daidzins and genistins in the products supplemented with 3% ethanol and 6% NaCl were hydrolyzed. Consequently, their corresponding daidzein and genistein contents were increased. This was in agreement with the report of Coward et al. (*14*) and Wang and Murphy (*5*) that genistein content was higher in the fermented soybean-based foods than in the unfermented foods. After 8 weeks of fermentation, daidzein and genistein contents in the products supplemented with 15% ethanol and 12.5% NaCl did not change, whereas daidzein and genistein contents in the products supplemented with 3% ethanol and 6% NaCl decreased slightly. In the ethanol and NaCl supplemented miso products, growth of the halophilic yeasts and LAB, present in the normally fermented miso products, was inhibited (Table 2). Because $\beta$-glucosidase-like enzymes were supposed to be present in the soybean koji to increase daidzein and genistein contents in the soybeans during koji preparation (Table 1), the conversion of daidzin to daidzein and of genistin to genistein during miso fermentation must originate from the koji. Thus, the koji mixed with steam-cooked soybeans for miso fermentation has contributed $\beta$-glucosidase-like enzymes to hydrolyze the glycosidic isoflavones. Because daidzein and genistein contents increased while the microbial growth and proteolysis were effectively inhibited in the miso supplemented with 15% ethanol and 12.5% NaCl for fermentation (Tables 2 and 3), the $\beta$-glucosidase-like enzymes from koji seem to be more resistant to ethanol and NaCl than were the indigenous microorganisms and proteolytic enzymes.

In conclusion, a one-tube procedure for the simultaneous extraction of isoflavones including daidzin, genistin, daidzein, and genistein from lyophilized and pulverized soybean powder or from miso homogenate and analysis with an HPLC was developed. When soybeans were soaked with water for 4 h, steam-cooked, inoculated with the conidia of *A. oryzae*, and incubated for 3 days for koji preparation, daidzein and genistein contents increased markedly in the water-soaked soybeans, whereas daidzin and genistin contents decreased. During the subsequent mold incubation, further increases of the daidzein and genistein contents in accordance with the decreases of daidzin and genistin contents were observed. It was obvious that *A. oryzae* had produced $\beta$-glucosidase-like enzymes to function in the hydrolysis of the glycosidic isoflavones. When the koji was mixed with water-soaked and steam-cooked soybeans, ground into paste, and supplemented with 15% ethanol and 12.5% NaCl or 3% ethanol and 6% NaCl for fermentation, fungal and LAB growth was completely inhibited after 2 weeks of fermentation. During fermentation, soluble protein contents increased much more rapidly in the miso supplemented with 3% ethanol and 6% NaCl than in the products supplemented with 15% ethanol and 12.5% NaCl. When the 4- and 8-week-fermented miso products were cooked with tofu for sensory evaluation, flavor ratings of the products supplemented with 3% ethanol and 6% NaCl were higher than that of a popular commercial product. After 4 weeks of fermentation, most of the daidzin and genistin had been hydrolyzed into daidzein and genistein. Apparently, the hydrolytic enzymes contributing to isoflavone transformation originated from soybeans after water soaking and from the koji mold. Therefore, the 4-week-fermented low-salt miso products (supplemented with 3% ethanol and 6% NaCl) were remarkably acceptable in flavor quality and their daidzein and genistein contents were significantly increased.

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