JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Distribution Profiles of Isoflavone Isomers in Black Bean Kojis Prepared with Various Filamentous Fungi

I-HSIN LEE AND CHENG-CHUN CHOU*

Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan

This study was conducted to compare the transformation of both isoflavone derivatives (aglycones, β-glucosides, and acetyl and malonyl gluclucosides) and β-glucosidase activity in kojis fermented with various generally recognized as safe filamentous fungi including *Aspergillus awamori*, *Aspergillus oryzae*, *Aspergillus sojae*, *Rhizopus azygosporus*, and *Rhizopus* sp. no. 2. Solid fermentation was performed to prepare the kojis by inoculating the steamed black beans with starter organism and culturing at 30 °C for 3 days. Results revealed that fermentation caused a marked increase in the content of aglycone (daidzein, glycitein, and genistein), the bioactive isoflavone, and a significant reduction in the content of β-glucoside isoflavone (daidzin, glycitin, and genistin), compared with the unfermented steamed black bean. The extent of increased aglycone and reduced β-glucoside isoflavone content varied with the starter organism used. Among the various black bean kojis prepared, the *Rhizopus* sp. no. 2 koji showed the highest level of enhancement in aglycone content. In the *Rhizopus* sp. no. 2 koji, the percentage of aglycone to total isoflavone increased from an initial ∼2.9 to ∼58.9% after fermentation. In comparison, the percentages found in kojis prepared with other starter organisms ranged from 18.9 to 38.9% after fermentation. Further preparations of black bean kojis with *A. awamori* at different cultivation temperatures (25, 30, and 35 °C) and various fermentation periods (1−5 days) revealed that koji prepared at 30 °C for 4 days showed the highest content of aglycones, with 7.7-, 5.7-, and 4.8-fold increases in the content of daidzein, genistein, and glycitein, respectively. In addition, the increase of aglycone content and the increase of β-glucosidase activity during the fermentation of this koji showed a similar trend.

KEYWORDS: Black bean koji; β-glucosidase; isoflavone; solid fermentation

INTRODUCTION

Black beans (*Phaseolus vulgaris* L.) are cultivated and consumed in many countries throughout the world and have both economic and nutritional importance. In China, *In-yu* black bean sauce and *in-si* or *tou-si*, the dried byproduct of the mash of black bean sauce, are traditional fermented products made from black bean that are widely used in Chinese meals (*1*). As early as the 16th century, the beneficial effects of black beans were recognized in China (*2*). More recently, Riberio and Salvadori (*3*) demonstrated that black beans reduced the incidence of DNA damage by cyclophosphamide. Takahashi et al. (*4*) suggest that black beans may be more effective in inhibiting low-density lipoprotein oxidation than soybean.

Similar to other leguminous plants, black beans are rich in isoflavones, which have been reported to help in the prevention of cancer, osteoporosis, postmenopausal syndrome, and hypercholesterolemia (*5*−*8*). Essentially, these isoflavones occur in four chemical forms, with daidzein, glycitein, and genistein serving as the three basic chemical structures for aglycones, whereas the three other forms are derivatives from each aglycone, namely, β-glucoside, acetyl glucoside, and malonyl glucoside (*9*). The chemical form is an important variable because it influences biological activity, bioavailability, and, therefore, the physiological contributions of isoflavones (*8*, *10*−*12*). Izumi et al. (*10*) reported that aglycones are more bioavailable than their respective glucosides, whereas conflicting opinions have been proposed. Setchell et al. (*13*) suggested that the sugar moiety in glucoside forms protects the isoflavone from being degraded in the intestine and indicated that the bioavailability of isoflavones is greater for the ingestion of β-glycosides than for aglycones. Zubik and Meydani (*14*) reported that the apparent bioabilability of genistein is not different when it consumed as either glycoside or aglycone. Besides, Ismail and Hayes (*15*) have reported that glycosides have to be in the nonconjugated form to be bioavailable. Furthermore, daidzin and genistin were considered to be the main compounds contributing to the objectionable aftertaste of soy foods (*16*).

The isoflavones in soybean and black bean occur primarily as β-glucosides, with a small percentage as the principal bioactive aglycone (*9*). However, some researchers have demonstrated that processing techniques alter the relative content

* Address correspondence to this atuhor at the Institute of Food Science and Technology, National Taiwan University 59, lane 144, Keelung Rd., Sec. 4, Taipei, Taiwam (telephone 886-2-3366-4111; fax 886-2-2362-0849; e-mail fstcchou@ccms.ntu.edu.tw).

10.1021/jf058139m CCC: $33.50
Published on Web 01/31/2006

of the isoflavone isomers in soy foods (*17*−*20*). Cooked soybeans, texturized vegetable protein, and soy milk, on average, contain >95% of total isoflavones as glucosides, whereas soybean-fermented products such as tofu and tempeh contain ca. 20 and 40%, respectively, of their isoflavones as aglycones (*21*). The higher genistein content observed in the fermented foods in comparison with the unfermented foods is generally attributed to the hydrolysis of genistin by microbial enzymes to form genistein during fermentation (*21*−*23*).

Isoflavone distribution profiles of various soy foods and the chemical modification of isoflavones during cooking and processing have been widely reported (*9*, *18*−*21*). In contrast, such information with regard to the black bean is still rather limited in the literature. Our study was thus carried out to investigate the transformation of isoflavone isomers in black beans during the solid fermentation of koji with various generally recognized as safe (GRAS) filamentous fungi. Additionally, using *Aspergillus awamori* as the starter organism, the effect of fermentation temperature and duration of fermentation on the transformation of isoflavones was examined.

MATERIALS AND METHODS

Chemicals. High-performance liquid chromatography (HPLC) grade glacial acetic acid was obtained from Alps Chemical Co., Ltd. (Taipei, Taiwan); acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Isoflavone standards daidzein, genistein, glycitein, daidzin, genistin, glycitin, acetyldaidzin, acetylgenistin, acetylglycitin, malonyldaidzin, malonylgenistin, and malonylglycitin were products of LC Laboratories (Woburn, MA). Glucosamine (hydrochloride), fluorescein, *p*-nitrophenyl β-D-glucopyranoside (*p*-NPG), and sodium carbonate were obtained from Sigma-Aldrich Co. (St. Louis, MO). Tween 80 was a product of Icatayama Chemical (Osaka, Japan).

Test Organism and Preparation of Inoculum. Various filamentous fungi commonly employed as the starter organisms for the preparation of traditionally oriental fermented food products were used as the test organisms in the present study. They included *Aspergillus oryzae* BCRC 30222, *Aspergillus sojae* BCRC 30103, and *Rhizopus azygosporus* BCRC 31158, obtained from the Culture Collection and Research Center (CCRC), Hsinchu, Taiwan, and *Aspergillus awamori* and *Rhizopus* sp. no. 2, obtained from Professor Yu, Institute of Food Science and Technology, National Taiwan University. To prepare the inocula, the test organism was inoculated onto potato dextrose agar (Difco, Detroit, MI) and incubated at 30 °C for 3 days. Spores of the test organism were harvested by flooding the surface of the agar with sterile distilled water containing 0.1% Tween 80. The spore suspension was adjusted with sterile distilled water to a concentration of $\sim 10^6$/mL and served as inoculum for the fermentation of black bean koji.

Preparation of Black Bean Koji. Whole black beans obtained locally were washed and then soaked overnight at room temperature in distilled water that was 6 times the weight of the beans. After the water had been decanted, the black beans were steam-cooked in an autoclave (121 °C, 15 min) and then cooled. Solid-state fermentation was performed by evenly spraying 1.0 mL of spore suspension ($\sim 10^6$/mL) into the steamed black bean substrate (50 g). After a thorough mixing, the inoculated black bean substrate was placed on a round 69-mesh screen and then incubated for 3 days at 30 °C and 95% relative humidity. During the cultivation period, the black beans were stirred and mixed after 17 and 25 h of cultivation to accelerate the release of fermentation heat. To examine the effect of fermentation temperature, kojis were prepared with *A. awamori* and incubated at 25, 30, or 35 °C for 3 days. To examine the effect of fermentation period, the kojis were fermented at 30 °C for a period of 1−5 days.

Analysis of Isoflavone. Isoflavones in the samples of unfermented steamed black bean and black bean koji were extracted according to the method described by Franke et al. (*24*). In brief, samples were first freeze-dried and homogenized. Dry powder (2.0 g) was extracted with 80% methanol (20.0 mL) by shaking (120 rpm) at 25 °C for 2 h and filtered through Whatman no. 42 filter paper. The filtrate was condensed to ~1−2 mL using a vacuum rotary evaporator at ~35 °C, combined with fluorescein as an internal standard (*19*), and readjusted with 80% methanol to a final volume of 20 mL. The filtrate was then filtered through a 0.45 μm Millipore PVDF filter (Schleicher & Schuell, GmbH, Dassel, Germany) and subjected to HPLC analysis for isoflavones according to the method described by Wang and Murphy (*9*) with minor modification. Essentially, the HPLC equipment used was a chromatograph (model 7200, Jasco Co., Tokyo, Japan) equipped with a YMC-Pack ODS-AM-303 column (250 × 4.6 mm, 5 μm, YMC Co., Ltd, Kyoto, Japan), a UV−vis detector (model UV-970, Jasco), and a SISC chromatography data processor (SISC Co., Davis, CA). A linear HPLC gradient was composed of (A) 0.1% glacial acetic acid in H_2O and (B) 0.1% glacial acetic acid in acetonitrile. After the 20 μL injection of sample onto the column (25 °C), solvent B was increased from 15 to 20% in 20 min and to 24% in 10 min and then held at 24% for 4 min; solvent B was then further increased to 35% in 10 min and held at 35% for 8 min and finally reduced to 15% in 5 min. The solvent flow rate was 1.0 mL/min. **Figure 1** shows a typical HPLC chromatograph. Contents of the isoflavones were calculated from standard curves of the area responses for authentic isoflavone standards normalized to the constant amount of fluorescein added to each sample. The contents were expressed as micrograms per gram of black bean or koji.

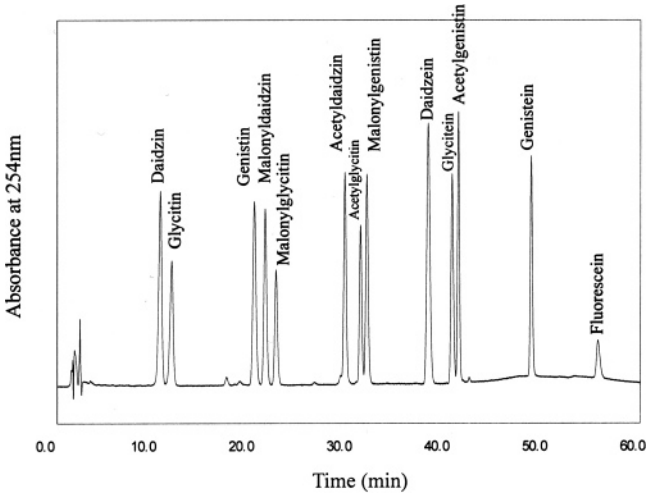

Figure 1. Typical HPLC chromatogram showing various isoflavones and fluorescein (internal standard).

Assay of β-Glucosidase Activity. β-Glucosidase activity was determined according to the method described by Bahl and Agrawal (*25*). Enzymes were extracted by adding 1.0 g of the freeze-dried sample to 10.0 mL of 0.1 M phosphate buffer solution (pH 6.0) and homogenized at room temperature for 1 min. The slurry was centrifuged at 15000 rpm and 4 °C for 20 min, and the supernatant was used as an enzyme source. Then 2.0 mL of 1 mM *p*-NPG, which had been tempered at 30 °C, was combined with 0.5 mL of enzyme solution and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 2.5 mL of 0.5 M sodium carbonate. The resultant color was measured at 420 nm. One unit of enzyme was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per minute under the assay condition.

Determination of Dry Weight. Dry weights of samples were determined according to the AOAC method (*26*).

Statistical Analysis. The mean values and the standard deviation were calculated from the data obtained with at least triplicate trials. Analysis of variance was carried out utilizing the SAS software package (*27*). Isoflavone content and β-glucosidase activity data were analyzed according to ANOVA factorial analysis with test organism, fermentation temperature, and fermentation period as factors depending on the experiment. Differences between the respective means were determined according to Duncan's multiple-range test.

RESULTS AND DISCUSSION

Isoflavone Isomers in Various Black Soybean Kojis. The contents of individual isoflavone isomers in the unfermented

Table 1. Isoflavone Contents of Black Bean Kojis Prepared with Various Starter Organisms

	content (μg/g of koji)					
isoflavone	unfermented	*A. awamori*	*A. oryzae*	*A. sojae*	*R. azygosporus*	*Rhizopus* sp. no. 2
daidzin	476 ± 9.8A	243 ± 2.0B	253 ± 19.7B	197 ± 23.4C	174 ± 5.5C	45 ± 3.7D
glycitin	166 ± 1.2A	79 ± 1.9D	98 ± 6.3B	89 ± 7.3C	94 ± 3.7BC	78 ± 2.6D
genistin	711 ± 22.9A	465 ± 7.2C	513 ± 41.0B	396 ± 24.6D	384 ± 3.3D	144 ± 7.2E
malonyldaidzin	141 ± 1.6A	69 ± 3.5D	99 ± 7.8B	41 ± 7.1E	79 ± 5.6C	77 ± 1.8CD
malonylglycitin	63 ± 4.1A	40 ± 1.1B	31 ± 11.6BC	12 ± 0.6D	31 ± 1.5BC	29± 1.4C
malonylgenistin	157 ± 2.8A	68 ± 4.5C	94 ± 11.6B	70 ± 5.0C	90 ± 6.6B	93 ± 2.2B
acetyldaidzin	69 ± 1.5B	51 ± 0.66C	64 ± 2.9B	57 ± 2.5C	85 ± 6.7A	52 ± 7.2C
acetylglycitin	25 ± 1.6A	15 ± 3.1B	16 ± 6.3B	19 ± 1.6AB	26 ± 5.3A	15 ± 1.0B
acetylgenistin	98 ± 3.8B	84 ± 1.3D	108 ± 4.7A	92 ± 3.7C	111 ± 4.2A	77 ± 1.2E
daidzein	24 ± 0.4E	161 ± 1.8D	150 ± 8.9D	291 ± 19.6B	271 ± 5.7C	422 ± 12.7A
glycitein	6 ± 0.2F	28 ± 0.8D	18 ± 1.0E	62 ± 2.3B	51 ± 0.1C	75 ± 3.1A
genistein	26 ± 0.3E	121 ± 0.6D	129 ± 8.5D	267 ± 10.1B	228 ± 2.6C	378 ± 7.2A
total	1962 ± 17.4A	1424 ± 9.0C	1573 ± 75.0B	1592 ± 1.1B	1625 ± 6.7B	1485 ± 28.9C

[a] Values are presented as means ± SD, and means in the same row with different letters were significantly different by Duncan's multiple-range test ($p < 0.05$).

steamed black bean and black bean kojis fermented with various *Aspergillus* spp. and *Rhizopus* spp. are shown in **Table 1**. It was found that the unfermented steamed soybean contained a total isoflavone content of 1962 μg/g. The glucoside forms (malonyl glucoside, acetyl glucoside, and β-glucoside) account for ~97.1% of the total isoflavone noted in the unfermented steamed black soybean; β-glucoside was noted to be the major form of isoflavone. On the other hand, the content of aglycone, contributing only ~2.9% of the total isoflavone content, was the lowest compared to malonyl glucoside, acetyl glucoside, and β-glucoside isoflavone. This observation is consistent with that previously reported for some soy-based foods (*28*).

Depending on the starter organism employed for fermentation, the prepared black bean kojis were found to have less total isoflavone content than the unfermented steamed bean. The hydrolytic cleavage of the glucose moiety from the isomers, which contribute to the mass of glucoside isoflavone, may contribute to the lower total isoflavone content observed in the prepared black bean kojis (*20*). By comparison of the distribution patterns of isoflavone isomers in the unfermented steamed black bean and the prepared kojis, the fermentation, similar to that observed on soybean, was noted to cause a marked increase in the content of aglycone isoflavone (daidzein, glycitein, and genistein) along with a major reduction in the content of β-glucosides. The extent of increased aglycone content and the reduction in the content of glucoside isoflavone due to fermentation varied with the starter organism employed. Among the various black bean kojis prepared, the aglycone content in the *Rhizopus* sp. no. 2 -prepared black bean koji showed the highest level of enhancement. The percentage of aglycone to total isoflavone in this koji showed an increase from ~2.9 to ~58.9%. The contents of daidzein, glycitein, and genistein increased from 24, 6.0, and 26 to 422, 75, and 378 μg/g, respectively, as noted in the finished koji. On the other hand, the percentages of aglycone to total isoflavone in the *A. awamori*, *A. oryzae*, *A. sojae*, and *R. azygosporus* black bean kojis were found to be 21.8, 18.9, 38.9, and 33.9%, respectively, which showed 6.6−13.7-fold increases compared to the unfermented black bean.

It was indicated that malonyl glucoside might convert to acetyl glucoside and β-glucoside isoflavone through decarboxylation and hydrolysis, respectively (*29*). Therefore, the slight increase of acetyl glucoside content noted in the *R. azygospours* koji might be due to the decarboxylation of malonyl glucoside; however, the real reason remains to be further examined.

The increased content of aglycone, the bioactive form of isoflavone, as observed in the present study, is consistent with previous studies of soybean fermented with *R. oligosporus* (*18*)-and *A. oryzae* (*23*). Wang and Murphy (*18*) reported that fermentation of soybean with *R. oligosporus* resulted in a 6.5-fold increase in the aglycone content in tempeh, a traditional Indonesian fermented product.

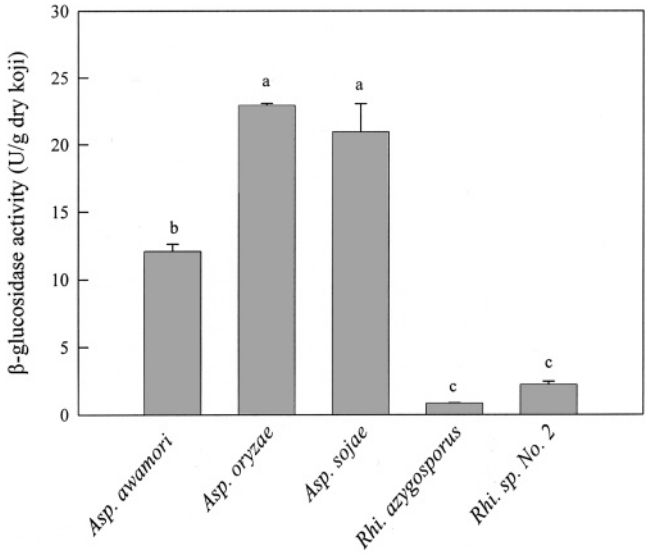

Figure 2. Activity of β-glucosidase in black bean kojis prepared with various starter organisms. Means (bar values) with different letters are significantly different by Duncan's multiple-range test ($p < 0.05$).

***β*-Glucosidase Activity in Various Black Bean Kojis.** György et al. (*30*) reported that *R. oryzae* produced enzymes capable of hydrolyzing the glucoside forms of isoflavone in soybean. β-Glucosidase has been reported to catalyze the hydrolysis of glucoside isoflavones with the formation of aglycones (*15*, *16*, *31*, *32*). Furthermore, Setchell (*33*) proposed that intestinal microflora such as *Bifidobacterium*, *Lactobacillus*, and *Bacteriedes* can produce β-glucosidase that hydrolyzes the glucoside components of isoflavone in the jejunum with the release of the bioactive aglycone forms. On the other hand, Ismail and Hayes (*15*), studying the activity of β-glucosidase from *Escherichia coli* and almonds, revealed that β-glucosidase can hydrolyze nonconjugated β-glucosides but not conjugated glucosides to their respective aglycones.

In the present study, all of the starter organisms examined were found to be capable of producing β-glucosidase and exhibited various activities in the prepared kojis (**Figure 2**). Therefore, the action of β-glucosidase produced by the starter organism during fermentation may be one of the factors contributing to the increase of aglycone content (**Table 1**). As shown in **Figure 2**, β-glucosidase activity detected in *A. oryzae*

Table 2. Isoflavone Contents of Black Soybean Kojis[a] Prepared with *A. awamori* at Different Temperatures

isoflavone	content (μg/g of koji)		
	25 °C	30 °C	35 °C
daidzin	360 ± 8.6A[b]	243 ± 2.0C	313 ± 4.5B
glycitin	62 ± 2.4B	79 ± 1.9A	46 ± 0.7C
genistin	383 ± 9.6B	465 ± 7.2A	325 ± 1.7C
malonyldaidzin	118 ± 11.9A	69 ± 3.5C	90 ± 2.1B
malonylglycitin	44 ± 10.8B	40 ± 1.1B	78 ± 5.0A
malonylgenistir	92 ± 16.4A	68 ± 4.5B	79 ± 4.4AB
acetyldaidzin	83 ± 7.6A	51 ± 0.7C	62 ± 2.8B
acetylglycitin	12 ± 0.8A	15 ± 3.1A	10 ± 3.4A
acetylgenistin	73 ± 0.9B	84 ± 1.3A	58 ± 0.9C
daidzein	76 ± 1.4C	161 ± 1.8A	108 ± 4.2B
glycitein	6 ± 1.3C	28 ± 0.8A	10 ± 0.6B
genistein	35 ± 1.0C	121 ± 0.6A	47 ± 1.6B

[a] Black soybean kojis were incubated at 95% relative humidity for 3 days. [b] Values are presented as means ± SD, and means in the same row with different letters were significantly different by Duncan's multiple-range test ($p < 0.05$).

and *A. sojae* kojis showed no significant difference ($p > 0.05$), but was significantly higher ($p < 0.05$) than that detected in the kojis prepared with other starter organisms. It is interesting to note that although the extent of isoflavone deglycosylation was highest in the *Rhizopus* sp. no. 2- koji (**Table 1**), the β-glucosidase activity detected under the present assay conditions was relatively lower when compared with those detected in the *Aspergillus* spp.-prepared kojis. Because optimal conditions for the activity of β-glucosidase vary with the source of enzyme (*34*, *35*), in the present study, β-glucosidase activity was assayed under a defined fix condition (30 °C, pH 6.0, and using *p*-NPG as substrate), which may not be optimal for all of the β-glucosidases, especially those from *Rhizopus* spp. This may thus lead to the low β-glucosidase activity detected in the koji prepared with *Rhizopus* spp. On the other hand, the possibility that factors other than β-glucosidase formed by starter organisms, especially *Rhizopus* spp., during fermentation also contribute to the increase of aglycone content in koji cannot be ruled out. This merits further investigation.

Effect of Temperature. Temperature is an important factor affecting the production of enzyme and metabolites by microorganisms during fermentation. Yin et al. (*36*) prepared sufu, a fermented tofu product, with *Actinomucor elegans* and indicated that sufu fermented at 26 °C was richer in isoflavone aglycones than that fermented at 32 °C. To examine the effect of cultivation temperature on the transformation of isoflavone isomers and production of β-glucosidase, kojis were prepared with *A. awamori* cultivated at 25, 30, and 35 °C, and the results are shown in **Table 2** and **Figure 3**. It was found that the contents of the glucoside form of isoflavones detected in the *A. awamori* koji, regardless of fermentation temperature (**Table 2**), were all less than the respective glucoside isoflavone content in the unfermented black bean (**Table 1**), whereas a significantly higher content of aglycones was observed in the prepared koji (**Table 2**). In addition, the koji prepared at 30 °C had the highest content of aglycone among the three temperatures.

Cultivation temperature was also found to affect significantly the production of β-glucosidase by *A. awamori* in the prepared koji. As shown in **Figure 3**, the koji prepared at 30 °C had the highest β-glucosidase activity as well.

Effect of Fermentation Period on Isoflavone Content and β-Glucosidase Activity in Koji. In this study, kojis were prepared by inoculating black bean with *A. awamori* and then cultivated for a period of 1−5 days to determine the effect of fermentation time on the transformation of isoflavone isomers.

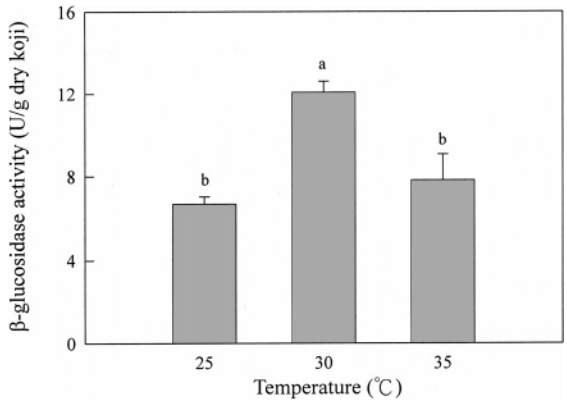

Figure 3. Activity of β-glucosidase in black bean kojis prepared with *A. awamori* at different temperatures. Means (bar values) with different letters are significantly different by Duncan's multiple-range test ($p < 0.05$).

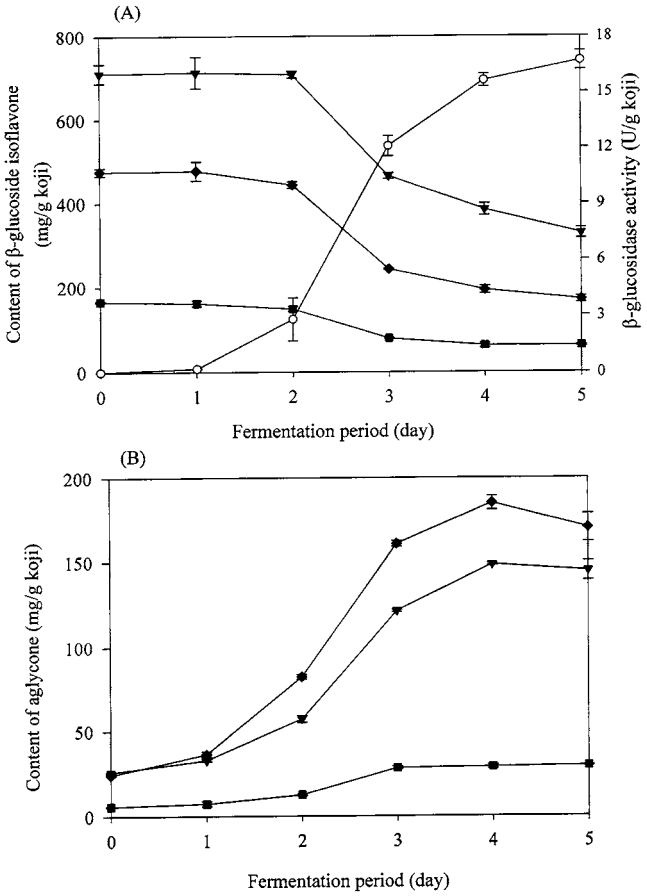

Figure 4. Changes of β-glucosidase activity and contents of β-glucoside isoflavone (**A**) as well as aglycones (**B**) during the fermentation of black bean kojis with *A. awamori*: (**A**) (○) β-glucosidase activity, (◆) daidzin, (▼) genistin, (■) glycitin; (**B**) (◆) daidzein, (▼) genistein, (■) glycitein.

Figure 4 shows the contents of β-glucoside and aglycone isoflavones in the prepared kojis. In addition, β-glucosidase activity detected in the koji is also shown in **Figure 4A**.

It was observed that the activity of β-glucosidase was rather low in the kojis prepared within a fermentation period of ≤2 days. Extending the cultivation period from 2 to 4 days resulted in a marked increase of β-glucosidase activity, whereas only a slight increase in the activity of β-glucosidase was noted in koji that had its cultivation period extended to 5 days.

As shown in **Figure 4A**, a significantly reduced content of β-glucoside isoflavones, especially daidzin and genistin, was

noted in the kojis with a cultivation period of 3−5 days. Meanwhile, a marked increase in the contents of daidzein and genistein was observed along with the marked increase of β-glucosidase activity in these kojis (**Figure 4B**). This finding is consistent with the reports of Chiou and Cheng (*23*) and Esaki et al. (*27*) and further demonstrates that an increase of aglycone content in koji is correlated with the β-glucosidase produced by the starter organism. Among the kojis fermented with *A. awamori* for different cultivation periods, koji prepared with 4 days of fermentation showed the highest content of aglycone, which increased form 56 μg/g before fermentation to 363 μg/g as noted in the finished koji with 7.7-, 5.7-, and 4.8-fold increases in the contents of daidzein, genistein, and glycitein, respectively. Furthermore, the content of daidzein in the 5-day koji was found to be significanly less ($p < 0.05$) than that in the 4-day koji as shown in **Figure 4B**. A similar phenomenon was observed by Esaki et al. (*31*) with soybean fermented at room temperature with *Aspergillus saitoi*. They attributed this phenomenon to the formation of 8-hydroxydaidzein and 8-hydroxygenistein at the expense of daidzein and genistein, respectively, through the catalytic action of hydroxylase produced by *A. saitoi* at the sporulation stage during fermentation. Klus et al. (*37*) also reported that the tempeh-producing microorganisms *Brevibacterium epidermidis* and *Micrococcus luteus* could transform glycitein to 4,6,6-trihydroxyisoflavone by an O-demethylation reaction and that *Microbacterium aborescens* could convert daidzein and glycitein to 4,6,7-trihydroxyisoflavone by a hydroxylation and O-methylation reaction, respectively. Whether *A. awamori*, the microorganism examined in the present study, also possesses these capabilities and thus caused the reduction of isoflavone aglycone content as noted for the 5-day koji merits further investigation.

This is the first report describing the transformation of isoflavone phytoesterogens during the fermentation of black bean. Fermentation of black bean with these test organisms markedly reduced β-glucoside isoflavone content, but enhanced the content of the bioactive isoflavone, aglycone, which has been linked to health benefits and well-being. The aglycone-enhancing effect, closely related to the activity of β-glucosidase, varied with the microorganisms involved in fermentation. Besides, fermentation temperature and length of fermentation period also affect the transformation of isoflavone isomers in black bean.

LITERATURE CITED

(1) Su, Y. C. Traditional fermented food in Taiwan. In *Proceedings of the Oriental Fermented Foods*; Food Industry Research and Development Institute: Taipei, Taiwan, 1980; p 15.

(2) Li, S. T. *Ben-Tsao Gong-Mu* (Chinase botanical encyclopedia); Great Taipei Publishing: Taipei, Taiwan, 1990.

(3) Riberio, L. R.; Salvadori, D. M. F. Dietary components may prevent mutation-related diseases in humans. *Mutat. Res.* **2003**, *544*, 195−201.

(4) Takahashi, R.; Ohmori, R.; Kiyose, C.; Momiyama, Y.; Ohsuzu, F.; Kondo, K. Antioxidant activities of black and yellow soybeans against low-density lipoprotein oxidation. *J. Agric. Food Chem.* **2005**, *53*, 4578−4582.

(5) Adlercreutz, H.; Mazur, W. Phyto-oestrogens and Western diseases. *Ann. Med.* **1997**, *29*, 95−120.

(6) Messina, M.; Barnes, S.; Setchell, K. D. Phyto-oestrogens and breast cancer. *Lancet* **1997**, *350*, 971−972.

(7) Booth, C.; Hargreaves, D. F.; Hadfield, J. A.; Mcgown, A. T.; Potten, C. S. Isoflavones inhibit intestinal epithelial and induce apoptosis in vitro. *Br. J. Cancer* **1999**, *80*, 1550−1557.

(8) Setchell, K. D. R.; Cassidy, A. Dietary isoflavones: biological effects and relevance to human health. *J. Nutr.* **1999**, *129*, 758S−767S.

(9) Wang, H. J.; Murphy, P. A. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. *J. Agric. Food Chem.* **1994**, *42*, 1674−1677.

(10) Izumi, T.; Piskula, M. K.; Osawa, S.; Obata, A.; Tobe, K.; Saito, M.; Kataoka, S.; Kubota, Y.; Kikuchi, M. Soy isoflavone aglycones are absorbed faster and in higher amounts than their clucosides in humans. *J. Nutr.* **2000**, *130*, 1695−1699.

(11) Xu, X.; Wang, H. J.; Murphy, P. A.; Cook, L.; Hendrich, S. Daidzein is a more more bioavailable soymilk isoflavone than is genistein in adult women. *J. Nutr.* **1994**, *124*, 825−832.

(12) Onozawa, M.; Fukuda, K.; Ohtani, M.; Akaza, H.; Sugimura, T.; Wakabayashi, K. Effects of soybean isoflavones on cell growth and apoptosis of the human prostatic cancer cell line LNCaP. *Jpn. J. Clin. Oncol.* **1998**, *28*, 360−363.

(13) Setchell, K. D. R.; Brown, N. M.; Desai, P.; Zimmer-Nechemias, L.; Wolfe, B. E.; Brashear, W. T.; Kirschner, A. S.; Cassidy, A.; Heubi, J. E. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J. Nutr.* **2001**, *131*, 1362S−1375S.

(14) Zubik, L.; Meydani, M. Bioavailability of soybean isoflavones from aglycone and glucoside forms in American women. *Am. J. Clin. Nutr.* **2003**, *77*, 1459−1465.

(15) Ismail, B.; Hayes, K. β-Glycosidase activity toward different glycosidic forms of isoflavones. *J. Agric. Food Chem.* **2005**, *53*, 4918−4924.

(16) Matsuura, M.; Obata, A. β-Glucosidase from soybeans hydrolyze daidzin and genistin. *J. Food Sci.* **1993**, *58*, 144−147.

(17) Barnes, S.; Peterson, T. G.; Cowards, L. Rationale for the use ofgenistein-containing soy martrixes in chemoprevention trails for breast and prostate cancer. *J. Cell Biochem. Suppl.* **1995**, *22*, 181−187.

(18) Wang, H. J.; Murphy, P. A. Mass balance study of isoflavones during soybeanprocessing. *J. Agric. Food Chem.* **1996**, *44*, 2377−2383.

(19) Coward, L.; Smith, M.; Kirk, M.; Barnes, S. Chemical modification of isoflavones in soyfoods during cooking and processing. *Am. J. Clin. Nutr.* **1998**, *68*, 1486S−1491S.

(20) King, R. A.; Bignell, C. M. Concentration of isoflavone phytoestrogens and their glucosides in Australian soya beans and soya foods. *Aust. J. Nutr. Diet.* **2000**, *57*, 70−78.

(21) Wang, H. J.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994**, *42*, 1666−1673.

(22) Nguyenle, T.; Wang, E.; Cheung, A. P. An investigation on the extraction and concentration of isoflavones in soy-based products. *J. Pharm. Biomed. Anal.* **1995**, *14*, 221−232.

(23) Chiou, R. Y Y.; Cheng, S. L. Isoflavone transformation during soybean koji preparation and subsequent miso fermentation supplemented with ethanol and NaCI. *J. Agric. Food Chem.* **2001**, *49*, 3656−3660.

(24) Franke, A. A.; Hankin, J. H.; Yu, M. C.; Maskarinec, G.; Low, S. H.; Custer, L. J. Isoflavone levels in soy foods consumed by multiethnic populations in Singapore and Hawaii. *J. Agric. Food Chem.* **1999**, *47*, 977−986.

(25) Bahl, O. M.; Agrawal, K. M. L. Glycosidases of *Phaseolus vulgaris*. *J. Biol. Chem.* **1968**, *243*, 98−102.

(26) AOAC. *Official Methods of Analysis*, 14th ed.; Sidney, W., Ed.; Association of Official Analytical Chemists: Washington, DC, 1984.

(27) SAS Institute. *SAS/STAT User's Guide*, version 8 ed.; SAS Institute: Cary, NC, 2001.

(28) Murphy, P. A.; Song, T.; Buseman, G.; Barua, K.; Beecher, G. R.; Trainer, D.; Holden, J. Isoflavones in retail and institutional soy foods. *J. Agric. Food Chem.* **1999**, *47*, 2697−2704.

(29) Barnes, S.; Coward, L.; Kirk, M.; Sfakianos, J. HPLC-mass spectrometry analysis of isoflavones. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 254−262.

(30) György, P.; Murata, K.; Ikehata, H. Antioxidants isolated from fermented soybeans (tempeh). *Nature* **1964**, *203*, 870−872.

(31) Esaki, H.; Watanabe, R.; Onozaki, H.; Kawakishi, S.; Osawa, T. Formation mechanism for potent antioxidative *o*-dihydroxyisoflavones in soybeans fermented with *Aspergillus saitoi*. *Biosci., Biotechnol., Biochem.* **1999**, *63*, 851−858.

(32) Esaki, H.; Watanabe, R.; Osawa, T.; Kawakishi, S. Transformation of genistein by the spores of *Aspergillus* spp. *J. Jpn. Soc. Food Sci.* **2004**, *51*, 210−213 (in Japanese).

(33) Setchell, K. D. R. Absorption and metabolism of soy isoflavones—from food to dietary supplements and adults to infants. *J. Nutr.* **2000**, *130*, 654S−655S.

(34) Saha, B. C.; Bothast, R. J. Glucose tolerant and thermophilic β-glucosidases from yeasts. *Biotechnol. Lett.* **1996**, *18*, 155−158.

(35) Belancic, A.; Gunata, Z.; Vallier, M. J.; Agosin, E. β-Glucosidase from the grape native yeast *Debaryomyces vanrijiae*: purification, characterization, and its effect on monoterpene content of a Muscat grape juice. *J. Agric. Food Chem.* **2003**, *51*, 1453−1459.

(36) Yin, L. J.; Li, L.; Liu, H.; Saito, M.; Tatsumi, E. Effects of fermentationtemperature on the content and composition of isoflavones and P-glucosidase activity in sufu. *Biosci., Biotechnol., Biochem.* **2005**, *69*, 267−272.

(37) Klus, K.; Borgerpapendore, G.; Barz, W. Formation of 6,7,4′-trihydroxyisoflavone (factor-II) from soybean seed isoflavones by bacteria isolated from tempe. *Phytochemistry* **1993**, *34*, 979−981.

Received for review August 22, 2005. Revised manuscript received November 7, 2005. Accepted November 9, 2005. This research was financially supported by The National Science Council, ROC (Taiwan) (NSC 94-2313-B-002-051).

JF058139M