JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Stability of Isoflavone Isomers in Steamed Black Soybeans and Black Soybean Koji Stored under Different Conditions

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Steamed black soybeans and black soybean koji, a potentially functional food additive, were stored at 4 or 25 °C with or without deoxidant and desiccant for 120 days. After storage, steamed black soybeans and koji showed various extents of reduction in isoflavone contents dependent on storage temperature, packaging condition, and the kind of isoflavone isomer. Generally, black soybeans and koji showed the highest residual of isoflavone when they were stored at 4 °C with deoxidant and desiccant. Under this storage condition, β-glucosides (daidzin, glycitin, and genistein), acetyl glucosides (acetyldaidzin, acetylglycitin, and acetylgenistin), manlonyl glucosides (malonyldaidzin, malonglycitin, and malonylgenistin), and aglycones (daidzein, glycitein, and genistin) in steamed black soybeans exhibited residuals of 100.1−100.9, 92.0−99.4, 90.0−94.0, and 77.2−78.8%, respectively, of their original contents after 120 days of storage. Meanwhile, the residuals found in black soybean koji were 77.8−90.0, 13.1−88.9, 66.7−85.5, and 76.4−80.6%, respectively.

KEYWORDS: Black soybeans; koji; isoflavone; storage

INTRODUCTION

Similar to soybean, black soybean [*Glycine max* (L.) Merr.] is rich in protein and contains plenty of isoflavone and vitamins B and E. In China, black soybean has been used to prepare traditional fermented food products such as *in-yu* and *tou si*, the dried byproduct of the mashed black soybean sauce (*1*). It provides a plentiful and inexpensive supply of protein and calories. Isoflavones have been reported to help in the prevention of cancer, osteoporosis, postmenopausal syndrome, and hypercholesterolemia (*2*−*5*). Despite the divided opinions on the effect of isoflavones in soy formula on hormonal activity of infants (*6*−*9*), *Rhizopus*-fermented black soybean in combination with rice was proposed to be used to formulate a nutritious weaning food (*10*). Additionally, Riberio and Salvadori (*11*) and Takahashi et al. (*12*), respectively, indicated that black soybeans reduced the incidence of DNA damage by cyclophosphamide and inhibited low-density lipoprotein cholesterol oxidation. We have also noted that black soybeans possessed antioxidative and antimutagenic properties (*13*−*15*). These functional properties of black soybeans increased after further fermentation with *Aspergillus awamori*. The fungi-fermented black soybeans (koji) contained a higher content of aglycone, the bioactive isoflavone, than did the unfermented black soybeans (*16*). Therefore, black soybeans and black soybean koji were suggested as potential and useful ingredients for the preparation of healthy food.

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To provide information that is required when black soybean koji are further processed, the stability of isoflavone contents in black soybeans and koji during storage was examined in the present study; specifically, black soybeans and black soybean koji were stored at 4 and 25 °C with or without deoxidant and desiccant for a period of 120 days. Changes in the content of various isoflavones including β-glucoside (daidzin, glycitin, and genistin), acetyl glucoside (acetyldaidzin, acetylglycitin, and acetylgenistin), manlonyl glucoside (malonyldaidzin, malonglycitin, and malonylgenistin), and aglycone (daidzein, glycitein, and genistein) were observed. Additionally, the stability of β-glucosidase activity in the black soybean koji during storage was investigated.

MATERIALS AND METHODS

Preparation of Steamed Black Soybeans and Black Soybean Koji. Black soybeans were obtained from a local market. Steamed black soybeans and koji were prepared according to the procedures described by Lee and Chou (*16*). Briefly, black soybeans, after washing and soaking overnight in distilled water and decantation of the water, were steam-cooked in an autoclave (121 °C, 15 min). Koji was then prepared with a solid fermentation by inoculating the steamed black soybeans with a spore suspension of *A. awamori* and incubating at 30 °C and 95% relative humidity for 3 days. The unfermented steamed black soybeans and the prepared koji were then freeze-dried and homogenized.

Storage of the Dried Steamed Black Soybeans and Koji. Each sample of the freeze-dried powder of steamed black soybeans or koji (20 g) was placed into a 100 mL brown glass bottle containing deoxidant (Agelong oxygen absorber, Sand-Tech Enterprise, Taipei, Taiwan), desiccant (Seca Pax desiccant, Trans World Container), both

10.1021/jf803702x CCC: $40.75
Published on Web 02/17/2009

Table 1. Change of Isoflavone Contents of Steamed Black Soybeans after 120 Days of Storage at 4°C under Different Packaging Conditions

	content of isoflavone after storage									
	initial		desiccant + deoxidant		desiccant		deoxidant		without added	
isoflavone	content (μg/g)	residual[a] (%)	content (μg/g)	residual (%)	content (μg/g)	residual (%)	content (μg/g)	residual (%)	content (μg/g)	residual (%)
β-glucosides										
daidzin	712.9 ± 7.7 A[b]	100.0	719.6 ± 3.5 A	100.9	709.3 ± 4.4 A	99.2	714.6 ± 14.2 A	100.2	705.1 ± 4.0 B	98.9
glycitin	249.8 ± 6.6 A	100.0	255.1 ± 8.3 A	102.1	247.8 ± 1.1 A	99.2	254.3 ± 9.7 A	101.8	247.2 ± 1.8 A	98.9
genistin	706.6 ± 7.9 A	100.0	708.9 ± 1.6 A	100.3	703.5 ± 2.3 A	99.6	705.8 ± 6.4 A	99.9	705.0 ± 4.4 A	99.8
malonyl glucosides										
daidzin	341.3 ± 5.0 A	100.0	320.9 ± 1.3 AB	94.00	304.7 ± 1.0 B	89.3	317.2 ± 3.2 AB	92.9	302.6 ± 2.4 C	88.6
glycitin	168.5 ± 2.9 A	100.0	151.6 ± 1.05 AB	89.98	142.3 ± 1.3 CD	84.4	150.8 ± 1.8 BC	89.5	141.9 ± 1.6 D	84.2
genistin	287.1 ± 7.4 A	100.0	269.3 ± 1.4 AB	93.80	251.5 ± 0.7 BC	87.6	261.4 ± 3.9 AB	91.1	243.4 ± 4.1 C	84.8
acetyl glucosides										
daidzin	80.9 ± 1.1 A	100.0	80.4 ± 4.2 B	99.4	77.3 ± 5.9 B	95.5	77.5 ± 3.4 B	95.9	74.1 ± 3.8 B	91.6
glycitin	103.4 ± 2.3 A	100.0	95.2 ± 0.4 B	92.0	89.5 ± 0.5 D	86.5.	93.2 ± 0.7 C	90.1	87.7 ± 0.6 E	84.7
genistin	108.6 ± 0.9 A	100.0	105.8 ± 5.1 B	97.4	99.9 ± 5.1 C	92.0	105.1 ± 0.8 B	96.8	98.1 ± 4.3 BC	90.4
aglycones										
daidzein	24.9 ± 0.4 A	100.0	19.6 ± 0.3 B	78.7	18.4 ± 0.5 B	73.8	18.6 ± 0.8 B	74.7	18.3 ± 0.8 B	73.5
glycitein	51.2 ± 0.9 A	100.0	39.5 ± 1.0 B	77.2	37.4 ± 1.9 B	72.9	37.6 ± 1.5 B	73.4	36.9 ± 1.3 B	72.0
genistein	26.2 ± 0.4 A	100.0	20.6 ± 0.8 B	78.8	19.4 ± 1.0 BC	74.2	19.8 ± 0.3 B	75.8	19.2 ± 0.3 C	73.3
total	2861.5 ± 16.9 A	100.0	2779.2 ± 1.2 B	97.2	2698.3 ± 6.0 C	94.3	2754.0 ± 36.2 B	96.3	2679.3 ± 15.0 C	93.6

[a] Residual (%) was obtained by dividing the isoflavone content of the treated sample with the initial isoflavone content of the sample. The isoflavone content of initial sample was regarded as 100%. [b] Values are presented as means ± SD ($n = 3$). Means of the same item in the same row with different letters were significantly different by Duncan's multiple-range test ($p < 0.05$).

deoxidant and desiccant, or nothing. They were then held at either 25 or 4 °C for a period of 120 days. The isoflavone content and dry weight of the samples were measured at predetermined time intervals.

Determination of Isoflavones. Determination of isoflavones in both the samples of unfermented steamed black soybeans and black soybean koji was conducted according to the procedures described previously (*16*). Briefly, the dried powder of the samples was extracted with 80% methanol at a ratio of 1:10 (w/v). The extract was then combined with fluorescein as an internal standard and subjected to HPLC analysis for isoflavones. 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 mhromatography 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 injection of the sample onto the column (25 °C), solvent B increased from 15 to 20% in 20 min, then increased to 24% in 10 min, was held at 24% for 4 min, then further increased to 35% 10 min later at which time it was held at 35% for 8 min, and then finally reduced to 15% after a further 5 min. The solvent flow rate was 1.0 mL/min. The content of the isoflavones was calculated from the 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 dried steamed black soybeans or koji.

Determination of β-Glucosidase Activity and Dry Weight. The procedures described by Lee and Chou (*16*) were followed to measure the activity of β-glucosidase activity. One unit of enzyme was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per minute under the assay condition. The dry weight of samples was determined according to the AOAC method (*17*).

Statistical Analysis. The mean value and standard deviation were calculated from the data obtained from the three separate experiments. Means were compared using Duncan's multiple-range test method in SAS, version 8 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Changes of Isoflavone Content in Steamed Black Soybeans after Storage. Lee et al. (*18*) indicated that the storage of soybeans during the high temperatures of summer influenced their isoflavone content, requiring the study of the effects of storage environment, such as humidity and temperature, on the transformation of isoflavone groups. **Table 1** shows the change of total isoflavone and individual isoflavone contents of steamed black soybeans stored at 4 °C under different packaging conditions for a period of 120 days. In general, a reduced content of total isoflavone and the individual isomers of acetyl and malonyl glucoside and aglycone in steamed black soybeans was noted, although the extent of reduction in the content of all the isoflavone isomers examined after storage varied with packaging condition and isoflavone isomer. Among the individual isoflavones, regardless of packaging condition, β-glucoside isoflavones including daidzin, glycitin, and genistin, having a content close to their initial values, showed the highest retention of 98.9−102.1% in the steamed black soybeans after 120 days of storage at 4 °C. Meanwhile, the residual of aglycone isoflavones was the least. Generally, steamed black soybeans stored with deoxidant and desiccant, among the various packaging conditions examined, showed the highest retention of total isoflavone and individual isoflavone contents, followed by that stored with either deoxidant or desiccant, whereas the retention was the least in black soybeans stored with neither deoxidant nor desiccant. For example, after storage at 4 °C for 120 days, the steamed black soybeans stored with desiccant and deoxidant exhibited total isoflavone and daidzein contents of 2779.2 and 19.6 μg/g, respectively. This accounted for ca. 97.2 and 78.7%, respectively, of their original contents. Meanwhile, the steamed black soybeans stored without added desiccant and deoxidant showed lower total isoflavone and daidzein contents of only 2679.3 and 18.3 μg/g, respectively, with lower residuals of 93.6 and 73.5%.

As shown in **Table 2**, a similar trend in the change of total and individual isoflavone isomers as that observed at 4 °C (**Table 1**) was also noted with the steamed black soybeans stored at 25 °C (**Table 2**). Generally, a reduced content of isoflavones was found in the black soybean at the end of the storage period. Besides, those stored with desiccant and deoxidant exhibited a relatively higher residual of isoflavone than those stored under other packaging conditions examined. However, the isoflavone content of the steamed black soybeans stored at 25 °C generally showed a greater extent of reduction with a relatively lower residual than those stored at 4 °C. For example, the steamed black soybeans stored without added desiccant and deoxidant showed total isoflavone and genistein contents of 2659.6 and

Table 2. Change of Isoflavone Contents of Steamed Black Soybeans after 120 Days of Storage at 25 °C under Different Packaging Conditions

	content of isoflavone after storage									
	initial		desiccant + deoxidant		desiccant		deoxidant		without added	
isoflavone	content (μg/g)	residual[a] (%)	content (μg/g)	residual (%)	content (μg/g)	residual (%)	content (μg/g)	residual (%)	content (μg/g)	residual (%)
β-glucoside										
daidzin	712.9 ± 7.7 A[b]	100.0	712.1 ± 4.0 A	99.9	706.2 ± 11.8 A	99.1	707.6 ± 9.5 A	99.3	704.4 ± 6.0 A	98.8
glycitin	249.8 ± 6.6 A	100.0	250.9 ± 1.5 A	100.5	247.2 ± 5.8 A	99.0	254.3 ± 3.4 A	100.0	247.2 ± 8.8 A	99.9
genistin	706.6 ± 7.9 A	100.0	702.9 ± 7.1 A	99.5	705.2 ± 11.0 A	99.8	701.3 ± 15.9 A	99.3	703.3 ± 2.1 A	99.5
malonyl glucoside										
daidzin	341.3 ± 5.0 A	100.0	315.7 ± 2.9 AB	92.5	296.6 ± 4.7 B	86.9	309.0 ± 3.5 AB	90.5	296.3 ± 5.2 B	86.8
glycitin	168.5 ± 2.9 A	100.0	148.4 ± 0.7 BC	88.0	144.4 ± 2.7 C	85.7	145.6 ± 1.4 B	86.4	141.2 ± 2.4 C	83.8
genistin	287.1 ± 7.4 A	100.0	253.9 ± 4.7 AB	88.4	244.7 ± 6.1 B	85.2	250.7 ± 2.6 AB	87.4	243.5 ± 10.7 B	84.8
acetyl glucoside										
daidzin	80.9 ± 1.1 A	100.0	76.7 ± 1.4 B	94.8	71.9 ± 1.1 C	88.9	75.5 ± 3.1 B	93.4	72.3 ± 2.5 C	89.4
glycitin	103.4 ± 2.3 A	100.0	92.3 ± 1.6 B	89.2	88.7 ± 0.9 C	85.8	91.1 ± 0.3 B	88.1	87.9 ± 1.3 C	85.0
genistin	108.6 ± 0.9 A	100.0	104.9 ± 0.4 B	96.6	103.5 ± 1.2 B	95.3	103.3 ± 4.5 B	95.2	96.6 ± 0.7 B	89.0
aglycone										
daidzein	24.9 ± 0.4 A	100.00	18.2 ± 1.0 B	73.0	17.3 ± 0.5 B	69.5	18.6 ± 0.8 B	74.7	18.3 ± 0.8 B	73.5
glycitein	51.2 ± 0.9 A	100.00	36.7 ± 1.7 B	71.7	35.2 ± 1.0 B	68.7	36.7 ± 1.7 B	71.6	34.7 ± 1.1 B	67.7
genistein	26.2 ± 0.4 A	100.00	19.4 ± 0.1 B	74.1	18.6 ± 0.0 BC	71.2	19.4 ± 1.9 B	73.9	18.3 ± 1.8 C	69.9
total	2861.5 ± 17.0 A	100.00	2732.0 ± 23.2 B	95.5	2679.6 ± 35.0 B	93.6	2708.2 ± 33.3 B	94.6	2659.6 ± 32.7 C	92.9

[a] Residual (%) was obtained by dividing the isoflavone content of the treated sample with the initial isoflavone content of the sample. The isoflavone content of initial sample was regarded as 100%. [b] Values are presented as means ± SD ($n = 3$). Means of same item in the same row with different letters were significantly different by Duncan's multiple-range test ($p < 0.05$).

Table 3. Change of Isoflavone Contents of Black Soybean Koji after 120 Days of Storage at 4 °C under Different Packaging Conditions

	content of isoflavone after storage									
	initial		desiccant + deoxidant		desiccant		deoxidant		without added	
isoflavone	content (μg/g of dry koji)	residual[a] (%)	content (μg/g of dry koji)	residual (%)	content (μg/g of dry koji)	residual (%)	content (μg/g of dry koji)	residual (%)	content (μg/g of dry koji)	residual (%)
β-glucoside										
daidzin	363.4 ± 7.3 A[b]	100.0	282.5 ± 3.7 B	77.7	261.9 ± 3.1 D	72.1	280.2 ± 2.5 BC	77.1	274.0 ± 1.2 C	75.4
glycitin	109.0 ± 4.9 A	100.0	97.8 ± 1.3 B	89.8	89.0 ± 0.2 C	81.6	95.4 ± 1.2 B	87.5	86.2 ± 0.5 C	79.2
genistin	577.4 ± 1.8 A	100.0	469.2 ± 7.1 B	81.3	436.5 ± 2.9 B	75.6	466.4 ± 2.0 B	80.8	462.4 ± 3.9 C	80.1
malonyl glucoside										
daidzin	59.1 ± 2.0 A	100.0	61.0 ± 1.3 A	103.3	49.8 ± 0.5 B	84.3	50.4 ± 0.1 B	85.3	50.3 ± 0.1 B	85.3
glycitin	77.2 ± 8.6 A	100.0	66.9 ± 2.8 A	86.6	51.8 ± 8.6 B	66.7	53.5 ± 2.2 B	69.3	50.7 ± 3.3 B	65.6
genistin	84.9 ± 0.8 A	100.0	79.4 ± 0.5 B	93.5	72.6 ± 0.7 C	85.5	78.0 ± 1.2 B	91.9	68.4 ± 1.7 D	80.5
acetyl glucoside										
daidzin	57.6 ± 2.4 A	100.0	10.4 ± 0.2 B	18.1	7.6 ± 0.4 C	13.1	8.2 ± 0.6 C	14.3	8.3 ± 0.1 C	14.5
glycitin	51.9 ± 1.0 A	100.0	46.0 ± 0.5 B	88.7	42.6 ± 0.1 C	82.2	46.0 ± 0.2 B	88.7	44.8 ± 0.3 D	86.3
genistin	91.9 ± 4.0 A	100.0	90.7 ± 5.1 AB	98.7	81.7 ± 5.1 D	88.9	89.6 ± 0.8 ABC	97.5	83.5 ± 4.3 C	90.8
aglycone										
daidzein	392.1 ± 11.2 A	100.0	335.0 ± 4.6 B	85.4	315.9 ± 1.5 C	80.6	323.8 ± 1.2 C	82.6	313.8 ± 1.8 C	80.0
glycitein	102.6 ± 0.9 A	100.0	83.5 ± 1.5 B	81.3	78.9 ± 0.3 BC	76.9	80.5 ± 1.7 BC	78.5	75.0 ± 0.4 C	73.1
genistein	274.3 ± 9.2 A	100.0	229.2 ± 5.6 B	83.6	209.7 ± 2.2 C	76.4	221.6 ± 1.0 B	80.8	211.3 ± 1.5 C	77.0
total	2100.3 ± 24.4 A	100.0	1851.5 ± 30.2 B	88.7	1747.6 ± 13.9 D	83.2	1793.5 ± 11.0 C	85.4	1728.8 ± 8.7 D	82.3

[a] Residual (%) was obtained by dividing the isoflavone content of the treated sample with the initial isoflavone content of the sample. The isoflavone content of initial sample was regarded as 100%. [b] Values are presented as means ± SD ($n = 3$). Means of same item in the same row with different letters were significantly different by Duncan's multiple range test ($p < 0.05$).

18.3 μg/g with residuals of 92.9 and 69.9%, respectively, after 120 days of storage at 25 °C. Meanwhile, those stored at 4 °C under similar packaging conditions were found to contain total isoflavone and genistein contents of 2679.3 and 19.2 μg/g, with residuals of 93.7 and 73.3%, respectively (**Table 1**).

The structure of isoflavones possessing a phenolic hydroxyl group tends to change due to oxidation in the presence of oxygen (*19*). It is therefore reasonable to expect that the available oxygen reduced in the presence of added deoxidant thus reduced the extent of oxidation of isoflavones. This may in turn contribute to the higher residuals of isoflavone noted in steamed black soybean stored with added deoxidant compared with those stored without deoxidant.

Interconversions may occur between the various forms of isoflavones. Malonyl glucoside and acetyl glucoside can be converted to their respective nonconjugated β-glucosides. Barnes et al. (*20*) indicated that malonyl glucoside might convert to acetyl glucoside and β-glucoside isoflavone through decarboxylation and hydrolysis, respectively. Additionally, the hydrolysis of glucosides by β-glucosidases may result in the formation of aglycones (*21*). Along with interconversion, the degradation and transformation of isoflavones to other derivatives may also occur (*22*−*24*). These reactions may all contribute to the change in the content of isoflavone observed during the storage period. However, the exact cause remained to be further investigated.

Among the three isoflavone isomers of aglycone, acetyl glucoside, and malonyl glucoside, generally, a lower residue residual of glycitein or glycitin was noted in steamed black soybean than in other isoflavone isomers after storage, regardless of storage temperature and packaging condition (**Tables 1** and

Table 4. Change of Isoflavone Contents of Black Soybean Koji after 120 Days of Storage at 25°C under Different Packaging Conditions

	content of isoflavone after storage									
	initial		desiccant + deoxidant		desiccant		deoxidant		without added	
isoflavone	content (μg/g of dry koji)	residual[a] (%)	content (μg/g of dry koji)	residual (%)	content (μg/g of dry koji)	residual (%)	content (μg/g of dry koji)	residual (%)	content (μg/g of dry koji)	residual (%)
β-glucoside										
daidzin	363.4 ± 7.3 A[b]	100.0	274.0 ± 0.8 B	75.4	258.6 ± 5.8 C	71.2	273.5 ± 1.6 B	75.3	272.4 ± 4.0 B	75.0
glycitin	109.0 ± 4.9 A	100.0	94.2 ± 0.4 B	86.5	88.6 ± 0.6 C	81.3	92.5 ± 0.9 BC	84.9	85.8 ± 0.6 D	78.8
genistin	577.4 ± 1.8 A	100.0	459.1 ± 2.3 B	79.5	436.7 ± 2.5 D	75.6	466.5 ± 4.8 C	80.8	457.9 ± 3.2 C	79.3
malonyl glucoside										
daidzin	59.1 ± 2.0 A	100.0	57.5 ± 0.3 A	97.4	51.4 ± 0.7 C	87.0	54.5 ± 2.0 B	92.4	48.7 ± 2.7 C	82.5
glycitin	77.2 ± 8.6 A	100.0	68.7 ± 0.5 AB	89.0	74.8 ± 9.8 A	96.9	69.8 ± 10.4 A	90.4	56.9 ± 3.9 BC	73.7
genistin	84.9 ± 0.8 A	100.0	76.1 ± 1.3 B	89.6	71.2 ± 2.6 C	83.8	74.9 ± 2.3 B	88.1	66.0 ± 1.8 D	77.7
acetyl glucoside										
daidzin	57.6 ± 2.4 A	100.0	10.2 ± 0.1 B	17.7	8.3 ± 0.2 BC	14.4	8.8 ± 0.1 BC	15.3	7.4 ± 0.2 C	12.9
glycitin	51.9 ± 1.0 A	100.0	44.8 ± 0.3 B	86.4	41.7 ± 0.3 C	80.3	45.9 ± 0.7 B	88.4	43.1 ± 0.7 D	83.1
genistin	91.9 ± 4.0 A	100.0	88.0 ± 0.4 AB	95.7	86.7 ± 1.2 AB	94.3	85.7 ± 4.5 B	93.2	85.9 ± 0.7 B	93.5
aglycone										
daidzein	392.1 ± 11.2 A	100.0	324.2 ± 1.4 B	82.7	316.4 ± 2.3 BC	80.7	317.1 ± 3.6 BC	80.9	311.0 ± 2.4 C	79.3
glycitein	102.6 ± 0.9 A	100.0	75.1 ± 0.3 B	73.2	72.6 ± 0.4 C	70.8	74.0 ± 1.0 BC	72.1	70.5 ± 0.4 C	68.7
genistein	274.3 ± 9.2 A	100.0	225.1 ± 5.9 B	82.1	209.0 ± 2.0 C	76.2	217.0 ± 2.1 BC	79.1	210.2 ± 5.3 C	76.6
total	2100.3 ± 24.4 A	100.0	1797.0 ± 9.2 B	85.6	1715.9 ± 9.6 C	81.7	1780.1 ± 25.5 B	84.86	1715.9 ± 18.7 C	81.7

[a] Residual (%) was obtained by dividing the isoflavone content of the treated sample with the initial isoflavone content of the sample. The isoflavone content of initial sample was regarded as 100%. [b] Values are presented as means ± SD ($n = 3$). Means of same item in the same row with different letters were significantly different by Duncan's multiple-range test ($p < 0.05$).

Table 5. β-Glucosidase Activity of Black Soybean Koji after 120 Days of Storage at 4 and 25 °C under Different Packaging Conditions

	β-glucosidase activity			
	4 °C		25 °C	
packaging condition	activity (units/g of dry koji)	residual[a] (%)	activity (units/g of dry koji)	residual (%)
desiccant + deoxidant	12.5 ± 0.0 a[b]	85.8	12.1 ± 0.2 a	82.6
desiccant	12.2 ± 0.5 a	83.7	11.5 ± 0.2 b	78.7
deoxidant	12.3 ± 0.5 a	83.9	11.7 ± 0.1 b	79.7
without added	11.7 ± 0.2 a	80.3	11.4 ± 0.3 b	78.1

[a] Residual (%) was obtained by dividing the activity of β-glucosidase activity of the treated sample with initial β-glucosidase activity of the sample. The initial β-glucosidase activity of the sample before storage was 14.62 units/g of dry koji. [b] Values are presented as means ± SD ($n = 3$), and means in the same column with different letters were significantly different by Duncan's multiple-range test ($p < 0.05$).

2). For example, the steamed black soybeans stored with desiccant and deoxidant at 25 °C showed an acetylglycitin residual of 89.2% at the end of the storage period. Meanwhile, higher residuals of 94.8 and 96.6%, respectively, were observed with the acetyldaidzin and acetylgenistin in the steamed black soybeans stored under similar conditions (**Table 2**). It has been suggested that the presence of a methoxy group at position 6 of the aromatic ring in glycetein, acetylglycetin, or malonylglycetin could have low bond dissociation energy, making them less stable compared to other respective isoflavone isomers (*25*). This instability may thus lead to the lower residual of glycetein, acetylglycetin, or malonylglycetin observed if compared with that of other respective isoflavone isomers.

Relative humidity has been reported to affect the stability of isoflavone (*26, 27*). In the present study, it was noted that steamed black soybeans stored at 4 °C with added desiccant generally resulted in a higher residual of isoflavone than those stored at 4 °C without added desiccant (**Table 1**). For example, total isoflavone and genestein showed residuals of 94.3 and 74.2%, respectively, in the steamed black soybeans sorted with desiccant at 4 °C for 120 days. Meanwhile, steamed black soybeans stored without desiccant showed relatively lower residuals of 93.6 and 73.3%, respectively, for total isoflavone and genistein. However, such a phenomenon was not observed when steamed black soybeans were stored at 25 °C (**Table 2**).

Changes of Isoflavone Content in Black Soybean Koji after Storage. **Tables 3** and **4** show the total and individual isoflavone contents of black soybean koji before and after the 120 days of storage at 4 and 25 °C, respectively. The initial content of the individual aglycone isomer (daidzein, glycitein, and genistein) found in the koji (**Tables 3** and **4**) is higher than the respective isoflavone isomer content of the steamed black soybeans (**Tables 1** and **2**). Besides, the black soybean koji also had a lower initial content of β-glucosides (daidzin, glycitin, and genistein) than the steamed black soybeans without fermentation. Apparently this discrepancy is due to fermentation when the catalytic action of β-glucosidase produced by microorganism as well as the hydrolysis and de-esterification of malonyl glucosides occurred in steamed black soybeans (*16*).

Similar to that observed for steamed black soybeans, the black soybean koji generally showed a reduced content of all isoflavone isomers and total isoflavone after 120 days of storage, regardless of storage temperature and packaging condition. Storage with deoxidant and desiccant also enabled the black soybean koji to retain a higher amount of the isoflavone. For example, the malonyldaidzin of black soybean koji stored with desiccant and deoxidant was the highest at 97.4% after storage at 25 °C compared to the lowest residual of 82.5% noted with that stored with neither desiccant nor deoxidant (**Table 4**). On the other hand, it is interesting to note there is a distinct difference in the change of β-glucoside and aglycone between the black soybean koji and steamed black soybeans during storage. Although no marked change in the contents of various

β-glucoside isoflavones was noted on steamed black soybeans after storage, regardless of storage temperature and packaging condition (**Tables 1** and **2**), a relatively marked reduction in content of these isoflavones was observed with black soybean koji (**Tables 3** and **4**). Furthermore, the black soybean koji generally exhibited a higher aglycone residual compared than that noted on steamed black soybeans after exposure to similar storage condition. For example, the residual of aglycone (daidzein, glycitein, and genistein) was 81.3−85.4% in black soybean koji stored with deoxidant and desiccant at 4 °C for 120 days (**Table 3**) compared to a lower residual of 77.2−78.8% found in black soybeans (**Table 1**). Besides, it is also interesting to note that the residual of acetyldaidzin of black soybean koji reduced markedly after storage, regardless of storage condition. The precise cause of this reduction merits further investigation. Additionally, it was also found that the content of total isoflavone reduced more in black soybean koji (**Tables 3** and **4**) than in steamed black soybeans (**Tables 1** and 2) under similar storage conditions.

Changes of β-Glucosidase Activity after Storage. β-Glucosidase is the enzyme that catalyzes the bioconversion of the isoflavone glucosides to their bioactive aglycone forms (*21*). Although only little β-glucosidase activity (0.6 unit/g of dry steamed black soybeans) was detected in the steamed black soybean, the black soybean koji exhibited a considerable β-glucosidase activity of ca. 14.6 units/g of dry koji, whereas a reduced activity was noted in black soybean koji after 120 days of storage regardless of packaging condition and storage temperature (**Table 1**). Among the various packaging conditions examined, black soybean koji stored with desiccant and deoxidant retained the highest β-glucosidase acitivities of 12.5 and 12.1 units/g of dry koji, respectively, at 4 and 25 °C and at the end of 120 days of storage. With a similar packaging method, black soybean koji usually exhibited a higher residual of β-glucosidase activity when stored at 4 °C than did that stored at 25 °C. For example, the residual β-glucosidase activity noted in koji stored at 4 °C with desiccant was found to be 83.7% compared with a lower residual of 78.7% observed in koji stored at 25 °C under similar packaging conditions. A similar phenomenon was also observed by Otieno et al. (*28*), who investigated the stability of β-glucosidase activity during the storage of soy milk fermented by *Bifidobacterium* and *Lactobacillus* spp. They found that β-glucosidase exhibited better stability at lower temperatures (−80 and 4 °C) than at higher temperatures (28 and 37 °C). Proteins generally showed better stability at 4 °C than at room temperature. Storage at room temperature often led to protein degradation and/or inactivity. This may account for the higher β-glucosidase activity noted in black soybean koji stored at 4 °C than at 25 °C. The catalytic action of β-glucosidase, which hydrolyzes nonconjugated β-glucosides but not conjugated glucosides into aglycones (*21*), may thus contribute to the relatively lower β-glucoside isoflavone residual and the higher aglycone residual noted in black soybean koji (**Tables 3** and **4**) than in steamed black soybeans (**Tables 1** and **2**). On the other hand, it is reasonable to expect that the extent of catalytic action exerted by β-glucosidase is higher at a higher temperature during the storage period. This might lead to less retention of β-glucoside noted in koji stored at 25 °C (**Table 4**) than at 4 °C (**Table 3**) under similar packaging conditions.

Data collected from the present study revealed that contents of various isoflavone isomers in steamed black soybeans and black soybean koji may reduce during storage. Although the retention of isoflavone varied with storage temperature, packaging condition, and individual isoflavone isomers, those stored with deoxidant and desiccant at 4 °C enabled steamed black soybeans and black soybean koji to retain the highest residual of isoflavone. Besides, black soybean koji retained a higher aglycone residual with a lower β-glucoside residual than did the steamed black soybeans after subjection to a similar storage condition. This information is useful to preserve the health benefits of steamed black soybeans or black soybean koji as they are further utilized as ingredients in the development of functional foods.

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Received for review November 27, 2008. Revised manuscript received December 26, 2008. Accepted December 28, 2008. This research was financially supported by The National Science Council, ROC (Taiwan) (NSC 96-2313-B-002-001).

JF803702X