

Full Length Research Paper

Isoflavone content and antioxidant activity of Thai fermented soybean and its capsule formulation

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Soybeans (*Glycine max*) are usually eaten as processed foods. Fermented soybeans are among the most popular of these processed foods. The aim of this study was to determine the effect of fermentation duration on isoflavone content and antioxidant activity of fermented soybeans. Capsule formulation of fermented soybeans was also studied. The Thai soybean variety, Rajamangala60, was fermented with *Aspergillus oryzae*. Isoflavone content and antioxidant activity were studied at 0, 12, 18, 36, 48, 96, 120, 168, 240, 360 and 480 h of fermentation duration. The results showed that isoflavone glycones (daidzin and genistin) decreased during fermentation, but aglycones (daidzein and genistein) increased. The highest amount of isoflavone aglycones was 384.30 ± 4.60 and 116.50 ± 1.56 mg/100 g fermented soybeans for daidzein and genistein, respectively. Antioxidant activity of fermented soybeans was evaluated by ABTS cation radical scavenging and ferric reducing antioxidant power (FRAP) methods. Antioxidant activity of fermented soybeans is increased during fermentation. Increases in isoflavone aglycones content and antioxidant activity were related to fermentation duration. The highest antioxidant activity of fermented soybean was found at the 240 h of fermentation with trolox equivalent antioxidant capacity (TEAC) 1.98 ± 0.09 μ g trolox/g fermented soybean and FRAP value of 0.623 ± 0.002 μ g FeSO₄/g fermented soybean. Soybeans fermented for 240 h were then formulated as capsules by a wet granulation method. They were then assessed for appearance, weight variation, disintegration time and antioxidative properties. The results showed that fermented soybean capsules conformed to USP32/NF27 criteria on weight variation and disintegration. Their antioxidant activity was lower than 240 h fermented soybeans, but still higher than the non-fermented ones ($p < 0.05$).

Key words: Antioxidant activity, *Aspergillus oryzae*, fermented soybean, radical scavenging, reducing power.

INTRODUCTION

Soybeans, *Glycine max* (L.) Merr. (Leguminosae) are one of the most important legumes (Yang et al., 2000). They

have been consumed in Asian countries for centuries as an important protein source to complement grain protein. Moreover, soybeans also contain many nutritious and functional phytochemicals such as isoflavones, phytic acids, saponins and oligosaccharides (Anderson and Wolf, 1995; Kwak et al., 2007). Soybeans can be eaten in their natural state, or as processed foods such as tofu, natto (Japanese fermented soybean), miso (fermented soybean paste), shoyu (Japanese fermented soy sauce), chungkookjang (Korean fermented soybean paste), kochujang (Korean fermented soy condiments), tempeh

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Abbreviations: FRAP, Ferric reducing antioxidant power; TEAC, trolox equivalent antioxidant capacity; ABTS, 2,2'-azinobis-(3-ethylbenthiazoline-6-sulfonic acid); HPLC, high performance liquid chromatography.

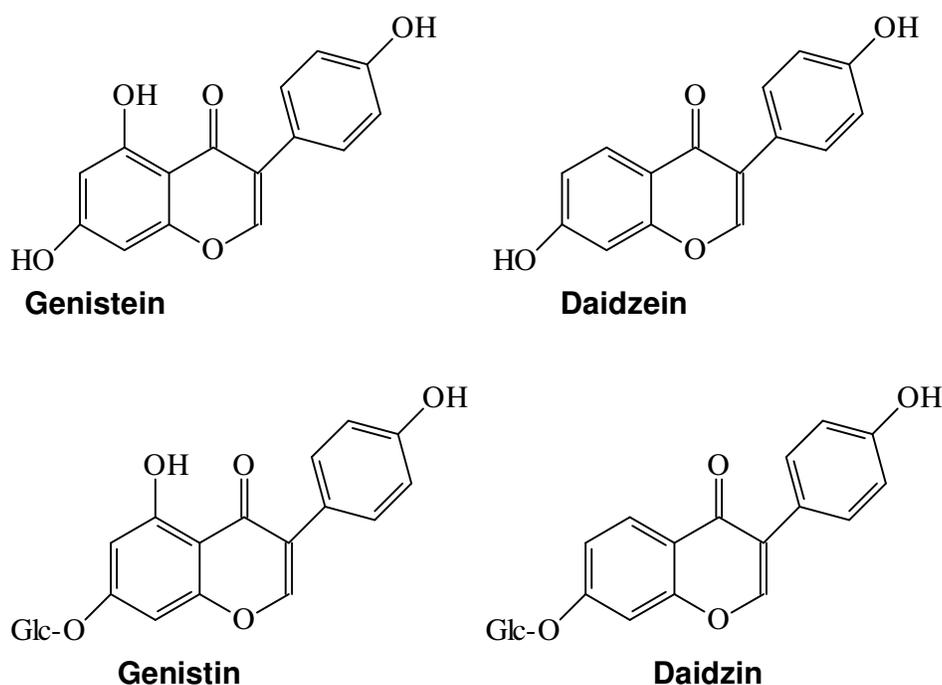


Figure 1. Chemical structure of Isoflavone aglycones (genistein and daidzein) and isoflavone glycones (genistin and daidzin). Glc is referred to a glucose molecule.

(traditional Indonesian fermented soybean), soy sauce, etc (Easaki et al., 1999; Kwak et al., 2007; Kim et al., 2008). Recently, many studies have revealed that fermented soybean foods do not lose their antioxidant activity (Berghofer et al., 1998; Easaki et al., 1998; Kim et al., 2008; Kwak et al., 2007). In contrast, their antioxidant activities are actually increased over non-fermented ones (Berghofer et al., 1998; Easaki et al., 1998; Kwak et al., 2007). Isoflavones are reported to exhibit many estrogenic, antioxidative, antiosteoporotic and anticarcinogenic activities (Cornwell et al., 2004). The chemical structures of isoflavone aglycones (daidzein and genistein) and glycones (daidzin and genistin) are shown in Figure 1.

Free radical-mediated damage may play a role in many disorders, such as chronic heart disease (CHD), diabetes and cancer. Free radicals are atoms or molecules that have one or more unpaired electrons in their atomic structures, so they are highly reactive. Free radicals can interact with cell membranes, and with macromolecules such as lipids, proteins and DNA, readily and rapidly, resulting in permanent damage. In human metabolism, oxygen is the major source of reactive oxygen species (ROS) which include the superoxide anion (O_2^-), the hydrogen peroxide (H_2O_2), the hydroxyl radical (OH^\cdot) and the peroxynitrite radical ($OONO^\cdot$). The imbalance between oxidant species production and antioxidant defenses leads to oxidative stress. In its more severe form, oxidative stress may result in cell death following

widespread macromolecule oxidation (Sridaya et al., 2009).

In Thailand, fermented soybeans are recognized as a health-promoting food. The Rajamangala60 variety is the most well-known in Thailand. Fermented Rajamangala60 soybean foods are particularly healthy for elderly persons. Therefore, a capsule formulation of Rajamangala60 fermented soybeans may be beneficial for the elderly as a health supplement.

As mentioned earlier, Thai fermented soybeans with different fermentation durations were screened for isoflavone content and antioxidant properties. That which showed the highest isoflavone content and antioxidant activity was selected for development as a nutritional supplement capsule to enhance antioxidative properties and free radical scavenging activity.

MATERIALS AND METHODS

Materials

ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate and TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) were purchased from Sigma Chemical (St. Louis, MO). Methanol, absolute ethanol, acetic acid, acetonitrile, sulfuric acid and hydrochloric acid were purchased from Fisher Scientific (Fair Lawn, NJ). Other chemical compounds were purchased from Wako Pure Chemical Industries (Osaka, Japan).

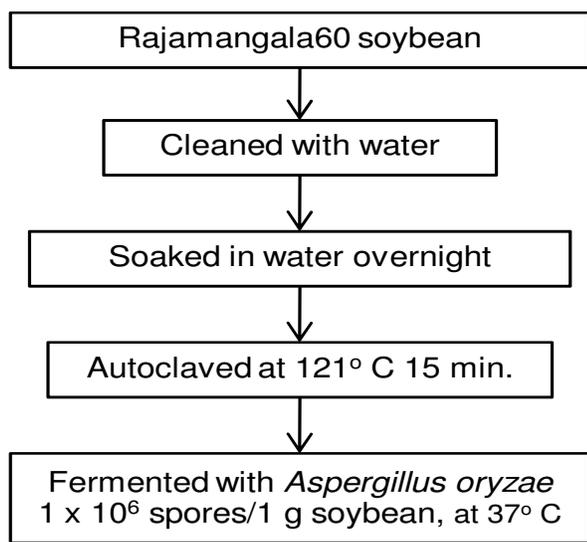


Figure 2. Flow diagram showing method of fermentation.

Method of fermentation

The method for fermentation was modified from the traditional Thai procedure. Briefly, Thai variety Rajamangala60 soybeans were cleaned and soaked in water overnight, then autoclaved at 121°C for 15 min. Sterilized soybeans were left to cool at about 40°C. Then *Aspergillus oryzae* was added at a concentration of about 1×10^6 spores per 1 g soybean. The soybean, *A. oryzae*, mixture was incubated at 37°C. The process of fermentation is shown in Figure 2.

Fermented soybean samples at 0, 6, 12, 18, 36, 48, 96, 120, 168, 240, 360 and 480 h after incubation were collected to test for isoflavone content and antioxidant activity.

Isoflavone extraction

Fermented soybeans were extracted by the following procedure: Each 10 g of sample was extracted with 100 ml of 80% methanol by stirring for 1 h at room temperature, and then centrifuging at $500 \times g$ for 30 min. The resulting supernatant was evaporated under controlled pressure using a rotary evaporator (Eyela, Tokyo, Japan) to dryness. The residue was then dissolved in 50% methanol and partitioned with 20 ml n-hexane three times to remove lipids.

Isoflavones analysis

The method for analysis of daidzein, genistein, daidzin and genistin was modified from Song et al. (1998). Briefly, each prepared sample solution was filtered through a 0.45 µm pore filter. An aliquot of 20 µl was used for analysis by high performance liquid chromatography (HPLC).

Samples were injected into an analytical Hypersil®-ODS column (Thermo Scientific, USA) 250 x 4.6 mm i.d. with 5 µm internal particle size. The mobile phase used was gradient elution of 0 - 60% acetonitrile in 0.1% trifluoroacetic acid for 55 min with a flow rate of 1 ml/min and detection by UV absorbance at 262 nm. The column temperature was controlled at 25°C.

In order to determine the contents of each isoflavone glycone

and aglycone, the standard curves were calculated using peak areas of different concentrations of standard daidzein, daidzin, genistein and genistin.

Antioxidant activity

ABTS radical cation decolorization assay

ABTS can produce stable free radicals, which are decolorized into their non-radical form when reacting with antioxidants. The method for determining ABTS radical scavenging activity was modified from Re et al. (1999). Briefly, ABTS^{•+} was generated by oxidation of 7 mM ABTS with 2.45 mM potassium persulfate ($K_2S_2O_8$), and then stored in a dark place at room temperature for 12-16 h. Then ABTS^{•+} stock solution was diluted with deionized water to obtain ABTS^{•+} working solution. The reactions between ABTS^{•+} working solution and different sample concentrations were initiated and stored at room temperature until the reaction was complete. After that, the reacted samples' absorbance was read at 734 nm. The results are expressed as trolox equivalent antioxidant capacity (TEAC) (µg trolox/1 g fermented soybean).

Ferric reducing antioxidant power ferric reducing antioxidant power (FRAP) assay

The method for determining the ferric reducing ability of each sample was modified from Benzie and Strain (1996). Briefly, FRAP reagent was prepared by mixing 0.1 M acetate buffer (pH 3.6) with 10 mM TPTZ and 20 mM ferric chloride in a ratio of 10:1:1 v:v:v. Then, 1.9 ml of FRAP reagent was added to 0.1 ml of each sample, and left at room temperature for 5 min until the reaction was complete. The reacted samples were read for absorbance at 593 nm using standard ferrous sulfate as control. An increase in the absorbance of the reaction mixture indicated an increase in the reducing power of the sample. The changes in absorbance A_{593nm} between the final reading selected and the absorbance of blank reagent was calculated for each sample and related to ΔA_{593nm} of a Fe^{2+} standard solution tested in parallel.

Capsule formulation

Preparation of granules

The fermented soybeans sample with the highest isoflavone content and antioxidant activity was selected to produce the hard gelatin capsule formulation. First, fermented soybean was ground into a fine mass using an electric grinder. Next, corn starch powder (USP) was slowly incorporated into the ground soybean and mixed until the desired dampened mass was obtained. Then a binding solution of 10% gelatin in water was added to the dampened mass slowly, drop-by-drop, until 2.5% gelatin in dampened mass was achieved. After that, the wet mass was forced through a #10 sieved (2000 µm), and dried at 40°C for 12 h using a hot air oven. The dried particles were then passed through a #12 sieve (1680 µm) to obtain granules.

Preparation of capsules

Size #12 granules (1680 µm) were filled into a size 0 capsule (outer diameter 7.65 mm, actual volume 0.68 ml) at a weight of 500 mg per capsule using a manual capsule filling machine. The processes of granule and capsule preparation were shown in Figure 3. Each capsule was sensory evaluated for external appearance such as

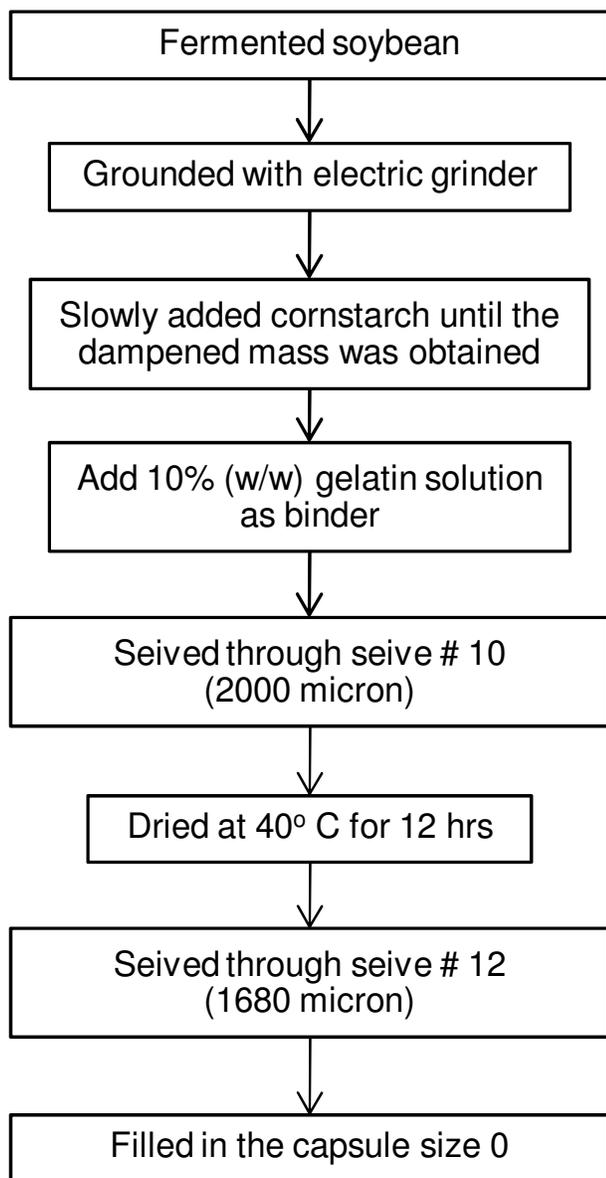


Figure 3. Flow diagram showing method of granule and capsule preparation.

color, capsule shape, capsule breakage, capsule leakage and odor. Capsules were then further assessed for quality following the official method of the United States Pharmacopeia/National Formulary USP32/NF27 edition (The United States Pharmacopeial Convention 2009).

Capsules quality control

Capsules were tested for disintegration following the official method of USP32/NF27 in general chapter part <2040>, disintegration and dissolution of dietary supplements. Capsules were also tested for weight variation following the official method of USP32/NF27 in general chapter part <2091>, weight variation of dietary supplement.

Isoflavone content and antioxidant activity of fermented soybean capsules

Ten capsules were dislocated, and the powder inside the capsules was extracted with the same method used in isoflavone extraction. Isoflavone content and antioxidant activity were determined using the same method as mentioned earlier.

RESULTS AND DISCUSSION

Analysis of isoflavone glycones and aglycones were performed by HPLC. HPLC chromatogram and isoflavone content are shown in Figure 4 and Table 1, respectively. In Figure 4, there are four main peaks. The glycones daidzin and genistin gave retention times at around 11 and 18 min, respectively, while the aglycones daidzein and genistein gave retention times at around 29 and 40 min, respectively. From the results, the following conclusion can be drawn. Firstly, the isoflavone glycones, (daidzin and genistin) slowly decreased in relation to a fermentation time. Although, there is an upsurge of daidzin content during the first 48 h of fermentation, later the daidzin content slowly decreased. Secondly, the isoflavone aglycones (daidzein and genistein) slowly increased in relation to a fermentation time. The highest amount of aglycones was found at 240 h after fermentation. There were no significant differences between all isoflavones content after 240 h after fermentation. These results are comparable to previous reports which indicated that isoflavone aglycones increases with fermentation duration (Kwak et al., 2007; Berghofer et al., 1998; Easaki et al., 1998; Easaki et al., 1999).

Antioxidant activities of fermented soybeans were evaluated by the ABTS cation radical scavenging and FRAP methods. Antioxidant activity results (Table 2) show that the antioxidant activity of fermented soybean increases with fermentation duration. In ABTS method, the antioxidant activity of each fermented soybean ranged from TEAC 0.74 ± 0.04 to 2.06 ± 0.21 μg Trolox/g fermented soybeans. TEAC of fermented soybeans at 0 - 18, 96 - 120 and 168 - 480 h of fermentation duration are not significantly different. In FRAP method, the antioxidant activity of each fermented soybean ranged from FRAP value of 0.295 ± 0.002 to 0.629 ± 0.001 μg FeSO₄/g fermented soybean. FRAP value of fermented soybeans at 12 - 18 and 240 - 480 h of fermentation duration are not significantly different. The highest antioxidant activities of fermented soybeans can be achieved after being fermented for 240 h. Both ABTS and FRAP methods show similar tendency.

The study also revealed that the isoflavone content and antioxidant activity of fermented soybean correlated with fermentation duration ($p < 0.005$). Isoflavone aglycones contents seen in Table 1 are related to the antioxidant activity of fermented soybean. Total aglycones (daidzein and genistein) were related with antioxidant activity

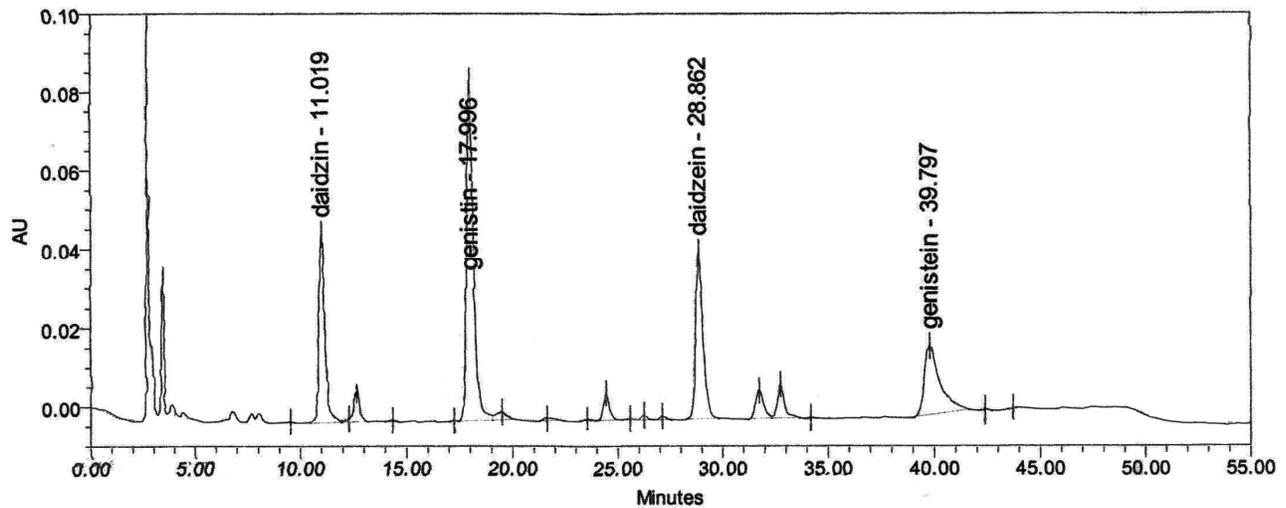


Figure 4. HPLC chromatogram of soy isoflavone of fermented soybean *A. oryzae* at day 10 after fermentation.

Table 1. Isoflavone content of fermented soybean with different fermentation duration.

Fermented duration (h)	Isoflavone content (mg/100 g fermented soybean) ¹			
	Daidzin	Genistin	Daidzein	Genistein
0	676.73 ± 8.17a	992.58 ± 11.07a	49.04 ± 0.55ac	42.15 ± 0.85a
12	669.00 ± 21.40a	603.38 ± 7.00b	42.04 ± 0.23ac	27.63 ± 0.85b
18	692.31 ± 0.30a	389.95 ± 9.37c	29.01 ± 0.23b	25.51 ± 0.23bc
36	790.80 ± 72.80a	276.40 ± 22.70d	44.61 ± 3.94c	20.86 ± 2.95c
48	1048.00 ± 10.40b	251.68 ± 1.73d	79.99 ± 1.43d	56.77 ± 1.41d
96	559.28 ± 3.43c	129.14 ± 2.14e	241.20 ± 3.64e	71.89 ± 1.40e
120	263.64 ± 2.42d	50.74 ± 1.01f	247.96 ± 4.15e	65.71 ± 3.09e
168	225.60 ± 79.50d	48.38 ± 0.74f	340.24 ± 4.19f	92.78 ± 2.16f
240	99.58 ± 12.83e	26.01 ± 0.19f	369.67 ± 6.05g	104.63 ± 1.91g
360	50.10 ± 25.40e	14.20 ± 9.16f	382.10 ± 3.01g	116.50 ± 1.56h
480	109.00 ± 2.97e	16.56 ± 3.01f	384.30 ± 4.60g	115.93 ± 0.96h

¹Value in table expressed as mean ± SD. Means in each column with different letters are significantly different ($p < 0.05$).

performed by ABTS method ($r = 0.521$, $p < 0.05$) and FRAP method ($r = 0.563$, $p < 0.05$). Therefore, it can be concluded that the higher isoflavone aglycone, the higher the antioxidant activity. This is same with previous report by Izumi et al. (2000) and Rao and Muralikrishna (2002) which reported that isoflavone aglycones have higher antioxidant activity than their bounded form.

The results indicate that, fermented soybean after day 10 (240 h) showed the highest isoflavone content and antioxidant activity. Therefore, fermented soybean at day 10 was selected to formulate as a capsule form using hard-gelatin capsules. The results of capsules quality are shown in Table 3, and the photographs of the capsules are shown in Figure 5. Capsules made from day 10

fermented soybean show a good appearance, with perfect capsule shape, no capsule breakage or content leakage, and with less odor than that of fermented soybean. Weight variation of these capsules ranged from 97 to 106% of average weight. None of the capsules was out of the range of 90 - 110% average weight (USP32/NF27 criteria). Thus, these capsules conform to USP/NF27 general chapter part <2091>. The fermented soybean capsules were further tested for disintegration according to the method of USP32/NF27 general chapter part <2040> for disintegration and dissolution of dietary supplements. Disintegration time of the capsules ranged from approximately 1 to 3 min, with an average disintegration time of 2.28 ± 0.94 min. In chapter <2040>,

Table 2. Antioxidant activities of fermented soybean with different fermentation duration determined by ABTS cation radical scavenging and FRAP method.

Fermented duration (h)	TEAC ($\mu\text{g Trolox/g}$ fermented soybeans) ¹	FRAP value ($\mu\text{g FeSO}_4/\text{g}$ fermented soybean) ²
0	0.74 \pm 0.04a	0.295 \pm 0.002a
12	0.79 \pm 0.02a	0.345 \pm 0.001b
18	0.74 \pm 0.05a	0.349 \pm 0.001b
36	1.04 \pm 0.01b	0.384 \pm 0.005c
48	1.26 \pm 0.03c	0.437 \pm 0.001d
96	1.61 \pm 0.04d	0.489 \pm 0.004e
120	1.58 \pm 0.12d	0.541 \pm 0.004f
168	1.97 \pm 0.14e	0.598 \pm 0.004g
240	1.98 \pm 0.09e	0.623 \pm 0.002h
360	2.02 \pm 0.15e	0.626 \pm 0.001h
480	2.06 \pm 0.21e	0.629 \pm 0.001h

Value in table expressed as mean \pm SD. Means in each column with different letters are significantly different ($p < 0.05$). ¹ TEAC: Trolox equivalent antioxidant capacity; ² FRAP: Ferric reducing antioxidant power.

Table 3. Weight variation, disintegration, antioxidant activity, and isoflavone contents of hard gelatin capsules made from day 10 fermented soybeans.

Tests	Results	Conform to USP32/NF27
Weight variation	None of the tablets is out of range 90 - 110% average weight	Yes
Disintegration (min)	2.28 \pm 0.94	Yes
ABTS cation radical scavenging activity (TEAC) ($\mu\text{g Trolox/g}$ fermented soybeans)	1.76 \pm 0.22*	-
Reducing power (FeSO_4 equivalent) ($\mu\text{g FeSO}_4/\text{g}$ fermented soybean)	0.43 \pm 0.01*	-
Isoflavone contents (mg/100 g fermented soybean)		
Daidzin	59.20 \pm 1.94*	-
Genistin	12.36 \pm 6.76*	-
Daidzein	270.68 \pm 13.44*	-
Genistein	76.93 \pm 18.16*	-

* The results are significantly lower than day ten fermented soybean ($p < 0.05$) but higher than non-fermented one ($p < 0.05$).

it is specified that the disintegration time of dietary supplements should not exceed 30 min. Thus, these capsules conform to USP/NF27 general chapter part <2040>. Indeed, all the results have demonstrated that these capsules conform to USP32/NF27. Capsules were additionally tested for isoflavone content and antioxidant activity. Isoflavone aglycones content and antioxidant activity of capsule were lower than day 10 fermented soybean ($p < 0.05$), but still higher than non-fermented soybeans ($p < 0.05$). The reduction of isoflavone content of the capsules may be as a result of the processes used in production: heating of the products may degrade certain phytochemicals, resulting in the decrease of

antioxidant activity. A greater surface area of the small-sized fermented soybean particles may lead to a reaction of antioxidative phytochemicals with the oxygen or radicals in the environment, thus causing the degradation of antioxidative phytochemicals. However, the sensory profiles (such as color and odor) of fermented soybeans which are developed into capsules are superior to fermented soybean. This makes the capsules easier to ingest.

In conclusion, the isoflavone content and profile of fermented soybeans are related to the duration of fermentation: isoflavone aglycones are increased with fermentation duration, while isoflavone glycones are

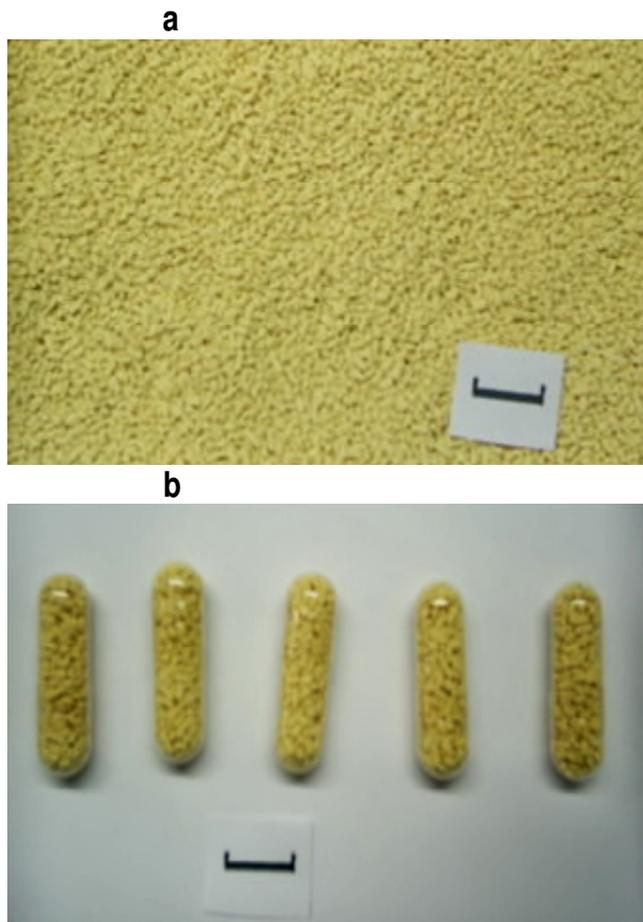


Figure 5. (a) Photograph of fermented soybean granules and (b) their capsules. One bar length indicates 1 cm.

decreased with fermentation duration. Antioxidant activity is increased with fermentation duration, and also increased with aglycones content. The formulation of fermented soybean capsules was successful. Soybeans at 10 days (240 h) after fermentation demonstrated the highest isoflavone contents and antioxidant activity. Further investigations will focus on improving the capsules' isoflavone content and antioxidant activity.

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REFERENCES

- Anderson RL, Wolf WJ (1995). Compositional changes in trypsin inhibitors, phytic acid, saponin, and isoflavones related to soybean processing. *J. Nutr.* 125: 24-31.
- Benzie IFF, Strain JJ (1996). The ferric Reducing Ability of Plasma (FRAP) as a measure of Antioxidant Power: The FRAP Assay. *Anal. Biochem.* 239: 70-76.
- Berghofer E, Grzedkowiak B, Mundigler N, Sentall WB, Welcak J (1998). Antioxidative properties of feta bean- soybean- and oat tempeh. *Int. J. Food. Sci. Nutr.* 49: 45-54.
- Cornwell T, Cohick W, Raskin I (2004). Dietary phytoestrogen and health. *Phytochemistry*, 65: 995-1016.
- Easaki H, Onozaki H, Morimitsu Y, Kawakishi S, Osawa T (1998). Potent antioxidative isoflavones isolated from soybeans fermented with *Aspergillus saitoi*. *Biosci. Biotechnol. Biochem.* 62(4): 740-746.
- Easaki H, Watanabe R, Onozaki H, Kawakishi S, Osawa T (1999). Formation mechanism for potent antioxidative *o*-Dihydroxyisoflavones in soybeans fermented with *Aspergillus saitoi*. *Biosci. Biotechnol. Biochem.* 63(5): 851-858.
- Kataoke s, Kubota Y and Kikuchi M (2000). Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans.
- Kim NY, Song EJ, Kwon DY, Kim HP, Heo MY (2008). Antioxidant and antigenotoxic activities of Korean fermented soybean. *Food Chem. Toxicol.* 46: 1184-1189.
- Kwak CS, Lee MS, Park SC (2007). Higher antioxidant properties of chungkookjang, a fermented soybean paste, may be due to increased aglycone and malonylaglycone isoflavone during fermentation. *Nutr. Res.* 27: 719-727.
- Rao M, Muralikrishna G (2002). Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (Ragi, *Eleusine coracana* Indaf-15), *J. Agric. Food Chem.* 50: 889-892.
- Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 26: 1231-1237.
- Song T, Barua K, Buseman G, Murphy PA (1998). Soy isoflavone analysis: quality control and a new internal standard. *Am. J. Clin. Nutr.* 68(1): 147S-147S.
- Srividya AR, Yadav AK, Dhanbal SP (2009). Antioxidant and antimicrobial activity of rhizome of *Curcuma aromatica* and *Curcuma zeodaria*, Leaves of *Arbutilon indicum*. *Arch. Pharm. Res.* 1(1): 14-19.
- The United States Pharmacopeial Convention (2009). The United States Pharmacopeia/National Formulary. USP32/NF27. Rockville, MD, USA. pp. 782-786.
- Yang JH, Mau JL, Ko PT, Huang LC (2000). Antioxidant properties of fermented soybean broth. *Food Chem.* 71: 249-254.