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ARTICLE

Enhanced aglycone production of fermented soybean products by *Bacillus* species

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ABSTRACT This study evaluated the effect of starter culture and fermentation period on the isoflavone content of protein-rich soybeans variety TG145. Initially, soybeans were washed, soaked in water for 16 h and autoclaved at 121°C for 40min. Three different bacterial starter cultures (~10^4 CFU/g) namely *Bacillus subtilis* BEST195, *B. subtilis* Asaichiban and *B. subtilis* TN51 were then added and the fermentation was allowed to proceed at 42°C for 24 h (*natto*-style) and 72 h (*thua nao*-style). The quantities of six major isoflavones (daidzin, genistin, glycitin, daidzein, genistein, and glycitein) were then determined in these fermented soybean products using reverse phase HPLC technique. Generally, our results clearly showed that the content of total isoflavones in the fermented products prepared by *Bacillus* starter cultures greatly increased ranging from 43 to 99% compared to that of the unfermented autoclaved soybeans. In addition, a dramatic increase of aglycones was also observed (> 400%) in the soybean products fermented by *Bacillus* subtilis strain TN51. This present study suggests a promising use of *Bacillus* starter cultures in improving isoflavone compounds especially the aglycones which would benefit for novel functional food development. **Acta Biol Szeged 53(2):93-98 (2009)**

KEY WORDS

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Bacillus
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Soybeans (*Glycine max* (L.) Merr.) are highly regarded as a healthy food in several Asian countries and are widely consumed as soymilk, *tofu* and fermented products. In these soybeans and soy-products, several phytochemicals are found and they appear to be the active compounds causing many beneficial health effects (Dixon 2004; Wiseman 2006). Soy isoflavones in particular are a group of natural heterocyclic phenols comprising of aglycones, β-glucosides, acetylglucosides, and malonylglucosides. These isoflavone compounds are of great importance due to their pharmacological and antioxidant properties. For example, several researchers have showed the beneficial use of isoflavones; these include prevention of mammary cancer (Gotoh et al. 1998), reduced risk of cardiovascular diseases (Teede et al. 2001), improvement of bone health and menopause symptoms (Potter et al. 1998; Ishimi et al. 2002), antimutagenic effects (Peterson et al. 1998; Park et al. 2003), and antidiabetic effects (Liu et al. 2006). Of these compounds, it appears that the glucoside forms are predominant in soybeans. However, it should be noted that the content and composition of isoflavones are variable depending on many parameters such as soybean variety (Wang and Murphy 1994; Lee et al. 2003, 2007), geographical plantation (Wang and Murphy 1994; Hoeck et al. 2000;, Lee et al. 2003), storage time (Lee et al. 2003), crop year (Wang and Murphy 1994), and food processing techniques (Jackson et al. 2002; Kao et al. 2004, Lee et al. 2007).

In Asia, there are several traditional fermented soybean products such as Japanese *natto*, Korean *chungkookjang*, Indian *kinema* and Thai *thua nao*. Interestingly, numerous studies have reported that the amount of aglycones is much higher in fermented soybeans compared to that of unfermented soybeans; such products include *natto* (Wei et al. 2008), *miso* (Yamabe et al. 2007), *sufu* (Yin et al. 2004, 2005), *douche* (Wang et al. 2007), and *chungkukjang* (Kwak et al. 2007). At present, the presence of aglycone isoflavones has attracted special interest due to their bioavailability, high rate of absorption in animals and humans (Izumi et al. 2000; Kano et al. 2006) and high antiproliferative activity on human cancer cells (Peterson et al. 1998). Especially for fermented soybeans, several studies have also revealed the role of microbes in soybean fermentation as the active agents to enrich isoflavone aglycones. This conversion of glucoside isoflavones to aglycones during the fermentation is achieved by the activity of microbial β-glucosidase enzymes (Chien et al. 2006;, Kuo et al. 2006;, Chun et al. 2007, 2008). As part of the programme to improve nutritional value of *thua nao*, we previously screened and identified a strong proteolytic bacterium *Bacillus subtilis* sp. TN51 from commercial *thua*

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nao products (Dajanta et al. 2009) and attempted to use such a strain as a pure starter culture in soybean fermentation. This study was therefore undertaken to investigate the content and composition of isoflavone compounds in unfermented soybean and fermented soybean prepared by *Bacillus subtilis* strains. The content of isoflavones of *thua nao* prepared in traditional manner was also determined.

Materials and Methods

Bacterial strains and culture conditions

Three bacterial strains used in this study were *Bacillus subtilis* TN51 isolated from *thua nao* products (Dajanta et al. 2009), *B. subtilis* BEST195 (Qiu et al. 2004) and *B. subtilis* ASA isolated from Asaichiban *natto* product. These bacteria were routinely cultured on nutrient agar and their stock cultures were maintained at -80°C in 20% glycerol. For inoculum preparation, the bacteria were grown in nutrient broth at 37°C for 24 h. The cells were then harvested, resuspended in sterile distilled water and properly adjusted to obtain a concentration of 10^4 CFU/mL. The suspension was served as the inoculum for soybean fermentation.

Preparation of fermented soybeans

Protein-rich soybean seeds variety TG145 supplied by the Field Crops Research Institute, Department of Agriculture, Chiang Mai, Thailand were used in this study. Initially, the dehulled soybeans were washed and soaked in tap water for 16h at ambient temperature (~25°C). After decanting the water, soaked soybeans were placed in a plastic bag and autoclaved at 121°C for 40 min. To initiate the fermentation, the autoclaved soybeans were inoculated with the *Bacillus* inocula. The fermentation process was allowed to proceed at 42°C for 24 h (*natto*-style) and 72 h (*thua nao*-style). In addition, *thua nao* products fermented in the traditional manner were also prepared; for this, soybeans were cooked by boiling for 2 – 3 h instead of autoclaving.

Extraction of isoflavones

Soybean isoflavones were extracted using the method of Achouri et al. (2005). Two grams of freeze-dried samples were dissolved in 10 ml of 80% methanol in water (v/v) in a 50 mL screw-cap tube, vortexed for 1 min, sonicated in a FB 15046 sonicator (Fisher Scientific, Germany) at 50 – 60 Hz of ultrasonic frequency for 15 min, and centrifuged at 3200 rpm for 30 min. The sample remains were subjected to repeated extractions with 10 mL of 80% methanol. Supernatants from two extractions were then combined, concentrated with an evaporator and dried by nitrogen gas flow at room temperature. Dried isoflavone extracts were subsequently dissolved with 3 mL of 80% methanol and filtered through a 0.2 µm filter membrane prior to HPLC injection.

HPLC analysis

Isoflavones were analysed by reverse-phase HPLC according to the method of Kim and Chung (2007). The Dionex HPLC system used was equipped with a P680 HPLC pump, ASI-100 automated sample injector, thermostatted column compartment TCC-100, Agilent Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm), and PDA-100 photodiode array detector. The mobile phase was composed of 0.1% (v/v) acetic acid in filtered MilliQ water (solvent A) and 0.1% (v/v) acetic acid in acetonitrile (solvent B). The injection volume was 20µl and the components were eluted using the following solvent gradient: from 0 to 50 min 15 – 35% solvent B; then held at 35% solvent B for another 10 min; and from 60 to 65 min re-equilibrated back to 15% solvent B. The flow rate was 1.0 mL/min and UV detector wavelength was set at 254 nm. Stock solutions of six isoflavone standards including daidzin, glycitin, genistin, daidzein, glycitein, and genistein (Plantech-UK) were prepared in 80% (v/v) methanol. Calibration curves were plotted by using peak area and corresponding isoflavone concentration. The identity and purity of isoflavones in the samples were proven by comparing the retention times and UV spectra of the standards.

Statistical analysis

Isoflavone values reported were the means of triplicate determinations with standard deviations (means ± SD). Analysis of variance (ANOVA) was also carried out using SPSS Version 15.0. Duncan's multiple range tests were introduced to determine the significant differences between the treatments ($P<0.05$).

Results and Discussion

A typical HPLC chromatogram of the isoflavone standards, unfermented soybeans, and fermented soybean products are represented in Figure 1. Due to their different chemical structures, these isoflavone compounds were successfully separated by the HPLC system established in this study. The chromatogram of isoflavones extracted from unfermented and fermented soybeans were similar to that of the pure isoflavone standards (Fig. 1). The reliability of the extraction method was also assessed by addition of known concentration of glycitein standard into unfermented soybean before extraction of isoflavones. Recovery percentages for glycitein were calculated as 98% (n = 5). By this means, the correlation coefficients (R^2) of all standard curves of isoflavone standards were over 0.99 (data not shown). Besides, the HPLC soybean chromatograms also showed several peaks of unidentified compounds (X1 – X6). Based on previous reports (Lee et al. 2004; Kim and Chung 2007), these unknown compounds were possibly malonyldaidzin, malonylglycitin, malonylgenistin, acetyldaidzin, acetylglycitin, and acetylgenistin. These compounds are also isoflavone derivatives and their content

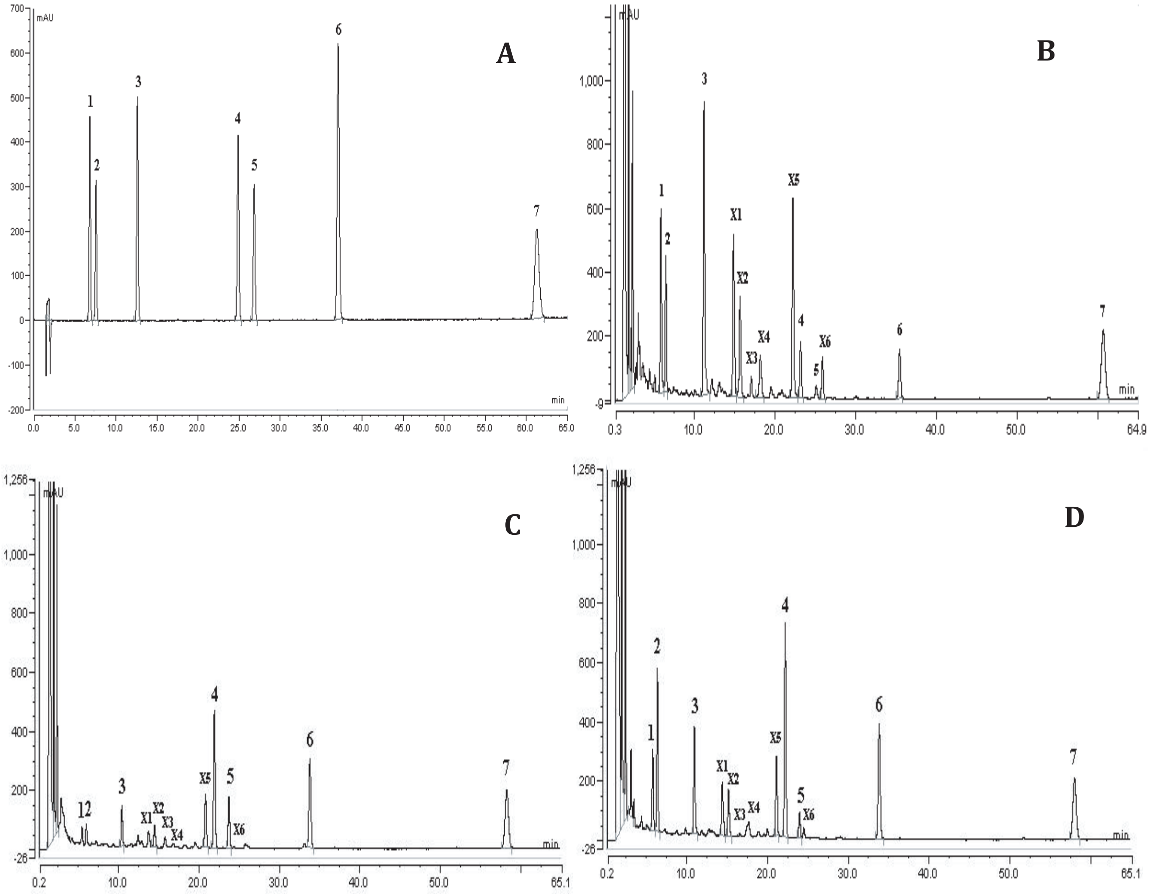

Figure 1. Representatives of HPLC chromatograms showing isoflavone content of (A) standard isoflavones (1 = daidzin, 2 = glycitin, 3 = genistin, 4 = daidzein, 5 = glycitein, 6 = genistein, and 7 = flavone); (B) autoclaved soybeans; (C) traditionally fermented soybeans; and (D) fermented soybeans prepared with B. subtilis TN51. X1, X2, X3, X4, X5, X6 are unidentified compounds that may be malonyldaidzin, malonylglycitin, malonylgenistin, acetyldaidzin, acetylglycitin, and acetylgenistin (see text for details).

appeared to be much higher in unfermented soybeans (Fig. 1B). These glucosides-based compounds have been reported in small amounts and thus not taken into account in our study. It is also evident that these compounds are abundant in soybeans but not in fermented soybeans.

The concentration and composition of isoflavones in unfermented cooked soybeans (boiled and autoclaved) are shown in Table 1. The total isoflavones are expressed as the sum of glucosides (daidzin + glycitin + genistin) and aglycones (daidzein + glycitein + genistein). According to Table 1, the data clearly show that cooking method also affect the isoflavone contents. For unfermented soybeans, autoclaving appears to promote the concentration of total isoflavones especially the glucoside forms. Boiled soybean showed lower level of glucoside isoflavones probably due to heat damage (boiling in water for 4 h). This event is in agreement as previously described by Jackson et al. (2002) and Kao et al. (2004) in that the process of soaking and heating soybeans in water led to a decrease of glucosides. Total glucosides were the largest proportion in both unfermented soybean samples and were accounted for 78 and 84% of the total isoflavones, respectively. This was slightly lower than the 88% proportion as reported by Wei et al. (2008) for unfermented autoclaved soybean. This minor discrepancy was probably due to the difference in the variety of soybean and isoflavones extraction method (Achouri et al. 2005; Yamabe et al. 2007). Genistin was shown to be the most predominant isoflavon in both types of cooked soybeans followed by daidzin and glycitin as in the amount of 116-167, 72-100, and 68-108 µg/g, respectively. For the aglycones group, daidzein (36-37 µg/g) was found at significantly higher level than genistein (23-25 µg/g) and glycitein (10-12 µg/g) ($P<0.05$).

Conversion of glucoside isoflavones into aglycones isoflavones during soaking and cooking has been reported previously. Kao et al. (2004) demonstrated that soaking temperature and time affect the content and conformation of

Table 1. Concentrations (µg/g, dry mass basis) of isoflavone components of unfermented soybeans and soybeans fermented with *Bacillus subtilis* strains at 42°C[a].

	Unfermented soybeans[b]		Fermented soybeans[b]			
Isoflavone			thua nao-style		natto-style	
	BSB	ASB	BTN	CTN	BNA	BNB
I. Glucosides						
Daidzin	72 ± 5Bd (22)	100 ± 19Bc (22)	68 ± 8Cd (11)	11 ± 2Ce (3)	169 ± 18Cb (20)	286 ± 21Aa (32)
Glycitin	68 ± 5Bd (21)	108 ± 15Bc (24)	191 ± 12Ab (30)	24 ± 6Ce (7)	279 ± 13Aa (32)	297 ± 10Aa (34)
Genistin	116 ± 7Ad (35)	167 ± 12Ac (37)	84 ± 8Be (13)	26 ± 7Cf (8)	214 ± 29Bb (25)	256 ± 8Ba (29)
Total Glucosides	255 ± 18d (78)	375 ± 42c (84)	343 ± 27c (54)	60 ± 14e (19)	663 ± 47b (77)	839 ± 23a (94)
II. Aglycones						
Daidzein	37 ± 3Cc (11)	36 ± 2Cc (8)	195 ± 9Aa (31)	135 ± 14Ab (42)	131 ± 4Db (15)	15 ± 2Dd (2)
Glycitein	10 ± 1Ec (3)	12 ± 1Dc (3)	32 ± 3Db (5)	63 ± 10Ba (20)	13 ± 2Fc (2)	7 ± 2Cc (1)
Genistein	25 ± 2Dc (8)	23 ± 1CDc (5)	70 ± 4BCa (11)	61 ± 7Bb (19)	57 ± 5Eb (7)	27 ± 3Cc (3)
Total Aglycones	73 ± 6d (22)	71 ± 4d (16)	297 ± 14a (46)	260 ± 22b (81)	201 ± 11c (23)	49 ± 7e (6)
Total Isoflavones	328 ± 24d (100)	447 ± 46c (100)	640 ± 31b (100)	320 ± 32d (100)	864 ± 37a (100)	888 ± 21a (100)

[a] Values are mean ± standard deviation (n = 3) and number in parentheses are percentage of each isoflavone relative to total isoflavones. Data in the same row (small letters) or same column (capital letters) with different superscripts were significantly different ($P<0.05$). [b] BSB = boiled soybean; ASB = autoclaved soybean; BNA = soybeans fermented by *Bacillus subtilis* Asaichiban; BNB = soybeans fermented by *B. subtilis* BEST 195; BTN = *B. subtilis* TN51, which isolated from local Thai fermented soybean (*thua nao*); CTN = control, traditional fermentation.

isoflavone compounds in soybean. After soaking soybean at 25, 35, and 45°C for 12 h, the concentration of aglycone isoflavones (daidzein, glycitein, and genistein) clearly increased, conversely there was a decrease of conjugated (malonylglucosides and acetylglucosides) and β-glucoside isoflavones. Such results suggested that malonylglucoside can be converted to acetylglucoside and further converted to glucoside or aglycone isoflavones during the soaking process. Also, Chein et al. (2005) indicated that the conformation of glucosides could be changed during moist heating, with the highest rate of conversion of malonylgenistin to genistin, followed by malonylgenistin to acetylgenistin, and acetylgenistin to genistin. In this study, soybean seeds were soaked in water at 25°C for 16 h and boiled in boiling water for 4 h or autoclaved at 121°C for 40 min; therefore, genistin predominates in all of the cooked soybeans.

Similar results were presented in this study for genistin and daidzein, which are the major glucoside and aglycone forms in unfermented soybean (Kim and Chung 2007) although Lee et al. (2007) showed higher amounts of genistein than daidzein in Ohio soybeans. A number of investigators reported that malonylgenistin is the most predominant in soybeans (Lee et al. 2004; Kim and Chung 2007). However, this study has not calculated the concentration of malonylglucoside and acetylglucoside isoflavones in soybeans.

Apart from physical factors, it is interesting to note that microbial fermentation also plays a key role in isoflavone content variation. In this study, fermentation of bacterial pure starter culture tends to promote the increase of total isoflavones (Table 1). Besides, the fermentation period appears to be another major factor affecting the isoflavone content as observed in the fermented soybeans prepared traditionally (*thua nao*).

All soybeans fermented with pure *Bacillus subtilis* strains showed significantly higher concentrations of total isoflavones than those fermented with mixed natural microorganisms ($P<0.05$). Soybeans fermented with pure culture of *B. subtilis* BEST195 and Asaichiban presented the highest amount of total isoflavone compounds, followed by soybean fermented with *B. subtilis* TN51 and mixed natural microorganisms: 888, 864, 640, and 320 µg/g, respectively. Traditionally fermented soybeans and unfermented cooked soybeans had the similar concentrations of total isoflavones, but differences in the form of isoflavones present. Besides, traditionally fermented soybeans contained higher concentration of aglycone isoflavones, including daidzein (135 µg/g), glycitein (63 µg/g), and genistein (61 µg/g), than unfermented cooked soybeans (36-37, 10-12, and 23-25 µg/g, respectively). Therefore, *B. subtilis* fermented soybeans appear to be a better source of bioavailable soy isoflavones as it has been reported that aglycone isoflavones are absorbed faster and in higher amounts than their glucosides in humans (Izumi et al. 2000; Kano et al. 2006).

Different isoflavone forms were distributed in fermented soybeans; glucoside isoflavone compounds showed the largest proportion in soybean fermented by *B. subtilis* Asaichiban, *B. subtilis* BEST195, and *B. subtilis* TN51, *i.e.* 77, 94 and 54% of total isoflavones, respectively. Of the glucoside isoflavones, glycitin was the most abundant (30-34% of total isoflavones). In contrast, the aglycone contents of traditionally fermented soybeans were very high and accounted for 81% of total isoflavones. After fermentation, total glucosides in fermented soybeans prepared by *B. subtilis* Asaichiban and *B. subtilis* BEST195 increased significantly from 375 µg/g in unfermented autoclaved soybean to 663 and 839 µg/g, respectively. The marked increase of total glucoside isoflavones is probably due to their conversion from malonylglucoside and acetylglucoside isoflavones during fermentation. Indeed, the peak area of malonylglucoside and acetylglucoside isoflavones in both fermented soybeans decreased remarkably when compared with unfermented autoclaved soybean (data not shown). Our results are in agreement with other previous work reporting that the glucoside isoflavone form was a major component in these fermented soybeans (Nakajima et al. 2005; Wei et al. 2008).

Aglycone isoflavones in fermented soybeans presented significantly higher levels than unfermented cooked soybean, excluding *B. subtilis* BEST195 fermented soybeans ($P<0.05$). Content of aglycone isoflavones in fermented soybeans varied with the starter culture; fermented soybeans prepared with *B. subtilis* TN51 showed the greatest abundance of total aglycone isoflavones (297 µg/g), followed by traditionally fermented soybeans (260 µg/g), and *B. subtilis* Asaichiban (201 µg/g), respectively. It has been reported that the increase of aglycone forms during soybean fermentation was observed when using *Bacillus* strains and suggested that β-glucosidase is a key enzyme for the conversion of isoflavone forms in soybean fermented foods *via* deglycosylation. Wei et al. (2008) reported that aglycone isoflavones concentration in *B. subtilis* BCRC14718 fermented soybeans increased significantly after fermentation for 24 h;, conversely, glucoside isoflavone forms were decreased significantly. Likewise, Ibe et al. (2001) reported that isoflavone glucosides in soybean were hydrolysed by β-glucosidase which was produced by *B. sutilis* IF9916, and Kuo et al. (2006) indicated that daidzin and genistin glucoside were converted into aglycone isoflavone, daidzein and genistein by means of deglycosylation by β-glucosidase produced by *B. subtilis* NTU-18 during fermentation of black soybean. Hydrolysis of glucoside isoflavones was started at 8 h after inoculating with *Bacillus* culture. Also, other isoflavone conjugates that are acetylglucoside and malonylglucoside isoflavones contained in soybean might be deglycosylated into aglycone forms; therefore, in this study decreased β-glucosides, malonylglucosides, and acetylglucosides peak areas were observed in fermented soybeans HPLC chromatograms (Fig. 1). It is not only *B. subtilis* that can produce β-glucosidase during soybean fermentation; other microorganisms used for fermented soybeans such as lactic acid bacteria and Bifidobacteria with soymilk (Chun et al. 2007, 2008), *Actinomucor elegans* with *sufu* (Yin et al. 2004, 2005), *Rhizopus* with *tempeh* (Miura et al. 2002), and *Aspergillus* with *miso* (Yamabe et al. 2007) were reported in previous literature. Chien et al. (2006) suggested that β-glucosidase deglycosylation caused a significant decrease of malonylglucoside and acetylglucoside along with a significant increase of aglycone isoflavone during fermentation of soymilk with lactic acid bacteria and bifidobacteria. Malonylglucoside isoflavones are easily converted to glucosides owing to breakdown of weak bonds between sugar and malonyl group caused by heat; moreover, the effect of β-glucosidase, which hydrolyses β-glucosidic linkages of oligosaccharides and other glucosides conjugated compounds to form isoflavone aglycones.

In general, daidzein aglycone isoflavone was found as a larger proportion than genistein and glycitein in fermented soybean. Moreover, *B. subtilis* TN51 fermented soybeans contained significantly higher daidzein and genistein isoflavones than other strains ($P<0.05$), while mixed natural microorganisms fermented soybean showed the highest content of glycitein. Wei et al. (2008) indicated that the concentration of daidzein in soybean fermented with *B. subtilis* BCRC14718 was higher than genistein throughout 48 h of fermentation time. Kuo et al. (2006) demonstrated faster rate of deglycosylation in daidzin (100%) than genistin (75%) by β-glucosidase which was produced from *B. subtilis* NTU-18.

Compared with other fermented soybean products, Thai soybean variety TG145 fermented with *B. subtilis* TN51 showed lower content of total isoflavone compounds than *chungkukjang* which was produced from Korean and Chinese soybean, but exhibited higher amount of aglycone forms. Furthermore, *B. subtilis* TN51 fermented soybean also produced larger amounts of all glucoside and aglycone isoflavone compounds than *doenjang* produced from Korean and Chinese soybeans (Lee et al. 2007). This is probably due to the different soybean variety, fermentation process, strain of microorganisms, and isoflavone extraction method (Miura et al. 2002; Yin et al. 2004, 2005; Yamabe et al. 2007; Wei et al. 2008).

Conclusion

This is the first study reporting content and composition of isoflavone compounds in Thai fermented soybean (*thua nao*). The results indicated that all of fermented soybeans such as *natto-* and *thua nao*-style products contained higher aglycone compounds than unfermented cooked soybeans. Moreover, soybean fermented with *B. subtilis* TN51 showed highest amount of daidzein and genistein. Aglycones are of great interest due to their beneficial properties on human health. Further work on fermentation improvement using *B. subtilis* TN51 as starter culture is being undertaken in which its use is expected to develop an aglycone-rich fermented soybean.

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