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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Hydrolysis of black soybean isoflavone glycosides by *Bacillus subtilis* natto

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Abstract Hydrolysis of isoflavone glycosides by *Bacillus subtilis* natto NTU-18 in black soymilk is reported. At the concentration of 3–5% (*w*/*v*), black soymilk in flask cultures, the isoflavones, daidzin, and genistin were highly deglycosylated within 24 h. Deglycosylation of isoflavones was further carried out in a 7-l fermenter with 5% black soymilk. During the fermentation, viable cells increased from 10^3 to 10^9 CFU ml^{-1} in 15 h, and the activity of β-glucosidase appeared at 8 h after inoculation and reached a maximum (3.3 U/ml) at 12 h, then decreased rapidly. Deglycosylation of isoflavone glycosides was observed at the same period, the deglycosylation rate of daidzin and genistin at 24 h was 100 and 75%, respectively. It is significantly higher than the previous reports of fermentation with lactic acid bacteria. In accordance with the deglycosylation of isoflavone glycosides, the estrogenic activity of the 24 h fermented black soymilk for ERβ estrogen receptor increased to threefold; meanwhile, the fermented broth activated ERα estrogen receptor to a less extent than ERβ. These results suggest that this fermentation effectively hydrolyzed the glycosides from isoflavone in black soymilk and the fermented black soymilk has the potential to be applied to selective estrogen receptor modulator products.

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Introduction

Among the foods consumed by humans, soybeans contain the highest concentration of isoflavones. These soy isoflavones (e.g., daidzein and genistein) are implicated in some health-enhancing properties such as the prevention of certain cancers (Cappelletti et al. 2000; Miura et al. 2002; Ravindranath et al. 2004), lowering the risk of cardiovascular diseases (Anthony et al. 1996; Goodman-Gruen and Kritz-Silverstein 2001), and an improvement of bone health (Cotter and Cashman 2003; Weaver and Cheong 2005). Black soybean [*Glycine max* (L.) Merrill], a variety type of soybean with a black seed coat, has been widely utilized as a tonic food and material in oriental medicine for hundreds of years. The black pigmentation is due to accumulation of anthocyanins in the seed coat (Choung et al. 2001) and leads to various black soybean pharmaceutical effects. Black soybeans also have a stronger inhibitory effect against LDL oxidation than yellow soybeans and this effect is dependent on the total polyphenol content in its seed coat (Takahashi et al. 2005). Reported biological functions of black soybean include antiviral (Yamai et al. 2003), antitumor (Liao et al. 2001), and radical-scavenging activity (Takahata et al. 2001).

Numerous studies have revealed that the biological effects of isoflavone are not due to the glycosides form but instead are mainly from their aglycones, such as daidzein and genistein (Kawakami et al. 2005; Hendrich 2002). However, most of isoflavones in nature food materials existed as a glycosylated form and cannot be easily absorbed in the intestines (Kawakami et al. 2005; Izumi et al. 2000). It has been demonstrated that in human, isoflavone aglycones are absorbed faster and in greater amounts than their glycosides (Izumi et al. 2000) because

when soy foods are consumed, the soy isoflavones are converted to their aglycones by enteric bacteria (Xu et al. 1995). It has also been revealed that isoflavone aglycones are abundant in fermented soy products such as miso, natto, and tempeh, and they are produced by *Saccharomyces rouxii*, *Bacillus subtilis* natto (Ibe et al. 2001; Toda et al. 1999), and *Rhizopus oligosporus*, respectively. Ibe et al. (2001) proved that *B. subtilis* natto possesses β-glucosidase with the ability to hydrolyze isoflavone glucoside in flask cultures. To establish a microbial process for deglycosylation of isoflavones is thought to be an effective pathway for supply of isoflavone aglycones. To promote the utilization of black soybean, *B. subtilis* natto NTU-18 possesses high β-glucosidase activity screened by our lab was used to ferment with this material. Deglycosylation of isoflavone glycosides by *B. subtilis* natto NTU-18 in black soymilk is performed in jar-fermenter cultures and the changes of estrogenic activity of the fermented black soymilk are reported.

Materials and methods

Chemicals

Genistein, daidzein, genistin, daidzin, *p*-nitrophenol, *p*-nitrophenyl phosphate, L-homoarginine, diethanolamine, and *p*-nitrophenyl-β-D-glucoside were purchased from Sigma Chemical (St. Louis, MO, USA). Liquid chromatography (LC) grade acetonitrile was purchased from Merck (Darmstadt, Germany). Nutrient broth (NB) and Bacto-agar were purchased from Difco (Detroit, MI, USA). Reagent grade absolute alcohol was purchased from Merck (Darmstadt, Germany).

Microorganism

A high β-glucosidase activity strain, *B. subtilis* natto NTU-18, was isolated from the commercial product for hydrolysis of isoflavone glycosides study. The strain is maintained and subcultured on NB slant at 4 °C.

Hydrolysis of isoflavone glycosides by fermentation

The black soybeans [*Glycine* max (L.) Merr.] (Tainan NO.3) were purchased from Shiuejia artel (Tainan, Taiwan). The black soybeans were milled with a grinder (CP-75 S, Ladyship, Kaohsiung, Taiwan) and the meal was used for black soymilk preparation. Seed culture of *B. subtilis* natto NTU-18 was prepared by transferring a loop of bacteria from the NB slant into a 500-ml Hinton flask containing 100 ml of NB medium. It was then cultured in shaker at 37 °C, 125 rpm for 12 h. After that, the broth was transferred for further cultures. Scale-up cultures were performed in a 7-l fermenter (BioFlo 110 Modular Benchtop Fermentor, New Brunswick Scientific, NJ, USA) and the initial working volume was 5 l of 5% (*w*/*v*) black soymilk (250 g of black soybean powder in 5 l H_2O). The aeration rate was 1.0 v.v.m., agitation speed was 800 rpm and the temperature was 37 °C. The deglycosylation rate of the four isoflavone glycosides was defined as, (increased molarity of the isoflavone aglycone/the initial molarity of the corresponding isoflavone glycones) ×100%.

Extraction of isoflavones

The extraction of isoflavones was based on the work of Rostagno et al. (2003). Briefly, 1.0 ml of 50% EtOH was added to the lyophilized powder of 1.0 ml fermented broth and then incubated in an ultrasonic bath at 60 °C for 30 min. After that, the mixture was centrifuged at 15,000×*g* for 5 min, and the supernatant was filtered through a 0.45-μm filter for the quantification of isoflavones using high-performance liquid chromatography (HPLC).

Analysis of isoflavones

HPLC analysis of isoflavones was based on the work of Wang and Murphy (1994). The HPLC system was consisted of a Shimadzu SCL-10Avp system controller, two Shimadzu LC-10ATvp liquid chromatograph pumps, and a Shimadzu SPD-M10Avp photodiode array detector. A Develosil ODS-5 column, 250 mm with 4.6 mm packing, was used. A linear HPLC gradient was composed of 0.1% (*v*/*v*) glacial acetic acid in H_2O (solvent A) and 80% (*v*/*v*) acetonitrile in 0.1% (*v*/*v*) glacial acetic acid water solution (solvent B). After the injection of 20 μl sample, solvent B was increased from 15 to 35% more than 30 min, and then returned to 15% for 10 min. The solvent flow rate was 1.0 ml min^{-1}. Elution was monitored by UV absorbance at 262 nm, and the spectral data from 190–800 nm were recorded over the time of the run on all samples. Peak areas were integrated for quantification. Isoflavones (daidzin, genistin, daidzein, and genistein) were identified by comparison of the HPLC retention time and the UV spectra of the authentic compounds. The isoflavone concentration of the samples was calculated by interpolation of the calibration curves prepared by various concentrations of the isoflavone standards.

Assay of β-glucosidase activity

The β-glucosidase activity was monitored throughout the fermentation process. The assay of β-glucosidase activity was modified from Choi et al. (2002). One milliliter of culture broth was centrifuged at 15,000×*g* for 5 min, then

the supernatant was discarded and the cell pellet was washed with sodium phosphate buffer (pH 7.0). The cell pellet was resuspended in 0.5 ml of sodium phosphate buffer (pH 7.0) containing 5 mM *p*-nitrophenyl-β-D-glucoside (PNPG) and incubated at 37 °C for 20 min. The reaction was stopped by addition of 0.5 ml 2 M Na_2CO_3 and centrifuged at 15,000×*g* for 5 min. This reaction supernatant was measured for the absorption at 405 nm. The standard curve was prepared by measuring A_{405} of various concentrations of *p*-nitrophenol, and the amount of *p*-nitrophenol produced in the reaction was calculated by interpolation A_{405} to the standard curve. A unit of enzyme activity was defined as the amount of enzyme that would liberate 1 μmol *p*-nitrophenol per minute.

Estrogenic activity assay

The transcriptional activation on estrogen receptor of the fermented black soymilk was examined by a transactivation assay in which a chimeric vectors carrying a chimeric receptor (with the ligand binding domain of the ER and the DNA-binding domain of the yeast transcription factor Gal4) construct and a Gal4-regulated reporter gene consisting of an alkaline phosphatase gene regulated by a basal promoter and four tandem Gal4 response elements construct. These were transiently cotransfected into CHO-K1 cells. CHO-K1 cells (American Type Culture Collection, Rockville, MD, USA) were grown at 37 °C in a 5% CO_2 atmosphere in Ham's F-12 medium supplemented with 10% fetal bovine serum (Gibco BRL, Rockville, MD, USA). At near confluence, cells were removed with trypsin-EDTA, seeded at a density of 2.5×10^4 cells/well into 96-well plates, and were then incubated at 37 °C for 24 h, 5% CO_2. The chimeric receptor constructs were pBK-CMV-Gal4-hERα-ligand-binding domain (Gal4-hERαLBD) and the pBK-CMV-Gal4-hERβ-ligand-binding domain (Gal4-hERβLBD), respectively. The vectors containing reporter gene was pBK-CMV-$(UAS)_4$-tk-alkaline phosphatase (AP) obtained from Dr. J.-A. Gustaffson, Department of Medical Nutrition, Karolinska Institute, Huddinge, Sweden. The correct in-frame fusions were confirmed by DNA sequencing. Using Lipofectamine 2000 (Gibco BRL) according to the manufacturer's instruction, CHO-K1 cells were transfected with 0.3 μg of the chimeric receptor construct pBK-CMV-Gal4-hERα (or β), and pBK-CMV-(UAS)4-tk-alkaline phosphatase (AP) (4:1 or 5:1) in 100 μl serum-free medium OPTI-MEM (Gibco BRL) containing 1 μl lipofectamine per well. After 5 h, the medium was changed to Ham's F-12 medium containing 10% serum replacement and vehicle (ethanol or DMSO), 1 nM 17β-estradiol (E_2) or appropriate concentration of samples. The procedure of transactivation was based on the report of Chao and Huang (2003). One milliliter of fermented black soymilk sampled from jar-fermenter cultures at set intervals was extracted with 1 ml of 95% ethanol. After vacuum dried and then dissolved in a minimal amount of 50% ethanol solution, the samples were diluted to an appropriate concentration with Ham's F-12 medium containing 10% serum replacement (TCM, Celox, St. Paul, MN, USA) immediately before use. Finally, 1 ml of the prepared sample contained 10 ng the vacuum dried. The procedure of the SEAP assay has been described previously (Durocher et al. 2000). After 48 h of transiently transfected cells, culture medium, 50 μl, was transferred to a new 96-well plate and mixed with an equal volume of SEAP assay solution containing 20 mM *p*-nitrophenyl phosphate (*p*NPP), 1 mM $MgCl_2$, 10 mM L-homoarginine, and 1 M diethanolamine, pH 9.8. Absorbance was read at 405 nm in 15 min. Folds of activation was calculated by taking the AP activity of vehicle-treated cells as 1 (Bowers et al. 2000). Cell viability was monitored using the MTT method (Durocher et al. 2000). Experiments were repeated at least three times in triplicate. Statistical analysis was carried out utilizing SAS 8.0 for Windows.

Results

Isoflavones in black soybean

The black soybeans were extracted with 50% ethanol and analyzed with HPLC for isoflavone quantification. The retention time of daidzin, genistin, daidzein, and genistein was 11.3, 14.4, 19.9, and 24.4 min, respectively. The absorption spectrum of daidzin, genistin, daidzein, and genistein within 190–800 nm was also investigated (data not shown). The maximum absorption of daidzin, genistin, daidzein, and genistein was 249, 259, 248, and 259 nm, respectively. Our results indicate that 1.0 g of the black soybean contained 636 μg of daidzin, 715 μg of genistin, 144 μg of daidzein, and 284 μg of genistein. Black soybeans contained the similar level of isoflavones in soybeans (Yen and Lai 2003), but black soybeans had higher level of isoflavone aglycones.

Hydrolysis of isoflavone glycosides in flask cultures of black soymilk

Deglycosylation of isoflavone glycosides by liquid cultures of *B. subtilis* natto NTU-18 in black soymilk was investigated. Deglycosylation efficiency of genistin and daidzin in different concentrations of black soymilk was shown in Fig. 1. After 24 h of being cultivated in 1, 3, and 5% black soymilk, the deglycosylation rate of daidzin and genistin was 51 and 73%, 111 and 79%, 113 and 80%, respectively. No residual genistin and daidzin were observed in the fermented broth after 24 h fermentation;

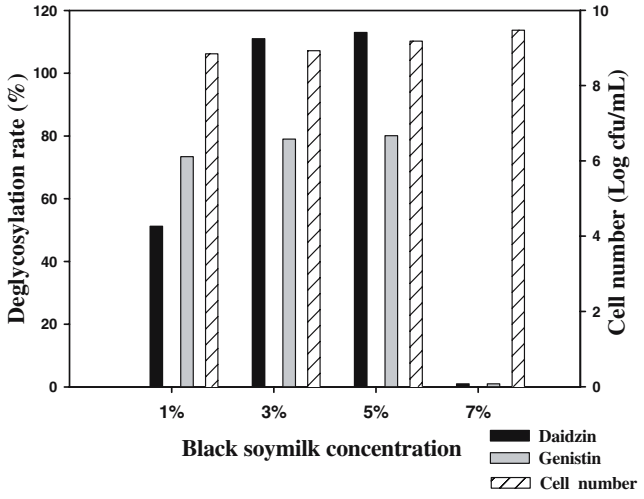

Fig. 1 Deglycosylation of daidzin and genistin in different concentrations black soymilk flask cultures of *B. subtilis* natto NTU-18. The culture conditions were 37 °C and 125 rpm for 24 h. Deglycosylation rate (%)=[increased molarity of aglycone isoflavone/initial molarity of glycone isoflavone] × 100%

however, lower amount of genistein and daidzein than their theoretical value were produced in 1% of black soymilk. Surprisingly, the hydrolysis of isoflavone glycosides in the 7% black soymilk was almost completely inhibited during the period we investigated although the viable cells in the concentrations of black soymilk tested in the experiments achieved the same level (more than 10^9 cfu ml^{-1}).

Deglycosylation of isoflavone glycosides in 5% black soymilk flask cultures was time coursed analyzed. As shown in Fig. 2, the β-glucosidase activity was started detectable at 8 h after inoculation and raised to the maximum (3.3 U ml^{-1}) at 12 h, then the β-glucosidase activity dramatically decreased to 0.3 U ml^{-1} in 3 h. In

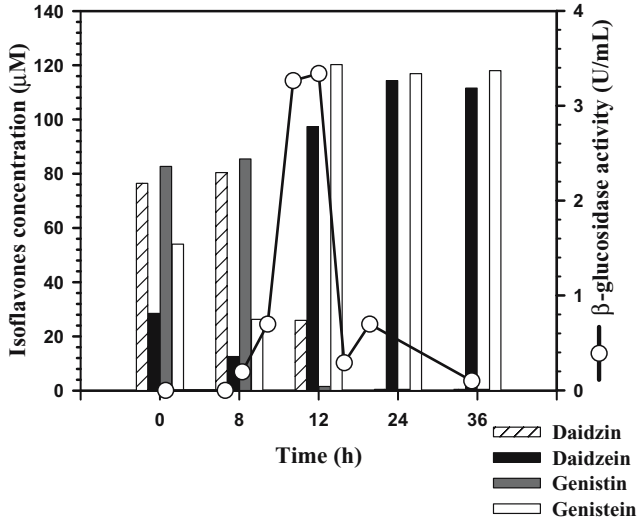

Fig. 2 β-glucosidase activity (-○-) and the hydrolysis of isoflavone glycosides in 5% black soymilk flask cultures of *B. subtilis* natto NTU-18

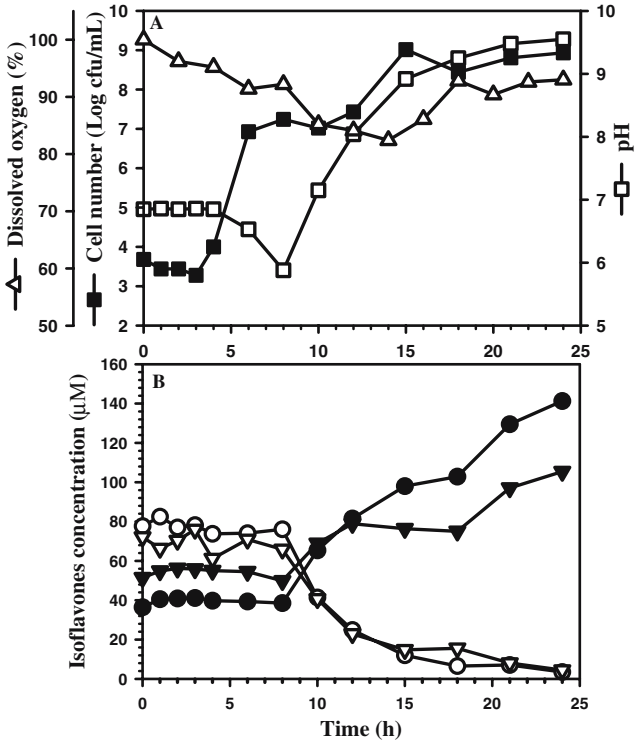

Fig. 3 Hydrolysis of isoflavone glycosides in fermenter cultures of *B. subtilis* natto NTU-18 in 5% black soymilk. **a** The profile of viable cells (■), pH (□), and dissolved oxygen (△). **b** The concentration change of daidzein (●), genistein (▼), daidzin (○), and genistin (▽). The culture conditions were 37 °C, 800 rpm of agitation speed, and 1 v.v.m. of aeration rate

accordance to the appearance of β-glucosidase activity, isoflavone glycosides were hydrolyzed to their aglycones. Within 12 h, daidzin decreased from 76.5 to 25.9 μM and genistin decreased from 82.7 to 1.5 μM. Meanwhile, daidzein increased from 28.5 to 97.4 μM and genistein increased from 54.0 to 120.3 μM. Daidzin and genistin were almost completely deglycosylated in 24 h by *B. subtilis* natto NTU-18 in 5% black soymilk flask cultures.

Hydrolysis of isoflavone glycosides in a 7-l jar fermenter

Hydrolysis of isoflavone glycosides was further investigated in a 7-l fermenter with a working volume of 5 l 5% black soymilk. As shown in Fig. 3, in 3 h of lag phase, the viable cell number, pH, and isoflavone glycosides did not change significantly. After this, 4×10^3 CFU ml^{-1} viable cells increased rapidly to 10^7 CFU ml^{-1} during 3–6 h, and entered a stationary phase during 6–13 h. Then, a second biomass accumulation during 12–15 h, viable cells increased from 10^7 to 10^9 CFU ml^{-1}, was observed. The medium's pH decreased from 6.9 to 5.9 during 4–8 h after inoculation and then increased to 9.5 during 8–24 h. The dissolved oxygen was sustained at high saturation through the culture by a high agitation speed and aeration rate.

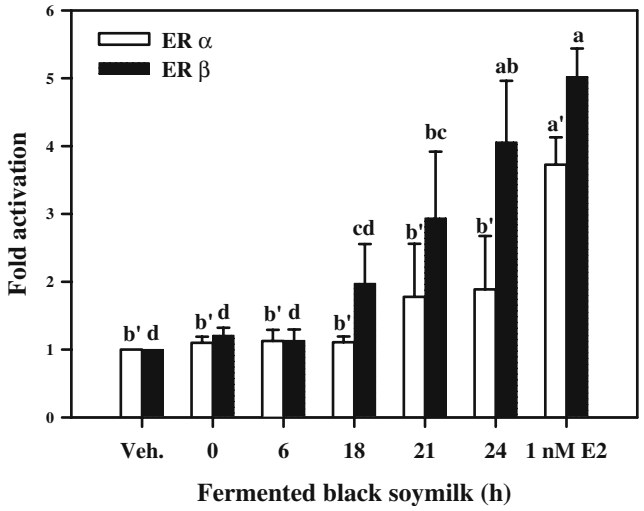

Fig. 4 Time course of transcriptional activation of 5% fermented black soymilk on estrogen receptor (GAL4-hERα orβ). The values are expressed as means±SE of triplicate well in a representative experiments. Data shown are representative of at least three separate experiments with similar results, and 17β-estradiol (1 nM) is the positive control. Values not sharing the same letter are significantly different from one another by Duncan's multiple range test ($p<0.05$). The meaning of a, b, c, and d represents the existence of significant difference of ERβ activities among assayed samples, while a′ and b′ represent for ERα

Regarding the hydrolysis of isoflavone glycosides, daidzin and genistin was started, hydrolyzed to daidzein and genistein at 8 h after inoculation, and 100% daidzin and 75% genistin was deglycosylated after 24 h of fermentation. The final concentration of daidzein and genistein in cultured broth at 24 h after inoculation achieved 141.3 and 105.4 μM, respectively (Fig. 3b).

Estrogenic activity of fermented black soymilk

Fermented black soymilk was sampled at set intervals and was extracted by ethanol for the measurement of their estrogenic activity. CHO-K1 cells were transiently transfected with expression vectors for ERα or ERβ, an UAS-alkaline phosphatase reporter, and treated with estrogenic activity determining samples (Fig. 4). Our data reveal that 18 h fermented black soymilk activated ERβ estrogenic receptor ($p<0.05$) to twofold that of vehicle treatment but did not activate ERα estrogenic receptor ($p>0.05$). For estrogenic activity assay of 21 and 24 h fermented black soymilk, they further increased to threefold and fourfold of ERβ estrogenic activity, respectively, and the ERα estrogenic activity both just slightly induced. At the same time, 1 nM of 17β-estradiol (E2) induced a fourfold ERα and a fivefold of AP activity. No ERα or ERβ estrogenic activity was detected in samples from 6 h fermented broth or nonfermented black soymilk. These data reveal that, after fermented with *B. subtilis* natto NTU-18 black soymilk exhibited a higher estrogenic activity toward ERβ and a lower ERα estrogenic activity.

Discussion

There are some reports indicating the deglycosylation of isoflavone glycosides with fermentation by lactic acid bacteria (Choi et al. 2002), *R. oryzae*, and basidiomycetes (Miura et al. 2002) through their β-glucosidase activities. *B. subtilis* natto was chosen to be used in this study because of their safety, grow rapidly and easy to be scale-up for mass cultures. In this study, *B. subtilis* natto NTU-18 hydrolyzed daidzin and genistin to daidzein and genistein, respectively, in 1–7% of black soymilk. There were no obvious peaks, as described by Toda et al. (1999), being formed in the HPLC analysis equipped with photodiode array (data not shown). *B. subtilis* natto NTU-18 showed higher deglycosylation efficiency of isoflavone glycosides at the initial concentration of 3–5% black soymilk than at the concentration of 1 and 7%. We also analyzed the distribution of β-glucosidase in *B. subtilis* natto NTU-18, including culture broth and intracellular and cell debris fraction of the strain. It was found that the cell debris fraction had the highest β-glucosidase activity. This suggests that the enzyme was cell-associated, agreed to the results reported by Ibe et al. (2001) and those using lactic acid bacteria (Choi et al. 2002; Matsuda et al. 1994).

Setlow et al. (2004) reported that *B. subtilis* possessed more than one kind of β-glucosidases. As shown in Fig. 2, the yield of daidzein was higher than the reduction of daidzin within 12 h of black soymilk fermentation. It suggests that not only daidzin and genistin, but also other isoflavone conjugates such as isoflavone 7-*O*-glucosides and isoflavone 7-*O*-glucoside-6″-*O*-malonates contained in soya might also serve as pools for the release of free aglycones.

B. subtilis natto NTU-18 showed two stages of growth when cultivated with 5% black soymilk in a jar fermenter (Fig. 3). In the first stage, the cells entered the exponential growth phase after 3 h of lag phase and accompanied with a decrease in pH, and no deglycosylation of isoflavone glycosides were observed in this period. This phenomenon suggests that in this stage, the cells took use of available carbon(s) other than isoflavone glycosides from the black soymilk. This might account for the observation that the deglycosylation of isoflavone glycosides was inhibited or postponed in a higher concentration (7%) of black soymilk that may provide more available carbon source other than isoflavone glycosides. When the more available carbon was exhausted, the growth of the cells entered the stationary phase of the first stage, and began to hydrolyze the

isoflavone glycosides to aglycones to obtain the free sugars. Then, the growth of the cells entered the second stage; therefore, the viable cells increased almost 100-fold and reached the maximum of 10^9 CFU ml^{-1} during 10–15 h after inoculation. Meanwhile, the dissolved oxygen of the fermented broth decreased to a minimal value in the whole fermentation. It is speculated that *B. subtilis* natto NTU-18 utilized glucose as its carbon and energy source via deglycosylation of isoflavone glycosides during the second stage. Ibe et al. (2001) have reported that *B. subtilis* natto has the ability of β-glucosidase to hydrolyze isoflavone glycosides. We further find initial concentration of black soymilk is an important factor for high efficiency of hydrolysis of isoflavone glycosides and carried out hydrolysis of black soymilk isoflavone glycosides in a jar fermenter. Choi et al. (2002) examined the hydrolysis of soybean isoflavone glycosides by lactic acid bacteria, and found that 70–80% of genistin was converted to genistein and 25–40% of daidzin to daidzein after fermentation. *B. subtilis* NTU-18 used in this study showed higher deglucosylation efficiency in jar-fermenter cultures than lactic acid bacteria examined by Matsuda et al. (1994) and Choi et al. (2002).

Isoflavones are most well-known phytoestrogen. It is well accepted that the estrogenic activities of soy isoflavones play an important role in their health-enhancing properties. The physiological responses to estrogen are known to be mediated within specific tissues by at least two estrogen receptors (ERs), ERα and ERβ. The ERβ is more effective than ERα in transactivation of the electrophile/antioxidant response element and therefore is a better inducer of antioxidant enzymes such as quinine reductase and glutathione S-transferase (GST) (Montano et al. 2000; Montano and Katzenellenbogen 1997). The estrogen receptor, ERβ, refers to clinically important issues such as bone stability and cardiovascular health (Enmark et al. 1997; Onoe et al. 1997). The ERα is highly expressed in breast and uterine tissue; thus, the induction of high ERα activity may enhance the growth of hormone-dependent cancer cells (Speirs et al. 1999). Selected estrogen receptor modulators (SERM) that are highly selective for ERβ is therefore highly anticipated. Various in vitro estrogenic activity assays such as competitive ligand-binding assays (Mroito et al. 2001), cell proliferation assays (Kayisli et al. 2002), or recombinant receptor/reporter gene assays (Legner et al. 1999; Saito et al. 2000; Klinge et al. 2003; McInerney et al. 1998) and yeast-base screens (Bovee et al. 2004) have been reported. These assay systems are based on the molecular mechanisms involved in the classical estrogen action. More recently, genetically modified yeast cells and human cell lines are being used for estrogenic activity measurement by transcription activation of reporter genes. These systems can be used to identify ER antagonists by giving them in combination with a nearly maximal effective dose of E2. Using human cell lines is more sensitive than yeast and may be able to identify estrogenic compounds that require cellular metabolism for activation into their estrogenic state (Legner et al. 1999).

Chimeric receptor–reporter gene constructs have proven utility in screening compound for estrogenic activity (Zacharewski et al. 1995). We used a chimeric receptor–reporter assay system to determine the estrogenic activity of fermented black soymilk. In Figs. 3 and 4, when isoflavone glycosides were not converted into isoflavone aglycones in the first growth stage, no significant ERα or ERβ estrogenic activity was detected in the first 6 h fermentation. While daidzin and genistin began to be hydrolyzed to daidzein and genistein, respectively, in the later stage, the black soymilk showed a higher ERβ estrogenic activity. Our results agree to the previous reports that isoflavone glycosides bound poorly to both ERα and ERβ, and induced transcription poorly (Mroito et al. 2001), and the isoflavone aglycones generally bind to and induce transcription of hERβ to a higher extent than of hERα (Mroito et al. 2001; Kuiper et al. 1996). From the view of extension of utilization of black soybean, this fermented black soymilk with estrogenic activity, relatively selective to ERβ, might be beneficial to postmenopause syndrome through positive estrogenic effects mainly in tissues expressing ERβ, such as the induction of antioxidant enzymes, the prevention of osteoporosis, and the reduction of cardiovascular disease risk. In summary of the results of above, this fermented black soymilk may be further applied to selective estrogen receptor modulator (SERM) products.

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