CHAPTER III

MATERIALS AND METHODS

1. Chemicals

All chemicals used in this study were analytical grades and listed as following:

Chemicals	Companies
Absolute ethanol	Merck
ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)	Fluka
Authentic standards of daidzin, genistin, daidzein and genistein	Sigma
Catechin	Sigma
DPPH (1,1-Diphenyl-2-picryl-hydrazyl)	Sigma
Folin Ciocalteu's phenol reagent	Sigma
Gallic acid	Sigma
Glacial acetic acid	Scharlau
Iron (III) chloride	BDH
Iron (II) sulfate heptahydrate	Scharlau
Methanol	Merck
Potassium chloride	Scharlau
Potassium dihydrogen phosphate	Scharlau
Phosphoric acid	Scharlau
Sodium acetate-3-hydrate	Fisher
Sodium carbonate anhydrus	Fluka
Sodium hydroxide	Scharlau

Chemicals	Companies
TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine)	Sigma
Trolox(6-hydroxy-2,5,7,8-tetramethyl chlorman-2-carboxylic acid)	Fluka
Sodium tungstate	J.T.Bake
1,1,3,3-Tetraethoxypropane	Carlo Erba
Sodium Molibdate	Fluka
p-nitrophenyl- β –D-glucopyranoside (*p*NPG)	Wako
p-nitrophenol	Wako

2. Preparation of microorganisms

Thirty three pure strains of *Aspergillus* were obtained from the BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, Thailand. All these microorganisms were isolated from fermented food products such as soy sauce, miso, koji and Japanese sake (Table 9). The freeze-dried culture was rehydrated with 1 mL of sterile distilled water. Few drops of cell suspension were inoculated onto potato dextrose agar (PDA) from Difco (Franklin Lakes, MD, USA) and incubated at 37^{o}C for 3 days. The growing colonies were transferred to the new PDA plate and incubated at 37^{o}C for 5 days. Spores of the fungi were harvested by flooding the surface of the agar with sterile distilled water and aseptically filtered through three layers of the sterile gauze. The turbidity of spore suspension was adjusted to 0.5 McFarland unit according to Pfaller et al. (1995) and used as inoculum for the fermentation of soybean.

Table 9. *Aspergillus* strains used in the screening test.

Aspergillus strains	Code number	Source of strains
A. oryzae	BIOTEC 49	Soy sauce
A. oryzae	BCC 3103	Koji
A. oryzae	BCC 3083	Soy sauce, Miso
A. oryzae	BCC 3373	Koji
A. oryzae	BCC 3088	Koji
A. oryzae	BCC 3087	Fermented soybean
A. oryzae	BCC 3048	Soy sauce
A. oryzae	BCC 3102	Koji
A. oryzae	BCC 13295	Unknown
A. oryzae	BCC 17102	Unknown
A. oryzae	BCC 17103	Unknown
A. oryzae	BCC 17104	Unknown
A. oryzae	BCC 14613	Sake koji
A. oryzae	BCC 14615	Koji
A. oryzae	BCC 14616	Koji
A. oryzae	BCC 6128	Unknown
A. oryzae	BCC 7238	Unknown
A. oryzae	BCC 7051	Unknown
A. sojae	BCC 3037	Soy sauce
A. niger	BCC 3344	Koji
A. niger	BCC 3025	Fermented salty-soybean
A. terricola	BCC 3026	Koji
A. flavas	BCC 3041	Koji
A. ornatus	BCC 3101	Koji
A. awamori	BCC 13292	Unknown
A. kawachii	BCC 13291	Unknown
A. japonicus	BCC 18313	Koji
Aspergillus sp.	BCC 17548	Koji
Aspergillus sp.	BCC 17549	Koji
Aspergillus sp.	BCC 17550	Koji
Aspergillus sp.	BCC 17551	Koji
Aspergillus sp.	BCC 17552	Koji
Aspergillus sp.	BCC 17553	Koji

3. Preparation of soybean broth

Soybean (*Glycine max* (L) Merr; SJ.2) was obtained from Limsakdakun Co.Ltd., Chiang Mai, Thailand. Whole soybean (100g) were ground into powder by blender (Tomex model A328), mixed with 1 L of distilled water, and then steamed at 121^{o}C for 30 min by an autoclave (Hirayama model HVA-85/110). Steamed soybean

mixture was cooled at room temperature. The supernatant was recovered by centrifugation using a centrifuge (Beckman model JE 25) at 12,000 xg at 4^{o}C for 15 min and referred as soybean broth.

4. Preparation of culture filtrates

Soybean broth (5 ml) was inoculated with 1 ml of *Aspergillus* spp. spore suspension with concentration of 10^6 spores/ml and incubated at 30^{o}C for 4 days in an incubator shaker (Innova model 4100). The culture filtrates were centrifuged using a centrifuge (Beckman model JE 25) at 12,000 xg at 4^{o}C for 15 min. The supernatant was recovered and used for analysis.

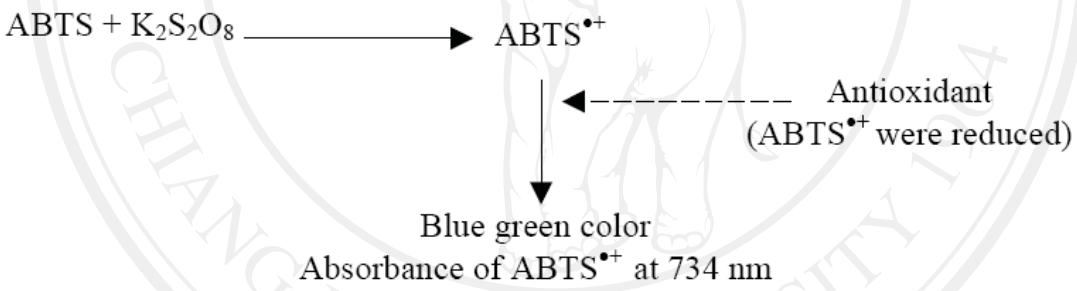
5. Preparation of fermented soybean

Whole soybean was soaked in water for 12 h and then autoclaved at 121^{o}C for 30 min. After cooling, the cooked soybean was inoculated with the spore suspension of *Aspergillus* at the level of 1×10^6 spores per gram of cooked soybean. The samples were then incubated at 30^{o}C. Samples were collected at 24 h interval up to 4 days. The samples were immediately grounded into powder in liquid nitrogen using a blender (Model BBL550XL, Hawii, USA). The samples were stored at -20^{o}C until used. To prepare methanol extract, powderized sample (1 g) was extracted in 5 ml methanol with shaking at 60 RPM in a waterbath for 12 h at 37°C (Lori et al, 1998). The fermented soybean extracts were recovered by centrifugation using a centrifuge model JE 25 (Beckman Coulter, Inc., CA, USA) at 12,000 xg at 4°C for 15 min. The methanol extract was vacuum concentrated at 40°C and dried by a freeze-dryer to dryness.

6. Antioxidative activities assays

6.1 ABTS radical-scavenging activity assay in fermented soybean broths and fermented soybean extracts

The ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation decolorization test is a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. The experiments were carried out using an improved ABTS decolorization assay with some adaptation (Re et al. 1999). It is applicable for both lipophilic and hydrophilic compounds. $ABTS^{\bullet+}$ was generated by oxidation of ABTS with potassium persulfate as shown in the following scheme :



Reagents

ABTS

Potassium persulfate

Standard trolox

Procedure

The scavenging effect on ABTS radical of fermented soybean broths and fermented soybean extracts were determined by the method of Roberta Re (1998). Fermented soybean powder was extracted with methanol (1:5, w/v). Extraction was

carried out by shaking in a waterbath for 12 h at 37 ^{o}C (Lori et al, 1998). The fermented soybean extracts were recovered by centrifugation using a centrifuge (Beckman model JE 25) at 12,000 xg at 4^{o}C for 15 min. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution (7 mM) in distilled water with 2.45 mM potassium persulfate . The mixture was allowed to stand in the dark at room temperature (25°C) for 12–16 h before use d. The $ABTS^{\bullet+}$ solution was diluted with distilled water to the absorbance of 0.70-0.90 at 734 nm . In the tested reaction, fermented soybean broths or fermented soybean extracts (20ul) or standard (trolox) were mixed with distilled water (80ul) and 2 ml of ABTS working solution. After 3 min incubation at room temperature, absorbance was then measured at 734 nm. Scavenging effect on ABTS radical ability of fermented soybean broths and fermented soybean extracts were expressed as mg trolox/ ml sample and mg trolox/g fermented soybean, respectively.

$ABTS^{\bullet+}$ working solution

+

Sample or Standard solution

↓

$ABTS^{\bullet+}$ λ_{max} 734 nm

Scheme 10. The protocol of ABTS free radical cation decolorisation assay

6.2 β-glucosidase activity assay in fermented soybean broths and fermented soybean extracts

Reagents

p-nitrophenyl- β-D-glucopyranoside (pNPG)

Phosphate citrate buffer

Sodium carbonate

Bovine serum albumin (BSA)

Procedure

β-glucosidase activity was determined by the method of Esaki et al (1999) by using *p*-nitrophenyl- *β* –D-glucopyranoside (pNPG) as a substrate. Fermented soybean powder was extracted with phosphate-citrate buffer pH 6.0 (1:5, w/v). Extraction was carried out by sonicating the mixture for 20 min at 4°C. The fermented soybean extract was recovered by centrifugation using a centrifuge (Beckman model JE 25) at 12,000 xg at 4°C for 15 min. The fermented soybean broth or fermented soybeans extract 0.5 ml was mixed with 2.0 ml of 1 mM pNPG in a 0.1 M phosphate-citrate buffer (pH 6.0). The reaction mixture was incubated at 30°C for 20 min in water bath, then stopped reaction by adding 2.5 ml of 0.5 M sodium carbonate. The resulting p-nitrophenol was immediately monitored at 420 nm. One unit of β-glucosidase was defined as the amount of enzyme which liberated 1 μmol of p-nitrophenol per min with specified condition.

The protein content of fermented soybean broths and fermented soybean extracts were determined by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as a standard.

6.3 Ferric reducing ability power assay (FRAP) in fermented soybean extracts

The ferric reducing ability power assay is a simple measurement of reducing ability of antioxidants. The FRAP assay is a method for assessing antioxidant power. Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. Fe^{2+}-TPTZ has intensive blue color and can be monitored at 593 nm (Benzie and Strain 1996).

Reagents

Acetate buffer, pH 3.6

2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)

Ferric chloride ($FeCl_3 \cdot 6H_2O$)

Ferrous sulfate ($FeSO_4$)

Procedure

Ferric reducing ability power (FRAP) was determined by the method of Benzie and Strain (1996). FRAP reagent was prepared by mixing 300 mM acetate buffer pH 3.6 with 10 mM TPTZ and 20 mM $FeCl_3 \cdot 6H_2O$. The mixture was mixed for 15 seconds and its absorbance was recorded at 593 nm. Fermented soybean extracts or standard ($FeSO_4$) solution was added in freshly prepared FRAP reagent. After 4 min of mixing, absorbance was then measured at 593 nm. The change in absorbance ($\Delta A_{593\ nm} = A_{593\ nm\ after} - A_{593\ nm\ befor}$) was calculated for each sample and related to $\Delta A_{593\ nm}$ of Fe^{2+} standard solution. The ferric reducing ability power of fermented soybeans was expressed as mg $FeSO_4$/g fermented soybean.

FRAP reagent
(10 mM TPTZ solution, 20 mM $FeCl_3$ solution)
+
Sample or Standard solution

↓ 4 min.

Fe^{2+} – TPTZ complex λ_{max} 593 nm

Scheme 11. The protocol of ferric reducing ability power assay (FRAP)

6.4 Total phenolic content assay in fermented soybean extracts

Reagents

Folin-Ciocalteu's phenol reagent

Standard gallic acid

Sodium carbonate solution

Procedure

Total phenolic content was determined by the method of Marinova et al (2005) using gallic acid as standard. Fermented soybean extract (1 mL) was added to 25 ml volumetric flask, containing 9 mL of deionized water. Folin-Ciocalteu's phenol reagent (1 mL) was added to the mixture and shaken. After 5 min, 10 mL of 7% Na_2CO_3 solution was added to the mixture. The solution was diluted to the final volume of 25 mL with distilled deionized water and mixed. After incubation for 90 min at room temperature, the absorbance against prepared reagent blank was measured at 750 nm. Total phenolic content of fermented soybean was expressed as mg gallic acid equivalents (GAE)/g fermented soybean.

6.5 Total Flavonoid content assay in fermented soybean extracts

Reagents

Standard catechin

Sodium hydroxide (NaOH)

Sodium nitrite ($NaNO_2$)

Aluminium trichloride ($AlCl_3$)

Procedure

Total flavonoid content was measured by the aluminum chloride colorimetric assay according to method described by Zhishen et al. (1999), using catechin as standard. Fermented soybeans extract (1 mL) was added to 10 mL volumetric flask containing 4 ml of distilled deionized water. To the flask was added 0.3 mL 5% $NaNO_2$. After 5 min, 0.3 mL 10% $AlCl_3$ was added. At 6^{th} min, 2 mL of 1M NaOH was added and the total volume was made up to 10 mL with distilled deionized water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content of fermented soybean was expressed as mg catechin equivalents (CE)/g fermented soybean.

6.6 DPPH radical scavenging activity assay in fermented soybean extracts

Reagents

1,1- diphenyl-2-pycrylhydrazyl (DPPH)

Methanol

Standard trolox

Procedure

The scavenging activity for the DPPH radical was determined using the method of Velazquez et al (2003). Varying amounts of fermented soybean extract were dissolved in 0.1 mL methanol and mixed with 2.9 mL of 0.1 mM DPPH in 80% methanol. The mixtures were left in dark for 30 min at room temperature and the absorbance was then measured at 517 nm. The inhibitory percentage of DPPH was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = [1-((A_{\text{sample}}-A_{\text{blank}})/ A_{\text{control}})] \times 100\%$$

where A_{sample} was the A_{517} of sample and $DPPH^{\bullet}$, A_{blank} was the A_{517} of sample without $DPPH^{\bullet}$, and A_{control} was the A_{517} of $DPPH^{\bullet}$ without sample. The effective concentration at which 50% of the DPPH radicals were scavenged or EC_{50} value was obtained by interpolation from linear regression analysis from the plot of scavenging activity against the concentration of sample.

6.7 Lipid peroxidation assay by Thiobarbituric Acid Reactive Substances (TBARS) in fermented soybean extracts

Reagents

Linoleic acid

Phosphate buffer

Methanol

Tetraethoxypropane

Thiobarbituric acid reactive substances (TBARS)

Procedure

Oxidation of linoleic acid was determined by modified the method of Haraguchi et al. (1992). Different amounts of fermented soybeans extract dissolved in 30 µL methanol were added to a reaction mixture consisting of 0.57 mL of 2.51% linoleic acid in methanol and 2.25 mL of 40 mM phosphate, pH 7.0. After 5 days of incubation at 40°C, 0.2 ml of reaction mixture was taken and added with 1 mL of TBA solution containing 0.375% TBA, 15% TCA and 0.25 N HCl and boiled for 10 min. The mixture was centrifuged at 5,500xg for 25 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer UV-1601 (Shimadzu, Kyoto, Japan). The TBARS value was calculated from the standard curve of 1,1,3,3-tetraethoxypropane.

6.8 Plasmid Relaxation assay in fermented soybean extracts

Reagents

pUC18 plasmid DNA

Tris-HCl buffer

Hydrogen peroxide (H_2O_2)

Ferric chloride ($FeCl_3$)

Ethylenediaminetetraacetic acid (EDTA)

Ethidium bromide

Agarose gel electrophoresis

Procedure

In vitro plasmid relaxation assay was determined by a modified version of the method of Ishikawa et al. (2004). DNA strand damages were measured by converting

circular double-stranded supercoiled plasmid DNA into nicked circular and linear forms. Reactions were performed in 50 µL of solution containing 10 µL of supercoiled pUC18 plasmid DNA (750 ng), 10 µL of 10 mM phosphate, pH 7.8, 5 µL of 3.5% hydrogen peroxide, 5 µL of 100 µM ferric chloride, and 20 uL of fermented soybean extracts at various concentrations (2.5, 5, 7.5, 10 mg/mL). The mixtures were incubated at 37°C for 30 min, and the reactions were stopped by adding 1 µL of 5 mM ethylenediaminetetraacetic acid (EDTA). Reaction mixtures (20 µL) were mixed with 5 µL of loading buffer containing 30% glycerol and 0.25% bromophenol blue. The mixture (15 µL) was loaded onto a 1% agarose gel. Electrophoresis was conducted using a constant voltage of 110 V. The gel was stained using ethidium bromide solution for 10 min. The DNA bands were visualized under UV light and captured by a CCD camera (Genegenious, Singene, UK).

6.9 Protein oxidation inhibition assay in fermented soybean extracts

Reagents

Bovine serum albumin (BSA**)**

Copper sulfate ($CuSO_4$)

Hydrogen peroxide (H_2O_2)

Coomassie blue

SDS polyacrylamide gel

Procedure

Protein oxidation inhibition assay was determined by the method of Shacter (2000) with a slight modification. Reactions were performed in 85 µL of solution consisting of 25 µL of 2.5 mg/mL BSA, 10 µL of 1.5 mM $CuSO_4$, 10 µL of 37.5 mM

H_2O_2 and 40 µL of fermented soybean extracts at various concentrations (0.25, 1.25, 2.5, 5 and 10 mg/mL). The mixtures were incubated at 37ºC for 30 min, and the reactions were stopped by adding 85 µL of gel loading buffer, and then boiled for 3 min. Samples were subjected to electrophoresis (Laemmli, 1970). Proteins (5 µg) were loaded into the gel made of 4% stacking and 12.5 % separating gels and then subjected to electrophoresis at a constant voltage of 75 V using a mini vertical Hoefer apparatus (Hoefer Scientific Instruments, San Francisco, CA., USA). After electrophoresis, the gels were fixed and stained with 0.125% Coomassie blue R-250 in 50% ethanol and 10% acetic acid and destained in 25% ethanol.

6.10 HPLC and LC-MS analysis for isoflavone compositions

Reagents

Standard compound of daidzin, daidzein, genistin, genistein, 8-hydroxydaidzein and 8-hydroxygenistein

Methanol

Acetic acid

Procedure

In order to verify the presence of isoflavone composition, powderized sample was extracted with methanol as previously described and filtered through a 0.45 µm membrane (Millipore Co., Bedford, MA, USA) prior to analysis by HPLC (Griffith and Collison, 2001). Reversed phase HPLC analysis was carried out with Hewlett-Packard HP 1100 series equipped with an autosampler, DAD detector, and HP ChemStation Software (Scientific Equipment Source, Pickering, Canada), using a BSD Hypersil C-18 (4.6 x 250 mm, 5 µm). For the analysis of isoflavones, the mobile

phase was composed of solvent A [H_2O: methanol: acetic acid, 88: 10: 2, (v/v)] and solvent B [methanol: acetic acid, 98: 2 (v/v)]. Following the injection of 20 μL of sample, solvent A was increased from 90% to 100% solvent A over 20 min, and then held at 35% for 10 min. The solvent flow rate was 1 mL/min and the eluted isoflavones were detected at 254 nm. The column temperature was controlled at 25°C. Quantitative data for daidzin, daidzein, genistin, genistein, 8-hydroxydaidzein (8-OHD) and 8-hydroxygenistein (8-OHG) were obtained from comparison with known standards. Mass spectrometry was used to confirm the presence of substances in fermented soybean with the authentic standards. MS analysis was carried out on a Hewlett-Packard Model LC/MSD SL, USA. The capillary voltage of 4000V (positive) and 3500V (negative) was used with this analysis. The flow rate of N_2 was set at 13 L/min at 350°C on 50 psi of nebulizer pressure. The scan ranges of mass spectral was 100-500 m/z in API-ES mode.

6.11 Statistical analysis

All experiments were run in duplicate with triplicate determinations. Analysis of variance (ANOVA) and mean comparison were performed by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was carried out using SPSS 11.0 for windows (SPSS Inc, Chicago, IL, USA).