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Fermentation Time and Extraction Solvents Influenced *in vitro* Antioxidant Property of Soluble Extracts of Mao-tofu Fermented with *Mucor* sp.

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Abstract: Mao-tofu fermented for 3, 5, 7 and 9 days by a strain of *Mucor* sp. was extracted with five solvents including 20, 40 or 60% (v/v) ethanol solutions and pH 4.5 or 6.5 water to investigate the impacts of fermentation time and extraction solvent on the *in vitro* antioxidant property of the extracts. Extraction yield and degree of hydrolysis of the protein fractions in the extracts were measured. Antioxidant properties of the extracts namely scavenging activity on 2,2-diphenyl-1-picrylhydrazyl radical and 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid), iron (III)-reducing activity and iron (II)-chelating activity were evaluated *in vitro*. The results showed that longer fermentation time of Mao-tofu gave the extracts a higher extraction yield, higher degree of hydrolysis of the protein fractions and higher antioxidant activity. The extract prepared with 60% ethanol solution behaved the highest antioxidant activity while that prepared with pH 4.5 water showed the lowest activity. Five extracts from Mao-tofu fermented for 9 days were analyzed by size exclusion chromatography and the molecular weights of their main protein fractions were found to be from 0.2 to 6.0 kDa. Amino acids analysis showed that total content of six hydrophobic amino acids among the five extracts showed no difference, while ethanol solution extracts had more Arg, Cys and Met but less Ala, Gly and His than that of water extract. Present result revealed that the soluble extracts of Mao-tofu had an improved *in vitro* antioxidant activity than soybean proteins and both fermentation time of *Mucor* sp. and solvent types influenced the *in vitro* antioxidant activity of the extracts.

Key words: Mao-tofu extract, antioxidant property, radical scavenging activity, iron (II)-chelating activity, iron (III)-reducing activity, *Mucor* sp.

INTRODUCTION

Reactive oxygen species such as free radicals superoxide anion radicals ($\cdot O_2^-$), hydroxyl radicals ($HO\cdot$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) and other oxygen-free radicals may be formed in the body and food system. These radicals not only induce lipid peroxidation that causes deterioration of foods, but also cause oxidative damage by oxidizing bio-molecules and leading to cell death and tissue damage (Valko *et al*., 2004). Scavenging these radicals by antioxidants is of particularly important. Unfortunately, some synthetic antioxidants such as butylhydroxyanisole and butylhydroxytoluene are questioned for their probable toxic and carcinogenic effects. Recently studies are interested in natural antioxidants from food sources. Intake of food-derived antioxidants might reduce oxidative damages of the body (Bagchi *et al*., 2000). Epidemiologic studies had showed that consumption of soybean-derived products could reduce cancer occurrence, osteoporosis and cardiovascular diseases in humans (Friedman and Brandon, 2001; Han *et al*., 2004). Some antioxidant components such as isoflavones and peptides had been identified in soybean foods (Liu *et al*., 2005; Wang *et al*., 2008) and increasing interest is paid for antioxidant peptides (Baydar *et al*., 2007; Rajapakse *et al*., 2005). Several traditional fermented soybean foods including miso, natto, tempeh, sufu and douchi had been proven to have antioxidant activity (Chen *et al*., 2007; Gibbs *et al*., 2004; Zhu *et al*., 2008). Fermentation, one of the oldest food processing techniques, is believed to give these foods some neutraceutical value.

Microbial fermentation is a potential means to produce natural antioxidants in various fermented soybean foods, for example, Indonesian tempeh produced by *Rhizopus oligosporus* (Chang *et al*., 2009) Chinese douchi by *Aspergillus oryzae* and *Mucor* sp. (Li *et al*., 2007; Wang *et al*., 2007) Japanese miso by *A. oryzae* and

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Saccharomyces rouxii (Hirota *et al*., 2000; Moktan *et al*., 2008), Chinese furu or sufu by *Aspergillus* spp. (Ren *et al*., 2006). Mao-tofu, one of traditional Chinese soybean foods, is farmhouse- fermented tofu (soybean protein curd) primarily by mould *Mucor* sp. characterized by a cover of white fungous mycelia. Fermentation of tofu with *Mucor* sp. shows some helpful impacts on the quality of final product. Furu, another type of fermented tofu product in China, shares some similarity to Mao-tofu in production and was found to have remarkable antioxidant activity (Ren *et al*., 2006). Based on this finding, there is a need to study antioxidant properties of Mao-tofu.

In the present study, the impacts of fermentation time of *Mucor* sp. and solvent types on *in vitro* antioxidant property of soluble extracts of Mao-tofu were studied. Tofu was fermented by a stain of *Mucor* sp. previous isolated from farmhouse-prepared Mao-tofu. Five solvents were used to extract soluble protein fractions from Mao-tofu fermented at different stages. The soluble extracts obtained were evaluated for their extraction yields and degree of hydrolysis of protein fractions and especially for their antioxidant properties including scavenging activity on two radicals (DPPH radical and $ABTS^{\cdot+}$), iron (III)-reducing and iron (II)-chelating activity. Meanwhile, amino acid and peptide profiles of some soluble extracts prepared were characterized and compared.

MATERIALS AND METHODS

Materials: Tofu used for Mao-tofu preparation was obtained from local market. 2,2-diphenyl- 1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), cytochrome C, insulin, oxidized L-glutathione and L-tyrosine were purchased from Sigma (St. Louis, MO, USA). Other chemicals used were all of analytical reagents. Water used was re-distilled water.

Preparation of Mao-tofu: A strain of *Mucor* sp. previously isolated from a Mao-tofu product collected from countryside in central China was identified as *Mucor micheli ex fries*. Spore suspension of *Mucor* sp. was prepared as a described method (Abbas *et al*., 2002) with Potato Dextrose Agar medium and stored at 4°C until Mao-tofu preparation.

The whole study began at Sep. 2009 and lasted to Oct. 2010. Preparation procedure of Mao-tofu followed the method described by Zhao and Zheng (2009) as follows: fresh tofu was cut into cubes (about 18×14×10 cm) by knife with smooth surface. After being sterilized at 100°C for 20 min to inactivate microorganisms or enzymes contaminated, the cooled cubes were cut into dices (about 3×3×3 cm) aseptically in laboratory and inoculated with *Mucor* inoculum (10^5 spores mL^{-1}) over their surface. The inoculum had been prepared before as a pure starter. All dices were put in sterilized bamboo trays separately to facilitate air circulation and mycelia development and cultured in an incubator at 20±1°C with a relative humidity about 73 to 76% for 9 days. During fermentation, some dices were random selected every 3, 5, 7 and 9 days as analysis samples and subjected to extraction procedure or chemical analysis.

Analysis of Mao-tofu: The prepared Mao-tofu samples were analyzed for their moisture content expressed as weight of water in 100 g sample (g 100 g^{-1}) by AOAC Methods 926.08 (AOAC 2000) or for total protein or water soluble protein content by Kjeldahl method 920.123 (AOAC 2000) on a Kjeltec 2300 Analyzer (Foss Tecator AB, Höganäs, Sweden). Conversion factor 5.71 was used to calculate total protein or water soluble protein content expressed as weight of proteins or soluble proteins in 100 g Mao-tofu (g 100 g^{-1}).

The content of water-soluble total proteins of the Mao-tofu samples was analyzed as reference method of Moatsou *et al*. (2004). Mao-tofu of 10 grams was homogenized with 50 mL distilled water by using a high speed homogenizer (Type DS-1, Shanghai Jingke Ltd., Shanghai, China) for 5 min, centrifuged at 4000 g for 20 min and filtered through filter paper (Whatman 40). Nitrogen in the obtained extract was estimated by Kjeldahl method as before. Water-soluble total proteins was also calculated and expressed as weight of water-soluble proteins in 100 g sample (g 100 g^{-1}).

Preparation of Mao-tofu extracts: The Mao-tofu samples (fermented for 3, 5, 7 or 9 days) were extracted with each of five extraction solvents including 20, 40, 60% (v/v) ethanol solution, pH 4.5 and pH 6.5 water, respectively to obtain soluble protein fractions. The extraction procedure applied was carried as follow: Mao-tofu of 75 g was mixed with one extraction solvent at room temperature to give final volume 250 mL in a plastic beaker, homogenized with a high speed homogenizer (Type DS-1, Shanghai Jingke Ltd., Shanghai, China) at 10000 rpm for 1.5 min, centrifuged at 4000 g for 20 min and then filtered through filter paper (Whatman 40) to obtain corresponding extract. The separated extract of ethanol solution was evaporated in a vacuum rotary evaporator at 50°C to remove ethanol and then adjusted to the previous volume with water. Nitrogen content in the obtained extract and the Mao-tofu sample were estimated by Kjeldahl method (AOAC, 2000) and used to calculate extraction yield of soluble protein fractions with Eq:

$$\text{Extraction yield (\%)} = \frac{P_E}{P_S} \times 100$$

where, P_E and P_S are total protein amount (mg) in the obtained extract and the Mao-tofu sample of 75 g, respectively.

Evaluation of degree of hydrolysis of protein fractions in extract: An OPA (o-pthaldialdehyde) method (Spellman *et al*., 2003) with some modifications was used to determine the amount of free amino groups of the protein fractions in the extract. The OPA reagent was prepared by combing following reagents to a final volume of 100 mL with water: 75 mL, 0.2 mol L^{-1} sodium borate buffer (pH 9.5), 5 mL 400 g SDS L^{-1}, 80 mg L^{-1} OPA methanol solution and 0.4 mL β-mercaptoenthanol. The reagent was prepared daily and protected from light. The assay was carried out by adding 3.0 mL analysis sample to 3.0 mL OPA reagent. The absorbance of the mixture was measured at 340 nm in an UV spectrophotometer (UV-2401PC, Shimadzu, Japan) and taken after 5 min. L-leucine solution (12 to 36 μg mL^{-1}) was used as standard. Nitrogen content of the extract was determined by Kjeldahl method as above. The Degree of Hydrolysis (DH) of the protein fractions of the extract was determined by assaying the amount of free amino groups of protein fractions by OPA method and calculated by using Eq. described by Pena-Ramos and Xiong (2001):

$$\text{DH (\%)} = \frac{h}{h_{tot}} \times 100$$

where, h is the number of broken peptide bonds per unit weight and h_{tot} is the total number of bonds per unit weight, which equals 7.8 meq g^{-1} proteins for soybean proteins (Pena-Ramos and Xiong, 2001).

Assaying radical scavenging activity on DPPH and $ABTS^+$: Scavenging activity of the extract prepared on DPPH radical was measured as per the method of Xie *et al*. (2008) with some modifications. An aliquot (0.5 mL) of sample solution at protein concentration of 0.5 mg mL^{-1} was mixed with 2.5 mL of DPPH (20 mmol L^{-1} in ethanol). The reaction mixture was incubated for 30 min in the darkness at room temperature. The absorbance of resulting solution was measured at 517 nm in the UV spectrophotometer. Ethanol was used as control. Radical-scavenging capacity of the extract was measured as a decrease in the absorbance of DPPH radical and expressed as inhibition percent by the following Eq:

$$\text{Inhibition (\%)} = \left(\frac{A_C - A_E}{A_C}\right) \times 100$$

where, A_E and A_C are the absorbance for the extract and control, respectively.

Scavenging activity of the extract on $ABTS^+$ radicals was measured as per the method of Erel (2004) with some modifications. $ABTS^+$ was produced by reacting 7 mmol L^{-1} ABTS aqueous solution with 2.45 mmol L^{-1} potassium persulfate in the darkness for 12 to 16 h at room temperature. Prior to assaying, ABTS solution was diluted with ethanol (about 1:89 v/v) and equilibrated at 30°C to give an absorbance at 734 nm of 0.700±0.02. After addition of 1.0 mL of diluted $ABTS^+$ solution to 10 μL of sample solution (protein concentration 4.0 mg mL^{-1}) in ethanol, the absorbance was taken in the UV spectrophotometer at 30°C exactly 1 min after initial mixing and up to 6 min. Appropriate solvent blanks were run in each assaying. Radical- scavenging capacity of the extract was expressed as inhibition percent by the following Eq:

$$\text{Inhibition (\%)} = \left(\frac{A_C - A_E}{A_C}\right) \times 100$$

where, A_E and A_C are the absorbance for the extract and control, respectively.

Measurement of iron (II)-chelating activity: Iron (II)-chelating activity of the extract was measured according to the method of Xu *et al*. (2009) with some modifications. First, 250 μL of 1 mg mL^{-1} sample solution (final protein concentration 100 μg mL^{-1}) was mixed thoroughly with X μL of freshly prepared 1 mmol L^{-1} $FeCl_2$ aqueous solution (final $FeCl_2$ concentration 10 to 60 μmol L^{-1}) and (1250-X) μL of Milli-Q water in a 5 mL test tube to give a final volume of 1.50 mL. The mixture was allowed to stand for 2 min and 1 mL of 0.5 mmol L^{-1} ferrozine aqueous solution (final ferrozine concentration 0.2 mmol L^{-1}) was added. The resulting mixture was allowed to react for 10 min and its absorbance at 562 nm was measured in the UV spectrophotometer. Etyhylene Diamine Tetraacetic Acid (EDTA) at a final concentration of 10 μmol L^{-1} was served as positive control. Iron (II)-chelating activity of the extract was expressed as chelating power and calculated with Eq:

$$\text{Chelating power (\%)} = \left(1 - \frac{A_E}{A_C}\right) \times 100$$

where, A_E and A_C are the absorbance for the extract and control [X μL of 1mmol L^{-1} $FeCl_2$ aqueous solution mixed with (2500-X) μL of Milli-Q water], respectively.

Determination of iron (III)-reducing activity: The ability of the extract to reduce iron (III) was determined as per the method of Yildirim *et al.* (2001). An aliquot of 1 mL sample solution (protein concentration 5 mg mL^{-1}) was mixed with 2.5 mL of 0.2 mol L^{-1} phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixtures were incubated at 50°C for 30 min and 2.5 mL of 10% trichloroacetic acid was added to stop reaction. The mixture was centrifuged at 1650 g for 10 min. Finally, 2.5 mL of the supernatant separated was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride solution. After a 10 min reaction time, the absorbance of the resulting solution was measured at 700 nm in the UV spectrophotometer, expressed as iron (III)-reducing power and used to reflected iron (III)-reducing activity of the extract. Higher absorbance of the mixture indicated higher iron (III)-reducing power of the sample solution.

Size exclusion chromatography analysis: A reference procedure described by Ren *et al.* (2008) with some modifications was applied to analyze peptide profiles of the prepared extract. Protein concentration of the sample solution was fixed at 2 mg mL^{-1}. Half of a milliliter of the solution was applied to a 10×300 mm Amersham Pharmacia Superdex-25 10/300 GL column (GE Amersham, USA) and analyzed in an AKTA Explorer 100 (GE Amersham, USA). The column was equilibrated and eluted with 0.25 mol L^{-1} phosphate buffer (pH 7.2) in isocratic mode at a flow-rate of 0.5 mL min^{-1} and a fixed pressure of 0.15 MPa. The elution was monitored with an UV detector at 215 nm. Cytochrome c (12.4 kDa), (5.7 kDa), oxidized L-glutathione (0.6 kDa) and L-tyrosine (0.2 kDa) were selected as standards.

Analysis of amino acid compositions: The prepared extract of 200 mg was hydrolyzed under reduced pressure in 6 moL L^{-1} HCl at 110°C for 24 h. Seventeen amino acids except for tryptophan (Trp) in the extract were analyzed by using a Hitachi amino acids analyzer 835-50 (Hitachi, Tokyo, Japan) with the recommended procedure provided by the producer. Total Hydrophobic Amino Acids (THAA) were calculated as the sum of eight amino acids including Ala, Val, Leu, Ile, Phe, Pro, Tyr and Met.

Statistical analysis: All experiments were carried out at least three batches. All data were expressed as Means±standard deviation from at least three independent analyses. Differences between the mean values of multiple groups were analyzed by one-way Analysis of Variance (ANOVA) with Duncan's multiple range tests. Microsoft Excel version 2003 software (Microsoft Corporation, Redmond, WA, USA) and SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA) were used to report the data.

RESULTS AND DISCUSSION

Fermentation, proteolysis and extraction of Mao-tofu: Fermentation of Mao-tofu progressed in two stages, mold mycelia growth on the surface of Mao-tofu first and then proteolysis by enzymes excreted from mycelia. As fermentation progressed, main compositions of Mao-tofu changed due to water evaporation and some proteins were degraded into peptides reflected by the increased amount of water soluble proteins in Mao-tofu (as shown in Table 1). Moisture content of Mao-tofu decreased from 84.46 to 73.64% during fermentation, while total protein or water-soluble protein content increase from 8.11 to 14.26 or from 0.33 to 7.56%, respectively. The variations of these indices shared similarity to the reported work (Zhao and Zheng, 2009).

When five solvents were employed to extract soluble fractions from Mao-tofu, both fermentation times of Mao-tofu and solvent types showed influences on the extraction yield of soluble protein fractions significantly, as declared in Table 2. The extraction yield of soluble protein fractions increased as fermentation time progressed, in spite of what extraction solvent was employed. At late stage of fermentation (9 days), the extraction yield of the soluble protein fractions was the highest (from 31.7 to 36.1%), which meant that nearly third of total proteins could be extracted from Mao-tofu by five solvents. Meanwhile, the extraction yield of the soluble protein fractions was impacted clearly by extraction solvent applied. Logically, pH 6.5 water could extract more protein fractions from Mao-tofu than pH 4.5 water, as isoelectric point of soybean proteins was about 4.5. The

Table 1: Some chemical characteristics of Mao-tofu fermented by *Mucor* sp. at different times[a]

	Fermentation times (Days)				
Parameters	0	3	5	7	9
Moisture (%)	84.46±0.05^{e}	80.24±0.05^{d}	77.48±0.04^{c}	74.89±0.05^{b}	73.64±0.08^{a}
Total proteins (g 100 g^{-1} sample)	8.11±0.09^{a}	10.45±0.05^{b}	12.03±0.03^{c}	13.31±0.07^{d}	14.26±0.04^{e}
Water soluble proteins (g 100 g^{-1} sample)	0.33±0.01^{a}	1.79±0.01^{b}	3.54±0.01^{c}	6.13±0.01^{d}	7.56±0.01^{e}

a: Different letters after the values in same line indicate that one-way ANOVA of means is significantly different ($p<0.05$)

practical data shown in Table 2 supported this conclusion. If an organic solvent ethanol was incorporated into water as extraction solvent, the polarity of the solvent was lowered, which would lead some protein fractions were insoluble but some small peptides or amino acids more soluble. When more ethanol was incorporated into water, the resulting extraction solvent would extract less soluble protein fractions than pH 6.5 water, as the data shown in Table 2 indicated. If ethanol level in the extraction solvent had an increasing step of 20% (v/v), extraction yield of soluble protein fractions might have a decrease about 2%.

The evaluation results about the DH of the soluble protein fractions in the extracts revealed that soybean proteins in Mao-tofu during fermentation were degraded. Fresh tofu was analyzed as a control. Table 3 shows that the DH of the soluble protein fractions in the extracts increased from 17 to 45% as fermentation progressed, revealing the soluble protein fractions in the extracts might mainly be small peptides or amino acids (especially when Mao-tofu was fermented for 9 days). As pH 4.5 water could extract less protein fractions from Mao-tofu, the resulting extract had the highest DH. Not contrary to expectations, the extract of pH 6.5 water had the lowest DH as more protein fractions could be extracted. It was noticed that the soluble protein fractions extracted by ethanol solution had larger DH as insolubility of protein fractions in ethanol solution.

It was reported that *B. subtilis* showed proteolytic activity to kinema, a soybean-fermented food and the content of free-amino acids (the products of protein degradation) was found to be 60-fold increased markedly (Kiers *et al*., 2000). Han *et al*. (2004) revealed that total free amino acids had about 4-fold increases during the ripening of sufu. Zhu *et al*. (2008) had proved the proteins degradation in Chinese traditional fermented okara during fermentation by SDS-PAGE, because their results showed that the proteins were gradually hydrolyzed during first 48 h and after then virtually disappeared from the gel. Our analysis results about DH and soluble proteins also indicated protein degradation in Mao-tofu induced by *Mucor* sp. and shared similarity to these reported works.

Scavenging activity of Mao-tofu extracts on two radicals: The prepared extracts were assayed for their radical scavenging activities on two radicals, DPPH radical and $ABTS^+$. A control, soybean protein isolates, was also evaluated and showed a weak activity of 3.79 or 4.97% on DPPH radical or $ABTS^+$. The results are listed in Table 4 and 5 and state clearly that all extracts had much better activity than the control. The longer fermentation time the higher activity of the extract. The data listed in Table 4 also show that (1) the extracts prepared with ethanol solutions exhibited better radical scavenging activity on DPPH radical than that prepared with pH 4.5 or 6.5 water; (2) the extracts prepared with 60% (v/v) ethanol solution showed the highest activity (from 25.4 to 45.2%) while that prepared with pH 4.5 water showed the lowest activity (from 14.2 to 27.7%) and (3) if ethanol level in extraction solvent had an increasing step of 20% (v/v), the scavenging activity of corresponding extract on DPPH radical usual had an increase about 3%. The extract prepared with pH 4.5 water had the lowest activity, which implied the role of protein fractions to scavenge DPPH radical. The data listed in Table 5 described scavenging activity of the extracts on $ABTS^+$ and it shared similarity to the characteristics described above for the data in Table 4, except that if ethanol level in

Table 2: Extraction yield (%) of protein fractions obtained from Mao-tofu with five solvents*

	Fermentation times of Mao-tofu (Days)				
Extract	0	3	5	7	9
20% EE	6.1±0.9^{bc}	20.9±0.8^{ab}	24.9±1.5^{bc}	28.7±0.6^{cd}	35.3±0.7^{bc}
40% EE	5.0±0.7^{ab}	18.7±2.0^{ab}	22.9±1.6^{ab}	25.6±1.0^{ab}	32.9±0.3^{ab}
60% EE	3.7±0.1^{a}	16.7±2.9^{a}	20.3±0.3^{a}	23.1±0.7^{a}	31.7±1.3^{a}
pH 4. 5 WE	5.4±0.6^{b}	20.8±2.9^{ab}	23.6±1.6^{ab}	26.4±1.6^{bc}	33.9±1.1^{abc}
pH 6.5 WE	7.6±0.1^{c}	24.2±1.9^{c}	26.9±0.7^{c}	29.8±1.2^{d}	36.1±1.1^{c}

*EE, Mao-tofu ethanol extract prepared with ethanol solution. WE, Mao-tofu water extract prepared with pH 4.5 or pH 6.5 water. Means with different letter(s) within same list are significantly different ($p<0.05$)

Table 3: Degree of hydrolysis (%) of protein fractions in Mao-tofu extracts prepared with five solvents*

	Fermentation times of Mao-tofu (Days)				
Extract	0	3	5	7	9
20% EE	17.4±0.6^{a}	22.8±0.9^{b}	23.8±0.5^{a}	32.1±0.7^{a}	38.5±0.1^{b}
40% EE	17.7±0.9^{a}	23.5±0.8^{b}	24.8±0.8^{ab}	32.4±1.5^{a}	38.3±1.2^{b}
60% EE	18.2±0.5^{a}	25.9±0.4^{c}	26.3±0.2^{b}	33.8±1.5^{a}	40.9±0.5^{b}
pH 4.5 WE	21.8±0.6^{b}	27.8±0.5^{d}	30.2±0.3^{c}	33.7±1.7^{a}	45.7±0.6^{c}
pH 6.5 WE	20.3±0.2^{b}	20.7±0.4^{a}	23.8±1.1^{a}	31.0±0.3^{a}	28.5±2.8^{a}

*EE, Mao-tofu ethanol extract prepared with ethanol solution. WE, Mao-tofu water extract prepared with pH 4.5 or pH 6.5 water. Means with different letter(s) within same list are significantly different ($p<0.05$)

Table 4: Radical scavenging activities (%) of Mao-tofu extracts on DPPH radical*

	Fermentation times of Mao-tofu (Days)			
Extract	3	5	7	9
20% EE	19.8±1.3^{bc}	21.5±1.2^{b}	24.0±1.4^{c}	37.0±1.7^{b}
40% EE	22.6±1.9^{cd}	24.6±1.9^{bc}	26.7±1.8^{bc}	42.8±2.0^{c}
60% EE	25.4±2.7^{d}	27.4±2.9^{c}	30.1±2.3^{c}	45.2±1.8^{c}
pH 4.5 WE	14.2±2.6^{a}	16.2±2.5^{a}	18.9±2.4^{a}	27.7±1.1^{a}
pH 6.5 WE	18.2±1.4^{b}	21.0±1.8^{b}	24.0±1.3^{b}	36.7±2.2^{B}

*EE, Mao-tofu ethanol extract prepared with ethanol solution. WE, Mao-tofu water extract prepared with pH 4.5 or pH 6.5 water. Radical scavenging activity (%) of soybean protein isolates on DPPH was 3.79±0.78. Means with different letter(s) within same list are significantly different ($p<0.05$)

Table 5: Radical scavenging activities (%) of Mao-tofu extracts on ABTS$^{\cdot+}$*

	Fermentation times of Mao-tofu (Days)			
Extract	3	5	7	9
20% EE	22.9±1.4^{c}	24.9±1.4^{bc}	33.8±1.2^{c}	37.0±1.6^{c}
40% EE	25.0±1.7^{c}	25.8±2.0^{c}	37.1±0.9^{d}	39.1±1.1^{cd}
60% EE	30.3±1.3^{d}	33.0±2.8^{d}	40.1±1.4^{e}	42.4±3.0^{d}
pH 4.5 WE	11.0±1.4^{a}	13.2±1.4^{a}	13.1±1.4^{a}	13.8±1.6^{a}
pH 6.5 WE	20.2±1.4^{b}	22.0±1.4^{b}	30.9±1.4^{b}	32.2±1.6^{b}

*EE, Mao-tofu ethanol extract prepared with ethanol solution. WE, Mao-tofu water extract prepared with pH 4.5 or pH 6.5 water. Radical scavenging activity (%) of soybean protein isolates on ABTS$^{\cdot+}$ was 4.97±0.43. Means withdifferent letter(s) within same list are significantly different ($p<0.05$)

extraction solvent had an increasing step of 20% (v/v), the scavenging activity of corresponding extract on ABTS^{+} behaved an irregular increasing profile. Based on these results, it could be concluded that lower polarity of extraction solvent was helpful to obtain Mao-tofu extract with higher antioxidant activity.

Zhu *et al*. (2008) compared the antioxidant activity of Water Extract of Soybean Koji (WESK) and okara koji (WEOK) on DPPH radical and ABTS$^{+\cdot}$ DPPH radical scavenging activity of WEOK or WESK was about 4.13 or 3.34 mg Trolox g^{-1}. Okara and soybean without fermentation exhibited an ABTS$^{+\cdot}$ scavenging activity of 13.5 and 16.4 mg Trolox eq g^{-1}, while fermented okara and soybean gave an enhanced ABTS^{+} scavenging activity of 34.8 and 35.7 mg Trolox eq g^{-1}, respectively. Their result was similar to our result, indicated that fermentation could improve antioxidant activity of the extracts. Wang *et al*. (2008) had studied the antioxidant properties of aqueous extracts of Douchi *in vitro* on DPPH radical and ABTS$^{+\cdot}$, in which IC_{50} values of the extract were 0.658 and 0.204 mg mL^{-1}, respectively. In present study, when Mao-tofu was fermented for 9 days, the extract prepared with 60% (v/v) ethanol solution at 0.5 mg mL^{-1} could scavenge DPPH radical about 45.2%, or at 4.0 mg mL^{-1} could scavenge ABTS$^{+\cdot}$ about 42.4%, implying that the extract of Mao-tofu had a closer activity on DPPH radical but a lower activity on ABTS$^{+\cdot}$ than aqueous Douchi extracts obtained by Wang *et al*. (2008).

Iron (ii)-chelating and iron (iii)-reducing activity of mao-tofu extracts: The assaying results about iron (II)-chelating activity of the extracts were expressed as iron (II)-chelating power and are given in Table 6. All extracts at protein concentration of 1mg mL^{-1} displayed effective iron (II)-binding ability. Iron (II)-chelating activity of the extracts increased as fermentation time of Mao-tofu increased. The extracts obtained from Mao-tofu fermented for 9 days exhibited the highest chelating power. Also, the extracts prepared with 60% (v/v) ethanol solution had the highest activity (from 25.5 to 76.5%) while that prepared with pH 4.5 water had the lowest activity (from 15.8 to 30.4%). Totally, the extract prepared with pH 6.5 water showed an alike activity to other two extracts prepared with 20 and 40% (v/v) ethanol solution, except for when Mao-tofu was fermented for 7 days. All extracts gave much better activity than soybean protein isolates (about 19.9%) except for pH 4.5 water extract of Mao-tofu fermented for shorter time. The result also indicated that fermentation of Mao-tofu gave final product better antioxidant properties.

Mao-tofu extracts also showed activity to reduce iron (III) into iron (II), which could be reflected from the data listed in Table 7. Longer fermentation time led the extracts a higher reducing activity. The extracts prepared with 60% (v/v) ethanol solution had the highest activity (with reducing power from 0.389 to 0.595) while that prepared with pH 4.5 water had the lowest activity (with reducing power from 0.262 to 0.351). When Mao-tofu was fermented for 7 or 9 days, the extract prepared with pH 6.5 water had a closed activity to other two extracts prepared with 20 and 40% (v/v) ethanol solution. All extracts had much better iron (III)-reducing power than soybean protein isolates (with a value of 0.165).

Lee *et al*. (2008) had evaluated the antioxidant properties of water extracts of Monascus-fermented Soybeans (MFS). The chelating ability of water extracts

Table 6: Iron (II) -chelating power (%) of Mao-tofu extracts prepared with five solvents*

	Fermentation times of Mao-tofu (Days)			
Extract	3	5	7	9
20% EE	20.2±2.3^{b}	24.6±1.2^{b}	28.0±1.9^{b}	72.0±1.6^{b}
40% EE	22.9±1.5^{bc}	26.2±1.7^{b}	32.7±1.9^{c}	74.4±2.3^{bc}
60% EE	25.5±1.8^{c}	30.8±1.7^{c}	61.5±2.1^{e}	76.5±2.4^{c}
pH 4.5 WE	15.8±2.6^{a}	18.4±1.1^{a}	22.8±1.6^{a}	30.4±1.6^{a}
pH 6.5 WE	20.8±1.5^{b}	25.8±0.5^{b}	54.7±3.4^{d}	75.7±2.4^{bc}

* EE, Mao-tofu ethanol extract prepared with ethanol solution. WE, Mao-tofu water extract prepared with pH 4.5 or pH 6.5 water. Iron (II) -chelating power (%) of soybean protein isolates was 19.9±1.6. Means with different letter (s) within same list are significantly different ($p<0.05$)

Table 7: Iron (III)-reducing power of Mao-tofu extracts prepared with five solvents

	Fermentation times of Mao-tofu (Days)			
Extract	3	5	7	9
20% EE	0.309±0.012^{ab}	0.351±0.018^{b}	0.405±0.033^{b}	0.466±0.025^{b}
40% EE	0.351±0.029^{bc}	0.406±0.019^{c}	0.445±0.030^{bc}	0.488±0.024^{b}
60% EE	0.389±0.040^{c}	0.455±0.018^{d}	0.490±0.038^{c}	0.595±0.053^{c}
pH 4.5 WE	0.262±0.018^{a}	0.318±0.013^{a}	0.330±0.012^{a}	0.351±0.018^{a}
pH 6.5 WE	0.333±0.022^{b}	0.378±0.009^{bc}	0.417±0.03 0^{b}	0.490±0.027^{b}

* EE, Mao-tofu ethanol extract prepared with ethanol solution. WE, Mao-tofu water extract prepared with pH 4.5 or pH 6.5 water. Iron (III) - reducing power (%) of soybean protein isolates was 0.165±0.04. Means with different letter (s) within same list are significantly different ($p<0.05$)

from MFS and soybeans on iron (II) at 5 mg mL^{-1} were 74.2 and 58.6%, respectively; meanwhile, the reducing power of MFS extract was 0.54 at 5 mg mL^{-1} but that of soybean extract was 0.55 at 10 mg mL^{-1}. Zhang *et al.* (2010) studied the chelating activity of soybean protein hydrolysates and found that high Molecular Weight (MW) hydrolysates had higher chelating activity against iron (II) than low-MW hydrolysates. The work of Moure *et al.* (2006) also showed that soybean protein hydrolysates had higher reducing power than the original proteins; for example, one hydrolysates fractions showed a reducing power of 0.292 at 5 mg mL^{-1}, but original proteins gave reducing power of 0.138 at same concentration. Compared to these reported results, when Mao-tofu was fermented for 9 days, the extract prepared with 60% (v/v) ethanol solution showed a chelating activity 76.5% at 1 mg mL^{-1} and a reducing power of 0.595 at 5 mg mL^{-1}. Our result showed that the soluble extract of Mao-tofu had a similar reducing power compared to the water extracts of MFS of Lee *et al.* (2008) and a higher chelating activity than the water extracts of MFS or soybean protein hydrolysates of Moure *et al.* (2006).

Peptide profiles and amino acid compositions of mao-tofu extracts: A primary analysis result from size exclusion chromatography showed peptide profiles of five extracts prepared from Mao-tofu fermented for 9 days, as previous results showed that these extracts had higher antioxidant activity. The results are given in Fig.1. Figure 1B showed some different peptide compositions of two water extracts and some large protein fractions existed in pH 6.5 water extract. Figure 1C showed the different peptide compositions of three ethanol extracts and 20% ethanol extract contained more larger protein fractions than 60% ethanol extract. The elution results of cytochrome c, insulin, oxidized L-glutathione and L-tyrosine are shown in Fig. 1A, which indicates an elution time of 4.4, 17.3, 23.6 and 38.5 min, respectively for these standards. The elution of the main fractions of five extracts began at ~16 min and most fractions were eluted at ~44 min. It was estimated therefore that the main fractions of five extracts had molecular weight ranging from 0.2 to 6.0 kDa, indicating that the main fractions in five extracts were free amino acids and small peptides. Also, Fig. 1C revealed that the extract of 60% ethanol solution had more amino acid and less large peptides.

The amino acid compositions of five extracts of Mao-tofu fermented for 9 days are listed in Table 8 except for Trp. Analysis results showed that five extracts had a similar THAA profile (from 39.26 to 39.91%). Meanwhile, three ethanol solution extracts had higher level of Arg, Cys and Met but lower level of Ala, Gly and His, compared to two water extracts. It could be assumed that the enriching of sulfur-containing amino acids (Met and Cys) and the decreasing of Ala and Gly in Mao-tofu extracts of ethanol solution might be accounted for their better antioxidant properties.

It was reported by Torruco-Uco *et al.* (2009) that protein hydrolysis by Alcalase released the peptides with more hydrophobic amino acids (Phe, Tyr, Met, etc) in their sequence, so the antioxidant activity of the hydrolysates was higher. Zhuang *et al.* (2010) reported that when jellyfish gelatin was hydrolyzed by different proteases, the hydrolysates with highest antioxidant activity had higher content of Met and Cys. Our work supported these

Table 8: Amino acids compositions (mol %) of five extracts prepared from Mao-tofu fermented by *Mucor* sp. for 9 days[a]

Amino acids	pH 4.5 WE	pH 6.5 WE	20% EE	40% EE	60% EE
Ala	9.03	8.84	8.51	8.21	8.13
Arg	2.69	2.86	3.07	3.08	3.05
Asp	9.12	9.34	9.58	9.52	9.47
Cys	0.59	0.65	0.83	0.75	0.79
Glu	26.33	26.44	26.09	26.78	26.53
Gly	9.98	9.57	9.15	9.14	9.07
His	0.85	0.78	0.62	0.62	0.62
Ile	4.14	4.19	4.26	4.22	4.24
Leu	5.73	5.66	5.77	5.71	5.76
Lys	3.66	3.61	3.61	3.71	3.62
Met	0.56	0.55	0.98	0.96	1.05
Phe	3.71	3.89	3.95	3.82	3.88
Pro	9.75	9.57	9.57	9.66	9.86
Ser	3.28	3.30	3.29	3.24	3.16
Thr	3.72	3.86	3.86	3.89	3.87
Tyr	2.40	2.35	2.27	2.20	2.37
Val	4.48	4.54	4.61	4.48	4.54
Total	100.00	100.00	100.00	100.00	100.00
THAA	39.78	39.59	39.91	39.26	39.83

a: EE, Mao-tofu ethanol extract prepared with ethanol solution. WE, Mao-tofu water extract prepared with pH 4.5 or pH 6.5 water. The data were expressed as mol ratio (%) of each amino acid to 17 amino acids except for Trp. THAA, total hydrophobic amino acids, is the sum of eight amino acids including Ala, Val, Leu, Ile, Phe, Pro, Tyr and Met

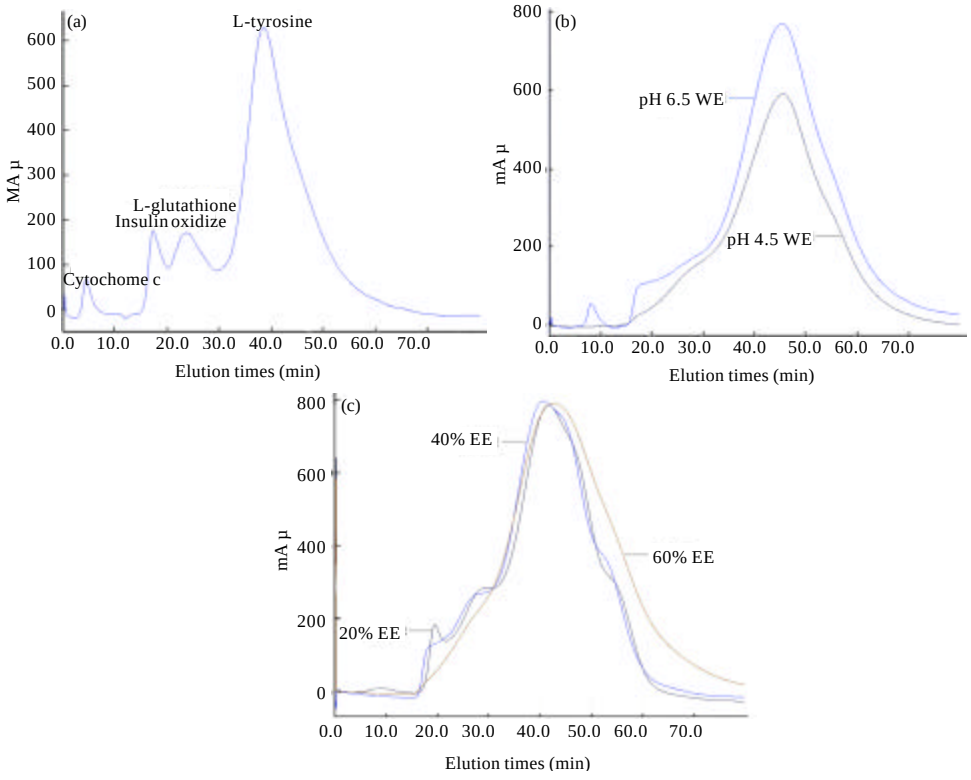

Fig. 1: Size exclusion chromatography analysis of the standards (A), water extracts (B) and ethanol solution extracts (C) of Mao-tofu fermented for 9 days. The standards analyzed were cytochrome c, insulin, oxidized L-glutathione and L-tyrosine. WE, water extract; EE ethanol extract. The analysis was carried at Superdex-25 column with eluting flow rate of 0.5 mL min^{-1} and monitored at 215 nm

works. The extracts we prepared with ethanol solutions were also found to be rich in Met and Cys and had higher antioxidant activity, although they were not rich in total hydrophobic amino acids.

CONCLUSION

Tofu was fermented with a strain of *Mucor* sp. to prepare Mao-tofu and five solvents were used to extract Mao-tofu. Both fermentation time and solvent types showed clearly impacted on extraction yield, degree of hydrolysis of protein fractions and especially on antioxidant property *in vitro* of the extracts including scavenging activity on DPPH radical and $ABTS^{+\cdot}$, iron (III)-reducing activity and iron (II)-chelating activity. Longer fermentation time of Mao-tofu would give the extracts higher extraction yield, higher degree of hydrolysis and higher antioxidant activity. All extracts had better antioxidant activity than soybean protein isolates and the extracts prepared with ethanol solution had higher activity. The extract prepared with 60% (v/v) ethanol solution had the highest activity while the extract prepared with pH 4.5 water showed the lowest activity. Other evaluation results indicated the main protein fractions in the extracts of Mao-tofu fermented for 9 days had molecular weights about 0.2 to 6.0 kDa and the extracts of ethanol solutions had more Arg, Cys and Met.

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