Antioxidant activity of fractionated foxtail millet protein hydrolysate

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Abstract: The objective of the study was to assess the antioxidant potential of defatted foxtail millet protein hydrolysates (DFMPH). Gel filtration (Sephadex G-25) chromatography was used to fractionate DFMPH. The amino acid composition, ABTS, DPPH, inhibition of linoleic acid autoxidation, metal-chelating free radical scavenging ability, and reducing power were tested to determine their antioxidant potency. The antioxidant activity of fraction (FIV) (85.71%) was closer to that of α-tocopherol (86.27%) but lower than that of BHT (butylated hydroxytoluene) (92.44%) in the linoleic acid oxidation system. Furthermore, FIV exhibited higher ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), DPPH (1,1-diphenyl-2-picrylhydrazyl) and metal-chelating activity than the other fractions with a significant difference ($P < 0.05$). Amino acid profile revealed that FIV, with the strongest antioxidant activity, had the highest hydrophobic amino acids content (51.94%) and hydrophobicity (8.62 kJ/moL amino acid residue "AAR"). Molecular weight of the fractions varied from 77-1042 Da. The data obtained by the in vitro systems established antioxidant potency of DFMPH fractions.

Keywords: Millet protein hydrolysate, fractionation, amino acid, molecular weight, antioxidant activity

Introduction

Free radicals are associated with the occurrence of several degenerative disorders including cardiovascular disease, diabetes, inflammatory diseases and aging. Apart from the lipid peroxidation in food systems, free radicals are thought to mediate diseases such as cancer, coronary heart diseases and Alzheimer's disease (Martinez-Cayuela, 1995; Huda-Faujan *et al*., 2007). Therefore, protection against free radicals is key factor in preventing deterioration of foods via lipid peroxidation and some deleterious diseases.

Antioxidant agents can act against free radicals either by retarding their formation (preventive antioxidants) or by inactivating radicals to remove them from the reaction medium (chain-breaking antioxidants) (Wang *et al*., 2007; Kim *et al*., 2007; Amadou *et al*., 2009). Currently, the natural antioxidant α-tocopherol and some synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole and propyl gallate are commonly used to act against free radicals in food and biological systems. However, the use of synthetic antioxidants in food products is under strict regulation owing to their potential health hazards (Prashant *et al*., 2005). Therefore the search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers. Numerous studies have shown that the majority of antioxidant activities in natural sources are due to compounds such as flavonoids, α-tocopherol, ascorbic acid and β-carotene (Zhang *et al*., 2009; Pérez *et al*., 2007; Amadou *et al*., 2009).

Bioactive peptides commonly contain 3-20 amino acids per peptide as inactive sequences within large proteins and are released when the parent protein is hydrolyzed by digestive enzymes (*in vitro* and *in vivo*), by microbial enzymes, or during food processing (Amadou *et al*., 2010). Enzymatic hydrolysis of proteins is one approach used to release bioactive peptides and is widely applied to improve functional and nutritional properties of protein sources (Jun *et al*., 2004). The biological activity of a peptide is widely recognized to be based on amino acid composition (Jun *et al*., 2004; Nurul *et al*., 2010). Peptides could be used in the formulation of functional foods and nutraceuticals to prevent damage related to oxidative stress in human disease conditions. Moreover, natural antioxidants are desirable because they can be used at higher concentrations without the toxic side effects associated with the use of synthetic equivalents (Prashant *et al*., 2005).

Foxtail millet is highly nutritious, non-glutinous, and like buckwheat and quinoa, is not an acid forming food so it is soothing and easy to digest. It is considered to be one of the least allergenic and most digestible grains available and it is a warming grain that helps to heat the body in cold or rainy seasons and climates (FAO, 2008; Kamara *et al*., 2009). Foxtail millet is tasty, with a mildly sweet, nut-like flavor and contains a myriad of beneficial nutrients. It is nearly 15% protein, contains moderate amounts of

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fiber, B-complex vitamins including niacin, thiamin, and riboflavin, the essential amino acid methionine, lecithin, and some vitamin E. It is particularly high in the minerals iron, magnesium, phosphorous, and potassium. The seeds are also rich in photochemicals, including phytic acid, which is believed to lower cholesterol, and phytate, that is associated with reduced cancer risk (FAO, 2008).

Research has been performed to develop techniques for converting protein hydrolysates into more useful products. However, researchers have not reported the occurrence of antioxidative peptides in millet protein hydrolysates. In the present study, the antioxidant activities of foxtail millet hydrolysates obtained with Alcalase 2.4L enzyme and fractioneted using Sephadex G-25 column chromatography was investigated by various assays, including the antioxidative, reducing activity and the scavenging effect on free radicals of the fractions. Finally, amino acid composition and molecular weight distribution of the fractionated hydrolysates were also investigated.

Materials and Methods

Materials

Foxtail millet was purchased from a local market in Wuxi, P. R. China. The seeds were milled using a laboratory scale hammer miller and the resulting flour was sieved through a 60 mesh screen. The foxtail millet flour (FMF) was dispersed in hexane at flour to hexane ratio of 1:5 (w/v) and stirred for 4 h at room temperature. Hexane extraction was performed twice. The hexane was decanted and the defatted foxtail millet flour (DFMF) was air dry for 24 h under a vacuum drier and stored at 5°C in sealed glass jars until used.

Protein hydrolysate was made and evaluated using a food grade enzyme (IKamara *et al*., 2010). The enzyme tested (Novo Nordisk's Enzyme Business in Beijing, China) was Alcalase 2.4L endonuclease from *Bacillus subtilis* with specific activity of 2.4 AU/g. All chemicals used in the experiments were of analytical grade.

Protein hydrolysis

DFMF was hydrolyzed with Alcalase 2.4L under conditions offering optimum hydrolysis (pH 8.0 and temperature 50°C). One hundred grams of DFMF was weighed into a vessel immersed in a water bath maintained at the above temperature and 700 mL of distilled water was added to make a suspension and was adjusted to pH 8.0 and preheated to 50°C; then enzyme (1% in reaction mix) was added with continuous stirring. Hydrolysis was carried out for 3 h. The hydrolysates was centrifuged at 11500 × g for 10 min at 4oC with a D-3756 Osterode am Harz model 4515 centrifuge (Sigma, Germany). The supernatant was carefully decanted and immediately heated for 5 min in a boiling water bath to inactivate the enzymes. Heat inactivation followed centrifugation to prevent gelatinization (Nkonge and Ballance, 1984). The defatted foxtail millet hydrolysate was lyophilized and stored at -20°C until used. All the experiments were performed in triplicate and the results are the average of three values

Gel filtration chromatography

Gel filtration chromatography was carried out on column (1.0×100 cm) packed with Sephadex gel, type G-25. A sample volume of 3.0 mL (10 mg of protein/mL solution) was applied to the column at room temperature and the elution carried out with 0.05 M phosphate buffer, pH 6.9, containing 0.15 M Nacl and run at a constant flow rate of 0.49 mL/min and the fraction collected in each test tube after 5 min was 2.45 mL.

Amino acid analysis

The dried samples were digested with HCl (6 M) at 110°C for 24 h under nitrogen atmosphere. Reversed phase high performance liquid chromatography (RP-HPLC) analysis was carried out in an Agilent 1100 (Agilent Technologies, Palo Alto, CA, USA) assembly system after precolumn derivatization with o-phthaldialdehyde (OPA). Each sample (1 µL) was injected on a Zorbax 80 A C18 column (i.d. 4.6 × 180 mm, Agilent Technologies, Palo Alto, CA, USA) at 40oC with detection at 338 nm. Mobile phase A was 7.35 mM/L sodium acetate/ triethylamine/ tetrahydrofuran (500:0.12:2.5, v/v/v), adjusted to pH 7.2 with acetic acid, while mobile phase B (pH 7.2) was 7.35 mM/L sodium acetate/methanol/acetonitrile (1:2:2, v/v/v). The amino acid composition was expressed as g of amino acid per 100 g of protein.

Determination of molecular weight (M_w)

Molecular weight distributions were determined by gel permeation chromatography (GPC) by using a high-performance liquid chromatography (HPLC) system (Waters 600, USA). A TSK gel 2000 SWXL column (7.8 i.d. × 300 mm, Tosoh, Tokyo, Japan) was equilibrated with 45% acetonitrile (v/v) in the presence of 0.1% trifluoroacetic acid. The hydrolysates (100 µg/µL) were applied to the column and eluted at a flow rate of 0.5 mL/min and monitored at 220 nm at room temperature. A molecular weight calibration

curve was prepared from the average retention time of the following standards obtained from (Sigma, Germany: cytochrome C (12500 Da), aprotinin (6500 Da), bacitracin (1450 Da), and tripeptide GGG.

Inhibition of linoleic acid autoxidation

The antioxidant activities of DFMPH fractions were measured based on the method of Osawa and Namiki, 1986 with some modifications. One milligram of sample was dissolved in 1.5 mL of 0.1 M phosphate buffer (pH 7.0), and added to 1 mL of linoleic acid (50 mM) dissolved with ethanol (99.5%) in a glass test tube which was sealed tightly with silicon rubber cap and kept at 60°C in the dark for 8 days. The degree of oxidation was evaluated by measuring the ferric thiocyanate values according to the method described by Mitsuta *et al*. (1966). The sample solution (100 µL) incubated in the linoleic acid model system described above was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 30% (w/v) ammonium thiocyanate, and 0.1 mL of 0.02 M ferrous chloride dissolved in 1 M HCl. After 3 min, the degree of color development, which represents linoleic acid oxidation, was measured at 500 nm. The antioxidant activities of butylated hydroxytoluene (BHT) and α-tocopherol were also assayed at the same concentration for comparison purposes. The inhibition percent of linoleic acid peroxidation was calculated as:

$$\text{Inhibition (\%)} = \left(\frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100 \quad (1)$$

DPPH radical scavenging activity assay

The method described by Wu *et al*. (2003), with a slight modification, was used to measure the DPPH radical scavenging activity. The sample fractions (FI, FII, FII and FIV) were dissolved in distilled water to obtain a concentration of 40 mg of protein/mL. Then 2.0 mL of sample was mixed with 2.0 mL of 0.15 mM DPPH that was dissolved in 95% ethanol. The mixture was then shaken vigorously using a mixer (QT-1 Mixer, Tianchen Technological Co. Ltd. Shanghai, China) and kept in the dark for 50-60 min. The absorbance of the resultant solution was recorded at 517 nm. The scavenging activity was calculated using the following equation:

$$\text{DPPH (\%)} = \left(\frac{\text{absorbance of DPPH} - \text{absorbance of sample}}{\text{absorbance of DPPH blank}}\right) \times 100 \quad (2)$$

Where the DPPH blank is the value of 2 mL of 95% ethanol mixed with DPPH solution, the DPPH sample is the value of 2 mL of sample solution mixed with DPPH solution, and the control sample is the value of 2 mL of sample solution mixed with 2 mL of 95% ethanol.

ABTS radical scavenging activity assay

ABTS radical scavenging activity of DFMPH fractions was determined using the method described by Re *et al*. (1999), with slight modification. A stock solution of ABTS radicals was prepared by mixing 5.0 mL of 7 mM ABTS solution with 88 µL of 140 mM potassium persulfate, and kept in the dark at room temperature for 12-16 hours. An aliquot of stock solution was diluted with phosphate buffer, PB (5 mM, pH 7.4) containing 0.15 M NaCl in order to prepare the working solution of ABTS radicals to an absorbance of 0.70 ± 0.02 at 734 nm. A 65 µL aliquot of the fractions dissolved in the same phosphate buffer (66.67 µg/mL final assay concentration) or only buffer (for the control) was mixed with 910 µL of ABTS radical working solution, incubated for 10 min at room temperature in the dark, and then absorbance was measured at 734 nm. The percent reduction of ABTS+ to ABTS was calculated according to the following equation:

$$\text{ABTS (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100 \quad (3)$$

Measurement of iron (II) chelating activity assay

The metal-chelating activity of the hydrolyzed fractions was assessed using the method of Dinis *et al*. (1994), with slight modification. 1 mL of peptide solution (5 mg/mL) was first mixed with 3.7 mL of distilled water. Then it was reacted with a solution containing 0.1 mL 2 mM $FeCl_2$ and 0.2 mL of 5 mM Ferrozine. After 10 min, the absorbance of the reaction mixture was measured at 562 nm. The metal-chelating ability of the hydrolyzed fractions was calculated as a percentage using the equation;

$$\text{Metal-chelating ability (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100 \quad (4)$$

Reducing power

The reducing power of the hydrolyzed fractions was measured according to the method of Duh *et a*l. (1998), with slight modification. Sample (0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg) was added to 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Then 2.5 mL of 10% trichloroacetic acid (TCA) was added to the reaction mixture. A volume of 2.5 mL from each incubated mixture was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1%

ferric chloride in a test tube. After a 10 min reaction time, the absorbance of the resulting solution was measured at 700 nm. Triplicate tests were conducted for each sample. Increased absorbance of the reaction mixture indicated increased reducing power. All the experiments were performed in triplicate and the results are the average of three values.

Statistical analysis

An analysis of variance (ANOVA) of the data was performed and statistical significance of the differences in mean values were evaluated by Duncan test at $P < 0.05$ using SPSS version 18.0 (SPSS Inc, Chicago, IL, USA).

Results and Discussions

DFMPH Fractionation

DFMPH was fractionated by gel filtration column chromatography on Sephadex G-25, four fractions were obtained (FI, FII, FIII and FIV), as shown in Figure 1. The four fractions were lyophilized and stored at -20°C until used.

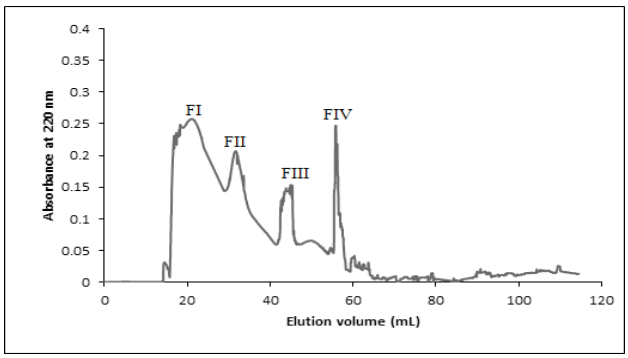

Figure 1. Gel filtration chromatographic profiles of DFMPH using Sephadex G-25

Amino acid composition

The fractions obtained were subjected to amino acid composition analyses in order to determine the possible effect of the amino acid profile on antioxidant activity. The fractions showed remarkable differences in their content of hydrophobic amino acids, Table 1. However, the total hydrophobic amino acids content in FIV was higher (51.93%) than FI, FII and FIII (51.02%, 40.13% and 48.37%) respectively. The amino acid compositions among all fractions revealed that they are rich in serine, proline, arginine, phenylalanine, leucine, alanine, aspartic acid and glutamic acid with a significant difference ($P < 0.05$). An increase in hydrophobicity will increase the solubility in lipid and therefore enhance their antioxidant activity (Rajapakse *et al*., 2005; Amadou *et al*., 2010; Zhang *et al*., 2009). Calculation of hydrophobicity was done according to Bigelow (1967). The result revealed that the hydrophobicity exhibited by FIV (8.62 kJ/moL amino acid residue "AAR") was higher than FI, FII and FIII (8.29, 6.38 and 6.73 kJ/moL AAR) respectively, (Table 1). Furthermore, the highest antioxidant and free radicals scavenging activities were apparently due to the high total amino acid content and thus the highest hydrophobicity. Peptides, derived from many protein sources with increased hydrophobicity have been reported to be related with antioxidative properties (Chen *et al*., 1998; Wu *et al*., 2003).

Table 1. Amino acid composition of DFMPH fractions (g/100 g protein)

Amino Acid (AA)	FI	FII	FIII	FIV
Essential AA				
Isoleucine (Ile)	4.69 ± 0.32b	3.87 ± 0.33a	3.73 ± 0.16a	4.04 ± 0.10a
Leucine (Leu)	11.76 ± 0.38b	10.70 ± 0.25a	14.52 ± 0.62c	14.67 ± 0.29c
Lysine (lys)	0.9 ± 0.08b	0.98 ± 0.35a	0.91 ± 0.13a	0.95 ± 0.13a
Methionine (Met)	2.46 ± 0.24a	3.55 ± 0.27b	3.59 ± 0.16b	3.42 ± 0.19b
Phenylalanine (Phe)	5.65 ± 0.31b	2.86 ± 0.14a	5.10 ± 0.47b	5.54 ± 0.53b
Threonine (Thr)	3.36 ± 0.38a	3.72 ± 0.46a	3.32 ± 0.57a	3.38 ± 0.40a
Valine (Val)	4.79 ± 0.29c	2.96 ± 0.24a	3.71 ± 0.39b	3.74 ± 0.38b
Histidine (His)	2.62 ± 0.72a	5.43 ± 0.78b	2.63 ± 0.61a	2.66 ± 0.46a
Tryptophan (Trp)	3.80 ± 0.73b	1.88 ± 0.65a	2.68 ± 0.37a	2.47 ± 0.30a
Non-Essential AA				
Alanine (Ala)	8.03 ± 0.61a	9.60 ± 0.42a	8.26 ± 0.80a	8.05 ± 1.31b
Arginine (Arg)	10.69 ± 1.04a	9.66 ± 0.55a	11.04 ± 0.76a	13.14 ± 0.55a
Aspartic acid (Asp)	6.25 ± 0.84a	7.36 ±1.22a	6.71 ± 0.92a	5.99 ± 0.84a
Cysteine (Cys)	1.10 ± 0.60a	0.88 ± 0.10a	0.80 ± 0.14a	0.79 ± 0.14a
Glutamic Acid(Glu)	23.28 ± 1.35a	27.19±1.61c	26.13±1.42bc	24.33 ± 0.98ab
Glycine (Gly)	2.53 ± 0.53a	3.06 ± 1.04a	2.82 ± 0.91a	2.68 ± 0.58a
Serine (Ser)	6.94 ± 0.80a	6.78 ± 0.77a	8.07 ± 0.67a	7.35 ± 0.45a
Tyrosine (Tyr)	2.93 ± 0.29b	1.55 ± 0.44a	2.59 ± 0.34b	2.73 ± 0.34b
Proline (Pro)	10.71 ± 0.45c	5.04 ± 0.75a	6.90 ± 1.02b	11.01 ± 0.55c
THAA	48.36 ± 2.02bc	38.62±0.84a	45.81± 1.73b	50.74 ± 2.01c
Hϕ (kJ/mol AAR)	8.29 ± 0.73b	6.38 ± 0.68a	6.73 ± 0.60a	8.62 ± 0.64b

The data are means and standard deviations of triplicate. Column with different letters indicate statistical differences ($P < 0.05$). THAA: Total Hydrophobic Amino Acid (Alanine, Isoleucine, Leucine, Methionine, Phenylalanine, Proline, Tyrosine and Valine). Hϕ (kJ/moL AAR) Hydrophobic Amino Acid Residue.

Molecular weight distribution

Gel permeation chromatography (GPC) using an HPLC system was used to determine the molecular weight distribution profiles of the DFMPH fractions. Figure 2 shows the molecular size distribution profiles of the fractions. The chromatographic data indicated that the various fractions were composed of lower molecular weight peptides whose peaks ranged from 77–1042 Da (Figure 2). In this study, results revealed that fraction FIV had the lowest molecular weight distribution with peaks mainly located at 99–420 Da (73.82%), 420–1040 Da (18.25%) and 1040–1040 Da (6.94%), this is probably associated with higher antioxidant activity. These findings are in agreement

with observations from other studies and support the fact that functional properties of antioxidative peptides are highly influenced by molecular weight distribution (Wang *et al*., 2007; Kim *et al*., 2007).

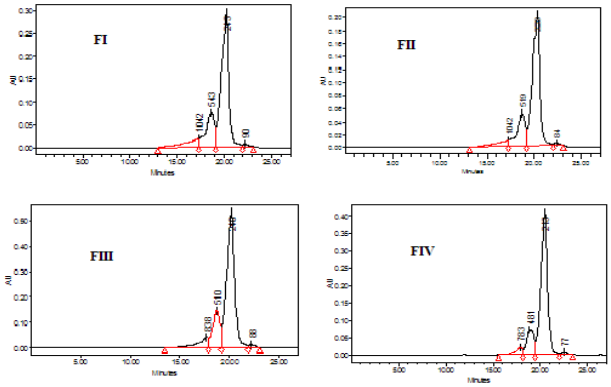

Figure 2. Molecular weight distribution of DFMPH fractions

Inhibition of linoleic acid autoxidation

Lipid peroxidation is thought to proceed via radical mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids (Palakawong *et al*., 2010). Peroxidation of fatty acids can cause deleterious effects in foods by forming complex mixture of secondary breakdown products of lipid peroxides. Intake of these foods can cause a number of adverse effects (Pérez *et al*., 2007; Wang *et al*., 2007) including biotoxicity. Therefore, the DFMPH fractions were further characterized for their antioxidant activity by assessing their ability to protect linoleic acid against oxidation. The antioxidant activity of the DFMPH fractions were investigated in a linoleic acid autoxidation system and compared with that of α-tocopherol and BHT. As shown in Figure 3, the oxidation of linoleic acid was markedly inhibited by the addition of DFMPH fractions. Among the four fractions, the highest antioxidant activity was found in FIV (85.71%), which exhibited a significant inhibition of linoleic acid peroxidation closer to that of α-tocopherol (86.27%) but lower than that of BHT (92.44%) (Figure 3). Previous studies have demonstrated that gel filtration chromatography could be an effective technique for separation and purification thus to enhance the bioavailability of peptides (Zhang *et al*., 2009; Amadou *et al*., 2010).

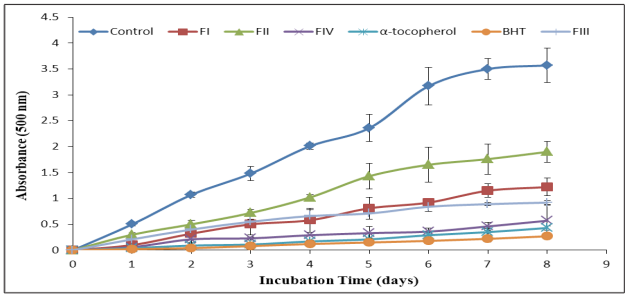

Figure 3. Lipid peroxidation measured in linoleic acid model system for 8 days in the presence of different DFMPH fractions. BHT and α-tocopherol were used as positive controls. Vertical bars indicate mean values ± SD

DPPH radical-scavenging activity assay

The DPPH free radicals, which are stable in ethanol, show maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donating substance such as an antioxidant, the radicals are scavenged and the absorbance is reduced. Thus, the DPPH radicals were widely used to investigate the scavenging activity of some natural compounds. DPPH is widely used to evaluate the antioxidant activity of natural compounds (Udenigwe *et al*., 2009). However, DPPH's scavenging activity indicates the ability of the antioxidant compound to donate electrons or hydrogen, thereby converting the radical to a more stable species (Bougatef *et al*., 2009. These antioxidants donate hydrogen to free radicals, leading to non-toxic species and therefore the inhibition of the propagation phase of lipid oxidation. Results shown in Table 2 revealed that FIV at 1.0 mg/mL exhibits the highest DPPH radical-scavenging activity (78.1%), which is similar to proteins fraction isolated from wheat gluten hydrolysate (Wang *et al*., 2007). However, fractions FI, FII and FIII showed considerable DPPH radical-scavenging activities with a significant difference ($P < 0.05$). The DFMPH fractions possibly contained some substrates, which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

Table 2. Free radicals scavenging effects of DFMPH fractions

Fractions	Antioxidant activity (%)		
	DPPH Radical[a]	ABTS[b]	Fe^{2+} Chelating[c]
FI	72.2 ± 0.39c	62.04 ± 0.15b	52.92 ± 0.34c
FII	46.95 ± 1.19a	57.04 ± 1.68a	34.24 ± 0.41a
FIII	61.09 ± 0.75b	74.6 ± 0.66c	51.16 ± 0.54b
FIV	84.1 ± 0.71d	83.21 ± 0.21d	66.38 ± 0.70d

[a]Scavenging effects were tested at 1.0 mg/mL, [b]ABTS were tested at 66.67 µg/mL and [c]Chelating activity were tested at 5 mg/mL. The data are means and standard deviations of triplicate. Rows with different letters indicate statistical differences ($P < 0.05$).

ABTS radical scavenging activity assay

ABTS radical scavenging is a method widely used for the screening of antioxidant activity and is reported as a decolorization assay applicable to both lipophilic and hydrophilic compounds (Miliauskasa *et a*l., 2004). However, reduction of the color indicated reduction of ABTS radical (O'Sullivan *et al*., 2011). The result of the different fractions exhibited good ABTS radical scavenging ability with a significant difference ($P < 0.05$). Table 2. From the result, FIV exhibited the highest ABTS radical scavenging ability (79.21%), followed by FIII (69.36%), FI (62.04%) and FII (57.18%) respectively, (Table 2). However, the antioxidative scavenging quality is closely related to the amino acid composition and lower molecular weight. These results indicated that FIV had a notable effect in scavenging for ABTS.

Metal-chelating activity assay

The chelating of ferrous ions by DFMPH fractions were estimated by the method of (Dinis *et al*., 1994). Ferrozine can quantitatively form complexes with Fe^{2+} ion. In the presence of chelating agents, the complex formation is disrupted resulting to a decrease in the red colour of the complex. Measurement of colour reduction makes possible the estimation of the metal chelating activity (Jun *et al*., 2004). A sample concentration of 5 mg/mL was used to determine the metal chelating properties of DFMPH fractions. The results in Table 2, show that samples from the various fractions interacted with iron. However, their chelating activities showed a significant difference ($P < 0.05$). Fraction FIV exhibited a higher chelating activity (66.38%) than FI, FII and FIII being 52.92%, 34.24% and 51.16%, respectively. Fraction FIV is a potential source of natural antioxidant.

Reducing power

Reducing power has been used to measure the potential antioxidant activities of bioactive compounds in different products, including peptides (Mitsuda *et al*., 1966; Amadou *et al*., 2010). In this assay, the presence of antioxidants caused the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form, and the yellow color of the test solution changed to various shades of green and blue depending on the reducing power of each compound. The Fe^{2+} was then monitored by measuring the formation of Perl's Prussian blue at 700 nm. Different studies have indicated that antioxidant activity and reducing power are related (Duh, 1998; Huda-Faujan *et al*., 2007). The reducing power of DFMPH fractions as a function of their concentration is shown in Figure 4. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration (Ferreira *et al*., 2007). The highest reducing power was found in FIV, followed by FIII, FII and FI respectively, (Figure 4). The reducing power of DFMPH fractions increased with increasing their concentrations. A similar observation has been reported by Huda-Faujan *et al*., 2007).

Conclusion

Different DFMPH fractions obtained by gel filtration exhibited different antioxidant and free radicals scavenging activities. The fractions can act as a hydrogen donor, a water-soluble free radical quencher, and a transitional metal ion sequesterant. Compared with the ABTS method, DPPH assay was a far more analytical in determining the radical scavenging ability of water-soluble peptides. The results showed that FIV had the highest antioxidant and free radicals scavenging activities. The molecular weight distribution of peptides from FIV revealed that its peaks were mainly located at 99–420 Da (73.82%), 420–1040 Da (18.25%) and 1040–1040 (6.94%). However, the lower molecular weight and the amino acid composition of DFMPH fractions were found to be strongly correlated with their antioxidant activity. The protein fractions derived from DFMPH are potential natural antioxidants in prevention of lipid peroxidation of polyunsaturated oils.

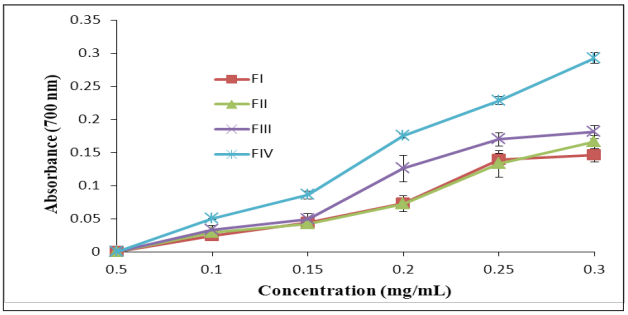

Figure 4. Reducing power of DFMPH fractions used at different concentrations

Acknowledgments

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