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Isolation and identification of antioxidative peptides from rice endosperm protein enzymatic hydrolysate by consecutive chromatography and MALDI-TOF/TOF MS/MS

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ABSTRACT

The defatted rice endosperm protein (REP) was, respectively, digested by five different protease treatments (Alcalase, Chymotrypsin, Neutrase, Papain and Flavorase), and Neutrase appears to be the most desirable for producing high quality antioxidant peptides from REP. Specially, the DPPH and hydroxyl radical scavenging activities of NHREP were higher than its superoxide radical scavenging activity, and the percentage inhibition of autooxidation of NHREP (80.09%) was similar to that of α-tocopherol (86.59%) on day 5. Furthermore, NHREP was purified consecutively, and the antioxidant peptides were identified to be Phe-Arg-Asp-Glu-His-Lys-Lys (FRDEHKK, 959.5 Da) and Lys-His-Asp-Arg-Gly-Asp-Glu-Phe (1002.5 Da) by MALDI-TOF/TOF MS/MS. Lastly, FRDEHKK was chemically synthesised. It significantly inhibited lipid peroxidation in an linoleic acid emulsion system more effectively than α-tocopherol, and enhanced the viability of *t*-BHP induced cytotoxicity up to 74.38% (for MRC-5) and 78.39% (for RAW264.7) at 80 μg/ml. Conclusively, it was feasible to produce natural antioxidants from REP.

1. Introduction

The formation of free radicals such as superoxide ($O_2^{-\cdot}$) and hydroxyl (·OH) is an unavoidable consequence in aerobic organisms during respiration. These radicals are very unstable and react rapidly with other groups or substances in the body, leading to cell or tissue injury (Ames, Shigena, & Hagen, 1993). On the other hand, oxidation also directly affects food quality, commonly associated with changes of food flavour and texture, so antioxidants are also important to the food industry. An antioxidant is defined as any substance that significantly delays or inhibits oxidation of a substrate when present at low concentrations compared to that of an oxidisable substrate (Ames et al., 1993; Horton, 2003; Lewis, 1993). Synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ), are used under strict regulation because of their toxic effects on human's enzyme systems (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Hatate, Nagata, & Kochi, 1990).

In contrast, the natural antioxidants have attracted increasing interests because of their safety and wide distribution properties in recent years (Hatate et al., 1990; Lewis, 1993; Mitsuta, Yasumoto, & Iwami, 1996). For instance, antioxidative activity has been seen in the hydrolysates of soy protein (Chen et al., 1996; Pena-Ramos & Xiong, 2002), yellowfin sole frame protein (Jun, Park, Jung, & Kim, 2004), bovine serum albumin (Hatate et al., 1990), egg-yolk protein (Park, Jung, Nam, Shahidi, & Kim, 2001), porcine myofibrillar proteins (Saiga, Tanabe, & Nishimura, 2003), hydrolysed fermented mussel sauce (Rajapakse, Mendis, Jung, Je, & Kim, 2005), porcine collagen hydrolysate (Li, Chen, Wang, Ji, & Wu, 2007), Alaska pollack frame protein (Je, Park, & Kim, 2005), whey proteins (Bayram, Pekmez, Arda, & Yalcin, 2008), chickpea protein hydrolysate (Li, Jiang, Zhang, Mu, & Liu, 2008), wheat gluten (Wang, Zhao, Zhao, & Jiang, 2007), mackerel hydrolysates (Wu, Chen, & Shiau, 2003), enzymatic hydrolysates of squid skin collagen (Nam, You, & Kim, 2008), pea protein hydrolysates (Humiski & Aluko, 2007), mackerel muscle protein (Yin, Tong, & Jiang, 2005), conger eel muscle protein (Ranathunga, Rajapakse, & Kim, 2006), grass carp muscle protein (Ren, Zhao, Shi, Wang, & Jiang, 2008) and rapeseed peptides (Zhang, Wang, & Xu, 2008).

The antioxidant activities of peptides are closely related to their amino acid constituents and their sequences. Peptides with one or more residues of Val, Leu, Ile, Tyr, Trp, His, Met, Gly and Phe show stronger antioxidant activities (Rajapakse et al., 2005). It is

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believed that the antioxidant activity was attributed to hydrogen-donating ability, lipid peroxyl radical trapping, or the metal ion-chelating ability of the imidazole group (Je et al., 2005; Nam et al., 2008; Ranathunga et al., 2006). However, further research is needed to clarify the structure–function relationship of antioxidant peptides.

Matrix-assisted laser desorption/ionisation coupled with time-of-flight tandem mass spectrometer analysis (MALDI-TOF/TOF MS/MS) has been used for determining isotopic exchange rates in specific regions of proteins (Li et al., 2007). It has undisputed advantages in the areas of sensitivity, protein solubility and molecular mass. The molecular mass and amino acids sequence were identified by the MALDI-TOF/TOF MS/MS in this paper.

Rice is one of the most main diets for human nutrients and energy in China. Rice endosperm protein (REP) is hypoallergenic and contains a good quantity of lysine, whose quality surpasses that of wheat and corn (Qureshi, Sami, Salser, & Khan, 2001). In China, with the expansion of rice starch production, REP (approximately 60–85% of by-products in the process of the rice starch) is available in large amounts and at low cost. If this protein by-product is recovered and utilised, it can represent a significant economic and social benefit.

Several studies have been performed to identify the potential antioxidative activity of the polyphenol extraction of rice bran (Kim, Kim, Kim, Kim, & Suh, 2008). However, to our knowledge, there has not yet been any research on antioxidative peptides from rice endosperm protein. Our objective was to determine whether the defatted rice endosperm protein may represent a source of antioxidative peptides upon hydrolysis. The antioxidant activities were evaluated by the scavenging effects on free radicals and the ability to inhibit lipid peroxidation in a liposome model system. Furthermore, the purification and identification of the antioxidative peptides was determined using consecutive chromatography and MALDI-TOF/TOF MS/MS.

2. Materials and methods

2.1. Materials

The rice endosperm protein (REP) was supplied by Hangzhou Westlake MSG Group Company Ltd. (Hangzhou, China). Alcalase 2.4 L (EC 3.4.21.62, from *Bacillus licheniformis,* 2.4 AU/g), and Chymotrypsin were donated by Novo Nordisk (Bagsvaerd, Denmark). Papain, Neutrase, and Flavorase were donated by Genetic biochemical products Ltd. (Wuxi, China). SP-Sephadex C-25, and Sephadex G-15 were purchased from Sigma Chemical Co. (St. Louis, USA). Linoleic acid, α-tocopherol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals and reagents used in the experiments were of analytical grade.

2.2. Preparation and hydrolysis of the rice endosperm protein (REP)

The rice endosperm protein (REP) was defatted using hexane for 8 h according to the AOAC standard procedure (AOAC, 1990), then freeze-dried (protein content of 65.39 ± 1.68%, measured by the Kjeldahl method). In succession, REP was hydrolysed with five different enzymes (Alcalase, Chymotrypsin, Neutrase, Papain, and Flavorase) based on their optimum hydrolysis conditions, respectively (Table 1). First, the defatted REP was dissolved in distilled water as a 5% (w/v) solution, the suspension was adjusted to the appropriate temperature and pH. Then the reaction was initiated by adding each of the five different enzymes, continued for 4 h and the pH of the mixture was kept constant by continuously adding 0.1 M NaOH solution. After the reaction, the mixture was heated to 85 °C for 10 min to inactivate the enzyme and centrifuged at 3500*g* for 10 min at 4 °C. The supernatant was freeze-dried and stored in the desiccator.

2.3. Determination of the degree of hydrolysis (DH)

The degree of hydrolysis (DH, %), defined as the per cent ratio of the number of peptide bonds broken (h) to the total number of bonds per unit weight (h_{tot}), was calculated from the amount of the base consumed by pH-stat assay (Adler-Nissen, 1986), The DH was calculated by the following equation:

$$\mathrm{DH} = \frac{h}{h_{\mathrm{tot}}} = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{M_p} \times \frac{1}{h_{\mathrm{tot}}} \times 100\% \quad (1)$$

where B is the base consumption in ml; N_b is the normality of the base; α is the average degree of dissociation of the α-NH_2 groups; M_p is the mass of protein ($N \times 5.95$) in g; h is the hydrolysis equivalents in meqv/g protein, and h_{tot} is the total number of peptide bonds in the protein substrate (9.20 meqv/g rice protein).

2.4. Measurements of the antioxidant activities

2.4.1. Scavenging effect on DPPH radical of the enzymatic hydrolysate from rice endosperm protein (EHREP)

The DPPH free radical, which is stable in ethanol, shows a maximum absorbance at 517 nm. When the DPPH radical encounters a proton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance is reduced. This test system is a simple method to measure the loss of absorbance that occurs when the DPPH radical is reduced by an antioxidant (Chen et al., 1996; Mitsuta et al., 1996). The DPPH free radical scavenging activity was measured following the method described by Chen

Table 1
Degree of hydrolysis (DH) and free radicals scavenging effects of the EHREPs.

Proteases	Optimal hydrolysis conditions		DH (%)	Free radicals scavenging effects of hydrolysate (%)		
	Temperature (°C)	pH		DPPH radical[a]	Hydroxyl radical[b]	Superoxide radical[c]
Papain	55	6.50	5.86 ± 0.47	44.31 ± 2.12	34.82 ± 1.26	33.05 ± 2.01
Chymotrypsin	45	7.50	23.21 ± 0.28	35.26 ± 1.34	44.74 ± 1.76	55.03 ± 1.54
Flavorase	50	6.00	2.01 ± 0.30	24.31 ± 1.66	28.42 ± 1.31	15.03 ± 1.92
Neutrase	37	7.00	14.98 ± 0.71	85.86 ± 1.33	82.93 ± 1.21	75.69 ± 1.23
Alcalase	50	8.00	11.25 ± 0.58	71.26 ± 1.06	57.06 ± 1.48	64.71 ± 2.17
α-Tocopherol	–	–	–	97.56.±1.02	98.01 ± 1.29	95.26 ± 2.01

Other hydrolysis conditions of each enzyme were same as 5% substrate concentration, the ratio of *E*/*S* of 2:100, 4 h of hydrolysis time. Every experiment was carried out in triplicate. Data were expressed as means ± standard errors.

[a] Scavenging effects were tested at 1.5 mg/ml.

[b] Scavenging effects were tested at 2.0 mg/ml.

[c] Scavenging effects were tested at 2.0 mg/ml.

et al. (1996). The dried hydrolysates were dissolved in 4 ml distilled water to form the sample solutions with various concentrations of 0%, 0.04%, 0.08%, 0.12%, 0.16%, 0.2% and 0.24% (w/v). One microlitre of 40 μM DPPH radical in ethanol was added to the sample, followed by the measurement at 517 nm after 30 min of incubation at 25 °C. α-Tocopherol was used as positive controls for this test. All measurements were made in triplicate and averaged. The abilities to scavenge the DPPH radicals were calculated according to the following equation:

$$\text{SE}\ (\%) = \left(1 - \frac{A_S}{A_C}\right) \times 100 \quad (2)$$

where SE means the scavenging effect, A_S is the absorbance of the sample at 517 nm, A_C is the absorbance of the control at 517 nm, the sample is composed of 4 ml of sample solution mixed with 1 ml of ethanol including 40 μM DPPH, and the control is composed of 4 ml of distilled water mixed with 1 ml of ethanol including 40 μM DPPH.

EC_{50} (meaning the medium effective concentration that causes a decrease in the initial radical concentration by 50%) is a parameter widely used to measure the antiradical efficiency. The lower the EC_{50} is, the higher the free radical scavenging ability is.

2.4.2. Superoxide radical scavenging activity of EHREP

The superoxide radical scavenging activity of EHREP was determined by the nitroblue tetrazolium reduction method (Zhang et al., 2008). One microlitre of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468 μM NADH in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of EHREP (0–6 mg/ml) was mixed. The reaction was started by adding 100 μl of phenazine methosulphate solution (PMS) (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. All measurements were made in triplicate and averaged. The abilities to scavenge the superoxide radical were calculated using the following equation:

$$\text{radical scavenging activity}\ (\%) = \left(1 - \frac{A_s}{A_c}\right) \times 100 \quad (3)$$

where A_s is the absorbance of the sample (EHREP at 1–6 mg/ml) at 560 nm and A_c is the absorbance of the control (EHREP at 0 mg/ml) at 560 nm.

2.4.3. Hydroxyl radical scavenging activity of EHREP

The hydroxyl radical scavenging effects of EHREP were assayed using the method of Zhang et al. (2008). The reagents were added to a test tube in the following order: 0.4 ml KH_2PO_4–KOH buffer (pH 7.5), 0.1 ml sample solution with various concentrations (0–6 mg/ml), and 0.1 ml of 1 mM EDTA, 10 mM H_2O_2, 60 mM 2-deoxy-D-ribose, 2 mM ascorbic acid, and 1 mM $FeCl_3$ (0.1 ml distilled water in place of the $FeCl_3$ solution as control). The reaction solution was incubated at 37 °C for 1 h. Next, 1 ml of 20% TCA was added to stop the reaction. The colour was developed by addition of 1 ml of 1% TBA into the reaction tubes, which were placed in boiling water for 15 min. The tubes were cooled to room temperature and then the absorbance was read at 532 nm. At each concentration of the hydrolysates from one batch, determinations were carried out in triplicate. The scavenging effects were calculated according to Eq. (3).

2.4.4. Test of inhibition of linoleic acid autoxidation

The antioxidative activities of NHREP and the synthetic antioxidant peptide were respectively measured in a linoleic acid model system according to the methods of Chen et al. (1996) with a few modifications. The sample (100 mg) was dissolved in 100 ml 50 mM phosphate buffer (pH 7.0), and added to a solution of 1.5 ml linoleic acid and 100 ml 99.5% ethanol. Then, the total volume was adjusted to 250 ml with distilled water. The mixture was incubated in a conical flask with a screw cap at 40 ± 1 °C in a dark room, and the degree of oxidation was evaluated by measuring the ferric thiocyanate values.

The ferric thiocyanate value was measured according to the method of Mitsuta et al. (1996). The reaction solution (25 μl) incubated in the linoleic acid model system described herein was mixed with 1.175 ml 75% ethanol, 0.025 ml 30% ammonium thiocyanate, and 0.025 ml 0.02 mol/l ferrous chloride solution in 3.5% HCl. After 3 min, the thiocyanate value was measured by reading the absorbance at 500 nm following colour development with $FeCl_2$ and thiocyanate at different intervals during the incubation period at 40 ± 1 °C. The antioxidant activities of BHT and α-tocopherol were also assayed at the same concentration for comparison purposes.

2.4.5. Cell culture and viability determination

Human embryonic lung fibroblasts (MRC-5) and mouse macrophage (RAW264.7) cells, were grown as monolayers at 5% CO_2 and 37 °C humidified atmosphere using EMEM medium supplemented with 10% foetal bovine serum, 2 mM glutamine and 100 μg/ml penicillin–streptomycin. To determine the radical-induced cytotoxicity, cells were seeded in a 96-well plate at a concentration of 1.3×10^4 cells/well and incubated with serum-free EMEM medium until 90% of confluence. Cells were treated with various concentrations of antioxidant peptide and incubated for 10 h. Cellular oxidation was accelerated by exposing cells to 200 μM *t*-butylhydroperoxide (*t*-BHP), and after 2 h, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method that assesses the ability of succinate dehydrogenase to convert MTT into visible formazan crystals. For each well, 250 μl of MTT (0.5 mg/ml final concentration) was added and incubated (37 °C) in the dark for 1 h. The formazan crystals formed were solubilised in DMSO and the optical density was measured at 570 nm by using an Emax microplate reader (Molecular Devices, Sunnyvale, CA). Relative cell viability was determined using the amount of MTT converted into formazan salt, and data were expressed as mean percentage of viable cells compared to the respective control culture.

2.4.6. Cellular ROS determination by DCFH-DA

Intracellular formation of ROS was assessed as described previously using an oxidation sensitive dye DCFH-DA as the substrate. RAW264.7 cells growing in fluorescence microtiter 96-well plates was loaded with 20 μM DCFH-DA in HBSS and incubated in the dark for 20 min. Cells were then treated with different concentrations of peptide and incubated for another 1 h. After washing the cells with PBS for three times, 300 μM H_2O_2 was added. The formation of 2′,7′-dichlorofluorescin (DCF) due to oxidation of DCFH in the presence of various ROS was read after every 30 min at the excitation wavelength (Ex) of 485 nm and the emission wavelength (Em) of 535 nm using a GENions® fluorescence microplate reader. Following maximum rate of fluorescence increase, each well was normalised to cell numbers using MTT cell viability assay. Dose dependant and time dependant effects of treatment groups were plotted and compared with that of fluorescence intensity from control and blank groups.

2.5. Purification of the antioxidative peptides from the Neutrase hydrolysate from rice endosperm protein (NHREP)

Firstly, the freeze-dried NHREP was dissolved in 20 mM sodium acetate buffer (pH 4.0) and fractionated by ion-exchange chromatography on a SP-Sephadex C-25 column (4 × 40 cm) equilibrated (in the abovementioned sodium acetate buffer) and eluted with a

linear gradient of NaCl concentrations from 0 to 1.0 mol/l. The fractions showing antioxidative activity were pooled and freeze-dried. Secondly, the firstly collected fractions were dissolved in 50 mM sodium phosphate buffer (pH 7.0) and loaded onto a Sephadex G-15 gel filtration column (2.5 × 90 cm) which had previously been equilibrated with the same buffer. The column was then eluted with the same buffer, and the fractions exhibiting antioxidative activity were pooled and freeze-dried. Thirdly, the secondly collected antioxidative fractions were dissolved in distilled water and separated using reversed-phase HPLC on Primesphere C_{18} (10 μm, 1.0 × 25 cm; Phenomenex, Macclesfield, UK) column using a linear gradient of acetonitrile (0–50% in 40 min) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 4.0 ml/min. The elution peaks were monitored at 215 nm, and their antioxidative activities were measured by the DPPH radical scavenging activity. At last, the antioxidative peptides were pooled and freeze-dried.

2.6. Identification of the antioxidative peptides by MALDI-TOF/TOF MS/MS analysis of amino acid sequences

The collected peaks with the higher antioxidative activities were filtered through a Minisart RC_4 filter (0.45 μm) and analysed by a matrix-assisted laser desorption ionisation (MALDI) mass spectrometer equipped with a delayed extraction source and a 355 nm pulsed nitrogen laser. An instrument Bruker Reflex IV MALDI was run in the linear mode using 20 kV acceleration. The 100 times diluted sample was mixed with 1 volume of matrix solutions (20 mg/ml of sinapinic acid in acetonitrile/water, 50:50, v/v). Finally, 0.5 μl of the mixture was deposited onto the MALDI target plate. All spectra were the results of signal averaging of 200 shots. The MALDI-TOF/TOF MS/MS was run in the positive refractor mode. The peptide sequencing was performed by processing the MS/MS spectra using BioTools (Version 3.0; Bruker Daltonics Inc., Billerica, MA, USA) as well as manual calculation.

Samples with peptides that contained Lys/Gln ambiguities were analysed again after dissolving the respective preparations in acetic anhydride (2:1 methanol/acetic anhydride) which resulted in selective acetylation of the ε-amino group of Lys.

2.7. Amino acid analysis

The antioxidative peptides (100 mg) were respectively subjected to acid hydrolysis with 5 ml of 6 M HCl under nitrogen atmosphere for 24 h at 110 °C. Each hydrolysate was washed into a 50 ml volumetric flask and made up to the mark with distilled water. The amino acids were subjected to RP-HPLC analysis (Agilent 1100, USA) after precolumn derivatisation with *o*-phthaldialdehyde (OPA). Methionine and cysteine were determined separately as their oxidation products according to the performic acid procedure prior to hydrolysis in 6 M HCl. Amino acid composition was reported as g amino acid per 100 g protein.

2.8. Peptide synthesis

The antioxidant peptide was synthesised (GL Biochem (Shanghai) Ltd.) for analysis of its properties, using solid phase peptide synthesis methods. The synthesised peptide was purified by HPLC on a kromasil C18-5 column. The molecular mass of the isolated peptide was determined by chromatography coupled to mass spectrometry (LC–MS/ESI).

2.9. Statistical analysis

All the tests for antioxidant activities of hydrolysates were conducted in triplicate. Data were expressed as means ± standard errors. The statistical analysis was performed using SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). The significant difference was determined with 95% confidence interval ($P < 0.05$).

3. Results and discussion

3.1. Radicals scavenging activities assessment for the enzymatic hydrolysates of the defatted rice endosperm protein (EHREP)

The radicals scavenging activities of the five EHREPs (obtained by Alcalase, Chymotrypsin, Papain, Flavorase and Neutrase), were respectively tested. Table 1 shows that the degree of hydrolysis of REP by Neutrase (14.98%) was slightly lower than that of Chymotrypsin (23.21%), but higher than those of Alcalase, Papain and Flavorase (11.25%, 5.86% and 2.01%, respectively). All the hydrolysates resulting from various enzymes were capable of scavenging DPPH radicals. Specifically, the DPPH radical scavenging activities of all the resultant hydrolysates increased with the concentrations, and they were 85.86 ± 1.33% (Neutrase), 71.26 ± 1.06% (Alcalase), 35.26 ± 1.34% (Chymotrypsin), 24.31 ± 1.66% (Flavorase), 44.31 ± 2.12% (Papain) and 97.56 ± 1.02% (α-tocopherol) at a concentration of 1.50 mg/ml, respectively. Moreover, the EC_{50} of Neutrase hydrolysate (1.00 mg/ml) was next to that of α-tocopherol (0.08 mg/ml) (data not shown). The results indicated that the Neutrase hydrolysate from the rice endosperm protein (NHREP) took on DPPH radical scavenging activity similar to α-tocopherol, and obviously higher than other hydrolysates.

It is well known that the radical system used for the antioxidant activity evaluation may influence the experimental results, and two or more radicals systems are required to investigate the radical scavenging activities of a selected antioxidant (Li et al., 2008). Therefore, the superoxide radical ($O_2^{-\cdot}$) and hydroxyl radical ($^{\cdot}OH$) scavenging capacities of the EHREPs were also measured. As shown in Table 1, the hydroxyl and superoxide radicals scavenging activities of the NHREP were 82.93 ± 1.21% and 75.69 ± 1.23% at a concentration of 2.00 mg/ml, higher than those of hydrolysates by Alcalase, Chymotrypsin, Flavorase and Papain. These results revealed that the NHREP was hydroxyl and superoxide radical scavengers. The hydroxyl radical possessed the strongest chemical activity among the reactive oxygen species (ROS), and easily reacted with biomolecules such as amino acids, proteins, and DNA (Chen et al., 1996; Pena-Ramos & Xiong, 2002; Rajapakse et al., 2005). Therefore, the hydroxyl radical scavenging activity of the NHREP would contribute to its antioxidant activities.

From the radicals scavenging activities assessment, we deduced that NHREP possibly contained some effective antioxidative peptides, which could convert free radicals to more stable products and terminate the radical chain reaction. Moreover, the needed reaction condition of Neutrase hydrolysis was moderate (pH 7.0 and 37 °C, Table 1), which helps to decrease the processing cost. Therefore, we chose Neutrase as the optimum enzyme to hydrolyse the defatted REP for antioxidant peptides. Furtherly, we focused on the antioxidant activity of the NHREP in the linoleic acid autoxidation assays and the active constituents of the potent antioxidative peptides from the NHREP.

3.2. The antioxidant activity of the NHREP in the linoleic acid emulsion model system

Peroxidation of fatty acids can cause deleterious effects in foods by forming complex mixtures of secondary breakdown products of lipid peroxides. Lipid peroxidation proceeds *via* radical-mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids (Rajapakse et al., 2005). The inhibitory effect of the NHREP on the peroxidation of linoleic acid model system, was measured in comparison with α-tocopherol and BHT as

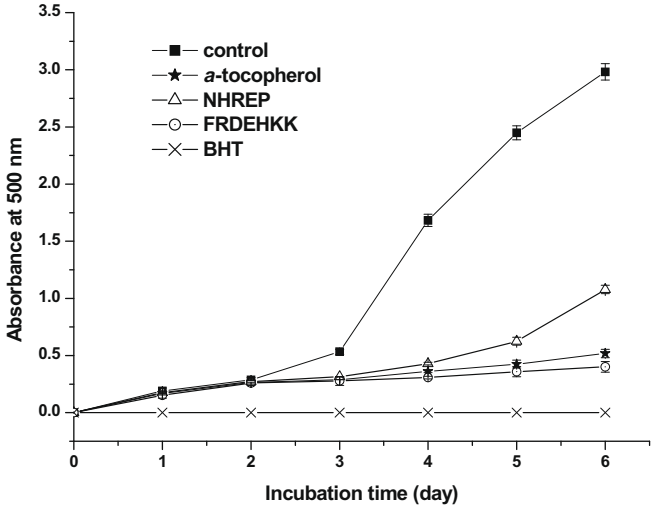

Fig. 1. Antioxidant activities of NHREP and FRDEHKK were evaluated in a linoleic acid emulsion system. Samples were incubated for 6 days. The degree of linoleic acid oxidation was measured by the ferric thiocyanate method every 24 h. BHT and α-tocopherol were used as positive controls. Each value is expressed as mean ± S.D. (n = 3). Lower absorbance at 500 nm represents higher lipid peroxidation inhibition.

positive controls (Fig. 1). As shown in Fig. 1, the autooxidation of linoleic acid without any antioxidants was increased fast from day 1 to day 6. However, NHREP, α-tocopherol and BHT all showed antioxidant activities in inhibition of linoleic acid peroxidation, and significantly prolonged the induction period of autooxidation of linoleic acid, as compared with the control ($P < 0.05$). The percentage inhibition of autooxidation in linoleic acid system of NHREP was 80.09%, similar to that of α-tocopherol (86.59%) but lower than that of BHT (99.69%) on day 5.

Natural antioxidants are less potent than synthetic antioxidants, but the latter were used with strict limits because of their very restrictive toxicological limits (Rajapakse et al., 2005). In addition, the incorporation of protein hydrolysates could confer other desirable nutritional and functional properties to foods (Li et al., 2008). From these results, we concluded that the NHREP seemed to contain some antioxidative peptides which have potential to be a substitute for the synthetic antioxidants.

3.3. Purification of antioxidant peptides from the NHREP and determination of their amino acid sequences

Since the NHREP exhibited obvious antioxidant activities, it was then separated sequentially with SP-Sephadex C-25 column, a Sephadex G-15 gel filtration column, and reversed-phase HPLC on Primesphere 10 C_{18}-HC 120. Throughout the purification process, the antioxidative activities were assessed using DPPH radical scavenging activity tests.

Fig. 2 illustrates the column chromatographic profiles of the NHREP and their DPPH radical scavenging effects. The NHREP was initially separated using ion-exchange chromatography on an SP-Sephadex C-25 column and fractionated into seven portions (A–G), with fractions A–C indicating the unbound fractions and D–G representing the bound fractions. Since the column used was a cation-exchanger, it signified that fractions A–C were neutral or weak acidic peptides and D–G were basic peptide fractions. As shown in Fig. 2a, F fraction was found to possess the strongest DPPH radical scavenging activity (81.09 ± 1.22%) among all the fractions. The lyophilised F fraction was then subjected to size exclusion chromatography on Sephadex G-15 and fractionated into four major portions, with the order of the antioxidant activities for the four fractions of F1 < F4 < F2 < F3 (Fig. 2b). Fraction F3 exhibited the strongest antioxidative activity. To identify the putative active peptides, this fraction was further separated by reversed-phase HPLC using a 0.1% TFA acetonitrile system and fractionated into F3a, F3b, F3c, F3d and F3e. The subfractions F3b and F3c possessed higher antioxidative activities (Fig. 2c). Therefore, we finally identified the amino acid sequences and the molecular weights of F3b and F3c by MALDI-TOF/TOF MS/MS.

The molecular mass of the antioxidant peptides were determined to be 959.5 Da and 1002.5 Da. The precursor ion scan mass spectrum of F3b and F3c, and the MS/MS spectrum of a single charged ion with m/z at 959.5 Da and 1002.5 Da are shown in Fig. 3. Because the Biolynx peptide sequencer failed to gain direct amino acid sequences from the MS/MS spectrum, each mass signal and corresponding fragmentation spectrum could be matched to a single peptide fragment by manual calculation (Ren et al., 2008). In fact, the manual analysis gave nine possible sequences of F3b: (a) Phe-Arg-Asp-Glu-His-Lys-Lys; (b) Phe-Arg-Asp-Glu-His-Gln-Gln; (c) Phe-Arg-Asp-Glu-His-Gln-Lys; (d) Phe-Arg-Asp-Glu-His-Lys-Gln; (e) Phe-Arg-Asp-Glu-His-Gln-Gly-Ala; (f) Phe-Arg-Asp-Glu-His-Gly-Ala-Gln; (g) Phe-Arg-Asp-Glu-His-Gly-Ala-Gly-Ala; (h) Phe-Arg-Asp-Glu-His-Lys-Gly-Ala; (i) Phe-Arg-Asp-Glu-His-Gly-Ala-Lys.

This is because the fragmentation is not complete and the molecular weight of the amino acids inside the peptide chain may represent the sum of two amino acids:

$$\text{Gln}\ (128)\ \text{or}\ \text{Lys}\ (128) = \text{Gly}\ (57) + \text{Ala}\ (71)$$

The amino acid composition of F3b were analysed to confirm the peptide sequence, and Table 2 presented the results. Gly, Glu, Phe, Lys, Asp, Arg and His were the major constituent amino acids of the F3b peptide, but the content of Ala were very low. Likewise, the occurrence of the Lys instead of the mass-related Gln in the sequence was verified by on-plate acetylation and subsequent mass spectrometric analysis (Susanne et al., 2009). For the ion signals at m/z 959.533, an additional shift of +42 Da (i.e. 1001.533) due to acetylation of a primary amine group of Lys was detectable (Fig. 3a). Thus, the sequences (b–i) shown above were impossible and the selected amino acid sequence Phe-Arg-Asp-Glu-His-Lys-Lys (a) agreed with the amino acids analysis of the peptide F3b.

Similarly, there were three possible sequences for F3c by manual analysis. They were: (a) Lys-His-Asn-Arg-Gly-Asp-Glu-Phe; (b) Gln-His-Asn-Arg-Gly-Asp-Glu-Phe; and (c) Gly-Ala-His-Asn-Arg-Gly-Asp-Glu-Phe.

From the amino acids compositions (Table 2), we found that Phe, Lys, Asp, Gly, Glu, Arg and His were the major constituent amino acids of the F3c peptide, but the content of Ala was very low. In Fig. 3b, an additional shift (+42 Da) for the ion signals of at m/z 1002.5217 and 1044.5409 was also detectable, due to acetylation of a primary amine group of Lys. Therefore, the selected amino acid sequence Lys-His-Asn-Arg-Gly-Asp-Glu-Phe (a) agreed well with the amino acids analysis of the peptide F3c.

Accordingly, the amino acid sequences of the antioxidant constituent were identified to be FRDEHKK (F3b, that is Phe-Arg-Asp-Glu-His-Lys-Lys) and KHNRGDEF (F3c, that is Lys-His-Asn-Arg-Gly-Asp-Glu-Phe) (Fig. 3a and b). The detected molecular masses of 959.5 Da and 1002.5 Da agreed well with the theoretical mass calculated by the sequences. Several antioxidative peptides from protein hydrolysates had also been reported and that their antioxidative activities depended upon their amino acid sequences. They were respectively RPDFDLEPPY from yellowfin soleframe protein (Jun et al., 2004), LGLNGDDVN from conger eel muscle protein (Ranathunga et al., 2006), QGAR from the porcine skin collagen (Li et al., 2007), PSKYEPFY from grass carp muscles (Ren et al., 2008), NADGLNGLEGLA and NGLEGLK from giant squid muscle protein (Rajapakse et al., 2005).

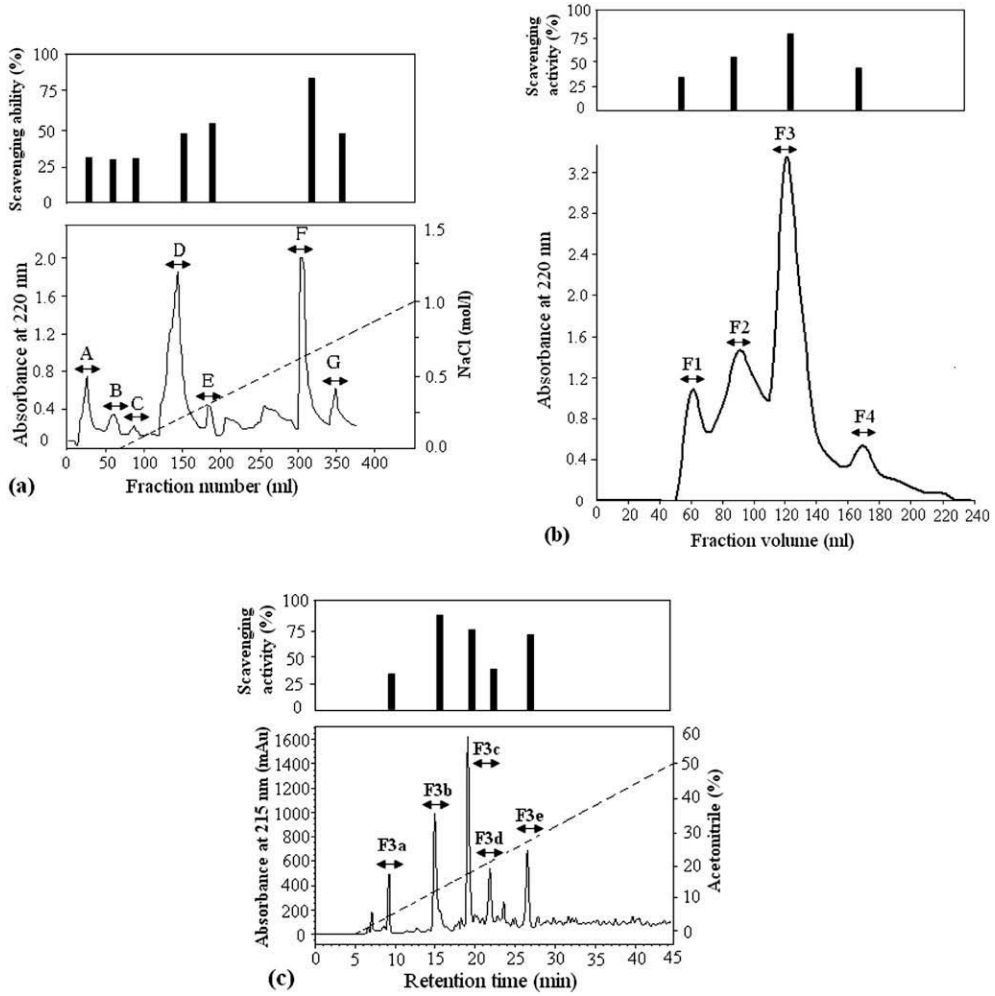

Fig. 2. Purification of NHREP. (a) The SP-Sephadex C-25 column chromatographic profiles of the NHREP and their DPPH radical scavenging effects with the final concentration of 0.017%. (b) The Sephadex G-15 column chromatographic profiles of F fraction, and their DPPH radical scavenging effects with the final concentration of 0.017%. (c) RP-HPLC chromatographic profiles the on Primesphere C18 (10 μm, 1.0 × 25 cm) column using a linear gradient of acetonitrile (0–50% in 60 min) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 4.0 ml/min with a final concentration of approximately 0.0005%.

3.4. Antioxidant activities of synthesised FRDEHKK

FRDEHKK, one of the antioxidant peptides from NHREP, was chemically synthesised using solid phase synthesis. The purity and molecular weight of the synthesised peptide was 98.52% and 960.25 Da, respectively (data not shown). Its antioxidant activity was further evaluated in the linoleic acid model system and radical-induced cell culture assay. In the linoleic acid model system, peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals, derived from the pre-existing lipid peroxide, were employed directly to initiate lipid peroxidation in the emulsified linoleic acid system (Je et al., 2005). As shown in Fig. 1, FRDEHKK significantly inhibited lipid peroxidation in the linoleic acid emulsion system and the activity was higher than that of α-tocopherol.

Cell culture experiments was performed using human embryonic lung fibroblasts and mouse macrophage, to assess the protective effects of FRDEHKK on radical-mediated cellular injuries and death. Cellular generation of alkoxyl and peroxyl radicals were simulated by exposing *t*-BHP for 2 h, and viability of cells were determined by an MTT assay (Qian, Jung, Byun, & Kim, 2008). According to the results (Fig. 4a), FRDEHKK did not exhibit any cytotoxic effect on the lung fibroblasts or the mouse macrophage at the tested concentrations (0–100 μg/ml). FRDEHKK significantly ($P < 0.05$) enhanced the viability of *t*-BHP induced cytotoxicity up to 74.38% (for the human embryonic lung fibroblasts) and 78.39% (for the mouse macrophage) at a concentration of 80 μg/ml.

In this study, FRDEHKK effectively quenched the free radicals generated by various methods. Therefore, we were interested in studying the direct effects of this peptide in scavenging cellular radicals. Fluorescent probes have been widely employed to monitor oxidative activity in cells (Qian et al., 2008). Fluorescence, emitted by DCF following ROS-mediated oxidation of DCFH, followed a time course increment up to 2 h as shown in Fig. 4b. Pre-treatment with FRDEHKK decreased the DCF fluorescence, and FRDEHKK (30–100 μg/ml) exerted a considerable radical scavenging effect throughout the incubation time. These results suggest that FRDEHKK can protect cells from oxidative damage by reactive oxygen species (ROS) according to previous studies by Qian et al. (2008), thus it may be developed into a potential bio-molecular candidate to inhibit ROS formation of cellular.

Previous works on antioxidative peptides had also concluded that peptides with 5–16 amino acid residues could inhibit autoxidation of linoleic acid, due to their higher accessibility to cross the intestinal barrier and interact more effectively with free radicals (Chen et al., 1996). Antioxidant peptides derived from different sources have exhibited varying potencies in scavenging free radicals. The antioxidative activity of a peptide or protein is dependent

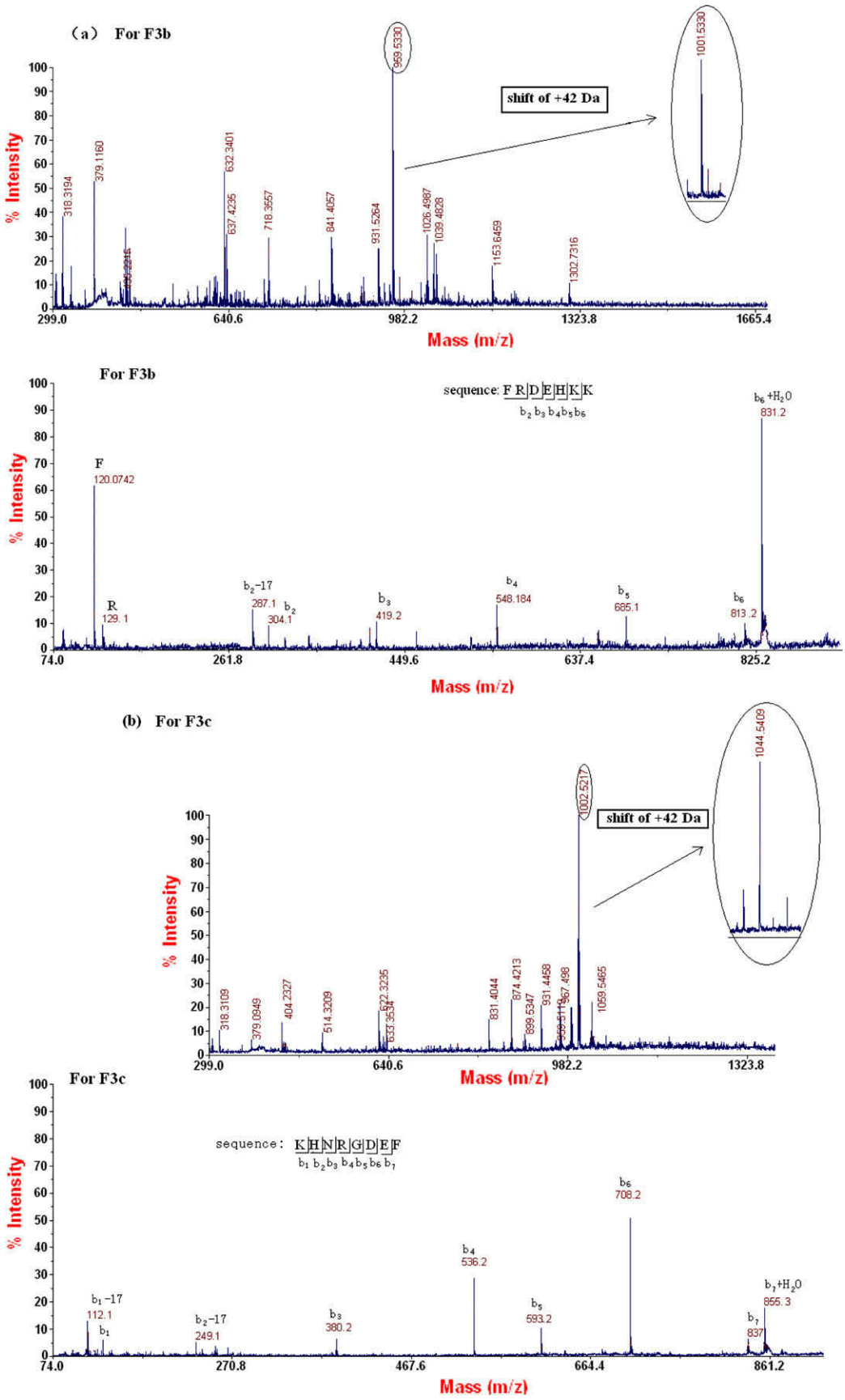

Fig. 3. Identification of the antioxidant peptides. (a) MALDI-TOF/TOF MS and MALDI-TOF/TOF MS/MS spectrum of the antioxidative constituent identified from the F3b. For clarity, only the b fragment ion is labelled. (b) MALDI-TOF/TOF MS and MALDI-TOF/TOF MS/MS spectrum of the antioxidative constituent identified from the F3c. For clarity, only the b fragment ion is labelled.

Table 2
Amino acids composition of F3b and F3c (g/100 g protein).

Amino acids	Composition (g/100 g protein)	
	F3b	F3c
Asp[a]	8.92	13.96
Glu[b]	16.18	14.27
Ser	1.70	1.92
His	9.65	10.50
Gly	4.56	4.74
Thr	0.95	1.98
Arg	10.96	11.80
Ala	1.62	1.63
Tyr	1.84	2.08
Cys-s	0.44	0.82
Val	1.63	1.06
Met	1.24	1.98
Phe	11.49	11.25
Ile	1.78	1.02
Leu	1.93	3.92
Lys	19.02	10.09
Pro	1.41	1.66
Trp	n.d[c]	n.d[c]

[a] Aspartic acid + asparagine.
[b] Glutamic acid + glutamine.
[c] Not determined.

on molecular size and chemical properties such as hydrophobicity and electron transferring ability of amino acid residues in the sequence (Qian et al., 2008). His and Lys have been generally accepted to be antioxidative, and exhibited higher antioxidative activities when incorporated into peptides (Chen et al., 1996; Halliwell, Gutteridge, & Aruoma, 1987; Wu et al., 2003). Moreover, the activity of histidine containing peptides has been reported to act against lipid peroxidation, due to presence of an imidazole ring which may be involved in hydrogen donation and lipid radical trapping ability (Chen et al., 1996).

The aromatic amino acid Phe is present within the antioxidant peptide sequence. Phe was reported to contribute to peroxidation inhibition by increasing the solubility of the peptide in lipid (Je et al., 2005; Ren et al., 2008). By donating protons easily to electron deficient radicals and maintaining their stability *via* resonance structures, Phe has also been shown to act positively as direct radical scavengers (Rajapakse et al., 2005).

It is also thought that acidic and/or basic amino acids play an important role in the chelation of metal ions by carboxyl and amino groups in their side chains (Rajapakse et al., 2005; Saiga et al., 2003). In this study, the two purified peptides also consisted of two acidic amino acid residues (Asp and Glu) and three basic amino acid residues (His, Lys and Arg). Therefore, we can further presume that the observed higher hydroxyl radical scavenging activity of NHREP could be due to the ion chelation activity of its inherent antioxidant peptides.

Additionally, the presence of Asp seems to play a vital role irrespective to its position as observed in several antioxidative peptide sequences (Li et al., 2007). Glu, Asp and Lys were also reported to interact with metal ions through their charged properties and to inactivate prooxidant activity of metal ions (Park et al., 2001; Wu et al., 2003). Thus, the presence of Asp-Gly residues in the purified peptides from NHREP may have contributed to its higher radical scavenging potential.

The strong activity shown by FRDEHKK must be related to the presence of the amino acids within its sequence. Besides, six constituent amino acids (Lys, His, Arg, Asp, Glu and Phe) of the identified peptide, mixed with the same concentration as FRDEHKK, did not show antioxidant activity. Therefore, the amino acid sequence of the peptide might play an important role on its activities. The same conclusion was also made by Ren et al. (2008).

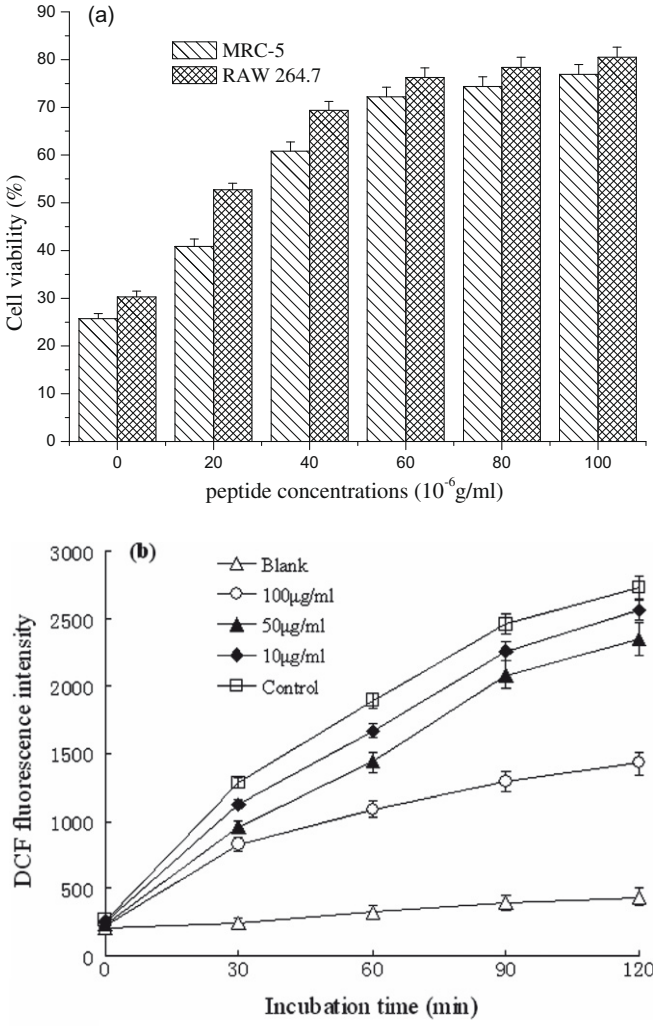

Fig. 4. Radical-induced cell culture assays. (a) Effects of FRDEHKK on *t*-BHP-induced cytotoxicity. Human embryonic lung fibroblasts (MRC-5) and mouse macrophage (RAW264.7) cells were cultured in EMEM medium, and cellular oxidative stress was artificially induced. Viability of cells treated with different concentrations of peptides was determined by MTT assay ($n = 3$). (b) Effects of FRDEHKK on the scavenging of cellular ROS. RAW264.7 cells were labelled with non-toxic fluorescence dye, DCFH-DA, and treated with different concentrations of FRDEHKK. Fluorescence intensities of DCF due to oxidation of DCFH by cellular ROS (generated by H_2O_2) were detected time-dependently (Ex = 485 nm and Em = 535 nm). Cells with no H_2O_2 stimulated were used as blank and with no peptide treated as control groups ($n = 3$).

4. Conclusion

The defatted rice endosperm protein (REP) was respectively digested by different protease treatments (Alcalase, Chymotrypsin, Neutrase, Papain and Flavorase) to obtain antioxidative peptides. The enzymatic hydrolysate of the defatted rice endosperm protein by Neutrase (NHREP) exhibited the highest antioxidant activities on 1,1-diphenyl-2-picrylhydrazyl (DPPH)/hydroxyl/superoxide radicals scavenging activities, and inhibited the autooxidation in a linoleic acid model system. Then two different peptides showing strong antioxidant activities were isolated from the hydrolysate using consecutive chromatographic methods including ion-exchange chromatography, gel filtration chromatography and reversed-phase high performance liquid chromatography. They were identified as Phe-Arg-Asp-Glu-His-Lys-Lys (FRDEHKK, 959.5 Da) and Lys-His-Asp-Arg-Gly-Asp-Glu-Phe (KHNRGDEF, 1002.5 Da) by MALDI-TOF/TOF MS/MS. At last, FRDEHKK was

synthesised and the antioxidant activities were measured in the linoleic acid model system and radical-induced cell culture assay. The results confirmed the antioxidant activities of FRDEHKK, and we concluded that it was feasible to produce natural antioxidants from REP. To our knowledge, it is the first record on the antioxidant potential of the enzymatic hydrolysate from the rice endosperm protein, which may be potent for the future food industry. Despite the need for further research, NHREP could be considered as suitable natural antioxidants to prevent oxidation reactions in food processing.

Acknowledgments

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