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An antioxidant peptide derived from Ostrich (*Struthio camelus*) egg white protein hydrolysates

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ABSTRACT

Ostrich (*Struthio camelus*) egg white (OEW) proteins were hydrolyzed using various proteases (α-chymotrypsin, pepsin, trypsin and papain). Antioxidant activities of hydrolysates were evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and iron chelating activity. The hydrolysate obtained by trypsin exhibited the highest antioxidant activity. This hydrolysate was passed through an ultrafiltration membrane with a 3 kDa-cut off, and the resulting filtrate was purified using reversed-phase high performance liquid chromatography (RP-HPLC). Eight peptide fractions were separated and their antioxidant activities were tested. The results showed that the F_6 fraction possessed the highest antioxidant activity in the inhibition of linoleic acid autoxidation (86.4% at 20 μg/ml), scavenging activity for DPPH radical (81% at 200 μg/ml) and 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonicacid) diammonium salt (ABTS) radical (37.6% at 90.9 μg/ml). In addition, the iron chelating activity, hydroxyl radical scavenging and reducing power of the F_6 fraction were 20% at 317.5 μg/ml, 28.6% at 163.9 μg/ml and 0.083 at 113.6 μg/ml, respectively. The peptide sequence was found to be LTEQESGVPVMK (with a molecular mass of 1317.65 Da) using mass spectrometry. The results suggest that the digestion of OEW proteins by trypsin protease could be exploited to produce natural antioxidants.

1. Introduction

Reactive oxygen species (ROS) are the products of normal body reactions that have various forms, such as superoxide anion radicals $(O_2\bullet)^-$, hydroxyl radicals (HO•), hydrogen dioxide ($HO_2\bullet$), hydrogen peroxide (H_2O_2), and singlet oxygen ($O_2\bullet$) (Gülçin, 2009; Gülçin, Berashvili, & Gepdiremen, 2005). Among all oxygen species, superoxide anion radical $(O_2\bullet)^-$, hydroxyl radicals (HO•), and hydrogen peroxide (H_2O_2) are supposed to be the most abundant ROS in biological systems (Cui, Luo, Xu, & Murthy, 2004). ROS is one of the causes of cellular damages, destruction of protein structures and DNA mutations (Philanto, 2006). Effects of ROS on biomolecules can directly lead to toxic cellular reactions in the body. Furthermore, they are one of the major motives of health disorders, including cancer, arthritis, diabetes, inflammation, coronary heart disease and processes of aging (Bernardini et al., 2011). Thus, it is necessary to scavenge free radicals engendered in the living body and to inhibit the formation of free radicals by preventing the process of foodstuff oxidation. It was also reported that some synthetic antioxidative agents, such as butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), propyl gallate and tert-butylhydroquinone (TBHQ) can be used in foods to suppress the formation of free radicals, preventing lipid-oxidation and improving shelf life (Wanita & Lorenz, 1996). However, the use of synthetic antioxidants affords potential risks to human health (Becker, 1993; Gülçin, 2012).

In the past few years, biomaterials with antioxidant activity have drawn the attention of researchers. Bioactive peptides with antioxidant activity are a group of biomaterials that are derived from protein hydrolysates of various food sources; such as dairy, meat, soy and egg (Dávalos, Miguel, Bartolomé, & Lopez-Fandino, 2004). Some bioactive peptides derived from egg proteins have been described. Two bioactive peptides with the sequences of FRADHPFL and RADHPFL were identified after the enzymatic hydrolysis of hen egg white ovalbumin, exhibiting antihypertensive activity (Miguel & Aleixandre, 2006). Recently, we have reported an ACE inhibitory peptide, and antioxidant, antimicrobial peptide from hen egg white lysozyme hydrolysate (Asoodeh, Memarpoor-Yazdi, & Chamani, 2012; Memarpoor-Yazdi, Asoodeh, & Chamani, 2012).

Ostrich egg whites are known as rich source of food proteins. In this study, protein hydrolysates from ostrich egg whites were prepared to extract antioxidant peptides. After purifying the antioxidant peptides using high performance liquid chromatography, the antioxidant activities were measured via different methods, including autoxidation of linoleic acid, the scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

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(ABTS), metal ion chelating activity, reducing power and hydroxyl radical scavenging activity.

2. Material and methods

2.1. Materials

Fresh ostrich eggs (*Struthio camelus*) were bought from a local market in Mashhad, Iran, and transported to laboratory. α-Chymotrypsin, pepsin, trypsin (from bovine pancreas, type II) and papain (from pawpaw sap) were purchased from Sigma (St. Louis, MO, USA). 1,1-diphenyl-2-pycryl-hydrazyl (DPPH), trifluoroacetic acid (TFA), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid) (ABTS), butylatedhydroxyanisole (BHA), linoleic acid, 3-(2-pyridyl)-5-6-diphenyl-1,2,4-triazine-4′, 4″-disulphonic acid sodiumsalt (ferrozine), Glutathione reduced (GSH) and 2,4,6-trinitrobenzenesulphonic acid (TNBS), were also purchased from Sigma (St. Louis, MO, USA). Trichloroacetic acid (TCA), potassium persulphate, potassium ferricyanide, sodium sulphite and ferrous chloride were obtained from Merck Chemicals Co. (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.2. Preparation of egg white protein hydrolysates

Ostrich egg white (100 ml) was discretely mixed with 400 ml of enzyme buffer which is described below and then homogenized for about 10 min. The egg white was separately hydrolyzed using various enzymes under optimum pH values at 37 °C. Trypsin and α-chymotrypsin (50 mM of sodium phosphate, pH 8.0), papain (50 mM of sodium phosphate, pH 6.0) and pepsin (50 mM of glycine-HCl, pH 2.0) were used for digestion. A ratio of 20:1 (w/w) egg white protein to protease was used to prepare hydrolysate at different intervals (0, 0.5, 1, 2, 4, 6, 8 and 10 h). To inactivate the proteases, the samples were placed in boiling water for 15 min. The hydrolysates were centrifuged at 8500×*g* and 4 °C for 15 min. The supernatants were fractionated through an ultrafiltration membrane with a 3 kDa cut-off using a bioreactor system under 40 PSI nitrogen gas to obtain fractions with less than 3 kDa molecular mass. Afterwards, the resultant solution was lyophilized.

2.3. Chromatographic separation

Ostrich egg white hydrolysates were dissolved in distilled water at concentration of 20 mg/ml and then 0.4 ml of the resulted solution was injected into chromatography column. A Knauer HPLC (Knauer HPLC, Berlin, Germany) apparatus equipped with smartline pump model 1000 and PDA detector model 2600 was used for peptide purification. The fractions which showed antioxidant activity were purified by RP-HPLC on a semi-preparative column (10×250 mm, manufactured by Macherey-Nagel GmbH & Co. Düren, Germany) using a linear gradient of acetonitrile (2–32%, v/v over 40 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 2.0 ml/min. Fractions corresponding to each peak were collected using the absorbance at 214 nm. Then, the fractionated ostrich egg white peptides were lyophilized and stored at −20 °C for subsequent analyses.

2.4. Determination of the degree of hydrolysis (DH)

To determine the DH value of the hydrolysates obtained by four enzymes (α-chymotrypsin, pepsin, trypsin and papain), free amino groups of hydrolysates were measured based on its reaction with trinitrobenzenesulphonic acid (TNBS) (Nalinanon, Benjakul, Kishimura, & Shahidi, 2011). A test sample of 150 μl of hydrolysate was mixed with 2.0 ml of 0.2 M phosphate buffer (pH 8.2) and 1.0 ml of 0.1% TNBS solution, followed by incubation in a water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulphite. The mixtures were cooled at room temperature for 15 min. The absorbance was measured at 420 nm and α-amino acid was expressed in terms of L-leucine. DH was calculated according to the equation:

$$DH = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

where L_t is the amount of α-amino acid released at time t. L_0 is the amount of α-amino acid in the original acid-solubilized protein substrate. L_{max} was determined in a sample which was 100% hydrolyzed in 6 M HCl at 100 °C for 24 h.

2.5. DPPH radical scavenging activity assay

The DPPH radical scavenging activity was determined by the method of Göçer and Gülçin (2011) with slight modification. One milliliter of either hydrolysate or peptide solution at different concentrations (0–1 mg/ml) was mixed with 4 ml of 0.15 mM DPPH (in 95% ethanol). The mixture was then shaken vigorously using a mixer. The reaction mixture was incubated for 30 min in the darkness at room temperature. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer. Ethanol and BHA was used as a control and standard sample, respectively. The radical scavenging capacity of the samples was measured as a decrease in the absorbance of DPPH radical and it was calculated using the following equation.

$$\text{Radical scavenging activity } (\%) = \left(A_{control} - A_{sample}\right)/A_{control} \times 100$$

The IC_{50} value was defined as an effective concentration of peptide fraction that is required to scavenge 50% of radical activity (Bursal & Gülçin, 2011). All experiments were carried out in triplicate.

2.6. Measurement of metal ion chelating activity

The ability of the various peptide fractions to chelate Fe^{2+} ion was assessed using the method of Gülçin et al. (2011). Briefly, 1 ml of peptide solution (0.0–1.0 mg/ml) was premixed with 2 ml of double distilled water and 0.05 ml of 2 mM $FeCl_2$. After 3 min at room temperature, the reaction between $FeCl_2$ and peptide was inhibited by the addition 0.1 ml of 5 mM ferrozine solution. The mixture was stirred and kept further at room temperature for 10 min. The absorbance of the resulting solution was measured at 562 nm. Double distilled water was used as a control and EDTA was also used as a standard metal chelating agent. The chelating capacity of peptide on Fe^{2+} was calculated as follows:

$$\text{Chelating ability } (\%) = \{1 - (\text{absorbance of the sample at } 562 \text{ nm}) / (\text{absorbance of control at } 562 \text{ nm})\} \times 100.$$

Furthermore, the IC_{50} value was defined as an effective concentration of peptide fraction that is required to chelate 50% of iron ions.

2.7. ABTS radical scavenging activity assay

ABTS radical scavenging activity of the peptides was determined according to the method of Tironi and Añón (2010) with some modifications. The $ABTS^+$ solution was produced by the reaction of 7 mM of ABTS solution in 2.45 mM potassium persulfate (final concentration). The mixture was kept in the dark at room temperature for 16 h before use. The solution was diluted with distilled water and equilibrated at room temperature to give an absorbance of 0.70 ± 0.02 at 734 nm in a 1 cm cuvette. Then, 200 μl of sample (1 mg/ml) was added to 2 ml of $ABTS^+$ solution and absorbance at 734 nm was measured at different times. Appropriate solvent blank (negative controls NC) was run

for each assay, while GSH was used as a positive control (PC). The scavenging percentage was calculated as follows:

$$\text{Scavenging activity } \% = [(Abs_0 - Abs_t) - (Abc_0 - Abc_t)/Abc_0] \times 100$$

where; Abs_t and Abs_0 = absorbance of the sample at t = 20 min and t = 0, respectively. Abc_t and Abc_0 = absorbance of the negative control at t = 20 min and t = 0, respectively (Gülçin, 2011).

2.8. Inhibition of linoleic acid autoxidation

Linoleic acid oxidation inhibition activity of peptides and hydrolysates was determined according to ferric thiocyanate method with minor modifications (Wu, Chen, & Shiau, 2003). Briefly, 2.0 mg of peptide sample was dissolved in 1.0 ml of 50 mM sodium phosphate buffer (pH 7.0) and was mixed with 1.0 ml of 50 mM linoleic acid in ethanol (95%). Furthermore, the sample was replaced with BHA and buffer for comparison as positive control (PC) and negative control (NC), respectively. The reaction mixture was incubated in a 5 ml conical flask with a screw cap at 60 °C in a dark room to accelerate oxidation. Then, the solutions 2.35 ml of 75% ethanol, 50 μl of 30% ammonium thiocyanate, and 50 μl of 20 mM ferrous chloride solution in 3.5% HCl were added to 50 μl of reaction mixture. The mixture was stirred for 5 min; the degree of linoleic acid oxidation was evaluated at different times with color development, which was measured at 500 nm. The percent of the oxidation inhibition was calculated as:

$$\text{Inhibition } \% = [1 - (Abs_t - Abs_0)/(Abc_t - Abc_0)] \times 100$$

where; Abs_t and Abs_0 = sample absorbance at t = 48 h and t = 0, respectively; Abc_t and Abc_0 = negative control absorbance at t = 48 h and t = 0, respectively.

2.9. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was assayed according to the method of Li, Jiang, Zhang, Mu, and Liu (2008) with slight modification. A mixture of 100 μl of 1,10-phenanthroline (5.0 mM), 100 μl of $FeSO_4$ (5.0 mM) and 100 μl of ethylenediaminetetraacetic acid (EDTA) (15 mM) were mixed with 70 μl of sodium phosphate buffer (0.2 M, pH 7.4). Then, 100 μl of sample (1 mg/ml) and 140 μl of H_2O_2 (0.01%) were added. The mixture was incubated at 37 °C for 60 min, and the absorbance was measured at 536 nm. Glutathione reduced (GSH) at 1 mg/ml was used as a control. To calculate hydroxyl radical scavenging, the following equation was used:

$$\text{Hydroxyl radical scavenging activity } \% = (A_s - A_0) \times 100/(A_c - A_0)$$

where: A_s is the absorbance of the sample; A_0 is the absorbance of the blank solution using distilled water instead of sample; and A_c is the absorbance of a control solution in the absence of H_2O_2.

2.10. Reducing power assay

The reducing powers of the peptides were measured according to the method of Ahmadi, Kadivar, and Shahedi (2007) with minor modification. Briefly, 500 μl of sample (1 mg/ml) was mixed with 500 μl of 0.2 M phosphate buffer (pH 6.6) and 500 μl of 1% potassium ferricyanide, and the mixtures was incubated at 50 °C. After 20 min of incubation, 500 μl of 10% trichloroacetic acid (TCA) was added to the reaction mixture, and centrifuged at 5000 *g* for 10 min. Then, 500 μl of supernatant was mixed with 500 μl of distilled water and 100 μl of 0.1% ferric chloride, and the absorbance was measured at 700 nm. Instead of sample, 500 μl distilled water was used as blank in this test. Increased absorbance of the reaction mixture indicates the stronger reducing power.

2.11. Identification of amino acid sequences of the most active peptide

The amino acid sequence and molecular mass of the most active peptide (F6) were determined using the tandem mass spectrometry. The peptide sample was diluted with the ratios of 1:10 and 1:100 with 2% acetonitrile containing 0.1% TFA and analyzed by MALDI-TOF-TOF mass spectrometer using a 5800 Proteomics Analyzer [Applied Biosystems at Proteomics International Pty Ltd., Nedlands, Western Australia]. MS/MS spectra were analyzed using PEAKS Studio Version 4.5 SP2 [Bioinformatics Solutions Inc., Waterloo, Canada].

3. Results and discussion

3.1. Effect of DH on the antioxidant activity of OEW proteins

All the OEW proteins were hydrolyzed under the optimum enzymatic conditions at various incubation times to obtain antioxidant peptides. The DH of hydrolysates from the OEW proteins is shown in Fig. 1a. The hydrolysis rates were increased during the time. The results suggested that all four selected proteases have different affinities for proteolytic cleavage. Among them, trypsin showed the highest activity to cleavage, while pepsin could not be a suitable protease to digest OEW proteins. The hydrolysates were tested for antioxidant activity using DPPH radical scavenging and iron chelating

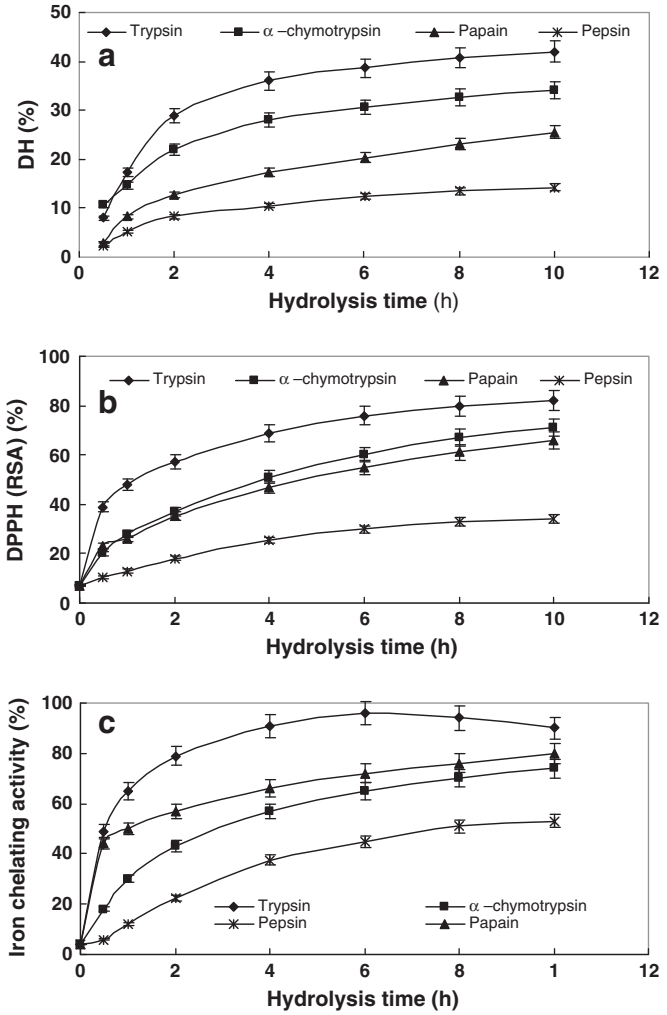

Fig. 1. (a) Degree of hydrolysis (DH) of OEW protein hydrolysates assayed for four enzymes at different hydrolysis times. (b) DPPH radical scavenging activity (RSA). (c) Iron chelating activity of hydrolysates. Results are the mean values of triplicate analyses. The standard errors were less than 5% of the means.

activity methods. As shown in Fig. 1b, the hydrolysates prepared by trypsin exhibit the highest ability to scavenge DPPH radicals. Furthermore, our results suggest that there is a direct relationship between the hydrolysis time, DH and the antioxidant activity.

Similar results were also observed for iron chelating activity in which it was enhanced with the increase in DH during the test (Fig. 1c). After 6 h hydrolysis, the tryptic hydrolysate showed the highest iron chelating activity (96% at a concentration of 317.5 μg/ml), and it exhibited 77% DPPH radical scavenging activity at 200 μg/ml. In a similar study, trypsin has been used to hydrolysis gelatin from *Dosidicus gigas* and results showed higher antioxidant activity than α-chymotrypsin and pepsin hydrolysates (Mendis, Rajapakse, Byun, & Kim, 2005). The ferrous ion chelating ability of gelatin and its hydrolysates showed a high chelating ability of 80% at 200 μg/ml concentration (Giménez, Alemán, Montero, & Gómez-Guillén, 2009). Giant squid muscle proteins have been hydrolyzed using pepsin, trypsin and α-chymotrypsin, and results showed that the tryptic hydrolysate was the highest lipid peroxidation inhibition activity after 6 h digestion (Rajapakse, Mendis, Jung, Je, & Kim, 2005). The results indicated that OEW protein hydrolysates (after 6 h) had a potential antioxidant activity.

3.2. HPLC fractionation

In has been observed that the peptides having molecular mass of approximately lower than 3 kDa have the highest antioxidant activity (Sarmadi & Ismail, 2010). Therefore, the OEW hydrolysate was passed through 3 kDa membrane to obtain low molecular peptides. The resulting trypsin hydrolysate permeate (<3 kDa) was further fractionated using RP-HPLC. This hydrolysate was separated into eight fractions (F_1–F_8) as indicated in Fig. 2a. Using the several RP-HPLC runs, sufficient amount of different fractions (2–5 mg) were gathered, and then antioxidant activity of isolated fractions was evaluated.

3.3. DPPH radical scavenging activity

The DPPH radical scavenging activity assay is one of the in vitro methods for the measurement of the capacity of an antioxidant to reduce free radicals. The degree of color changes is correlated with the sample antioxidant activity (Xie, Huang, Xu, & Jin, 2008). Resultant fractions from trypsin hydrolysates were tested for DPPH radical scavenging activity, and results are depicted in Fig. 2b. This activity was compared with BHA as a synthetic antioxidant. The results show that three peptides named F_3, F_4 and F_6 at 200 μg/ml can reduce the signal intensity of DPPH and their radical scavenging activities were 75%, 78% and 81%, respectively. Among them, the F_6 fraction showed the highest scavenging effect on DPPH. Furthermore, the respective IC_{50} values were determined and the IC_{50} value of the F_6 fraction was 137 μg/ml (final concentration) (Table 1). A DPPH radical scavenging 79.7% was reported for alfalfa leaf peptide at 1.6 mg/ml concentration (Xie et al., 2008) which indicated that the isolated fraction was more potent than leaf peptides.

3.4. Iron chelating activity

Among the transition metals, iron is known as the effective factor in lipid oxidation due to its high reactivity. The ferrous iron via the

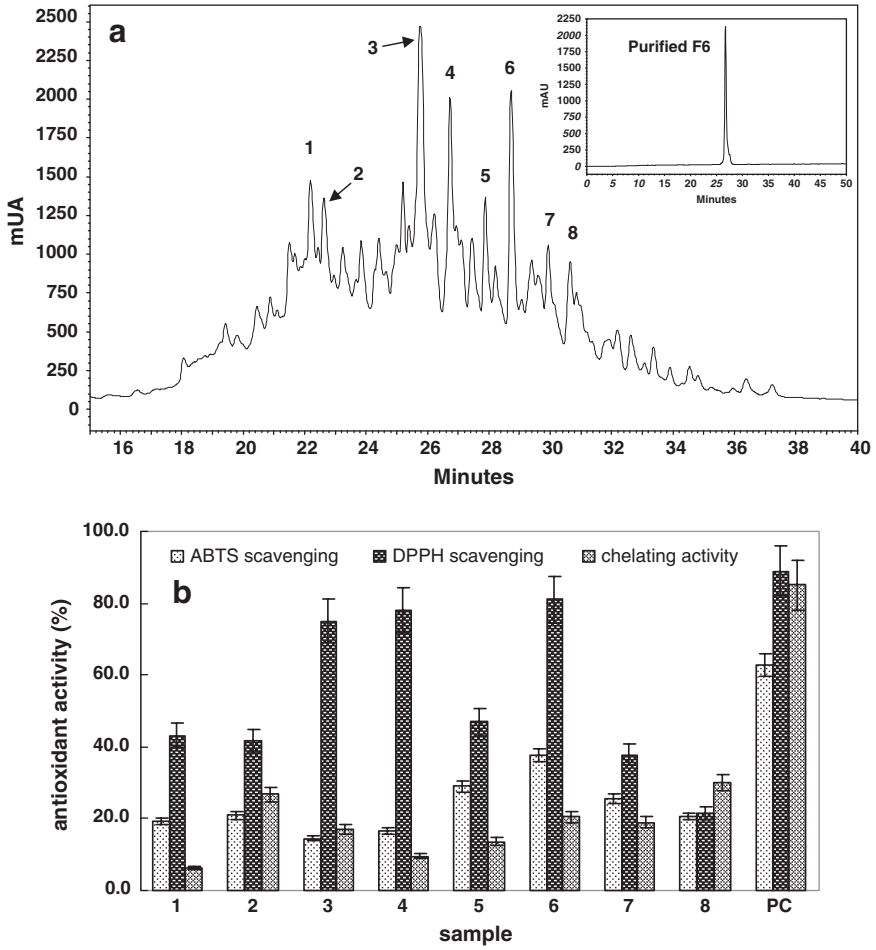

Fig. 2. (a) Separation of antioxidant peptides from OEW trypsin hydrolysate by RP-HPLC system. Eight fractions were separated. (b) Antioxidant activity of peptide fractions (1–8) from OEW protein hydrolysates. Positive controls (PC) for ABTS radical scavenging, DPPH radical scavenging and iron chelating activities are GSH, BHA and EDTA, respectively. Bars represent the standard deviation from triplicate determinations.

Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\bullet$) generates hydroxyl radical. In addition, Fe^{3+} ion produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+} ion (Gülçin, 2006). In the presence of chelating agents, the ferrozine-Fe^{2+} complexes are disrupted, resulting in a decrease in the red color of the complex (Rajapakse et al., 2005). Iron chelating activity of peptide fractions was measured as antioxidant activity (Fig. 2b). In this study, EDTA was used as a positive control to compare the results and it exhibited the highest chelating activity (85%). The results showed that iron chelating activities of three potent fractions F_2, F_6 and F_8 at concentration 317.5 μg/ml were 26.72%, 20.49% and 30%, respectively. IC_{50} values of these peptide fractions were found to be 619, 825 and 526 μg/ml, respectively (Table 1). In this study, the tryptic hydrolysate exhibited a very high metal iron chelating capacity (96%), whereas the purified peptide registered to show a less than 30% activity. This suggests that synergism among different peptides plays an important role in the chelating activity of OEW hydrolysate.

3.5. ABTS radical scavenging activity

ABTS radical is relatively stable but readily reduced by antioxidants. The scavenging activity against cationic ABTS radical indicates the ability of peptide fractions to act as electron donors or hydrogen donors in free radical reactions (Prior, Wu, & Schaich, 2005). To assay the ABTS radical scavenging of peptide fractions, cationic ABTS radical decolorization was carried out. The results showed that the F_6 fraction revealed the highest scavenging activity (37.6% at 90.9 μg/ml). On the other hand, the GSH as a positive control at the same concentration exhibited the highest scavenging activity (62.7%) on ABTS, and its ability was fast in the initial stage (0–10 min) and then gradually decreased (20 min) (Fig. 2b). The ABTS scavenging activity of eleven amaranth peptide fractions has been reported, and among them, two fractions exhibited higher antioxidant activity than the other fractions. Their activities were 66.8 and 83% (both at 310 μg/ml), but the other fractions (at concentration lower than 200 μg/ml) registered less than 37%. (Tironi & Añón, 2010). Different ABTS scavenging activities may have been resulted in the low solubility of some peptides in aqueous buffers. Our peptides showed a higher DPPH radical than ABTS radical scavenging activity (Fig. 2b). Therefore, these peptides have more efficiency to scavenge free radicals, which are soluble in lipids.

3.6. Inhibition of linoleic acid autoxidation

Lipid oxidation is the main cause of deterioration of food quality, leading to rancidity and shortening of shelf life (Philanto, 2006). Lipid oxidation products react with proteins causing their oxidation. Carbohydrates are also susceptible to oxidation, but they are less sensitive than lipids and proteins (Parkin & Damodaran, 2003). Hence, inhibition of linoleic acid autoxidation is currently investigated. The results of the inhibitory effect of selected peptides on linoleic acid autoxidation are presented in Table 1. The values of inhibitory effect of peptides were in the range of 32 to 86%. Additionally, changes in the absorbance 500 nm over the time for every fraction are shown in Fig. 3. The maximum level of oxidation was observed for a negative control (NC) (35 mM phosphate buffer pH 7.8) after 48 h. During the incubation, no change in the oxidation level was observed for BHA, whereas, NC showed the highest change in the oxidation level. Among the eight fractions, F_2, F_6 and F_8 fractions at concentration of 20 μg/ml exhibited 86.4%, 77.3% and 84.1% inhibitory activity, respectively. As shown in Fig. 3, fractions could be grouped as presenting high (F2, F6, F8), intermediate (F3, F4, F7) and low (F1,F5) antioxidant activity after 48 h, despite the differences are not evident for up to 24 h. Antioxidant activity of the peptides depended on amino acid compositions and their sequences (Memarpoor-Yazdi et al., 2012). The presence of hydrophobic amino acids in the sequence could be responsible for the high antioxidant activity. The hydrophobicity of amino acids in the sequences leads to more interactions between the peptide and the fatty acids, resulting in protection against oxidation (Mendis et al., 2005). Our result indicated that OEW proteins exhibit a noticeable antioxidant property to inhibit the peroxidation of linoleic acid.

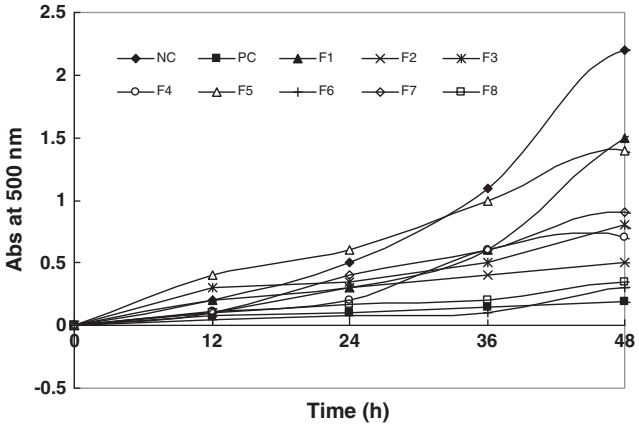

Fig. 3. Linoleic acid oxidation inhibition assay of OEW peptide fractions. PC: positive control (BHA) and NC: negative control (buffer).

3.7. Hydroxyl radical scavenging assay

Hydroxyl radical is one of the ROS that easily reacts with biomolecules, such as amino acids, proteins and DNA. Therefore, removal of hydroxyl radicals can protect humans against some diseases (You, Zhao, Cui, Zhao, & Yang, 2009). In this study, hydroxyl radical was generated by 1, 10-phenanthroline/(EDTA)/H_2O_2 system to determine the

Table 1
Antioxidant activity of peptide fractions from OEW hydrolysates and positive controls.

Sample	Inhibition of linoleic acid outoxidation (%)	Hydroxyl radical scavenging (%)	Reducing power (OD700 ± 0.003)	IC_{50} (μg/ml)	
				DPPH radical scavenging	Chelating activity
Fraction 1	31.8 ± 095	9.8 ± 0.28	0.087	235 ± 9.20	nd
Fraction 2	86.4 ± 3.02	23.2 ± 0.77	0.073	244 ± 9.01	619 ± 26.61
Fraction 3	63.6 ± 2.03	22.2 ± 0.74	0.067	161 ± 6.60	1000 ± 38
Fraction 4	68.2 ± 2.52	68.8 ± 2.48	0.082	156 ± 6.55	857 ± 30.8
Fraction 5	36.4 ± 1.24	29.5 ± 1.00	0.069	207 ± 7.86	nd
Fraction 6	77.3 ± 2.93	28.6 ± 1.08	0.083	137 ± 4.79 [a]	825 ± 32.17 [b]
Fraction 7	59.1 ± 1.77	22.3 ± 0.58	0.068	271 ± 10.84	812 ± 28.05
Fraction 8	84.1 ± 3.36	64.3 ± 2.50	0.058	480 ± 15.30	526 ± 17.90
BHA	91.4 ± 2.93	nm	nm	nm	nm
GSH	nm	nm	0.961	nm	nm

nd: non detected, nm: not measured, standard deviations were less than 5%.
[a] 137 μg/ml or 104 μM.
[b] 825 μg/ml or 626 μM.

hydroxyl radical scavenging capacity of peptides. As shown in Table 1, among eight fractions, the F_4 (68.8%) and F_8 (64.3%) fractions at 163.9 μg/ml were more effective than the others. Rajapakse et al. (2005) reported that the peptide from fermented mussel sauce (HFGBPFH) with a molecular mass of approximately 962 Da showed a stronger hydroxyl radical scavenging (96%) at 200 μg/ml concentration. Prepared hydrolysates from *Misgurnus anguillicaudatus* by papain exhibited 56.1% hydroxyl radical scavenging (You et al., 2009). Therefore, all isolated peptides from the OEW trypsin hydrolysate possessed a considerably hydroxyl radical scavenging activity.

3.8. Reducing power

Reducing power is usually used for the measurement of the activity of peptide fractions and protein hydrolysate. Reducing the Fe^{3+}/ferricyanide complex to the ferrous form was used to determine the reducing power of peptides (You et al., 2009). Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung, Chang, Chao, Lin, & Chou, 2002). The reducing powers of all peptide fractions were little below the absorbance 0.1. Low values of the absorbance may be due to little concentration of the peptide fractions in the mixture solution (Table 1). However, the result showed that the F_1, F_4 and F_6 fractions at 113.6 μg/ml possessed stronger antioxidant activity than the others, and the values of the absorbance were observed to be 0.087, 0.082 and 0.083, respectively. In addition, the values of absorbance for GSH as positive control and H_2O as negative control were found to be 0.942 and 0.033, respectively. In a similar study, Vaštag, Popovic, Popovic, Krimer, and Pericin (2011) hydrolyzed pumpkin oil cake proteins by the combination of alcalase and flavourzyme, and reported that reducing power of all hydrolysates sample (at concentration 80.2 μg/ml) in different degree hydrolysis (DH) was below 0.1.

3.9. Peptide identification

All peptide fractions were tested for antioxidant activity in which the F_6 fraction showed the highest antioxidant activity. The molecular mass and the sequence of the active peptide were determined by MALDL-TOF-TOF mass spectrometer. The peptide sequence was provided by Proteomics International Pty Ltd and found to be Leu-Thr-Glu-Gln-Glu-Ser-Gly-Val-Pro-Val-Met-Lys with a molecular mass of 1317.65 Da (figure not shown). Due to preparing of hydrolysate by trypsin, the resulted sequence has been terminated by lysine residue. Recently, two antioxidant peptides (DPPH scavenger) were isolated from the rotifer *Brachionus rotundiformis* by enzymatic hydrolysis. The two antioxidant peptides were identified to be Leu-Leu-Gly-Pro-Gly-Leu-Thr-Asn-His-Ala (1076 Da), and Asp-Leu-Gly-Leu-Gly-Leu-Pro-Gly-Ala-His (1033 Da). The two peptides were evaluated using DPPH radical scavenging activity and the IC_{50} values of these peptides were 189.8 and 167.7 μM, respectively (Byun, Lee, Park, Jeon, & Kim, 2009). In our study, IC_{50} value of the fraction 6 was 104 μM. An antioxidant hepta-peptide sequence, (His-Phe-Gly-Asp-Pro-Phe-His, MW 962 Da) was purified from fermented mussel sauce (Rajapakse et al., 2005). This peptide at concentration 54 μM inhibited the lipid peroxidation; whereas, the fraction 6 at concentration 15.17 μM inhibited 77.3% the lipid peroxidation indicating that the identified peptide has a higher antioxidant effect. A peptide with the sequence of Val-Lys-Glu-Ala-Met-Ala-Pro-Lys derived from casein, exhibited inhibition of enzymatic and non enzymatic lipid peroxidation (Rival, Boeriu, & Wichers, 2001). A peptide from the extracellular death factor in *Escherichia coli* with the sequence of H-Asn-Asn-Trp-Asn-Asn-OH also exhibited 36% hydroxyl radical scavenging activity at concentration of 300 μM (Gao et al., 2010), whereas, in this study, the fraction F_6 has a hydroxyl radical scavenging 28.6% at 124 μM. Bioactive peptides are directly dependent on their structural, compositional and sequential properties (Rajapakse et al., 2005). It was reported that peptides containing Val, Leu, Ile, Ala, Phy, and Lys residues at the N-terminus and Pro in the sequence show antioxidant activity (Xie et al., 2008). Therefore, antioxidant activity of the F_6 fraction might be due to the positioning of Leu and Lys residues at N and C-termini, respectively. Furthermore, it is assumed that hydrophobic Leu residue at the C or N-terminus can increase the interaction between peptide and fatty acids (Ranathung, Rajapakse, & Kim, 2006). The peptide of horse mackerel viscera protein hydrolysate showed 89.2 and 59.1 percentage of DPPH and hydroxyl radical scavenging activity. The amino acid sequence of purified peptide was determined as Ala-Cys-Phe-Leu (518.5 Da), and it exhibited also high activity on the inhibition of peroxidation α-linoleic acid (Sampath Kumar, Nazeer, & Jaiganesh, 2011). Moreover, the abundance of hydrophobic residues, such as Leu, Val, Pro and Met, in peptide sequences seems to be responsible for the higher antioxidant effects by increasing peptide solubility in lipids and interaction with radical species (Mendis et al., 2005). Thereupon, the F_6 fraction can readily react with lipid-soluble DPPH radical, but not with water-soluble ABTS radical. Hence, the antioxidant activity of F_6 fraction is related to the inhibition of linoleic acid autoxidation (77.3%) and DPPH radical scavenging (81%) is higher than the ABTS radical scavenging activity (37.6%). Tironi and Añón (2010) reported that some peptide fractions from amaranth proteins exhibited a strong activity to inhibit the oxidation of the linoleic acid, while the same fractions could not be stronger on the ABTS radicals. Hydrophobic peptides generally protect linoleic acid easily by donating photons to hydrophobic peroxyl radicals (Mendis et al., 2005). The peptide was also rich in Glu, Val, and Gln (41%) residues which exerted scavenging effect of free radical. Furthermore, Glu is an effective cation chelator that forms complex with calcium, iron and zinc (Baumy, Guenot, Sinbandhit, & Brule, 1989).

4. Conclusion

In the present study, antioxidant activity of OEW hydrolysates and their derived-peptides were measured. To the best of our knowledge, this report is the first that introduces a novel antioxidant peptide from OEW hydrolysates. The antioxidant activities of four hydrolysates were increased with increasing the degree of hydrolysis (DH). Among different hydrolysates, trypsin hydrolysate was found to possess the highest antioxidant activity. The F_6 fraction was the most active peptide than the others. Results of amino acid composition showed that the presence of hydrophobic amino acids, such as Leu, Val, Pro and Met could be important in the antioxidant activity. Further studies on the purification of angiotensin-I converting enzyme inhibitory peptides from OEW proteins are ongoing in our laboratory.

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References

Ahmadi, F., Kadivar, M., & Shahedi, M. (2007). Antioxidant activity of *Kelussiao doratissima* Mozaff. in model and food systems. *Food Chemistry*, *105*, 57–64.

Asoodeh, A., Memarpoor-Yazdi, M., & Chamani, J. (2012). Purification and characterisation of angiotensin I converting enzyme inhibitory peptides from lysozyme hydrolysates. *Food Chemistry*, *131*, 291–295.

Baumy, J. J., Guenot, P., Sinbandhit, S., & Brule, G. (1989). Study of calcium-binding to phosphoserine residues of a-casein and its phosphopeptide (1–25) by P-31 NMR. *The Journal of Dairy Research*, *56*, 403–409.

Becker, G. L. (1993). Preserving food and health: Antioxidants make functional, nutritious preservatives. *Food Processing*, *12*, 54–56.

Bernardini, R. D., Harnedy, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A. M., et al. (2011). Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. *Food Chemistry*, *124*, 1296–1307.

Bursal, E., & Gülçin, İ. (2011). Polyphenol contents and in vitro antioxidant activities of lyophilised aqueous extract of kiwifruit (*Actinidia deliciosa*). *Food Research International*, *44*, 1482–1489.

Byun, H., Lee, J., Park, H., Jeon, J., & Kim, S. (2009). Antioxidant peptides isolated from the marine rotifer, *Brachionus rotundiformis*. *Process Biochemistry*, *44*, 842–846.
Chung, Y. C., Chang, C. T., Chao, W. W., Lin, C. F., & Chou, S. T. (2002). Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. *Journal of Agricultural and Food Chemistry*, *50*, 2454–2458.
Cui, K., Luo, X., Xu, K., & Murthy, M. R. (2004). Role of oxidative stress in neurodegeneration: recent developments in assay methods for oxidative stress and nutraceutical antioxidants. *Progress in Neuropsychopharmacology and Biological Psychiatry*, *28*, 771–799.
Dávalos, A., Miguel, M., Bartolomé, B., & Lopez-Fandino, R. (2004). Antioxidant activity of peptides derived from egg white proteins by enzymatica hydrolysis. *Journal of Food Protection*, *67*, 1939–1944.
Gao, Y., Chen, K., Zhang, B., Li, X., Chen, L., Li, Y., et al. (2010). Antioxidant and free radical-scavenging activity of the extracellular death factor in *Escherichia coli*. *Peptides*, *31*, 1821–1825.
Giménez, B., Alemán, A., Montero, P., & Gómez-Guillén, M. C. (2009). Antioxidant and functional properties of gelatin hydrolysates obtained from skin of sole and squid. *Food Chemistry*, *114*, 976–983.
Göçer, H., & Gülçin, I. (2011). Caffeic acid phenethyl ester (CAPE): Correlation of structure and antioxidant properties. *International Journal of Food Sciences and Nutrition*, *62*(8), 821–825.
Gülçin, Ï. (2006). Antioxidant and antiradical activities of L-carnitine. *Life Sciences*, *78*, 803–811.
Gülçin, Ï. (2009). Antioxidant activity of L-adrenaline: A structure-activity insight. *Chemico-Biological Interactions*, *179*, 71–80.
Gülçin, Ï. (2011). Antioxidant activity of eugenol: A structure-activity relationship study. *Journal of Medicinal Food*, *14*(9), 975–985.
Gülçin, Ï. (2012). Antioxidant activity of food constituents—An overview. *Archives of Toxicology*, *86*(3), 345–396.
Gülçin, Ï., Berashvili, D., & Gepdiremen, A. (2005). Antiradical and antioxidant activity of total anthocyanins from Perillapankinensisdecne. *Journal of Ethnopharmacology*, *101*, 287–293.
Gülçin, Ï., Topal, F., Çakmakçı, R., Bilsel, M., Gören, A. C., & Erdogan, U. (2011). Pomological features, nutritional quality, polyphenol content analysis, and antioxidant properties of domesticated and 3 wild ecotype forms of raspberries (*Rubus idaeus* L.). *Journal of Food Science*, *76*(4), C585–C593.
Li, Y. H., Jiang, B., Zhang, T., Mu, W. M., & Liu, J. (2008). Antioxidant and free radical scavenging activities of chickpea protein hydrolysate (CPH). *Food Chemistry*, *106*, 444–450.
Memarpoor-Yazdi, M., Asoodeh, A., & Chamani, J. (2012). A novel antioxidant and antimicrobial peptide from hen egg white lysozyme hydrolysates. *Journal of Functional Foods*, *4*, 278–286.
Mendis, E., Rajapakse, N., Byun, H., & Kim, S. (2005). Investigation of jumbo squid (*Dosidicus gigas*) skin gelatin peptides for their in vitro antioxidant effects. *Life Sciences*, *77*, 2166–2178.
Miguel, M., & Aleixandre, A. (2006). Antihypertensive peptides derived from egg proteins. *American Society for Nutrition*, *136*, 1457–1460.
Nalinanon, S., Benjakul, S., Kishimura, H., & Shahidi, F. (2011). Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. *Food Chemistry*, *124*, 1354–1362.
Parkin, K. L., & Damodaran, S. (2003). Oxidation of food components. *Encyclopedia of Food Science and Nutrition*, *4288*, 4294.
Philanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal*, *16*, 1306–1314.
Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, *53*, 4290–4302.
Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y., & Kim, S. K. (2005). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Research International*, *38*, 175–182.
Ranathung, S., Rajapakse, N., & Kim, S. K. (2006). Purification and characterization of antioxidative peptide derived from muscle of conger eel (Conger myriaster). *European Food Research and Technology*, *222*, 310–315.
Rival, S., Boeriu, C., & Wichers, H. (2001). Caseins and casein hydrolysates. Antioxidative properties and relevance to lipoxygenase inhibition. *Journal of Agricultural and Food Chemistry*, *49*, 295–302.
Sampath Kumar, N. S., Nazeer, R. A., & Jaiganesh, R. (2011). Purification and biochemical characterization of antioxidant peptide from horse mackerel (Magalaspis cordyla) viscera protein. *Peptides*, *32*, 1496–1501.
Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. *Peptides*, *31*, 1949–1956.
Tironi, V. A., & Añón, M. C. (2010). Amaranth proteins as a source of antioxidant peptides: Effect of proteolysis. *Food Research International*, *43*, 315–322.
Vaštag, Z., Popovic, L., Popovic, S., Krimer, V., & Pericin, D. (2011). Production of enzymatic hydrolysates with antioxidant and angiotensin-I converting enzyme inhibitory activity from pumpkin oil cake protein isolate. *Food Chemistry*, *124*, 1316–1321.
Wanita, A., & Lorenz, K. (1996). Antioxidant potential of 5-npentadecyl resorcinol. *Journal of Food Processing and Preservation*, *20*, 417–429.
Wu, H. C., Chen, H. M., & Shiau, C. Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International*, *36*, 949–957.
Xie, Z., Huang, J., Xu, X., & Jin, Z. (2008). Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. *Food Chemistry*, *111*, 370–376.
You, L., Zhao, M., Cui, C., Zhao, H., & Yang, B. (2009). Effect of degree of hydrolysis on the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates. *Innovative Food Science and Emerging Technologies*, *10*, 235–240.