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ORIGINAL ARTICLE

Purification and identification of antioxidant peptides from egg white protein hydrolysate

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Abstract Egg white proteins were hydrolysed separately using five different proteases to obtain antioxidant peptides. The antioxidant activity of egg white protein hydrolysates was influenced by the time of hydrolysis and the type of enzyme. Of the various hydrolysates produced, papain hydrolysate obtained by 3-h hydrolysis (PEWPH) displayed the highest DPPH radical scavenging activity. PEWPH could also quench the superoxide anion and hydroxyl radicals, effectively inhibit lipid peroxidation and exhibit reducing power. Then, PEWPH was purified sequentially by ultrafiltration, gel filtration, RP-HPLC and two fractions with relatively strong antioxidant activity were subsequently subjected to LC–MS/MS for peptide sequence identification. The sequences of the two antioxidant peptides were identified to be Tyr-Leu-Gly-Ala-Lys (551.54 Da) and Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe-Gln (974.55 Da), and they were identified for the first time from food-derived protein hydrolysates. Last, the two purified peptides were synthesized and they showed 7.48- and 6.02-fold higher DPPH radical scavenging activity compared with the crude PEWPH, respectively. These results indicate that PEWPH and/or its isolated peptides may be useful ingredients in food and nutraceutical applications.

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Introduction

Oxidation is an essential reaction in all living organisms and the formation of free radicals is unavoidable during oxidative metabolic process. Overproduction of free radicals is believed to be involved in the initiation or progress of several chronic diseases such as diabetes, cardiovascular diseases, neurodegenerative disorders and cancer (Butterfield et al. 2002; Bidchol et al. 2009). In foods, the development of rancid flavor and undesirable chemical compounds are the results of free radical-mediated oxidation of fatty acids and lipids. Further, oxidation of food lipids leads the deterioration of quality, shortens the shelf life of foods and may cause disease conditions following consumption of potentially toxic reaction products (Kim et al. 2007). Therefore, it is important to inhibit the oxidation and the formation of free radicals occurring in the living body and foodstuffs.

Recently, many protein hydrolysates and their isolated peptides, such as soybean protein (Chen et al. 1996), bullfrog skin (Qian et al. 2008), fish skin gelatin (Sampath Kumar et al. 2011a), egg white proteins (Dávalos et al. 2004), rice endosperm protein (Zhang et al. 2009), loach protein (You et al. 2010) and rapeseed protein (Pan et al. 2009), have been found to possess antioxidant capacity. The antioxidant activity of protein hydrolysates or peptides have been ascribed to the cooperative effect of a number of properties, including their ability to scavenge free radicals, to act as metal-ion chelator, oxygen quencher or hydrogen donor and to inhibit the lipid oxidation (Moure et al. 2006). These food-derived antioxidants are considered to be safer and without the side effects associated with the synthetic

antioxidants. What is more, protein hydrolysates present nutritional and functional properties beside their antioxidant activity (Xie et al. 2008; Chen et al. 2011).

A myriad of studies have been carried out to fractionate and purify the active peptides to identify the prominent antioxidant components from protein hydrolysates (Chen et al. 1995; Guo et al. 2009; Tang et al. 2010; Zhang et al. 2011). Although many interpretations have been put forth to explain the antioxidant properties of peptides, the relationship between the structure of the isolated peptides and their specific antioxidant activity has not been fully elucidated. However, it has been widely accepted that antioxidant peptides usually contain 3–16 amino acid residues and the antioxidant activity is more related to their composition, structure and hydrophobicity. Moreover, presence of proper amino acids and their correct positioning in peptide sequence play an important role in the antioxidant activity of peptides (Chen et al. 1996; Tang et al. 2010; Sarmadia and Ismaila 2010).

Egg white proteins are widely used as functional and nutritional ingredients in food products and their hydrolysates obtained by protease treatment are water soluble and have high nutritional value (Li-Chan et al. 1995). Some bioactive peptides from egg white proteins have also been reported (Dávalos et al. 2004; Miguel et al. 2004; Pellegrini et al. 2004; Liu et al. 2010a). In the present study, an optimum protease was selected to hydrolyze egg white protein for the antioxidant peptides and the possible mode of action of the antioxidative hydrolysate discussed. Ultrafiltration, gel filtration chromatography and RP-HPLC were used to purify the antioxidant peptides. Finally, two new egg white-derived antioxidant peptides were isolated from the hydrolysate, and their amino acids sequences were determined by LC–MS/MS.

Materials and methods

Materials

Dried egg white (DEW), spray-dried at an exhaust temperature of 60–70°C after desugarization by yeast, was provided by Hanovo (Dalian, China). Papain and neutrase were procured from Sinopharm Chemical Reagent Co, (Shanghai, China). Alcalase, flavourzyme was obtained from Novozymes North America Inc. (Franklinton, NC, USA). Trypsin, linoleic acid, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

Preparation of egg white protein hydrolysates (EWPHs)

The dried egg white was dissolved in distilled water at a concentration of 30 mg/ml and heated at 90°C for 10 min, then hydrolyzed separately using trypsin at pH 7.4, 45°C, papain at pH 6.0, 50°C, neutrase at pH 7.0, 50°C, flavourzyme at pH 7.0, 50°C and alcalase at pH 8.6, 50°C for 5 h. During hydrolysis, the pH was maintained the optimal value with 1 M NaOH or HCl. Samples were taken at 0.5-, 1-, 2-, 3-, 4- and 5-h intervals and inactivation of enzyme was achieved by heating in boiling water for 10 min. Then, the hydrolysates were centrifuged at 4,000 g for 15 min and the supernatants were collected. Finally, the supernatants were lyophilized (FDU-1100, EYELA, Japan) and stored at −20°C until use. The recovery of hydrolysate was calculated as the amount of protein present in the hydrolysate relative to the initial amount of protein present in the reaction mixture, following Chen et al. (2011).

$$\text{Recovery of hydrolysate} = C_1V_1/C_2V_2 \times 100\%$$

where C_1 and C_2 are the values of the nitrogen content of supernatant and total hydrolysates before centrifugation, respectively (grams per milliliter). V_1 and V_2 are the values of the volume of supernatant and total hydrolysates before centrifugation, respectively (milliliters).

Antioxidant activity

DPPH radical scavenging activity

The scavenging effect of EWPH on the DPPH free radical was measured as Shahidi et al. (2006) with some modifications. Briefly, a volume of 2 ml of EWPH was added to 2 ml of 0.1 mM DPPH in 99.7% ethanol. The mixture was vortexed (Vortex MaxiMix_II, Barnstead, Dubuque, IO, USA) for 10 s and left for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm (Hitachi U-2800 Spectrophotometer, Japan). The scavenging effect can be expressed in the following formula as

$$\text{DRSA}(\%) = [1 - (A_t - A_b)/A_0] \times 100\%$$

where A_t is the value of 2 ml of EWPH mixed with 2 ml of 0.1 mM DPPH, A_b is 2 ml of EWPH mixed with 2 ml of 99.7% ethanol and A_0 is 2 ml of dH_2O mixed with 2 ml of 0.1 mM DPPH.

The EC_{50} value was defined as an effective concentration of peptide that is required to scavenge 50% of radical activity. The lower the EC_{50}, the higher the free radical scavenging ability.

Hydroxyl radical scavenging activity assay

Hydroxyl radical scavenging activity was determined using the α-deoxyribose oxidation method (Chung et al. 1997). A reaction mixture containing each 0.2 ml of 10 mmol/l $FeSO_4$-EDTA and 0.5 ml of 10 mmol/l α-deoxyribose was

mixed with 0.2 ml of the sample solution (distilled water with the same volume was used as a control). 0.1 mol/l sodium phosphate buffer (pH 7.4) was added into the reaction mixture until the total volume reached 1.8 ml. Then 0.2 ml of 10 mmol/l H_2O_2 was added into the reaction mixture and incubated at 37°C for 1 h. After incubation, each 1 ml of 2.8% trichloroacetic acid (TCA) and 1.0% thiobarbituric acid (TBA) were added. Then, the mixture was placed in a boiling water bath for 15 min. Absorbance was measured at 532 nm.

$$\text{Hydroxyl radical scavenging activity}(\%) = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100\%.$$

Superoxide anion scavenging activity assay

Superoxide anion scavenging activity was determined by measuring the inhibition of the auto-oxidation of pyrogallol (Marklund and Marklund 1974). Briefly, 1.0 ml of sample was mixed with 1.8 ml of 50 mM Tris–HCl buffer (pH 8.2). The mixture was incubated at 25°C for 10 min, and then 0.1 ml of 10 mM pyrogallol (dissolved in 10 mM HCl) was added. The absorbance of the solution at 320 nm was measured up to 4 min. The oxidation rate of pyrogallol for samples was calculated as the slope of the absorbance line (ΔA_1). The autoxidation rate of pyrogallol for control was measured with 1.0 ml of distilled water (ΔA_0). The superoxide anion scavenging activity was calculated as

$$\text{Superoxide anion scavenging activity}(\%) = [(\Delta A_0 - \Delta A_1)/\Delta A_0] \times 100\%.$$

Reducing power

Reducing power of freeze-dried and spray-dried EWPH was measured following Yen and Chen (1995). Sample was dissolved in distilled water at different concentration. An aliquot (1 ml) of sample solution was mixed with 2.5 ml of 0.2 M sodium phosphate buffer and 2.5 ml of 10 mg/ml potassium ferricyanide aqueous solution. Then the mixture was incubated at 50°C for 20 min. Then, trichloro-acetic acid (2.5 ml of a 10% solution) was added to the mixture and centrifuged at 3,000×g for 10 min. The supernatant (2.5 ml) was mixed with water (2.5 ml) and 0.1% ferric chloride aqueous (0.5 ml), and absorbance was measured at 700 nm (Hitachi U-2800 Spectrophotometer, Japan). Increased absorbance of the reaction mixture indicated increased reducing power.

Lipid peroxidation inhibition

Lipid peroxidation inhibition activity was measured in a linoleic acid emulsion system (Kim et al. 2007). Simply, a sample was dissolved in 5 ml of 50 mM sodium phosphate buffer (pH 7.0) and added to a solution of linoleic acid (0.065 ml) in 99.5% ethanol (5 ml). The final volume of the solution was adjusted to 12.5 ml with distilled water. The mixture was incubated in dark (60°C) for 7 days. The degree of lipid peroxidation was measured using the ferric thiocyanate method. For that, 0.1 ml of reaction mixture was mixed with 4.7 ml of 75% ethanol at every 24-h interval. Subsequently, 0.1 ml of 30% ammonium thiocyanate aqueous solution and 0.1 ml of 0.02 M ferrous chloride aqueous solution were added, and absorbance was measured at 500 nm (Hitachi U-2800 Spectrophotometer, Japan) after 3 min. Increased absorbance of the reaction mixture indicated increased oxidation of linoleic acid.

$$\text{Inhibition of lipid peroxidation}(\%) = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100\%.$$

Purification of antioxidant peptide

Ultrafiltration

For purification of antioxidant peptides, 3 h-papain hydrolysate (PEWPH) was subjected to ultrafiltration (Vivaflow 50, sartorius stedim, Germany). PEWPH was first fractionated through a 10,000 MWCO membrane and the permeate fraction and then through a 3,000 MWCO membrane. All fractions recovered were lyophilized and named as PEWPH-I (Mw > 10 kDa), PEWPH-II (3 kDa < Mw < 10 kDa) and PEWPH-III (Mw < 3 kDa).

Gel filtration chromatography

The fraction with the highest antioxidant activity after ultrafiltration was further separated by gel filtration on ÄKTATM explorer system (GE Healthcare, USA) with Superdex peptide 10/300 (10 × 300 mm, GE Healthcare, USA) and its working molecular weight range was 100–7,000 Da. The elution buffer was 0.05 M sodium phosphate buffer (pH 7.0), flow rate 0.5 ml/min and monitored at 215 nm. The active fractions were pooled and lyophilised immediately.

Reversed-phase high-performance liquid chromatography

The fraction exhibiting strong antioxidant activity after gel filtration chromatography was further purified on ÄKTATM explorer system (GE Healthcare, USA) with a Source™ 5RPC ST 4.6/150 column (Amersham Pharmacia). The column was eluted by a linear gradient of acetonitrile (0–45%) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min. The eluted peaks were detected at 215 nm and the fraction was then lyophilised.

Amino acid sequence determination of the isolated active peptides

The purified peptides were redissolved in 20 μl of 0.1% formic acid and was injected onto a Zorbax 300 SB C18 peptide trap (Agilent technologies, Wilmington, DE, USA) and desalted with 0.2% formic acid at a flow rate of 10 μl/min for 20 min. Peptides were eluted from the trap and separated on a reversed-phase C18 column (0.15 mm × 150 mm, Column Technology Inc.) with a linear gradient of 0–50% mobile phase B (0.1% formic acid–84% acetonitrile) in mobile phase A (0.1% formic acid) over 60 min at 65 μl/min. LC–MS/MS experiments were performed with a LTQ linear ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) equipped with a microspray source. The LTQ mass spectrometer operated in the data-dependent mode with the following parameters: spray voltage (3.4 kV), spay temperature 160°C, full scan *m/z* range (400–1,800). The MS/MS spectra from the LTQ dataset were searched against the upper large database using SEQUEST algorithm. All SEQUEST searches were performed on the Bioworks 3.2 software (Thermo Finnigan). The purified peptides were synthesised (Beijing SBS Genetech Co., Ltd.) for the analysis of their antioxidant activity, using solid phase peptide synthesis methods.

Statistical analysis

All the experiments were carried out in triplicate and data were analyzed using SPSS 18.0. One-way analysis of variance and least significant difference (LSD) were used to differentiate mean values.

Results

Preparation of EWPHs and their antioxidant activity

Egg white proteins were separately hydrolyzed with papain, trypsin, flavourzyme, alcalase and neutrase at optimal conditions. The antioxidant activity of the hydrolysates was evaluated using a DPPH radical scavenging assay. As shown in Fig. 1, the antioxidant activity of EWPHs was influenced by the time of hydrolysis and the type of enzyme. For trypsin hydrolysate, the antioxidant activity increased with the increasing time of hydrolysis, while for EWPHs prepared by the other four enzyme the antioxidant activity increased with the hydrolysis time at first and then decreased gradually. Among the hydrolysates resulting from various enzymes under different hydrolysis time, the highest antioxidant activity was observed in the papain hydrolysate at 3 h hydrolysis, which presented 73.14% DPPH scavenging activity ($P < 0.05$). The recovery of PEWPH was 50.62%. Therefore, the 3-h papain hydrolysate (PEWPH) was selected for further study.

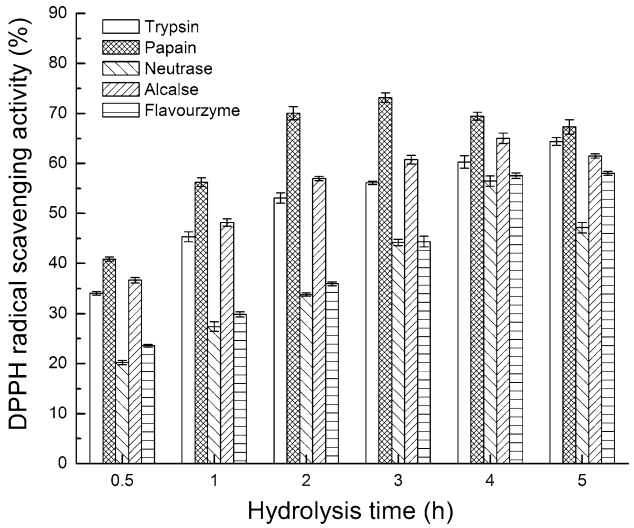

Fig. 1 DPPH scavenging activity of various enzymatic hydrolysates from egg white protein

Antioxidant activity of PEWPH

To evaluate the antioxidant activity of PEWPH, radical scavenging effects, reducing power and lipid peroxidation inhibition activity were investigated in comparison with ascorbate and BHA as positive controls. As shown in Table 1, PEWPH exhibited concentration-dependent DPPH, superoxide anion and hydroxyl radicals scavenging activities and the scavenging effect increased with increasing concentration. Moreover, the scavenging activity of PEWPH against DPPH and hydroxyl radicals was more effective than superoxide anion radical ($P < 0.05$). In reducing power test, a higher absorbance indicates a higher reducing power. The reducing power of PEWPH was also concentration-dependent as the radical scavenging activities (Table 1). However, PEWPH showed lower radical scavenging activities and reducing power than ascorbate and BHA ($P < 0.05$).

The inhibitory effect of PEWPH on the lipid peroxidation was measured in linoleic acid model system. As Fig. 2 shows, the autooxidation of linoleic acid without any antioxidants was increased fast from day 1 to day 7. However, PEWPH, ascorbate and BHA could effectively inhibit linoleic acid peroxidation and significantly prolong the induction period of the autooxidation of linoleic acid, as compared with the control ($P < 0.05$). The lipid peroxidation inhibitory activity of ascorbate and BHA decreased with time, while PEWPH was more stable over time. The lipid peroxidation inhibitory ratio of PEWPH was 69.93% (0.5 mg/ml) on the 7th day, which was higher

Table 1 Free radical scavenging activity and reducing power of PEWPH and the purified peptides (P1 and P2)

Antioxidant	Concentration (mg/ml)	DRSA (%)	HRSA (%)	SRSA (%)	Reducing power[a]
PEWPH	40	89.69 ± 2.41	83.58 ± 0.25	50.48 ± 1.75	0.824 ± 0.006
	20	83.26 ± 2.27	74.24 ± 1.05	34.87 ± 0.57	0.668 ± 0.021
	10	73.12 ± 0.98	53.14 ± 0.36	27.87 ± 0.65	0.366 ± 0.012
	5	59.90 ± 1.45	39.95 ± 0.79	20.13 ± 0.32	0.215 ± 0.004
BHA	1	90.17 ± 1.54	74.25 ± 1.05	100.00 ± 0.00	1.758 ± 0.064
	0.1	50.26 ± 0.75	42.16 ± 1.63	100.00 ± 0.00	0.964 ± 0.036
Ascorbate	1	92.04 ± 2.84	58.37 ± 0.42	100.00 ± 0.00	2.145 ± 0.075
	0.1	76.25 ± 1.24	18.25 ± 0.74	93.46 ± 1.03	1.574 ± 0.068
P1	1	73.08 ± 1.15	57.03 ± 1.24	36.27 ± 1.06	0.502 ± 0.034
	0.5	56.82 ± 0.56	45.14 ± 0.74	27.96 ± 1.24	0.327 ± 0.021
P2	1	66.27 ± 1.43	52.24 ± 0.58	32.05 ± 0.42	0.451 ± 0.012
	0.5	53.73 ± 2.06	41.07 ± 1.18	23.13 ± 0.75	0.267 ± 0.024

Ascorbate and BHA were used as positive control

DRSA DPPH radical scavenging activity, *HRSA* hydroxyl radical scavenging activity, *SRSA* superoxide anion scavenging activity

[a] Absorbance at 700 nm

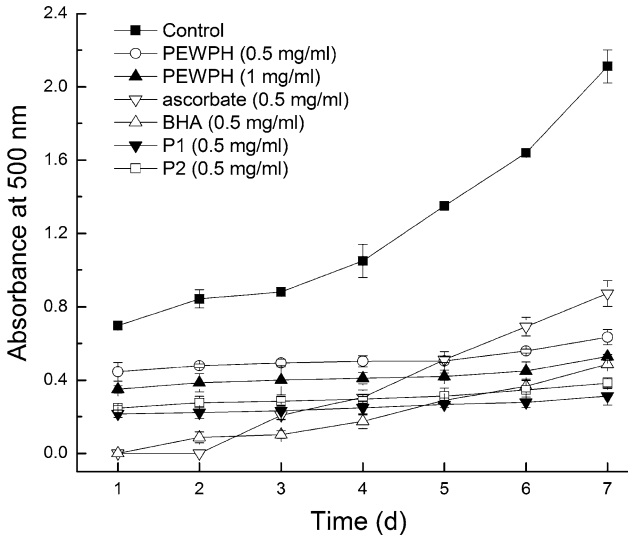

Fig. 2 Lipid peroxidation inhibition activity of PEWPH and the purified peptides (P1 and P2) in linoleic acid emulsion system. Ascorbate and BHA were used as positive control

than that of ascorbate (58.66%, 0.5 mg/ml), but lower than that of BHA (76.94%, 0.5 mg/ml).

Purification and identification of antioxidant peptide

PEWPH, having the highest antioxidant activity, further separated three MW groups using UF membranes (MWCO = 10 and 3 kDa) into PEWPH-I (MW > 10 kDa), PEWPH-II (MW = 3–10 kDa) and PEWPH-III (MW < 3 kDa). A greater DPPH radical scavenging activity (78.74% at 5 mg/ml) was observed with low-molecular-weight peptides (PEWPH-III) (table was not shown). PEWPH-III was then separated into four fractions (F1–F4) by gel filtration on ÄKTATM explorer system with Superdex peptide 10/300 column. Each fraction was pooled, lyophilised and its antioxidant activity was assayed. As shown in Fig. 3, all fractions displayed antioxidant activity and F3 possessed the highest DPPH radical scavenging activity. To further purify the antioxidant peptides, F3 was separated by reversed-phase HPLC on ÄKTATM explorer system with a Source™ 5RPC ST 4.6/150 column. Chromatography with the column produced more than 20 hydrophobicity-based subfractions shown as peaks (Fig. 4a), of which 14 peaks were collected and tested for scavenging activity against DPPH radical. As shown in Fig. 4b, all fractions showed varying degrees of DPPH radical-scavenging activity and sub-fraction 3 (F3-3) and 14 (F3-14) were identified to be highly potent. Therefore, F3-3 and F3-14 were subsequently subjected to LC–MS/MS for peptide sequence identification and their amino acid sequences were Tyr-Leu-Gly-Ala-Lys (551.54 Da) and Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe-Gln (974.55 Da), respectively (Fig. 5a, b, respectively). Following sequence interpretation and database searching, the MS–MS spectrum was matched to Ovalbumin (23–27) and Ovalbumin (128–136), respectively.

Antioxidant activity of purified peptides

Two peptides were synthesized on the basis of the sequence of the two purified peptides and named as P1 (Tyr-Leu-Gly-Ala-Lys) and P2 (Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe-Gln), respectively. Their antioxidant activity was evaluated using the radical scavenging activities, reducing

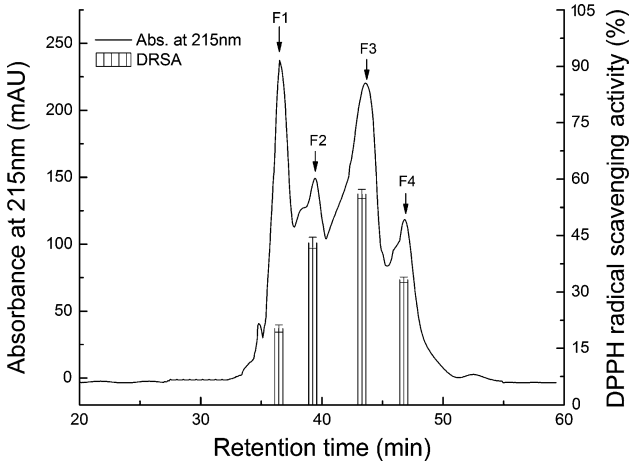

Fig. 3 Gel chromatogram of antioxidant peptides from PEWPH-III on the Superdex Peptide 10/300 GL column (10 × 300 mm) and the DPPH radical scavenging activity of the eluted peaks

power and lipid peroxidation inhibitory activity. As shown in Table 1 and Fig. 2, the two purified peptides significantly inhibited lipid peroxidation in the linoleic acid emulsion system and the activity was higher than that of ascorbate and BHA as positive control. They also exhibited reducing power and efficiently quenched different sources of free radical: DPPH, hydroxyl and superoxide radicals. The purification of PEWPH has greatly increased the antioxidant activity. P1 and P2 showed 7.48- and 6.02-fold increase in scavenging activity for DPPH radical as compared with the crude PEWPH, respectively (Table 2).

Discussion

Enzymatic hydrolysis is one of the approaches for the effective release of antioxidant peptides from protein sources. The antioxidant activity of protein hydrolysates depends on the protein substrate, proteases and hydrolysis conditions employed (Sampath Kumar 2011b). During hydrolysis, a wide variety of smaller peptides and free amino acids are generated, depending on enzyme specificity and the hydrolysis time. Changes in size, level and composition of free amino acids and small peptides affect the antioxidant activity (Wu et al. 2003). Due to the specific cleavage positions on polypeptide chain, papain, trypsin, flavourzyme, alcalase and neutrase are capable of producing antioxidant peptides when they are incorporated into hydrolyze natural protein by

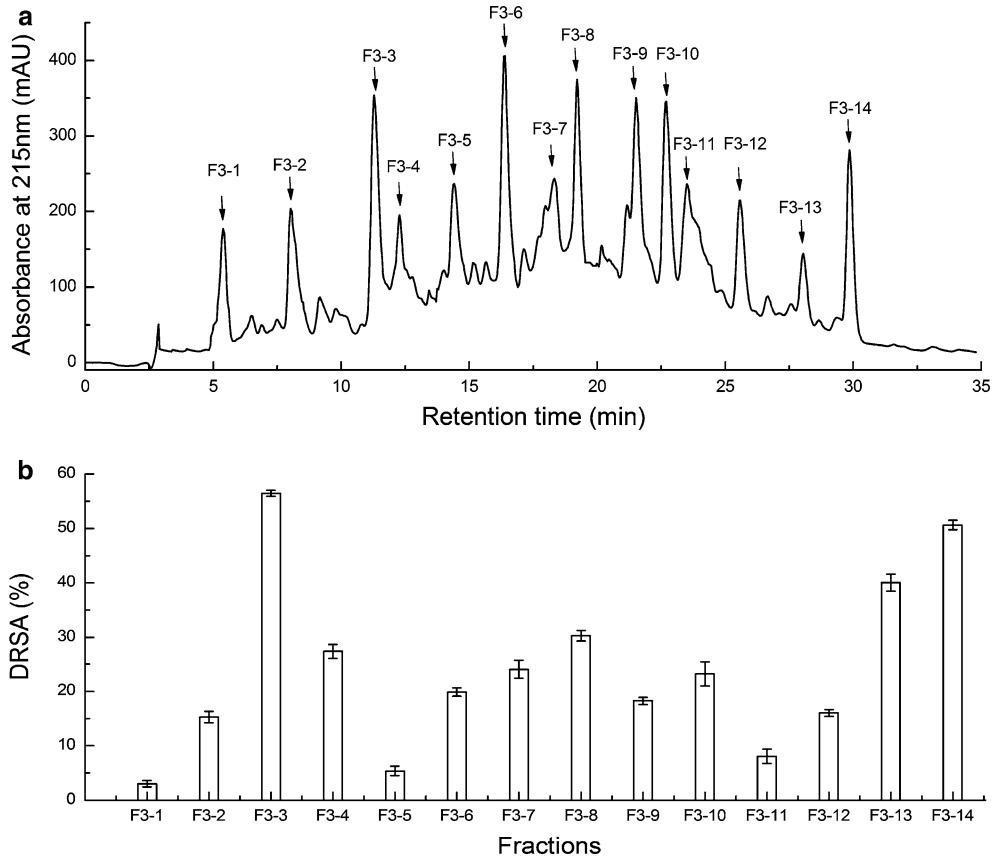

Fig. 4 RP-HLPC chromatogram of antioxidant peptides from gel chromatography fraction 3 on the Source^{TM} 5RPC ST 4.6/150 column (**a**) and the DPPH radical scavenging activity of the eluted peaks (**b**)

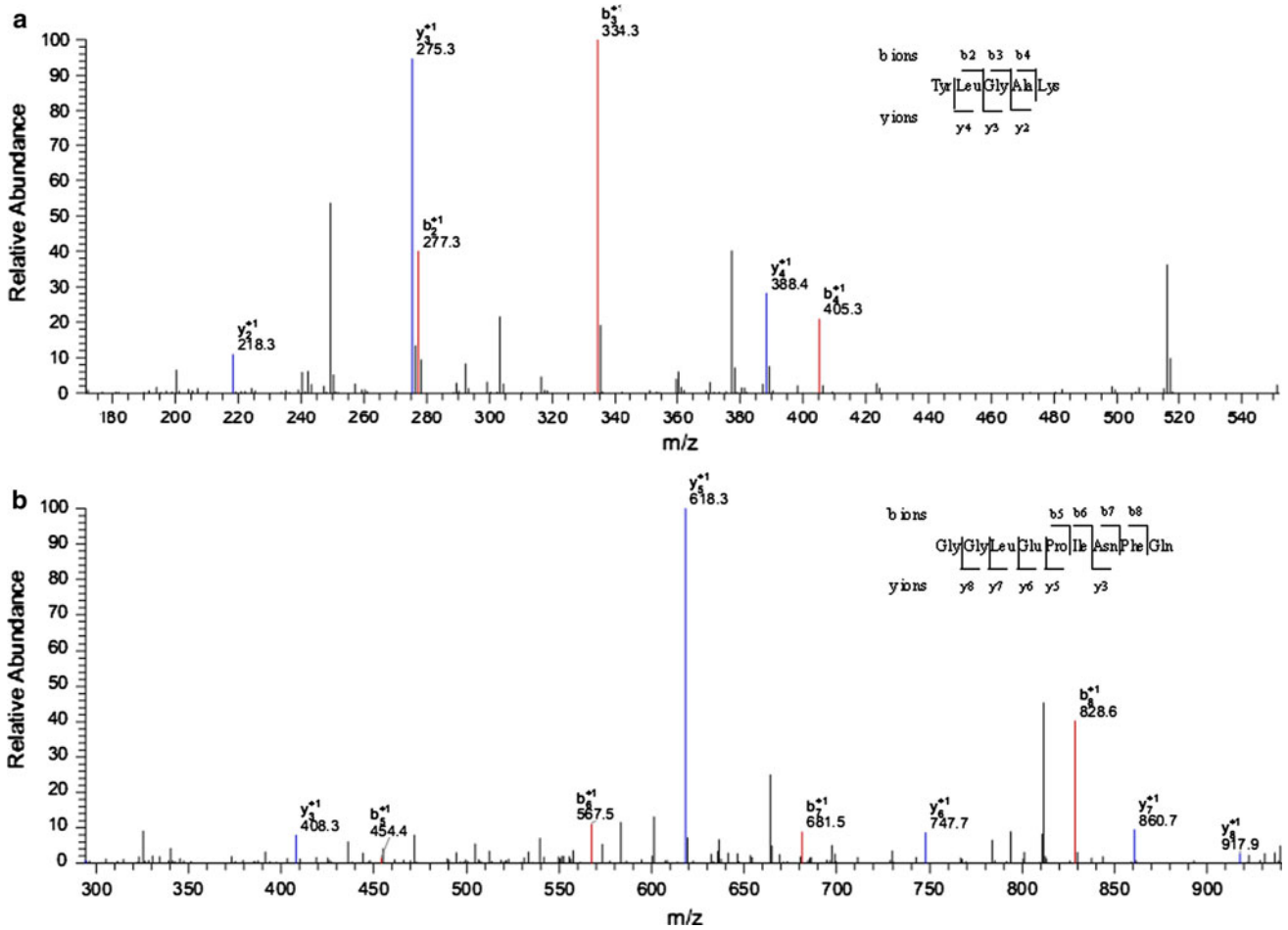

Fig. 5 Characterization of the antioxidant peptides: **a** MS/MS spectrum of F3-3; **b** MS/MS spectrum of fraction F3-14

Table 2 DPPH radical scavenging activity and purification fold in the stages of purification from egg white protein

Antioxidant	DPPH radical scavenging activity (EC_{50}, mg/ml)	Purification (fold)
PEWPH	2.35 ± 0.31	1
Ultrafiltration (PEWPH-III)	1.37 ± 0.08	1.71
Gel filtration (F3)	0.67 ± 0.09	3.51
RP-HPLC		
P1	0.31 ± 0.02	7.58
P2	0.39 ± 0.03	6.02

referring to literature (Lee et al. 2011; Mendis et al. 2005; Moure et al. 2006; Tang et al. 2010; Zhang et al. 2009). In the present study, egg white proteins was separately hydrolyzed with these five proteases, and papain hydrolysis for 3 h (PEWPH) revealed the most potent free radical scavenging ability than other protease hydrolysates. Therefore, papain was selected as an optimum protease to hydrolyze egg white protein for the antioxidant peptides and PEWPH was employed for further study.

Since the antioxidant mechanisms are diverse, the antioxidant activity of protein hydrolysates is better characterised using different assays, based on different mechanisms and using different media (Moure et al. 2006). Reducing power assay is often used to evaluate the ability of antioxidant to donate electron or hydrogen. Free radicals form stable substances by accepting donated electron and the free radical chain reactions are thus interrupted (Yen and Chen 1995). Furthermore, radical quenching is a primary mechanism of antioxidants to inhibit oxidative processes (Kim et al. 2007). The present study demonstrated that PEWPH possesses hydrogen/electron donating activity, which could make it interact with free radicals and terminate the radical chain reaction or prevent the formation of radical. PEWPH could also effectively inhibit lipid peroxidation probably by scavenging lipid-derived radicals (R·, RO· or ROO·) to stop the chain reactions or/and form a protecting membrane surrounding lipid droplets against oxidation initiators, while BHA and ascorbate afford their

protective actions in lipid peroxidation via reactions with lipid radicals, which converted the free radicals into more stable species. With more free radical generated with the increasing incubate time, more and more BHA and ascorbate were consumed. Therefore, the lipid peroxidation inhibitory activity of PEWPH was more stable over time than that of BHA and ascorbate as like the report by Zhang et al. (2011).

The antioxidant activity of PEWPH was similar to that of papain-derived hydrolysate of loach (*Misgurnus anguillicaudatus*) protein (You et al. 2010) and higher than that of porcine blood plasma protein hydrolysates prepared with alcalase (Liu et al. 2010b). However, the antioxidant potencies of PEWPH were not as great as those of the use of synthetic (BHA) or vitamins (ascorbate) in food products. But PEWPH was naturally derived from food protein sources that were consumed daily, and it was considered to be milder and safer without the side effects associated with the synthetic antioxidant. Furthermore, the hydrolysis by papain enhanced the functional properties of hydrolysate from egg white proteins (Chen et al. 2011). The incorporation of protein hydrolysates could confer desirable nutritional and functional properties to foods.

To elucidate the peptides that are responsible for the antioxidant activity in the PEWPH, PEWPH was purified sequentially by ultrafiltration, gel filtration and RP-HPLC. Finally, two new antioxidant peptides with the sequence of Tyr-Leu-Gly-Ala-Lys (P1) and Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe-Gln (P2) were obtained. The antioxidant activity of P1 and P2 has been increased after purification (7.48 and 6.02-fold higher than the crude PEWPH, respectively). You et al. (2010) has isolated an antioxidant peptide Pro-Ser-Tyr-Val from loach protein hydrolysate (LPH) by ultrafiltration and consecutive chromatographic methods. The hydroxyl radical scavenging activity of the purified peptide was 9.14-fold higher than that of crude LPH. Antioxidant peptide Asp-Val-Cys-Gly-Arg-Asp-Val-Asn-Gly-Tyr, purified by ultrafiltration and four-step RP-HPLC from duck processing by-products hydrolysate, showed 7.71-fold higher scavenging activity compared with the crude hydrolysate (Lee et al. 2010). The results further confirm the general finding that short peptides with 5–16 amino acids exhibit greater antioxidant activity than their parent native proteins or crude hydrolysates. Higher antioxidative properties by low-molecular-weight peptides are thought to be due to their low molecular weight as they can easily react with lipid radicals and thereby reduce radical-mediated lipid peroxidation (Chen et al. 1995; Wu et al. 2003).

Dávalos et al. (2004) has purified three highly potent antioxidant peptides with the sequence of Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu, Tyr-Arg-Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe and Tyr-Gln-Ile-Gly-Leu from egg white hydrolysate prepared by pepsin. The common feature of P1 and these three peptides was that they all contained Tyr at the N terminus and all consisted Leu in the sequence. The presence of a hydroxyl group in the aromatic structure of Tyr may make it act as a chain-breaking antioxidant following a hydrogen atom transfer mechanism. The antioxidative potency of peptides containing Leu has been attributed to its long aliphatic side-chain group that conceivably is capable of interaction with acyl chains of susceptible fatty acids (Zhu et al. 2008). Therefore, we speculated that Leu and N terminus Tyr probably played important roles in the antioxidant activity of P1, while P2 and Tyr-Arg-Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe have a same fragment Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe, which might be the reason for the antioxidant activity of P2. Moreover, in the sequence of the P1 and P2, there are three and four hydrophobic amino acids, representing 60 and 44% of the peptide chain, respectively. Since hydrophobicity of antioxidants is important for accessibility to hydrophobic targets (Chen et al. 1996), the presence of hydrophobic amino acids in the two purified peptide sequence were thought to be critical for the lipid oxidation inhibition by increasing solubility of peptides in lipid.

Additionally, Gly and Pro have been suggested to play an important role in radical scavenging ability of some peptides. The side-chain of Gly consists of a single hydrogen atom and may confer high flexibility on the peptide backbone. The pyrrolidine ring of Pro tends to interrupt the secondary structure of the peptide imposing conformational constraints (Rajapakse et al. 2005; Alemán et al. 2011). Glu and Lys were reported to interact with metal ions through their charged properties and to inactivate prooxidant activity of metal ions (Wu et al. 2003). Tyr, Phe, Gly and Glu have been shown to act positively as direct radical scavengers due to their ability to quench unpaired electrons or radicals by supporting protons (Zhang et al. 2009). Mendis et al. (2005) purified a radical scavenging peptide with the sequence His-Gly-Pro-Leu-Gly-Pro-Leu from the hydrolysate of fish skin gelatin; they suggested that the presence of His, Leu, Gly and Pro played an important role in the activity. In this study, P1 consisted of Tyr, Gly and Lys and P2 consisted of Phe, Glu, Pro and two Gly. Thus, it could be further presumed that the observed higher radical scavenging activity of the two purified peptides could be attributed to the presence of these amino acids.

Conclusions

In the present study, two new antioxidant peptides were purified from egg white protein hydrolysate prepared with papain (PEWPH) and their amino acid sequences were identified to be Tyr-Leu-Gly-Ala-Lys and Gly–Gly-Leu-Glu-Pro-Ile-Asn-Phe-Gln. Based on the current results,

PEWPH and/or its isolated peptides may be a promising antioxidant for functional food ingredient and pharmaceuticals. Smaller size, presence of some favorable amino acids and their specific positioning in the sequence could have been attributed to the antioxidant activity of the two peptides from PEWPH. Further research should be done in order to establish a precise structure–activity relationship of the two antioxidant peptides. Moreover, purifying and identifying antioxidant peptides in the other fractions collected by gel filtration are also needed.

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Conflict of interest The authors declare that they have no conflict of interest.

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