Asian-Aust. J. Anim. Sci.
Vol. 22, No. 11 : 1587 - 1593
November 2009

www.ajas.info

Antioxidative Activities of Hydrolysates from Duck Egg White Using Enzymatic Hydrolysis

Yi-Chao Chen*, Hsi-Shan Chang, Cheng-Taung Wang[1] and Fu-Yuan Cheng[2]

Graduate Institute of Animal Science, National Chung-Hsing University, 250, Kao-Kung Road, Taichung 402, Taiwan

ABSTRACT : Duck egg white (DEW) hydrolysates were prepared by five enzymes (papain, trypsin, chymotrypsin, alcalase, and flavourzyme) and their antioxidant activities investigated in this study. DEW hydrolyzed with papain (DEWHP) had the highest peptide content among the five enzymatic treatments. Besides, the peptide content of DEWHP increased when the enzyme to substrate ratio (E/S ratio) increased. It was suggested that higher E/S ratio contributed to elevate the degree of hydrolysis of DEW effectively. Similar results were also obtained by Tricine-SDS-PAGE. In addition, SDS-PAGE patterns indicated papain was the only one amongst all enzymes with the ability to hydrolyze DEW. In antioxidant properties, DEWHP showed more than 70% of inhibitory activity on linoleic acid peroxidation and superoxide anion scavenging. Moreover, the Fe^{2+} chelating effect of DEWHP was greater than 90%, while no significant difference was observed in DPPH radical scavenging and reducing ability. The results of peptide contents, antioxidant activities and electrophoresis suggested that the higher the peptide content, the stronger the antioxidant activities in DEWHP. (**Key Words :** Duck Egg White, Hydrolysis, Antioxidant Activities)

INTRODUCTION

Free radicals and other reactive oxygen species (ROS) are generated by exogenous chemicals or endogenous metabolic processes in food system or human body (Wang et al., 2006). The oxidative stress caused by free radicals and ROS would damage lipid and protein that resulted in cell death and tissue injury damage (Kehrer, 1993). A lot of studies reported the accumulation of peroxidants was associated with cancer, cardiovascular disease, hypertension, neurodegenerative disorders (such as Alzheimer's disease) and aging. Indeed, oxidative damage plays an important role in human diseases. Thus, ingestion of antioxidant supplements or food containing antioxidants may reduce oxidative damage in human (Lin and Yen, 1999).

Bioactive peptides could be produced by enzymatic hydrolysis of food proteins and shown to antioxidant activities against the peroxidation of lipids or fatty acids. The peptides derived from pork proteins (Carlsen et al., 2003), milk casein (Pihlanto, 2006), soy proteins (Chen et al., 1996) and egg-yolk proteins (Sakanaka et al., 2004) have been demonstrated to exhibit *in vitro* antioxidant activity. These products are accepted and recognized as safe and healthy foods by consumers. Recently, foods are considered not only as a source of nutrients but also a source of bioactive compounds. Thus, the development of food possessing bioactive functions has drawn attentions in recent time.

Duck egg is one of the important livestock productions in Taiwan. However, only few were served as fresh table eggs. Most duck eggs were processed in the manufactures, and provided as traditional Chinese-style egg products including Pidan (alkali-pickled duck egg) and salted duck egg. So far any other processed products of duck eggs are actually rare. The industry of duck egg production would be affected, once the demands of Pidan and salted duck egg decreased. Like hen egg white, proteins of DEW offered a balanced content of essential amino acids and excellent nutritional values due to about 11% of aqueous solution of proteins in Duck egg white (DEW)(Friedman, 1996). Additionally, DEW proteins not only have high nutritional values, but also possess several unique functional properties such as foaming, emulsifying and gelling that are used widely in various processed foods (Mine, 1995; Raikos et al., 2007). Many researches tried to alter functional

* Corresponding Author: Yi-Chao Chen. Tel: +886-6-5911211, Fax: +886-6-5912474, E-mail: ycchen@mail.tlri.gov.tw

[1] Council of Agriculture, Executive Yuan, 37, Nanhai Road, Taipei 100, Taiwan.

[2] Department of Hospitality Management, Toko University, 51, Sec. 2, University Road, Puzih City, Chiayi County 613, Taiwan.

Received February 17, 2009; Accepted June 29, 2009

characteristics of egg white protein by chemical or enzymatic modifications (Kato et al., 1993; Lee and Chen, 2002). However, few studies had been reported about the bioactivity properties of DEW hydrolysates by enzymatic hydrolysis. Therefore, the objectives of this study were to investigate enzymatic hydrolysis of DEW and to evaluate their antioxidant properties.

MATERIALS AND METHODS

Prepare of duck egg white (DEW)

Duck eggs were obtained from Taiwan Livestock Research Institute animal industry's poultry farm and were cleaned, broken and separated. A 600 ml albumen was homogenized (IKA type-T25BS1, Germany) at low speed for 60 s. After homogenization, the albumen was diluted 10 times with 0.1 M phosphate buffer (pH 7.0), and then used for the experiment.

Enzymatic hydrolysis of duck egg white

Papain, trypsin, chymotrypsin, alcalase and flavourzyme (from Sigma-Aldrich Co., USA) were used in this experiment. The enzymes were added at enzyme to substrate ratios (E/S ratios) of 1%, 2%, 3%, 4% and 5%. The optimal temperature and pH parameter were listed as follow: papain (40°C, pH 7.0); trypsin and chymotrypsin (40°C, pH 8.0); alcalase and flavourzyme (50°C, pH 8.0). The resulting hydrolysates were heated in boiling water for 5 min to inactivate the enzymes, and then centrifuged at 4°C and 5,000 rpm for 10 min. The supernatant was filtered by Whatman No.1 and the filtrate was stored at 5°C for analysis.

Peptides content of hydrolysates

The peptides content of hydrolysates were determined using the *o*-phthaldialdehyde (OPA) method described by Nielsen et al. (2001). The OPA reagent was prepared as follow: 3.81 g sodium tetraborate decahydrate and 100 mg sodium dodecyl sulfate (SDS) were dissolved in 70 ml distilled water. Eighty mg of OPA was dissolved in 2 ml ethanol and 88 mg dithiothreitol (DTT) was dissolved in the distilled water. The above-mentioned solutions were mixed well and adjusted the total volume to 100 ml with distilled water. 1 ml OPA reagent was added to 50 μl hydrolysates sample and mixed thoroughly. The mixtures were incubated at room temperature for 2 min before reading the absorbance of 340 nm. Serine (10-100 μg/ml) was used as a standard.

Degree of hydrolysis

Degree of hydrolysis (DH) determined using the method of Pedroche et al. (2007) and expressed in terms of percent hydrolysis. The DH was calculated as the following equation:

$$\text{DH (\%)} = \text{peptides in hydrolysate} / \text{total peptides in DEW} \times 100$$

Total peptides content in DEW was carried out by strong acid for complete hydrolysis and measuring by OPA method. Briefly, 10 ml 6N HCl was added to 1 ml sample, and then the mixture was placed in 110°C for 18 h in order to hydrolyze sample completely. Subsequently, HCl of the hydrolysate was removed by vacuum concentration, and then adjusted to the original sample volume (1 ml) by distilled water and measured by OPA method.

Total antioxidant activity in a linoleic acid system

Total antioxidant activity of duck egg white hydrolysates was determined using the linoleic acid system (Yen et al., 2000). The linoleic acid emulsion was prepared by mixing 0.5608 g linoleic acid, 0.5608 g Tween 20 and 100 ml 0.02 M phosphate buffer (pH 7.0). Sample (0.5 ml) was mixed with 2.5 ml of 0.02 M linoleic acid emulsion and 2 ml of 0.2 M phosphate buffer (pH 7.0). The reaction mixture was incubated at 37°C for 72 h in the dark, and the degree of oxidation was measured every 24 h according to method of the ferric thiocyanate (FTC)(Mitsuda et al., 1966). 0.1 ml of reaction mixture was transferred to a test tube and subsequently added 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 mM ferrous choride (in 3.5% HCl). Then, the solution was stirred for 3 min, and read the absorbance at 500 nm. The inhibition of linoleic acid peroxidation was calculated as:

$$\text{Inhibitory activity (\%)} = [1-(\text{absorbance of sample at 500 nm}) / (\text{absorbance of control at 500 nm})] \times 100$$

Scavenging effect on DPPH free radicals

The scavenging activity on α,α-diphenyl-β-picrylhydrazyl (DPPH) free radicals was measured according to the method of Shimada et al. (1992). The hydrolysate (1 ml) was mixed with 0.25 ml of 1 mM DPPH methanolic solution (prepared freshly). The mixture was shaken vigorously and incubated for 30 min in the dark at room temperature. The absorbance of reaction mixture was measured at 517 nm. The capability to scavenge DPPH radicals was expressed as (%) = [1-(absorbance of sample at 517 nm)/(absorbance of control at 517 nm)]×100.

Chelating effects on ferrous ions

Determination of chelating effects on ferrous ion was carried out as described by Dinis et al. (1994). Briefly,

sample (1 ml) was added to a solution of 2 mM ferrous chloride (0.1 ml) and 3.7 ml methanol. The reaction was initiated by adding 5 mM ferrozine (0.2 ml) and then incubated for 10 min at room temperature. Absorbance of the resulting solution was measured at 562 nm. The capability of chelating ferrous ions was calculated as (%) = [1-(absorbance of sample at 562 nm)/(absorbance of control at 562 nm)]×100.

Scavenging effects on superoxide anion

The scavenging activity on superoxide radicals was determined using the PMS-NADH superoxide generating system (Robak and Gyglewski, 1988). Sample (0.5 ml) was mixed with 0.5 ml of NBT (300 μM), 0.5 ml of NADH (936 μM), and 0.5 ml of PMS (120 μM) in 0.1 M phosphate buffer (pH 7.4). The mixture was vortex mixed and incubated for 5 min at room temperature. The absorbance was read at 560 nm. The scavenging capability on superoxide radical was calculated as (%) = [1-(absorbance of sample at 560 nm)/(absorbance of control at 560 nm)]×100.

Reducing power

The reducing power of hydrolysates was determined as described by Oyaziu (1986) with a slight modification. Sample (0.2 ml), 0.2 M, pH 6.6 phosphate buffer (0.2 ml) and 1% potassium ferricyanide (0.2 ml) were mixed well and incubated at 50°C for 20 min. Then 0.2 ml of 10% trichloroacetic acid (TCA) was added into mixture, and centrifuged at 3,000 rpm for 10 min. The supernatant (0.5 ml) was mixed with distilled water (0.5 ml) and 0.1 ml of 1% ferric chloride. After incubation at room temperature for 10 min, the absorbance was read at 700 nm. High absorbance of the reaction mixtures indicates strong reducing power.

Electrophoresis analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Desert et al. (2001), using 15% acrylamide for protein separating. Electrophoresis was carried out at 75 V in the stacking gel and at 100 V in the separating gel. Protein bands were stained with Coomassie brilliant blue R-250 (Bio-Rad, USA). The broad range of molecular weight standards from 250 to 10 kDa (Bio-Rad No.161-0363, USA) was used as the marker protein. Furthermore, the low molecular weights of hydrolysates were separated by a 16.5% Tricine-SDS-PAGE as described by Schägger and Von Jagow (1987). Electrophoresis was carried out at 75 V in the stacking gel and at 150 V in the separating gel. The polypeptide of molecular weight standards from 26.6 to 1.42 kDa (Bio-Rad No.161-0326, USA) was used as the marker protein.

Statistical analysis

In this study, the results were analyzed using the general linear-model procedure from the Statistic Analysis System software package version 8.1 (Statistic Analysis Institute, 1998). Significant differences between means were analyzed by Duncan's multiple range tests. Each experiment was conducted in triplicate.

RESULTS AND DISCUSSION

Preparation of DEW hydrolysates

Primary amino groups (or peptides) and SH-compounds (DDT or beta-mercaptoethanol) would react with OPA reagent and the complex had a peak absorbance at 340 nm (Nielsen and Dambmann, 2001). In this study, the OPA method was applied to monitor the hydrolysis of DEW with various enzymes. Change of peptide contents in different DEW hydrolysates were shown in Figure 1. Results showed that papain was the only one could hydrolyze DEW among five enzymes. When the E/S ratio of papain was 1%, the peptides contents of DEW hydrolysates would reach the peak (43.9 μg/ml) at 1 h hydrolysis and then maintained without increasing. When the E/S ratio was raised to 2%, the peptides contents of hydrolysate was 59.3 μg/ml at 1 h

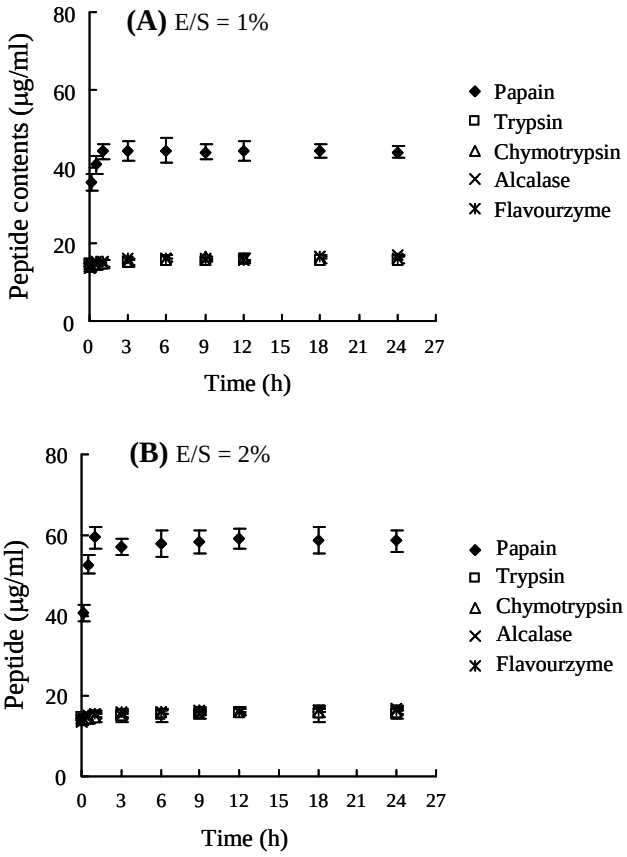

Figure 1. Changes of peptide contents in different duck egg white hydrolysates. (A) E/S = 1%; (B) E/S = 2%.

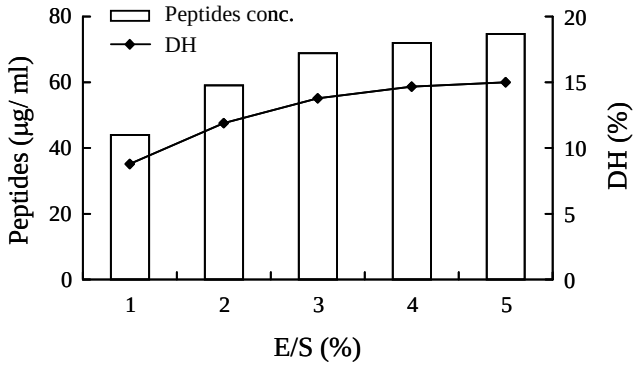

Figure 2. Changes in the peptides content and degree of hydrolysis of duck egg white hydrolysates by papain (DEWHP) by various E/S ratios of papain.

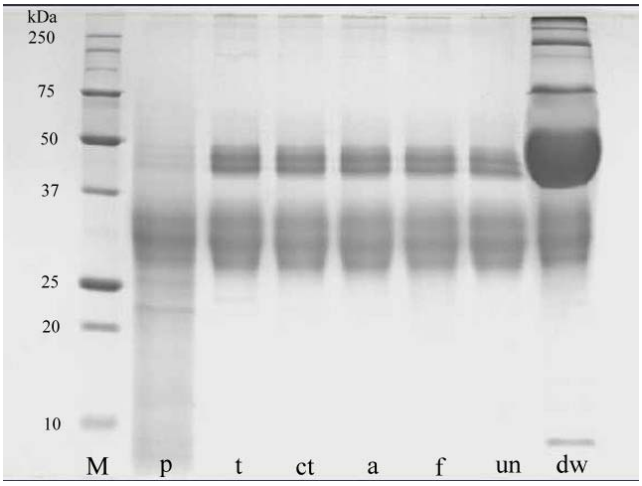

Figure 3. SDS-PAGE (15%) analysis of duck egg white hydrolysates obtained from different enzymes. M: marker, p: papain, t: trypsin, ct: chymotrypsin, a: alcalase, f: flavourzyme, un: untreatment, dw: fresh duck egg white. Lane p, t, ct, a, f (with enzymes treatment) and lane un (without enzymes treatment) were all through heat process. Lane dw was fresh duck egg white without heat.

hydrolysis and then the trend was similar to that of 1% papain. It was revealed that when the E/S ratio of papain was 1 or 2% for DEW hydrolysis, no more peptides were released after 1 h hydrolysis. However, increase of E/S ratio contributed to the increased release of peptides in DEW hydrolysis. That suggested E/S ratio was more important than the extension of hydrolysis time for the DEW hydrolysis. Similar results have been reported by Cheng et al. (2008) who hydrolyzed the by-products from meat processing factory, chicken leg bone, with Alcalase, trypsin and pepsin, and pointed out that enzymatic hydrolysis of protein substrates exhibited an initial fast reaction (0-2 h) followed by slowdown.

It is known that peptides are usually released from protein during food processing or enzymatic hydrolysis (Korhonen and Pihlanto, 2003). Guo et al. (2009) studied the optimum conditions of hydrolysis for the angiotensin-I converting enzyme (ACE) inhibitory peptides from whey protein using response surface methodology, and also revealed that a relatively high E/S ratio was desirable to promote the degree of hydrolysis. Therefore, we attempted to improve the degree of hydrolysis of DEW by increasing E/S ratio of papain. The peptides content and DH of the hydrolysates of DEW by various E/S ratios were showed in Figure 2. Higher peptides contents were obtained following the increases of E/S ratio. But the percentage of peptides contents increased slowly when the E/S ratio was higher than 3%. Similar results were found in DH.

The composition and structure of DEW were similar to those of chicken. Most of albumen is water soluble and globular proteins (Watanabe et al., 1981; Powrie and Nakai, 1985) and is difficult to break using proteases because of its quaternary structure where numerous peptides are buried (Lee et al., 1999). In addition, egg albumen was known to contain inhibitors against various enzymes such as ovomucoid, ovoinhibitor, and etc. (Adler-Nissen, 1985) as a consequence, it was difficult to hydrolyze egg albumen. However, papain, a cysteine protease, was useful in tenderizing meat and other proteins by cleaving peptide bonds of basic amino acids. It can degrade large molecules of proteins into small fragments. Lee and Chen (2002) hydrolyzed chicken egg white using papain and obtained several functional properties such as solubility, foaming capacity and cake volume. In current study we selected five proteases and tried to hydrolyze the native DEW, and found that only papain was able to hydrolyze DEW. The other proteases did not work in DEW hydrolysis (they belonged to serine proteases). In other words, cysteine protease applied to DEW hydrolysis was more efficient than serine protease. Moreover, higher E/S ratio attributed to obtain more peptides in DEW hydrolysis.

Electrophoresis analysis of DEW hydrolysates

SDS-PAGE analysis of DEW hydrolysates using various enzymes was shown in Figure 3. In the protein profiles, there were no obvious differences among the control (lane un) and DEW with enzymatic treatments (lanes t, ct, a and f), except DEWHP. The protein fragments more than 50 kDa of molecular weights were disappeared in DEW hydrolysates comparing with fresh duck egg white (lane dw) due to stop enzymes activities by heating that resulted in large protein fragments aggregating and filtered out. In contrast, the proteins fragments between 25-37 kDa appeared to be more stable under heating. In DEWHP, the migration of 50-40 kDa and proteins below 25 kDa was observed and suggested only papain was able to hydrolyze DEW into small protein fragment, and degraded proteins

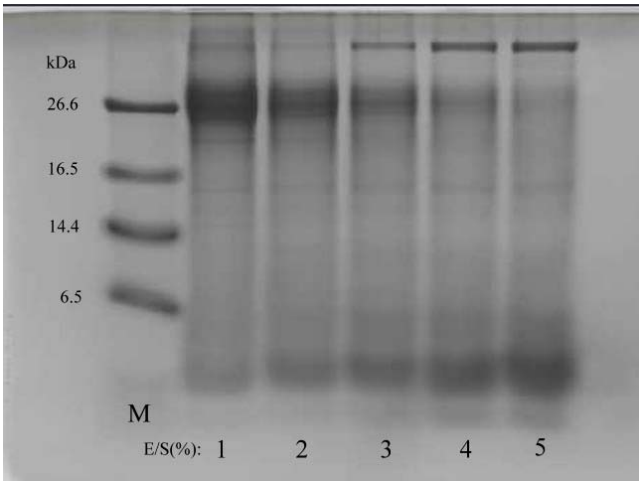

Figure 4. Tricine-SDS-PAGE (16.5%) analysis of DEWHP by various E/S ratios of papain. M: marker. Lane 1-5 represented the DEWHP obtained from papain with 1-5% of E/S ratios.

about 50 kDa into <25 kDa. To improve the resolution of small proteins in the DEWHP, the 16.5% Tricine-SDS-PAGE was utilized and shown in Figure 4. The intensities of bands about 26.6 kDa gradually decreased while those of <6.5 kDa increased gradually with the increase of E/S ratio. This indicated that the small proteins fragments of DEWHP (<6.5 kDa) were produced by the degradation of protein fragments of 26.6 kDa. In addition, it was also observed that the intensity of protein band above 26.6 kDa increased depending on increase of E/S ratios.

Antioxidant activity of DEWHP

Total antioxidant activity was measured by ferric thiocyanate (FTC) method that was adopted to evaluate inhibition activity of lipid peroxidation in the linoleic acid emulsion system. The inhibition activity of DEW hydrolysates obtained from papain hydrolysis for 1 h (DEWHP) on lipid peroxidation was presented in Table 1. DEWHP and BHT treatments significantly attenuated the peroxidation of linoleic acid comparing to control ($p<0.05$). Higher E/S ration resulted in stronger total antioxidative activity (The inhibitory activities of DEWHP on peroxidation of linoleic acid were 30.35%, 49.14%, 70.14%, 73.67% and 74.48% when E/S ratios were from 1% to 5%, respectively). The antioxidative activity of DEWHP sharply increased when the E/S ratio from 1% to 3%. However, no significant differences were obtained from DEWHP when the E/S ratio was greater than 3% ($p>0.05$). Those were similar to results of peptides contents in DEWHP, which revealed the antioxidative activity was positively related to peptide contents. The results of our work were consistent with Sakanaka et al. (2004) who studied on the antioxidative activity of egg-yolk protein hydrolysates.

Evaluation of the antioxidant properties of DEWHP

A broad variety of *in vitro* assays has been developed to evaluate the antioxidant activities of protein hydrolysates. The different antioxidant properties of DEWHP were summarized in Table 1. Free radical scavenging ability was a main mechanism in antioxidant (Pihlanto, 2006). DPPH was a stable compound with free radical residues and used extensively as a substrate to evaluate the proton-radical scavenging action of antioxidants (Duh and Yen, 1997). DPPH scavenging effects of DEWHP with E/S ratio from 1 to 5% were about 30 to 35%, while BHT (0.1%) was used as a positive control and showed 95.02%. Wu et al. (2003) stated that DPPH scavenging effect of mackerel hydrolysates was about 80%. In this study, DEWHP did not exhibit strong effect on DPPH scavenging. The same observations were discovered in reducing power ability (Table 1). Some literatures reported that reducing power could offer an electron donor to end the chain reaction of free radicals associated with antioxidant activity (Yen et al., 2000; Lin and Chen, 2004; Liu et al., 2005). Though no consistent result was found in our work. In scavenging

Table 1. Inhibition of linoleic acid peroxidation, DPPH radicals scavenging effects, superoxide anion scavenging effects, reducing ability and Fe^{2+} chelating effects of DEWHP obtained from various E/S ratios of papain

E/S (%)	Inhibition of linoleic acid autoxidation	DPPH radicals scavenging effects	Superoxide anion scavenging effects	Fe^{2+} chelating effects	Reducing ability OD_{700}
	%				
1	30.25[e]	35.19[b]	51.20[f]	88.81[d]	0.170[b]
2	49.14[d]	33.63[c]	63.21[e]	90.28[c]	0.120[b]
3	70.76[c]	31.53[d]	70.57[d]	91.99[b]	0.115[b]
4	73.67[b]	31.83[d]	72.29[c]	92.65[b]	0.110[b]
5	74.48[b]	30.51[e]	73.87[b]	92.67[b]	0.106[b]
BHT[1]	84.88[a]	95.02[a]	-	-	2.241[a]
SOD[1]	-	-	85.41[a]	-	-
EDTA[1]	-	-	-	98.70[a]	-

[1] As a positive control: BHT (1 mg/ml); SOD (0.5 mg/ml); EDTA (0.5 mg/ml).
[a-e] Means on the same column with different superscripts are significantly different ($p<0.05$).

Table 2. The correlation coefficients between the amounts of the peptides and the antioxidative properties of DEWHP

	Peptides	FTC[1]	DPPH	$SupO^{2-}$	Fe^{2+}	Ra
E/S ratio	0.9373[2]	0.9223	-0.9481	0.9213	0.8994	-0.8558
	0.0187[3]	0.0257	0.0141	0.0262	0.0377	0.0643
Peptides	-	0.9909	-0.9702	0.9989	0.9896	-0.9707
		0.001	0.0062	<0.001	0.0013	0.006

[1] FTC = Inhibition of linoleic acid autoxidation by FTC method; DPPH = DPPH radicals scavenging effects; $SupO^{2-}$ = Superoxide anion scavenging effects; Fe^{2+} = Fe^{2+} chelating effects; Ra = Reducing ability.
[2] Pearson correlation coefficient. [3] Probability.

activity of superoxide anion radical, DEWHP would effectively scavenge superoxide anion radical (>70%) when the E/S ratio exceed 3% and the scavenging effects was exhibited dose-dependent. However, the scavenging activity of DEWHP was not higher than that of SOD (0.05%), which was used as a positive control. Superoxide anion radical (O_2^-) is known to be generated in most biological systems and harmful to cellular components as a precursor contributing to tissue damage (Halliwell, 1994). Its toxic role could be eliminated by superoxide dismutase (SOD), which converts superoxide anion radical possesses into hydrogen peroxide and oxygen (Lee et al., 2005). Therefore, antioxidants with scavenging activity on superoxide anion radical seemed to possess protecting activity against cellular damages. The results in a current study agreed with previous researches that conducted on fermented soymilk (Wang et al., 2006) and the hydrolysates of tuna protein (Je et al., 2007).

Metal ions such as Fe^{2+} and Cu^{2+} were reported to generate free radicals that could initiate lipid peroxidation and start a chain reaction (Halliwell and Gutteridge, 1990). Therefore, chelating ability of Fe^{2+} could inhibit metal-catalyzed lipid oxidation. The DEWHP exhibited significant Fe-chelating effects (>90%) when the E/S ratios were over 2%. Amino acids and proteins are reported to have metal binding ability (Nelson and Potter, 1979), which agrees with our results.

The correlation coefficient was estimated in order to understand the quantitative effects of the peptides contents upon the antioxidant properties (Table 2). The peptides contents were positively correlated with antioxidant properties including inhibitory activity of linoleic acid, superoxide anion scavenging effect and Fe^{2+} chelating, while negatively correlated with DPPH scavenging effect and reducing power. In addition, DEWHP had low DPPH scavenging effects (30%) and reducing power (0.106-0.170), which suggesting that the antioxidant activity of DEWHP was not related to the free radical scavenging activity or reducing power of the peptides. In conclusion, our work showed papain was the only one with ability to hydrolyze duck egg white among all enzymes, and higher E/S ratio contributed to elevate the degree of hydrolysis of DEW effectively. Moreover, the results of peptides contents, antioxidant activities and electrophoresis suggested that peptides contents are directly related to the antioxidant activity in DEWHP. However, further study is necessary to characterize the antioxidant peptides in DEWHP.

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