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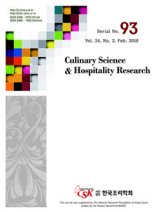

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Increase of Antioxidant Activities of Egg White Protein Hydrolysate by Fractionation without Using Toxic Chemicals

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KEYWORDS

Antioxidant,
DPPH,
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ORAC,
Peptide,
Metal-chelating,
Isoelectric point,
Autofocusing,
Protein hydrolysates.

ABSTRACT

The objectives of the present study were to examine the antioxidant activity of autofocusing fractions from egg white protein hydrolysates and obtain higher antioxidant peptide fraction, which could be applied to the food model system. Alkaline protease hydrolysate of egg white protein exerted higher antioxidant activities than other protein hydrolysates and were fractionated on the basis of the amphoteric nature of sample peptides by preparative isoelectric focusing without toxic solvents and reagents, which is termed autofocusing. Neutral and basic fractions showed higher 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity than the acidic fractions. The acidic and neutral fractions showed higher hydroxyl (OH) radical scavenging activity and oxygen radical absorbance capacity (ORAC) values than the basic fractions. The acidic fractions showed higher metal chelating activity than basic fractions. Antioxidant activities of some autofocusing fractions except for ORAC showed higher compared to the crude hydrolysate. These results suggest that peptides fractions from egg white protein are effective antioxidant, and that autofocusing could be useful to increase antioxidant activity for application to food system.

1. INTRODUCTION

Lipid oxidation is one of the most important factors that influence the quality and acceptability of foodstuffs and prepared foods. Products of lipid oxidation are responsible for unacceptable off-flavors and off-odor during cooking, handling, processing and storage. Namely, lipid oxidation causes shortens shelf life and quality losses (Kingston, Monahan, Buckley, & Lynch, 1998).

For many years, antioxidants have been used safely and effectively to retard oxidative deterioration of foods. Most antioxidants used as food additive, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are synthetic antioxidants. Although they have strong antioxidant activity, various potential side effects and toxic effects on the human enzyme system have been reported (Park, Kwon & Rha, 2014; Wanita & Lorenz, 1996,). Therefore, developments of safe and nontoxic natural antioxidants have been popular topics in recent decades. Numerous studies have been conducted to investigate the antioxidant properties of food protein hydrolysates from plant and animal sources. For example, antioxidant activity has been found in enzymatic hydrolysates

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of soybean (Pena-Ramos & Xiong, 2002), peanut (Sirtori, Isak, Resta, Boschin & Arnoldi, 2012), and rice (Zhang et al., 2010). Antioxidant peptides from animal resources are mainly extracted from enzymatic hydrolysates of fish protein (Ren et al., 2008), milk protein (You et al., 2010), whey protein (Bayram, Pekmez, Arda, & Yalcin, 2008), egg yolk protein, egg white protein (Memarpoor-Yazdia, Asoodehb, & Chamania, 2012), and meat protein (Zhang et al., 2010). Studies have shown wide effective in protein hydrolysates to suppress lipid oxidation in cooked meat patties (Park et al., 2012; Peña-Ramos & Xiong, 2003; Salminen, Kivikari & Heinonen, 2006; Wang & Xiong 2005).

Egg white consists of approximately 40 proteins and serves as important dietary nitrogen source (Miguel & Aleixandre, 2006). The egg white proteins show enzymatic and non-enzymatic biological activities, such as antimicrobial, vitamin and metal-binding activities, and etc (Ibrahim, 1997). It has been demonstrated that enzymatic hydrolysates of egg white proteins exert some biological activities such as attenuation of colitis (Kobayashi et al., 2015) and so on. However, there are limited studies on antioxidant activity of egg white hydrolysate.

Therefore, the primary objective of this study was to compare the antioxidant activity of enzymatic hydrolysates of egg white proteins with other animal source protein hydrolysates.

The antioxidant activity was determined using four different methods, including the scavenging activity on the 1,1-diphenyl-2-picrylhydrazyl (DPPH), metal ion chelating activity, oxygen radical absorbance capacity, and hydroxyl radical(OH) scavenging activity. Furthermore, egg white protein hydrolysates prepared by using two commercial enzymes were fractionated by autofocusing, a preparative isoelectric focusing based on amphoteric nature of sample peptide, to obtain higher antioxidant peptide fraction, which could be applied to the food model system.

2. MATERIALS AND METHODS

2.1. Chemicals

Chemicals. Linoleic acid, butylated hydroxytoluene (BHT), DPPH, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,3,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). A cod roe peptide and surimi peptide were kindly provided by Nippon Suisan Kaisha, Ltd. (Tokyo, Japan). Egg white peptide (commercial name; RunPep was kindly provided by Pharma Foods International Co., Ltd. Tween 20 was purchased from Nacalai Tesque (Kyoto, Japan). Alkaline protease (commercial name; Orientase 22BF) was kindly provided by HBI enzyme Inc (Hyogo, Japan).

2.2. Preparation of Egg White Protein Hydrolysates (EWPs) from Hen Egg White

Hen eggs were obtained from a local market and brought to laboratory, stored at 4℃ < 2h before experimental work. The hen eggs were broken and the egg whites were collected. A total of 100 g of egg whites was mixed with 100 mL deionized water, and then were denatured by 20 min of heating at 70℃. After adjusting the pH to 8.0 with 1 M NaOH solution, and the temperature to 60℃, alkaline protease Orientase 22BF (0.1%, w/w) was added and protein digestion was carried out for 180 min. After the hydrolysis, the enzyme was inactivated by boiling for 15 min. The hydrolysate was centrifuged in a refrigerated centrifuge (Tomy Seiko Co., Ltd., Tokyo, Japan) at 2,300 rpm for 30 min, and the supernatant was then freeze-dried and the powder was stored −20℃ for following experiments.

2.3. Fractionation of Peptides in RunPep (RPs) and Egg White Protein Hydrolysates (EWPs)

Peptides in EWPs were fractionated by autofocusing by the method of Hashimoto et al.(2006). using a tank (975 mm in length × 200 mm inner width × 120 mm in height) with 10 sample compartments (66.5 mm in length × 80 mm in width × 80 mm in height). Sample compartments 5 and 6 were filled with 500 mL of sample water solution (5%), and the other compartments were filled with deionized water. Autofocusing of peptides was performed at a constant voltage of 500 V (DC) for 24 h. All fractions were collected after autofocusing and adjusted to pH 7.0 and subsequently freeze-dried prior to testing for antioxidant activities *in vitro* systems.

2.4. Amino Acid Analysis

To monitor the fractionation of peptides by autofocusing,

Table 1. Enzyme characteristics

Enzyme	Source	Optimum condition		Type
		Temperature (℃)	pH	
Orientase 22BF	*Bacillus* sp.	50～60	8.0～9.0	Complex

amino acid analysis of the autofocusing fractions was performed according to the methods of Bidingmeyer et al. (1984). with slight modification.

2.5. DPPH Radical-Scavenging Activity

DPPH radical-scavenging activity of some protein hydolysates and the autofocusing fractions in aqueous solutions were tested as follows. Samples, BHT, and Trolox (15 or 50 mg for each) were dissolved in 2 mL of 10 mM sodium phosphate buffer, pH 7.0. To the sample solutions, 2 mL of ethanol and 1 mL of DPPH solution (0.5 mM DPPH in ethanol) were added. The decrease in absorbance at 517 nm of DPPH radical was measured using a Beckman DU-640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) 30 min after the addition of DPPH solution.

2.6. OH Radical-Scavenging Activity

The OH radical-scavenging activity of some protein hydolysates and the autofocusing fractions were measured by a chemiluminescence method, based on the emission of light by reaction of luminol with Fenton reaction-induced OH radical, with the antioxidant measurement kit Radical Catch (ALOKA Co., Ltd., Tokyo, Japan); 50 μL of the cobalt solution and 50 μL of the luminol solution were mixed with 20 μL of sample solution (0.5, 1.25, 2.5, and 5% peptide) and incubated for 5 min at 37℃. Generation of OH radicals was initiated by the addition of 50 μL of H_2O_2 solution. Light emission at 430 nm was measured for 120 s immediately after the initiation. Ultrapure water (Simplicity systems; Millipore, Billerica, MA) was used as a control. Light emissions from 80 to 120 s were integrated. The rate of decrease in light emission as compared to the control was expressed as the antioxidant activity. Regression lines were fitted to where the linear correlations were observed between light emission and gradient concentration of the samples. IC_{50} values (mg/mL) were determined as the concentration of samples where light emission decreased by a half of control using the regression lines.

2.7. ORAC

The antioxidant activity was measured using the oxygen radical absorbing capacity (ORAC) assay according to the method of Huang et al. with slight modification. The ORAC assay can detect the scavenging activity of AAPH-generated peroxyl radicals induced by a sample, which is monitored by the prevention of loss of fluorescein degradation by peroxyl radicals. In brief, fluorescein and AAPH were dissolved in 75 mM potassium phosphate buffer, pH 7.4. Samples (1 mg) were dissolved in 75 mM phosphate buffer, pH 7.4. A 200 μL aliquot of fluorescein (94.4 nM), 20 μL of serially diluted samples (0.025, 0.01, and 0.005%), and 75 μL of AAPH (31.7 mM) solutions were dispended into a 96-well plate. Trolox (50, 25, 12.5, and 6.25 μM) solution prepared in the 75 mM phosphate buffer was used as the positive control. Degradation of fluorescein was measured as a decline in fluorescence (Ex., 485 nm; Em., 520 nm), which was measured every 2 min for 90 min at 37℃ using a Powerscan HT microplate fluorescence reader (DS Pharma Biomedical, Osaka, Japan). The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence decline curve. The net AUC of the sample was calculated by subtracting the AUC of the blank. The regression equation between net AUC and Trolox concentration was determined, and ORAC values were expressed as micromoles of Trolox equivalents (TE) per 1 g of peptide using the Trolox standard curve.

2.8. Ferrous Iron (Fe^{2+})-Chelating Activity

The chelating activity of some protein hydolysates and the autofocusing fractions for ferrous ions were measured according to the ferrozine method with minor modifications. A 3 mL aliquot of each sample solution was mixed with 0.1 mL of 2 mM ferrous chloride ($FeCl_2$). After 5 min, the reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), and the mixture was incubated at room temperature for 30 min. A mixture of 3 mL of water, $FeCl_2$, and ferrozine was used as the blank. A mixture of 3 mL of sample with 0.1 mL of 2 mM $FeCl_2$ and 0.2 mL of water was used as the control. After 30 min incubation, absorbance was read at 561 nm. The percent metal chelating ability was calculated according to the following formula:

$$\text{Chelating ability}(\%) = \frac{[A_{blank} - (A_{sample} - A_{control})]}{A_{blank}} \times 100$$

where A_{blank}=absorbance of the blank, A_{sample}=absorbance of the sample, and $A_{control}$=absorbance of the control sample at 561 nm.

2.9. Statistical Analysis

Statistical comparisons were made using Fisher's PLSD

method after one-way analysis of variance (ANOVA) using StatView 4.11 (Abacus Concepts Inc., Berkeley, CA). The results were considered significantly different at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Antioxidant Activities of Protein Hydrolysates

As shown in Table 2, RPs and EWPs had higher DPPH radical-scavenging activities than other protein hydrolysates. Also, the RPs and the EWPs showed the higher OH radical -scavenging activities, ORAC values and chelating activity than the other hydrolysates.

3.2. Fractionation of Peptides in the RPs and EWHs

Peptides in the RPs and the EWPs showing higher antioxidant activities than the other protein hydrolysates were fractionated by autofocusing. Fig. 1(a) and Fig. 1(b) show pH gradient and distribution of peptides in the autofocusing fractions after the focusing of RPs and EWPs, respectively. In the case of RPs, more than 85% of the peptides were distributed between Fr. 3 and 6 (3<pH<9). Approximately 12% peptides were recovered in basic fractions (pH>12 ; Fr. 7～10). A low % of peptides were recovered in the acidic fractions (pH<3; Fr. 1～2). In the case of EWPs, more than 83% of the peptides were distributed between Fr. 4 and 8 (3<pH<8). Approximately 11% peptides were recovered in Fr. 9 and Fr. 10. Approximately 7% peptides were recovered in the Fr. 1, Fr. 2, and Fr. 3. The peptides in the acidic and basic fractions were characterized by a higher content of acidic and basic amino acids, respectively (Figure 2a and Figure 2b). On the basis of pH profile and amino acid composition, as Fig. 1 and 2, autofocusing fractions were combined in equal amounts as follows: RPs1 (strong acidic, Fr. 1～3), RPs2 (acidic, Fr. 4), RPs3 (weak acidic, Fr. 5), RPs4 (weak basic, Fr. 6), RPs5 (basic, Fr. 7-10), EWPs1 (acidic, Fr.1-6), EWPs2 (neutral, Fr. 7-8), EWPs3 (basic, Fr. 9-10).

3.3. DPPH Radical Scavenging Activity of Autofocusing Fractions

When DPPH radical encounters a proton-donating substance such as an antioxidant, the radical is scavenged, and

Table 2. Antioxidant activities of some protein hydrolysates

Protein hydrolysates	OH radical scavenging activity IC_{50} value (mg/mL)	ORAC trolox equivalents (μmole/g peptides)	Metal chelating activity (%)	DPPH radical scavenging activity (%)
CRPs	12.67±0.61[a]	688.95±28.07[a]	5.65±1.31[a]	12.47±0.72[a]
SMPs	19.49±0.70[b]	867.80±31.73[b]	6.68±1.83[a]	13.25±0.83[a]
RPs	11.58±0.51[c]	853.10±35.79[b]	23.74±1.70[b]	49.64±1.40[b]
EWPs	5.80±0.74[d]	1,835.22±30.98[c]	9.03±2.16[c]	18.28±0.52[c]

Each value represents the mean four replicates±standard deviations.
Different lettters above bars represent statistically significant differences ($p<0.05$).

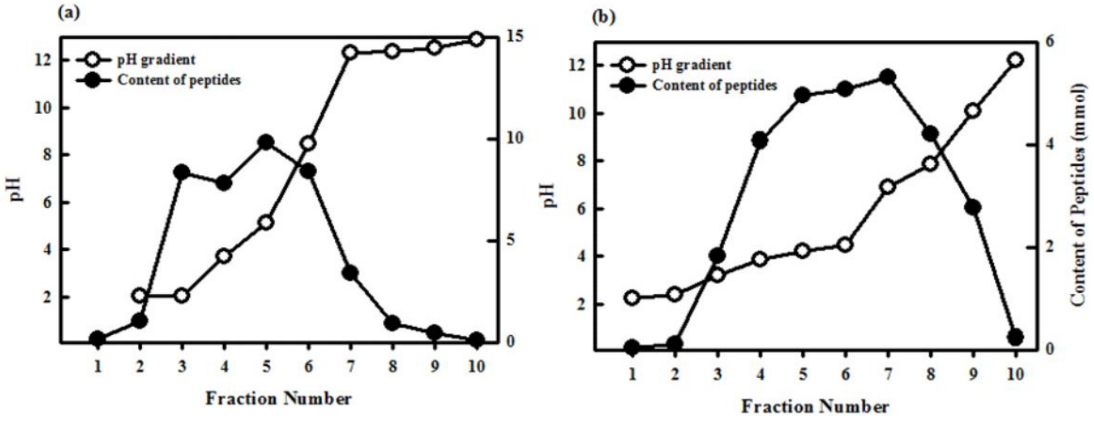

Fig. 1. Contents of peptide and pH gradient of autofocusing of RPs (a) and EWPs (b).

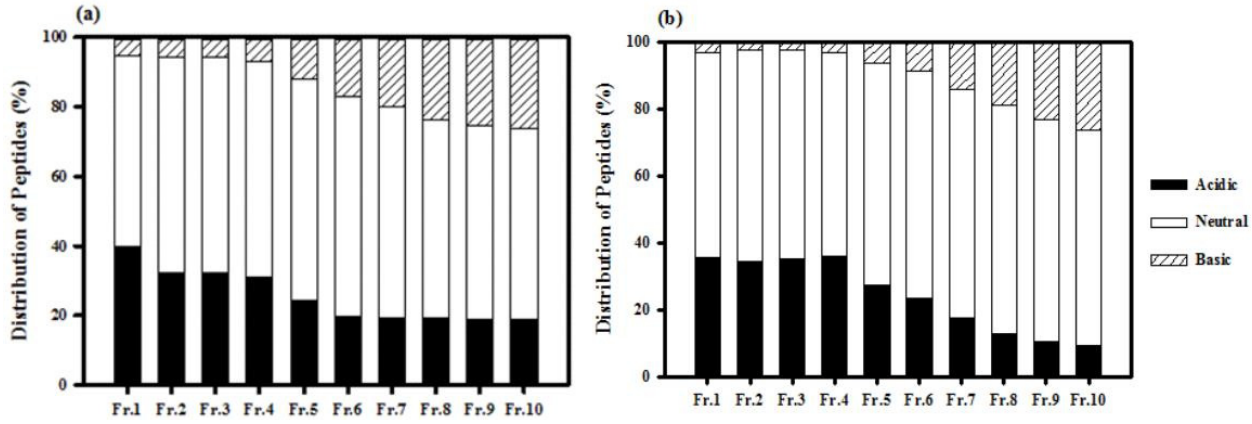

Fig. 2. Amino acid compositions of autofocusing fractions of RPs (a) and EWPs (b).

the absorbance is reduced by changing the color. Therefore, DPPH is often used as a substrate to evaluate the antioxidant activity of an antioxidant (Jung, Kim, Jeong & Choi, 2017; Liu, Kong, Xiong & Xia, 2010).

Fig. 3 shows the DPPH radical-scavenging activity of the autofocusing fractions from the RPs and EWPs. The EWPs and their autofocusing fractions exhibited higher antioxidant activity compared to RPs and their autofocusing fractions. In the case of RPs, the DPPH radical-scavenging activity was higher in the strong acidic fractions (RPs1) than that in the other fractions. Autofocusing fractions except for RPs1 had a lower DPPH radical-scavenging activity than the crude hydrolysate before fractionation. In the case of EWPs, all fractions showed higher DPPH radical scavenging activity than RPs fractions. Especially, neutral (EWPs2) and basic fractions (EWPs3) of EWPs showed significantly higher than that of the crude hydrolysate, and the neutral (EWPs2) and basic fraction (EWPs3) exhibited high DPPH radical scavenging activity equivalent to BHT and Trolox, synthetic antioxidants. BHT and Trolox reduced radical by almost 93 and 91%, respectively. Thus, we can infer that RPs, EWPs, and their fractions contained antioxidant peptides that were capable of scavenging radicals. It has been reported that the high level of DPPH free radical scavenging activity of protein hydrolysates was associated with a high amount of hydrophobic amino acids of peptides (Rajapakse, Mendis, Jung & Kim, 2005; Ren et al., 2008; Zang, Duan & Zhuang, 2012; Zarei et al., 2014). From the DPPH free radical scavenging activities assessment, we deduced that some hydrophobic amino acids possibly exist in the antioxidant peptides. Future studies in identification of amino acids sequence of peptide in the active fractions are necessary to understand the mechanism of antioxidant activity.

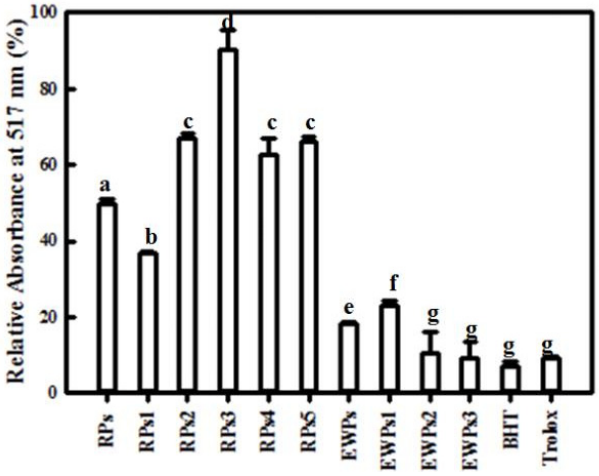

Fig. 3. DPPH radical-scavenging activity of fractions of RPs (a) and EWPs (b) with different isoelectric point (pI). The concentration of each sample is 1%. Each value represents the mean four replicates±standard deviations. Different lettters above bars represent statistically significant differences (p<0.05).

3.4. OH Radical Scavenging Activity of Autofocusing Fractions

OH radical is the most reactive radical that can induce severe damage to adjacent biomolecules and thereby cause aging, cancer, and other diseases. Therefore, removal of OH radical could be one of the most effective defenses against these various diseases (Jeong, Park, Lam & de Lumen, 2003; Wang, Huang, Chen, Huang & Zhou, 2015).

Fig. 4 shows the OH radical-scavenging activity of the autofocusing fractions from the RPs and EWPs. Among the ten peptide fractions, strong basic fractions (pI>10.0) were less effec-

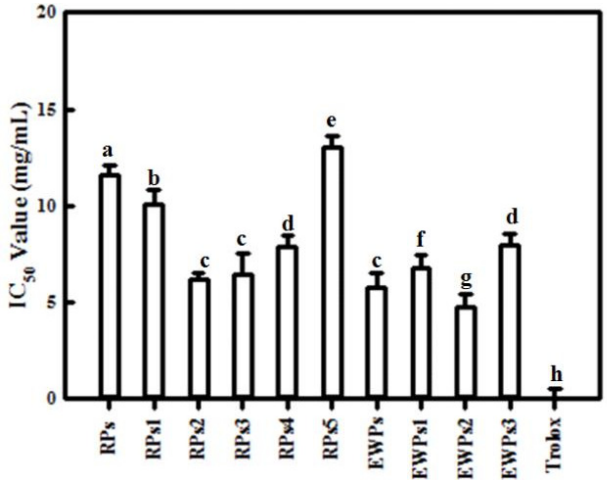

Fig. 4. OH radical-scavenging activity of fractions of RPs (a) and EWPs (b) with different isoelectric point (pI). Each value represents the mean four replicates±standard deviations. Different lettters above bars represent statistically significant differences (p<0.05).

tive than the others (pI<8.5). All autofocusing fractions of EWPs obtained by the alkaline protease hydrolysis were exhibited higher OH radical scavenging activity than RPs and their peptide fractions.

In the case of RPs, the OH radical-scavenging activity was higher in the acidic (RPs2) and weak acidic fractions (RPs3) than that in the other fractions, and the OH radical scavenging activity of the RPs increased after fractionaion by autofocusing. In the case of the EWPs, only neutral fraction (EWPs2) had significantly higher antioxidant activity than the crude hydrolysate before fractionation. The results about the comparison of the DPPH radical scavenging activity between EWPs and autofocusing fractions were almost consistent with the results of OH radical scavenging activity. Especially, the neutral fraction (EWPs2) of EWPs exhibited the highest DPPH and OH radical scavenging activities. Thus, we can infer that EWP contained some antioxidant peptides with neutral pI, which could convert free radicals to more stable products and terminate the radical chain reactions.

It has been demonstrated that addition of some protein hydrolysates such as whey and soyprotein hydrolyzed potato protein, can suppress oxidation in cooked meat patties(Peña-Ramos & Xiong, 2003; Wang & Xiong 2005). Also, our previous study demonstrated that addition of 1% autofocusing fractions of wheat gluten hydrolysates can suppress TBARS, an indicator of development of off-flavors in cooked pork meat patties (Park et al., 2012). Therefore, the effect of autofocusing fractions from EWP on lipid oxidation during cooking and storage in cooked pork meat patties should be evaluated to find a relationship between *in vitro* and in food system.

3.5. Oxygen Radiacl Absorbing Capacity(ORAC) of Autofocusing Fractions

Fig. 5 shows the oxygen radical absorbing capacity (ORAC) of the autofocusing fractions from the RPs and EWPs. In the case of RPs, ORAC was higher in the acidic (RPs2), weak acidic(RPs3), and weak basic fractions (RPs4) than that in the other fractions. The all autofocusing fractions had a lower the oxygen radical absorbing capacity than the crude hydrolysate before fractionation. In the case of EWPs, neutral fraction (EWPs2) had higher than other autofocusing fractions. The EWPs autofocusing fractions had lower ORAC values than crude EWPs in a similar manner to RPs ORAC values. Namely, the RPs and EWPs had higher ORAC values than that of autofocusing fractions. This suggests that synergism among different peptides plays an important role in the ORAC of EWPs and RPs. The antioxidant capacity in egg-white proteins hydrolysates was also reported by other authors (Davalos, Miguel, Bartolome & Lopez-Fandino, 2004; Tsuge, Eikawa, Nomura, Yamamoto & Shgisawa, 1991). Davalos et al. (2004) demonstrated radical scavenging activity (ORAC value) of peptides produced by enzymatic hydrolysis of crude egg white with pepsin. They isolated and characterized few peptides exhibiting antioxidant activity the amino acid sequence of

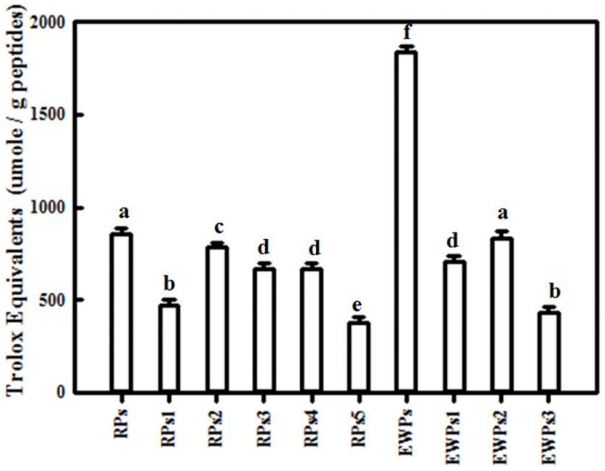

Fig. 5. ORAC activity of fractions of RPs (a) and EWPs (b) with different isoelectric point (pI). Each value represents the mean four replicates± standard deviations. Different lettters above bars represent statistically significant differences (p<0.05).

which corresponded to ovalbumin fragments. In the present study, no correlation was found between ORAC values and the other antioxidant activities. Therefore, more than one assay should be used to measure the antioxidant activity of peptides. Future studies in antioxidant activity of peptide fractions in food model are necessary to find the correlations and to clarify of mechanism of antioxidant activity between *in vitro* and in food system.

3.6. Ferrous Iron (Fe^{2+})-Chelating Ability of Autofocusing Fractions

Among the transition metals, iron is known as the effective factor in lipid oxidation due to its high reactivity. The ferrous iron via the Fenton reaction ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH^-+OH^{\cdot}$) generates hydroxyl radical. In addition, Fe^{3+} ion produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+} ion (Gulcin, 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 (Kim & Lee, 2007; Rajapakse et al., 2005). Metal chelating activity of peptide fractions was measured as antioxidant activity (Fig. 6).

As shown in Fig. 6, chelating ability of protein hydolysates increased after fractionation. In the case of RPs, strong acidic fraction (RPs1) exhibited an effective chelating ability on Fe^{2+}(32.18%). The basic fraction (RPs5) exhibited the highest chelating ability on Fe^{2+} (44.23%). In the case of EWPs, all autofocusing fractions showed higher chelating ability than that of crude EWPs before fractionation. Especially, the acidic fraction (EWPs1) showed the highest chelating ability on Fe^{2+} (43.39%). It is reported that mineral ions in the sample migrate into the cathode compartment during autofocusing (Hashimoto et al., 2006). Therefore, divalent mineral ions are likely to be removed autofocusing fractions, specially the acidic fraction (Park et al., 2012).

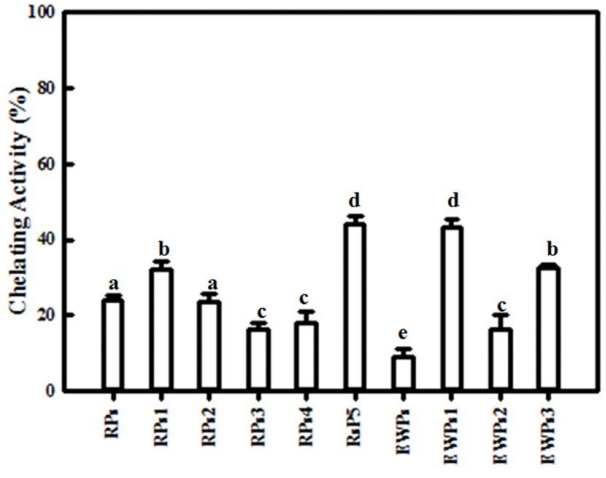

Fig. 6. Metal-chelating activity of fractions of RPs (a) and EWPs (b) with different isoelectric point (pI). Each value represents the mean four replicates±standard deviations. Different letters above bars represent statistically significant differences (p<0.05).

The neutral fraction (EWPs2) of EWPs had higher the DPPH, OH radical scavenging activity, and the ORAC values compared with the others. Whereas, the metal chelating activity of the neutral fraction (EWPs2) was lower than the other fractions.

4. CONCLUSIONS

In conclusion, present study reported for the first time the antioxidant activities of peptides fractions obtained from egg white protein hydrolysates by isoelectric focusing. The peptide fractions in egg white protein hydrolysate were successfully fractionated by autofocusing for evaluating antioxidant activities of each peptide fraction *in vitro* system. The antioxidant activities of egg white protein hydrolysates except for ORAC increased after fractionation by autofocusing. Especially, the acidic fraction from egg white protein hydrolysates had a greater efficiency in scavenging various free radicals compared to that for the other fractions.

By using the autofocusing fractions of egg white protein hydrolysates, a study of the suppression of lipid oxidation during cooking and storage in cooked pork patties is in progress, which would support better understanding of the mechanism underlying the antioxidant activity of protein hydrolysates in the food model. The application of these peptide fractions in foods models should be further studied to estimate the correlations between *in vitro* and in food. Also, future studies in identification of peptide in the active fractions are necessary to understand the mechanism of antioxidant activity. The autofocusing approach for fractionation of protein hydrolysates may be useful for the preparation of potent antioxidant compounds and, could be applied to the food system because of its low-cost and biocompatiblity, as requiring no harmful reagents. In the present study, alkaline protease is an effective enzyme to obtain antioxidant peptides from egg white proteins. The autofocusing fractions of the enzymatic hydrolysates could be a suitable natural antioxidant to prevent lipid oxidation and retard development of off-flavors.

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