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Antioxidant Activity of Some Protein Hydrolysates and Their Fractions with Different Isoelectric Points

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Antioxidant activities of commercially available enzymatic hydrolysates of milk and plant proteins were examined. Among them, soy protein and wheat gluten hydrolysates showed strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and antioxidation activity against linoleic acid oxidation in emulsion systems. Peptide fractions with higher antioxidant activities than crude enzymatic hydrolysates of gluten and soy protein were prepared without toxic solvents and reagents. Peptides in these plant protein hydrolysates were fractionated on the basis of the amphoteric nature of sample peptides by preparative isoelectric focusing without adding chemically synthesized carrier ampholytes, which is termed autofocusing. The acidic fractions from both protein hydrolysates showed stronger DPPH radical scavenging activities than the basic fractions, while the basic fractions strongly suppressed 2,2′-azobis (2-amidinopropane) dihydrochloride-induced oxidation of linoleic acid in an emulsion system. These acidic and basic peptide fractions would be useful to examine the mechanism underlying the antioxidant activities of peptides in food.

KEYWORDS: Lipid; oxidation; lipid oxidation; antioxidant activity; peptide; protein hydrolysates; autofocusing; DPPH

INTRODUCTION

Lipid oxidation is a very important subject in food storage and in human heath. Lipid oxidation is the main factor causing the deterioration of food during storage and processing, as it can induce undesirable changes in color, flavor, texture, and nutritional profile as well as produce potentially toxic reaction products. Namely, oxidation of lipids in foods causes quality losses and shortens shelf life. In addition, lipid oxidation in the human body may play a significant role in coronary heart disease, atherosclerosis, cancer, and the aging process (*1, 2*). The extent and speed of oxidation depend on several factors, such as temperature, the presence of prooxidants and antioxidants, and the molecular nature and status of the lipids in the products (*3, 4*).

To suppress the oxidative deterioration of foods, synthetic and natural antioxidants are used. In general, natural antioxidants have received considerable interest from the food industry due to consumer preference because of the concern over the safety of synthetic antioxidants (*5, 6*). It is well-known that polyphenols (*7, 8*), carotenoids (*8*), vitamins C (*8, 9*) and E (*9*), phytate (*10*), etc. have antioxidant activities. Recent literature suggests that plant foods rich in phytochemicals may have potential health benefits due to their antioxidant activity (*11, 12*). In addition, some proteins and peptides, such as casein (*13*), maize zein (*14*), enzymatic hydrolysates of soy (*15, 16*), gelatin (*17*), elastin (*5*), and egg white proteins (*18*), have antioxidant activities. The demand for the use of amino acids, peptides, or proteins as antioxidants in foods is increasing due to the low costs, safety, and their inherent high nutritional values (*5*).

High-performance liquid chromatography (HPLC) has been used extensively for laboratory-scale isolation of the peptides in protein hydrolysates and identification of antioxidant peptides (*19*). On the basis of activity-guided fractionation, six antioxidant peptides have been identified from soy protein hydrolysate (SP). These peptides are derived from β-conglycinin and are composed of 5−16 amino acid residues, including Pro, His, or Tyr within the sequences and Val or Leu at the amino-terminal position (*15*). Therefore, these peptides are potential food additives that can suppress lipid oxidation. However, the high cost of the preparative HPLC apparatus and chemicals has been a stumbling block in the isolation or preparation of antioxidant peptides in sufficient quantities to examine their value in the food system (*20*). In addition, acetonitrile, methanol, and trifluoroacetic acid, which have been extensively used for peptide preparation by the HPLC system, are not suitable solvents for food processing because of their toxicity (*21*). Thus,

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it is difficult to isolate or synthesize adequate amounts of antioxidant peptides for food ingredients. As crude enzymatic hydrolysates of food proteins have been used to examine their antioxidant activities in food systems, the mechanism underlying the antioxidant activities of peptides in foods is still unclear. We recently developed a low-cost and biocompatible approach to peptide fractionation based on the amphoteric nature of sample peptides in our laboratory (*21*). This approach is termed autofocusing and is a potential industrial peptide fractionation process (*22*). The objective of the present study was to prepare a peptide fraction with a high antioxidant activity by autofocusing, which could be applied to the food system.

MATERIALS AND METHODS

Chemicals. Linoleic acid, butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industry (Osaka, Japan). Casein hydrolysates (FE120ICT and CE90GMM) were purchased from DMV Japan (Tokyo, Japan). SP (Hinute DC-6) was kindly provided by Fuji Oil (Osaka, Japan). Gluten hydrolysate (GP) (WGH) was kindly provided by Nisshin Pharma (Tokyo, Japan). Ascorbic acid and Tween 20 were purchased from Nacalai Tesque (Kyoto, Japan).

Fractionation of Peptides in Protein Hydrolysates. Peptides in hydrolysates of soy protein and gluten were fractionated by autofocusing, which is conducted on the basis of the amphoteric nature of the samples using an apparatus with 10 sample compartments (66.5 mm in length × 80 mm in width × 80 mm in height) (*22*). Sample compartments 5 and 6 were filled with 500 mL of 10% sample solution, and the other compartments were filled with deionized water. A direct electric current at 500 V was applied for 24 h.

Determination of DPPH Radical-Scavenging Activity. The DPPH radical-scavenging activity of some protein hydrolysates in the aqueous solutions was tested as follows. Each protein hydrolysate (50 mg) was dissolved in 2 mL of 10 mM sodium phosphate buffer, pH 7.0. To the solution, 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 was measured using a Beckman DU Series 640 spectrophotometer (Beckman, CA) 30 min after the addition of DPPH solution.

Antioxidant Activity against Linoleic Acid Oxidation in the Emulsion System. A linoleic acid emulsion was prepared by mixing 0.28 g of linoleic acid and 0.28 g of Tween 20 in 50 mL of 20 mM sodium phosphate buffer, pH 7.0. The oil and aqueous phases were mixed and homogenized for 3 min in a high-speed blender (Nihon Irikagakukiki Seisakusho, Tokyo, Japan) operated at 22000 rpm. The droplet was further homogenized using an ultrasonic homogenizer (Nihonseki, Tokyo, Japan) that was operated at maximum power for 2 min.

An aliquot of 0.5 mL of each sample solution (30, 75, 150, and 300 mg/mL) was mixed with 2.5 mL of the linoleic acid emulsion and 2 mL of the sodium phosphate buffer. As an accelerator of oxidation, 50 μL of AAPH solution (50 mg/mL) was injected into the emulsion. BHT (0.03%) was used as a reference compound. The mixture was incubated at 40 °C in a capped tube at approximate intervals (0, 75, 150, 300, 450, 600, and 850 min).

Lipid peroxide, an oxidation product of linoleic acid, was determined by the method of Mitsuda et al. (*23*) and Osawa et al. (*24*) with slight modifications. To 0.1 mL of each sample solution were added 4.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate. Then, 0.1 mL of 0.02 M ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture. Precisely 3 min after the addition of the ferrous chloride solution, the absorbance of the red $Fe(SCN)^{2+}$ formed by the Fenton reaction was measured at 500 nm.

Amino Acid Analysis. To monitor the fractionation of peptides by autofocusing, amino acid analysis of the autofocusing fractions was performed according to the methods of Bidingmeyer et al. (*25*) with slight modifications (*26*).

Statistical Analysis. Statistical comparisons were made by using Fisher's PLSD method after one-way analysis of variance (ANOVA) by using StatView 4.11 (Abacus Concepts Inc., Berkeley, CA). The results were considered significantly different with $p < 0.05$.

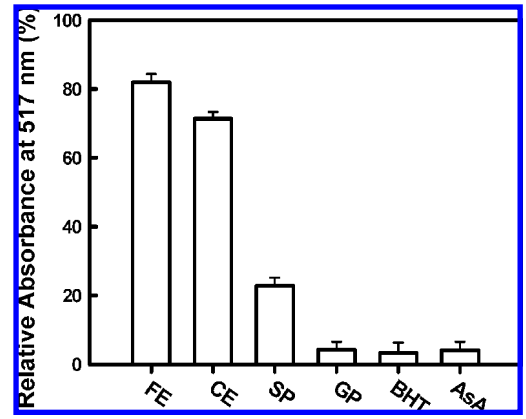

Figure 1. DPPH radical-scavenging activity of some protein hydrolysates. The concentration of each sample is 1%. The concentrations of BHT and ascorbic acid (AsA) are 0.1 and 0.01%, respectively. Each value represents the mean of four replicates and standard deviations in error bars. FE, caseins hydrolysates (FE120ICT); CE, caseins hydrolysates (CE90GMM); SP, soy protein hydrolysate; and GP, gluten hydrolysate.

RESULTS

Antioxidant Activities of Protein Hydrolysates. When the DPPH radical is scavenged, the absorbance is decreased. The results are expressed as a percentage of the initial absorbance of the DPPH radical in the absence of sample. As shown in **Figure 1**, SPs and GPs have higher DPPH radical-scavenging activities than casein protein hydrolysates. In particular, a final concentration of 1% GP showed the DPPH radical-scavenging activity equivalent to 0.1% BHT or 0.001% ascorbic acid.

The antioxidant activity of GPs and SPs against AAPH-induced linoleic acid oxidation in the emulsion system was also evaluated. As shown in **Figure 2**, SPs and GPs suppressed the linoleic acid oxidation in a dose-dependent manner. The formation of the lipid peroxide was completely suppressed by the addition of SPs and GPs to give 3 and 1.5% in the emulsion system, respectively.

Fractionation of Peptide in the SPs and GPs. Peptides in the SPs and GPs were fractionated by autofocusing. **Figure 3a** shows a pH gradient and a distribution of peptides in the autofocusing fractions after the focusing of GP. More than 90% of the peptides, with pI less than 6, were distributed between fractions 4 and 7. On the other hand, in the basic fractions, less than 7.1% peptides were recovered (fractions 8−10), which were characterized by a higher content of basic amino acids and a lower content of acidic amino acids in comparison to the main fraction (**Figures 3a** and **4a**). Similar results were obtained for the fractionation peptides in the SP (**Figures 3b** and **4b**).

Antioxidant Activities of Autofocusing Fractions. Figure 5 shows the DPPH radical-scavenging activity of the autofocusing fractions from the GPs and SPs. In both cases, the DPPH radical-scavenging activity was higher in the acidic fractions (pI < 6.0) than that in the basic fractions (pI > 8.0). In the case of GPs (**Figure 5a**), autofocusing fractions except for fraction 1, of which the content was negligible, had a lower DPPH radical-scavenging activity than the crude hydrolysate before fractionation. On the other hand, the DPPH radical-scavenging activity of fractions 2−4 of the SP was significantly higher than that of the crude hydrolysate (**Figure 5b**).

Figure 6 shows the antioxidant activity of the autofocusing fractions from the SPs and GPs in the emulsion system. The

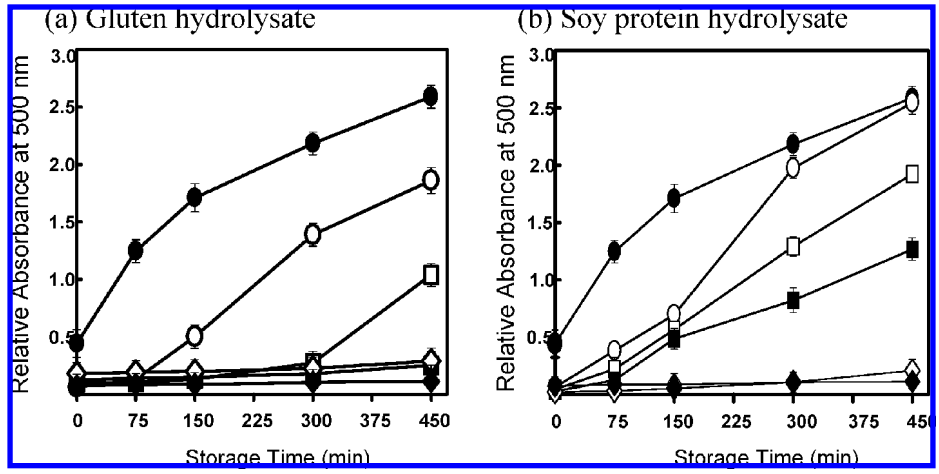

Figure 2. Inhibitory effect of GPs (**a**) and SPs (**b**) on linoleic acid oxidation in emulsion system. Each value represents the mean of four replicates and standard deviations in error bars. Control (●) and 0.3 (○), 0.75 (□), 1.5 (■), 3.0 (◊), and 0.03% (♦) BHT at 40 °C.

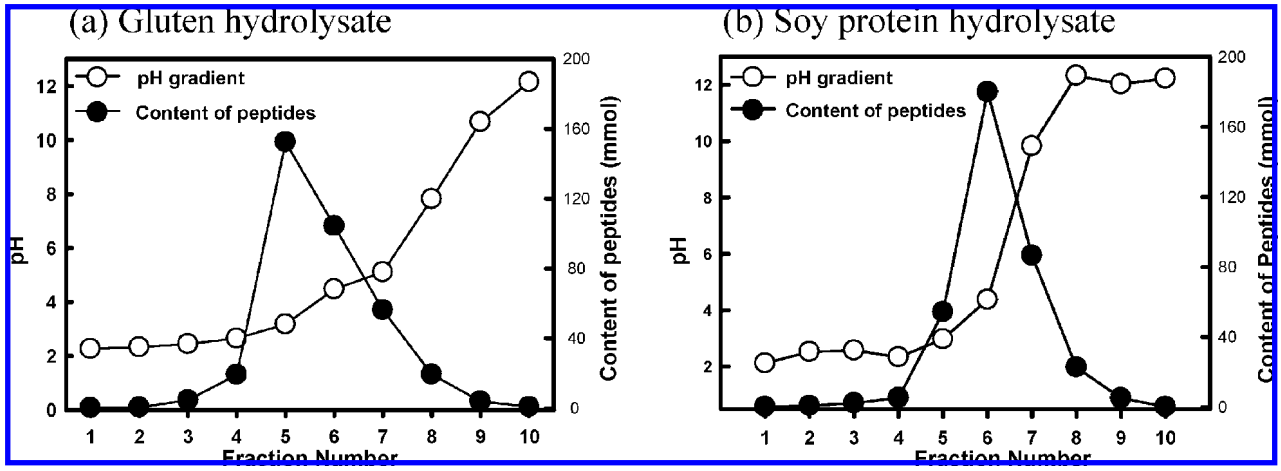

Figure 3. Contents of peptide and pH gradient of autofocusing fractions of GPs (**a**) and SPs (**b**).

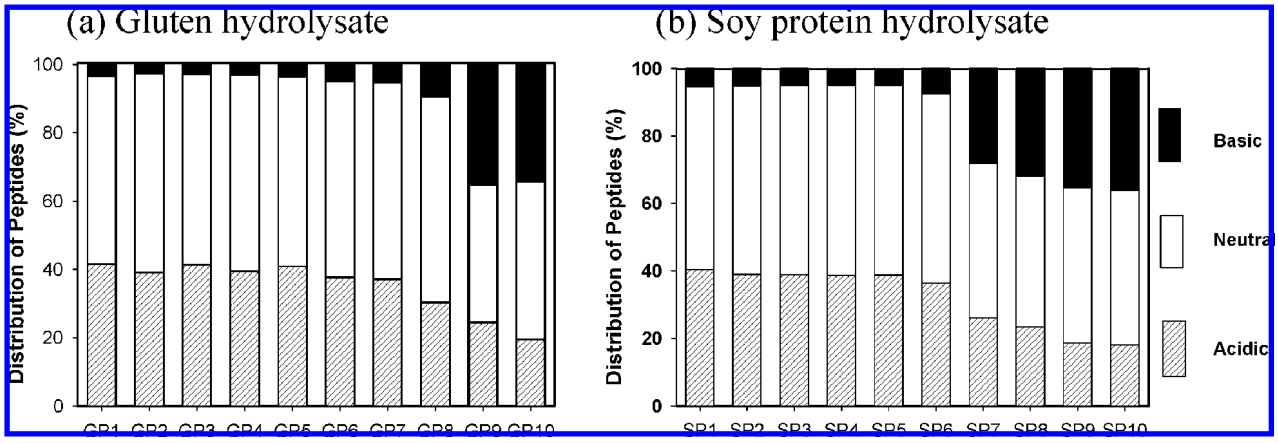

Figure 4. Amino acid compositions of autofocusing fractions of GPs (**a**) and SPs (**b**).

same amount of each autofocusing fraction was added to the reaction mixture. The lipid peroxide was linearly formed up to 300 min, when fractions with relatively low antioxidant activity were added. By addition of fractions with relatively high antioxidant activity, lipid formation was completely suppressed up to 300 min or even after 600 min, respectively. Then, the formation of lipid oxidation at 300 and 600 min is shown in the following sections. All autofocusing fractions showed antioxidant activity against AAPH-induced linoleic acid oxidation. In the case of the GP (**Figure 6a**), fractions 7−10 and crude hydrolysate delayed lipid oxidation up to 300 min. On the other hand, only the basic fractions (fraction 9−10) showed a significantly higher antioxidant activity as compared to the crude hydrolysate up to 600 min. In the case of the SP (**Figure 6b**), except for fraction 6, the autofocusing fractions had significantly higher antioxidant activity than the crude hydrolysate. Fractions 3−4 and 7 delayed lipid oxidation up to 300 min. Fractions 2 and 8−10 completely suppressed the formation of lipid peroxide up to 600 min, while fractions 8−10 showed low DPPH radical scavenging activity (**Figure 5b**).

Effects of the Combination of Fractions on Lipid Oxidation in an Emulsion. Some autofocusing fractions were

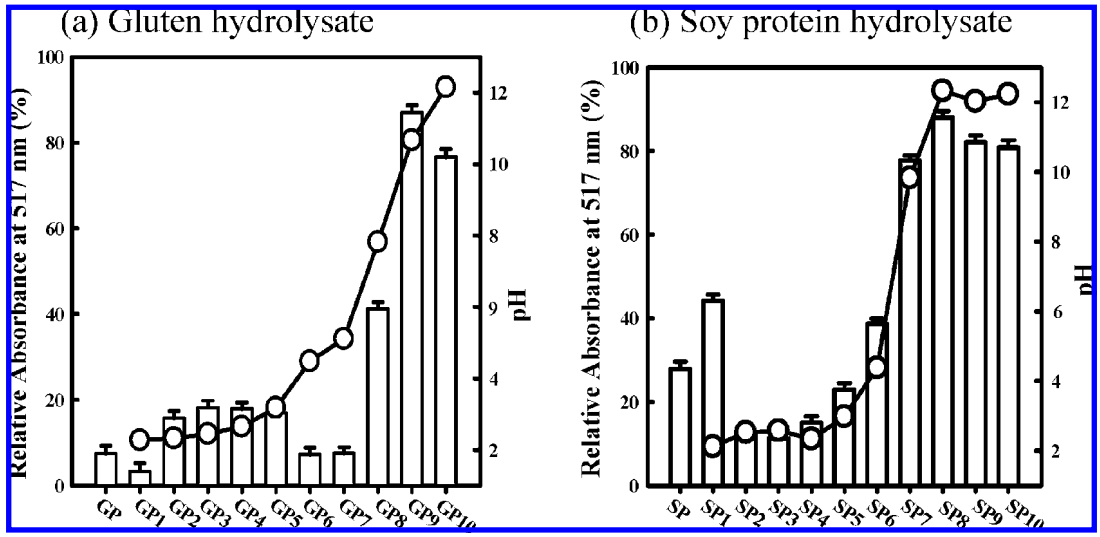

Figure 5. DPPH radical-scavenging activity of GPs (**a**) and SPs (**b**) with different isoelectric points (pI). The concentration of each sample is 1%. Each value represents the mean of four replicates and standard deviations in error bars.

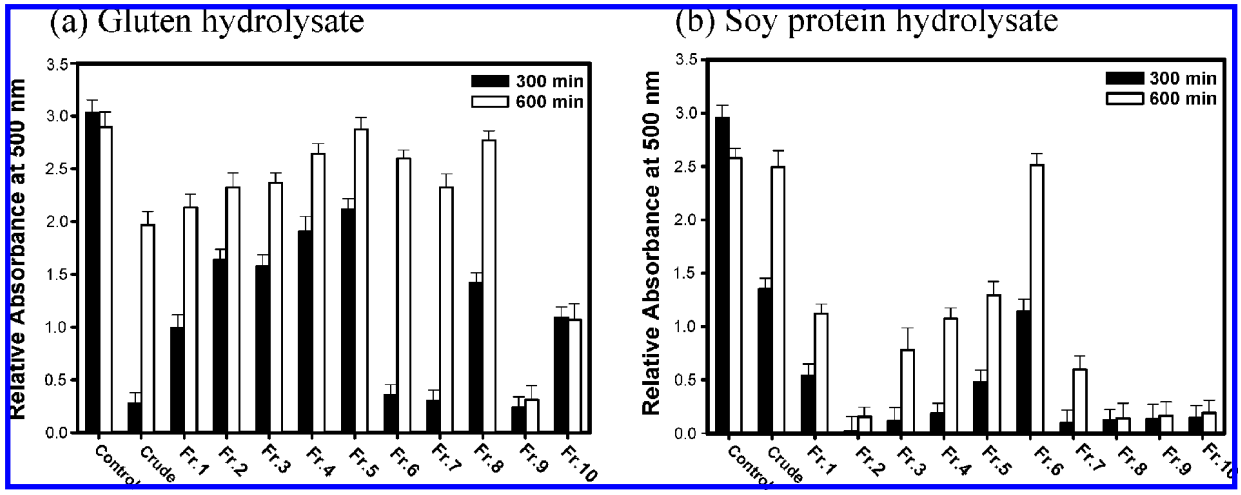

Figure 6. Inhibitory effect of autofocusing fractions of GPs (**a**) and SPs (**b**) on linoleic acid oxidation in an emulsion system. The concentration of each sample is 0.75%. Each value represents the mean of four replicates and standard deviations in error bars.

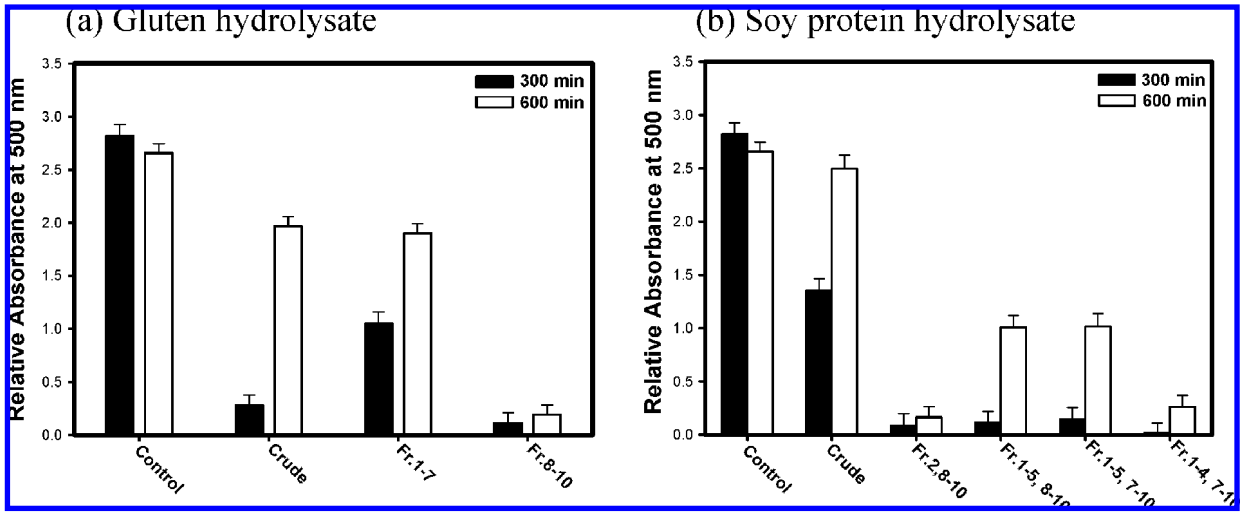

Figure 7. Inhibitory effect of combined fractions of GPs (**a**) and SPs (**b**) on linoleic acid oxidation in an emulsion system. The concentration of each sample is 0.75%. Each value represents the mean of four replicates and standard deviations in error bars.

combined in the same amounts, and their antioxidant activities were examined. In the case of GP, fractions 1−7 and 8−10 were combined and used as acidic and basic fractions, respectively. As shown in **Figure 7a**, the basic fractions showed a higher antioxidant activity than the acidic fractions. In the case of the SP, fractions 2 and 8−10 were combined, as they showed high antioxidant activity, respectively (**Figure 6b**). The combined fractions completely suppressed linoleic

acid oxidation (**Figure 7b**). Other combinations were also made by removal of fractions with low antioxidant activity (**Figure 6b**). Removal of fractions 6, 6 and 7, and 5 and 6 can increase the antioxidant activity as compared to the crude hydrolysate (**Figure 7b**).

DISCUSSION

The in vitro oxidation model using AAPH as the initiator has been widely used to evaluate antioxidant activity (*27*). This system has been considered to mimic the oxidation in the biological system. The DPPH radical is a stable free radical compound and has a strong absorbance at 517 nm. When the DPPH radical is scavenged, the absorbance decreases, which allows simple evaluation of radical scavenging activity (*28*). As shown in **Figures 1** and **2**, SPs and GPs showed antioxidant activity by using these two methods. These hydrolysates used in this study are prepared with protease and would contain small amounts of some proteases. There is a possibility that peptide or protein derived from protease might directly affect the antioxidant activity, while its contents are small.

After fractionation by autofocusing, some acidic fractions with high DPPH radical scavenging activity from both hydrolysates unexpectedly showed lower antioxidant activity against the AAPH-induced linoleic acid oxidation in the emulsion system.

The expected mechanisms underlying antioxidant activity against the AAPH-induced linoleic acid oxidation in the emulsion system are as follows: first, hydrogen-donating abilities to the produced AAPH radicals; second, suppression of hydrogen release from linoleic acid; third, prevention of oxygen binding to lipid radicals; and fourth, hydrogen-donating abilities to alkoxyl, peroxyl, and lipid radicals. Then, possible explanations for the low antioxidant activity against the emulsion system by the acidic fraction are as follows. First, the acidic fraction can donate a hydrogen atom to the DPPH radical, whereas it can not scavenge other radicals in the emulsion system and can not directly suppress the release of hydrogen and binding of oxygen to linoleic acid. Second, the acidic peptides can not approach a droplet of linoleic acid in the emulsion system due to an electrostatic effect (*29, 30*). In the former case, the acidic fraction can suppress oxidation in food and biological systems only when oxidation is induced by DPPH radical-like substances. In the latter case, the acidic fraction may suppress oxidation of neutral lipid in food and/or biological systems.

By using autofocusing, adequate amounts of peptide fractions with high DPPH radical scavenging activity or high antioxidant activity against AAPH-induced linoleic acid oxidation in the emulsion system can be prepared, respectively (**Figures 5** and **6**). By using these fractions, a study of the suppression of lipid oxidation in some food models such as raw and cooked minced meat patties is now in progress, which would facilitate better understanding of the mechanism underlying the antioxidant activity of peptides in the food system. By combining some autofocusing fractions, a peptide fraction with higher antioxidant activity against the AAPH-induced linoleic acid oxidation in the emulsion system as compared to the crude hydrolysate can be prepared (**Figure 7**). If the antioxidant activity of peptides in food can be increased by autofocusing, this technique could be used for the preparation of peptide-based food additives to suppress oxidation, as this technique does not require expensive and harmful solvents and has an inherent potential for further scale-up.

ABBREVIATIONS USED

FE, caseins hydrolysates (FE120ICT); CE, caseins hydrolysates (CE90GMM); SP, soy protein hydrolysate; GP, gluten hydrolysate.

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