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Effects of Peptide Fractions with Different Isoelectric Points from Wheat Gluten Hydrolysates on Lipid Oxidation in Pork Meat Patties

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ABSTRACT: Wheat gluten hydrolysate (WGH) was fractionated on the basis of the amphoteric nature of sample peptides by preparative isoelectric focusing (autofocusing). Cooked pork patties were stored at 4 and 20 °C in the dark. WGH and autofocusing fractions suppressed the oxidation of lipids in the patties. The acidic (pI < 3.0) and basic (pI > 9.0) autofocusing fractions suppressed lipid oxidation in the cooked patties to a greater extent than other fractions and WGH. Each autofocusing fraction was evaluated by 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radical scavenging activities, β-carotene bleaching, oxygen radical absorbance capacity, and Fe^{2+} chelating assays; however, none of the in vitro assays predicted the suppressive effect of WGH on lipid oxidation in the cooked patties. These findings suggest that the microdistribution of peptides in food systems and their interaction with food matrix compounds play a significant role in the suppression of lipid oxidation in meat patties rather than radical scavenging activity.

KEYWORDS: *lipid oxidation, antioxidant, isoelectric point, DPPH, OH radical, ORAC, peptide, metal chelating, meat*

INTRODUCTION

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. Namely, the oxidation of lipids in foods results in loss of quality and shortens shelf life.[1,2] In addition, lipid oxidation produces potentially toxic reaction products for human health. The extent and speed of oxidation depend on several factors, such as the 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 have been used. In general, natural antioxidants have received considerable interest from the food industry, due to consumer concerns over the safety of synthetic antioxidants.[5,6] It has been reported that some proteins and enzymatic hydrolysates of food proteins exert antioxidant activity in food systems.[1,7−10] Consequently, the demand for peptides or proteins as antioxidants in foods is increasing due to the their low cost, safety, and inherent high nutritional values.[5]

Antioxidant peptides have been identified from enzymatic hydrolysates of soy,[11,12] gelatin,[13] and egg white proteins.[14] In those studies, the antioxidant activity was evaluated by in vitro assays, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, hydroxyl (OH) radical scavenging, β-carotene bleaching, oxygen radical absorbance capacity (ORAC), and Fe^{2+} chelating assays. It has been assumed that peptides with in vitro antioxidant activities are responsible for the antioxidant activities in food systems.

Wheat gluten, an important byproduct of the wheat starch industry, is produced worldwide in enormous quantities and recognized as a good source of dietary protein.[15] Enzymatic hydrolysates of wheat gluten (wheat gluten hydrolysate; WGH) have been demonstrated to have in vitro antioxidant activities. Recently, we fractionated peptides in WGH by preparative isoelectric focusing, based on the amphoteric nature of sample peptides, whereby peptide fractions of sufficient amounts for addition to food systems could be prepared. This approach is referred to as autofocusing and has potential for industrial peptide fractionation, as it is a low-cost and biocompatible approach, requiring no harmful reagents.[16,17] We demonstrated that the acidic autofocusing fractions from WGH showed stronger DPPH radical scavenging activities than the basic fractions, while the basic fractions strongly suppressed 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH)-induced oxidation of linoleic acid in an emulsion system.

The objectives of the present study were to examine the antioxidant activity of autofocusing fractions from WGH and elucidate their antioxidant properties in a food system. For these purposes, the effect of autofocusing fractions from WGH on lipid oxidation in pork meat patties and the in vitro antioxidant activities of the autofocusing fractions were investigated.

MATERIALS AND METHODS

Chemicals. Linoleic acid, butylated hydroxytoluene (BHT), DPPH, and AAPH were purchased from Wako Pure Chemical Industries (Osaka, Japan). A WGH (commercial name; WGH composition: water, 2.9%; protein, 77.5%; natrium, 0.22%; and lipid, 0.2%) was kindly provided by Nisshin Pharma (Tokyo, Japan). Tween 20 was purchased from Nacalai Tesque (Kyoto, Japan).

Fractionation of Peptides in WGH. Peptides in WGH were fractionated by autofocusing using an apparatus (975 mm in length × 200 mm inner width × 120 mm in height) with 10 sample compartments

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(66.5 mm in length × 80 mm in width × 80 mm in height).[18] Sample compartments 5 and 6 were filled with 500 mL of 10% sample solution, 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 from autofocusing were collected and adjusted to pH 7.0 and subsequently freeze-dried prior to testing for antioxidant activities in vitro and in food systems.

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.[19] with slight modification.[20]

DPPH Radical-Scavenging Activity. DPPH radical-scavenging activity of WGH 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.

OH Radical-Scavenging Activity. The OH radical-scavenging activity of WGH 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 °C. 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 WGHs. IC_{50} values (mg/mL) were determined as the concentration of WGH where light emission decreased by a half of control using the regression lines.

ORAC. The antioxidant activity was measured using the oxygen radical absorbing capacity (ORAC) assay according to the method of Huang et al.[21] 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 °C 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.

β-Carotene–Linoleic Acid Bleaching Assay. The antioxidant activity was assayed using the β-carotene bleaching method.[22−24] β-Carotene (2 mg) was dissolved in 20 mL of chloroform. A 4-mL aliquot of the solution was added to a conical flask with 40 mg of linoleic acid and 400 mg of Tween 20. The chloroform was removed using a rotary evaporator at 50 °C. Distilled water (100 mL) was added to the β-carotene emulsion and mixed, and aliquots (3 mL) of the β-carotene emulsion and 0.2 mL of the sample solution (WGHs and autofocusing fractions in distilled water) were placed in capped culture tubes (final WGH concentration, 0.03%) and mixed well. The tubes were immediately placed in a water bath and incubated at 50 °C. Oxidation of the β-carotene emulsion was monitored by reading the absorbance (470 nm) at 20 min intervals for 120 min. The control consisted of 0.2 mL of distilled water instead of the sample solution.

Ferrous Iron (Fe^{2+})-Chelating Activity. The chelating activity of WGH and the autofocusing fractions for ferrous ions were measured according to the ferrozine method with minor modifications.[25] 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 a 30 min incubation, absorbance was read at 561 nm. The percent metal chelating ability was calculated according to the following formula:

$$\text{chelating ability (\%)} = \left[A_{\text{blank}} - \left(A_{\text{sample}} - A_{\text{control}}\right)\right] / A_{\text{blank}} \times 100$$

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

Preparation of Raw and Cooked Meat Patties. Fresh meats (pork fillet and boned rib of pork) with approximately 10% fat were obtained from a local market and used within 1 h of purchase. The two types of meat were mixed by gently blending for 2 min in a mixer (Sanyo Electric Co., Ltd., Osaka, Japan) with 1% (w/w) of WGH samples. Patties were shaped manually into an approximately 5 cm (diameter) × 0.4 cm (thickness) geometry. Patties were fried in a Teflon-coated electric frying pan set at 150 °C for 2 min per side, with a total cooking time of 4 min. The final internal temperature of meat patties was 55 °C. Cooked patties were placed on polystyrene trays, wrapped with retail commercial oxygen-permeable cling film, and stored at 4 and 20 °C in the dark for 72 and 48 h until analyses, respectively.

Thiobarbituric Acid Reactive Substances (TBARS). Lipid oxidation of the cooked meat samples was determined at 0, 24, and 48 h of storage. Samples were taken immediately after cooking and at 24 h intervals for lipid oxidation measurements. The method of Lemon[25] was modified according to Raghavan et al.[26] for measuring TBARS value. The sample (4 g) was extracted with 8 mL of 10% trichloroacetic acid (TCA) solution by homogenization using a Biohomogenizer (Biospec Products, Bartlesville, OK) at high speed for 1 min. The samples were centrifuged at 2300 rpm in a high speed refrigerated centrifuge (Tomy Seiko Co. Ltd., Tokyo, Japan) for 30 min. An aliquot (2 mL) of the supernatant was mixed with 2 mL of 0.02 M TBA solution containing 0.04% BHT and heated in a boiling water bath for 20 min. Color development was measured at 532 nm. A standard curve was generated using tetraethoxypropane. The amount of TBARS was expressed as milligrams of malondialdehyde (MDA) equivalents per kilogram meat patty.

Sensory Evaluation. Descriptive sensory evaluation was performed by a 20 member trained panel that consisted of selected faculty, staff, and graduate students from Kyoto Prefectural University. Pork patties with added WGH (0, 1, and 5%), salt (0 and 1%), and fat (10%) were prepared. Samples were sectioned from the cooked patties and served along with water to panelists. Panelists were instructed to rinse their mouth with water after tasting each sample and relax for 20−30 s before tasting the next sample. Empty cups were provided for expectoration of the samples.

Evaluated were the following sensory attributes: color, flavor, taste, texture, and overall acceptability. Scores were assigned using a 7-point scale as follows: −3 = dislike extremely, −2 = dislike very much, −1 = dislike moderately, 0 = neither like nor dislike, 1 = like moderately, 2 = like very much, and 3 = like extremely. Panelists were instructed to mark on the scale where they felt best described the sensory attributes of samples. These studies were approved by the experimental ethics committees of Kyoto Prefectural University (Kyoto, Japan).

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 $p < 0.05$.

RESULTS

Fractionation of Peptides in the WGH. Peptides in the WGH were fractionated by autofocusing. Figure 1 shows the

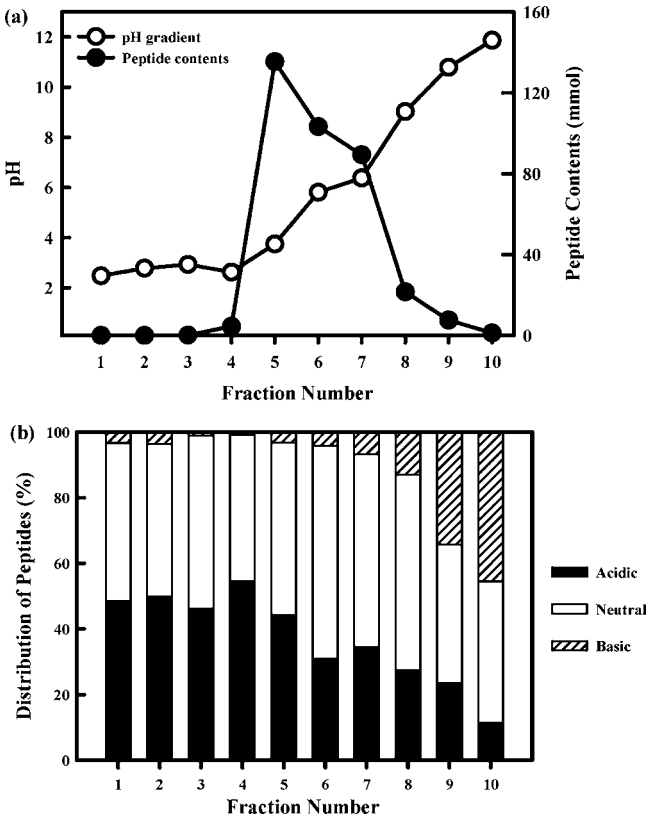

Figure 1. (a) Peptide contents and pH gradient of autofocusing fractions of WGH. (b) Amino acid composition of autofocusing fractions of WGH.

pH gradient and the distribution of peptides in the autofocusing fractions (Fr.). More than 85% of the peptides were distributed between Fr. 5 and 7 (4 < pH < 7). Approximately 10% of the peptides were recovered in basic fractions (pH > 8.0; Fr. 8−10). A low % of peptides were recovered in the acidic fractions (pH < 4.0; Fr. 1−4). The peptides in the acidic and basic fractions are characterized by a higher content of acidic and basic amino acids, respectively (Figure 1b). On the basis of pH profile and amino acid composition, as shown Figure 1, autofocusing fractions were combined in equal amounts as follows: GP1 (acidic, Fr. 1−4), GP2 (weak acidic, Fr. 5), GP3 (neutral, Fr. 6−7), and GP4 (basic, Fr. 8−10).

Radical-Scavenging Activity. Figure 2 shows the DPPH radical-scavenging activity of the WGH and autofocusing fractions. WGH and all fractions almost completely scavenged DPPH radicals at 1% sample solution (data not shown), which indicates higher activity than observed with other peptides samples.[12] At 0.3% sample solution, crude WGH showed the highest scavenging activity, while acidic and basic fractions showed lower scavenging activity than crude WGH. BHT and Trolox reduced radical activities by almost 90 and 95%, respectively.

As shown in Figure 3, WGH and all fractions scavenged OH radicals in a similar manner to DPPH radicals (Figure 2).

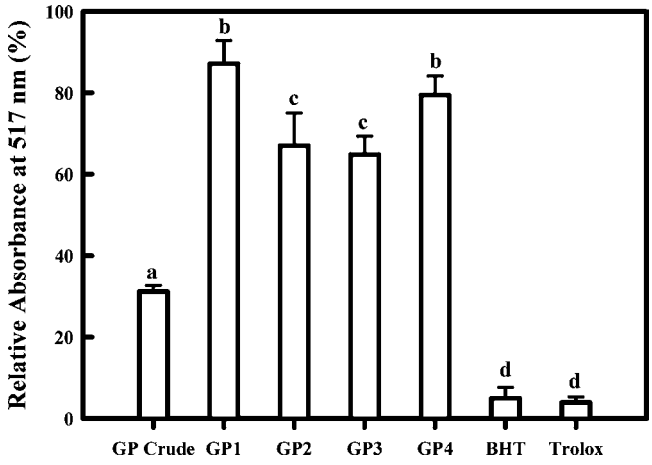

Figure 2. DPPH radical scavenging activity of WGHs with different isoelectric points. Each value represents the mean of four replicates ± standard deviations. Different letters above bars represent statistically significant differences ($P < 0.05$).

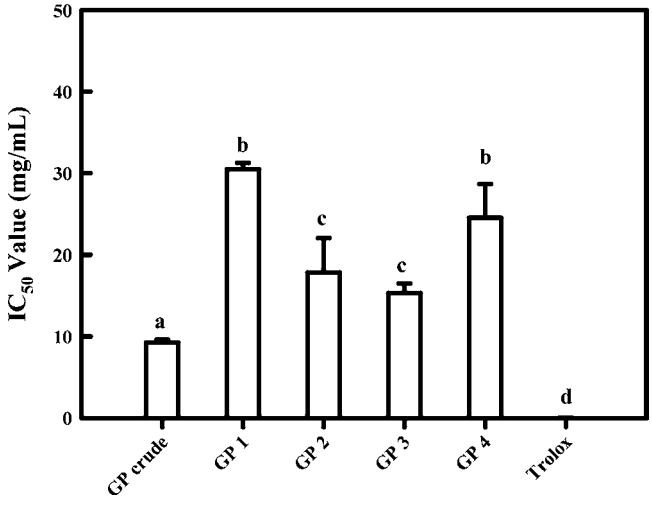

Figure 3. OH radical scavenging activity of WGHs with different isoelectric points (pI). Each value represents the mean of four replicates ± standard deviations. Different letters above bars represent statistically significant differences ($P < 0.05$).

As shown in Figure 4, acidic and neutral fractions showed lower and higher ORAC values than crude WGH and the other fractions, respectively.

Evaluation of Antioxidant Activity by β-Carotene Bleaching. As shown in Figure 5, WGH and all fractions significantly suppressed discoloration of β-carotene at 60 and 120 min as compared with the control. The acidic fraction showed the lowest antioxidant activity among the samples.

Ferrous Iron (Fe^{2+})-Chelating Ability. As shown in Figure 6, all autofocusing fractions showed higher chelating ability than crude WGH. Specifically, acidic and weakly acidic fractions showed high chelating activity. Mineral ions in the sample migrate into the cathode compartment during autofocusing.[18] Therefore, divalent mineral ions are likely to be removed by autofocusing, thereby increasing the mineral chelating ability of autofocusing fractions, especially the acidic fraction.

Effect of WGH Addition on the Sensory Properties of Patties. Minced pork meat was mixed with crude WGH [1 and 5% (w/w)] and salt and then cooked. Sensory properties of the cooked meat patties were evaluated by panelists. As shown

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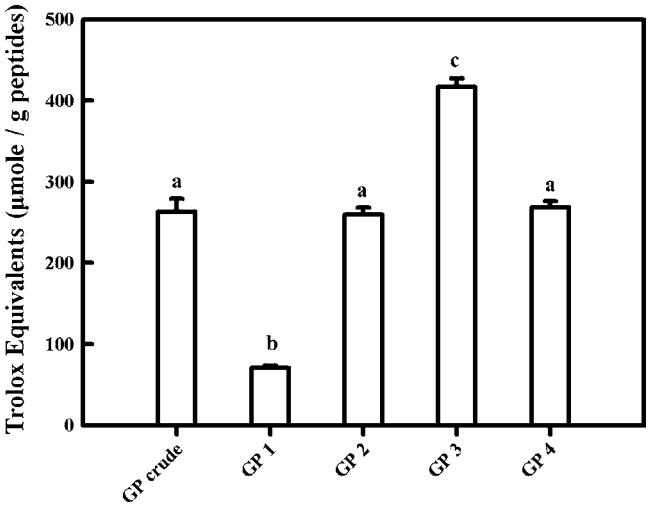

Figure 4. ORAC activity of WGHs with different isoelectric points (pI). Each value represents the mean of four replicates ± standard deviations. Different letters above bars represent statistically significant differences ($P < 0.05$).

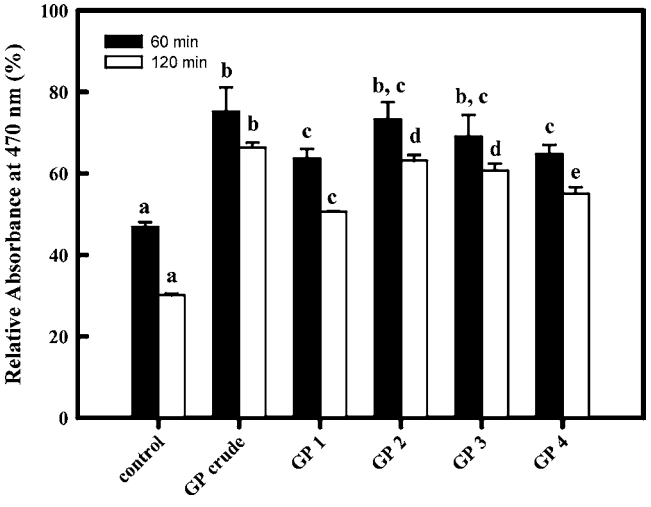

Figure 5. Inhibitory effect of autofocusing fractions of WGHs on linoleic acid oxidation in an emulsion system. Each value represents the mean of four replicates ± standard deviations. Different letters above bars represent statistically significant differences ($P < 0.05$).

in Table 1, there were no significant differences ($p > 0.05$) in the sensory properties of patties (color, flavor, taste, texture, and overall acceptability) with the addition of up to 5% crude WGH in the presence of salt. Panel acceptance was higher for cooked patties containing 1% WGH and 1% salt than patties containing 5% WGH and 1% salt, although differences were not statistically significant. These results indicate that the addition of crude WGH in pork patties (up to 5% but preferably 1%) did not adversely affect the color, smell, taste, texture, and overall acceptability of cooked pork. In the following experiments, crude WGH and its autofocusing fractions were added to meat patties at a concentration of 1% (w/w).

Antioxidant Activity in Raw and Cooked Patties. MDA levels of the cooked pork patties after storage at 4 and 20 °C are shown in Figure 7. TBARS values increased with storage time and temperature. The crude WGH and all autofocusing fractions significantly suppressed MDA formation at both storage temperatures as compared to control. These results demonstrate

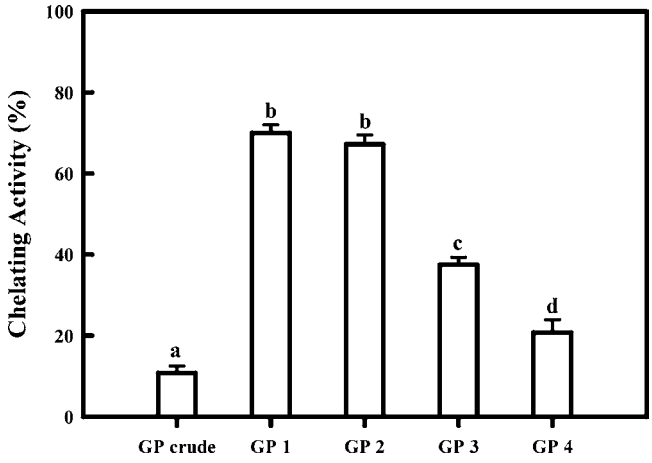

Figure 6. Metal-chelating activity of WGHs with different isoelectric points (pI). Each value represents the mean of four replicates ± standard deviations. Different letters above bars represent statistically significant differences ($P < 0.05$).

Table 1. Panel Sensory Evaluation Scores of Cooked Patties Made with WGH[a]

	level of WGH (%) (with salt concn)					
sensory attributes	0 (0.5)	0 (1)	1 (0.5)	1 (1)	5 (0.5)	5 (1)
color	0.13	−0.27	0.53	0.63	0.50	0.60
smell	0.23	0.23	0.07	0.50	−0.30	−0.30
taste	0.60	0.50	0.37	1.00	0.10	0.20
texture	0.53	0.33	−0.13	1.03	0.55	0.75
overall acceptability	0.53	0.23	0.17	1.03	0.07	0.10

[a] $N = 20$; −3 to 3 point scoring scale (−3 = dislike extremely, and 3 = like extremely).

that WGH and its autofocusing fractions can retard lipid oxidation in a muscle food system. After 72 and 48 h at 4 and 20 °C, respectively, all autofocusing fractions showed significantly ($P < 0.05$) higher antioxidant activity than crude WGH. Among them, acidic and basic fractions showed higher activity than other fractions after 48 h at 20 °C.

DISCUSSION

It has been demonstrated that the addition of some enzymatic hydrolysates of food proteins, such as milk protein, can suppress oxidation in food systems.[9] The present study also demonstrates that addition of 1% crude WGH can suppress lipid oxidation in cooked pork meat patties during room temperature and chilled storage. The addition of 1% crude WGH did not adversely affect the sensory properties of patties. Therefore, WGH has the potential for use as a practical natural antioxidant in muscle food.

In food systems, only crude enzymatic hydrolysates of food proteins have been evaluated for antioxidant activity due to the difficulty in fractionating sufficient amounts of peptide.[12] In the present study, peptides in crude WGH were successfully fractionated by autofocusing for evaluating an antioxidant activity of each fraction in the meat patties. The results indicated that some autofocusing fractions, especially acidic and basic fractions, showed higher antioxidant activity than crude WGH. To elucidate the underlying properties of the antioxidant activity of autofocusing fractions against lipid oxidation in meat patties, individual fractions were evaluated using in vitro antioxidant

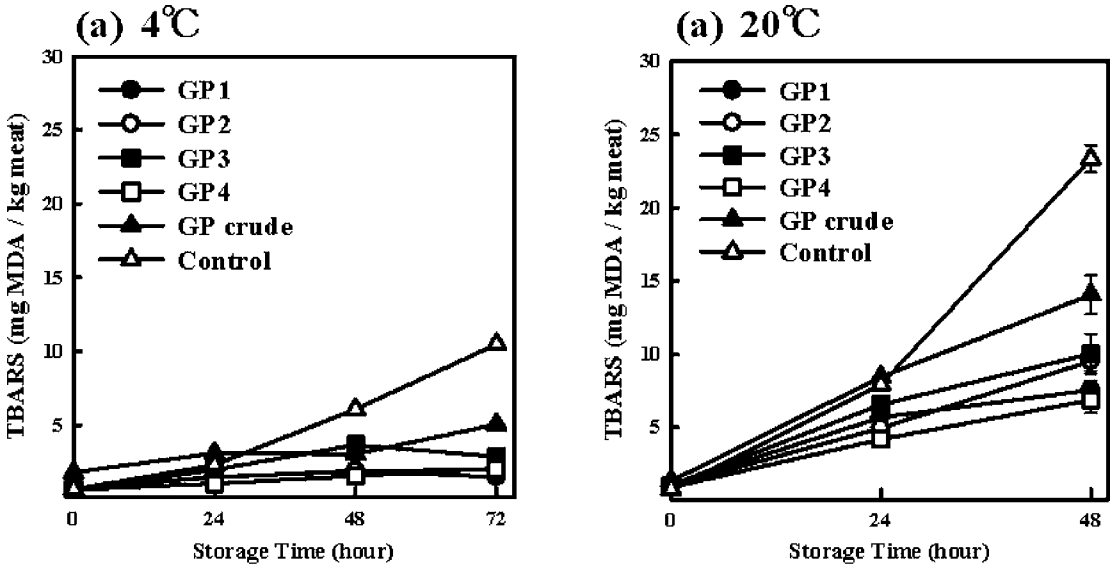

Figure 7. Effects of the addition of WGHs on lipid oxidation in cooked pork patties stored at 4 and 20 °C. Each value represents the mean of four replicates ± standard deviations. (a) Stored at 4 °C and (b) stored at 20 °C.

assays. Recent investigations have demonstrated that the reported antioxidant activity of the same sample frequently depends on the assay system employed;[27,28] thus, using at least two methods is recommended. In this study, three radical-scavenging methods, as well as Fe^{2+} chelating ability assay, were used. In addition, the suppression of linoleic acid oxidation in a model emulsion system was examined.

The unpaired electrons of free radicals react with electrons from polyunsaturated fatty acids, inducing an autoxidation reaction. Radical-scavenging activity is thought to predict antioxidant activity in food, as scavenging free radicals can stop the autoxidation reaction. In the present study, crude WGH exhibited higher DPPH and OH radical-scavenging activities than autofocusing fractions. For peroxyl radical-scavenging activity, a different tendency was observed. That is, the crude WGH was inferior to the neutral fractions (GP3) and showed an antioxidant activity similar to that of GP2 and GP4 (the basic fraction). However, the antioxidant activity of crude WGH against peroxyl radical was much higher than that of GP1 (the acidic fraction), which is in agreement with the results of other radicals. These results about the comparison of antioxidant activity against free radicals between crude WGH and autofocusing fractions were almost consistent with the results of antioxidant activity to linoleic acid oxidation in the emulsion system, in which crude WGH was more efficient than autofocusing fractions for retarding the lipid oxidation. Therefore, radical-scavenging activities by peptides can be related to the inhibition of linoleic acid oxidation in the present simple emulsion system, although Alamed et al.[29] reported that ORAC and DPPH assays could not predict the ability of antioxidants to inhibit lipid oxidation in corn oil-in-water emulsions.

In the case of lipid oxidation in complex food systems such as meat patties, however, such correspondence between the radical-scavenging ability and prevention of lipid oxidation could not be observed. In the meat patties, the autofocusing fractions, especially the acidic and basic fractions, exerted higher antioxidant activity against lipid oxidation as compared to crude WGH. Contrastingly, the acidic and basic fractions generally exhibited low radical-scavenging activities and lower ability to retard linoleic acid oxidation in the simple emulsion system as described above. We consider the reasons why such contradiction occurs between the meat patties system and the radical scavenging assay or model emulsion system as follows.

In the meat patties experiments of the present study, TBA test was used. This method is normally used for detecting the decomposition products of peroxides such as aldehydes.[30] It is possible that the acidic and basic peptide fractions can prevent the generation of aldehydes by protecting the peroxides from decomposition. Because aldehydes are usually responsible for the deterioration of sensory quality of food products including lipids, these peptide fractions may be useful for extending the shelf life of foods.

The second factor is the chelating effect. Pork meat contains heme and nonheme iron, which can catalyze the oxidation of unsaturated fatty acids during cooking by Fenton's reaction. Therefore, Fe^{2+} chelating ability can suppress lipid oxidation in pork meat patties. The results of Figure 6 suggest the possibility that the acidic peptide fraction (GP1) retards the lipid oxidation in pork meat patties, taking advantage of a high chelating activity. However, for the basic peptide fraction (GP4), the high antioxidant activity against lipid oxidation in meat patties cannot be ascribed to the chelating activity, because the chelating activity of this peptide fraction is the lowest among all of the peptide fractions.

The third factor is related to the microdistribution of peptides in the food matrix. In the meat patties, lipids are distributed as fat globules emulsified with proteins and phospholipids. In this situation, fatty acids in triacylglycerols in fat globules and phospholipids suffer from oxidation. Both proteins and phospholipids contain the negative and positive charged groups in the molecules, and those charged groups attract opposite charged groups of other compounds. The acidic and basic peptide fractions, rich in negative and positive charges, respectively, may be easily attracted to the surface of fat globules via electrostatic interaction, thereby preventing the lipids from oxidation efficiently.

In addition to the mechanisms as described above, the acidic and basic fractions might inhibit enzymes present in meat such as peroxidase and lipase. The peptide fractions might also modulate the activity of endogenous antioxidants such as

tocopherol, ascorbic acid, carotene, ubiquinone, glutathione, etc. In the complex food systems such as meat patties, several factors affect the results about protection of lipid oxidation by peptides synergistically or competitively. Further studies in simpler model systems are necessary to understand the mechanism of antioxidant activity of the autofocusing peptide fractions in foods.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

WGH, wheat gluten hydrolysate; MDA, malondialdehyde; OH, hydroxyl; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ORAC, oxygen radical absorbance capacity; TBARS, thiobarbituric acid reactive substances; TE, Trolox equivalents

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