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Preparation and Antioxidative Properties of a Rapeseed (*Brassica napus*) Protein Hydrolysate and Three Peptide Fractions

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This study investigated the possibility of converting the insoluble rapeseed meal protein into functionally active ingredients for food applications. The rapeseed (*Brassica napus*) meal protein isolates were first digested by Alcalase and Flavourzyme, and the resultant rapeseed crude hydrolysate (RSCH) exhibited a dose-dependent reducing antioxidant power and hydroxyl radical scavenging ability. RSCH could also inhibit the malonyldialdehyde (MDA) generation by 50% in blood serum at 150 mg/mL. RSCH was further separated into three fractions (RSP1, RSP2, and RSP3) by Sephadex gel filtration according to their different molecular weights. The amino acid compositions and antioxidant potentials were assessed for RSP1−3 fractions. All three fractions showed inhibiting effects on superoxide anion generation to various extents. They could also inhibit the autohemolysis of rat red blood cells and MDA formation in rat liver tissue homogenate. The results suggested that rapeseed peptide hydrolysate may be useful as a human food addition as a source of bioactive peptides with antioxidant properties.

KEYWORDS: Antioxidant properties; protein hydrolysates; rapeseed (*Brassica napus*); radical scavenger; lipid oxidation

INTRODUCTION

Many biofunctional components have been identified in foods with the belief that they could potentially exert beneficial effects on human health (*1*). Bioactive peptides are small protein fragments that have biological effects once they are released during gastrointestinal digestion in the organism or by previous in vitro protein hydrolysis. So far, bioactive peptides with antimicrobial, antihypertensive, immunomodulatory, opioid, antioxidant, or hypocholesterolemic activity have been described (*2*−*5*).

Rapeseed (*Brassica napus*) is one of the most important oilseed crops worldwide, representing the third largest source of edible oil according to the U.S. Department of Agriculture (*6*). Rapeseed is also the world's second leading source of protein meal. Defatted rapeseed meal contains about 32% protein (*7*), which could potentially be used as a food ingredient instead of being wasted or being used for animal feed. Because rapeseed constitutes an interesting raw material for the preparation of protein isolates and hydrolysates, researchers have tried to utilize rapeseed protein isolates and its defatted meal as a source of bioactive peptides to enhance the value of the rapeseed hydrolysates. For example, Marczak et al. (*8*) used subtilisin to produce antihypertensive peptides from rapeseed protein. Yust et al. (*9*) reported the HIV protease inhibitory activity of rapeseed protein hydrolysates produced using Alcalase.

Among bioactive components, those with antioxidant effects have been the most extensively studied. Natural antioxidants including rosemarinic acid, catechin, tocopherols, ascorbate, and various phenolic extracts from plants have been widely used in processed foods. The search for natural antioxidants has extended beyond the above traditional sources, and a number of studies have shown that peptides and protein hydrolysates of plant and animal origin could possess significant antioxidant activity. For example, the hydrolysates of soy protein (*10*, *11*), whey protein (*12*, *13*), zein protein (*14*), potato protein (*15*), wheat protein (*16*, *17*), gelatin (*18*), and egg albumin (*19*) have been shown to possess antioxidant activities, which is determined by their size, amino acid composition, and configuration of peptides.

Aerobic organisms must deal with free radicals that are generated from sequential reduction of oxygen during the normal course of aerobic metabolism. These radicals may cause cellular damage leading to a number of pathological conditions including atherosclerosis, arthritis, diabetes, and carcinoma, if produced in an uncontrolled manner (*20*). Meanwhile, during food processing

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and storage, radical-mediated oxidation of fats and oils is one of the most important reasons for the deterioration of oil-containing foods. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as synthetic antioxidants are widely used to retard lipid peroxidation, which, however, have increasingly caused public concerns due to their potential health hazards. Therefore, there has been a growing interest in the identification and characterization of natural antioxidants for both human health and food preservation during the past few decades. Antioxidant activity has been identified in some protein hydrolysates such as those from fish protein (*21*), egg yolk protein (*22*), wheat germ protein (*16*), milk proteins (*23*), and porcine hemoglobin (*24*).

The present study reports the antioxidant activities of rapeseed peptides obtained from the rapeseed Huaza3 variety, which is widely planted in China as it is high in oil and low in erucic acid and glucosinolate. After the rapeseed protein isolate was digested by Alcalase and Flavourzyme, the rapeseed crude hydrolysate (RSCH) was shown to possess antioxidant activities revealed by its reducing power and scavenging effects on hydroxyl free radicals. Next, we produced three rapeseed peptide fractions by gel filtration chromatography, and their respective abilities to inhibit the production of reactive oxygen species and prevent malondialdehyde (MDA) production in rat liver homogenate were demonstrated in vitro.

MATERIALS AND METHODS

Enzymatic Hydrolysis and Preparation of RSCH. Crude commercial defatted and dehulled meal of rapeseed variety Huaza3 after oil-pressing procedure was obtained from a local factory and used in this study as starting material. After grinding, rapeseed protein was 1:30 w/v dissolved in 850 mM saline and centrifugated (Hitachi, SCR20BC) at 3000*g* at room temperature for 20 min. The pellet was dissolved in distilled water and centrifuged again to get the albumin fraction. After freeze-drying, this fraction was adjusted to 5% w/v concentration using distilled water and used for further analysis. Albumin isolate, prepared as described above, was then hydrolyzed by sequential treatment with Alcalase (Novozymes) and Flavourzyme (Novozymes) using a hydrolysis reactor. A digestion with Alcalase for 1 h was followed by incubation with Flavourzyme for another 2 h. A three-factorial response surface design optimization with degree of hydrolysis (DH) as response values was determined for Alcalase. The three parameters, enzyme concentration [0.2, 0.3, and 0.4 Alcalase units (AU)/g substrate], substrate percent (3, 5, and 7%), and temperature (40, 50, and 55 °C) were fitted to generate optimum concentrations of enzyme, substrate concentration, and temperature for achieving optimum degree of hydrolysis. Box−Behnken surface response using the JMP 7.0 statistical software (SAS Institute Inc.) was used to evaluate the interactions between parameters to generate optimum values for enzymatic hydrolysis. Hydrolysis parameters were then used as follows: protein isolate concentration, 5% w/v; enzyme concentration, 0.2 AU/g of substrate for Alcalase and 50 LAPU/g of substrate for Flavourzyme; pH 8 and temperature 50 °C for both enzymes. Hydrolysis was conducted in a 1000 mL reaction vessel equipped with a stirrer, thermometer, and pH electrode. Hydrolysis was stopped by heating at 80 °C for 10 min. Hydrolysates were clarified by centrifugation at 3000*g* for 10 min. The DH, defined as the percentage of peptide bonds cleaved, was calculated by determining free amino groups by reaction with TNBS according to the method of Adler-Nissen (*25*). The hydrolysate was subjected to the Kjeldahl procedure to determine crude protein content, as outlined by the *Official Methods of Analysis* of the AOAC (*26*).

G-25 Gel Filtration Chromatography and Preparation of Rapeseed Peptide (RSP1−3) Fractions. Rapeseed protein hydrolysate (0.2 mL; 100 mg/mL) was injected into a Sephadex G-25 gel filtration column at a flow rate of 0.5 mL/min, and eluted peptide fractions were pooled and molecular masses determined by reference to a calibration curve created by running molecular mass markers on the Sephadex G-25 under running conditions identical to those for the test samples. Molecular mass standards were bacitracin (1422 Da), oxidized glutathione (612 Da), and reduced glutathione (307 Da).

Amino Acid Analysis. Peptide samples (10 mg) were treated with 4 mL of 6 M HCl in tubes sealed under nitrogen for 24 h at 110 °C for hydrolysis. Amino acids were determined by high-performance liquid chromatography (HPLC) of the derivatives obtained by reaction with diethyl ethoxymethylenemalonate, according to the method of Alaiz et al. (*27*).

Reducing Power Assay. The reducing power of the peptide fractions was measured according to the method of Oyaizu (*28*) with minor modifications. Various concentrations of the hydrolysates in 2.5 mL of 0.2 M phosphate buffer at pH 6.6 were added to 2.5 mL of potassium hexacyanoferrate(III). The mixture was incubated at 50 °C for 20 min, and then 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture followed by vortex mixing for 2 s. After centrifugation at 3000*g* for 10 min, 2.5 mL of the mixture was measured for absorbance at 700 nm wavelength. A high absorbance was indicative of strong reducing power.

Hydroxyl Radical Scavenging Assay. The hydroxyl radical scavenging effects of the rapeseed peptide fractions were assayed using the method of Halliwell et al. (*29*). The reagents were added to a test tube in the following order: 0.4 mL of KH_2PO_4−KOH buffer (50 mM, pH 7.5), 0.1 mL of sample solution at various concentrations, and 0.1 mL of 1 mM EDTA, 10 mM H_2O_2, 60 mM 2-deoxy-D-ribose (0.1 mL of distilled water in place of the 2-deoxy-D-ribose solution as sample blank), 2 mM ascorbic acid, and 1 mM $FeCl_3$. The reaction solution was incubated at 37 °C for 1 h. Next, 1 mL of 25% HCl was added to stop the reaction. The color was developed by the addition of 1 mL of 1% thiobarbituric acid (TBA) into the reaction tubes, which were placed in boiling water for 15 min. The tubes were cooled to room temperature, and then the absorbance was read at 532 nm. At each concentration of the hydrolysate from one batch, determinations were carried out in triplicate. The scavenging effects were calculated according to the formula $^{\bullet}OH$ scavenge (%) = $[A_0 - (A_1 - A_2)]/A_0 \times 100$, where A_0, A_1, and A_2 represent the absorbance of the control, the sample, and the sample blank, respectively.

Superoxide Radical Scavenging Activity. Superoxide radicals were generated in vitro by xanthine oxidase. The scavenging activity of the rapeseed peptides was determined using the nitroblue tetrazolium (NBT) reduction method. In this method, O_2^- reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. The capacity of the samples to scavenge the superoxide radicals was assayed as follows: The reaction mixture contained 0.5 mL of 0.8 mM xanthine and 0.48 mM NBT in 0.1 mM phosphate buffer (pH 8.0) and 0.1 mL of the sample solution to give various concentrations. After heating to 37 °C for 5 min, the reaction was initiated by adding 1.0 mL of xanthin oxidase (XOD) (0.049 U/mL) and was carried out at 37 °C for 20 min; the reaction was stopped by adding 2.0 mL of 69 mM sodium dodecyl sulfonate (SDS). The absorbance of the reaction mixture was measured at 560 nm. The results were calculated as the percentage inhibition according to the formula % (inhibition) = $[(A_0 - A_1) - (A_2 - A_3)]/(A_0 - A_1) \times 100$, where A_0, A_1, A_2, and A_3 are the absorbance of the control, the blank control, the sample, and the blank sample, respectively.

Rat Red Blood Cell Hemolysis Inhibition Test. The hemolysis test was performed according to the method described by Sakurai and others (*30*). In brief, an aliquot (200 μL) of RSCH and RSP1−3 fractions with a series of concentrations were mixed with 5 mL of washed rat erythrocytes (1×10^{10} cells/mL), respectively, suspended in 128 mM NaCl, and incubated at 37 °C for 24 h. After incubation, the mixture was diluted 1:5 with 128 mM NaCl, and the unlysed cells were then pelleted by 1000*g* centrifugation for 10 min. The A_{540} of the resulting supernatants was measured to determine the release of hemoglobin. Efficiency of hemolyis inhibition was expressed as a percentage calculated according to the equation inhibition (%) = $(1 - A_1/A_0) \times 100$, where A_0 and A_1 represent the absorbance of the control and the sample, respectively.

Lipid Peroxidation Assay. For the in vitro studies, approximately 100 mg of rat liver was homogenized in ice-cold 20 mM Tris-HCl (pH 7.4) to produce a tissue homogenate. The homogenate was centrifuged at 2500*g* for 30 min at 4 °C, and the protein concentration was determined using a standard commercial kit (Bio-Rad Laboratory Ltd.) with bovine serum albumin as a standard. The homogenate (200 μL) was mixed with 100 μL of rapeseed peptide preparations (128 mM NaCl instead in blank

control) and incubated at 37 °C for 1 h. After cooling on ice, MDA levels in liver were measured with a commercial MDA kit (Jiancheng Bioengineering Institute, Nanjing, China). In acidic medium, MDA reacted with TBA upon boiling, and the resultant MDA−TBA adducts was pink in color and measured at 532 nm. The results were expressed as MDA formation per milligram of protein. In another two groups of experiments, 0.1 mL of 10 mM H_2O_2 or 1 mM $FeSO_4$ was added into the incubation mixture, respectively, to evaluate the iron-chelating ability and H_2O_2-quenching ability of RSPs.

Rat Intraperitoneal Injection Experiment. Wistar rats (35 ± 2 g) at 4 months of age were divided into three groups (five females and five males per group) including control group, high-dose group (150 mg of RSCH/kg·day), and low-dose group (50 mg of RSCH/kg·day). The rats were housed under normal laboratory conditions (27 ± 2 °C, 12/12 h light/dark cycle) with free access to standard rat feed and water. The rapeseed peptide was intraperitoneally injected to the animals at 50 or 100 mg/kg (control group received 128 mM NaCl instead). On the 16th day, the rats were killed by decapitation. Blood was collected in test tubes with heparin sodium (50 U/mL blood), and serum was collected by centrifuging the blood at 1000*g* for 15 min at 4 °C. Serum was removed immediately for MDA assay according to the procedures described below. Blood collection from animal experiments was approved by the institutional Animal Ethics Committee.

Statistical Analysis. Each data point represents the mean of three samples. Data were subjected to analysis of variance (ANOVA) followed by Duncan's multiple-range posthoc test, and the significance level of $P < 0.05$ was employed.

RESULTS AND DISCUSSION

Preparation of Rapeseed Protein Hydrolysates. In Huaza3 rapeseed variety, albumins were found to be 37% in total protein according to the Bradford assay result in this study. We chose the albumin part to continue with enzymatic hydrolysis. Alcalase, a commercially available serine protease that prefers uncharged residue sites for action, was chosen as the first food-grade proteolytic enzyme to obtain the rapeseed peptide hydrolysates. During the enzymatic process of protein isolate hydrolysis, the specificity of the enzyme, the operating conditions, and the extent of hydrolysis are the main factors that may influence the nature and the composition of peptide mixtures produced and consequently the functional properties of the resulting hydrolysates. Recent results (*31*) showed that the protease employed can greatly affect the biological activity of the obtained hydrolysates when used as additive in insect cell cultures devoid of serum. A three-factorial response surface design optimization with degree of hydrolysis as response values was determined. The prediction profile designated 0.38 AU enzyme concentration and 4.87% substrate concentration at 50 °C for obtaining the optimum DH of 14%.

Flavourzyme was used in this study as the second proteolytic enzyme after the Alcalase treatment. After the hydrolysis with Alcalase, the number of N-terminal sites was increased for the action by Flavourzyme, which is a fungal complex of exopeptidases and endoproteases. In addition, it can also decrease the bitterness of its digested products. The DH of protein hydrolysates is an important parameter that relates well with the bioactive, functional, and immunological properties (*32*, *33*). After the 2 h digestion by Flavourzyme, the DH increased progressively to 30% and the bitterness decreased by 60% compared to the Alcalase hydrolysates (data not shown). The degree of hydrolysis obtained in this study was lower than that of the sunflower protein hydrolysates digested by the same enzyme regimen treatment (Alcalase plus Flavourzyme), 30% reported here versus 60 and 50%, respectively, in those two studies (*34*, *35*).

Separation of Three Rapeseed Peptide Fractions. The final rapeseed hydrolysates obtained by sequential incubation with Alcalase and Flavourzyme were applied to a G-25 gel filtration column to obtain peptides with a homogeneous size (**Figure 1**). The obtained profile is typical of a protein hydrolysate formed by a pool of peptides of gradually decreasing molecular masses. Peaks were not detected in the chromatograms corresponding to the nonhydrolyzed rapeseed protein, which indicates the efficiency of the enzymatic hydrolysis. Rapeseed hydrolysate was characterized by a profile with separated peaks that suggests the presence of peptides quite heterogeneous in size. Three major fractions named RSP1, RSP2, and RSP3, respectively, were separated, recovered, and studied. The overall recovery after gel filtration is 54%, and three fractions took 30.4, 41.2, and 28.4%, respectively. All three fractions reacted positively with biuret and ninhydrin, which indicated their peptide identity. The eluted fractions after RSP3 reacted only with ninhydrin, but not with biuret, which indicated that they are mainly composed of free amino acids along with low molecular saccharide and salts.

We also analyzed the amino acid composition of three peptide fractions and found they were quite different as shown in **Table 1**. Because after the enzyme hydrolysis Trp is totally destroyed and Gln and Asn were transformed into Glu and Asp, respectively, 17 amino acids were included in **Table 1**. A number of studies have demonstrated a good correlation between certain amino acid residues or short peptides with radical scavenging ability of protein hydrolysates or peptides (*36*, *37*). **Table 1** shows that three RSP fractions are rich in Arg, Glu, Phe, Leu, Tyr, and Pro, most of which reportedly have relation to antioxidant properties either in their free forms or as residues in proteins and peptides (*13*, *38*, *39*). Hydrophobic amino acids accounted for <35% for all three RSP fractions, so the abundance of charged or polar N- and C-terminal groups in peptides produced would enable them to be readily soluble in aqueous solution.

Antioxidant Properties of RSCH. Hydrolysates obtained from the enzymatic hydrolysis of various food proteins are known to possess antioxidant potential. During the past two decades, studies have described the antioxidant properties of animal and plant protein hydrolysates (*40*−*43*). In the attempts to evaluate the antioxidant potential of rapeseed meal enzyme-treated protein hydrolysate, we first determined whether RSCH as a whole had any potential as antioxidative nutraceuticals by reducing activity and $^{\bullet}OH$ scavenging measurements.

To determine the reducing activity of RSCH and RSP1−3, we used the ferric reducing antioxidant assay, which is based on the ability of an antioxidant to reduce Fe^{3+} to Fe^{2+} in a redox-linked colorimetric reaction, which involves one electron transfer. In our study, we found that the reducing power of RSCH was positively correlated with the concentrations used in the assay (**Figure 2**). The presence of reducing power indicates that the RSCH extract has electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reactions.

Among the oxygen radicals, the hydroxyl radical is the most reactive and causes great damage to living cells due to its ability to react with various molecules such as phospholipids, DNA, and organic acids. The scavenging effect of RSCH against hydroxyl radicals was shown as the inhibition rate (**Figure 3**). Rapeseed hydrolysate showed a very potent dose-dependent hydroxyl radical scavenging activity, and its activity increased with increasing concentration. At a dose of only 0.1 mg/mL, RSCH scavenged >9% of hydroxyl free radicals produced, and at the 100 and 250 mg/mL concentration, RSCH exhibited 80 and 87% scavenging activities, respectively.

We next investigated further whether the presence of RSPs has any in vivo impact on cellular antioxidative enzyme levels. After abdominal injection of RSCH, we measured the MDA production level in rat blood serum. The blood serum MDA level

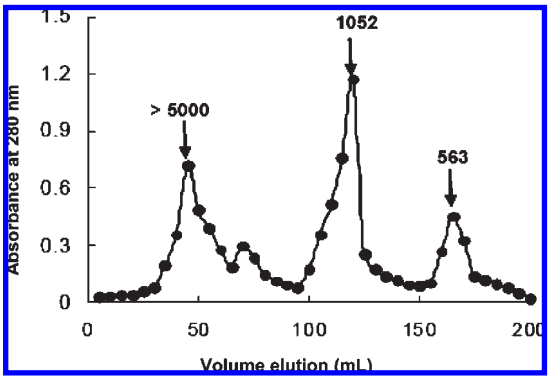

Figure 1. Elution profile of the double enzyme digested rapeseed meal hydrolysates on the Sephadex G-25 column. Each fraction group was collected according to their different molecular weight ranges, condensed, and freeze-dried. Sample size, 20 mg; flow rate, 0.5 mL/min; detection, 280 nm. G-25 The arrows indicate the predicted molecular mass markers of three major elution peaks designated RSP1, RSP2, and RSP3.

Table 1. Free Amino Acid Compositions of RSP1−3 Fractions on Sephadex G-25 Gel Filtration Column after Preparation of Rapeseed Meal Hydrolysate by Two Different Enzymes[a]

amino acid composition[a] (% v/v)	RSP1	RSP2	RSP3
essential amino acids			
His	5.4 ± 0.3	7.1 ± 0.3	5.8 ± 0.3
Ile	3.2 ± 0.2	3.1 ± 0.3	4.1 ± 0.2
Leu	6.0 ± 0.3	7.4 ± 0.4	7.2 ± 0.5
Lys	6.4 ± 0.4	3.1 ± 0.3	3.6 ± 0.2
Met	1.1 ± 0.2	2.6 ± 0.2	1.8 ± 0.2
Cys	4.3 ± 0.2	1.5 ± 0.2	1.4 ± 0.1
Phe	4.2 ± 0.3	11.2 ± 0.5	7.1 ± 0.2
Thr	3.6 ± 0.3	4.4 ± 0.2	3.8 ± 0.2
Val	3.5 ± 0.2	3.1 ± 0.3	3.5 ± 0.2
nonessential amino acids			
Tyr	2.5 ± 0.2	9.7 ± 0.4	6.9 ± 0.3
Ala	2.6 ± 0.2	2.9 ± 0.3	2.3 ± 0.2
Arg	21.4 ± 0.7	9.1 ± 0.5	18.0 ± 0.5
Asp[b]	6.9 ± 0.3	8.1 ± 0.4	10.0 ± 0.4
Glu[c]	13.3 ± 0.5	12.7 ± 0.7	10.6 ± 0.4
Gly	3.3 ± 0.2	5.3 ± 0.3	4.9 ± 0.3
Pro	8.5 ± 0.3	5.2 ± 0.3	6.2 ± 0.3
Ser	3.7 ± 0.2	3.2 ± 0.3	3.0 ± 0.3

[a] Data correspond to the average and SD of three independent experiments. [b] Aspartic acid + asparagine. [c] Glutamic acid + glutamine.

in normal rat was significantly higher than that of the RSCH-injected rat (11.18 ± 2.01 versus 9.75 ± 1.67 nM for the low-dose group, $P < 0.05$, and 5.94 ± 0.43 nM for the high-dose group, $P < 0.01$) (**Table 2**). Therefore, RSCH injection at both 50 and 150 mg/kg·day could significantly decrease the rat blood serum MDA level.

Superoxide Anion Scavenging Activity of RSPs. Superoxide radicals have been observed to kill cells, inactivate enzymes, and degrade DNA, cell membranes, and polysaccharides (*44*). These radicals may also play an important role in the peroxidation of unsaturated fatty acids and possibly other susceptible substances (*45*). Therefore, after we detected the antioxidant activities in rapeseed hydrolysates, we further studied the scavenging effects of RSCH and RSP1−3 fractions on superoxide radicals because it is one of the most important ways of clarifying the mechanism of antioxidant activity. The superoxide scavenging activity was measured using the xanthine−xanthine oxidase system, and the results are indicated as the inhibition rate of superoxide activity (**Figure 4**). RSCH and RSP 1−3 fractions

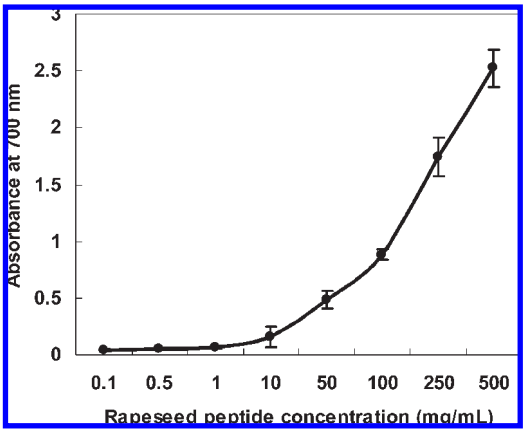

Figure 2. Reducing power of double enzyme digested rapeseed meal hydrolysate (RSCH) measured by the ability to donate electrons and reduce Fe^{3+} to Fe^{2+} ions. The formation of a Fe^{2+}/ferricyanide complex was measured as the absorbance at 700 nm. Data were expressed as means with standard deviations ($n = 3$).

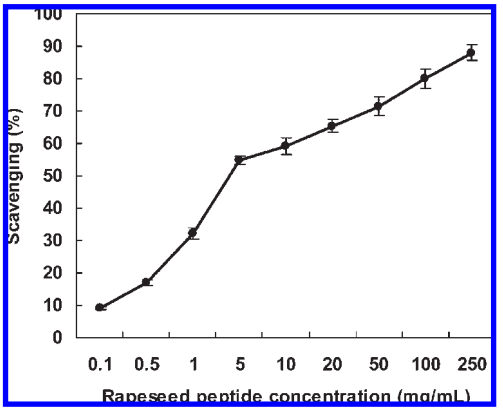

Figure 3. Scavenging effects on hydroxyl radicals of double enzyme digested rapeseed meal hydrolysates (RSCH). Data were expressed as means with standard deviations ($n = 3$).

Table 2. MDA Formation in Rat Blood Serum 16 Days after Rat Abdominal Injection of RSCH ($n = 10$, $X \pm SD$)[a]

group	n	MDA in serum (nmol/mL)
control	10	11.18 ± 2.02
RSCH, 50 mg/kg·day	10	9.75 ± 1.67[b]
RSCH, 150 mg/kg·day	10	5.94 ± 0.43[c]

[a] Values are the average of 10 replicate determinations. [b] $P < 0.05$ compared to control group. [c] $P < 0.01$ compared to control group.

exhibited superoxide scavenging activity. The highest inhibition rates of RSCH and RSP1−3 were 80, 90, 35, and 80%, respectively, at concentrations of 0.5 (RSCH), 0.5 (RSP1), 0.05 (RSP2), and 2 mg/mL (RSP3) (**Figure 4**).

Effects of RSPs on the Hemolysis of Rat Red Blood Cells (RBCs). Research shows that RBCs undergo autohemolysis daily in vivo, and in the current study, we used an in vitro model to mimic this situation. RBCs undergo hemolysis due to membrane destruction during their oxidation. Results indicated that at certain concentrations, four RSPs including RSCH and RSP1−3 could prevent RBC hemolysis (**Figure 5**). The highest inhibition rate was observed for RSCH at 0.5 mg/mL with inhibition of 40% and for RSP1 at 2 mg/mL with inhibition of 26%. In **Figure 5**, a diminishment of inhibition effect was

observed for all four peptide preparations, and this indicated that in the preparation, the active components responsible for the inhibiting effect probably got lost during the gel filtration procedure.

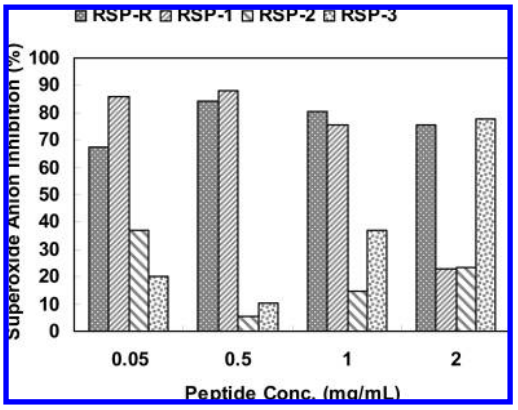

Figure 4. Superoxide anion scavenging activity of RSPs using the xanthine−xanthine oxidase system. The results are indicated as the inhibition rate of superoxide activity. Data were expressed as means with standard deviations (n = 3).

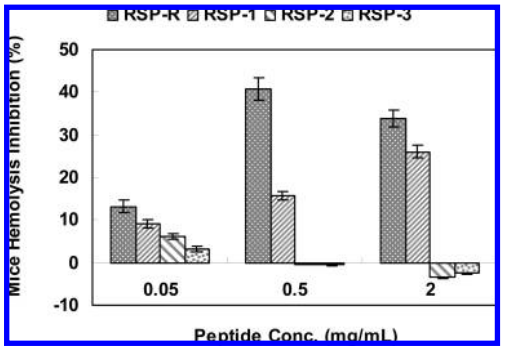

Figure 5. Inhibiting effects of RSPs on the autohemolysis of rat blood cell. The results are indicated as the inhibition rate of hemolysis. Data were expressed as means with standard deviations (n = 3).

Effects of RSPs on MDA Generation in Rat Liver Tissues. Lipid oxidation in the human body may play a significant role in coronary heart disease, atherosclerosis, cancer, and the aging process (*46*). In this study, the antioxidant activity in terms of prevention of lipid oxidation was tested for RSPs using a rat liver tissue homogenate model system. RSCH and RSP1−3 were added to the rat liver homogenates at different concentrations, and MDA generation in the homogenates was monitored to indicate the degree of lipid oxidation (**Table 3**). At the doses tested (0.05, 0.5, and 2 mg/mL), four RSP preparations were able to inhibit the MDA formation by 8−57% (**Table 3**). Fe^{2+} and H_2O_2 are two inducers of free radicals. When the mice liver tissue was incubated with Fe^{2+} or H_2O_2, the MDA content increased significantly ($P < 0.05$). It was found that in the presence H_2O_2, RSPs could still decrease liver tissue MDA levels in a dose-dependent manner and the inhibition extent was quite similar (**Table 3**). Becausse the content of MDA in the system was much higher when H_2O_2 was involved, RSPs showed a higher antioxidant activity in an induced oxidation system than in a noninduced oxidation system using mouse liver homogenates. In contrast, in the presence of Fe^{2+}, the percentages of RSP inhibition on liver tissue MDA levels were much less. The inhibition of 2 mg/mL RSCH on the Fe^{2+}-induced MDA formation was only 9%, significantly lower than in the same system without Fe^{2+} (57%) (**Table 3**). Therefore, RSPs was less potent in the Fe^{2+}-induced oxidation system than in the same system without inducers. In conclusion, these data showed that different RSP preparations are potent scavengers of peroxyl radicals, but not excellent iron chelators. Because RSPs showed potent free radical scavenging activity, it is reasonable to hypothesize that RSPs exerted their protection against phospholipid oxidation through strong radical scavenging activity.

ROS are constantly generated for various physiological functions in the human body. An imbalance caused by excessive oxidants may result in oxidative damage to many large biomolecules, such as lipids, DNA, and proteins (*47*). In our current study, we evaluated the antioxidant potential of double enzyme-treated rapeseed meal protein hydrolysates (RSCH) and three gel filtration fractions (RSP1−3) and determined whether these RSPs have any potential commercial use as

Table 3. Inhibition Effect of Rapeseed Peptide Preparations on Noninduced and $FeSO_4$- and H_2O_2-Induced Lipid Peroxidation (MDA Production) in Rat Liver Tissue Homogenate in Vitro (n = 10, $X \pm$ SD)[a]

		inoculation in vitro		Fe^{2+}-induced		H_2O_2-induced	
sample	dose (mg/mL)	MDA (nmol/mg)	inhibition ratio (%)	MDA (nmol/mg)	inhibition ratio (%)	MDA (nmol/mg)	inhibition ratio (%)
control	0	3.3 ± 0.2	0	6.4 ± 1.4	0	5.0 ± 1.0	0
RSCH	0.05	3.1 ± 0.2	8.4	5.2 ± 1.1	19.7	3.3 ± 0.7	33.3
	0.50	2.7 ± 0.2	20.7	5.7 ± 1.3	10.9	3.7 ± 0.8	26.3
	2.00	1.4 ± 0.1	57.5	5.9 ± 0.2	9.2	1.5 ± 0.1	70.7
RSP1	0.05	3.0 ± 0.3	9.3	5.8 ± 0.5	9.5	4.1 ± 0.3	17.2
	0.50	2.5 ± 0.2	24.0	6.0 ± 1.2	6.4	3.1 ± 0.2	38.9
	2.00	1.8 ± 0.1	45.5	5.4 ± 1.2	16.8	2.6 ± 0.2	48.1
RSP2	0.05	3.0 ± 0.2	10.8	6.4 ± 1.5	0.6	4.4 ± 0.5	11.0
	0.50	3.0 ± 0.3	11.7	6.3 ± 1.6	3.0	4.9 ± 0.4	1.6
	2.00	2.8 ± 0.3	15.9	6.3 ± 1.5	1.9	3.9 ± 0.3	21.6
RSP3	0.05	3.0 ± 0.4	9.0	6.3 ± 1.4	1.6	4.6 ± 0.4	7.8
	0.50	2.7 ± 0.3	19.5	6.4 ± 1.2	1.4	3.8 ± 0.4	23.5
	2.00	2.2 ± 0.2	33.2	6.2 ± 1.1	3.1	3.1 ± 0.2	37.3

[a] Values are the average of 10 replicate determinations.

antioxidative nutraceuticals. Our data showed that RSPs could effectively scavenge superoxide radicals and hydroxyl radicals and inhibit the generation of MDA in liver tissue homogenate, which could be the major forms of ROS generated in the human body. Thus, RSPs might serve as promising agents to reduce oxidative damage to biomolecules by modulating the effects of reactive oxidants. We intend in our future studies to identify the most antioxidative fractions and determine ways to increase the yield of these specific fractions in a cost-effective and environmentally friendly manner. Meanwhile, it will be interesting to evaluate in vitro the antioxidant activity of RSPs in the human digestive system by pepsin and pancreatin. With the increase of DH in the human digestive system, electron-dense amino acid side residue chain groups will become more exposed. Furthermore, peptide scissions and an increased availability of free amino acids during digestion provided an additional source of protons and electrons to maintain a high redox potential. These physicochemical changes can lead to an enhanced radical scavenging capacity and need further work.

LITERATURE CITED

(1) Kitts, D. D. Bioactive substances in food: identification and potential uses. *Can. J. Physiol. Pharmacol.* **1993**, *72*, 423–434.

(2) Hartmann, R.; Meisel, H. Food-derived peptides with biological activity: from research to food applications. *Curr. Opin. Chem. Biol.* **2007**, *18*, 163–169.

(3) Smacchi, E.; Gobbetti, M. Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. *Food Microbiol.* **2000**, *17*, 129–141.

(4) Schlimme, E.; Meisel, H. Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Nahrung* **1995**, *39*, 568–587.

(5) Pihlanto, A. Bioactive peptides: functionality and production. *Agro Food Industry Hi-Tech* **2006**, *17*, 24–26.

(6) FAO/WHO/UNU Agricultural Bulletin Board on Data Collection. *Disemination and Quality of Statistics*; World Health Organization: Geneva, Switzerland, 2002.

(7) Klockeman, D. M.; Toledo, R.; K.A., S. Isolation and characterization of defatted canola meal protein. *J. Agric. Food Chem.* **1997**, *45*, 3867–3870.

(8) Marczak, E. D.; Usui, H.; Fujita, H.; Yang, Y. J.; Yokoo, M.; Lipkowski, A. W.; Yoshikawa, M. New antihypertensive peptides isolated from rapeseed. *Peptides* **2003**, *24*, 791–798.

(9) Yust, M. D. M.; Pedroche, J.; Megias, C.; Giron-Calle, J.; Alaiz, M.; Millan, F.; Vioque, J. Rapeseed protein hydrolysates: a source of HIV protease peptide inhibitors. *Food Chem.* **2004**, *87*, 387–392.

(10) Chen, H. M.; Muramoto, K.; Yamauchi, F.; Fujimoto, K.; Nokihara, K. Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *J. Agric. Food Chem.* **1998**, *46*, 49–53.

(11) Pena-Ramos, E. A.; Xiong, Y. L. Antioxidative activity of soy protein hydrolysates in a liposomal system. *J. Food Sci.* **2002**, *67*, 2952–2956.

(12) Pena-Ramos, E. A.; Xiong, Y. L. Antioxidative activity of whey protein hydrolysates in a liposomal system. *J. Dairy Sci.* **2001**, *84*, 2577–2583.

(13) Pena-Ramos, E. A.; Xiong, Y. L. L.; Arteaga, G. E. Fractionation and characterisation for antioxidant activity of hydrolysed whey protein. *J. Sci. Food Agric.* **2004**, *84*, 1908–1918.

(14) Kong, B.; Xiong, Y. L. Antioxidant activity of zein hydrolysates in a liposome system and the possible mode of action. *J. Agric. Food Chem.* **2006**, *54*, 6059–6068.

(15) Wang, L.; Xiong, Y. L. Inhibition of lipid oxidation in cooked beef patties by hydrolyzed potato protein is related to its reducing and radical scavenging ability. *J. Agric. Food Chem.* **2005**, *53*, 9186–9192.

(16) Zhu, K.; Zhou, H.; Qian, H. Antioxidant and free radical scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase. *Process Biochem.* **2006**, *41*, 1296–1302.

(17) Wang, J. S.; Zhao, M. M.; Zhao, Q. Z.; Jiang, Y. M. Antioxidant properties of papain hydrolysates of wheat gluten in different oxidation systems. *Food Chem.* **2007**, *101*, 1658–1663.

(18) Mendis, E.; Rajapakse, N.; Kim, S. K. Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *J. Agric. Food Chem.* **2005**, *53*, 581–587.

(19) Davalos, A.; Miguel, M.; Bartolome, B.; Lopez-Fandino, R. Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *J. Food Prot.* **2004**, *67*, 1939–1944.

(20) Halliwell, B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence. *Lancet* **1994**, *344*, 721–724.

(21) Wu, H. C.; Chen, H. M.; Shiau, C. Y. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Res. Int.* **2003**, *36*, 949–957.

(22) Sakanaki, S.; Tachibana, Y.; Ishihara, N.; Juneja, L. R. Antioxidant activity of egg-yolk protein hydrolysates in a linoleic acid oxidation system. *Food Chem.* **2004**, *86*, 99–103.

(23) Pihlanto, A. Antioxidative peptides derived from milk proteins. *Int. Dairy J.* **2006**, *16*, 1306–1314.

(24) Chang, C. Y.; Wu, K. C.; Chiang, S. H. Antioxidant properties and protein compositions of porcine haemoglobin hydrolysates. *Food Chem.* **2007**, *100*, 1537–1543.

(25) Adler-Nissen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzensulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256–1262.

(26) Association of Official Analytical Chemists (AOAC).*Official Methods of Analysis*, 16th ed.; AOAC: Gaithersburg, MD, 1995.

(27) Alaiz, M.; Navarro, J. L.; Giron, J.; Vioque, E. Amino acid analysis by high-performance liquid chromatography after derivatization with diethylethoxymethylenemalonate. *J. Chromatogr.* **1992**, *591*, 181–186.

(28) Oyaizu, M. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr.* **1986**, *44*, 307–315.

(29) Halliwell, B.; Gutteridge, J. M. C.; Aruoma, O. I. The deoxyribose method: a simple test tube assay for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem.* **1987**, *165*, 215–219.

(30) Sakurai, J.; Fujii, Y.; Torii, K.; Kobayashi, K. Dissociation of various biological activities of *Clostridium perfringens* α toxin by chemical modification. *Toxicon* **1989**, *27*, 317–323.

(31) Deparis, V.; Durrieu, C.; Schweizer, M.; Marc, I.; Goergen, J. L.; Chevalot, I.; Marc, A. Promoting effect of rapeseed proteins and peptides on Sf9 insect cell growth. *Cytotechnology* **2003**, *42*, 75–85.

(32) van der Ven, C.; Gruppen, H.; de Bont, D. B. A.; Voragen, A. G. J. Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. *Int. Dairy J.* **2002**, *12*, 813–820.

(33) Alting, A. C.; Meijer, R. J. G. M.; van Beresteijn, E. C. H. Selective hydrolysis of milk proteins to facilitate the elimination of the ABBOS epitope of bovine serum albumin and other immunoreactive epitopes. *J. Food Prot.* **1998**, *61*, 1007–1012.

(34) Villanueva, A.; Vioque, J.; Sanchez-Vioque, R.; Clemente, A.; Bautista, J.; Millan, F. Production of an extensive sunflower protein hydrolysate by sequential hydrolysis with endo- and exo-proteases. *Grasas Aceites* **1999**, *50*, 472–476.

(35) Megias, C.; Pedroche, J.; Yust, M. M.; Giron-Calle, J.; Alaiz, M.; Millan, F.; Vioque, J. Affinity purification of copper-chelating peptides from sunflower protein hydrolysates. *J. Agric. Food Chem.* **2007**, *55*, 6509–6514.

(36) Chen, H. M.; Muramoto, K.; Yamauchi, F.; Nokihara, K. Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *J. Agric. Food Chem.* **1996**, *44*, 2619–2623.

(37) Hernandez-Ledesma, B.; Amigo, L.; Recio, I.; Bartolome, B. ACE-inhibitory and radical-scavenging activity of peptides derived from beta-lactoglobulin f(19−25). Interactions with ascorbic acid. *J. Agric. Food Chem.* **2007**, *55*, 3392–3397.

(38) Saito, K.; Jin, D. H.; Ogawa, T.; Muramoto, K.; Hatakeyama, E.; Yasuhara, T.; Nokihara, K. Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry. *J. Agric. Food Chem.* **2003**, *51*, 3668–3674.

(39) Saiga, A.; Tanabe, S.; Nishimura, T. Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. *J. Agric. Food Chem.* **2003**, *51*, 3661–3667.

(40) Yamaguchi, N.; Naito, S.; Yokoo, Y.; Fujimaki, M. Application of protein hydrolysate to biscuit as antioxidant. *J. Jpn. Soc. Food Sci.* **1980**, *27*, 56–59.

(41) Jun, S. Y.; Park, P. J.; Jung, W. K.; Kim, S. K. Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *Eur. Food Res. Technol.* **2004**, *219*, 20–26.

(42) Yee, J. J.; Shipe, W. F.; Kinsella, J. E. Antioxidant effects of soy protein hydrolysates on copper-catalyzed methyl linoleate oxidation. *J. Food Sci.* **1980**, *45*, 1082–1083.

(43) Bishov, S. J.; Henick, A. S. Antioxidant effect of protein hydrolysates in a freeze-dried model system. *J. Food Sci.* **1972**, *37*, 873–875.

(44) Moldovan, L.; Moldovan, N. I. Oxygen free radicals and redox biology of organelles.. *Histochem. Cell Biol.* **2004**, *122*, 395–412.

(45) Nice, D. J.; Robinson, D. S. Inhibition of lipid autoxidation by bovine superoxide dismutase. *Food Chem.* **1992**, *45*, 99–103.

(46) Sakanaki, S.; Tachibana, Y.; Ishihara, N.; Juneja, L. R. Antioxidant properties of casein calcium peptides and their effects on lipid oxidation in beef homogenates. *J. Agric. Food Chem.* **2005**, *53*, 464–468.

(47) Temple, N. J. Antioxidants and disease: more questions than answers. *Nutr. Res. (N.Y.)* **2000**, *20*, 449–459.

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