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Chromatographic Separation and Tandem MS Identification of Active Peptides in Potato Protein Hydrolysate That Inhibit Autoxidation of Soybean Oil-in-Water Emulsions

Yu Cheng,[†] Jie Chen,[†] and Youling L. Xiong*[,‡]

[†]State Key Laboratory of Food Science and Technology and School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China, and [‡]Department of Animal and Food Sciences, University of Kentucky, Lexington, Kentucky 40546

A previously developed antioxidative potato protein hydrolysate was fractionated using gel filtration. The efficacy of different fractions for inhibiting lipid oxidation in soybean oil-in-water emulsions, neutralizing 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+\bullet}$) radicals, and chelating prooxidative metal ions was investigated. Low-molecular-weight fraction Peak 3 (709 Da) exhibited the strongest antioxidant activity and radical scavenging activity. Active peptides based on the $ABTS^{+\bullet}$ scavenging assay were isolated by RP-HPLC and purified by UPLC. The amino acid sequence determination by MS/MS identified eight prominent peptides in the antioxidative Peak 3 fraction, of which Thr-Tyr, Tyr-Phe-Glu, Tyr-Ser-Thr-Ala, and Asn-Tyr-Lys-Gln-Met matched the sequence of papatin.

KEYWORDS: Potato protein hydrolysate; antioxidant; emulsion; lipid oxidation; peptide sequence

INTRODUCTION

Oil-in-water (O/W) emulsions are common biphasic dispersions that exist in food products, such as beverages, sauces, and soups. Of particular prevalence are emulsions prepared from vegetable oils which are rich in unsaturated fatty acids. Oxidation is a common problem for this type of food emulsions and is a main cause for rancidity off-flavors. To improve the oxidative stability of O/W emulsions, various antioxidants are incorporated. Recent studies have shown that many protein hydrolysates can act as antioxidants to inhibit lipid oxidation in model systems, for example, liposome suspensions (*1*−*3*) and unsaturated free fatty acid dispersions (*4*, *5*). Antioxidant activity of protein hydrolysates against lipid oxidation in more complex food systems, e.g., meat emulsions, has also been reported (*6*−*10*).

In a previous investigation (*11*), we produced an antioxidative protein hydrolysate from potato protein using Alcalase. The activity of this potato protein hydrolysate (PPH) to inhibit lipid oxidation in soybean O/W emulsions was demonstrated (*12*). When incorporated into emulsions as a coemulsifier with Tween 20, PPH substantially enhanced the oxidative stability. In situ studies of the PPH in emulsified meat (batters) confirmed remarkable capability of the protein hydrolysate to minimize oxidation changes in meat batters (*7*).

The mode of action of peptides in general and PPH in particular has been subjected to extensive studies. It has been reported that radical scavenging activity, a main mechanism for the inhibition of oxidation by protein hydrolysates, was related to the peptide size (*13*, *14*). In general, low molecular weight peptides have stronger activity against radicals (*1*, *15*, *16*). Such peptides are capable of chelating prooxidative metal irons, thereby minimizing oxidative potential (*17*, *18*).

The objective of the present study was to identify the main peptides present in antioxidative PPH that acted to inhibit oxidation in a soybean O/W emulsion system. This was accomplished by the initial separation of peptides into different molecular weight fractions using gel filtration, followed by the purification using reversed phase HPLC (RP-HPLC). The individual peptides were subsequently isolated and their amino acid sequences identified using ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS).

MATERIALS AND METHODS

Materials. Potato protein concentrate (81% protein) was obtained from AVEBE ba (Veendam, The Netherlands). Alcalase (endoproteinase from *Bacillus licheniformis*, 2.4 AU/g) was donated by Novozymes North America Inc. (Franklinton, NC). TEP (1,1,3,3-tetraethoxypropane), ABTS [2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)], BHA (butylated hydroxyanisole), and hexanal were purchased from Sigma-Aldrich Co. (St. Louis, MO). Soybean oil was provided by Eastocean Oils and Grains Industries Co. (Zhang Jiagang, Jiangsu, China). All other chemicals were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China), and they were of analytical grade unless otherwise specified. Distilled deionized water was used for the preparation of all solutions.

Preparation of Protein Hydrolysate. PPH was prepared by 1-h hydrolysis of intact potato protein (4%, w/v) in an aqueous solution at pH 8.0 and 50 °C using Alcalase (*11*). The 1-h protein hydrolysate has been shown to strongly inhibit lipid oxidation in meat emulsion systems (*7*, *11*). The enzyme/protein substrate ratio was 1/100. After hydrolysis, the broth was adjusted to pH 7.0 with 1 M NaOH and then heated at 80 °C for 15 min to inactivate the enzyme. The hydrolysate was freeze-dried, pulverized, placed in sealed bottles, and stored at 4 °C.

*Corresponding author. Phone: 859-257-3822. Fax: 859-257-5318. E-mail: ylxiong@uky.edu.

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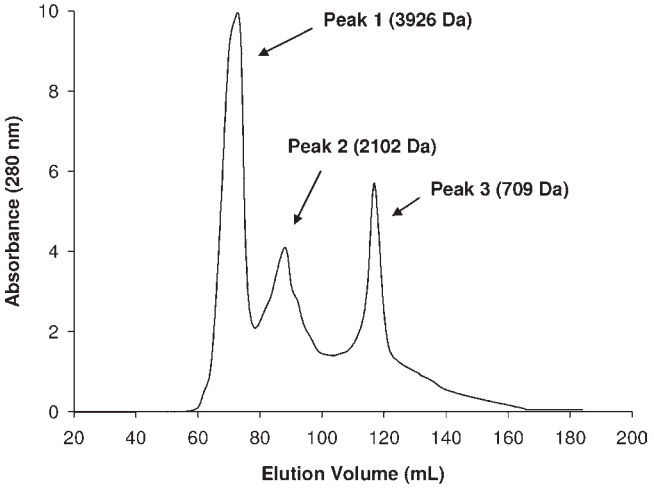

Figure 1. Sephadex G15 gel filtration of potato protein hydrolysate ($n = 20$).

Peptide Fractionation. PPH was fractionated on the basis of molecular weight (MW) using gel filtration. Specifically, the PPH solution (5%, w/v, in deionized water, pH 7.0) was subjected to low-pressure size exclusion chromatography with a 2.6 × 120 cm Sephadex G-15 column (GE Healthcare China Ltd., Shanghai, China). Three fractions (Peak 1, Peak 2, and Peak 3) were collected (**Figure 1**). To estimate their MWs, these fractions were injected into a high-performance Waters 1525 HPLC system (Waters Corporation, Milford, MA) with a Shodex PROTEIN KW-802.5 column (8 × 300 mm) (Showa Denko KK, Japan) (*19*). On the basis of the MW−elution time regression plot generated with MW standards (200−12400 Da), Peaks 1, 2, and 3 were found to consist of peptides with mean MWs of 3926, 2102, and 709 Da, respectively. Identical fractions of 20 separate gel filtration runs were combined to obtain sufficient amounts of peptide samples. The amino acid compositions of different fractions were determined by HPLC using the OPA derivation method (*19*).

Radical Scavenging Activity (RSA). The ABTS radical cation ($ABTS^{+\bullet}$) assay was used for RSA analysis (*20*). $ABTS^{+\bullet}$ was produced by reacting ABTS stock solution (7 mM) with 2.45 mM potassium persulfate (final concentration). The $ABTS^{+\bullet}$ scavenging activity of different peptide fractions was measured by mixing 20 μL of samples with 1980 μL of diluted $ABTS^{+\bullet}$ solution and reading the absorbance (734 nm) after 10 min. A standard curve was prepared by reacting 20 μL of a series of concentrations of Trolox (0−3 mM) with 1980 μL of diluted $ABTS^{+\bullet}$ solution; the RSA of peptide samples was expressed as the Trolox equivalent (μM).

Fe^{2+} Chelating Activity. Metal chelating activity of PPH fractions was evaluated as described by Wang and Xiong (*11*). One-milliliter of 20 μM $FeCl_2$ was mixed with 2 mL of 500 μM ferrozine. After the addition of 0.5 mL of peptide sample solutions and subsequent 10-min incubation, the absorbance was read at 562 nm. The Fe^{2+} chelating activity was calculated as [1 − (sample solution absorbance/blank solution absorbance)] × 100.

Emulsion Preparation. O/W emulsions were prepared by the homogenization of 10% (w/w) of soybean oil in 90% (w/w) of 0.01 M phosphate buffer (pH 7.0) containing 11.1 mg/mL of Tween 20 and different MW fractions (5 mg/mL for Peak 1 and Peak 2; 1 mg/mL for Peak 3) using the procedures described previously (*12*). Briefly, fine emulsions were obtained by blending coarse emulsions using a high-performance disperser (Ika-Werke Gmbh & Co., Staufen, Germany) at 21,500 rpm for 2 min followed by passing through an ATS AH-basic high-pressure (320 bar) valve homogenizer (ATS Engineering Inc., Brampton, Canada) twice. All emulsions were remarkably stable; the particle size showed no significant change even after 2 weeks of storage at 37 °C.

Characteristics of Emulsion Particles. The emulsions prepared above were diluted to a lipid concentration of 0.05% (w/w) using a 0.01 M phosphate buffer solution (pH 7.0) to avoid multiple scattering effects prior to analysis. The particle size distribution and ζ-potential of the emulsions were measured using a Mastersizer Nano-ZS instrument (Malvern Instruments, Worcestershire, UK). The emulsion droplet size, expressed as the mean diameter Z-average, and the ζ-potential were obtained as the average of three freshly prepared emulsion samples with duplicate measurements. Partitioning of peptides at the interface of emulsions was determined according to Huang (*21*) as detailed by Cheng (*12*).

Emulsion Oxidative Stability. Emulsions were oxidized by incubation at 37 °C in darkness up to 14 days as described previously (*12*). Lipid oxidation was monitored by the indicators of hexanal and 2-thiobarbituric acid-reactive substances (TBARS). For hexanal determination, emulsion samples (3 mL) collected from different storage times were placed in 30-mL head space vials and sealed with poly(tetrafluoroethylene) butyl rubber septa. The head space conditions were as follows: sample temperature, 70 °C; equilibration time, 30 min; transfer line temperature, 130 °C; and injection time, 0.04 min. Aldehydes were separated using temperature programming (40 °C for 5 min, followed by heating to 180 at 2 °C/min) on a PEG-20 M capillary column (0.32 mm × 30 m) in a Shimadzu GC-2010 gas chromatograph (Shimadzu Corporation, Tokyo, Japan) equipped with a PerkinElmer Turbo Matrix 16 head space sampler (Perkin-Elmer Inc., Covina, CA). Concentrations were determined from peak areas using an authentic hexanal standard (Sigma-Aldrich Co., St. Louis, MO).

For TBARS, the procedure of Mei et al. (*22*) was followed. Mixtures of the TBA reagents and emulsion samples were heated in a boiling water bath for 15 min, cooled to room temperature, and centrifuged at 4000*g* for 25 min. Absorbance of the supernatant was read at 532 nm. Sample TBARS concentrations were determined from the standard curve prepared using TEP.

Isolation and Identification of Active Peptides. PPH fractions from gel filtration that exhibited strong $ABTS^{+\bullet}$ scavenging activity were further purified using preparative Waters 1525 HPLC (Waters Corporation, Milford, MA) on a Sunfire Pre C18 column (1.9 × 15 cm) with a linear gradient of methanol (10−95% in 50 min) at a flow rate of 6 mL/min. The elution fractions were collected and concentrated using a rotary evaporator, and subsequently freeze-dried. In order to obtain sufficient amounts of peptide samples, the reversed phase HPLC was run 10 times, and the same fractions from different runs were combined. The accuracy of elution time was 0.01 min.

Purified peptide fractions from the RP-HPLC were tested for $ABTS^{+\bullet}$ scavenging activity (*20*). The fractions that exhibited the strongest radical scavenging activity were subjected to ultraperformance liquid chromatography for peptide separation in an Acquity UPLC BEH C18 column (2.1 × 120 mm) (Waters Corporation, Milford, MA). The mobile solvent flow rate was set at 0.3 mL/min, and a linear gradient (0 to 20%, acetonitrile and formic acid in 20 min for mobile phases A and B, respectively) was used. Eluted peptides were analyzed by tandem mass spectrometry (MS/MS) for sequence identification using a Waters Synapt Mass Quadrupole Time-of-Flight Mass Spectrometer (Waters Corporation, Milford, MA) through an electrospray ionization source. An automated data acquisition using the information-dependent mode was performed on Synapt MS under the control of MassLynx software (Waters Corporation, Milford, MA). The spectra were interpreted using the peptide sequencing module of the MassLynx software. Properties of identified peptides were calculated using Peptide Property Calculator of Innovagen (Innovagen AB, Se-22370 Lund, Sweden). The peptide sequences were matched to the published sequences of potato proteins from the National Center for Biotechnology Information (NCBI) database.

Statistical Analysis. All experiments were replicated three times on different days unless stated otherwise, and triplicate sample analyses were performed within each replication. Data were subjected to one-way analysis of variance (ANOVA) using the Origin 7.5 statistics program (OriginLab Corporation, Northampton, MA). Differences ($P < 0.05$) between means were identified by LSD's test.

RESULTS AND DISCUSSION

$ABTS^{+\bullet}$ Scavenging and Fe^{2+} Chelating Activities of PPH Fractions. The $ABTS^{+\bullet}$ scavenging activities of different PPH MW fractions are compared at 1 and 5 mg/mL protein concentration levels (**Figure 2A**). At 1 mg/mL, the lowest MW fraction (Peak 3, 709 Da) exhibited the strongest activity, which was two times greater than the PPH mixture and equal to that of BHA. The $ABTS^{+\bullet}$ scavenging activity of fraction 1 (Peak 1), which had the highest average MW (3926 Da), was approximately 32% of that of fraction 3 ($P < 0.05$). At 5 mg/mL, fraction 3 had $ABTS^{+\bullet}$

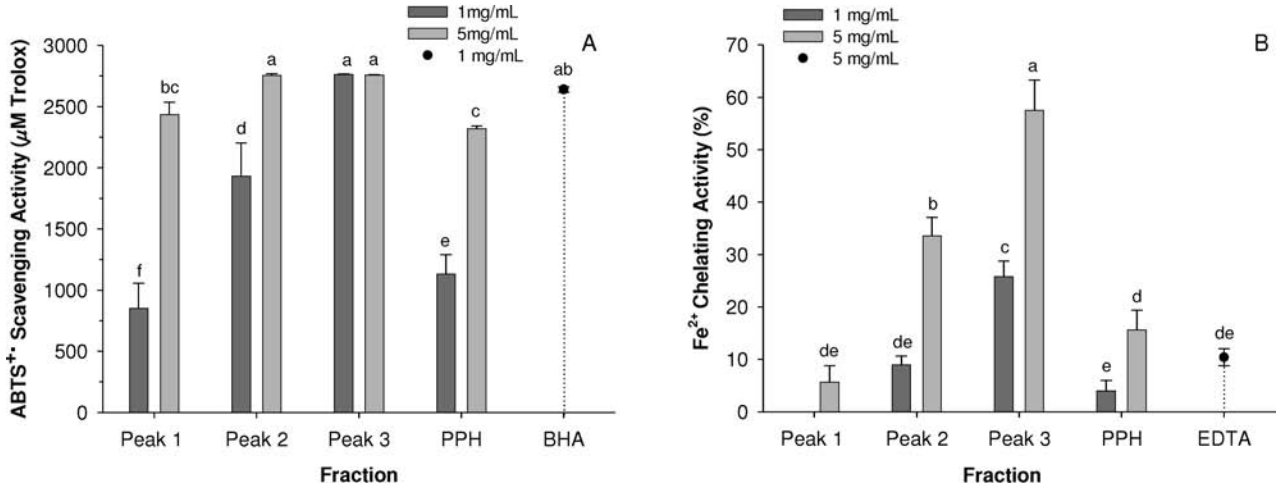

Figure 2. $ABTS^{+\bullet}$ scavenging activity (**A**) and metal chelating activity (**B**) of PPH fractions (Peak 1, Peak 2, and Peak 3) obtained from gel filtration. The protein concentration for each fraction was 1 and 5 mg/mL, while the concentration for BHA and EDTA was 1 and 5 mg/mL, respectively. Means (±SD) ($n = 3$) within the same graph without a common letter differ significantly ($P < 0.05$).

scavenging activity identical to that at 1 mg/mL, suggesting saturation. However, the activity of fractions 1 and 2 increased by 1.8 and 0.4-fold ($P < 0.05$), respectively, when the peptide concentration was raised from 1 to 5 mg/mL. The results indicated that the most efficient and responsible radical scavenging peptides in PPH were those with relatively low MWs. The mean MW of 709 Da in fraction 3 corresponded to penta- or hexapeptides, in agreement with literature reports that a typical antioxidative peptide comprises 2−9 amino acids (*23*). Comparison of the amino acid profiles of different PPH fractions indicates substantially greater amounts of tyrosine and phenylalanine residues present in the Peak 3 fraction (**Table 1**). Both amino acids, known to be reactive to radicals (*24*, *25*), likely contributed to the activity of the small-MW peptide fraction. The presence of tyrosine in β-lactoglobulin hydrolysate and in its constituting peptides has been linked to the inhibition of lipid oxidation in emulsions in which the β-lactoglobulin hydrolysate was used as antioxidant (*10*).

PPH fractions were also compared for their Fe^{2+} chelating activity (**Figure 2B**). At 1 mg/mL, the Peak 1 fraction did not exhibit any activity, while the Peak 3 fraction had stronger activity than any other fractions or the PPH mixture ($P < 0.05$). When the protein concentration was raised to 5 mg/mL, the metal chelation activity of fractions 2 and 3 increased by 2.6 and 1.2-fold ($P < 0.05$), respectively. The remarkably strong Fe^{2+} sequestering capacity of Peak 3 was further demonstrated by comparing with EDTA, whose efficacy was only about 20% of that of Peak 3 at 5 mg/mL assay concentrations.

The strong metal chelation activity of the Peak 3 fraction (709 Da) can be explained because low-MW peptides tend to have greater negative charge (carboxyl groups)-to-mass ratios than larger peptides, enabling them to bind metal ions more efficiently. The result was in accordance with the literature reports that small peptides from enzyme hydrolyzed proteins, for example, catfish (*17*) and buckwheat (*1*) proteins, are generally more capable of chelating metal ions when compared with high-MW peptides. Furthermore, as shown in **Table 1**, histidine was more abundant in fraction 3 than in fractions 1 and 2. Histidine, which was used as a tag for peptide separation using metal affinity chromatography (*26*, *27*), was correlated to the metal chelating activity of antioxidant peptides (*28*, *29*).

Inhibition of Lipid Oxidation in Emulsions. The impact of PPH fractions on the oxidative stability of O/W emulsions was evaluated by means of hexanal and TBARS. Hexanal is one of the major secondary oxidation products of linoleic acid and considered to be a good oxidation indicator for oils rich in linoleic acid, such as soybean oil (*30*). In our previous study (*12*), we had demonstrated that the oxidative stability of soybean oil emulsions could be enhanced by PPH at a concentration of 5 mg/mL or above. Therefore, the protein concentration used in the present study was 5 mg/mL for all fractions except fraction 3 (Peak 3) because at 1 mg/mL, it already displayed remarkably strong radical scavenging activity (**Figure 2A**).

Table 1. Amino Acid Composition of PPH Fractions From Gel Filtration

amino acid (g/100 g protein powder)		PPH	Peak 1	Peak 2	Peak 3
Asp[a]	D/N	9.30	10.64	8.06	3.90
Glu[b]	E/Q	9.33	10.32	9.15	3.28
Ser	S	4.18	3.82	3.86	2.58
His	H	1.46	1.00	1.46	2.53
Gly	G	3.58	3.82	3.41	2.23
Thr	T	4.58	4.36	4.81	2.41
Arg	R	3.12	3.61	2.20	1.68
Ala	A	4.10	3.93	4.53	2.15
Tyr	Y	1.58	1.81	3.69	8.86
Cys	C	0.44	0.27	0.36	0.16
Val	V	4.37	4.87	4.92	2.45
Met	M	1.77	1.32	2.38	1.62
Phe	F	4.36	3.26	4.84	7.12
Ile	I	3.46	3.89	4.30	2.54
Leu	L	7.43	6.70	8.61	4.18
Lys	K	5.25	7.27	3.44	1.96
Pro	P	3.17	3.69	2.85	2.10
Total amino acid		71.48	74.58	72.88	51.79

[a] Aspartic acid + asparagine. [b] Glutamic acid + glutamine.

Results from hexanal and TBARS analyses are presented in **Figure 3**. The hexanal content in the head space of the control emulsion (no PPH) rose sharply (6-fold, $P < 0.05$) from day 1 to day 3, followed by a rapid decline (**Figure 3A**). In contrast, the hexanal content in the head space of all peptide-treated emulsions increased very little throughout the entire 14-day storage. The presence of PPH and its MW peptide fractions inhibited the production of hexanal through the removal of radicals and stabilization of metal ions, thereby preventing the initiation or propagation of unsaturated fatty acids in the soybean O/W emulsion. It cannot be ruled out, however, that the low levels of

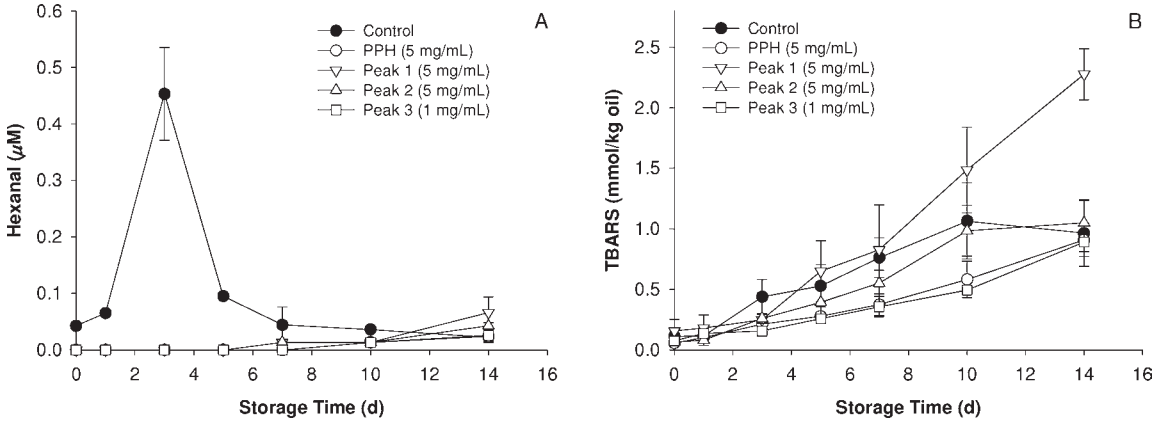

Figure 3. Formation of hexanal (n = 2) (**A**) and TBARS (n = 3) (**B**) during the storage (37 °C) of soybean O/W emulsions prepared with Tween 20 (10 mg/mL) + PPH (5 mg/mL) and PPH fractions Peak 1 (5 mg/mL), Peak 2 (5 mg/mL), and Peak 3 (1 mg/mL) obtained from gel filtration.

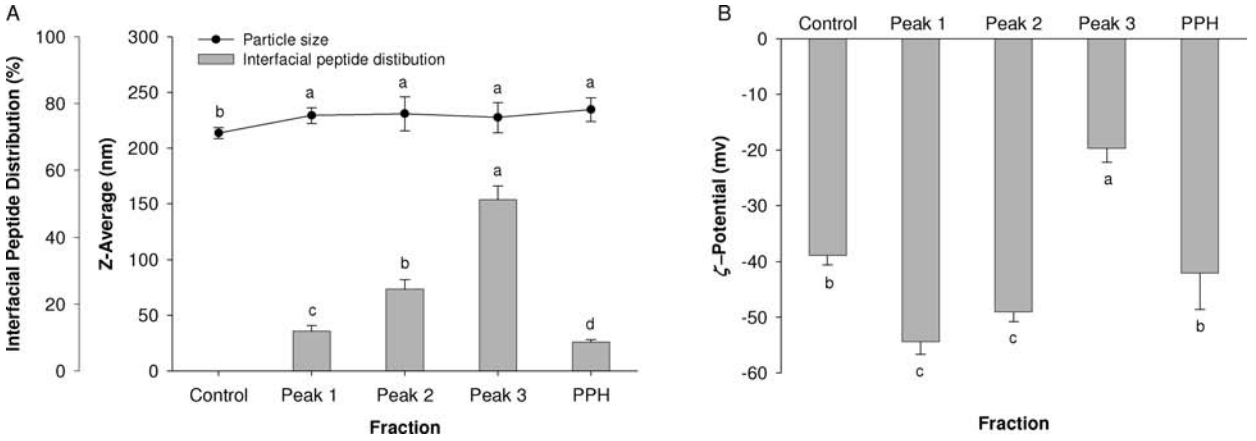

Figure 4. Particle size (n = 3), interfacial peptide distribution (n = 3) (**A**), and ζ-potential (n = 3) (**B**) of soybean O/W emulsions prepared with Tween 20 (10 mg/mL) + PPH (5 mg/mL) and PPH fractions Peak 1 (5 mg/mL), Peak 2 (5 mg/mL), and Peak 3 (1 mg/mL) obtained from gel filtration.

hexanal detected in the head space of all treated emulsion samples could also be due to the possibility that lipid carbonyls form complexes with amino groups of protein side chains (*31*−*34*). If formed, hexanal in the soybean O/W emulsion could react with peptides in different PPH fractions to become nonvolatile, hence undetectable. This phenomenon has been observed in washed turkey meat where hexanal and propanal when added to washed meat became less detectable (*35*).

In the TBARS analysis, the potential underestimation of carbonyl (hexanal) formation by the head space GC method is avoided because the TBARS assay involved the application of heat (boiling) under acidic conditions that liberates protein-bound MDA (*36*). When PPH fractions with different MWs were added to the soybean O/W emulsion, mixed PPH and the Peak 3 fraction inhibited TBARS production during storage (**Figure 3B**). The TBARS content in the 10-day emulsions was reduced 45.2% and 53.6% by PPH and Peak 3, respectively, when compared with the control. The degree of TBARS inhibition by PPH in the present study was identical to that noted in our previous study (*12*) at the same protein concentration (5 mg/mL). However, while the Peak 2 fraction showed a tendency to suppress TBARS, the Peak 1 fraction promoted TBARS production after 10 days ($P < 0.05$).

In order to explain the variation in antioxidant efficacy between PPH fractions, peptide deposition at the interface and the surface charge of emulsion droplets was measured (**Figure 4**). For the Peak 3 fraction, the peptides were not only strongly active against the radical species as shown previously but also were readily loaded at the interface with 51% being distributed at the interfacial membrane when compared with only 12% for the Peak 1 fraction and 24% for the Peak 2 fraction (**Figure 4A**). These data were consistent with the radical scavenging and metal ion chelation analysis results and served to explain why Peak 1 and Peak 2 peptide fractions were relatively ineffective, while the Peak 3 fraction was remarkably efficient in maintaining the oxidative stability of soybean O/W emulsions. However, no significant particle size difference was observed between emulsions (230 ± 2 nm) treated with different peptide fractions. The oil droplet size difference between peptide-treated emulsions and the peptide-free control emulsion (diameter 230 nm versus 213 nm) suggested that the interfacial membrane of treated emulsions was on average 17 nm larger in thickness. Surprisingly, this physical difference did not seem to have an apparent effect on the protection of encapsulated oil against oxidation because the production of TBARS during emulsion storage (**Figure 3B**) was not correlated to the size of oil droplets.

The emulsions with the Peak 1 and Peak 2 peptide fractions had the greatest negative ζ-potential (**Figure 4B**), suggesting that they had a strong tendency to attract prooxidative metal ions that were present as trace compounds in the materials used to prepare the emulsions (oil, emulsifiers, etc.). For example, dry PPH contained 0.9 ppm iron and 0.2 ppm copper (unpublished data). However, the emulsion droplets with the Peak 3 fraction had a much lower ζ-potential which would reduce the accessibility of cationic prooxidants. As shown earlier (**Figure 2B**), the Fe^{2+} chelating activity of Peak 1 and Peak 2 peptide fractions was low relative to that of the Peak 3 fraction and PPH. Because of their

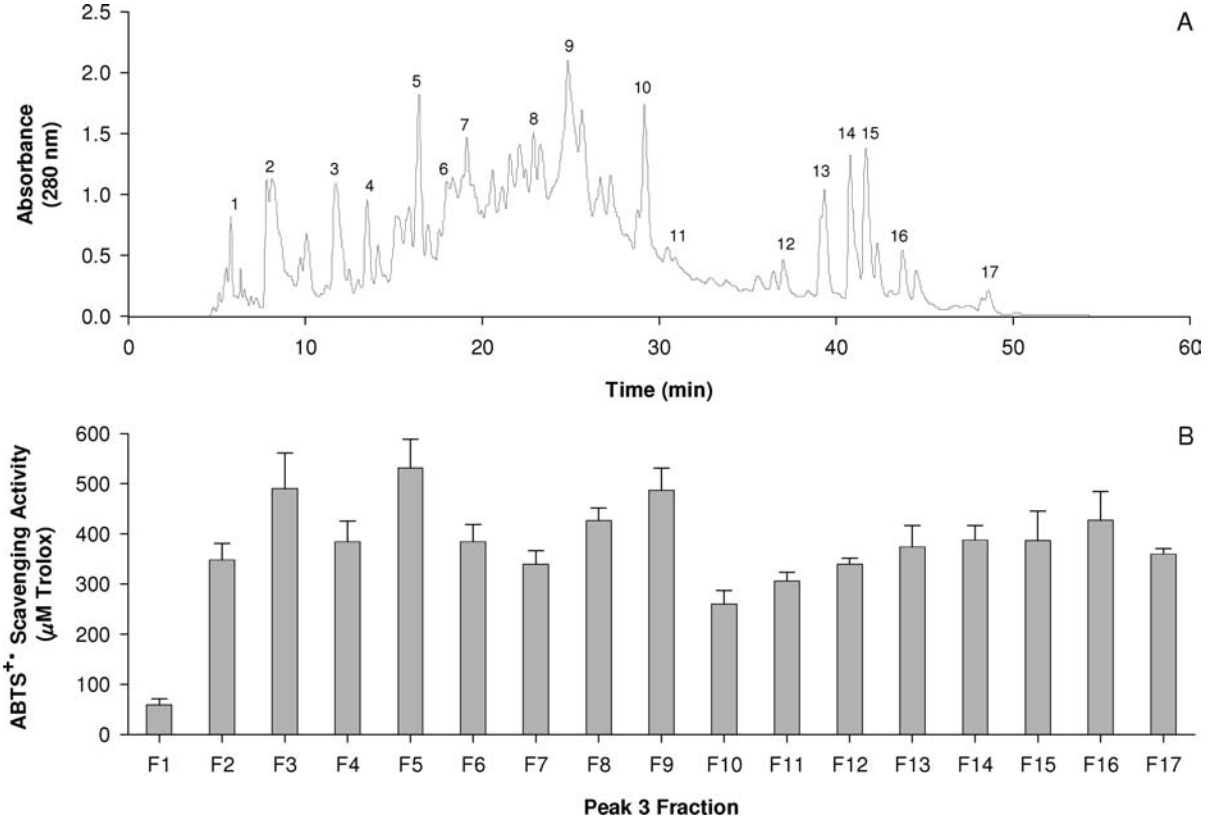

Figure 5. Preparative RP-HPLC spectra of Peak 3 obtained from a PPH mixture using gel filtration with 17 isolated fractions labeled by numbers (**A**) and the $ABTS^{+\bullet}$ scavenging activity of these individual correspondent fractions at the protein concentration of 200 μg/mL (**B**).

Table 2. Antioxidative Peptides From Selected Peak 3 Fractions That Were Isolated and Identified Using UPLC-MS/MS

fraction	peptide		MW	prob. (%)
P3−F3	TY	Thr-Tyr	282.1	100
	VY	Val-Tyr	280.1	99
	NYKQM	Asn-Tyr-Lys-Gln-Met	682.3	92
	YSTA	Tyr-Ser-Thr-Ala	440.2	99
P3−F5	YFE	Tyr-Phe-Glu	457.2	97
	SWN	Ser-Trp-Asn	405.2	99
P3−F9	HVCYMF	His-Val-Cys-Tyr-Met-Phe	798.3	92
	MWL	Met-Trp-Leu	448.2	100

weak Fe^{2+} binding capacity, Peak 1 and Peak 2 would be less capable of preventing metal ions from contacting lipid peroxides to initiate their decomposition and ensuing formation of secondary oxidation products.

Peptide Purification and Sequence Identification. Peak 3, the most antioxidative fraction from the gel filtration of PPH, was subjected to preparative HPLC separation and purification followed by tandem MS to identify the most reactive peptides. The RP-HPLC yielded a total of 17 fractions, designated as P3−F1 through P3−F17 (**Figure 5A**). Of these individual fractions, P3−F3, P3−F5, and P3−F9 demonstrated strong $ABTS^{+\bullet}$ scavenging activity (**Figure 5B**). These three fractions were subsequently submitted to ultraperformance liquid chromatography−tandem mass spectrometry (UPLC-MS/MS) for peptide separation and sequence identification.

The eight prominent peptides with a probability of certainty greater than 90% were identified. The sequences of these peptides are shown in **Table 2**. These peptides, consisting of 2−6 amino acid residues, tyrosine being common in most, were in the 280−800 Da MW range. Many reports have also associated the antioxidant activity of protein hydrolysates to short peptides, for example, peptides from yellow stripe trevally meat hydrolysates (656 and 617 Da) (*15*), zein hydrolysates (252 and 502 Da) (*13*), porcine plasma protein hydrolysate (441 Da) (*37*), and buckwheat protein hydrolysate (362 and 456 Da) (*38*).

Table 3. Properties of Antioxidative Peptides from Selected Peak 3 Fractions That Were Isolated and Identified Using UPLC-MS/MS[a]

fraction	peptide	pI	hydrophilic residues/total residues (%)	average hydrophilicity	net charge at pH 7.0 (rounded)
P3−F3	TY	5.9	0	−1.3	0
	VY	5.9	0	−1.9	0
	NYKQM	9.7	60	0	1
	YSTA	5.9	25	−0.7	0
P3−F5	YFE	3.3	33	−0.6	−1
	SWN	14.0	67	−1	1
P3−F9	HVCYMF	7.1	0	−1.5	0
	MWL	6.0	0	−2.2	0

[a] Values were calculated using Peptide Property Calculator of Innovagen (Innovagen AB, Se-22370 Lund, Sweden).

The identified peptides in the antioxidant PPH fractions comprised both hydrophobic and hydrophilic amino acids; however, the majority of the amino acids in these peptides were hydrophobic, rendering all peptides an overall slightly negative hydrophilicity (**Table 3**). The prevalent distribution of hydrophobic groups versus hydrophilic side chains would favor the partitioning of peptides at the emulsion interface, thereby enhancing the oxidation stability of emulsions through the stabilization of invading radicals and prooxidative metal ions before they reach the lipid core.

The MS/MS spectra of isolated and identified peptides from antioxidative Peak 3 fractions (F3, F5, and F9) are displayed in

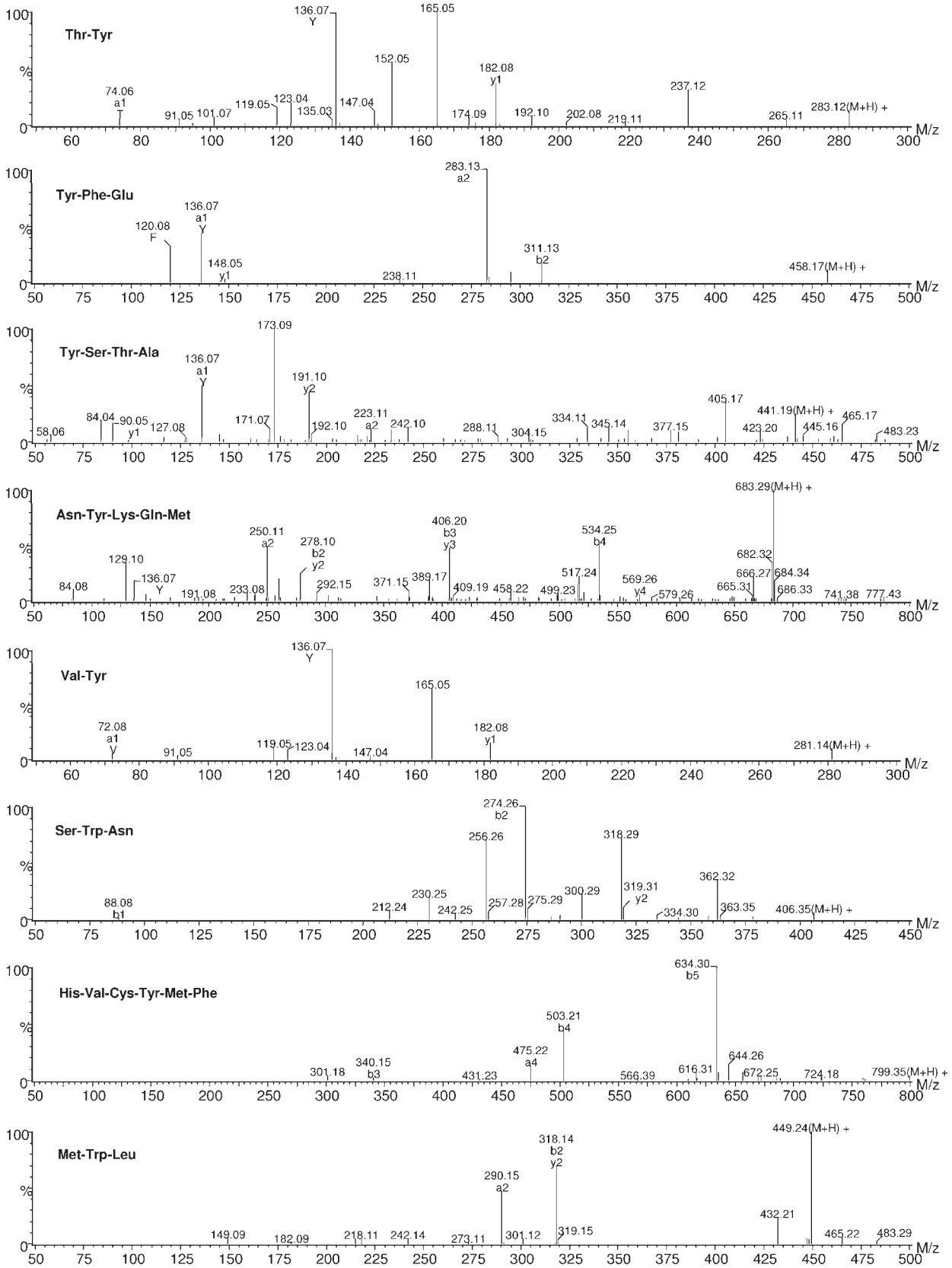

Figure 6. Tandem mass spectra of prominent peptides isolated from individual antioxidative Peak 3 fractions using RP-HPLC followed by UPLC.

Figure 6. When peptide ions collide in MS, the amide bond in peptide ions is cleaved between the carbonyl and the amide nitrogen to form a y ion and a b ion (*39*). On the basis of the distinct spectral signals at the specific mass/charge (m/z) ratios of y ions and b ions, the sequences of these peptides can be determined. The spectra of identified peptide fragments were characterized by different y ions or b ions, along with some secondary a ions (**Figure 6**).

Four of these peptides matched segments in the sequence of patatin, a major component of potato protein (**Table 4**).

Table 4. Identified Antioxidative Peptides in PPH That Matched the Peptide Sequences of Patatin

peptide	matched sequence in patatin[a]	
TY	f270−271	GTTSEFDK**TY**
	f361−362	**TY**EEALKRFA
YFE	f105−107	DIVPF**YFE**HG
YSTA	f185−188	YDIC**YSTA**AA
NYKQM	f249−253	KFASIKSL**NYKQM**LLLSLGT

[a] From National Center for Biotechnology Information (NCBI).

Depending on the enzymes used, different antioxidative peptides can be produced. For example, using pancreatin and Amano-P, peptides with the sequences of Phe-Gly-Glu-Arg, Phe-Asp-Arg-Arg, and Phe-Gly-Glu-Arg-Arg were obtained from potato protein (*40*). Phenylalanine and arginine were the common amino acids in those peptides.

However, in our peptides prepared from potato protein with Alcalase, tyrosine, which exhibited strong radical scavenging activity and antioxidant activity in linoleic acid emulsions (*41*), was the common amino acid. Tyrosine was inside the sequence and at both termini of the peptides. Therefore, its exact position did not seem to be a critical factor for the elicitation of a peptide's antioxidant potential. Peptide Tyr-Phe-Glu, which was isolated from P3−F5 (**Figure 5**), was the most abundant and most active peptide of all. The combination of two aromatic amino acids, which are primary targets of oxygen radicals (*24*), seemed to allow the tripeptide to readily absorb free radicals, forming a relatively stable complex with low resonance energy.

In conclusion, a low-MW peptide fraction (709 Da) prepared from PPH exhibited strong radical scavenging and metal chelating activities and was capable of improving the oxidative stability of soybean O/W emulsions. The eight prominent peptides identified from the low-MW fraction comprised 2−6 amino acids of which tyrosine was a common residue in most of them. The presence of these antioxidative oligopeptides served to protect emulsified oil from free radical and pro-oxidant attack, thereby preventing lipid oxidation. The results provide the basis for the preparation of small antioxidative peptides from potato protein for potential food emulsion applications.

ACKNOWLEDGMENT

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