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Synergistic Inhibition of Lipid Oxidation by Pea Protein Hydrolysate Coupled with Licorice Extract in a Liposomal Model System

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ABSTRACT: Fourteen pea protein hydrolysates (PPHs) were prepared using different proteases and tested for antioxidant activity in a liposomal model system under oxidative stress (100 μM $FeCl_3$/2 mM ascorbate). Almost all PPHs inhibited lipid oxidation, and those prepared from heated protein with Flavourzyme (Fla-PPH) or Protamex (Pro-PPH) were the most effective. Remarkable synergistic effects were observed on both Fla-PPH and Pro-PPH with licorice extract (LE). Electron microscopy revealed a self-assembled network that appeared to provide crucial protection of liposome against oxidation. The presence of LE enhanced the antioxidant potential by producing a more compact network apparently via PPH−LE complexation. Zeta-potential measurements suggested electrostatic interactions are important driving forces for the accumulation of active peptides at the liposome interface. Peptides rich in leucine, lysine, glutamic acid, glutamine, valine, or proline with a hydrophobic N-terminus, as identified by mass spectrometry, were implicated in the antioxidative protection.

KEYWORDS: *pea protein hydrolysate, licorice extract, lipid oxidation, synergism*

INTRODUCTION

Peptides and protein hydrolysates as potential alternatives to traditional antioxidants have drawn constant attention of food ingredient manufacturers because, unlike small-molecule antioxidants, peptides tend to be multifunctional and can modify the physical properties of foods besides inhibiting lipid oxidation.[1,2] Plant- and animal-derived protein hydrolysates, which are composed of peptides, have been widely reported to manifest inhibitory activity against lipid oxidation in both model and food systems.[3−6] The antioxidant mechanisms of protein hydrolysates in lipid emulsion systems involve both chemical and physical pathways. Peptides can act as hydrogen or electron donors, radical stabilizers, or metal ion chelators,[7] and many can also form physical barriers around lipid droplets to block the penetration and diffusion of lipid oxidation initiators.[8,9] However, due to the size reduction and increased charges, peptides are conceivably more effective in an aqueous environment than in the lipid phase.

Phenolic compounds extracted from plants and spices are another important group of natural antioxidants for food quality preservation. They can act as singlet oxygen quenchers and metal ion chelators to prevent the initiation of lipid oxidation or as reducing agents or hydrogen and electron donors to disrupt radical-chain reactions.[10] Generally, as nonionizable or less polar compounds, phenolic molecules have the propensity to concentrate at the oil−water interface and in the lipid phase where oxidative reactions are prevalent.[11] As such, phenolics are often found to be effective at retarding lipid oxidation in food emulsion systems.[12−14]

The efficacy of an antioxidant is dictated by its structure; in a heterogeneous food system, the amphiphilicity of the antioxidant can be a critical determinant. Due to their different affinities for aqueous and lipid solutions, peptides and phenolic compounds when used in combination in biphase (oil/water) systems may function either additively or synergistically, giving rise to the overall oxidative stability. Indeed, synergistic antioxidative effects have been reported on protein hydrolysates derived from soybean and yeast when coupled with simple phenolic compounds, such as BHA and BHT, in lipid systems.[15,16] However, knowledge of cooperative antioxidative effects of protein hydrolysates with naturally occurring plant phenolics in lipid emulsions or similar biphasic systems is limited.

The objective of the present study was to investigate the hypothetical cooperative effect of pea protein hydrolysates (PPHs) in combination with a licorice extract (LE) for the inhibition of lipid oxidation using a liposomal model system. To generate different peptides, a variety of proteolytic enzymes were employed. Pea (*Pisum sativum* L.) protein was used as the protein source because recent studies suggested that this low-allergenicity protein could be an excellent choice for the preparation of bioactive peptides with notable radical-scavenging activity.[17,18] On the other hand, licorice (*Glycyrrhiza glabra*), an herb that has been used for centuries as a food ingredient and medicinal additive, is also known for its remarkable antiradical activity.[19,20] To elucidate the reaction mechanism, peptides and peptide fractions responsible for the antioxidative effect of PPHs as well as the active compounds in LE were identified through mass spectrometry. The physical mode by which PPHs and LE acted concertedly to inhibit lipid oxidation was also explored.

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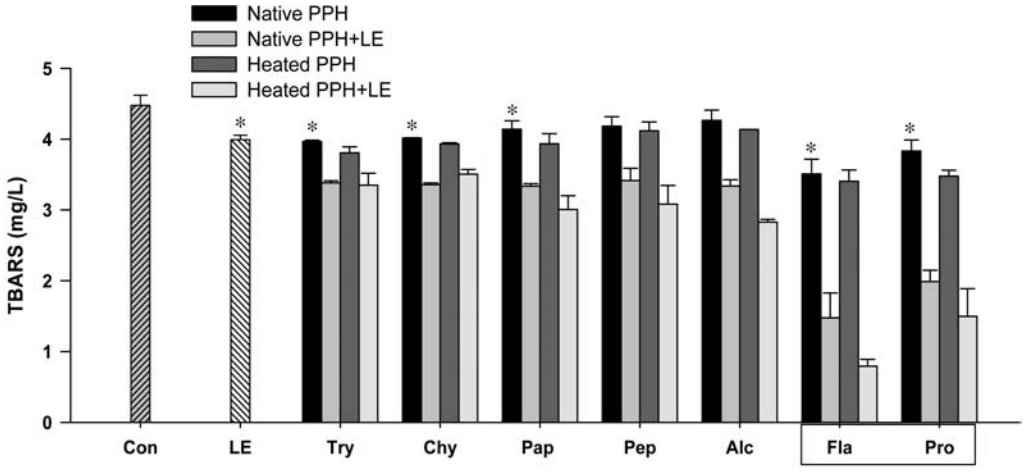


	synergism (%)[a]						
	Try	Chy	Pap	Pep	Alc	Fla	Pro
Native	9.0	15.6	28.0	26.6	38.8	51.6	54.6
Heated	−2.7	−5.9	30.0	39.5	50.0	57.7	50.2

Figure 1. Inhibition of lipid oxidation (TBARS) by PPHs (1 mg/mL) prepared from native and heated PPI with trypsin (Try), chymotrypsin (Chy), papain (Pap), pepsin (Pep), Alcalase (Alc), Flavourzyme (Fla), and Protamex (Pro) and their cooperative effects with LE (12.5 μg/mL). Con, control (without antioxidant); LE, licorice extract. Asterisks indicate significant difference ($P < 0.05$) from control.

MATERIALS AND METHODS

Materials. Pea protein isolate (PPI) was extracted from pulverized yellow peas (*P. sativum* L.) using the isoelectric precipitation method that involved initial extraction at pH 8.0 followed by precipitation at pH 4.5.[21] After neutralization (pH 7.0), the PPI suspension was lyophilized to yield a dry powder with 92% protein. Trypsin, chymotrypsin, papain, pepsin, ascorbic acid, and crude soybean phosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Alcalase, Flavourzyme, and Protamex were donated by Novozymes North America Inc. (Franklinton, NC, USA). Licorice (*G. glabra*) extract with a 25% total phenolics content was obtained from a commercial ingredient supplier. All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Nanopure deionized water was used for the preparation of all solutions.

PPH Preparation. PPHs were prepared according to the method of Peña-Ramos and Xiong.[22] A 2% (w/v) protein solution of PPI with or without preheating (90 °C, 5 min) was hydrolyzed for 0.5 h with trypsin, chymotrypsin, papain, and pepsin (pure enzymes) at 37 °C, or Alcalase, Flavourzyme, and Protamex (crude enzymes) at 50 °C. The enzyme−protein ratio was 1:100 for all proteases. The hydrolysis pH was adjusted to the optimal pH for each protease. After inactivation of proteases (80 °C, 15 min), all solutions were neutralized to pH 7.0, followed by centrifugation at 9000*g* for 10 min to remove insoluble particles. Samples were freeze-dried and stored at 4 °C before use.

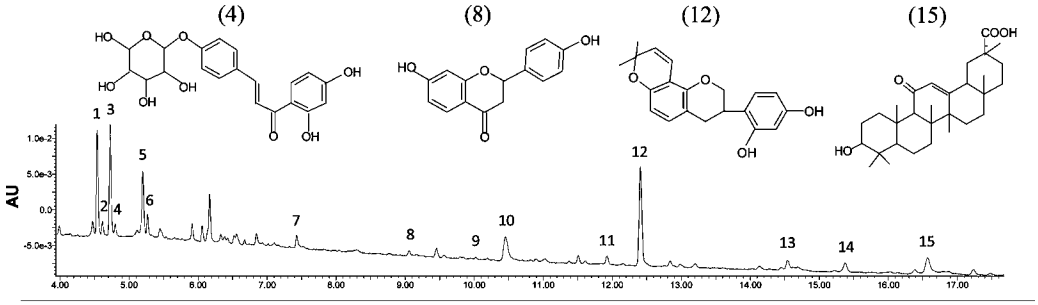
Amino Acid Composition. Amino acid composition analysis was carried out using an 1100 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with an ODS Hypersil C18 column (4.6 mm × 250 mm). The mobile phases were 20 mM sodium acetate (solvent A) and 20 mM sodium acetate/methanol/acetonitrile at a 1:2:2 vol ratio (solvent B). The flow rate was 1.0 mL/min. Precolumn reaction with *o*-phthalic dicarboxaldehyde yielded amino acid derivatives. Before analysis, PPH samples were digested with 6 M HCl for 24 h at 110 °C in sealed tubes for acidic hydrolysis and with 5 M NaOH for 22 h at 110 °C for tryptophan analysis.[23]

Purification and Identification of Active Peptides. All PPH samples were screened for antioxidant potential by means of TBARS testing (described later), and those that exhibited strong TBARS inhibition were subjected to fractionation via low-pressure size exclusion chromatography (LPSEC) with a Sephadex G-75 column (Pharmacia XK 26/120, Piscataway, NJ, USA) using a 0.01 M PBS (pH 7.0) elution buffer. Molecular weight (MW) distribution of each peptide fraction was estimated from a calibration curve generated from the retention volumes of the following standards: bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), aprotinin (6.5 kDa), and bacitracin (1.4 kDa) (Sigma Co., St. Louis, MO, USA). Data were fitted in the exponential decay model (modified single with three parameters) of the Sigma Plot ver. 10 software (Systat Software Inc., Chicago, IL, USA).

All fractions from LPSEC were tested for inhibition of TBARS. The fractions that showed the strongest inhibition were further purified using preparative Waters 1525 HPLC (Waters Corp., Milford, MA, USA) on a reversed-phase Hedera ODS-2 Pre C18 column (20 mm × 200 mm, Hanbon Sci. & Tech., Jiangsu, China) with a linear gradient elute of acetonitrile (10−60% in 40 min) at a flow rate of 5 mL/min. The elution fractions from 30 individual runs were collected, combined, and concentrated using a rotary evaporator and then freeze-dried.

Each peptide fraction from the above reversed-phase HPLC was tested for inhibitory activity against TBARS; the most effective fractions were subjected to LC-MS/MS analysis using a Waters Synapt mass quadrupole time-of-flight mass spectrometer (Waters Corp.) coupled with a Waters Acquity UPLC system through an electrospray ionization (ESI) source. The mobile solvent (acetonitrile and 0.1% formic acid for mobile phases A and B, respectively) flow rate was set at 0.3 mL/min, where a linear gradient elute (0−30% in 50 min for A) was used. The automated data acquisition using the information-dependent mode was performed on Synapt MS under control by MassLynx software (Waters Corp.). The spectra were interpreted using the peptide sequencing module of the MassLynx software. Properties of the identified peptides were derived using a peptide property calculator from Innovagen AB (Lund, Sweden), which is also available at http://www.innovagen.se/peptide-design-tools.asp.

Identification of Active Compounds in LE. The LE compound separation was carried out using a Waters Acquity UPLC system equipped with an Acquity photodiode array (PDA) detector and a UPLC CHH C18 column (2.1 mm × 120 mm) (Waters Corp.). The mobile solvents were (A) acetonitrile and (B) 0.1% formic acid. The gradient was linear from 5 to 50% A for 10 min, from 50 to 100% A for 20 min, and from 100 to 5% A for 5 min. The flow rate was 0.3 mL/min. Eluted compounds, detected by UV at 280 nm, were then subjected to a Waters Synapt mass quadrupole time-of-flight mass spectrometer using the ESI source. Data were collected and processed with MassLynx software. MS spectral data of licorice as reported in the



peak no.	compounds	class	[M-H]$^-$ (m/z)
(1)	Liquiritin apioside	Flavanone	549
(2)	Unknown		497
(3)	Neolicuroside	Chalcone	549
(4)	Isoliquiritin	Chalcone	417
(5)	Unknown		542
(6)	Unknown		565
(7)	Licorice glycoside A	Chalcone	725
(8)	Liquiritigenin	Flavanone	255
(9)	Glycyrrhizic acid	Triterpene	821
(10)	Glabrene	Isoflavene	321
(11)	Neoglycyrol	Coumarin	365
(12)	Glabridin	Isoflavan	323
(13)	Glabrol	Isoflavan	391
(14)	Unknown		337
(15)	18β-Glycyrrhetic acid	Triterpene	469

Figure 2. (Top) UPLC profile of licorice extract (LE) by PDA and ESI-MS detectors and (bottom) compounds identified in LE. The structures of representative compounds are shown in the upper panel.

literature were used as references to establish the active compounds in LE.[24,25]

Inhibition of Lipid Oxidation in a Liposomal Model System. Liposomes were prepared from crude soybean phosphatidylcholine.[7] Briefly, the dispersion of the phospholipid (0.2 mg/mL) in 0.12 M KCl and 5 mM histidine buffer (pH 6.8) was homogenized and then sonicated at 4 °C for 20 min to produce a liposome suspension that was physically stable for several weeks when kept in the refrigerator. To test the antioxidative activity, PPHs, peptide fractions, and their mixtures with LE were added to the liposome suspension, and lipid oxidation was initiated by iron-redox cycling (100 μM $FeCl_3$/2 mM ascorbate) and allowed to proceed by incubation at 37 °C for 1 h. After the addition of 2-thiobarbituric acid (TBA) and trichloroacetic acid, samples were heated in a boiling water for 15 min and then cooled to room temperature. Absorbance was read at 532 nm, from which the concentration of TBA-reactive substances (TBARS), expressed as milligrams of malonaldehyde per liter of liposome suspension (mg/L), was calculated.[26] Synergism of PPH (or peptide fraction) samples and LE in inhibiting TBARS was expressed and calculated as

$$\text{synergism (\%)} = \frac{(C - M) - [(C - H) + (C - L)]}{C - M} \times 100$$

where C, H, L, and M represent inhibition of TBARS by control (no antioxidant), PPH (or peptide fraction), LE, and mixed PPH (or peptide fraction) and LE, respectively.

Microstructure of Liposome Particles under Oxidative Stress. Transmission electron microscopy (TEM) was used to visualize the physical protective action of PPHs on liposome particles under oxidative stress. One drop of liposome suspension and one drop of staining solution (2% phosphotungstic acid, v/v) were applied onto a copper grid of 200 mesh and allowed to stabilize for 10 min. Images were captured using an ES500W Erlangshen CCD camera (Gatan, Inc., Pleasanton, CA, USA) mounted to a JEM-2100 electron microscope (JEOL, Tokyo, Japan).

Statistical Analysis. All data were expressed as the mean ± standard deviation of at least three independent experiments with repeated measures done on different days. Data were processed using the general linear model's procedure of Statistix software 9.0 (Analytical Software, Tallahassee, FL, USA). Significant differences ($P < 0.05$) between means were identified using the least significance difference (LSD) procedure.

RESULTS AND DISCUSSION

Inhibition of Lipid Oxidation. As shown in Figure 1, hydrolyzed pea protein samples, except those prepared from native PPI with pepsin and Alcalase, significantly suppressed lipid oxidation (TBARS). The most notable inhibition was imparted by PPHs prepared from preheated PPI with Flavourzyme and Protamex (Fla-PPH and Pro-PPH), which showed 24.0 and 22.3% TBARS reductions from the control ($P < 0.05$), respectively. The slightly stronger efficacy by PPHs produced from preheated protein was in accordance with our previous findings that they had stronger 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) and hydroxyl radical ($^{\bullet}OH$) scavenging activities than those from native PPI.[27] Presumably, PPHs reduced TBARS formation by stabilization of radicals and probably also by sequestration of prooxidative Fe^{2+}. In addition, PPHs might serve as a physical barrier around liposome particles to block the penetration and diffusion of oxidation initiators, similar to the action of some proteins in emulsions.[8]

As expected, LE also showed significant ($P < 0.05$) inhibition of TBARS formation in the liposome system (Figure 1). The antioxidant activity is generally attributed to the presence of phenolic compounds in the herb extract. Indeed, 15 compounds were identified in LE, most of which were well-

Table 1. Amino Acid Composition (Grams per 100 g) of Intact (PPI) and Hydrolyzed (PPHs) Pea Protein Isolate[a]

amino acid	PPI	Try	Chy	Pap	Pep	Alc	Fla	Pro
Asx[b]	11.3	11.2	11.3	11.4	11.7	11.5	11.1	11.2
Glx[b]	19.7	20.2	20.3	22.6	22.7	22.1	28.2	27.2
Ser	4.6	4.7	4.7	4.8	4.5	4.6	4.7	4.6
His	2.5	2.5	2.5	2.6	2.6	2.4	2.8	2.7
Gly	3.8	4.2	3.8	3.8	3.8	3.7	3.7	3.6
Thr	3.1	3.1	3.2	2.9	2.9	2.9	2.2	2.4
Arg	8.6	8.8	8.7	9.2	9.0	8.9	9.9	9.7
Ala	3.8	3.9	3.8	3.7	3.6	3.6	3.3	3.4
Tyr	3.2	3.1	3.2	2.9	2.9	2.9	1.9	2.1
Cys	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.2
Val	5.5	5.2	5.3	4.9	5.0	4.9	4.2	4.4
Met	1.2	1.1	1.1	1.0	0.9	1.0	0.6	0.6
Phe	5.7	5.6	5.7	5.4	5.5	5.7	4.9	5.1
Ile	5.0	4.9	4.9	4.3	4.8	4.6	3.9	4.0
Leu	8.5	8.3	8.4	7.7	8.1	7.9	6.5	6.8
Lys	7.6	7.7	7.7	7.8	7.5	7.7	8.0	7.9
Pro	4.9	4.7	4.7	4.1	3.9	4.6	3.4	3.6
Trp	0.8	0.7	0.7	0.6	0.6	0.5	0.5	0.5
hydrophobic R groups[c]	37.3	36.4	36.5	33.4	34.3	34.8	28.5	29.9
ionizable R groups[d]	49.7	50.3	50.4	53.7	53.4	52.7	60.1	58.7

[a]PPHs were prepared from PPI with trypsin (Try), chymotrypsin (Chy), papain (Pap), pepsin (Pep), Alcalase (Alc), Flavourzyme (Fla), and Protamex (Pro). [b]Asx, aspartic acid + asparagines; Glx, glutamic acid + glutamine. [c]Hydrophobic R groups include amino acids Ala, Val, Met, Phe, Ile, Leu, Pro, and Trp. [d]Ionizable R groups include amino acids Lys, Arg, His, Asx, and Glx.

known active compounds reported in the literature, including liquiritigenin, isoliquiritin, liquiritin apioside, licorice glycoside A, glabridin, glycyrrhizic acid, and 18β-glycyrrhetic acid (Figure 2). In particular, the phenolics play an important role in the antioxidant activity of LE.[28] Interestingly, when PPHs and LE were applied together, a synergistic antioxidative effect was generated for most samples except those prepared from heated protein with trypsin and chymotrypsin that showed an antagonism. The most remarkable synergism was displayed by Fla-PPH and Pro-PPH samples when used together with LE, up to 57.7 and 50.2%, respectively (Figure 1).

Physicochemcial Mechanism. The antioxidant activity of protein hydrolysates was likely dependent upon the characteristic amino acid composition and the sequence of the peptides derived, which was dictated by the protease specificity. As shown in Table 1, PPI had a relatively high content of Asx and Glx, but a low content of sulfur-containing amino acids, Met and Cys, with an overall amino acid composition being closely similar to that reported in the literature.[17] As expected, the supernatant fraction of Fla-PPH and Pro-PPH, which manifested the strongest TBARS inhibition, had a considerably higher Glx, His, Arg, and Lys content than PPI or other PPHs, suggesting an increased proportion of ionizable R groups (by about 10%) and consequent stronger electrostatic interactions between peptide side chains in these two PPHs. Both acidic and basic amino acid side-chain groups contribute to the inhibition of lipid oxidation.[6,29] Protein structural unfolding induced by moderate heating prior to hydrolysis could facilitate peptide bond cleavage, resulting in greater exposures of reactive amino acid side-chain groups capable of neutralizing radicals.[22,30] This seemed to explain the slightly stronger antioxidant activity seen in many of the PPHs prepared from heated PPI relative to those prepared from unheated PPI.

An important reason for the synergistic action of PPH and LE appeared to be different affinities of peptides and phenolics for liposome particles, because the polarity of a compound is a critical factor governing its distribution within an emulsion.[31] As bulky and amphiphilic molecules, peptides in PPH would partition both at the polar surface of the liposome and in the aqueous phase to inhibit lipid peroxidation through scavenging free radicals and sequestering prooxidative metal ions. In contrast, smaller organic compounds present in LE having polar groups −OH, −COOH, and −O-glc− (glycoside) would accumulate at the polar surface of liposome and, additionally, diffuse through the phospholipid bilayer, allowing their reaction with radicals formed both inside and outside liposome. The complementary processes of PPH and LE, as a result, would delay the initiation and suppress the propagation of lipid radical chain reactions, thereby inhibiting the TBARS formation. Medina et al.[32] reported that the synergistic effects of some natural phenolic compounds on the antioxidant activity of lactoferrin were related to their different affinities toward liposomes as well as oil-in-water emulsions. Furthermore, the degree of partition of PPH versus LE on the liposome particle might affect the fluidity of the interfacial "membrane" made up of lipid bilayer surrounded by PPH and LE, which would significantly influence their antioxidative activity.[33] The accumulation of peptides on the surface of liposomes and partitioning of LE at the surface as well as in the interior of liposomes would synergistically lead to a decreased membrane fluidity. The increased membrane stiffness consequently deterred the access of oxidants and restricted the mobility and diffusion of radicals within the liposome particle, as suggested by Liang et al.,[34] contributing to the remarkable oxidative stability of liposomes.

A further explanation of the synergistic effect was the interaction of PPH and LE compounds. The formation of PPH−LE complexes may alter the locations of PPH and LE on the liposome particles, which is likely to lead to higher concentrations of PPH and LE partitioning at the oil−water interface. This hypothesis is supported by Almajano and Gordon,[35] who noted that the synergistic antioxidative effects

Table 2. Inhibition of TBARS in a Liposome Oxidizing System by the Combination of Fla-PPH or Pro-PPH and LE at Various Concentrations[a]

	licorice extract			
PPH	0 μg/mL	7.5 μg/mL	12.5 μg/mL	17.5 μg/mL
control (no PPH)	4.5 ± 0.1 a, A	4.1 ± 0.2 b, A	4.0 ± 0.1 c, A	1.5 ± 0.1 d, A
Flavourzyme				
0.25 mg/mL	4.2 ± 0.0 a, AB	4.0 ± 0.3 ab, A	3.7 ± 0.0 b, A	0.9 ± 0.1 c, B
0.5 mg/mL	4.1 ± 0.1 a, ABC	3.6 ± 0.2 ab, A	3.5 ± 0.3 b, B	0.6 ± 0.1 c, C
1.0 mg/mL	3.7 ± 0.2 a, BC	2.7 ± 0.2 b, B	2.3 ± 0.1 c, C	0.6 ± 0.0 d, C
2.0 mg/mL	3.0 ± 0.2 a, CD	2.1 ± 0.2 b, BC	1.0 ± 0.3 c, D	0.6 ± 0.0 c, C
4.0 mg/mL	2.8 ± 0.3 a, DE	1.9 ± 0.2 b, C	0.7 ± 0.0 c, D	0.6 ± 0.1 c, C
6.0 mg/mL	2.4 ± 0.5 a, E	1.5 ± 0.4 ab, C	0.7 ± 0.0 b, D	0.7 ± 0.0 b, C
Protamex				
0.25 mg/mL	4.3 ± 0.1 a, A	4.1 ± 0.1 ab, A	3.9 ± 0.1 b, AB	1.1 ± 0.1 c, B
0.5 mg/mL	4.1 ± 0.0 a, AB	4.0 ± 0.0 a, A	3.4 ± 0.4 b, BC	0.8 ± 0.1 c, C
1.0 mg/mL	3.9 ± 0.0 a, AB	3.5 ± 0.1 a, B	2.6 ± 0.2 b, C	0.6 ± 0.1 c, C
2.0 mg/mL	3.3 ± 0.0 a, BC	2.6 ± 0.0 b, C	1.2 ± 0.0 c, D	0.6 ± 0.1 d, C
4.0 mg/mL	3.0 ± 0.2 a, CD	2.2 ± 0.1 b, D	0.9 ± 0.1 c, D	0.7 ± 0.1 c, C
6.0 mg/mL	2.6 ± 0.4 a, D	1.2 ± 0.0 b, E	0.8 ± 0.1 bc, D	0.7 ± 0.0 c, C

[a]Means ± standard deviations (n = 3) in the same row with different lowercase letters (a–d) differ significantly ($P < 0.05$). Means ± standard deviations (n = 3) in the same column with different uppercase letters (A–E) differ significantly ($P < 0.05$).

of BSA with some water-soluble phenolic compounds in inhibiting lipid oxidation were attributed to the formation of protein–phenol complexes, which could concentrate at the oil–water interface due to the surface-active nature of the protein. The PPH–LE complexes can be formed through noncovalent interactions, including both multisite interactions (several phenolic compounds bound to one peptide molecule) and multidentate interactions (one phenolic compound bound to several peptide sites or molecules), with proline, histidine, and arginine being the most active binding sites.[36] Hydrogen bonds between phenolic hydroxyl and peptide carbonyl, as well as hydrophobic interactions between phenolic rings and the planar peptide hydrophobic surfaces, are thought to be major stabilizing forces.[36,37] Compared to proteins, which have a compact structure, peptides that contain large regions of random coil have higher affinities for phenolic compounds due to the more exposed and available carbonyl oxygens and hydrophobic residues, allowing the formation of more entangled and complex products.[38] The remarkable synergistic effects displayed in Fla-PPH and Pro-PPH could be attributed to their large peptide sizes,[27] favoring multisite interactions with phenolics. Electrophoresis revealed that these two PPHs consisted mostly of peptides ranging from 10 to 30 kDa, whereas in other PPH samples, the peptides were mostly <15 kDa (data not shown).

It should be noted that, depending on the proteases used, the combination of PPH and LE might produce an antagonism (a negative synergism as shown in Figure 1). This adverse effect may be interpreted as the formation of PPH–LE complexes or the competitive adsorption of PPH onto the liposomes that decreased the accumulation of LE at the interface, where LE was expected to play a key role in retarding lipid oxidation.[11] Protease specificity was ostensibly a dominant factor. For example, chymotrypsin selectively cleaves peptide bonds formed by aromatic amino acids (tyrosine, tryptophan, and phenylalanine). Strong hydrophobic interactions between terminal (free) aromatic rings of peptides and phenolic rings of LE would likely weaken their individual partitioning at the interface. For free peptides, their competitive adsorption onto the lipid droplets through aromatic amino acid residues could also interfere with the absorption of LE within the liposomes.

Because both peptides and liposome (phosphatidylcholine) carry negative and positive charges at pH 6.8, it is reasonable to propose that electrostatic interactions are a driving force for the localization of PPH around the liposome interface. To substantiate this proposal, zeta-potential was measured. The zeta-potentials of liposomes and PPHs (Fla-PPH and Pro-PPH) at pH 6.8 were found to be −38 and −13 mV, respectively (data not shown), which confirmed the susceptibility of liposomes to Fe-catalyzed oxidation and the ability of PPHs to alleviate the impact due to sequestration of the cationic prooxidative metal ion. After incubation of PPHs with liposomes for 1 h at the same pH, regardless of the presence of LE, the zeta-potential increased to −30 mV ($P < 0.05$), suggesting the interaction of PPHs with the charged surface of liposomes.[39] This result also indicated that most of the negatively charged side chains of PPHs tended to orient toward the outer layer of the self-assembled network, which reinforced their antioxidant protection against cationic prooxidants and anionic radicals, such as superoxide ($O_2^{\bullet -}$).

To gain a further insight into the concerted actions of PPH and LE, the inhibition of TBARS was tested with a series of concentrations of Fla-PPH and Pro-PPH (0.25–6 mg/mL) in combination with LE (7.5–17.5 μg/mL). Results showed that the degree of synergism increased with increasing concentrations of PPH and LE (Table 2), suggesting higher amounts or effective doses of antioxidants were accumulating at the oil–water interface to stabilize radicals. It is noteworthy that TBARS formation was inhibited more remarkably by increasing the concentration of LE than by that of PPHs, suggesting that LE was more effective than PPH at retarding lipid oxidation in the liposomal system. This was probably because phenolic compounds were preferentially concentrated at the oil–water interface.[11] The noticeable synergism was observed at concentrations of no less than 1 mg/mL and 12.5 μg/mL of PPH and LE, respectively.

Physical Protection by PPH-LE Networks. Whereas chemical antioxidant mechanisms are generally considered as the primary means by which peptides inhibit the oxidative

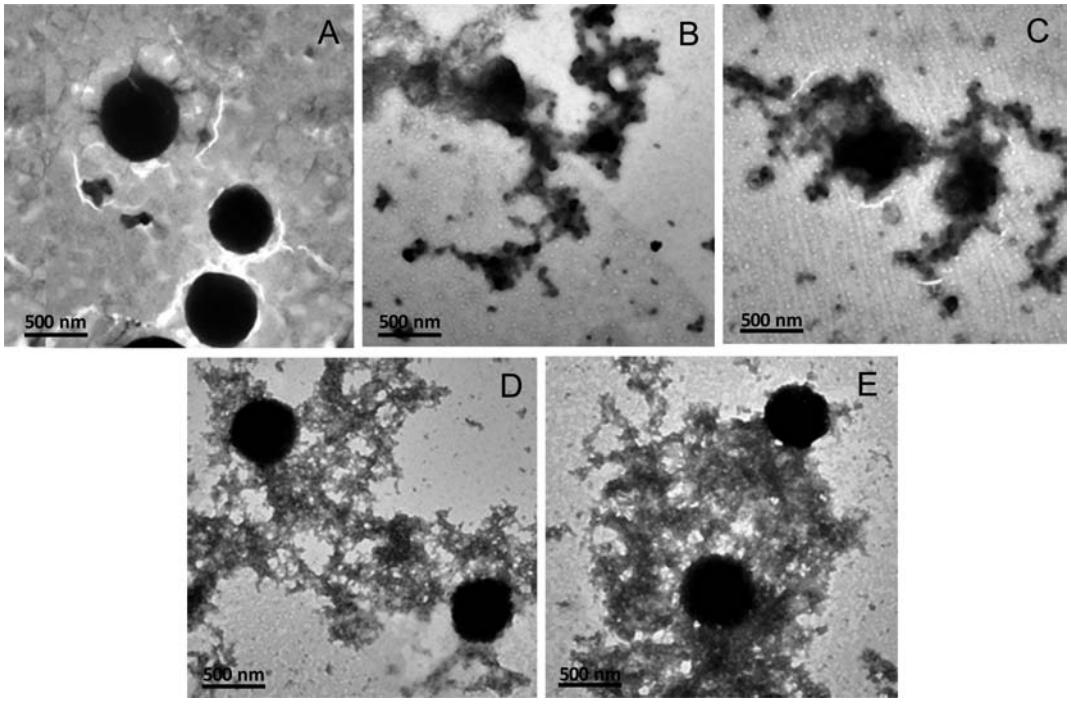


Figure 3. TEM micrograghs of liposomes under different conditions: (A) control (nonoxidized); (B) oxidized; (C) oxidized in the presence of LE; (D) oxidized in the presence of Fla-PPH; (E) oxidized in the presence of Fla-PPH and LE. PPH and LE concentrations were 1 mg/mL and 12.5 μg/mL, respectively.

process in a food emulsion system, physical effects as a complementary and sometimes important alternative mechanism are increasingly recognized.[2,40] It was plausible that the concentration of PPH−LE complexes at the interface would lead to the structuring of a resistant coating around the liposome particles to provide an effective physical barrier against oxidants. To test this hypothesis, TEM was applied to visualize the distribution of peptides in Fla-PPH (which exhibited the strongest TBARS inhibition) in oxidized liposome solutions, and the micrographs are displayed in Figure 3. Without oxidative stress, liposomes appeared as almost perfect round-shaped particles with a smooth boundary (A). When oxidized, these particles became deformed and disintegrated, and the particle boundary became largely indistinguishable (B). The addition of LE somewhat alleviated the oxidative stress as liposomes were noticeably less damaged (C) when compared with those oxidized (B). Yet, when PPH was incorporated into the oxidizing system, a salient self-assembled network around liposome was observed (D). By virtue of entrapment, this proteinaceous network provided protection to allow liposomes to largely retain their native round structure, a result that seemed to be indicative of reduced oxidative impact. Notwithstanding, oxidative damage was unavoidable due to the loose structure of the network and the insufficient adsorption of PPH onto the liposome interface. The better protective effects of Fla-PPH and Pro-PPH against lipid oxidation could be attributed to their large peptide scales, which facilitated the formation of a more compact protective network around liposomes. Tong et al.[30] also reported that high MW fractions of whey protein were more effective than low MW fractions in inhibiting lipid peroxide production.

When PPH and LE were applied together, a more compact and rigid network around liposome was generated (E), which corresponded to the lowest TBARS formation (or the highest synergism). The micrograph supported the hypothesis of PPH−LE complex formation, which gave rise to a highly interactive, entangled, and cross-linked network around liposomes. In fact, by virtue of neutralizing hydroxyl radicals, peptides with unpaired electrons can readily polymerize to form aggregated matrices.[41] In addition, the physical filling effect of LE or LE aggregates by self-association[36] might also contribute to the enhancement of the network compactness and rigidity. Due to the physical hindrance of the PPH−LE network, it was difficult for Fe^{2+} and other water-soluble oxidants to gain access to the liposome to initiate and accelerate lipid peroxidation. Meanwhile, hydroperoxides and peroxide radicals generated within a liposome particle could not easily transfer to other liposome particles to sustain the radical chain reactions, which may be another mechanism for the synergistic inhibition of lipid oxidation with the PPH−LE combination treatment.

Peptide self-assembly is a type of aggregation by which several individual peptides form nanoscale structures through noncovalent interactions.[42] β-Sheet conformation transition was considered to be the trigger of this action.[43] A number of studies have demonstrated the important role of electrostatic interactions in peptide self-assembly, which could be manipulated by pH adjustment.[43−45] As can be deduced from Table 1, there were strong electrostatic interactions between peptide side chains in PPHs due to the preponderance of ionizable R groups (>50%), especially for Fla-PPH (60.1%) and Pro-PPH (58.7%). At pH 6.8, both the terminal groups and ionizable R groups would be ionized; therefore, intensive electrostatic interactions along with hydrophobic association and hydrogen bonds would drive the peptides to an aggregated state ultimately leading to a defined network. However, weak protein networks were generated due to an imperfect repulsion−attraction balance between peptide side chains so that oxidants and prooxidants were still capable of approaching liposomes to initiate the oxidative damage. It has been reported that fine nanostructures were formed by peptide β-lg f1−8 at

pH 10, which resulted from well-balanced repulsion−attraction forces between neighboring peptide side chains.[42]

Identification of Active Peptides. Fla-PPH and Pro-PPH, which exhibited the strongest inhibition of lipid oxidation of all, were subjected to Sephadex G-75 LPSEC that yielded five peptide fractions each (Figure 4). On the basis of the MW calibration curve, which had an adjusted multiple regression coefficient (R^2) of 0.90 and a standard error of estimation (SEE) of 2.7, the estimated molecular masses of the fractions were 98 kDa (FF1), 16.8 kDa (FF2), 5.2 kDa (FF3), 4.1 kDa (FF4), and 2.0 kDa (FF5) for Fla-PPH and 26.1 kDa (PF1), 7.9 kDa (PF2), 4.1 kDa (PF3), 3.6 kDa (PF4), and 2.0 kDa (PF5) for Pro-PPH. The inhibition of TBARS by these fractions and their cooperative effects with LE were tested, and the results are summarized in Figure 5. FF1, FF3, and PF3 exhibited relatively high potencies to suppress TBARS, in agreement with the aforementioned finding that large and intermediate peptide fractions were more conducive to forming protective coatings around liposomes than small peptides. Also in excellent corroboration with previous results for PPHs, a synergistic antioxidative effect was observed on peptide fractions (except for PF1 and PF5) with LE, and the synergism produced by FF5 and PF3 was the most remarkable. Despite the highest synergistic effect, FF5 when used alone showed very weak inhibition of TBARS; therefore, FF3 and PF3 were chosen for further purification and separation with reversed-phase HPLC. A total of nine individual peaks were resolved from both fractions, and they were designated FF3-F1−F9 and PF3-F1−F9 (Figure 6). Fractions FF3-F6, FF3-F7, PF3-F8, and PF3-F9 were found to be strongly inhibitory of lipid oxidation in the TBARS assay and, consequently, were subjected to UPLC-MS/MS for peptide sequence identification.

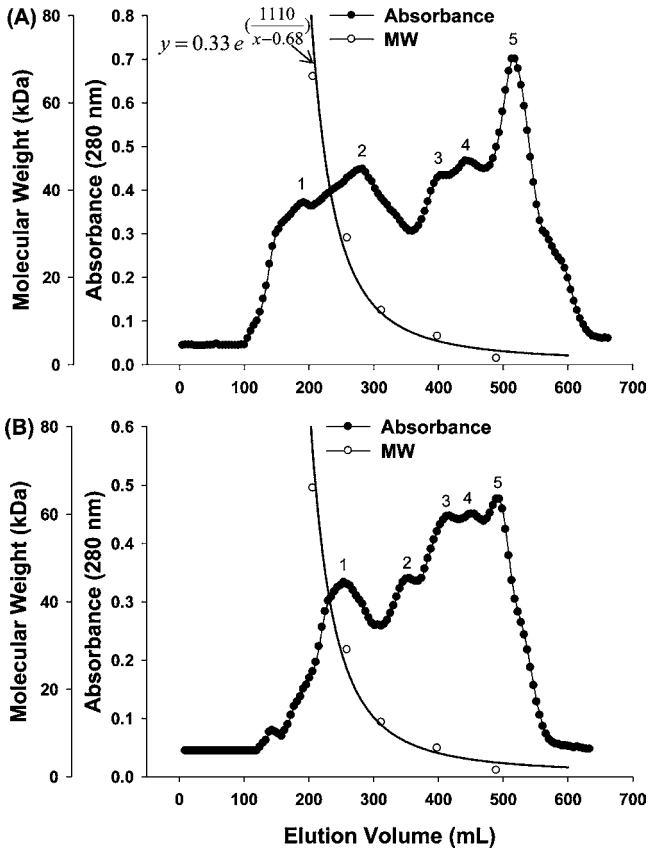
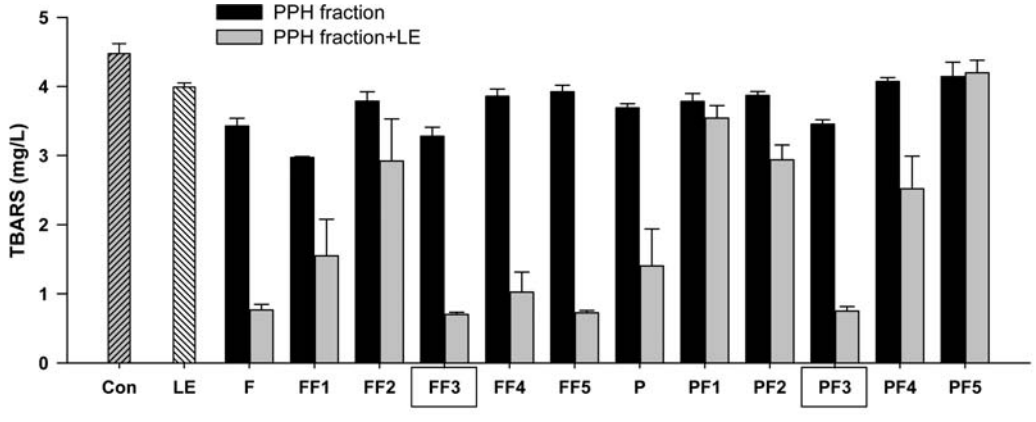


Figure 4. Sephadex G-75 gel filtration of Fla-PPH (A) and Pro-PPH (B), and regression plot of MW versus elution volume.

Table 3 lists 10 peptides identified with high confidence, of which, LQEEDNVISQ, ITPERTLQLQDLDIFVN, and LANRDDNEDLVGVL present in PF3-F8 matched the sequence of pea vicilin [National Center for Biotechnology Information (NCBI) database]. These peptides were of the general molecular size of 1000−2000 Da, ranging from 9 to 17 amino acid residues, well in line with literature reports that bioactive peptides generally consist of 2−20 amino acids.[46] Leucine (L), lysine (K), glutamic acid (E), glutamine (Q), valine (V), and proline (P) were the most prevalent amino acids in these peptides, which were all considered to be important amino acids for peptide antioxidant activity.[3,29,47] It is noteworthy that most of the peptides had a hydrophobic amino acid (leucine, isoleucine, threonine, or phenylalanine) at



	F	FF1	FF2	FF3	FF4	FF5	P	PF1	PF2	PF3	PF4	PF5
Syn (%)	58.0	32.0	24.6	55.4	68.0	72.3	51.3	−26.0	29.0	59.6	54.4	−194.6

Figure 5. Inhibition of lipid oxidation (TBARS) by gel filtration fractions from Fla-PPH and Pro-PPH and their cooperative effects with LE in a liposome oxidizing system. Con, control (without antioxidant); LE, licorice extract; F, Fla-PPH; P, Pro-PPH; FF1−FF5 and PF1−PF5, five gel filtration fractions from Fla-PPH and Pro-PPH, respectively. PPH and LE assay concentrations were 1 mg/mL and 12.5 μg/mL, respectively. Syn, synergism.

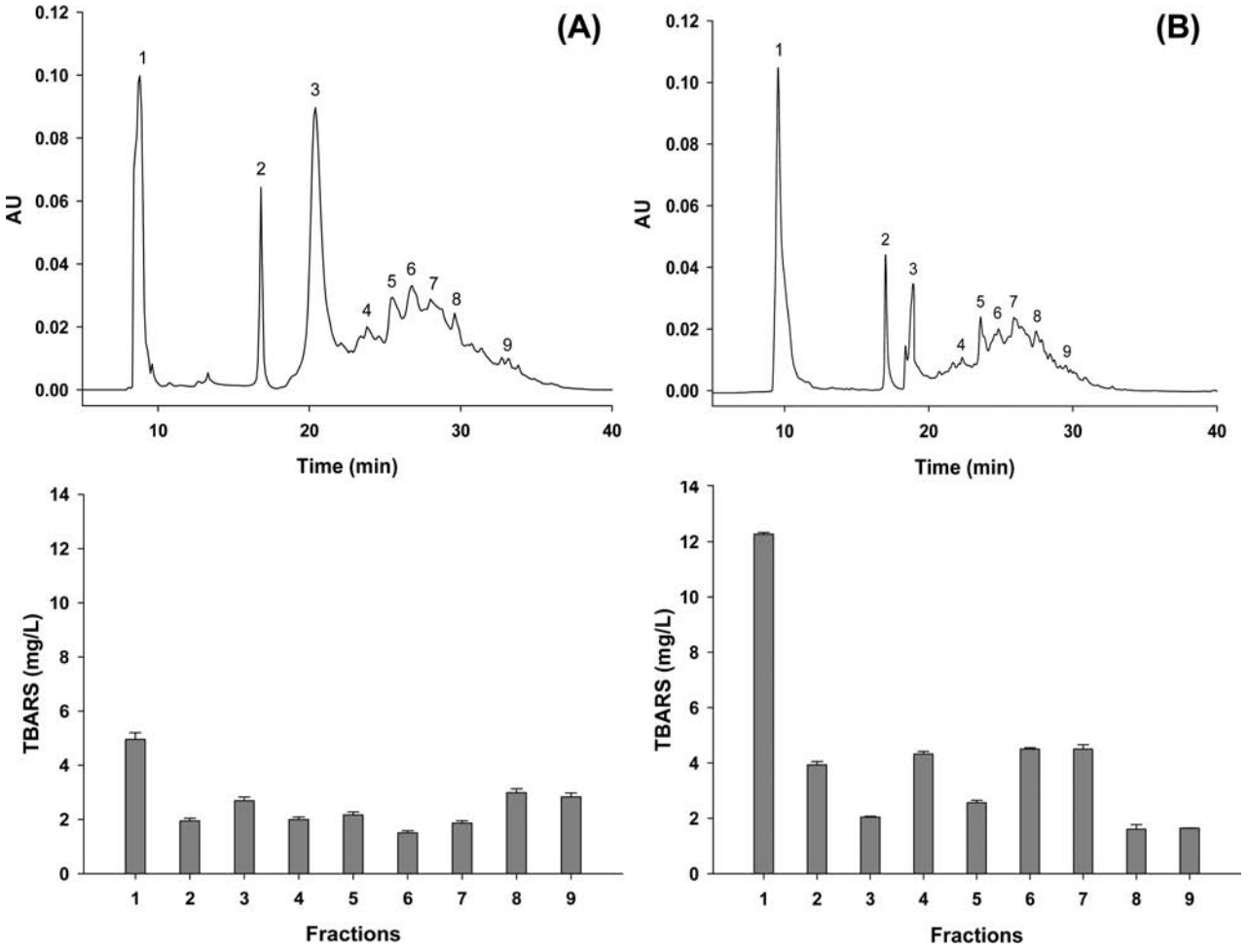


Figure 6. Preparative reversed-phase HPLC spectra of FF3 (A) and PF3 (B) from Fla-PPH and Pro-PPH (upper), and inhibition of lipid oxidation (TBARS) by each fraction in a liposome oxidizing system (lower). Fraction protein concentrations = 0.5 mg/mL.

Table 3. Characteristics of Antioxidative Peptides Isolated from FF3 and PF3 That Were Identified by MS/MS[a]

fraction	peptide sequence	MW	prob (%)	p*I*	net charge at pH 7.0
FF3-F6	GRPKPGKLQ	979.6	94	11.6	3
	FVTGLSEKLLS	1192.7	90	6.9	0
	FLSKGAASNVLSK	1320.7	85	10.6	2
FF3-F7	LARAFRATVDRVKK	1630.0	88	12.1	4
	TQLKPEFLQPFENPEPL	2026.0	80	4.0	2
PF3-F8	LQEEDNVISQ	1173.5	100	2.9	3
	ITPERTLQLQDLDIFVN	2015.3	100	3.7	2
	LANRDDNEDLVGVL	1541.8	100	3.5	3
PF3-F9	LALPVNLLKSFLALL	1624.0	89	10.1	1
	LTELKPRTLQELTLF	1801.0	95	7.0	0

[a]FF3 and PF3 were peptide fractions (gel filtration) from Fla-PPH and Pro-PPH, respectively (see Figure 5), which were further separated (HPLC) into nine fractions including F6, F7, F8, and F9 (see Figure 6).

the N-terminus. It is suggested that N-terminal hydrophobic amino acids play a unique role by promoting the interaction between peptides and fatty acids to favor the partition of peptides onto the oil droplet, thereby accentuating a peptide's antioxidative efficacy as the preferred target for lipophilic radical intermediates.[2,5] In addition, the prevalent proline increased the affinity of PPH (peptides) toward LE (phenolics),[36] which also contributed to the synergistic antioxidative action. The MS spectrum of one of the prominent peptides, identified as LANRDDNEDLVGVL (Leu-Ala-Asn-Arg-Asp-Asp-Asn-Glu-Asp-Leu-Val-Gly-Val-Leu), matching the vicilin 47K precursor in pea protein, is presented in Figure 7.

Most of the identified peptides were charged peptides, which was in agreement with the amino acid composition analysis and zeta-potential measurement. The charged side chains favored not only the adsorption of PPH onto the liposome interface and the PPH self-assembled action but also the sequestration of prooxidative metal ions and anionic radicals ($O_2^{\bullet-}$). Also, positively charged basic peptides would attract negatively charged glycyrrhizic acid and 18β-glycyrrhetic acid (deprotonated at pH 7) in LE, which would help increase the amount of antioxidants at the liposome interface. Park et al.[6] reported that both acidic and basic fractions of wheat gluten hydrolysate (WGH) suppressed lipid oxidation in cooked pork patties to a greater extent than other fractions and WGH. Charged peptide fractions have also been found to strongly inhibit lipid peroxidation in linoleic acid model systems and in autoxidation soybean oil-in-water emulsions.[29,48]

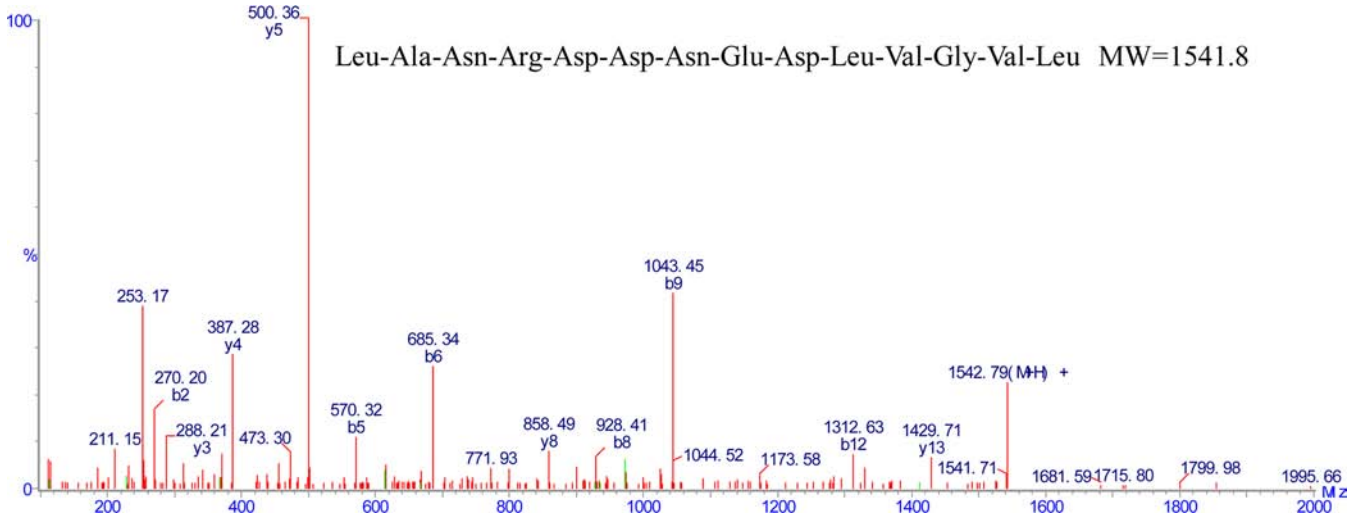


Figure 7. MS/MS spectrum of a prominent peptide (LANRDDNEDLVGVL) from antioxidative fraction PF3-F8.

In conclusion, pea protein hydrolysates prepared with commercial proteases in general and with Flavourzyme and Protamex in particular, when used in combination with licorice extract, provided superior protection of phospholipid-based liposome against radical-initiated oxidation over their separate uses. The synergism was attributed to the abundant charged amino acid side-chain groups and the dominating presence of a hydrophobic N-terminus of constituting peptides in the protein hydrolysates and the preponderance of phenolic compounds in licorice extract, which allowed the efficient removal of radicals in both aqueous and lipid phases. Whereas self-assembled networks formed around lipid droplets appeared to be a prevalent physical mechanism by which peptides diminished the potency of oxidants, the peptides' association with phenolic and other active components from licorice extract afforded a more compact and rigid protective network, providing liposomes with a stronger defense against oxidative stress. Hence, for maximum protection of food emulsions and antioxidant ingredient developments, it is highly desirable that enzymatic pea protein hydrolysates and licorice extract be used together. Future studies should focus on different concentration ratios of the two individual antioxidant sources to establish optimum ingredient blends for specific food applications.

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