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Interfacial Adsorption of Peptides in Oil-in-Water Emulsions Costabilized by Tween 20 and Antioxidative Potato Peptides

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ABSTRACT: Previous studies have shown that soybean oil-in-water (O/W) emulsions prepared with potato protein hydrolysate (PPH) are remarkably stable against oxidative changes. It was hypothesized that partitioning of peptides at the emulsion interface plays an important role in this phenomenon. The present study was conducted to examine the structural characteristics of the interfacial membrane. As revealed by atomic force microscopy, oil droplets costabilized with PPH and Tween 20 were more uniform than those stabilized with Tween 20 only (control). Confocal laser scanning microscopy images indicated the existence of peptides directly anchored into the interfacial membrane. The adsorbed peptides were mostly short oligopeptides composed of two to seven amino acids, of which Ser-Phe-Asp-Leu(Ile)-Lys matched the sequence of patatin. The adsorption of these peptides appeared to both improve the integrity of the interface and contribute to the oxidative stability of the emulsions. Furthermore, cryogenic transmission electron microscopy illustrated the morphology of the interfacial membrane as a noncontinuous short fibril structure. Partitioning of antioxidative peptides in the interfacial membrane provided steric hindrances and electrostatic effects to inhibit oxidation.

KEYWORDS: *antioxidative peptides, lipid oxidation, emulsion, cryo-TEM, AFM*

INTRODUCTION

Oil-in-water (O/W) emulsions are colloidal dispersion systems that embody a variety of products in the chemical and cosmetic industries. In the food industry, O/W foods, for example, creams and salad dressings, are also produced and widely consumed. The stability of an O/W emulsion is critically influenced by the biophysics of the interfacial membrane of the oil droplets in addition to the properties of the continuous phase.

In recent years, peptides produced by enzyme hydrolysis of proteins have attracted much attention because many exhibit strong antioxidative activity. When incorporated into "biphasic" O/W dispersions or heterogeneous foods, such as emulsion-type meat products, antioxidative peptides and protein hydrolysates are able to inhibit lipid oxidation.[1−4] Furthermore, protein-stabilized O/W emulsions are used as a convenient delivery system for food nutrients and functional ingredients that are susceptible to oxidation, such as bioactive lipid.[5] Hence, understanding the behavior of the protein membrane or coating in an O/W emulsion system is important, because the knowledge could be applied to guide the development of process strategies to maximize food product stability and nutritive value.

It has been reported that partitioning of tocopherols and anthocyanins in food emulsions critically contributes to the oxidative stability of emulsions.[6−8] Whereas protein hydrolysates dispersed in the aqueous phase are able to inhibit lipid oxidation in O/W to a certain extent,[9,10] controlling the interface characteristics of emulsions is thought to be an effective strategy to prevent emulsified lipid from oxidation.[5,11] Although the emulsion core is the primary site where lipid hydroperoxides are generated, they are prone to concentration at the interface due to their amphipathic nature.[11,12] Likewise, the high degree of amphiphilicity of peptides would predispose them to diffusion toward the interface, where they would adsorb, accumulate, and react with radicals and nonradical oxidants, thereby enhancing the oxidative stability of emulsions. In our previous investigation,[13] we noted that both the peptides dispersed in the aqueous phase and those partitioning in the interface contributed to the oxidative stability of soybean O/W emulsions. However, the physical behavior of the peptides distributed at the interface was not elucidated.

The objective of the present study was to investigate the adsorption behavior of the peptides at the O/W interface and the characteristics of the resulting emulsion membrane by means of biophysical methods. Specifically, atomic force microscopy (AFM), confocal laser scanning microscopy (CLSM), and cryogenic transmission electron microscopy (cryo-TEM) were applied to examine the microstructure and morphology of the interfacial membrane. Finally, adsorbed peptides with strong radical scavenging activity were separated, sequenced, and identified using UPLC-Q-TOF-ESI-MS to reveal their structural nature.

MATERIALS AND METHODS

Materials. Potato protein concentrate (81% protein) was obtained from AVEBE ba (Veendam, The Netherlands). Alcalase (proteinase from *Bacillus licheniformis*, 2.4 AU/g), Nile Red, Nile Blue, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were pur-

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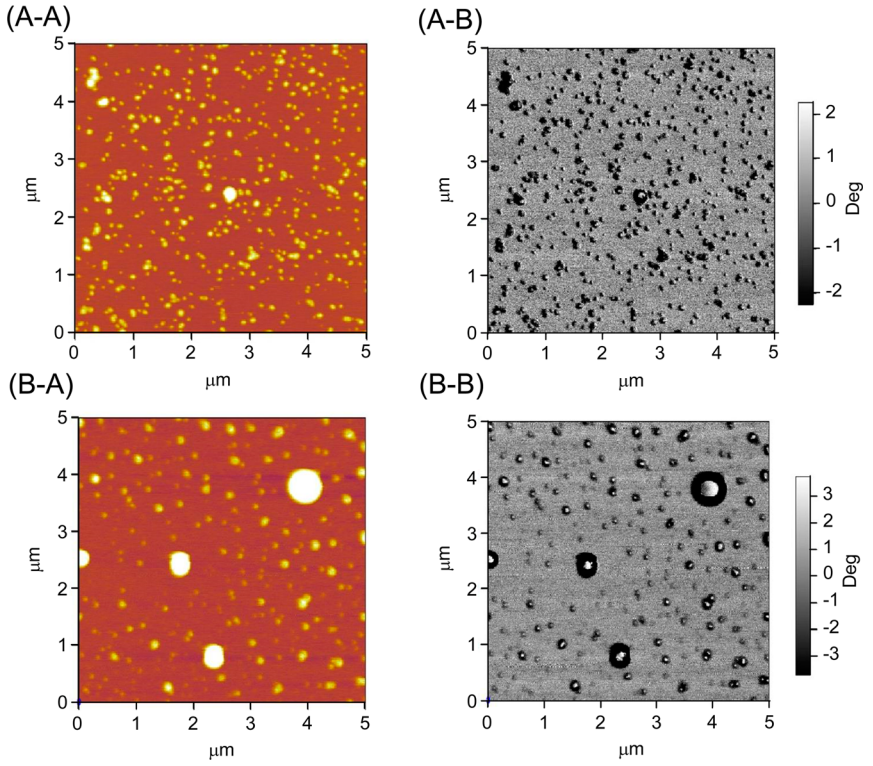

Figure 1. Atomic force microscopy images of fresh soybean O/W emulsions (10% w/w oil) prepared with 11.1 mg/mL Tween 20 + 20 mg/mL PPH using height retrace mode (A-A) and phase retrace mode (A-B) or with Tween 20 only using height retrace mode (B-A) and phase retrace mode (B-B) .

chased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China), and they were of analytical grade unless otherwise specified. Deionized water was used for the preparation of all solutions.

Preparation of Potato Protein Hydrolysate (PPH). PPH was prepared through 1 h of hydrolysis of intact potato protein (40 mg/mL) in an aqueous solution at pH 8.0 and 50 °C using Alcalase.[3] The enzyme/protein substrate was at a weight ratio of 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, placed in sealed plastic bags, and stored at 4 °C. This PPH has been shown to be strongly antioxidative in an O/W emulsion due to its potent radical-scavenging capability.[3,14]

Emulsion Preparation. O/W emulsions were prepared from the mixture of 10% (w/w) soybean oil (Eastocean Oils and Grains Industries Co., Zhang Jiagang, Jiangsu, China) and 90% (w/w) 0.01 M phosphate buffer (pH 7.0) containing 11.1 mg/mL Tween 20 + 20 mg/mL PPH (treatment) or 11.1 mg/mL Tween 20 alone (control). In treatment emulsion, Tween 20 was used as coemulsifier because, unlike lecithin, Tween 80, or some other surfactants, it helps stabilize O/W emulsion droplets without affecting the oxidative stability.[13] Fine emulsions were produced by blending the mixture using a T18 Ultra-Turrax high-performance disperser (Ika-Werke Gmbh & Co., Staufen, Germany) at 21500 rpm for 2 min followed by passing through an ATS AH-basic high-pressure (32 MPa) valve homogenizer (ATS Engineering Inc., Brampton, Canada) twice.[13] The average particle size of the emulsions was determined by dynamic light scattering (DLS) (Mastersizer Nano-ZS, Malvern Instruments, Worcestershire, UK).

Emulsion Microstructure. Emulsion samples were diluted 10 times for the examination of the microstructure. For AFM, 2 μL of a diluted emulsion was dried on freshly cleaved mica. AFM images were obtained on Asylum MFP-3D-SA (Asylum Research, Santa Barbara, CA, USA) using the tapping mode and the phase mode. The surface of mica was scanned in air using OMCL-AC160TS cantilevers at a set point of 0.63 V, a 1 Hz scan rate, and a drive frequency of 336.3 kHz.

For CLSM, the procedure of Kerstens et al.[15] was followed. An aliquot of of 2 μL diluted emulsions was placed on a microscope slide, covered with a coverslip, and observed using a Carl Zeiss LSM 710 confocal system (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with an oil immersion 63× objective. Images were captured by a digital camera connected to the microscope. Photographs of the emulsions were analyzed using software ZEN 2008 Light Edition (Carl Zeiss MicroImaging GmbH) and recorded. The oil droplets in an emulsion were stained using Nile Red in such a way that 2.5 mL of emulsion was mixed with 25 μL of 0.1 mg/mL dye solution prepared with ethanol. On the other hand, peptides were stained with Nile Blue through mixing 2.5 mL of emulsion with 12.5 μL of the dye solution in a dye/protein ratio of 1:100. Nile Red and Nile Blue were excited at 488 and 639 nm, respectively. Emitted light was collected between 653 and 750 nm for Nile Red and between 503 and 613 nm for Nile Blue in two independent channels.

For cryo-TEM, 2 μL of a diluted emulsion was placed on a lacy carbon film supported by a copper grid and allowed to stabilize for 10 min. A filter paper was used to remove liquid for thin liquid films formation. The grid was then immersed in liquid nitrogen (−180 °C) for quenching. After 1 min, the specimen was mounted into the prechilled cryogenic sample holder (Gatan-626 Cryo-tansfer, Gatan, Inc., Tokyo, Japan), followed by being loaded to a JEOL JEM-2100 transmission electron microscope (JEOL Ltd., Tokyo, Japan) immediately. The acceleration voltage of 200 kV was used and the temperature of the holder was kept below the −170 °C during operation. The images of the specimen were captured using a digital camera connected to the microscope.

Recovery of Interfacial Peptides. PPH peptides present at the interfacial membrane were collected by means of centrifugation. Emulsion samples were placed in 50 mL tubes and centrifuged for 45 min at 50000*g* at 20 °C to separate into a cream layer and a serum layer. The cream layer was carefully removed using a syringe and dispersed in the same volume of 0.01 M phosphate buffer (pH 7.0). After centrifugation again under the same conditions as above to remove loosely bound peptides, the cream layer was collected and

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extracted using chloroform/methanol mixture (2:1, v/v).[16] The methanol phase was dried, and the peptides were dissolved in water before analysis.

Identification of Peptides at the Interface. The peptide mixture obtained above was fractionated using Sephadex G15 gel filtration, and 50 fractions (2 min each) were collected. To obtain sufficient amounts of peptide samples, identical fractions from 10 separate gel filtration runs were combined and lyophilized. Each individual peptide fraction was tested for $ABTS^{\bullet+}$ scavenging activity at the protein concentration of 20 μg/mL; the fractions that exhibited strong radical scavenging aqctivity were subjected to solid phase extraction (SPE) to remove Tween 20 using a 3 mL Cleanert PCX SPE column (Agela Technologies Inc., Tianjin, China) preconditioned with 6 mL of methanol followed by 6 mL of deionized water. Peptide fractions loaded in the column were washed with 8 mL of deionized water and then 8 mL of a methanol/water solution (5:95, v/v) to remove Tween 20. Afterward, the samples were eluted with 3 mL of ammonia−water/methanol (5:95, v/v) and then dried by purging with nitrogen gas.

The purified peptide fractions were dissolved in 1 mL of water/acetonitrile (1:1, v/v). Ten microliters of the sample solutions was loaded and eluted in an Acquity UPLC BEH C18 column (2.1 × 120 mm) (Waters Corp., Milford, MA, USA). The mobile solvent (acetonitrile and formic acid for mobile phases A and B, respectively) flow rate was set at 0.3 mL/min, where a linear gradient (10−20% in 20 min for acetonitrile) was used. Eluted peptides were subjected to mass spectrometry sequencing using a Waters Synapt Mass Quadrupole TOF mass spectrometer (Waters Corp.) through an electrospray ionization source. Automated data acquisition using the information-dependent mode was performed using MassLynx software (Waters Corp.). The spectra were interpreted using the peptide sequencing module of the MassLynx software.

Properties of identified peptides were calculated using peptide property calculator from Innovagen (Innovagen AB, Lund, Sweden), which is also available at http://www.innovagen.se/peptide-design-tools.asp. The sequences identified were matched to the published sequences of potato proteins from the National Center for Biotechnology Information (NCBI) database.

Evaluation of Radical Scavenging Activity. The peptide fractions from the above gel filtration were diluted to 20 μg/mL and then subjected to the ABTS radical scavenging assay.[14] Briefly, samples (20 μL each) were reacted with 1980 μL of diluted $ABTS^{\bullet+}$, and the absorbance (734 nm) after 10 min was read. Radical scavenging activity was expressed as Trolox equivalent (μM) based on a standard curve generated with a series of concentrations of Trolox (0−3 mM).

Statistical Analysis. All experiments were repeated a minimum of three times ($n \geq 3$) in independent replication trials, each with duplicate or triplicate sample analyses. Data were subjected to one-way analysis of variance (ANOVA) using the Origin 7.5 statistics program (OriginLab Corp., Northampton, MA, USA). Differences ($P < 0.05$) between means were identified by Tukey's test.

RESULTS AND DISCUSSION

Emulsion Physical Stability. The emulsions prepared either with Tween 20 alone or with Tween 20 + PPH had DLS-measured particle sizes of 215.3 and 218.1 nm, respectively. The value for the latter emulsion was larger than expected, which could have resulted from clustering of emulsion particles or peptide aggregates. Nevertheless, both the control and PPH treatment emulsions were very stable, showing no creaming even after 2 weeks of storage at 37 °C. During this period, the average particle size for both emulsions also did not show any significant change ($P > 0.05$).

AFM. The microscope images of emulsions prepared with Tween 20 with or without 20 mg/mL PPH are displayed in Figure 1. They represent height retrace and phase retrace obtained under the tapping mode. The emulsions prepared with combined Tween 20 and PPH (Figure 1A-A,A-B) consisted of numerous smaller and more uniformly distributed oil droplets when compared with those prepared with Tween 20 alone (Figure 1B-A,B-B). The particle numbers per measurement area (5 × 5 μm) for the two different emulsion systems were 365 and 137, respectively. This remarkable disparity was attributed to the surface activity of peptides present in PPH allowing more efficient dispersion of oil in the aqueous system.[13] In a typical O/W emulsion, the emulsifier concentration and type affect the particle size of emulsion droplets. Therefore, when PPH was combined with Tween 20, the peptides could impart additional emulsifying power by a sheer concentration effect. The imaging analysis result confirmed that the particle size measured by DLS in the PPH + Tween emulsion was likely an overestimate due to interactions between peptide-covered oil droplets and between peptides themselves. Because PPH alone was not capable of producing a stable emulsion (result not shown), there existed a cooperativity between Tween 20 and peptides.

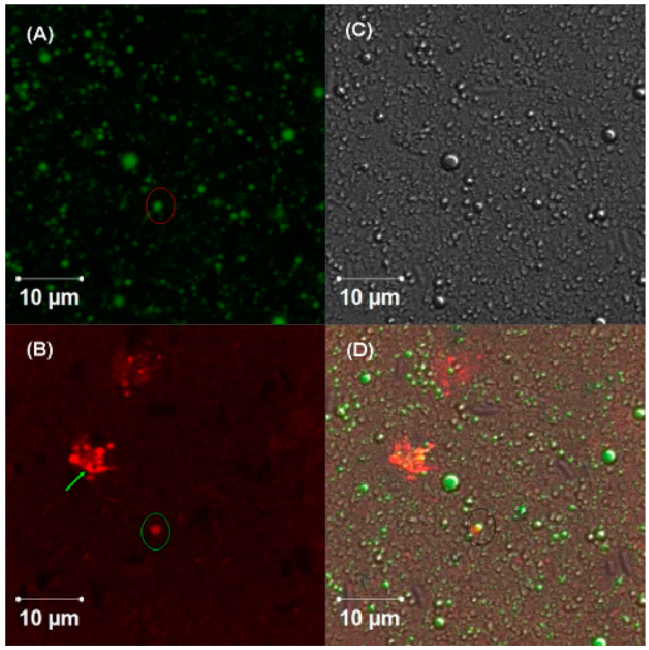

Figure 2. Confocal laser scanning micrographs of soybean O/W emulsions (10% w/w oil) prepared with 11.1 mg/mL Tween 20 + 20 mg/mL PPH: (A) image from Nile Red fluorescence (highlighting the oil phase); (B) image from Nile Blue/protein fluorescence; (C) phase contrast image without fluorescence; (D) overlay image combining fluorescence from both dyes. The arrow identifies a circular dark hole in the Nile Blue/protein image corresponding to a single large oil droplet. Scale bar represents 10 μm.

Phase angle shift, which is able to enhance the contrast of the AFM images, is a derivation of the tapping mode.[17,18] A phase image, taken by detecting the phase angle shift, is useful in distinguishing different chemical or physical features of the sample.[18−20] In phase images, dark and bright regions indicate different levels of hardness of the material.[20] It has been reported that interfacial characteristics and particle size affect not only the physical stability but also the chemical activity of emulsions.[21,22] An O/W emulsion could be regarded as a particle consisting of an oil core and a emulsifier shell. Because free oil inside an emulsion droplet is expected to have a higher fluidity than surface oil molecules with surfactants and the area-to-volume ratio is $3r^{-1}$ (where r is radius), a large oil droplet would be softer than a small oil droplet. The poorly covered

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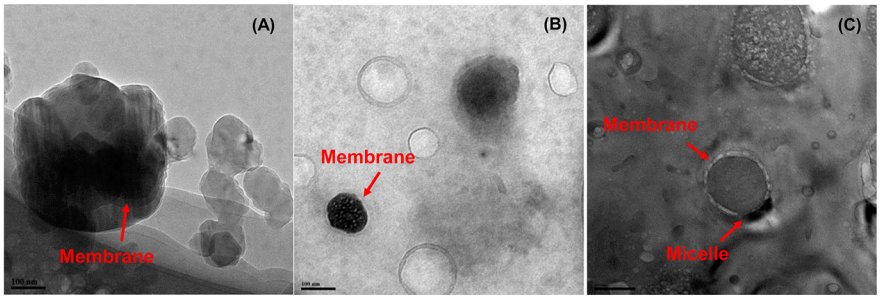

Figure 3. Cryo-TEM images of soybean O/W emulsions (10% w/w oil) prepared with (A) 11.1 mg/mL Tween 20, (B) 20 mg/mL PPH, and (C) 11.1 mg/mL Tween 20 + 20 mg/mL PPH. Bar = 100 nm.

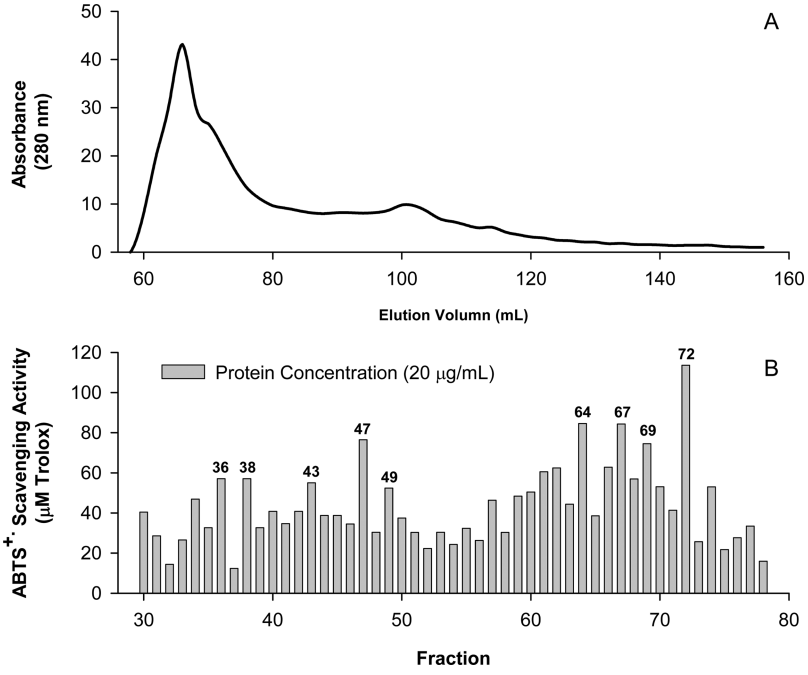

Figure 4. (A) Gel filtration spectra of PPH in the interface of soybean O/W emulsion prepared using 11.1 mg/mL Tween 20 + 20 mg/mL PPH; (B) $ABTS^{\bullet+}$ scavenging activity of gel filtration fractions from interfacial PPH. Every 2 mL of liquid was collected for one tube.

core region would be soft and appear bright, whereas the shell region would be hard and appear dark.

Indeed, in the Tween 20-stabilized oil emulsion, the emulsion droplets displayed a quite noticeable bright region in the surface center of the particles, indicating a soft matrix. In comparison, the emulsion prepared with combined PPH and Tween 20 exhibited a relatively dark region in the center of the oil droplets, indicating a harder or more intact membrane due to peptides. However, other parts of the surface of the emulsion droplets were not uniformly dark, suggesting that the distribution of PPH as a coemulsifier at the interface was not consistent. Moreover, the intensity of darkness appeared to be variable between oil droplets. If surface dilatational rheology measurements were carried out to determine the surface rheology, the results of AFM would be more convincing and mechanistic.

Inadequately covered (i.e., relatively exposed) surfaces of the emulsion droplets, notable for Tween 20 emulsions, would predispose the O/W emulsions to oxidative stress. When subjected to radicals and other oxidant species, the less well-protected oil particles would be prone to chemical degradation through oxidative chain reactions. Because peptides are emphateric ions, the less abundant presence of peptides at the interface would mean fewer charge repulsions against pro-oxidaitve metal and other oxidizing ionic species such as superoxide anion. Peptides located at the interface protect oil from oxidative stress through their radical scavenging activity and metal chelating actions. In addition, peptides dispersed in the aqueous phase are capable of enhancing the oxidative stability of emulsions by neutralizing radicals and sequestering pro-oxidaitve metal ions.[13,14,23]

CLSM. To substantiate that the physical location of peptides is an important factor for the inhibition of emulsion oxidation, CLSM images were presented to illustrate the distribution of peptides in different emulsion samples (Figure 2). Although CLSM was not able to display all particle sizes, we had found some typical particles to support out hypothesis. In these images, oil and peptides were labeled with fluorescent Nile Red and Nile Blue, respectively, enabling the delineation of peptide coating around the oil droplets. At the excitation wavelength of 639 nm, Nile Red-stained oil gave rise to a green color (Figure 2A), whereas at 488 nm, peptides stained with Nile Blue exhibited a red color (Figure 2B). The emulsion prepared with Tween 20 only showed numerous particles of varying sizes that emitted a green color. Because Tween 20 produces no fluorescence signals, these particles were oil droplets. On the

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Table 1. Peptides Identified by UPLC-Q-TOF-MS from Selected Fractions of PPH

fraction	peptide sequence	M_w	prob %
36	ALEPRP	681.4	95
38	VTLLADKQ	886.5	83
	KPSQSLQ	786.4	100
	DLKSV	560.3	74
	RLCAK	589.3	90
43	LTEPGR	671.4	75
	NSVDLQ	674.3	92
47	KNGKAM	647.3	87
	LFVRAS	691.4	74
49	DF	280.1	100
64	PLAL	412.3	100
	RPALM	586.3	97
67	SFDL(I)K	608.7	95
69	TEVL	460.2	99
	GPVNLL	611.4	99
	SFY	415.2	99
	SFDL(I)K	608.7	100
72	PDE	359.1	100
	AQRVP	569.3	100
	ENYKT	653.3	96
	RTQ	403.2	94

Table 2. Properties of Peptides Identified by UPLC-Q-TOF-MS from Selected Fractions

fraction	peptide sequence	p*I*	hydrophilic residues/total number of residues (%)	average hydrophilicity	net charge at pH 7.0
36	ALEPRP	7.0	33	0.6	0
38	VTLLADKQ	10.1	38	0	1
	KPSQSLQ	14.0	71	0.3	2
	DLKSV	6.7	60	0.6	0
	RLCAK	10.1	40	0.5	2
43	LTEPGR	7.0	33	0.6	0
	NSVDLQ	6.7	67	0.1	0
47	KNGKAM	10.6	50	0.7	2
	LFVRAS	11.0	33	−0.5	1
49	DF	3.1	50	0.3	−1
64	PLAL	6.0	0	0	−1
	RPALM	11	20	−0.1	1
67	SFDL(I)K	6.7	60	0.4	0
69	TEVL	3.3	25	−0.2	−1
	GPVNLL	6.0	17	−0.8	0
	SFY	5.9	33	−1.5	0
	SFDL(I)K	6.7	60	0.4	0
72	PDE	3.0	67	2	−2
	AQRVP	11.0	40	0.2	1
	ENYKT	6.9	60	0.7	0
	RTQ	14.0	67	0.9	2

other hand, when PPH was also present during the emulsion sample preparation, the product showed several conspicuous and many faint red droplets, indicting the presence of peptides surrounding the interface. In particular, a salient oil droplet and a remarkable cluster of red-colored particles are displayed (pointed by arrows), and these were peptide-coated particles (Figure 2B). Therefore, in these emulsions, Tween 20 and PPH coexisted at the interface for improved emulsion stability. Interestingly, apparently fewer oil droplets were visible in Nile Blue-stained samples (Figure 2B) than in Nile Red-stained samples (Figure 2A). This could be due to the lesser sensitivity of Nile Blue reacting with proteins than Nile Red reacting with oil.

Similar phenomena have been observed by Gallier et al.,[24] who used CLSM to analyze the membrane of milk fat globules. When some components absorbed in the membrane were replaced by other components with no fluorescence, their position in the membrane might turn to dark pores. The most prominent particle present in Figure 2A,B (arrow) was the same particle. When these two images were superimposed (Figure 2C), the color of the particle turned yellow, which was the overlay of green and red.[15] This was clear evidence that peptides in PPH were adsorbed at the interface as coemulsifiers. It was also shown in Figure 2B that some emulsion particles were surrounded by the aqueous environment consisting of peptides. This is in agreement with the result we reported previously with common transmission electron microscopy.[13]

On the basis of the results of AFM and CLSM, peptides from PPH were present at the interface, and they were distributed either through direct adsorption on the surface of oil droplets or by reinforcing the interface covered by Tween 20. Both scenarios are in agreement with the findings of Girardet et al.[25] that β-casein(f114−169) may cover the interface with Tween 20 or adsorb around the Tween 20 covering in the interface. Thus, the peptides partitioning in the interface filled in the space between individual Tween 20 molecules and prevented the exposure of oil to oxidants by means of physical obstacles in addition to chemical mechanisms. Peptides absorbed around Tween 20 might improve the interfacial charges and modify the electric double layers of the interface. This would lead to a stronger steric hindrance and enhanced electrostatic repulsions between oil droplets and oxidizing compounds as mentioned above to prevent oxidative chain reactions.

Cyro-TEM. Whereas CLSM yielded strong evidence that peptides in PPH were adsorbed at the interface of the soybean O/W emulsions, the structural details of the interfacial membrane were still missing. Hence, cyro-TEM, which operates in a nondestructive mode allowing emulsion samples to be examined in their intact state, was used to analyze the morphological characteristics of the interfacial membrane. The cyro-TEM images of the emulsion prepared with Tween 20 only exhibited no clear membrane (Figure 3A), whereas the emulsion prepared with PPH only showed a relatively uniform interfacial membrane-like structure (Figure 3B). On the other hand, the interface of the emulsion prepared using combined Tween 20 and PPH displayed a structurally heterogeneous membrane made up of several fragments with different lengths (Figure 3C). It appeared that the interface membrane was not composed of a single component; both Tween 20 and peptides were present. These two emulsifiers appeared to adsorb on the surface of the oil droplets alternatively but in an irregular pattern.

The dark segment in Figure 3C might be micelles of Tween 20 surrounding the surface of the emulsifier layer. A similar interfacial structure identified as a casein micelle sheath was found by other researchers who applied cyro-TEM to analyze the ultrastructure of dairy products.[26] It is possible that Tween 20 micelles, together with peptides from PPH, acted as physical barriers to prevent the penetration of peroxides and metal pro-oxidants, thereby inhibiting the oxidation of the oil.[12] Although the interfacial membrane of the emulsions prepared with combined Tween 20 and PPH appeared to be less tight than

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Table 3. Identified Interfacial Peptides That Matched the Peptide Sequences of Potato Protein

peptide sequence	matched protein[a]	sequence of matched protein[a]
DF	phosphoenolpyruvate carboxylase	f121 LKKKG**DF**GDE
		f431 LL**DF**LRQVST
PLAL(I)	NAD(P)H-quinone oxidoreductase subunit 1	f171 YEI**PLAL**CVL
	lipoxygenase 1	f461 QDNGSLK**PLAI**ELSFPHPDG
ENYKT	ATP synthase subunit b	f121 L**ENYK**NE**T**IP
SFY	glucose-1-phosphate adenylyltransferase	f371 DF**SFY**DRSAP
TEVL(I)	pyruvate kinase, cytosolic isozyme	f461 AKATDSES**TEVI**LEAALKSA
GPVNLL	lipoxygenase 1	f831 RS**GPVN**APYT**LL**FPTSEGGL
SFDL(I)K	patatin	f151 AIS**SFDIK**TN
PDE	isocitrate dehydrogenase	f80 **PDE**ARVKEFNL
AQRVP	ribulose-phosphate 3-epimerase	f121 LMIVEPD**QRVP**DFIKAGADI

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

the thin film in the emulsions prepared with Tween 20, the former clearly exhibited higher oxidation stability. This was not surprising because, in addition to the proposed physical effect, peptides can act as strong radical scavengers and reducing agents.[13,14] Emulsions stabilized by α-lactalbumin and β-lactoglobulin, which exhibited similar thickness when absorbed in the emulsion interface, showed different oxidative stabilities for their different structures and properties in the interface.[27,28] It has been demonstrated that antioxidants partitioning at the O/W interface are more effective than antioxidants present in the aqueous phase in suppressing free radical.[29]

Identification and Characterization of Interfacial Peptides. As PPH distributed in the interface was still a mixture of peptides, it was fractionated using G15 gel filtration. Fractions 36, 38, 43, 47, 49, 64, 67, 69, and 72, which exhibited notably higher ($P < 0.05$) ABTS radical scavenging ability than most other fractions and represented a broad fraction distribution (Figure 4), were further analyzed. These selected fractions were subjected to UPLC-Q-TOF-MS for peptide separation and sequence identification. They were also characterized for physical properties to help explain their physical role in the mechanism of oxidation inhibition by PPH at the interfacial membrane. As displayed in Table 1, altogether 17 peptides were identified. The molecular weights ranged from 200 to 800 Da, corresponding to dipeptides to heptapeptides. The anchorage of peptides at the interface was thought to be independent of peptide length;[30] short peptides were able to participate at the interface and might fill the gap between monolayer Tween 20 molecules. Lysine, arginine, glutamic acid, aspartic acid, proline, and leucine were the most abundant amino acids in the peptides identified. The free amino group in lysine and arginine and free carboxyl group in glutamic acid and aspartic acid, which possess a pair of free electrons, were able to chelate pro-oxidative metal ions such as Fe^{2+} and Cu^{2+} through electrostatic coordination. The sequestration action would lead to less catalysis activity of metal ions and, hence, reduced lipid oxidation.

Proline and leucine, which were prone to oxidation, were contained in the peptides that exhibited high ABTS radical scavenging ability. It has been widely reported that many bioactive peptides are made up of two to nine amino acids. Proline, lysine, and arginine, along with most hydrophobic amino acids, were commonly present in antioxidative peptides.[31] This was not surprising because, during the course of neutralizing free radicals as antioxidants, these amino acid residues were sacrificed.[32] The hydrophobic amino acids that were identified in the peptides were leucine, methionine, phenylalanine, and tyrosine. These amino acids can effectively associate with hydrophobic group in lipids or Tween 20 through hydrophobic effect, shielding the oil droplets from oxidant attack.

Of the total amino acid constituents, the ratio of hydrophilic residues to total number of residues was 45% (Table 2). A similar ratio also applied to hydrophobic residues. The well-balanced hydrophilicity−hydrophobicity explained excellent partitioning of these identified peptides at the interface. Furthermore, the average hydrophilicity of the peptides displayed a small part of the peptides being negatively charged but a large part carrying positive charges. That could lead to different partitions of the peptides. In general, strongly hydrophobic peptides would effectively partition in the oil−water interface with Tween 20, whereasa strongly hydrophilic peptides would have the propensity to form a secondary layer surrounding the inner layer made up of hydrophobic peptides and Tween 20 by weak interactions. Most of the hydrophilic peptides exhibited a net positive charge. The ability of peptides to repel metal ions or other oxidizing ionic species from lipid in the microenvironment of the interface would improve the oxidative stability of emulsified lipid.[4,5]

The identified peptides extracted from the emulsion interface were matched with the known sequence of potato proteins available in the database (BLAST program in NCBI). A portion of several peptides matched the peptide sequences of potato protein either partially or totally (Table 3). Although most of the proteins are not the major proteins in potato, the peptide SFDIK matched the peptide sequence of patatin, which is the most abundant potato protein. However, the peptide SFDIK was not among the antioxidative peptides identified in our previous work.[14,23] This indicated that antioxidative peptides identified in our previous studies might be mostly located in the aqueous phase of the emulsions and act as an active blocking agent to prevent the passage of oxidants to the interface. It was clear that antioxidative peptides, including SFDIK, in the interfacial membrane functioned through both physical and chemical mechanisms.

In conclusion, the partition of peptides in the soybean O/W emulsion membrane was demonstrated using several biophysical methods. The results implied that peptides may be present in two different modes: (1) as an integral part of the interfacial membrane through partitioning and (2) forming a surrounding layer immediately adjacent to the inner interface layer through weak forces. The peptides comprising the interface were mostly small peptides made up of hydrophobic and hydrophilic amino acids, of which peptide SFDIK matched

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the sequence of patatin. The antioxidant mechanism of peptides in the emulsion interface seemed to be both physical action including steric hindrance and possibly electrostatic effects and chemical action including radical scavenging capability. Further research should be carried out to measure the interfacial rheology in relation to the complex membrane structure so as to produce oxidation-resistant food emulsions.

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Notes

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