

Effect of enzyme types on the stability of oil-in-water emulsions formed with rice protein hydrolysates

Xin Pan,^a Yong Fang,^{a*} Lingling Wang,^a Minhao Xie,^{a,b} Bing Hu,^c Yiqing Zhu,^a Ermin Zhao,^b Fei Pei,^a Fei Shen,^a Peng Li,^a Xinchun Shen^a and Qiuhui Hu^a

Abstract

BACKGROUND: Common oil-in-water plant-based emulsions are allergenic and unstable to environmental stress, leading to increased consumer concerns about the food industry. To solve the problem of safety and instability, we investigated the influence of environmental stress on the stability of emulsions containing various rice protein hydrolysates, and compared the performance to whey protein, a common food emulsifier.

RESULTS: Rice protein hydrolysates were obtained by enzymatic hydrolysis with different proteases (neutrase, trypsin and alcalase). We evaluated the stability of emulsions produced with different hydrolysates according to storage, pH, ionic strength and thermal processing. Trypsin hydrolysates formed emulsion as stable as emulsion containing whey protein against a range of environmental stress containing pH (pH 6 to 7), salt ($< 150 \text{ mmol L}^{-1} \text{ NaCl}$) and temperature ($30\text{--}90^\circ\text{C}$). Moreover, a higher partition coefficient of protein in emulsion showed that the trypsin hydrolysates were easy to adsorb at the oil–water droplet interface, indicating its higher stability.

CONCLUSION: The results obtained in the present study suggest that trypsin hydrolysates could be utilized as natural emulsifiers to stabilize emulsion instead of traditional animal-based emulsifiers, opening many opportunities with respect to hypoallergenic emulsion systems in the food industry.

© 2019 Society of Chemical Industry

Keywords: rice; protein hydrolysates; emulsion; stability; whey protein

INTRODUCTION

There is increasing interest in using plant-based ingredients as natural emulsifiers instead of their synthetic counterparts, such as Tweens or Spans, and animal proteins, such as caseinate or whey protein, as a result of consumer concerns.¹ Proteins have native structural advantages in terms of their emulsifying capacity compared to other natural emulsifiers (e.g. quillaja saponin, and lecithin) as a result of the presence of hydrophobic and hydrophilic amino acids along with their polypeptide chains.² However, food allergens may exist in potential industrial emulsifiers obtained from plant-based proteins, including pea proteins,³ soy proteins⁴ and wheat proteins.⁵ Rice protein, with few known allergens reported in the existing databases,⁶ may be a potential alternative to other protein emulsifiers of plant origin.

In recent years, rice protein has been considered to be useful in food applications as a result of some bioactivities, such as an anti-carcinogenic capability and an anti-allergic ability.⁷ However, the poor solubility and instability of rice proteins under neutral conditions hampers their wide application as emulsifiers in the food industry.⁸ Thus, increasing treatments, such as freeze-milling,⁹ glycosylation¹⁰ and enzyme treatments,¹¹ have been used to enhance the emulsifying abilities of rice proteins by modification of their structure. Accordingly, enzymatic modification has

been applied to modify the molecular properties and improve the emulsifying capacity of proteins as a result of mild reaction conditions.^{12,13}

Recent studies concerning emulsions prepared with rice protein hydrolysates have focused on physical stability during storage. Cheetangdee¹⁴ found that the alcalase hydrolysates of rice bran protein had a pronounced effect on improving emulsion formability and long-term dispersibility. Tween 20 combined with rice protein hydrolysates systems can improve the storage stability

* Correspondence to: Y. Fang, College of Food Science and Engineering, Nanjing University of Finance and Economics/Collaborative Innovation Center for Modern Grain Circulation and Safety, Nanjing 210023, China. E-mail: fangyong10@nufe.edu.cn

^a College of Food Science and Engineering, Nanjing University of Finance and Economics/Collaborative Innovation Center for Modern Grain Circulation and Safety, Nanjing, China

^b Department of Food Science, University of Massachusetts Amherst, Amherst, MA, USA

^c College of Food Science and Technology, Nanjing Agricultural University, Nanjing, China

and inhibit the lipid oxidation of a oil-in-water emulsion for long storage.¹⁵ Furthermore, Xu *et al.*¹⁶ investigated the effect of limited trypsin hydrolysis on rice glutelin and found that rice glutelin hydrolysates at a degree of hydrolysis (DH) of 2% were most resistant to salt addition and thermal processing. Nevertheless, enzymes with specific cut sites led to a change in the exposed hydrophobic area in the protein, affecting their functional properties, especially the emulsifying property. Therefore, it was necessary to investigate the effect of enzyme cutting sites on the stability of the emulsions produced with rice protein hydrolysate with respect to constructing hypoallergenic emulsion systems.

The present study aimed to investigate the effect of hydrolysis treated with different enzymes on the surface hydrophobicity of rice proteins, as well as to evaluate the stability of the emulsions formed with rice protein hydrolysate exposed to environmental stress (e.g. pH, ionic strength and temperature) and its performance was compared with whey protein. Moreover, interfacial hydrolysate partitioning was measured to further explore the mechanism of stability.

MATERIALS AND METHODS

Materials

Rice protein (90%, w/w, dry basis) from rice endosperm was purchased from Pioneer Biotech Co., Ltd (Xi'an, Shanxi Province, China). Neutrase ($\geq 6000 \text{ U mg}^{-1}$), trypsin ($\geq 2500 \text{ U mg}^{-1}$) and 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from Sigma (St Louis, MO, USA). Alcalase 2.4 L FG (2.4 AU g^{-1} ; alkaline protease) was purchased from Novo (Novo Nordisk, Bagsvaerd, Denmark). Whey protein isolate (90%, w/w, dry basis) was purchased from Henan Qينو Food Ingredients Co., Ltd (Zhengzhou, He'nan Province, China). Food grade soybean oil (Luhua, Shandong, China) was purchased from a local market. All other chemicals used were of analytical grade and were obtained from Sigma.

Preparation of rice protein hydrolysates

Rice proteins were hydrolyzed by different proteases in accordance with the method of Phoon and Narsimhan.¹⁷ Rice proteins (10 g) were dissolved in 150 mL of distilled water and stirred for 0.5 h. The mass ratio of enzyme to substrate was 1:250 and rice proteins were hydrolyzed by neutrase at 50 °C and pH 7.0, as well as by other proteases (trypsin and alcalase) at 50 °C and pH 8.0. During the enzymatic hydrolysis process, the pH was kept stable by the addition of 0.5 mol L⁻¹ NaOH. After 4 h of hydrolysis, the mixtures were heated to 90 °C for 10 min to inactivate the enzyme and cooled promptly to ambient temperature. Finally, the pH of mixture was monitored to pH 7.0 and centrifuged at 12000 × g for 15 min, followed by the freeze-drying of supernatant.

Determination of the DH

DH was defined as the number of hydrolyzed peptide bonds (*h*) relative to the number of peptide bonds per unit weight (*h*_{tot}) expressed as a percentage, and determined by the pH-stat method,¹⁸ according to:

$$\text{DH (\%)} = \frac{h}{h_{\text{tot}}} \times 100 = \frac{BN_b}{\alpha M_p h_{\text{tot}}} \times 100$$

where *B* is the base (NaOH) consumption in mL, *N_b* is the normality of the base, *M_p* is the mass of hydrolyzed protein (g), *h*_{tot} is the total number of peptide bonds in the protein substrate (7.40 mEq g^{-1} rice protein) and α is the average degree of dissociation of the $\alpha\text{-NH}_2$ groups released during hydrolysis, expressed as:

$$\frac{1}{\alpha} = 10^{\text{pK}-\text{pH}} + 1$$

where pH and pK are the values at which proteolysis is conducted.

Surface hydrophobicity

The surface hydrophobicity of rice protein hydrolysates was examined using the method of Kato and Nakai.¹⁹ Rice protein and hydrolysates were dissolved with phosphate buffer (10 mmol L^{-1} , pH 7.0) to obtain densities at a range from 0.01 mg mL^{-1} to 0.1 mg mL^{-1} . Then, $25 \mu\text{L}$ of ANS (8.0 mmol L^{-1}) was added to protein solution (5 mL), followed by incubation for 10 min at room temperature. The fluorescence intensity was determined using a fluorescence spectrophotometer (F-7000; Hitachi, Tokyo, Japan), utilizing excitation and emission wavelengths of 390 and 470 nm. The slope of the relative fluorescent intensity sample concentration curves, as the surface hydrophobicity index, was calculated according to linear regression analysis.

Protein solubility

Solubility of rice protein and hydrolysates were determined by the method of Lowry *et al.*,²⁰ with some modifications. The pH of hydrolysates solution at a concentration of 10 mg mL^{-1} was adjusted from 3.0 to 9.0 using 0.5 mol L^{-1} HCl or NaOH. The solutions were centrifuged at $5000 \times g$ for 15 min, and the supernatant was taken for protein content analysis. The solubility of the protein was calculated as the percentage of protein in the supernatant with respect to total protein content.

Circular dichroism studies

The secondary structure of rice protein hydrolysates was measured by far-UV circular dichroism (Model MOS-450; French Biologic Company, Grenoble, France). The protein samples (1 mg) were dissolved in 10 mL of phosphate buffer (10 mmol L^{-1} , pH 7.0). Then, the solutions were poured into a 1 mm quartz cuvette. The circular dichroism spectra in the range (190 nm to 250 nm) were recorded at a nitrogen flow rate of 100 nm min^{-1} . The protein secondary structure content was obtained online using the SELCON3 method in DICHROWEB (<http://dichroweb.cryst.bbk.ac.uk>).

Preparation of emulsions

Emulsions were produced using the method of Niu *et al.*²¹ Hydrolysates samples were dissolved in phosphate buffer (10 mmol L^{-1} , pH 7.0) to obtain a concentration of 20 mg mL^{-1} , followed by stirring for 1 h. A mixture with 10% (v/v) soybean oil and 90% (v/v) hydrolysates solution was homogenized by a shear mixer (T18; IKA, Staufen, Germany) at $10000 \times g$ for 2 min. Fine emulsions were prepared by a high-pressure homogenizer (DSJ-20L; ATS Engineek Inc., Milan, Italy) at a pressure of 60 MPa for three cycles. The positive control emulsion containing 20 mg mL^{-1} of whey protein isolate was prepared. The pH of freshly prepared emulsions was adjusted to 7 using 0.5 mol L^{-1} NaOH or HCl to obtain initial emulsions, and

0.1 mg mL⁻¹ sodium azide was added into emulsions to prevent microbial growth.

Emulsion characterization

Droplet size and particle charge measurements

The droplet mean particle diameter and ζ -potential of the emulsions were determined using a MasterSizer 3000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK). The phosphate buffer (10 mmol L⁻¹, pH 7.0) was added into the emulsions to avoid multiple scattering before measurement. The refractive indices used were 1.33 for buffer. All of the measurements were performed in triplicate.

Microstructure of emulsions

The microstructure of the emulsions was observed after 1 day of storage at ambient temperature. The micrographs were taken with a microscope at 400 $\times$ magnification (Zeiss Axio Scope A1; Carl Zeiss, Oberkochen, Germany). One drop of sample was placed on a microscope slide and covered with a glass cover slip.

Emulsion stability

The stability of the initial emulsions was tested under storage and environmental stress. To test the effect of storage, size distribution and microstructure were used to characterize the stability of the emulsions during 7 days storage. To test the effect of pH, emulsions were adjusted to pH from 3 to 7 with 0.5 mol L⁻¹ HCl or 0.5 mol L⁻¹ NaOH. To test the effect of ionic strength, emulsions containing NaCl from 0 to 200 mmol L⁻¹ were prepared by mixing the original emulsions with NaCl. To test the effect of temperature, emulsions were heated to temperature ranging from 30 to 90 °C for 30 min and promptly cooled to room temperature. The emulsions treated with environmental stress were stored at room temperature for 24 h before the measurement of particle size and ζ -potential.

Partition of protein in emulsions

The partition of protein in emulsions was determined using the method described in our previous study.²² The serum and cream layer of emulsions were obtained by centrifuging at 15000 $\times g$ for 30 min, followed by filtering of the serum layer using a 0.22-mm syringe filter. The protein concentration in the water phase was measured by the procedure described by Lowry *et al.*²⁰ Then, the partition coefficient (PC) for hydrolysates in the continuous phase was determined by the method of Huang *et al.*²³ and calculated as:

$$PC = \frac{V_w}{V_1} \times \left(\frac{W_t}{W_w} - 1 \right)$$

where V_w is the volume of hydrolysates solution, V_1 is the volume of oil, W_t is the total amount of protein (mg mL⁻¹) and W_w is the protein concentration of the aqueous phase (mg mL⁻¹). The percentage of protein in the aqueous phase was calculated as (W_w/W_t) $\times$ 100%.

Statistical analysis

Analysis of variance was performed using SAS, version 9.4 (SAS Institute, Cary, NC, USA). Means were compared by Duncan's test. $P < 0.05$ was considered statistically different.

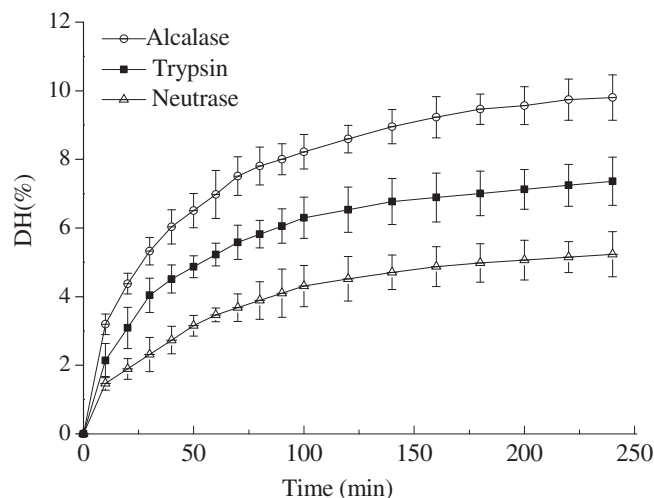


Figure 1. DH of rice protein hydrolysates digested by different enzymes.

RESULTS

Characterization of rice protein hydrolysates

Degree of hydrolysis

The DH of protein hydrolysates digested by different enzymes under different hydrolysis time is shown in Fig. 1. When the hydrolysis reaction progressed, DH of enzymes-treated protein rose continuously. However, the increasing rate of DH slowed down at a hydrolysis of 150 min. Then, the DH reached gentle values after a period of hydrolysis. Moreover, alcalase hydrolysates always exhibited a significantly ($P < 0.05$) higher DH compared to the other hydrolysates. After 4 h of hydrolysis, DH reached stable values: 4.23% for neutrase hydrolysates, 6.36% for trypsin hydrolysates and 9.81% for alcalase hydrolysates.

Effect of enzymatic hydrolysis on the surface hydrophobicity of rice protein

Figure 2 shows the effect of DH on the surface hydrophobicity of rice protein hydrolysates. With an increase in DH, the surface hydrophobicity index of trypsin hydrolysates and alcalase hydrolysates increased markedly, reaching significantly ($P < 0.05$) high values (333.43 and 439.06) at moderate hydrolysis. Then, the surface hydrophobicity index decreased greatly with a higher DH. However, the highest DH of neutrase hydrolysates showed the highest surface hydrophobicity index (359.21), and differed significantly ($P < 0.05$) compared to the other DH. The surface hydrophobicity of the hydrolysates varied with enzyme type and DH. However, all protein hydrolysates at a DH of 4% had the highest surface hydrophobicity index compared to the other DH under same enzyme treatments. To investigate the effect of enzyme cites on the stability of emulsions prepared with hydrolysates, all subsequent experiments were controlled with a DH of 4%.

Effect of enzymatic hydrolysis on the secondary structure of rice protein

As shown in Table 1, enzymatic hydrolysis changed the secondary structure of rice proteins. The β -sheet and β -turn contents of rice protein were decreased from 29.8% to 20.9% and from 22.0% to 16.9% by alcalase hydrolysis. However, the β -sheet contents of trypsin hydrolysates and neutrase hydrolysates were 22.3% and 24.6%, and the values were higher than alcalase hydrolysates. Additionally, the α -helix and random coils contents in alcalase hydrolysates were increased to 23.1% and 39.1%.

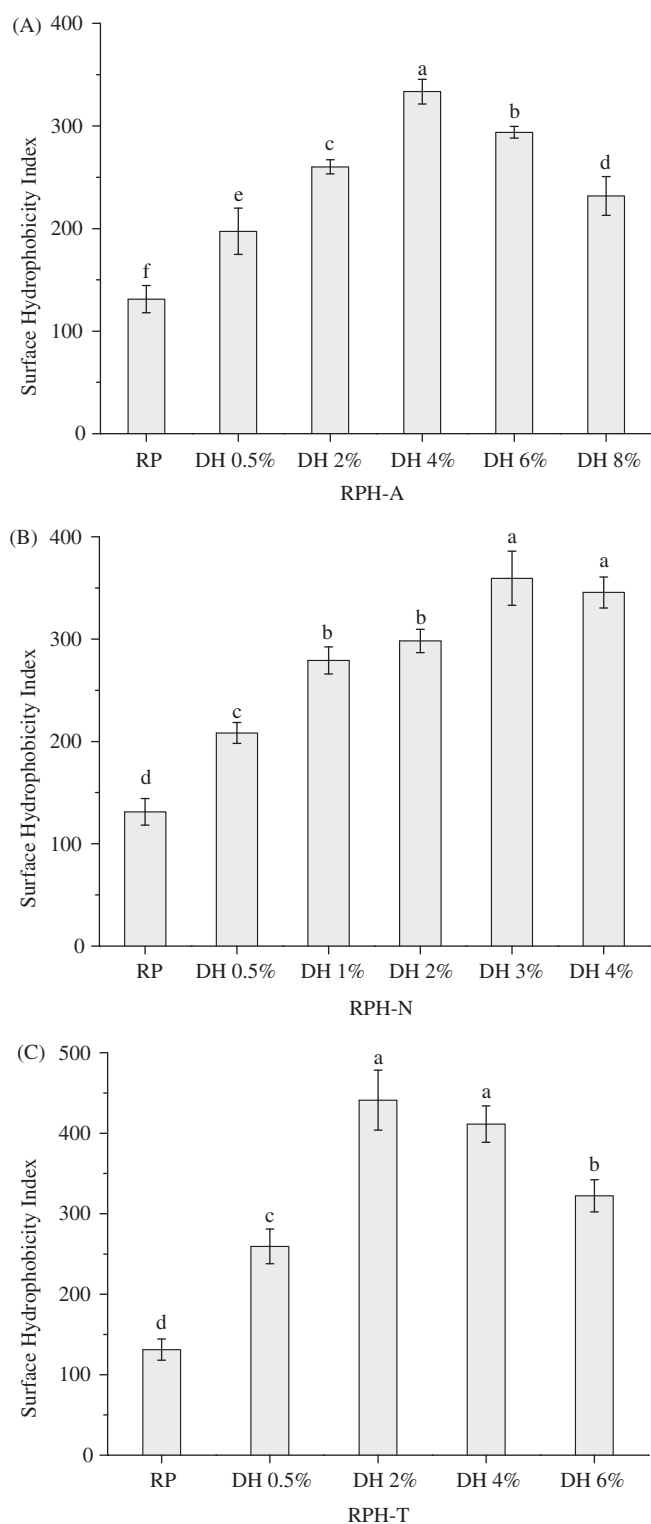


Figure 2. The surface hydrophobicity of RPH-A (A), RPH-N (B) and RPH-T (C) at different DH. Results are the mean $\pm$ SD of triplicate determinations. Different lowercase letters indicate a significant difference ($P < 0.05$). RPH-A, rice protein alcalase hydrolysates; RPH-N, rice protein neutrase hydrolysates; RPH-T, rice protein trypsin hydrolysates.

Table 1. Secondary structure content of rice protein hydrolysates determined by circular dichroism

Samples	Secondary structures (%)			
	α -helix	β -sheet	β -turn	Random coils
RP	16.9	29.8	22.0	31.3
RPH-A	23.1	20.9	16.9	39.1
RPH-N	19.7	24.6	20.1	35.6
RPH-T	22.4	22.3	18.1	37.2

Results are the average of triplicate determinations. RP, rice protein; RPH-A, rice protein alcalase hydrolysates; RPH-N, rice protein neutrase hydrolysates; RPH-T, rice protein trypsin hydrolysates.

Table 2. Change of particle sizes in rice protein hydrolysates emulsions during storage and partitioning of peptides in continuous phase (serum)

Sample	Mean particle size (μ m)		Proteins in the aqueous phase (%)	Partition coefficient (aqueous phase)
	Day 1	Day 7		
RPH-A	0.34 $\pm$ 0.01 a	0.45 $\pm$ 0.04 a*	73.79 $\pm$ 3.01 b	3.19
RPH-N	0.27 $\pm$ 0.04 b	0.47 $\pm$ 0.05 a*	76.38 $\pm$ 2.28 b	2.78
RPH-T	0.27 $\pm$ 0.01 b	0.31 $\pm$ 0.02 b*	68.58 $\pm$ 3.13 c	4.12
WPI	0.24 $\pm$ 0.03 b	0.30 $\pm$ 0.02 b*	82.57 $\pm$ 2.08 a	1.90

Results are the mean of triplicate determinations $\pm$ standard deviation. Different lowercase letters indicate significant differences ($P < 0.05$) among the emulsions prepared with different protein, whereas as asterisk (*) indicates a significant difference ($P < 0.05$) between days 1 and 7 for each sample. RPH-A, emulsion formed with rice protein alcalase hydrolysates; RPH-N, emulsion formed with rice protein neutrase hydrolysates; RPH-T, emulsion formed with rice protein trypsin hydrolysates; WPI, emulsions formed with whey protein isolate.

Effect of enzyme types on the stability of emulsions prepared with rice protein hydrolysates

Storage time

Table 2 shows the change in particle sizes in emulsions during 7 days of storage. The initial size of the emulsions stabilized with alcalase hydrolysates was 0.34 μ m and differed significantly ($P < 0.05$) compared to the other emulsions. In Fig. 3, mainly smaller droplets from emulsions produced with trypsin hydrolysates and whey protein isolate can be seen. Other emulsions possessed some obvious droplet aggregation after 1 day of storage. During storage, the droplet sizes of emulsions were increased, and emulsions prepared with trypsin hydrolysates exhibited the lowest change in droplet sizes (0.04 μ m), followed by emulsion produced with whey protein isolate (0.06 μ m). To further investigate the size distributions during storage, the multimodal distribution of emulsions prepared with alcalase hydrolysates and whey protein isolate was observed in Fig. 4 during storage. The size distribution of emulsion stabilized with trypsin hydrolysate was monomodal, and the rightshift was less than that of the other emulsions.

pH

As shown in Fig. 5(A), the particle size of the emulsions exhibited similar trends as the size of droplet increased first and then decreased from pH 3.0 to 7.0. The emulsion containing alcalase hydrolysates exhibited the highest size at a pH of 4.0

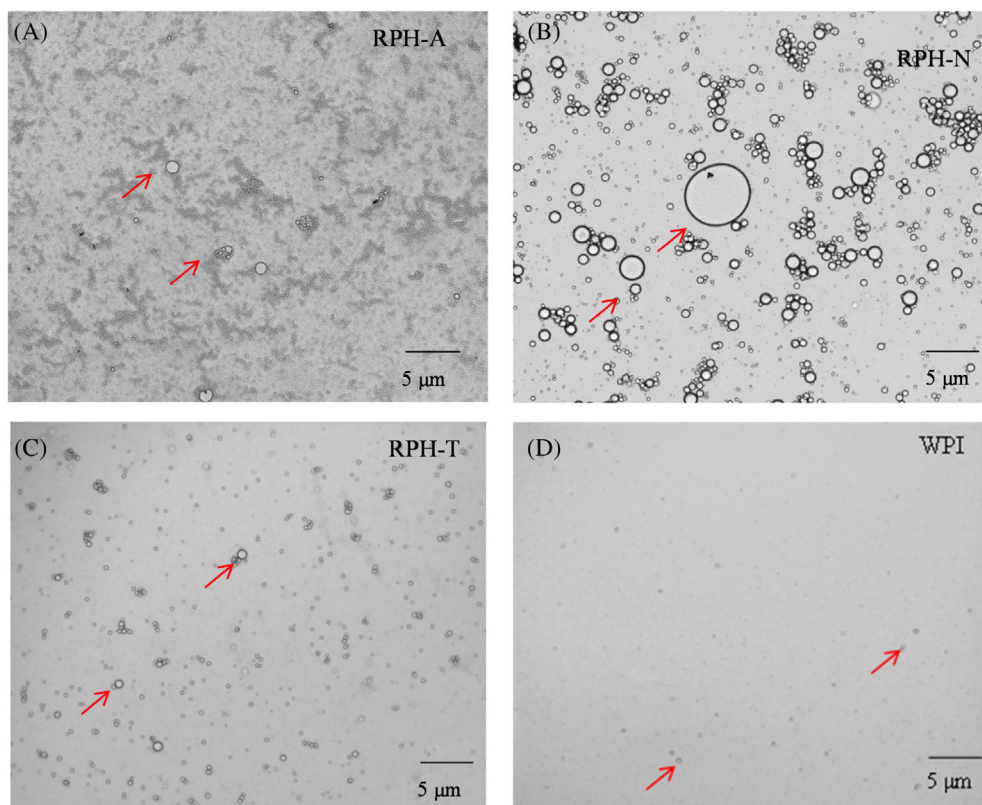


Figure 3. Microscopy images of emulsions formed with RPH-A (A), RPH-N (B), RPH-N (C) and WPI (D) observed at 400 $\times$ magnification. Red arrows indicate large oil droplets of the emulsion. RPH-A, emulsion formed with rice protein alcalase hydrolysates; RPH-N, emulsion formed with rice protein neutrase hydrolysates; RPH-T, emulsion formed with rice protein trypsin hydrolysates; WPI, emulsions formed with whey protein isolate).

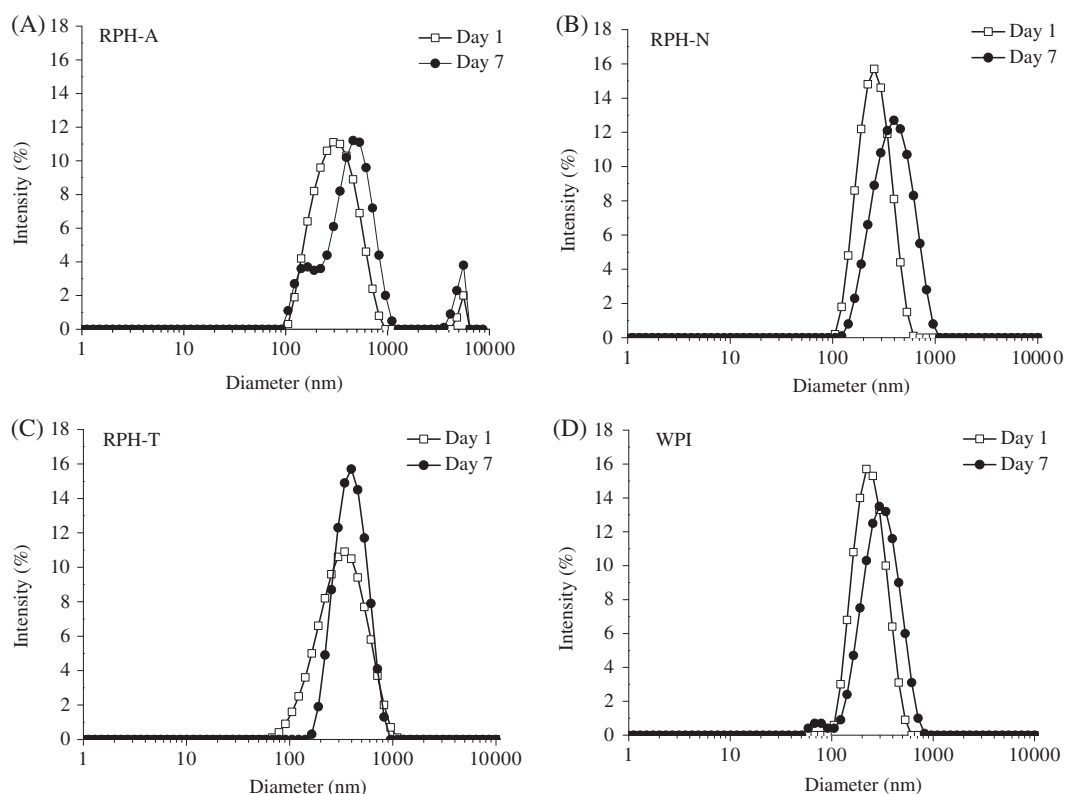


Figure 4. Size distribution of emulsions formed with RPH-A (A), RPH-N (B), RPH-T (C) and WPI (D), after room temperature storage for 1 and 7 days. RPH-A, emulsion formed with rice protein alcalase hydrolysates; RPH-N, emulsion formed with rice protein neutrase hydrolysates; RPH-T, emulsion formed with rice protein trypsin hydrolysates.

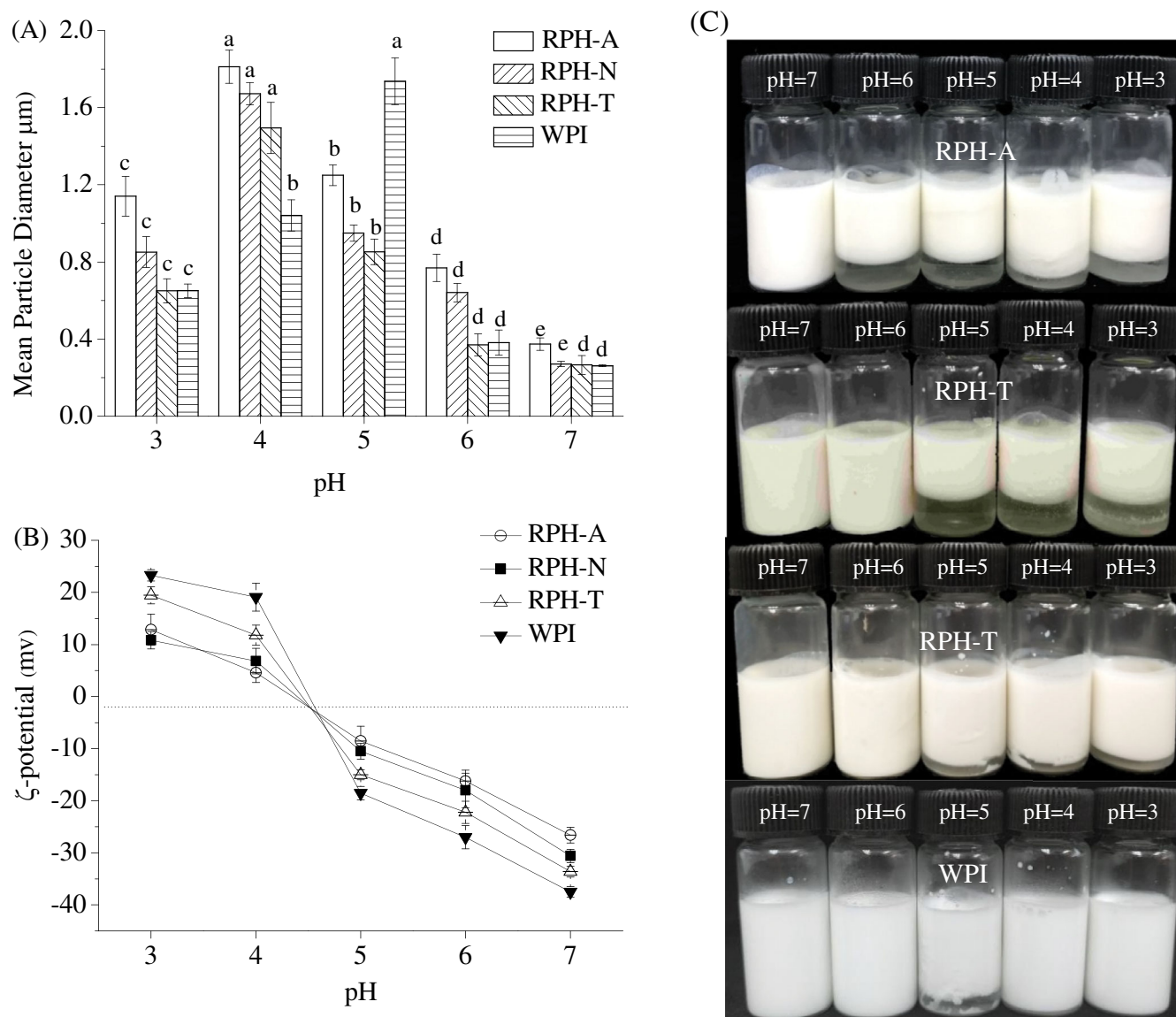


Figure 5. Influence of pH on the mean particle size (A), ζ -potential (B) and photographs (C) of oil-in-water emulsions formed with rice protein hydrolysates and whey protein isolate during storage for 1 days. Results are the mean $\pm$ SD ($n = 3$); different lowercase letters indicate a statistically significant difference ($P < 0.05$) among the same samples treated with different pH. RPH-A, emulsion formed with rice protein alcalase hydrolysates; RPH-N, emulsion formed with rice protein neutrase hydrolysates; RPH-T, emulsion formed with rice protein trypsin hydrolysates; WPI, emulsions formed with whey protein isolate.

($1.81 \pm 0.09 \mu\text{m}$), followed by emulsion formed with neutrase hydrolysates ($1.67 \pm 0.06 \mu\text{m}$) and emulsion produced with trypsin hydrolysate ($1.49 \pm 0.13 \mu\text{m}$), which differed significantly ($P < 0.05$) compared to the size of the emulsions at other pH values. Similarly, the size of emulsion prepared with whey protein increased greatly at pH 4.0 ($1.04 \mu\text{m}$) and pH 5.0 ($1.73 \mu\text{m}$) around the isoelectric point, and the emulsion maintained relative stability at other pH values.

To explore the mechanism of emulsion instability, the electrical characteristics of the protein-coated droplets were measured at different pH values (Fig. 5B). Correspondingly, the ζ -potentials of emulsion containing whey protein isolate were $+23.29 \text{ mV}$ at pH 3.0 and -37.46 mV at pH 7.0 and the magnitude charges were higher than for the other emulsions ($+19.45 \text{ mV}$ at pH 3.0, -33.60 mV at pH 7.0). In addition, the charges of emulsions near a pH in the range 4.0–5.0 were zero. Moreover, a visual observation of the emulsions is shown in Fig. 5(C), supporting the particle size

measurements. The emulsions containing trypsin hydrolysates and whey protein isolate, which exhibited less phase separation, were more stable to creaming than the other hydrolysates at low pH.

NaCl

As shown in Fig. 6(A), there was no significant difference ($P > 0.05$) in the particle size of the emulsions containing trypsin hydrolysates with an increased NaCl ranging from 50 to 150 mmol L^{-1} . Moreover, the size of the emulsions prepared with alcalase hydrolysates reached maximum values ($1.01 \mu\text{m}$) at a concentration of 200 mmol L^{-1} NaCl. The addition of NaCl destabilized the emulsions and decreased the absolute value of charge on the droplets (Fig. 6B). The emulsions prepared with whey protein isolate exhibited the highest change in ζ -potential, from -37.56 mV at 0 mmol L^{-1} NaCl to -23.36 mV at 200 mmol L^{-1} NaCl.

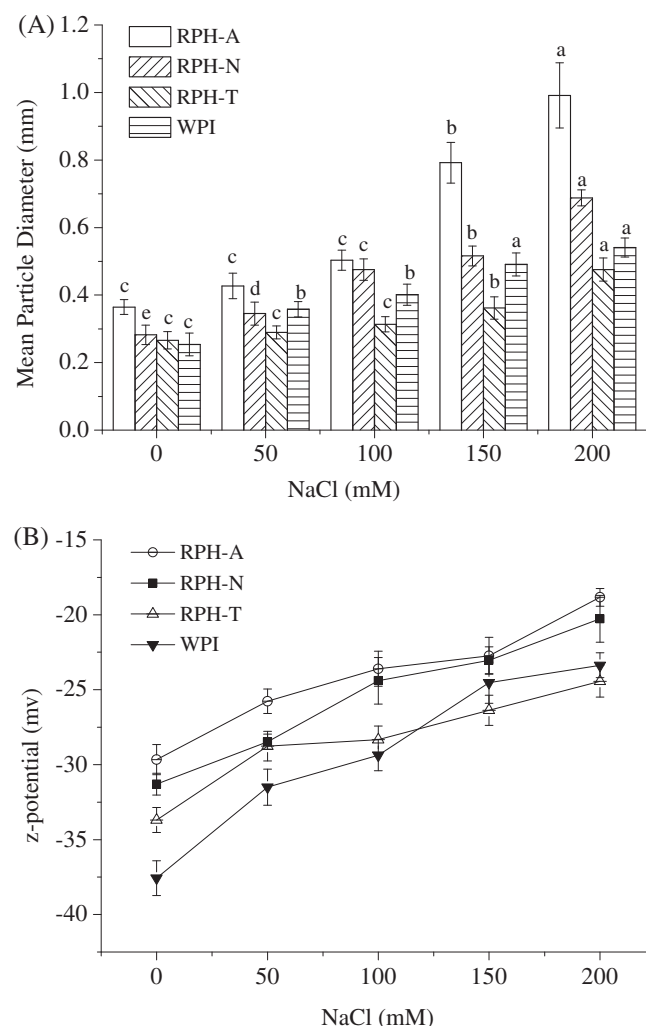


Figure 6. Influence of ionic strength on the mean particle size (A) and ζ -potential (B) of the oil-in-water emulsions formed with rice protein hydrolysates and whey protein isolate. Results are the mean $\pm$ SD ($n = 3$); different lowercase letters indicate a statistically significant difference ($P < 0.05$) among the same samples treated with different NaCl concentrations. RPH-A, emulsion formed with rice protein alcalase hydrolysates; RPH-N, emulsion formed with rice protein neutrase hydrolysates; RPH-T, emulsion formed with rice protein trypsin hydrolysates; WPI, emulsions formed with whey protein isolate.

Thermal treatment

Figure 7 shows the influence of temperatures on the particle size of the emulsions. The emulsions containing trypsin hydrolysates and whey protein isolate had the lowest variation in mean size, from 30 °C (263.7 nm and 256.5 nm) to 90 °C (281.4 nm and 274.5 nm). However, the thermal treatments obviously increased the size of the emulsions containing alcalase hydrolysates from 374.8 nm (30 °C) to 522.3 nm (70 °C) and little change in ζ -potential of the emulsion under heat treatment was observed (data not shown).

Effect of enzyme type on the partition of protein in emulsions

The distribution of proteins at the oil–water interface *versus* in the continuous phase was measured to further evaluate the stability in individual groups of emulsions. Table 2 shows the protein in the aqueous phase of emulsion prepared with different enzymes. The protein in the aqueous phase of emulsions formed with trypsin

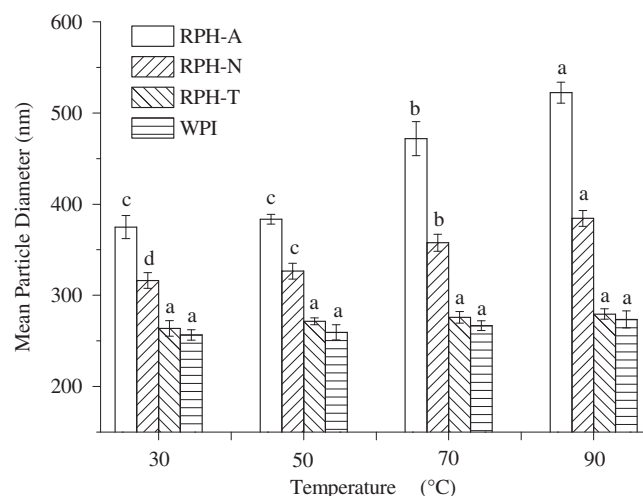


Figure 7. Influence of thermal processing on the mean particle size of the oil-in-water emulsions formed with rice protein hydrolysates and whey protein isolate. Results are the means and standard deviation ($n = 3$); different lowercase letters indicate a statistically significant difference ($P < 0.05$) among the same samples treated with different temperatures. RPH-A, emulsion formed with rice protein alcalase hydrolysates; RPH-N, emulsion formed with rice protein neutrase hydrolysates; RPH-T, emulsion formed with rice protein trypsin hydrolysates; WPI, emulsions formed with whey protein isolate.

hydrolysates was 68.58% and this differed significantly ($P < 0.05$) compared to the other emulsions. In addition, the partition coefficient (4.12) in emulsions produced with trypsin hydrolysates was also higher compared to that of the other emulsions. Interestingly, the emulsions containing whey protein isolate showed larger percentages of protein in the continuous phase and smaller partition coefficients.

DISCUSSION

Generally, rice protein can be used as a food additive in the food industry as a result of its high bioactivity and abundant amino acids. However, the poor solubility of rice protein has limited its application as an emulsifier. In the present study, enzymes treatments were used to modify the protein structure and improve the solubility of rice protein. As shown in Fig. 1, hydrolysates obtained by different enzyme treatments had different DH and this might be attributed to specific enzyme cutting sites. Alcalase possessed a broad specificity for releasing peptides, including end aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leu and Ala) and basic (Lys) residues,²⁴ leading to its higher DH compared to other enzyme treatments. With the hydrolysis time increasing, the reduction of substrate molecules could decrease the increasing rate of DH.²⁵ Then, the DH values of hydrolysates can be kept stable after a period of hydrolysis (Fig. 1). Moreover, the rice protein structure was destroyed and partly unfolded during the progression of hydrolysis.²⁶ The hydrophobic amino acids buried within the interior of protein were exposed and rice protein aggregation was reduced, leading to an improvement of surface hydrophobicity index²⁷ (Fig. 2) and solubility (see Supporting information, Fig. S1).

The hydrophobic effect is considered as the main force that maintains the tertiary structure of the protein. It is important to investigate surface hydrophobicity to further reveal the spatial structure and functional characteristics of the protein.²⁸ The

increase in surface hydrophobicity in the protein enhanced its affinity for the droplet surface, and this led to a stronger binding ability of the protein and oil droplet.¹⁹ Hence, surface hydrophobicity plays an important role in determining the emulsifying properties of proteins and peptides.²⁹ The different protein hydrolysates at a DH of 4% in Fig. 2 were considered to exhibit better emulsion stability; therefore, all subsequent experiments were controlled with a DH of 4%. Evangelho *et al.*³⁰ have reported that the surface hydrophobicity of the alcalase hydrolysates of black bean protein was higher, and the emulsion prepared with these hydrolysates showed a better storage stability. Nevertheless, the surface hydrophobicity index began to decrease with a higher DH, which might be a result of the decrease in peptide size in hydrolysates affecting the affinity of the ANS probe toward the protein.³¹ In addition, during the analysis of the secondary structure, β -sheets as protein structure ("trains") are relatively stable, whereas the α -helix, β -turn and random coils are relatively flexible and open.¹⁶ In Table 1, enzymatic hydrolysis decreased the β -sheet regions and increased the α -helix, β -turn and random coil regions, indicating that the secondary structure in the protein attains a more flexible and extended form after enzyme treatment.³² These changes in surface hydrophobicity and secondary structure in the protein showed that the protein hydrolysates had more flexible structures than the native protein. Accordingly, the hydrolysates might be adsorbed more easily to oil–water interfaces and could be utilized as excellent emulsifiers.³³

When selecting a natural emulsifier for a specific use, stability is an important criterion for consideration under conditions of practical application in food products (e.g. pH, ionic strength and temperature).³⁴ As shown in Fig. 5(A), the sizes of emulsions containing hydrolysates near pH 4 were higher compared to the others and this might be because all protein emulsions are unsteady with respect to aggregating near the isoelectric point of the proteins.³⁵ As shown in Fig. 5(B), the ζ -potential of hydrolysate emulsions was from -40 mV to 30 mV at a pH from 7.0 to 3.0 . Xu *et al.*³⁶ have reported that the carboxyl groups and amino groups are negatively or positively charged at different pH values, resulting in a variation in the number of anionic and cationic groups. Additionally, zero values of ζ -potential occurred between pH 4.0 and pH 5.0 , implying that rice protein hydrolysates stabilize emulsions by electrostatic repulsion rather than by steric repulsion.³⁷ Then, the net droplet charge was too low to prevent van der Waals attraction near the isoelectric point, leading to an increase in size as a result of droplet aggregation.³⁸ However, the magnitude of ζ -potential of the emulsion prepared with whey protein isolate was highest at pH 5 and its size was also higher compared to the other emulsions. Wang *et al.*³⁹ have reported emulsions with a ζ -potential in the range -30 to 30 mV are instable and tend to coagulate or flocculate. Thus, the diffusion rate of the interface protein in emulsion was slower as a result of the large repulsive force between the droplets. Similarly, the interaction between the protein molecules in the interface layer became weak, and the oil droplets might not be well wrapped, resulting in a higher size of emulsion.³⁴ Accordingly, the hydrolysate emulsions exhibited their highest sizes at pH 4 rather than at other pH values as a result of a higher absolute value of ζ -potential (Fig. 5B) compared to that at pH 5.0 . Moreover, this could be because protein hydrolysis can change the type and number of dotted groups in the protein, resulting in the isoelectric point of the rice protein shifting from 5 to 4 .⁴⁰

Accordingly, Fig. 6 shows the effect of NaCl on the stability of emulsions. In Fig. 6(A), the sizes of emulsions prepared with

protein hydrolysates are increased with a higher concentration of NaCl. This might be a result of the addition of Na^+ into the emulsions screening the electrostatic repulsion between the negatively charged droplets, resulting in the aggregation of the droplet. The size of emulsions produced with trypsin hydrolysates and alcalase hydrolysates did not change significantly ($P > 0.05$) at a concentration of NaCl of less than 100 mmol L^{-1} . At a low concentration of NaCl, the electrostatic repulsive forces acting between the droplets are sufficient to prevent any attractions (e.g. van der Waals and hydrogen bonds) and this could inhibit droplet aggregation.³⁶ Nevertheless, the size of the emulsion containing neutrase hydrolysates was markedly and significantly increased, which might be linked to the content of anionic proteins after enzyme treatment. When the addition of NaCl concentration was above a critical level, the attractive forces were stronger than the electrostatic repulsive forces, resulting in droplet aggregation and an increase in size (Fig. 5B).⁴¹ Similarly, NaCl added into emulsions can interact with the $-\text{COO}^-$ on the surface of the droplet and lead to an increase in the magnitude of the ζ -potential by the electrostatic effect.⁴² Moreover, Fig. 6 shows the effect of thermal treatments on the size of emulsions and found that emulsions produced with trypsin hydrolysates and whey protein isolate were stable to thermal processing. However, the thermal treatments obviously increased the size of neutrase hydrolysates, and droplet aggregation might be the result of a reduction in the electrostatic repulsion or a change in structures (e.g. non-polar or sulfhydryl groups).¹⁶

To better understand the mechanism of stabilizing hydrolysate emulsions, we investigated the interfacial peptide partitioning. As shown in Table 2, we found that emulsions containing whey protein isolated with the lowest partition coefficient showed better stability compared to hydrolysate emulsions. In general, the interface distribution of protein in emulsions was affected by the physicochemical properties of protein hydrolysates (i.e. size, molecular weight distribution, charge).⁴³ The hydrolysis can decrease the molecular weight and increase the amount of charged groups of the intact polypeptides. Then, the increasing charges in peptides (i.e. the charge:mass ratio) strengthens their association with the oil phase. The trypsin hydrolysates emulsions exhibited better stability as a result of the increasing surface charges in hydrolysates against their hydrophobic interaction. Zhao and Xiong⁴⁴ have reported a mechanism of stabilizing emulsion by surface hydrolysates via the formation of a flexible membrane around the oil droplet, providing physical protection of the emulsion. In the present study, electrostatic interaction, as well as the flexibility of peptide chains, can play an important role in stable surface membrane formation.

CONCLUSIONS

In summary, the results of the present study show that enzyme hydrolysis could improve the surface hydrophobicity and flexibility of rice protein. Moreover, trypsin hydrolysates at moderate DH could be used as plant-based emulsifiers (comparable to whey protein isolate) against environmental stress, containing ionic strength at low levels and temperatures. However, trypsin hydrolysate emulsions were mainly dominated by the electrostatic interactions against aggregation and this led to poor pH stability. Therefore, further studies are required to modify hydrolysate emulsions and improve the stability of the emulsions over a wide range of environmental conditions.

ACKNOWLEDGEMENTS

This work was financially supported by the National Key Research and Development Program of China (Grant no. 2017YFD0401105), the National Natural Science Foundation of China (31471680, 31972020) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

REFERENCES

- McClements DJ, Bai L and Chung C, Recent advances in the utilization of natural emulsifiers to form and stabilize emulsions. *Annu Rev Food Sci Technol* **8**:205–236 (2017).
- Lam RSH and Nickerson MT, Food proteins: a review on their emulsifying properties using a structure–function approach. *Food Chem* **141**:975–984 (2013).
- Stone AK, Avarmenko NA, Warkentin TD and Nickerson MT, Functional properties of protein isolates from different pea cultivars. *Food Sci Biotechnol* **24**:827–833 (2015).
- Nishinari K, Fang Y, Guo S and Phillips GO, Soy proteins: a review on composition, aggregation and emulsification. *Food Hydrocolloid* **39**:301–318 (2014).
- Wang Y, Gan J, Zhou Y, Cheng Y and Nirasawa S, Improving solubility and emulsifying property of wheat gluten by deamidation with four different acids: effect of replacement of folded conformation by extended structure. *Food Hydrocolloid* **72**:105–114 (2017).
- Hirano K, Hino S, Oshima K, Nadano D, Urisu A, Takaiwa F *et al.*, Evaluation of allergenic potential for rice seed protein components utilizing a rice proteome database and an allergen database in combination with IgE-binding of recombinant proteins. *J Agric Chem Soc of Jpn* **80**:564–573 (2016).
- Fabian C and Ju YH, A review on rice bran protein: its properties and extraction methods. *Crit Rev Food Sci Nutr* **51**:816–827 (2011).
- Mun S, Shin M and Kim YR, Emulsifying properties of proteins isolated from various rice cultivars. *Food Bioprocess Technol* **9**:813–821 (2016).
- Wang T, Wang L, Wang R and Chen Z, Effects of freeze-milling on the physicochemical properties of rice protein isolates. *LWT - Food Sci Technol* **65**:832–839 (2015).
- Yue L, Fang L, Luo C, Chen Z, Jian M, Charles S *et al.*, Functional properties of the Maillard reaction products of rice protein with sugar. *Food Chem* **117**:69–74 (2009).
- Hamada JS, Characterization and functional properties of rice bran proteins modified by commercial exoproteases and endoproteases. *J Food Sci* **65**:305–310 (2010).
- Gheli S, Sørensen ADM, García-Moreno PJ, Hajfathalian M and Jacobsen C, Physical and oxidative stability of fish oil-in-water emulsions fortified with enzymatic hydrolysates from common carp (*Cyprinus carpio*) roe. *Food Chem* **237**:1048–1057 (2017).
- Liu H, Li Y, Diao X, Kong BH and Liu Q, Effect of porcine bone protein hydrolysates on the emulsifying and oxidative stability of oil-in-water emulsions. *Colloid Surface A* **538**:757–764 (2018).
- Cheetangdee N, Effects of rice bran protein hydrolysates on the physicochemical stability of oil-in-water emulsions. *J Oleo Sci* **63**:1231–1241 (2014).
- Cheetangdee N and Benjakul S, Antioxidant activities of rice bran protein hydrolysates in bulk oil and oil-in-water emulsion. *J Sci Food Agr* **95**:1461–1468 (2015).
- Xu X, Wei L, Liu C, Luo L, Chen J, Luo S *et al.*, Effect of limited enzymatic hydrolysis on structure and emulsifying properties of rice glutelin. *Food Hydrocolloid* **61**:251–260 (2016).
- Phoon PY and Narsimhan G, Effect of hydrolysis of soy β -conglycinin on the oxidative stability of O/W emulsions. *Food Hydrocolloid* **35**:429–443 (2014).
- Adler-Nissen J, In enzymatic hydrolysis of food proteins. *Can Med Assoc J* **172**:1783–1785 (1986).
- Kato A and Nakai S, Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochim Biophys Acta* **624**:13–20 (1980).
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275 (1951).
- Niu F, Zhou J, Niu D, Wang C, Liu Y, Su Y *et al.*, Synergistic effects of ovalbumin/gum arabic complexes on the stability of emulsions exposed to environmental stress. *Food Hydrocolloid* **47**:14–20 (2015).
- Pan X, Fang Y, Wang LL, Shi Y, Xie MH, Xia J *et al.*, Covalent interaction between rice protein hydrolysates and chlorogenic acid: improving the stability of oil-in-water emulsions. *J Agric Food Chem* **67**:4023–4030 (2019).
- Huang SW, Frankel EN, Aeschbach R and German JB, Partition of selected antioxidants in corn oil-water model systems. *J Agric Food Chem* **45**:1991–1994 (1997).
- Doucet D, Otter DE, Gauthier SF and Foegeding EA, Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. *J Agric Food Chem* **51**:6300–6308 (2003).
- Chalamaiah M, Jyothirmayi T, Diwan PV and Kumar BD, Antioxidant activity and functional properties of enzymatic protein hydrolysates from common carp (*Cyprinus carpio*) roe (egg). *J Food Sci Tech* **52**:5817–5825 (2015).
- Paraman I, Hettiarachchy NS and Schaefer C, Glycosylation and deamidation of rice endosperm protein for improved solubility and emulsifying properties. *Cereal Chem* **84**:593–599 (2007).
- Paraman I, Hettiarachchy NS, Schaefer C and Beck MI, Hydrophobicity, solubility, and emulsifying properties of enzyme-modified rice endosperm protein. *Cereal Chem* **84**:343–349 (2007).
- Ai M, Tang T, Zhou L, Ling Z, Guo S and Jiang A, Effects of different proteases on the emulsifying capacity, rheological and structure characteristics of preserved egg white hydrolysates. *Food Hydrocolloid* **87**:933–942 (2019).
- Sun C, Wu T, Liu R, Liang B, Tian Z, Zhang E *et al.*, Effects of superfine grinding and microparticulation on the surface hydrophobicity of whey protein concentrate and its relation to emulsions stability. *Food Hydrocolloid* **51**:512–518 (2015).
- Evangelho JAD, Vanier NL, Pinto VZ, Berrios JJD, Dias ARG and Zavareze EDR, Black bean (*Phaseolus vulgaris* L.) protein hydrolysates: physicochemical and functional properties. *Food Chem* **214**:460–467 (2017).
- Diftis N and Kiosseoglou V, Improvement of emulsifying properties of soybean protein isolate by conjugation with carboxymethyl cellulose. *Food Chem* **81**:1–6 (2003).
- Yong YH, Yamaguchi S and Matsumura Y, Effects of enzymatic deamidation by protein-glutaminase on structure and functional properties of wheat gluten. *J Agric Food Chem* **54**:6034–6040 (2006).
- Zang X, Yue C, Wang Y, Shao M and Yu G, Effect of limited enzymatic hydrolysis on the structure and emulsifying properties of rice bran protein. *J Cereal Sci* **85**:168–174 (2019).
- McClements DJ and Gumus CE, Natural emulsifiers-biosurfactants, phospholipids, biopolymers, and colloidal particles: molecular and physicochemical basis of functional performance. *Adv Colloid Interface Sci* **234**:3–26 (2016).
- Du Y, Shi S, Jiang Y, Xiong H, Meng WW, Zhao Q *et al.*, Physicochemical properties and emulsion stabilization of rice dreg glutelin conjugated with κ -carrageenan through Maillard reaction. *J Sci Food Agr* **93**:125–133 (2013).
- Xu X, Zhong J, Chen J, Liu C, Luo L, Luo S *et al.*, Effectiveness of partially hydrolyzed rice glutelin as a food emulsifier: comparison to whey protein. *Food Chem* **213**:700–707 (2016).
- Wu MH, Yan HH, Chen ZQ and He M, Effects of emulsifier type and environmental stress on the stability of curcumin emulsion. *J Disper Sci Technol* **38**:1375–1380 (2016).
- Ozturk B, Argin S, Ozilgen M and McClements DJ, Formation and stabilization of nanoemulsion-based vitamin E delivery systems using natural biopolymers: whey protein isolate and gum arabic. *Food Chem* **188**:256–263 (2015).
- Wang Y, Li D, Wang LJ and Adhikari B, The effect of addition of flaxseed gum on the emulsion properties of soybean protein isolate (SPI). *J Food Eng* **104**:56–62 (2011).
- Jamdar SN, Rajalakshmi V, Pednekar MD, Juan F, Yardi V and Sharma A, Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chem* **121**:178–184 (2010).
- Qian C, Decker EA, Xiao H and McClements DJ, Comparison of biopolymer emulsifier performance in formation and stabilization

- of orange oil-in-water emulsions. *J Am Oil Chem Soc* **88**:47–55 (2011).
- 42 Israelachvili JN, Intermolecular and surface forces-intermolecular and surface forces (third edition). *Q Rev Biol* **2**:59–65 (2011).
- 43 Schröder A, Berton-Carabin C, Venema P and Cornacchia L, Interfacial properties of whey protein and whey protein hydrolysates and their influence on O/W emulsion stability. *Food Hydrocolloid* **73**:129–140 (2017).
- 44 Zhao J and Xiong YL, Interfacial peptide partitioning and undiminished antioxidative and emulsifying activity of oxidatively stressed soy protein hydrolysate in an O/W emulsion. *LWT – Food Sci Technol* **61**:322–329 (2015).