Fabrication of emulsions prepared by rice bran protein hydrolysate and ferulic acid covalent conjugate: Focus on ultrasonic emulsification

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ABSTRACT

The aim of the paper was to investigate the effect of ultrasonic emulsification treatment on the fabrication mechanism and stability of the emulsion. The covalent conjugate made with rice bran protein hydrolysate (RBPH) and ferulic acid (FA) was used as the emulsifier. The effects of high intensity ultrasound (HIU) power with different level (0 W, 150 W, 300 W, 450 W and 600 W) on the stability of emulsion were evaluated. The results showed that ultrasonic emulsification can significantly improve the stability of the emulsions ($p < 0.05$). The emulsion gained better stability and emulsifying property at 300 W. It was able to fabricate emulsion with smaller particle size, more uniform distribution and higher interfacial protein content. It was confirmed by fluorescent microscopy and cryo-scanning electron microscopy (cryo-SEM) furtherly. And it was also proved that the emulsion treated by proper HIU treatment at 300 W had better storage stability. Excessive HIU treatment (450 W, 600 W) had negative effects on the stability of emulsion. The stability of emulsion (300 W) against different environmental stresses was further explored, which established a theoretical basis for the industrial application of emulsion in food industry.

1. Introduction

Emulsions are dispersion systems consisting of two or more immiscible liquids. They are applied in the food industry widely, such as, the processing of milk, butter, milk drinks and ice cream are used the emulsion systems [1]. However, the physical instability phenomena, such as coalescence, austenitic ripening and flocculation often occur during the processing, storage or consumption of emulsions [2–4]. The chemical instability phenomena, such as decomposition of emulsifiers and oxidation of oils also appear at the same time [5]. These changes of emulsification and thermal stability limit the application of emulsion in the food industry. Hence, it is necessary to find a suitable emulsifier and an emulsification method to prepare a uniform and stable emulsion.

There is an increasing worldwide demand for the health-care functional emulsions in recent years. Emulsions made with a single emulsifier can not encapsulate functional substances well, they also have poor storage stability. Researchers have already paid attention to compound emulsifiers. Pan et al. [6] studied the covalent interaction between rice protein hydrolysate and chlorogenic acid, which improved the physical stability and oxidative stability of emulsion. Liu et al. [7] carried out the study on the stability of porcine bone protein hydrolysate and tannin complex emulsion. Rice bran protein (RBP) is recognized as a high-quality vegetable protein, the composition ratio of RBP meets human requirements [8]. Due to its hypoallergenic nature, it can be added as an ingredient in infant and elderly formula products [9]. However, as there are disulfide bonds in RBP, the solubility of RBP is poor, which greatly limits the application of RBP [10,11]. In our previous study [12], the rice bran protein hydrolysate (RBPH) pretreated by high hydrostatic pressure (HHP) could solve this issue, and the solubility and emulsifying properties were improved.

Ferulic acid (FA) is a major phenolic acid in paddy, which has high antioxidant, antibacterial, anti-inflammatory and hypocholesterolemic activities [13]. Our previous studies showed that the covalent conjugate of RBPH-FA had a good modification effect on RBPH and could improve its emulsifying and antioxidant properties [14]. Some studies have shown that the addition of polyphenols could change the relevant

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properties of the emulsion, and the polyphenols were taken to the oil droplet surface through the adsorption of protein, which has a synergistic effect with the antioxidant properties of protein, thus improving the stability of the whole system [7].

The appropriate emulsification method has an important influence on the properties and stability of the emulsions. As a novel technology for food processing, ultrasonic emulsification has attracted the interest of researchers in recent years because of its green, safe and convenient characteristics [15]. Ultrasonic emulsification is based on cavitation. Ultrasound can generate high temperature, high pressure, strong shear force and mechanical force, which can gain a more stable emulsion with fewer emulsifier [16]. Zhang et al. [17] prepared soy protein isolate and pectin emulsion gel with ultrasound treatment, the result showed that ultrasound treatment could enhance the emulsion gel stability well. Kalsa et al. [18] found that in the emulsion system of whey protein isolate and xanthan gum, the particle size of the emulsion decreased and the stability was improved significantly after different intensity ultrasound treatment. Thomas et al. [19] made double emulsions with lecithin and soy protein isolate in skim milk, and compared the emulsification ability of ultrasonication and high pressure homogenization. It was found that the particle size of ultrasonic treated double emulsion was slightly lower than that of high pressure homogenization, and the stability was significantly improved [19].

It is useful to enhance the physicochemical stability of emulsions with the molecular modified protein as emulsifier. Previous studies in our laboratory have shown that RBPH-FA conjugate has better antioxidant and emulsifying properties, and it can be used as a new type emulsifier in emulsions [12,14]. Although there are many studies on the functional properties of protein–polyphenol complexes, there are few reports on the application of RBPH-FA conjugate in emulsions, and the study of ultrasonic emulsification on this conjugate emulsion has not been reported.

In this study, the RBPH-FA covalent conjugate was used as emulsifier, the conjugate emulsion was fabricated by high intensity ultrasound (HU). The effects of ultrasound power on the emulsifying properties, interfacial adsorption capacity, physical and environmental stabilities of the emulsion were investigated. Chasing for a better way, that is good to the ultrasound utilization in food processing.

2. Materials and methods

2.1. Materials

Fresh rice bran was purchased from Heilongjiang Great Northern Wilderness Agribusiness Group Co., Ltd. (Harbin, Heilongjiang, China). RBP was self-extracted in laboratory. The protein content of this RBP was 91.67 %, which was determined by the Kjeldahl method (N% × 5.95). Trypsin (3 × 10^4 U/g) and FA were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Soybean oil was provided by Harbin Huikang Food Co., Ltd. (Harbin, Heilongjiang, China). Chemicals and reagents were of analytical grade. The sample solutions were prepared with deionized water.

2.2. Preparation of conjugate

2.2.1. HHP pretreatment

RBPH solutions (10 mg/mL) were pretreated under 200 MPa in an HHP instrument (Ren-He Electromechanical Engineering Co., Shenyang, China) at 25 °C according to our previous experiments [12].

2.2.2. Preparation of RBPH

HHP-pretreated RBPH solutions (10 mg/mL) were hydrolyzed by trypsin (pH 8.0, 37 °C) at normal pressure for 60 min. The mass ratio of enzyme to protein substrate was 1:20. The enzyme was inactivated for 10 min at 90 °C after hydrolysis. Hydrolysates were obtained from the separated supernate, lyophilized and stored at −20 °C until use [12].

2.2.3. Preparation of RBPH – FA conjugates

RBPH – FA conjugates were prepared by alkali-induced covalent conjugation according to our previous research [14]. The pH of RBPH solution was adjusted to 9.0. RBPH – FA conjugates were prepared by mixing RBPH solution with FA (1.5 mg/mL) for 24 h at room temperature under the condition of avoiding light. The mixed solutions were dialyzed for 48 h in a dialysis bag (3 kDa) and then freeze-dried until analysis.

2.3. Preparation of conjugate emulsions

The emulsion was prepared on the basis of Zhang et al. [17]. RBPH-FA conjugate was dispersed in phosphate buffer solution (PBS) (10 mmol/L, pH 7.0) with sustained stirring at room temperature for 3 h. 10 % (v/v) soybean oil was added to the RBPH-FA conjugate solution. The coarse emulsion was prepared by homogenizing 3 min at 20000 rpm using homogenizer (Ultra-Turrax T18, Angni Co., Shanghai, China). The coarse emulsion was emulsified by ultrasonic generator (Scientz-II D, Scientz Biotechnology Co., ltd., Ningbo, China) with different ultrasound powers (0–600 W) for 3 min. The 6 mm ultrasonic probe was placed in the coarse emulsion, the position was 1 cm away from the emulsion bottom. The ultrasonic parameters were set as follows: frequency 20 kHz, pulse period 6 s (4 s on, 2 s off). The final samples were stored in refrigerator with 4 °C till analysis.

2.4. Contact angle and interfacial tension

The contact angle and interfacial tension were determined by sitting drop method. The thin film (diameter 10 mm) was made by smearing conjugate emulsion on the glass slides. One drop of water was placed on the surface of the film by a high-precision syringe, and the image of the droplet was recorded after falling from the syringe through the camera. The profile of the droplet was solved numerically and was suitable for the Laplace-Young equation, from which the interfacial tension can be determined.

2.5. Emulsifying properties

The EAI and ESI of emulsions were measured by Liu et al. [7]. Fetched 20 μL of the emulsion sample, added it into sodium dodecyl sulfate (SDS) solution (4 mL), mixed them together thoroughly. The absorbance of the mixture was measured by ultraviolet spectrophotometer at 0 and 180 min.

The EAI and ESI were obtained as follows:

\[
EAI(m^2/g) = \frac{2 \times 2.303 \times A_0 \times 100}{10000 \times 0.1 \times 1 \times 0.005}
\]

\[
ESI(\%) = \frac{A_{180}}{A_0} \times %
\]

In this formula, A₀ and A₁₈₀ represent the absorbances at 0 and 180 min.

2.6. Turbidity

The determination of emulsion turbidity refers to Wang et al. [20] with a little modification. All the samples of emulsion were diluted 100 times with pH 7.0 PBS. PBS (pH 7.0) was the blank control, the absorbance at 600 nm wavelength was measured by UV spectrophotometer, and the turbidity was calculated as follows:

\[
T = \frac{2.302 \times A \times V}{I}
\]

where A is the absorbance of the diluted emulsion at 600 nm, V is the dilution factor, I is the optical path (0.01 m).
2.7. Particle size distribution (PSD)

The PSD analysis was performed by a Particle Size Analyzer (Microtrac S3500, Microtrac Inc., Krefeld, Germany).

2.8. ζ-Potential measurements

The ζ-potential of emulsion was measured by Zetasizer Nano ZS (Malvern Instrument Ltd., Malvern, Worcestershire, UK).

2.9. Distribution of interfacial protein

The distribution of interfacial protein was measured according to Huang et al. [21]. The emulsions were separated by centrifugation (15000g, 45 min,25°C) to obtain cream phase and serum phase. Then, the serum phase was separated again with the same parameters as the first time. The serum phase got from the second separation was filtered by 0.22 μm filter. After filtration, the protein in collected serum was non-adsorbed protein. The quantity of non-adsorbed protein was measured by Lowry’s method. The quantity of adsorbed protein was calculated by the value of total protein in the emulsion minus that of the non-adsorbed protein.

2.10. Microstructure of emulsions

2.10.1. Inverted fluorescent microscopy

Microstructure of HIU treated RBPH-FA conjugate emulsion was observed by an inverted fluorescent microscope (LEICA DMi8, Leica Microsystems, Germany). Under the condition of avoiding light, the emulsions were stained with Nile red for 30 min to observed the microstructure.

2.10.2. Cryo-scanning electron microscopy (Cryo-SEM)

The microstructure of HIU treated RBPH-FA conjugate emulsion was scanned by Cryo-scanning electron microscope (S-3400 N, Hitachi, Japan). Before scanning, the sample was needed to be loaded on double-sided adhesive tape and a thin coating of splattered gold was applied to the ruptured emulsion area. The acceleration voltage was 5 kV and the magnification was 6000 × and 10000 × respectively.

2.11. Emulsion storage stability

Emulsions fabricated with RBPH-FA conjugate at different HIU power treatment were stored at room temperature for 14 days. Then, particle size and ζ-potential were detected.

2.12. Emulsion stability against environmental stresses

2.12.1. Temperature stability

The conjugate emulsion (300 W) was subjected to different temperature condition from 30 to 90 °C for 30 min and stored for 24 h. And then, particle size and ζ-potential were measured to examine the temperature stability.

2.12.2. Ionic strength stability

5 M of NaCl solution was added to the conjugate emulsion (300 W), adjusting NaCl concentration in emulsion to 0, 50, 100, 200 and 300 mM. Then, emulsions at different salt ionic strength were placed at 25 °C for 24 h. The ionic strength stability was assessed by the particle size and ζ-potential.

2.12.3. pH stability

In this section, 1 M HCl and 1 M NaOH was used. The pH of the conjugate emulsion (300 W) was adjusted to 3–9, and the samples were stored for 24 h at 25 °C. Then, the measurements of particle size and ζ-potential were conducted.

2.13. Statistical analysis

Three replicate trials were conducted for each experiment, and the experimental results were expressed as mean value ± standard deviation by SPSS 22.0 software. Significant differences (p < 0.05) were taken by Duncan’s test.

3. Results and discussion

3.1. Effect of HIU treatment on emulsifying properties of RBPH-FA conjugate emulsion

Emulsifying activity and emulsifying stability are important indicators to characterize the emulsion emulsification properties and steady state of emulsion. Therefore, the effects of HIU treatment on the emulsifying activity index (EAI) and emulsifying stability index (ESI) of RBPH-FA covalent conjugate emulsion were evaluated. As shown in Fig. 1, the EAI and ESI of HIU treated emulsions were significantly (p < 0.05) higher compared with sample without HIU treatment (0 W). As the ultrasound power increased, the EAI and ESI of the emulsions firstly increase and then decrease. The emulsion presented better emulsifying property when treated at 300 W with the EAI and ESI of 86.64 m²/g and 94.23 %, respectively. This was owing to the cavitation effect and physical shear force generated by ultrasonic treatment that broke the oil droplets of emulsion into small particles, and then the particle size of emulsion turned to be smaller, this could improve the emulsification of emulsion [22]. Data in the research showed the emulsifying properties of emulsions began to decrease slightly at 450 W and 600 W. This was because excessive ultrasound power can affect the interfacial protein structure, leading to flocculation of interfacial proteins and aggregation of emulsion droplets, thus reducing the emulsifying properties of the emulsion [23]. Overall, HIU treatment improved the emulsifying properties of emulsion. Qayum et al. [24] demonstrated the ESI and EAI of α-lactalbumin emulsions were significantly increased by ultrasonic emulsification.

3.2. Effect of HIU treatment on PSD of RBPH-FA conjugate emulsion

Food emulsions are one kind of polydisperse emulsions comprised many droplets of different sizes [25]. The PSD is an effectual index to assess the stability of emulsions, it can also affect the physicochemical properties of emulsions [26]. The larger the emulsion droplet size is, the more unstable the system shows. The PSD is shown in Fig. 2. The fresh
3.3. Effect of HIU treatment on ζ-potential of RBPH-FA conjugate emulsion

The charge of food emulsion droplets is due to the adsorption of some biopolymer or mineral ions on the interface of oil droplet [31]. ζ-potential is a key indicator of the emulsion stability. Previous studies had shown that RBPH-FA conjugates can provide higher absolute ζ-potential values versus conventional proteins [14]. McClements et al. [32] noted that the distribution of some specific bioactive substances on the emulsion interface increased the charge density of droplet surface. Emulsions with low absolute ζ-potential values had low intermolecular repulsion and tended to aggregate or flocculate, while emulsions with the high absolute ζ-potential values had high intermolecular repulsion and better emulsion stability. In Fig. 3, the ζ-potential values of all emulsion samples were negative. The lowest absolute value of ζ-potential was 13.37 mV (0 W), and the absolute value of ζ-potential increased significantly (p < 0.05) of all the HIU treated samples. The absolute value of ζ-potential of the emulsions tended to increase and then decrease with the increase of ultrasound power. When the ultrasound power was 300 W, the absolute ζ-potential of the sample reached the maximum of 29.2 mV. However, with the increase of ultrasound power, the absolute ζ-potential of the sample began to decrease gradually. This was similar to the finding of Wang et al. [23]. The particle size of the emulsion decrease as the cavitation effect and high shear force provided by HIU treatment. Thus, the emulsifiers distributed on the interface increased, which led to absolute value of the ζ-potential increase. While too high ultrasound power led to flocculation and aggregation of emulsifiers taking place between the emulsion droplets and at the interface [33], thus made ζ-potential of the emulsion decreased. This was also consistent with the trend of emulsion interfacial proteins in this study. The RBPH-FA conjugate emulsion had the highest absolute value of ζ-potential at 300 W. This indicated that the emulsion surface had a high electrostatic repulsion, which can prevent the droplets from aggregating, and the emulsion was stable [34].

3.4. Effect of HIU treatment on interfacial protein distribution

The interfacial protein concentration is an important parameter affecting the stability of the emulsion, it can efficiently influence the anti-agglutination ability of the emulsion [35]. The adsorption of protein particles can form a strong film by adsorbing on interface between water and oil phase, which can reduce the interfacial tension and improve the stability of the emulsion [36]. The effects of HIU treatment on the distributions of RBPH-FA conjugate (molecular modified protein) in the emulsions are shown in Fig. 4. Compared with sample of 0 W (8.03 ± 0.32 %), the adsorbed protein distribution on the interface increased significantly after HIU treatment. It reached to the maximum at 300 W, with the value of 28.82 ± 1.23 %. Whereas, the distribution of non-adsorbed protein in aqueous phase decreased from 91.97 ± 3.54 % to 71.78 ± 2.38 %. This was attributed to the smaller particle size of droplets and the increased specific surface area of them after HIU treatment, which increased the protein adsorbed on the surface of the oil droplets, thus reduced the surface tension on the oil-water interface [37]. While the interfacial protein content decreased and the non-
3.5. Effect of HIU treatment on turbidity of RBPH-FA conjugate emulsion

The turbidity is used to evaluate the changes on the stability of the conjugate emulsion after ultrasound treatment. As shown in Fig. 5, with the increase of ultrasound power, the turbidity decreased significantly \( (p < 0.05) \) compared to the sample at 0 W. It reached the minimum at 300 W. This was because the droplets of the emulsion were broken into smaller ones under the strong effect of ultrasound, the surface area was turned to be larger, which increased the light scattering. The flocculation and aggregation of emulsion led to the increase in system turbidity at 450 W and 600 W. It was possible that excessive HIU treatment caused the aggregation of interfacial protein, which affected the turbidity. Thorarinsdottir et al. [39] showed that turbidity could be affected by the aggregation of protein. The particle size of emulsion could affect the turbidity, and there was a positive correlation between them. The larger the average particle size was, the higher the turbidity showed [40]. This was coherent with the results of our particle size study.

3.6. Effect of HIU treatment on contact angle and interfacial tension of RBPH-FA conjugate emulsion

The contact angle (\( \theta \)) can reflect the wetting degree of the protein at the interface of the emulsion, which is an indicator of emulsion stability [41]. When \( \theta \) is acute, the protein particles are hydrophilic, which is more conducive to the formation of oil-in-water emulsion; conversely, they tend to form water-in-oil emulsion. As shown in Fig. 6 (A), the contact angles of all emulsion samples were acute, that indicated the samples were hydrophilic. Compared to sample of 0 W \( (66.08 \pm 0.32^\circ) \), \( \theta \) of all the HIU treated samples decreased obviously, with the smallest \( \theta \) \( (24.34 \pm 0.16^\circ) \) at 300 W. However, the contact angle increased with the increasing power. Wang et al. [23] demonstrated that ultrasound treatment can provide better wettability for soy protein-pectin composite emulsions and stabilize oil-in-water emulsions.

Interfacial tension can characterize the emulsion stability. The lower the interfacial tension is, the more stable the emulsion is [30]. As Fig. 6 (B) shown, the interfacial tension decreased after HIU treatment. At 300 W, the interfacial tension value was the smallest, and the contact angle was also the smallest in comparison to other samples, indicating that the emulsion was more stable at 300 W. After ultrasonic treatment, the particle size decreased, the interfacial protein content and the wettability of the emulsifier increased, and the interfacial tension decreased, it could enhance the anti-flocculation and anti-aggregation ability of the emulsion. All these indicators proved that the proper HIU treatment had positive effects on the stabilization of the RBPH-FA conjugates emulsion.

3.7. Effect of HIU treatment on microscopic morphology of RBPH-FA conjugate emulsion

3.7.1. Observation under inverted fluorescent microscope

The microstructure of the RBPH-FA conjugate emulsion stabilized by HIU treatment is shown in Fig. 7 (A). The emulsions were stained with Nile red, which was represented as a red region in Fig. 7 (A). The particle size of HIU treated sample was smaller and more uniform than that of the untreated sample (0 W). Ultrasonic emulsification was able to fabricate stable RBPH-FA conjugate emulsion, and the particle size distribution could be affected by ultrasound power. The emulsion treated with 300 W had the smallest particle size, and the distribution was more uniform. Taha et al. [15] demonstrated the significant reduction in particle size of soybean isolate protein emulsions after ultrasound treatment. Due to the physical effects of ultrasonic treatment, such as acoustic cavitation, microfluidization and capillary surface waves, emulsion droplets can be destroyed efficiently, the size of droplets can be reduced, and made an appropriate steric hindrance [42]. When the ultrasound power was excessive (450 W, 600 W), the particle size of the emulsion droplets increased. This observation could be attributed to the disruption of the interfacial layer by excessive HIU.
treatment, resulted in flocculation and aggregation of the emulsion droplet; on the other hand, the thermal effect caused by excessive HIU treatment was also one of the reasons for emulsion instability.

3.7.2. Cryo-SEM analysis

The Cryo-SEM images of the RBPH-FA conjugate emulsion stabilized by HIU treatment are shown in Fig. 7 (B). The droplets of all the samples were spherical, and the diameters were between 2 and 10 μm. Compared with the untreated sample (0 W), the droplet size decreased significantly after HIU treatment. The size of droplet became significantly smaller after ultrasonication compared to the untreated sample (0 W). This was owing to HIU treatment could destroy the oil droplets and form stable emulsions [43]. The particle size was the minimum at 300 W, which indicated that the most suitable ultrasonic treatment power was 300 W. Ashokkumar et al. [44] pointed out that appropriate ultrasonic power treatment could generate stronger nonlinear acoustic pressure pulsations, this made the bubbles crush effectively and reduce the size of droplet. It can be seen the particle size at 450 W and 600 W became larger. Excessive treatment by ultrasound could decrease the protein distribution density on the oil–water interface, alter the intermolecular force [33]. This would cause the aggregation and the uneven distribution of droplets, and made the emulsion unstable.

3.8. Effect of HIU treatment on storage stability of RBPH-FA conjugate emulsion

All the emulsion samples were stored at 25 °C for 14 days, and the particle size was an indicator to reflect the stability change of the emulsions. From Fig. 8, it can be observed that the fresh emulsions were in a uniform state with no serum separation. After 14 days of storage, the emulsions at 0 W and 150 W exhibited obvious phase separation, while the emulsion at 300 W was more uniform than others, the emulsions at 450 W and 600 W had slight phase separation. The particle size of the stored emulsions was significantly larger compared to the fresh emulsions, and the largest particle size (27.07 ± 0.67 μm) was observed in the untreated emulsion (0 W). Compared with the untreated sample, the particle size of the stored emulsions decreased significantly with the increase of ultrasonic treatment power (p < 0.05). The particle size was the smallest (8.19 ± 0.29 μm) at 300 W. This indicated that ultrasonic emulsification treatment was able to impact the storage stability of the emulsions.

HIU treatment can cause the conjugate to be rapidly distributed on the oil–water interface, providing a certain space hindrance and preventing emulsion aggregation. On the other hand, ultrasonic emulsification made the oil droplets smaller, which contributed greatly to the stability of the overall emulsion system. Ozturk et al. [45] revealed that the smaller the emulsion droplet was, the better the gravity separation stability turned. This was consistent with the results of this study. After storage, the particle size of the samples treated at 450 W and 600 W were larger than sample treated at 300 W, which may be attributed to hydrophobic association [46].
3.9. Effect of HIU treatment on stability of RBPH-FA conjugate emulsions against environmental stresses

3.9.1. Temperature stability

Thermal treatment is a common processing method in food industry, it can inhibit bacteria, sterilize and prolong the shelf life of food effectively. Therefore, it is necessary to study the stability of emulsions after thermal processing. In Fig. 9, the particle size of the conjugate emulsion (300 W) increased with the temperature increasing. The particle size increased at 30 °C (3.14 ± 0.14 μm) and 50 °C (3.31 ± 0.24 μm), but the difference was not significant. This indicated that the emulsion remained stable between 30 and 50 °C relatively. This may be caused by the fact that RBPH-FA conjugates dispersed on the oil–water interface well during ultrasonic emulsification, forming a thicker interface layer. Ultrasonic treatment made the conjugates arrange orderly on the oil–water interface, reducing the exposure of hydrophobic end of proteins [47]. The particle size of the emulsion increased significantly at 70 °C and 90 °C, the values were 5.02 ± 0.21 μm and 5.41 ± 0.27 μm, respectively. This was due to the aggregation of the conjugates caused by the high temperature, which destroyed the interface layer, thus making the emulsion oil droplets to aggregate together. Thermal treatment can expose the hydrophobic groups of the conjugate or form disulfide bonds, leading to emulsion aggregation [48]. It was evident from the present experimental data that thermal treatment can affect ζ-potential of emulsions. With the increase of temperature, the absolute value of the emulsion ζ-potential decreased from 28.73 ± 1.2 to 22.76 ± 1.86 mV. Thermal treatment broke the interfacial layer of the emulsion and caused the conjugate to aggregate. Thus, the electrostatic repulsion between molecules was reduced, making the emulsion unstable.

3.9.2. Ionic strength stability

The stability of RBPH-FA conjugate emulsion after ultrasonic emulsification (300 W) under different salt ionic strength is shown in Fig. 10. The emulsion droplet size was the smallest (2.72 ± 0.07 μm) and the absolute value of ζ-potential was the largest (28.03 ± 1.95 mV) without the addition of NaCl, indicating that this emulsion was the most stable among all the samples. Neutral emulsions without salt addition were more stable as there was a strong electrostatic repulsion between droplets [49]. With the increase of ionic strength, the droplet size
increased gradually. It reached to $6.13 \pm 0.22 \, \mu m$ at 300 mM of NaCl.
And the emulsion showed obvious phase separation when NaCl concentration was over 100 mM. Ionic strength can change the spatial structure of protein and lead to the denaturation and aggregation of protein molecules, thus affected the processing stability of the emulsion [50]. Fig. 10 also shows the absolute value of the conjugate emulsion $\zeta$-potential decreases with the increase of NaCl concentration, from $28.03 \pm 1.95$ to $22.73 \pm 1.50 \, mV$. This was attributed to the electrostatic shielding effect of salt ions [51]. Additionally, the aggregation of proteins caused by the addition of salt ions can also lead to this phenomenon [33].

3.9.3. pH stability
The particle size and $\zeta$-potential of RBPH-FA conjugate emulsion (300 W) at different pH values is shown in Fig. 11. At pH 3, the size of the conjugate emulsion was the largest ($12.31 \pm 1.15 \, \mu m$). The particle size decreased gradually with pH increasing, and the size was $2.81 \pm 0.27 \, \mu m$ at pH 9. But there was no significant difference at pH 7 and pH 8. $\zeta$-Potential was $7.93 \pm 2.37 \, mV$ at pH 3 and $-12.43 \pm 0.7 \, mV$ at pH 4. According to our research (unpublished), the isoelectric point of the conjugate was between pH 3 and 4. The net charge of the particles decreased near the isoelectric point. This could lead to the electrostatic repulsion less than the hydrophobic interaction and van der Waals force, therefore made the emulsions unstable [52]. The absolute value of the conjugate emulsion $\zeta$-potential increased with the pH increasing. Our findings were consistent with Wang et al. [53]. Hence, the electrostatic repulsion between molecules may be the reason for the stability of the emulsion [47].

4. Conclusions
HIU treatment was used to fabricate the emulsion with RBPH-FA conjugate as an emulsifier. The results showed that HIU treatment can significantly improve the stability of the emulsion. Appropriate HIU treatment (300 W) can reduce the emulsion droplet size, increase the interfacial protein distribution, reduce the interfacial tension and
improve the emulsification, thus enhancing the stability of the emulsion. Excessive HIU treatment (450 W, 600 W) would induce flocculation and aggregation of emulsion droplets. Overall, ultrasonic emulsification is an effective way to form emulsions. The experimental results of this study can expand the application of plant-based emulsifiers, promote ultrasonic treatment as a green processing method and make the emulsion better applied in the food processing industry.

**CRediT authorship contribution statement**

Shirang Wang: Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. Tengyu Wang: Investigation, Software, Formal analysis, Writing – review & editing. Xiaoyi Li: Software. Yingju Cui: Validation. Yue Sun: Investigation. Guoping Yu: Conceptualization, Supervision, Project administration, Writing – review & editing, Funding acquisition. Jianjun Cheng: Resources, Methodology, Project administration.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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