Water-in-oil microemulsion: effect of *Desmodium intortum* protein isolate–emulsifier interaction, and its stability

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**ABSTRACT**

Water-in-oil (W/O) microemulsions exhibit delivery matrix and exhibit poor lipid solubility. Therefore, increasing the stability of W/O microemulsions is necessary to broaden their applications. In our experiment, a protein microemulsion system was constructed and its stability was investigated. The effects of surfactants, co-surfactants, quality ratio of them (Km) and hydrophilic-lipophilic balance (HLB) values were determined by using the pseudo-ternary phase diagrams and the area of microemulsions (MEs) as the indexes. The results showed that MEs’ stability was the best when the limonene was used as oil phase, Tween 20 + span 80 as the surfactant, and anhydrous alcohol as co-surfactant, if and only if HLB = 6.0 and Km = 3:1. Under this condition, the area of micro-emulsion was the largest, and the quality of microemulsion was better when the mass ratio of oil phase and emulsifier was 7:5. The protein microemulsion was a water-in-oil type, with a particle size of 1.41 ± 0.03 μm, containing 1127.37 ± 30.87 μg/g of proteins. Moreover, the temperature, light, and sample observation tests showed that the solution’s protein retention rate was lower than those in the microemulsion. Low-concentration NaCl, less than 9% of glucose and maltose exerted no significant influence on the protein microemulsion stability.

**Introduction**

Water-in-oil (W/O) microemulsions can be used as a packaging and delivery matrix for water-soluble nutrients or drugs (such as peptides, proteins, vitamins, and flavors). However, destabilizing effects often occur as the stability of W/O microemulsions is affected by many factors, thereby limiting the application of microemulsions. Therefore, the development of relatively stable W/O microemulsions and W/O microemulsions products with more stable quality during transportation or storage is important.

In recent years, food-grade microemulsions have overcome low solubility and biological utilization rate, which has become the carrier of bioactive substances. Proteins and polysaccharides can be used as emulsifiers and stabilizers in food processing. Many studies have reported that adding protein to the internal water phase helps improve the emulsion’s long-term stability, because the protein can form an elastic interface film at the surface. By studying the rheological properties of the
emulsifier and protein mixture on the interface, the emulsion can be clarified by the stability mechanism of the system.\textsuperscript{[9,10]} The emulsion stability can be improved by adding proteins to increase the dispersed phase’s viscosity or to gel the water phase.

A ternary phase diagram is used to determine the composition of the microemulsion and the proportion of the oil phase, surfactant, and cosurfactant. Once the proportion is determined, a similar microemulsion can be produced.\textsuperscript{[11]} The commonly used food-grade surfactants are soybean lecithin, Tween, span, and sucrose fatty acid ester.\textsuperscript{[12–14]} Some studies have shown that the emulsifying effect on the stability of microemulsion of combined surfactant is better than that of a single surfactant, the stability of microemulsion is better. Short- and middle-chain alcohols, such as ethanol, glycerol, and n-butyl alcohol, are multi-selected as cosurfactants in microemulsions.\textsuperscript{[3,6,11,14]}

This study aimed to determine whether the stability of W/O blank microemulsions can be improved by incorporating the protein isolate of Desmodium intortum into the aqueous phase based on the protein–emulsifier interface interaction. The W/O microemulsion was introduced into the complete W/O blank microemulsion to prepare W/O microemulsions with different protein concentrations for proteins and emulsifiers to compete for adsorption. We analyzed the addition of isolated proteins to W/O blank microemulsions and their impact on stability from the aspects of interfacial protein content, interfacial tension, micromorphology, particle size, ζ-potential value, rheological properties of the emulsion and oil-water interface film, and overall emulsion stability.

Materials and methods

Materials and reagents

Tween-20 and 80, span 20 and 80, Isopropyl palmitate (IPP), limonene (LIM), Triglyceride octyl anemate (TOA), glycerol, polyethylene glycol 400, butanol, methylene blue dye, Sudan red dye, methyl alcohol, copper-covered network, alcohol, NaOH, HCl, NaCl, glucose, maltose and 2% phosphotungstic acid, were purchased from Qingdao Youuso chemical technology Co. LTD. Protein standard monomers, namely, methionine (Chromadex 00017345, 99%), were obtained from Nanjing Senbaiga Biotechnology Co. LTD. Chromatographic-grade formic acid and n-butanol (purity of 99%) were purchased from Sigma-Aldrich Co. (Billerica, MA, USA). Deionized water was used throughout the experiment.

Basic components in desmodium intortum

Calcium measurements were based on GBT6436-2018, and total phosphorus was measured according to GBT6437-2018. Crude fat was determined by the Soxhlet extraction method and automatic fat analyzer. Crude protein content, total flavonoids, polysaccharides, and water-soluble dietary fiber were determined by the Kjeldahl, aluminum nitrate coloration, phenol-sulfuric acid, and alcohol precipitation methods, respectively.\textsuperscript{[15,16]}

Preparation of protein isolate from desmodium intortum

The protein was isolated from Desmodium intortum leaves using the classic “alkali-soluble acid precipitation method.” The preparation process conditions were determined based on the results obtained from the prior process optimization experiments. The sample was dried, crushed, sieved (40 mesh), and degreased with petroleum ether. Degreased powder and deionized water were mixed at a liquid-liquid ratio of 40 mL/g, and the pH of the solution was adjusted with NaOH (1 mol/L) to 10. The solution was magnetically stirred for 2 h at room temperature and centrifuged (5600 g, 30 min). The protein supernatant was collected, and the pH was adjusted to 2.5 (isoelectric point) with HCl (1 mol/L). The solution was allowed to stand for 2 h and centrifuged (5000 g, 20 min) to obtain the protein precipitate. Finally, the protein precipitate was washed until HCl and NaOH are completely
removed. The obtained protein solution was subjected to dialysis through a 0.2 μm nylon membrane and desalting treatment at 4°C for 48 h.\cite{18-20}

**Preparation of W/O blank microemulsion**

The W/O blank microemulsion was prepared using the method of a previous laboratory research. The surfactant, cosurfactant and oil phase were mixed using a magnetic stirrer. Deionized water was added while stirring, and the mixture changed from clear to cloudy. A transparent microemulsion was obtained.\cite{17,18}

Microemulsion was prepared by using limonene as oil phase, Tween 80/Span 20 as compounded surfactant, and absolute butanol as co-surfactant. The mass ratio of Tween 80 and Span 80 was 3.2:7.5, \(K_m = 3:1\), and HLB = 7.5. The compounded surfactant was used as emulsifier.

**Preparation of microemulsion with different protein concentrations**

The isolated protein were weighed and dissolved with 10 mM phosphate buffer (pH 7.0) to prepare a protein solution with concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 g/100 mL. The solution was stirred at room temperature for 3 h; added with 0.01% stock sodium nitrate, which was used as a preservative; and then placed in the refrigerator at 4°C for 12 h to fully hydrate.\cite{19-21}

**Physical and chemical properties of protein microemulsion**

**Particle size and zeta-potential value**

The emulsion was diluted 100-fold with 5 mM phosphate buffer solution (pH 7.0), and the potential and particle sizes of the emulsion oil droplets were determined using a Malvern Mastersizer (Zetasizer Nano S90, Malvern instrument co., LTD, USA). The measurement was repeated more than three times for each sample and the average value was taken.\cite{22}

**Emulsion rheological properties**

During the ratio of Oil to water was 3:1, the apparent viscosity of the emulsion was measured using a TA rheometer (SNB-1A, Shanghai Fang Rui instrument co., LTD, China). The accessary was an aluminum plate with a diameter of 60 mm and the plate gap was 1 mm. The test conditions were as follows: static shear mode, sampling delay of 1 min, measurement temperature of 25°C, temperature during the measurement controlled by a constant temperature circulating water system, and shear rate range of 1–100 S.\cite{23}

**Physical properties of protein microemulsion**

Protein microemulsion was centrifuged at 2000 rpm for 30 min. This microemulsion was not stratified. Methylene blue dye and Sudan red dye were used in the same volume of microemulsion. The microemulsion configurations were determined according to the red and blue dye diffusion velocities. When the red dye spread rapidly, the microemulsion can be a W/O and vice versa. The microemulsion was diluted 10 times and oscillated for mixing. The particle size distribution of microemulsion was measured using a nanoparticle analyzer at 25°C; 0.5 mL deionized water was dripped into the microemulsion and mixed at 30°C to determine the electrical conductivity. A rheometer was used to determine the viscosity of the microemulsion at a shear rate of 60 s\(^{-1}\) 25°C. The pH of the microemulsion was measured using a pH meter at 25°C.\cite{24-27}
**Microscopic morphology observation of desmodium intortum protein microemulsion**

Freeze-dried Desmodium intortum protein microemulsion powder is evenly dispersed on the surface of the conductive adhesive. After gold spraying, protein microemulsion powder was observed by electron microscope S-4800 emission scanning electron microscope was used to observe it with 5000X amplification under the condition of 3.0kV voltage.

**Protein microemulsion demulsification method**

Anhydrous sodium sulfate (2.5 g) was added into 2 mL protein microemulsion and heated to 50°C. When the oil and water formed layers after 20 min, the microemulsion system was damaged. After demulsification, 3 mL of 0.1% trifluoroacetic acid–butanol solution was extracted thrice as the upper liquid. The extracted liquid was collected in a beaker. The trifluoroacetic acid–butanol solution was dried at 40°C to obtain the proteins. \(^{[30]}\)

**HPLC method for determining the content of desmodium intortum protein in microemulsion**

As established in our laboratory, HPLC was used to determine the protein content in the microemulsion. \(^{[22]}\) The specific conditions were as follows: mobile phase (acetonitrile: water, which contains 0.1% trifluoroacetic acid), 75:25; flow rate, 1 mL/min; and column temperature, 30°C. Proteins showed good linear relationship in the range of 0.089–9.37 mg/mL concentration. According to this method, the regression equation was \(Y(A) = 127349X + 4157\) (\(R^2 = 0.9994\)). A microemulsion of approximately 2 mL Desmodium intortum protein was demulsified, and 0.1% trifluoroacetic acid–acetonitrile solution was added to dissolve the proteins. Reconstituted extracts were filtered through a 0.2 μm nylon membrane. Filtering was conducted prior to determination. The peak area was generated into the standard curve equation to calculate the relative protein content. \(^{[28,29]}\)

**Temperature test to determine the protein retention rate**

Approximately 50 mL of protein solution and protein microemulsion with the same concentration were added into beakers, which were subsequently placed in 4°C, 25°C, and 60°C without light for 18 days. The protein retention rate was determined every 3 days. \(^{[22,30]}\)

**Light test to determine the protein retention rate**

Approximately 50 mL of protein solution and protein microemulsion with the same concentration were added into beakers, adjusted to obtain a pH of 3 by adding 1 mol/L HCl, and stored for 35 days under natural light, ultraviolet light, and dark conditions. Demulsification was conducted on the protein microemulsion and protein retention rate was determined every one week. \(^{[7,22]}\)

**Sample observation test to determine the protein retention rate**

Approximately 50 mL of protein solution and protein microemulsion with the same concentration were added into beakers and stored at 4°C and 25°C for three months. Microemulsion form was observed at 0, 30, 60, and 90 days. The protein retention rate was determined after demulsification. \(^{[22,31]}\)

**Influence of food additives on protein microemulsion stability**

Protein microemulsion was placed in nine test tubes. Each tube contained 10 mL microemulsion. Afterward, 0–0.234 g/g NaCl, glucose and maltose (0–0.09 g/g) were added and dissolved completely. The test tubes were placed under 25°C for 1 h to determine the microemulsion particle size. \(^{[14,22–24]}\)
Statistical analysis

Experiments were performed in triplicate, and the results were expressed as means standard deviation. The graphs were generated using OriginPro 9.5, Adobe Illustrator CS6, MetaboAnalyst 3.0, and PathVisio 3.3.0. Statistical analysis was performed using SPSS 21.0 software (IBM, Armonk, NY, USA), and the data were expressed as the mean values ± standard deviation (X ± s). ANOVA with a Tukey post hoc test was employed to assess the multiple comparisons.

Results and discussion

Composition analysis of Desmodium intortum

According to Table 1, the contents of crude fat, crude ash, total phosphorus, total calcium, water-soluble dietary fiber crude protein, flavones and polysaccharide in the leaves of Desmodium intortum were all higher than those in the stems, the highly difference in crude protein content was 1.74% (P< .01). The contents of polysaccharide and crude fiber, in the leaves of Desmodium intortum were lower than those in stems, among which the content highly difference of crude fiber was 14.04% (P< .01).

Effect of different factors on microemulsion formation zone

Butanol, methyl alcohol, and propylene glycol were used as cosurfactants to prepare the microemulsion, as shown in Figure 1A. An appropriate cosurfactant can be placed between the surfactants to benefit the formation of a mixed adsorption layer to promote the formation of microemulsion. These cosurfactants are all short-chain unitary alcohol. Therefore, short-chain unitary alcohol used as a cosurfactant, together with an increasing number of carbon atom, should result in an excellent performing emulsion. Nevertheless, with extensions in the carbon chain, the weak short-chain unitary alcohol polarity causes considerable toxicity to humans.[3,10,22] Therefore, considering safety and convenience, we used butanol as the surfactant Table 2.

When butanol was used as cosurfactant, it existed in the interface membrane under low concentration. With the increased butanol concentration, oil and water phases showed a remarkable

| Table 1. Component content comparison of Desmodium intortum. |
|---------------------------------|--------|--------|
| Component content (%)          | stems  | leaves |
| Crude fat                      | 0.68 ± 0.03 | 1.26 ± 0.02** |
| Crude ash                      | 5.87 ± 0.02 | 10.52 ± 0.04** |
| P                              | 0.14 ± 0.02 | 0.18 ± 0.02 |
| Ca                             | 0.19 ± 0.02 | 0.29 ± 0.03* |
| Water-soluble dietary fiber    | 9.23 ± 0.27 | 17.43 ± 0.35** |
| Crude protein                  | 2.18 ± 0.06 | 3.92 ± 0.12* |
| Polysaccharide                 | 4.19 ± 0.15 | 3.40 ± 0.13* |
| Flavones                       | 0.153 ± 0.004 | 0.225 ± 0.003* |
| Crude fiber                    | 22.80 ± 0.30 | 8.76 ± 0.20** |

Note: Compared to stems, * in the table represent a significant difference of leaves in the component content (p< 0.05), ** in the table represent a highly significant difference in the component content (p< 0.01).

| Table 2. Measurement the conductivity and viscosity of Desmodium intortum protein microemulsion. |
|---------------------------------|--------|--------|---------|---------|
| Sample                          | Particle size (μm) | Evident morphology (μs/cm) | Evident morphology (mpa s) | pH value |
| Desmodium intortum protein microemulsion | 0.014 | 127.62 | 29.94 | 5.7 |
Figure 1. Effect of factors on the formation of microemulsion. Note: A: cosurfactant; B: Km; C: surfactant; D: HLB; B: oil
Figure 1. Continued.
Figure 2. HPLC chromatogram of Methionine (protein standard) and nanoemulsion.

Figure 3. Droplet size distribution curves of protein microemulsion.
relationship, thereby increasing the microemulsion stability. However, butanol can reduce the surface tension, adjust the HLB value of surfactant, and increase the strength of the interface film. With the increase of butanol, the connection between aqueous phase and oil phase was closer, and the stability of microemulsion increased.\(^{[2,5,22]}\) The microemulsion region was optimal only at a Km value of 3:1. Moreover, the microemulsion system was considerably stable (Figure 1B).

According to Figure 1C, when tween 20/span 80 was used as surfactant, microemulsion area was the largest. Stability was lost when a component changed in the microemulsion system. Tween 20/span 80 was utilized as surfactant to adjust the microemulsion system well.

Effects of tween 20 and span 80 composite emulsifiers with different HLB values on the microemulsion stability and region were investigated, as shown in Figure 1D. When the HLB was 7.5, the microemulsion area was optimal. The low HLB value of the surfactant showed weak hydrophilicity, which was unfavorable to the formation of hydrogen bonds between the water phase and surfactant. Such weak hydrophilicity reduced the water load in the microemulsion and subsequently influenced the microemulsion phase areas. When the surfactant content was high, it was easy to form microemulsions, and Km value had little influence on the formation of microemulsions. It was speculated that when the surfactant content was very high, the water is infinitely solubilized.\(^{[1,15,22]}\)

The microemulsion area was optimal when HLB was 7.5 and with isopropyl palmitate as the oil phase (Figure 1E). Oil phase size is an important factor in microemulsion formation because oil and surfactant both penetrate to form an oil film, which affects the microemulsion formation. An oil phase with an appropriately long chain easily embeds into the interface between the oil and water layers to form a stable micellar microemulsion. Thus, this phase is conducive to the formation of microemulsion.\(^{[20,22]}\) When the surfactant content was high, it was easy to form microemulsions, and Km value has little influence on the formation of microemulsions. It was speculated that when the surfactant content was very high, the water is infinitely solubilized.\(^{[3,16,25]}\) The nonionic surfactants such as Span and Tween are widely used in the preparation of solution- or emulsion-based films. Rodriguez analysis the combined effect of plasticizers and span-tween on the physical properties of starch based edible films. incorporated with different amounts of span-tween, shellac and emulsifier to develop biocomposite films. The above results

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**Figure 4.** Transmission electron microscopy images of protein microemulsion.
found that span and tween with high HLB values have slightly shorter alkyl chain-length than low HLB values.\textsuperscript{[23,24]}
Evident morphology of *Desmodium intortum* protein microemulsion

Sudan red dye showed faster diffusion velocity than that of methylene blue dye in the microemulsion, thereby confirming that the *Desmodium intortum* protein microemulsion was a W/O type. Based on HPLC chromatogram results, the retention time of the protein standard (methionine) was 15.057 min that of the protein in nanoemulsion was 15.024 min, with good separation effect from other components (Figure 2). Therefore, the content of protein in the microemulsion was calculated as 1127.37 ± 30.87 μg/g. The protein microemulsion particle size was

![Figure 6. Effects of light on protein and its microemulsion (A: proteins solution, B: protein microemulsion).](image-url)
1.41 ± 0.03 μm (Figure 3), those distribution was direct, narrow, and normally distributed, which suggested an ideal microemulsion preparation. The transmission electron microscopy result of protein microemulsion was shown in Figure 4. Most of the particles were in rod-like structure with a particle size about 2 μm, while a small part of the particles were in spherical structure with a particle size about 1 μm, and there was adhesion between adjacent particles. This is mainly due to the electrostatic shielding effect, which increases the interaction between particles and promotes the aggregation of proteins. Moreover, other physical and chemical properties of the Desmodium intortum protein microemulsion, including conductivity (127.62 μs/cm), viscosity (29.94 mPa.s) and pH value (5.7) were measured. According to the microemulsion quality standard system, particle size distribution, conductivity, viscosity and pH value are the most common indexes to evaluate the quality of a microemulsion [28–30].

**Temperature stability of desmodium intortum protein microemulsion**

Figure 5A showed that desmodium intortum proteins could be preserved for a long time under 4°C. Protein retention rate remained higher than 85% after 15 d. As temperature increased, the protein stability decreased. Temperature has a significant effect on the stability of protein. A rise in temperature promotes the conversion of the positive ions of protein into chalcone and consequently leads to protein degradation. [4,11,22] When the protein solution was under 60°C, the protein retention rate was only 13% after 15 d (p < .01). According to Figure 5B, Proteins exhibited a significantly increased retention rate of 63.5% at 60°C for 15 d after microemulsion embedding (p < .01). The improved microemulsion embedding can improve the thermal stability of proteins.

**Light stability of desmodium intortum protein microemulsion**

According to Figure 6A, the protein retention rate slowly decreased under dark storage. The retention rate remained of 62.5% after 35 days (p > .05). This rate rapidly decreased under natural and UV light (p < .05). This result illustrated that light decreases proteins, which could be because exposure to light energy changes the protein molecular structure and eventually causes protein degeneration. [22,28] Figure 6B showed that the protein retention rate generally increased after microemulsion embedding. This observation suggested that microemulsion embedding can increase light stability (p < .05), possibly because the value of the refractive index of protein microemulsion was similar to that of the aqueous solution, which was higher than that of the protein solution. The protein microemulsion could accept light energy, but light energy was absorbed in the oil phase and cosurfactant and away from the microemulsion, and this could guarantee the protein stability. [16,22]

The appearance of protein microemulsion was unchanged under 4°C and 25°C. This microemulsion was dark red and uniformly transparent with good liquidity. [8,22] This result showed that protein stability was enhanced after microemulsion and the storage stability was improved under 4°C (Figure 7A). Figure 7B illustrated that the protein retention rates decreased in the protein microemulsion and protein solution, and that the decrease rate was high under 25°C. The decrease in protein retention rate in the microemulsion was lower than that in the protein solution.

**Food additives of desmodium intortum protein microemulsion**

Figure 8A showed that 0–0.234 g/g NaCl exerted no evident effect on the stability of protein microemulsion and that the microemulsion particle size was almost the same. In 0.176 and 0.234 g/g NaCl, the size of protein microemulsion was significantly increased (p < .05), which may be because NaCl enhanced the interaction with water to damage the emulsion system. [13,22]
Sugar is a polar organic compound. Large sugar concentrations will lower water activity and decrease emulsion stability. Figure 8B and Figure 8C showed that when less than 9% of glucose and maltose were combined, the protein microemulsion particle size were almost the same. This result suggested that sugar exerted no significant effect on the stability of microemulsion.

Figure 7. Change in protein retention rate in three-month storage (A: 4°C, B: 25°C).
Figure 8. Effect of food additives on the stability of protein microemulsion. Note: A: NaCl, B: glucose, C: maltose. Different letters indicate significant difference ($p < .05$) according to different contents.
Conclusion

Pseudo-ternary phase diagram was created to determine the optimal formula of protein microemulsion: limonene as oil phase, tween 20/span 80 distribution as surfactant, butanol as cosurfactant, HLB = 7.5, and Km = 3:1. This microemulsion was an W/O type with good liquidity. Protein content in microemulsion was 1127.37 µg/g, the particle size was 1.41 µm, and the droplets were round balls with a uniform size. During temperature, light, and sample observation experiments, the decrease in protein retention rate in the microemulsion was lower than that in the protein solution. Common food additives, such as low-concentration NaCl (less than 0.117 g/g) and less than 0.09 g/g concentration of glucose and maltose, exerted no evident effect on the stability of protein microemulsions.

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Disclosure statement

The authors declare that they have no conflict of interest.

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