Food Science of Animal Products

Research Article

Characteristics of multiple emulsions stabilized by myosin from bighead carp (*Aristichthys nobilis*)

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Received: September 1, 2024; Revised: September 19, 2024; Accepted: October 8, 2024

Abstract: Myosin from bighead carp (*Aristichthys nobilis*) and span 60 were used to successfully prepare multiple emulsions through a one-step emulsification method. The characteristics of multiple emulsions stabilized by span 60 and myosin from bighead carp were analyzed. The absolute value of the zeta potential of the multiple emulsions increased with the increase of myosin concentration from 0.5 to 4 g/100 mL. Meanwhile, the size of multiple emulsions reduced with the increase of myosin concentration. The optical microscope and laser confocal microscope also verified that the size of multiple emulsions decreased and the emulsion droplets in the emulsion became uniform with the increase of protein concentration. However, the myosin was not enough to cover the oil droplets, and the droplet size increased and aggregation occurred at the concentration of 0.5 g/100 mL. The flocculation index and creaming index showed that increasing protein concentration could enhance the stability of various emulsions stabilized by span 60 and bighead carp myosin and the emulsions were stable for a longer period at 2–4 g/100 mL myosin addition. Furthermore, the stability index of multiple emulsions stabilized by myosin at 4 g/100 mL changed relatively few compared to the others. These results suggest that the emulsion had the best stability when the protein concentration was 4 g/100 mL. These results indicate that bighead carp myosin in conjunction with span 60 could be used as an emulsifier to prepare stable multiple emulsions. This study may be helpful for the development of multiple emulsions constructed using fish proteins.

Keywords: *Aristichthys nobilis*; myosin; multiple emulsions; stability

Citation: Y. H. Ma, J. X. Hou, Y. Cao, et al., Characteristics of multiple emulsions stabilized by myosin from bighead carp (*Aristichthys nobilis*), Food Sci. Anim. Prod. 2 (2024) 9240089. https://doi.org/10.26599/FSAP.2024.9240089.

1 Introduction

Emulsions are used to form a protective film at the oil and water interface through surfactants to achieve the state of oil-in-water (O/W) or water-in-oil (W/O)[1]. Proteins are amphiphilic and form a adsorption layer at the water and oil interface to reduce the interface tension for stabilizing emulsions[2]. Throughout the process of emulsion encapsulation, single emulsions typically exhibit unstable properties and have significant limitations in their ability to encapsulate water-soluble compounds. However, multiple emulsions can encapsulate both hydrophilic and hydrophobic compounds with one or many small dispersed droplets[3]. The structure of the multiple emulsions enables it to act as a carrier of polar and non-polar active substances and has a protective and slow-release effect for the encapsulated substances.

Bighead carp (*Aristichthys nobilis*) is one of the most important economic fishes in China[4], which is rich in protein and an ideal source of essential amino acids for the human body[5]. Up to 2019, the global production of bighead carp reached 3.14×10^6 t[6]. Fish proteins possess the function of preventing oil droplet aggregation and lipid oxidation with good emulsifying properties[7]. The emulsifying properties of myofibrillar protein (MP) from fish could form stable emulsions[8]. Grass carp MP particles can be effectively adsorbed on the surface of oil droplets to form a stable interfacial film, thereby achieving the formation of small oil droplets[9]. Myosin as an important component of MP under Arg-assisted ultrasonic treatment can improve the stability of emulsions by inducing the exposure of native myosin and facilitating the formation of ordered structures of interfacial myosin[10]. Compared to untreated MP, high pressure homogenization-treated bighead carp MP demonstrate emulsifying activity and foaming capacity with low salts[11]. Wang et al.[12] showed that bighead carp myosin particles can be used to form stable Pickering emulsions at the protein concentration of 4 g/100 mL and the oil-water ratio is 6:4 (*V*/*V*). It has been found that the multiple emulsions prepared by the one-step emulsification method have a better effect than that prepared by the two-step emulsification method[13]. One-step emulsification is suitable for industrial mass production, and the system is relatively stable without interference from the second-step emulsification process[14]. However, whether myosin from bighead carp can be used to construct multiple emulsions stabilized by myosin is still unclear.

Therefore, this study aimed to explore the influence of different protein concentrations on the characterization of multiple emulsions. Multiple emulsions were prepared by one-step emulsification with myosin from bighead carp and span 60. The properties of the emulsion were characterized by zeta potential, particle size and particle size distribution, optical microscopy and confocal microscopy. Finally, the stability of multiple emulsions constructed by myosin was evaluated by flocculation index (FI), creaming index (CI) and multi-heavy light scattering. This study could be helpful for the further development and utilization of fish proteins.

2 Materials and methods

2.1 Materials

Bighead carp was purchased from New Huadu Shopping Mall (Xiamen, China). Soybean oil was purchased from Yihai Kerry Arawana Holdings Co., Ltd (Shanghai, China). Potassium chloride, sodium chloride, sodium hydroxide, and magnesium chloride hexahydrate were purchased from Xilong Scientific Co., Ltd (Guangzhou, China). Fluorescein isothiocyanate (FITC), Nile red and β-mercaptoethanol (β-ME) were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. All chemical products were of analytical grade.

2.2 Extraction of myosin from bighead carp

Bighead carp myosin was prepared according to the method of Wang et al.[12] with slight modifications. The white muscle of bighead carp was mixed with buffer (pH 7.5, 0.1 mol/L KCl and 20 mmol/L Tris-HCl) at a ratio of 1:10 (g/mL) and homogenized for 2 min, and filtered to remove the myofilm after 15 min at 4 °C and centrifuged at 10 000 r/min for 20 min to collect the precipitate. The precipitate was suspended in 5-fold volume of 20 mmol/L Tris-HCl buffer (pH 6.8, 0.45 mol/L KCl, 5 mmol/L β-ME, 0.2 mol/L $Mg(CH_3COO)_2$ and 1 mmol/L ethyleneglycol-bis(2-aminoethylether)-*N*,*N*,*N*',*N*'-tetraaceticacid) and ATP-Na^{2+} was added to control the final concentration to 10 mmol/L, and left at 4 °C for 90 min. The supernatant was diluted with 7 times the volume of 1 mmol/L $KHCO_3$, left for 2 h, and the precipitate was collected after centrifugation at 10 000 r/min for 20 min. The collected precipitate was suspended in 2.5-fold volume of 20 mmol/L Tris-HCl buffer (pH 7.5, containing 0.5 mol/L KCl, 5 mmol/L β-ME) at 4 °C for 10 min, diluted by adding 5-fold volume of 1 mmol/L $KHCO_3$ and $MgCl_2{\cdot}6H_2O$ was added until the final concentration was 20 mmol/L to stand at 4 °C for 24 h. After centrifugation at 10 000 r/min for 20 min, the precipitate was collected to obtain the purified myosin, which was freeze-dried and prepared for use.

2.3 Preparation of multiple emulsions stabilized by myosin

The preparation of multiple emulsions stabilized by myosin using a one-step emulsification method[15]. Myosin was weighed and dispersed in distilled water at protein concentrations of 0.5, 1, 2, 3, and 4 g/100 mL as the aqueous phase. The myosin suspension was stirred for 4 h and refrigerated at 4 °C for 24 h. Span 60 was dispersed in soybean oil at a concentration of 1.5 g/100 mL and dissolved in a water bath at 80 °C as the oil phase. After dropped to 25 °C, the oil phase was mixed with the aqueous phase at a ratio of 50:50 (*V*/*V*) and homogenized at 22 000 r/min for 2 min in a homogenizer (FJ-200, Specimen, Shanghai, China) to obtain the multiple emulsions stabilized by myosin.

2.4 Measurement of zeta potential

The zeta potential of multiple emulsions stabilized by myosin was determined at room temperature using a nanoparticle size analyzer (NS-90Z, Omec, Zhuhai, China). Multiple emulsions stabilized by myosin were diluted 100 times with ultrapure water for the test.

2.5 Measurement of droplet size and droplet size distribution

The nano-laser particle size meter (Topsizer, Omec, Zhuhai, China) was used to measure the particle size and particle size distribution of multiple emulsions stabilized by myosin. At a temperature of 25 °C, the multiple emulsions were diluted to 10 mL with ultra-pure water and 1 g/100 mL SDS as dispersant respectively, and measured. Before measuring, washed and activated the calibration function of the instrument.

2.6 Microscopic observation

An optical microscope was to used observe the microscopic structure of the emulsion. Turned on the power of the microscope and adjusted the illumination brightness and aperture size to ensure suitable light. Then, 0.5 mL of bighead carp myosin emulsions were taken successively and diluted with 2.5 mL of distilled water for observation.

2.7 Confocal laser scanning microscopy (CLSM) observation

The micromorphology of myosin multiples was observed using CLSM (TCSSP8, Leica Microsystems GmbH, Wetzlar, Germany). A 0.5 mL of the emulsion was taken and 2.5 mL of distilled water was added, after which 50 μL of Nile red was taken for the staining mixing operation under light-proof conditions. During observation using CLSM, parameters such as focal length and light intensity were adjusted to obtain high-resolution images and three-dimensional reconstructed data[16].

2.8 Determination of FI

The volume-averaged particle size ($d_{4,3}$) of myosin multiple emulsion was measured by nano-laser particle size analyzer using ultrapure water and 1 g/100 mL SDS as dispersants, respectively. The FI of the emulsion was calculated using Eq. (1):

$$\mathrm{FI}\ (\%) = \left(\frac{d_{4,3\text{-w}}}{d_{4,3\text{-s}}} - 1\right) \times 100 \tag{1}$$

Where $d_{4,3\text{-w}}$ and $d_{4,3\text{-s}}$ are the volume-average droplet size (μm) of multiple emulsions stabilized by myosin and myosin in 1 g/100 mL SDS dispersion, respectively.

2.9 Determination of CI

The freshly prepared multiple emulsions stabilized by myosin were stored at 4 °C for 30 days under light-proof conditions, and the changes in the emulsions were observed and photographed[17]. The CI was calculated following Eq. (2):

$$\mathrm{CI}\ (\%) = \frac{h_s}{h_t} \times 100 \tag{2}$$

Where h_s is the height of the cream layer (cm); h_t is the total height of the emulsion (cm).

2.10 Determination of multiple light scattering

The samples were measured using the MS20 Stability Analyzer (MS20, Bruker Alicona, Beijing, China) and placed in special vials with a uniform emulsion height of 4.5 cm. The sample bottles were placed in the measurement position of the Multi Light Scatterometer. The relevant parameters were set according to the requirements. The instrument scanned once per 2 min according to the set parameters and continued scanning for 6 h, scanning height was 4.5 cm. The stability index is directly obtained from the instrument.

2.11 Statistical analysis

Each experiment was replicated three times and the results were recorded as mean ± standard deviation. Data were graphed by Origin Pro 2021 software, and the significant differences between data were analyzed by analysis of variance (ANOVA) using SPSS version 24 software. The significance between different samples was tested by Duncan's multiple comparisons ($P < 0.05$).

3 Results and discussion

3.1 Zeta potential of multiple emulsions stabilized by myosin

Zeta potential plays a critical role in the stability of the emulsion system to reflect the electrostatic interaction between the charged particles in the emulsions[18]. As shown in Figure 1, the absolute zeta potential of the multiple emulsions stabilized by myosin was lowest at a protein concentration of 0.5 g/100 mL and highest at 4 g/100 mL. There was significant difference in the absolute values of zeta potentials of multiple emulsions stabilized by myosin at the concentration between 3 and 4 g/100 mL ($P < 0.05$). The absolute zeta potential of the multiple emulsions stabilized by myosin increased with increasing myosin concentrations. Acidic groups in myosin molecules like carboxyl groups might be dissociated to result in negatively charged proteins[19]. Negatively charged protein molecules repel each other in the emulsions due to electrostatic repulsive force, preventing protein aggregation and precipitation[20]. The electrostatic interactions between the protein molecules could be helpful for the stability of emulsions[21].

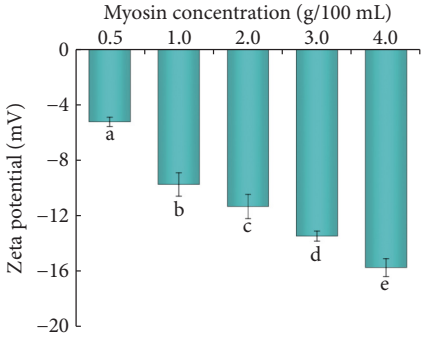
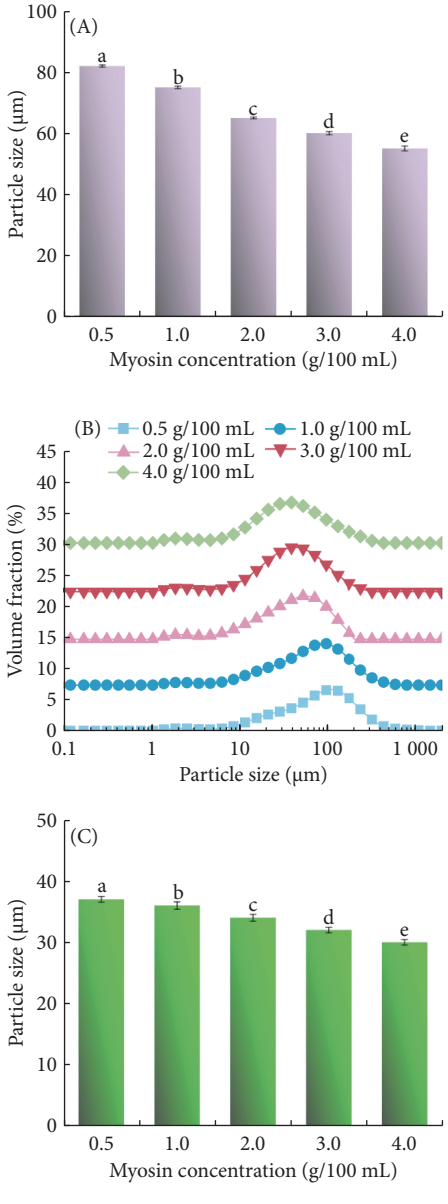


Figure 1 Zeta potential of multiple emulsions stabilized by myosin at different protein concentrations. Different lowercase letters denote significant differences ($P < 0.05$).

Multiple emulsions stabilized by myosin at a protein concentration of 0.5 g/100 mL exhibited a low absolute zeta potential, indicating poor stability. The absolute value of the zeta potential of multiple emulsions stabilized by myosin gradually increased with the increase in myosin concentration, indicating that the stability of the myosin multiple emulsions was strengthening. It could demonstrate a positive correlation between myosin concentration and multiple emulsion stability. Protein concentration is positively correlated with the stability of multiple emulsions stabilized by myosin as previous reports about emulsions stabilized by myofibrillar proteins[22].

3.2 Particle size and particle size distribution of multiple emulsions stabilized by myosin

The particle size and particle size distribution of multiple emulsions stabilized by myosin are in Figure 2. The particle size of multiple emulsions stabilized by myosin was negatively correlated with myosin concentration (Figure 2A). With the increase in myosin concentrations (0.5–4 g/100 mL), the protein interfacial layer might wrap the oil droplets and become thickened to reduce aggregation. The peak of the particle size distribution of multiple emulsions stabilized by myosin shifted towards a small size as the myosin concentration increased (Figure 2B). Meanwhile, as shown in Figure 2C, the particle size of all the emulsions reduced when the emulsions were dispersed with 1 g/100 mL SDS as a dispersant. This indicated that the flocculation occurred between the emulsion droplets. By Figure 2D, the peak of the particle size distribution of multiple emulsions stabilized by myosin moved towards a small size with increasing myosin concentration, similar to water as a dispersed phase in agreement with multiple emulsions stabilized by casein[23]. The increase in protein concentration resulted in a decrease in particle size and a peak shift of the particle size distribution of multiple emulsions stabilized by myosin toward small size.



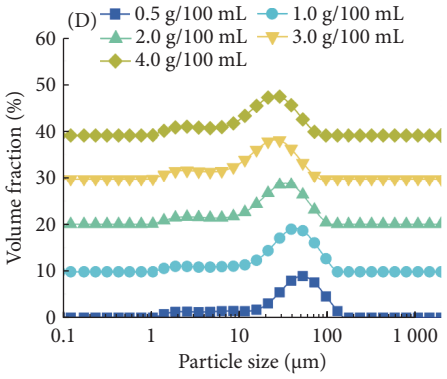


Figure 2 Particle size and particle size distribution of multiple emulsions stabilized by myosin at different protein concentrations. (A) and (B) are the particle size and particle size distribution of emulsion in the aqueous phase, (C) and (D) are the particle size and particle size distribution of emulsion in the SDS solution. Different lowercase letters denote significant differences ($P < 0.05$).

3.3 Microstructure of multiple emulsions stabilized by myosin

The microscopic morphology of the multiple emulsions stabilized by myosin at different protein concentrations is shown in Figure 3. It could be seen that dense small droplets were dispersed in large spherical oil droplets. Multiple emulsions stabilized by myosin at different protein concentrations exhibited a "three-phase, two-membrane" multicompartmental structure, which is also observed in the preparation of double emulsions of loaded proanthocyanidins stabilized by whey isolate protein complexes[24]. The particle size of the emulsions decreased as the protein concentration increased, in line with the results in Figure 2. The relatively large size of the emulsions in the multiple emulsions stabilized by myosin with low protein amount fractions indicated the significant aggregation of the emulsion droplets. As the protein amount fraction increased, the size of the multiple emulsions stabilized by myosin further decreased and a large number of tiny droplets could be observed inside emulsion droplets, which demonstrated the multiple emulsion structure.

Compared with optical microscope, CLSM has a prominent advantage in displaying multiple emulsion characteristics to present the subtle structure and characteristics of multiple emulsions accurately[25]. As shown in Figure 4, myosin could not fully cover the surface of the oil droplets when the concentration of myosin was below 1 g/100 mL and the emulsion droplets tended to aggregate and fuse. When the myosin concentration increased to 2 g/100 mL, the size of multiple emulsions stabilized by myosin decreased and

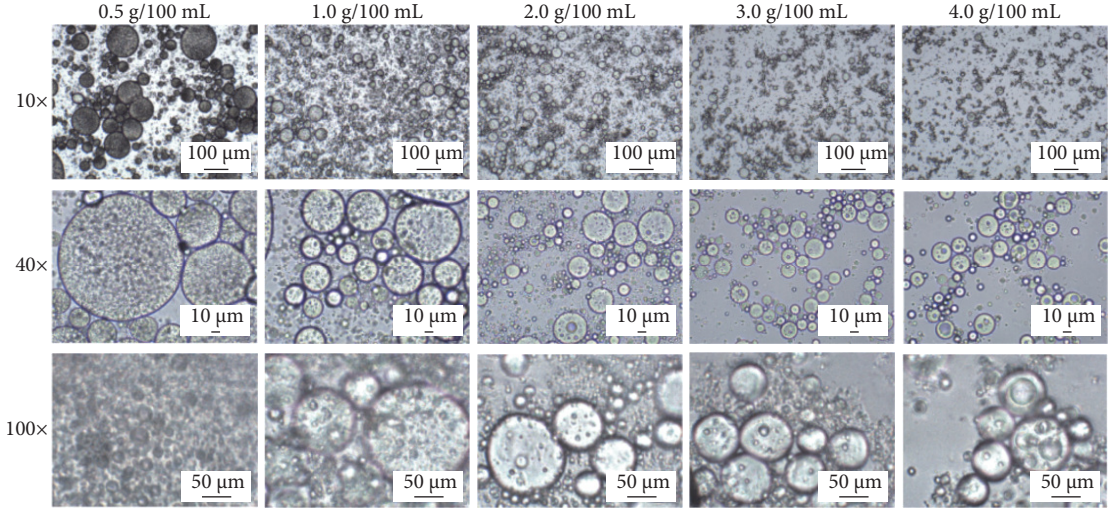


Figure 3 Optical microscopy image of multiple emulsions stabilized by myosin at different protein concentrations.

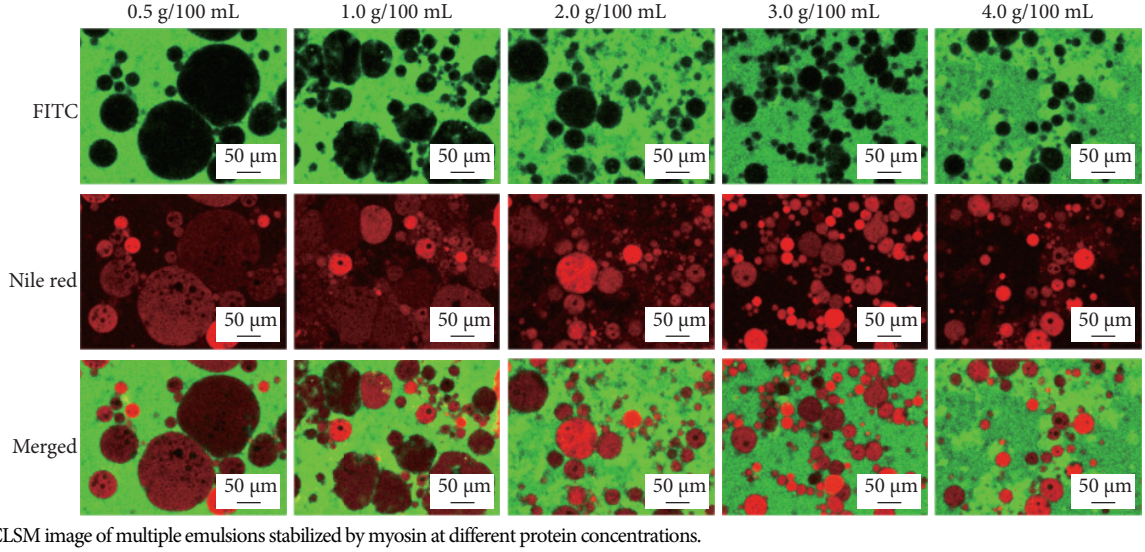


Figure 4 CLSM image of multiple emulsions stabilized by myosin at different protein concentrations.

the distribution became uniform. This was because an adequate number of protein molecules were able to quickly attach to the surface of newly formed emulsion droplets during the homogenization process. The electrostatic repulsion and spatial resistance between the emulsion droplets could prevent them from re-aggregating with each other. Similar results were found in the preparation of grape seed proanthocyanidin-loaded W/O/W emulsion gels[26]. The trend of the emulsion droplets was in agreement with the results observed through optical microscopy (Figure 3).

3.4 FI of multiple emulsions stabilized by myosin

FI is used to measure the degree of aggregation of emulsions[27]. The FI of multiple emulsions stabilized by myosin is shown in Figure 5. The FI of multiple emulsions stabilized by myosin exhibited a down trend with the increase in myosin concentration. Specifically, the FI of the emulsions was high at a low myosin concentration (0.5 g/100 mL), indicating that the emulsion droplets had weak interactions to flocculate. This was due to the fact that there were few protein molecules in the emulsion at low protein concentrations, which could not effectively cover and stabilize oil, resulting in aggregation between the emulsion droplets[28]. As previous reports, emulsions stabilized by perilla seed protein show the same trend, attributing to the fact that protein molecules can be adsorbed on the surface of the emulsion droplets to form a stable film at higher protein concentrations to effectively prevent aggregation[29].

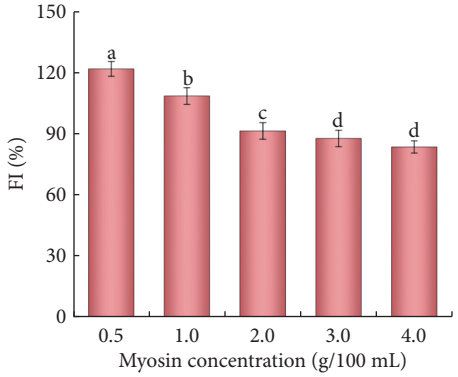


Figure 5 FI of multiple emulsions stabilized by myosin at different protein concentrations. Different lowercase letters denote significant differences ($P < 0.05$).

3.5 Storage stability of multiple emulsions stabilized by myosin

CI is an important index to reflect the storage stability of emulsions for determining the stability of the emulsions[30]. Figure 6 shows that the CI of multiple emulsions stabilized by myosin. At a myosin concentration of 0.5 g/100 mL, the CI of multiple emulsions stabilized by myosin reached 8.5% after 1 h and increased to 25.70% after 15 d. At a myosin concentration of 1 g/100 mL, the CI of multiple emulsions stabilized by myosin reached 22.8% after 30 d. At myosin concentrations of 2–4 g/100 mL, multiple emulsions stabilized by myosin remained stable for 30 d without creaming. The storage stability of multiple emulsions stabilized by myosin was inversely correlated with the size. The small size of emulsions is beneficial for the physical and chemical stability of the emulsion system. Additionally, lots of myosin was closely arranged at the oil-water interface to form the interfacial film with viscoelasticity and mechanical strength to protect emulsion droplets. At a lower particle concentration, the particles at the surface of the emulsion droplets have too small coverage areas to form an intact protective film, leading to a decrease in the stability of the emulsions[31]. These results showed that myosin was able to act as a solid particle emulsifier adsorbed at the oil-water interface and provided an effective barrier to stabilize multiple emulsions and prevent droplet aggregation. The emulsions were stable for a long time at myosin additions of 2–4 g/100 mL.

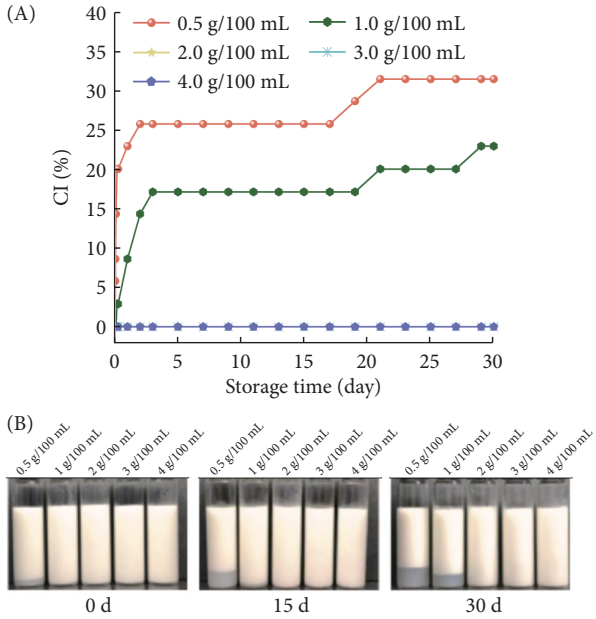


Figure 6 (A) CI and (B) storage stability of multiple emulsions stabilized by myosin at different protein concentrations.

3.6 Multiple light scattering of multiple emulsions stabilized by myosin

Emulsion droplets can collide with each other under gravity and Brownian motion to result in instability phenomena such as delamination, flocculation, and aggregation[32]. Multiple light scattering technology could fast and effectively estimate fluid stability[33]. Figure 7 shows the backscattered light index of multiple emulsions stabilized by myosin at different protein concentrations. A gradual decrease in backscattered light of multiple emulsions stabilized by myosin at the protein concentration of 0.5 g/100 mL with time at the front end of the liquid (0–15 mm) (Figure 7A).

However, the backscattered light of multiple emulsions stabilized by myosin at the protein concentration of 0.5 g/100 mL gradually increased with time at the back end of the liquid height (15–45 mm). Combined with the observation after 6 h, there was an obvious creaming phenomenon at the front end of the liquid, indicating that a stable myosin multiple emulsion could not be formed at this protein concentration. The backscattered light of multiple emulsions stabilized by myosin at the front end of the liquid (0–8 mm) decreased with time and the backscattered light increased with time at the back end of the liquid height (8–45 mm) at the protein concentration of 1 g/100 mL (Figure 7B). A slight creaming phenomenon appeared at the front end of the liquid after 6 h. The backscattered light of multiple emulsions stabilized by myosin at 2–4 g/100 mL decreased progressively with time throughout the liquid (0–45 mm) (Figures 7C–E). There was no significant difference in the front-end vs. back-end contrast, indicating that multiple emulsions stabilized by myosin were stable.

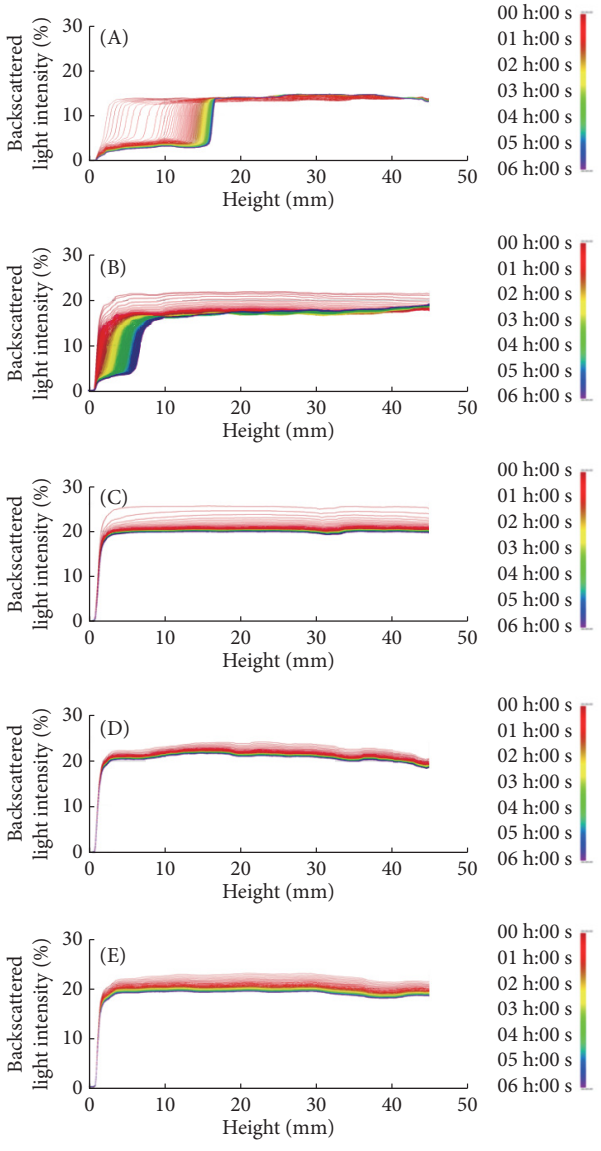


Figure 7 Backscattered light intensity of multiple emulsions stabilized by myosin at different protein concentrations. (A) 0.5, (B) 1, (C) 2, (D) 3, and (E) 4 g/100 mL, respectively.

The stability index and slope exhibit the stability of the emulsions[34]. Figure 8 shows the stability index of multiple emulsions stabilized by myosin at different protein concentrations. The stability index of the multiple emulsions stabilized by myosin, except for those with a myosin concentration of 0.5 g/100 mL, were all around 0.5 during the scanning time of 1 800 s, indicating that the initial emulsion had good stability performance. The stability index of multiple emulsions stabilized by myosin after 1 h of storage increased with the extension of storage time. The change in the stability index of multiple emulsions stabilized by myosin decreased with the increase in protein concentration at the same time. After 6 h, the stability index of the protein concentration of 0.5 and 1 g/100 mL varied greatly, and the stability of multiple emulsions stabilized by myosin was poor, which was consistent with the results in Figure 6. The multiple emulsions stabilized by myosin at 2 g/100 mL had a large change in stability index at the beginning. The stability index of multiple emulsions stabilized by myosin at 4 g/100 mL changed relatively a few compared to the others, indicating the stability of multiple emulsions stabilized by myosin at 4 g/100 mL was better than the others.

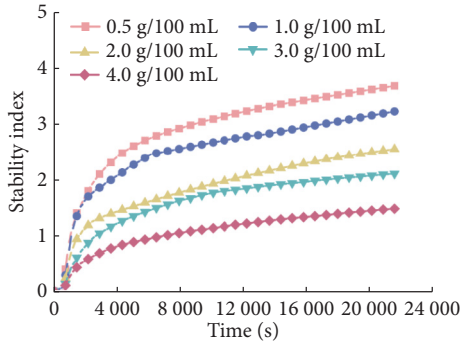


Figure 8 Stability index of multiple emulsions stabilized by myosin at different protein concentrations.

4 Conclusion

Multiple emulsions could be constructed using bighead carp myosin and span 60 by the one-step emulsification method. The absolute value of the zeta potential of multiple emulsions stabilized by myosin increased and the particle size decreased with the increase in myosin concentrations. The above results showed that multiple emulsions stabilized by myosin at 4 g/100 mL had the best stability. The size of multiple emulsions stabilized by myosin at the concentrations of 2–4 g/100 mL possessed small and uniform droplets. Protein molecules adsorbed on the surface of the oil droplets formed a stable film to prevent aggregation between emulsion droplets. In addition, bighead carp myosin could maintain the stability of multiple emulsions stabilized by myosin. These results indicated that myosin from bighead carp could be utilized to form stable multiple emulsions to broaden the application of fish proteins.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was funded by the Natural Science Foundation of Fujian Province in China (2021J01835), the Seed Industry Innovation and Industrialization Project of Fujian Province (2021FJSCZY01), Fujian Province Project for Promoting High Quality Development of Marine and Fishery Industry (FJHYF-L-2023-19; FJHYF-L-2023-29), and Opening Project of Fujian Provincial Engineering Technology Research Center of Marine Functional Food (2024MFF02002).

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