Modifying the physicochemical properties, solubility and foaming capacity of milk proteins by ultrasound-assisted alkaline pH-shifting treatment

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1. Introduction

Liquid milk proteins are often spray dried into powders that can be easily store, transport, and handle. These proteins are typically incorporated into food products as commercial ingredients in powder, popularly as concentrates or isolates of milk protein or its fractions [1]. Milk protein concentrate (MPC) has the same ratio of casein to whey protein as natural milk, which contains protein content varying from 50 % to 85 % [2]. Micelle casein concentrate (MCC), an emerging protein component mainly comprising of casein micelles and small amounts of whey protein and lactose [3,4], is reported to have the property of poor solubility and low rehydration [5]. Whey protein isolate (WPI) is made from whey, a by-product in the dairy industry, with unique functional and nutritional properties and wide applications in the food industry [6].

The conformational structure of protein has a key influence on its functionalities [7]. Therefore, the functional characteristics of milk protein can be effectively improved by modifying the molecular structure [8,9]. The pH-shifting is an emerging chemical treatment method that has been widely used to modify animal and plant proteins [10–13]. The treatment of pH-shifting allows the protein molecule to undergo unfolding and refolding processes, which results in the formation of a new structure, called a “molten globule”, causing the changes in the protein’s structure and function [12,14]. Subjecting proteins to acid or alkaline pH-shifting has been observed to an increase in functionality of amaranth protein [15] and ginkgo seed proteins [11] due to alterations of the structural conformations. Further studies showed that the synergy of ultrasonic technology with pH-shifting was superior to pH-shifting alone. Jiang et al. [16] found that pH-shifting alone treatment was not as effective as ultrasound combined with pH-shifting treatment in increasing the solubility of pea proteins. In addition, ultrasound has been applied to improve the functional properties of dairy products and


** Abbreviations:** MPC, milk protein concentrate; MCC, micellar casein concentrate; WPI, whey protein isolate; ANS, 8-anilino-1-naphthalenesulfonic acid; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; \( \theta \), surface hydrophobicity index; CD, circular dichroism; FC, foam capacity; FS, foam stability; MW, Molecular weight distribution.

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milk materials. Also, physical methods can be combined with pH-shifting to induce further conformational changes to the proteins. The studies showed that the solubility of the treated pea [16] and rapeseed proteins [7] was 7 times higher than that of the control. pH-shifting at extreme acid or alkaline pH condition significantly enhanced the foaming properties of chickpea protein isolate, while the use of ultrasound further intensifies this change [17].

To the best of our knowledge, no studies of the changes in physicochemical properties, solubility, and foaming capacity of three different compositions of milk proteins caused by the combined method of ultrasound and alkaline shifting have been published yet. This study aimed to determine whether this combined approach can improve the functional properties of milk proteins, especially those containing a high amount of casein with poor solubility. Meanwhile, this study also assessed the potential of this unfolding-sonication-refolding treatment in modifying milk protein ingredients.

2. Materials and methods

2.1. Materials

MPC80 (80 % protein, 6 % moisture, 2.5 % fat, 9 % lactose and 8 % ash) and MCC (micellar casein 48450) (90 % protein, 6 % moisture, 2 % fat, and 7 % ash) were obtained after dilution with PBS buffer (0.1 mol/L, pH 7.4). Then 20 mL of 8 mmol/L ANS solution was added per 4 mL of sample dilution and kept for 30 min under agitation to allow the unfolding of the protein molecules. After that, solutions were sonicated at 20 kHz and 300 W for 5 min (pulse on-time 2 s and off-time 3 s) on an ultrasound equipment (JY92-IIN, Ningbo Scientz Biotechnology Co., Ltd) fitted with a 12 mm horn. Ultrasonication is carried out in a circulating water bath at 30 °C. Subsequently, the pH of solutions was adjusted to 7.0 ± 0.02 using 1 mol/L HCl and kept at room temperature for 1 h. Native samples of MPC, MCC and WPI without treatment were at pH 7.06 ± 0.01, 7.17 ± 0.02, 6.28 ± 0.02 respectively, and served as the control of our study. All samples are stored at 4 °C for analysis.

2.2. Ultrasound-assisted pH-shifting treatment

The ultrasound-assisted pH-shifting treatment was applied in milk protein solutions following the method described by Jiang et al. [16] with some modifications. MPC, MCC and WPI powders were dispersed in Mill-Q water (Direct-Q® 5 UV, Millipore S.A.S, Massachusetts, USA) respectively and stirred overnight to obtain a solution with a concentration of 30 mg/mL. Then, pH of solutions was adjusted up to 8.0, 9.0, 10.0, 11.0, 12.0 (+±0.02) with the addition of 2 M NaOH and kept for 30 min under agitation to allow the unfolding of the protein molecules. After that, solutions were sonicated at 20 kHz and 300 W for 5 min (pulse on-time 2 s and off-time 3 s) on an ultrasound equipment (JY92-IIN, Ningbo Scientz Biotechnology Co., Ltd) fitted with a 12 mm horn. The protein was analyzed with the SDS-PAGE method using a MiniPROTEAN Tetra Cell system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the method of Laemmli [18]. After the protein solutions were diluted ten times, 10 μL aliquots were added to 10 μL of sample buffer (0.05 mol/L Tris-HCl, pH 8.0, containing 0.1 g/L SDS and 100 mL/L glycerol), and heated in boiling water for 3 min. Then, 10 μL of the mixture was loaded into each well. SDS-PAGE was performed using a 5 % stacking gel and a 12 % resolving gel at 130 V/90 min. SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 for 2 h and unstained in 7.5 % acetic acid until the bands were clear.

2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein was analyzed with the SDS–PAGE method using a MiniPROTEAN Tetra Cell system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the method of Laemmli [18]. After the protein solutions were diluted ten times, 10 μL aliquots were added to 10 μL of sample buffer (0.05 mol/L Tris-HCl, pH 8.0, containing 0.1 g/L SDS and 100 mL/L glycerol), and heated in boiling water for 3 min. Then, 10 μL of the mixture was loaded into each well. SDS-PAGE was performed using a 5 % stacking gel and a 12 % resolving gel at 130 V/90 min. SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 for 2 h and unstained in 7.5 % acetic acid until the bands were clear.

2.4. Particle size and zeta potential

To avoid multiple scattering effects, samples were diluted with Mill-Q water to a concentration of 3 mg/ml. Based on dynamic light scattering (DLS), the particle size distribution of samples was determined using a Zetasizer Nano ZS90 instrument (Malvern Instruments Limited, Worcestershire, UK) according to the method of Liu et al. [19]. The zeta potential of the samples was determined using the Malvern with Laser Doppler Velocimetry based on the method of Wu et al. [20].

2.5. Turbidity measurement

The protein solutions were diluted with Mill-Q water at a volume ratio of 1:4. The turbidity was measured through transmission of light (860 nm) with a 1 cm pathlength quartz cuvette on an UV756 spectrophotometer (Yoke Instrument Co. Shanghai China) [21].

2.6. Surface hydrophobicity

Surface hydrophobicity was determined using the ANS as a fluorescence probe referring to the method of Han et al. [22] with a slight modification. Protein solutions ranging from 0.01 to 1.0 mg/mL were obtained after dilution with PBS buffer (0.1 mol/L, pH 7.4). Then 20 μL of 8 mmol/L ANS solution was added per 4 mL of sample dilution and left in the dark at 25 °C for 15 min. The fluorescence intensity of the resulting solutions was measured with a fluorescence spectrophotometer (F-2700, Hitachi Limited, Japan) at excitation and emission wavelength of 390 nm and 470 nm, respectively, with a slit width of 5 nm. A plot of fluorescence intensity against protein concentration was fitted using linear regression with the slope being the surface hydrophobicity index (H₃).

2.7. Circular dichroism spectroscopy

Far-UV circular dichroism (CD) was used to investigate the secondary structure of proteins. Protein solutions (0.3 mg/mL) were evaluated at 25 ± 0.1 °C in the spectral range from 190 to 260 nm with a Chirascan spectrometer (Applied Photophysics Company, UK), using a quartz cuvette with an optical path of 0.1 cm.

2.8. Solubility measurements

Solubility measurements were performed according to the method of Wu et al. [4] with slight modifications. Briefly, aliquots were centrifuged at 700 × g for 10 min at 25 °C. The supernatant and the uncentrifuged protein solution (~2 g) were placed on an aluminum dish respectively, and heated in the oven (DHG-8070A, Zhongyiguoke Technology, Beijing, China) at 105 °C for 24 h and then weighed. The solubility is calculated as in the formula (1).

\[
\text{Solubility} = \frac{\text{weight of dried centrifuged supernatant}}{\text{weight of dried uncentrifuged solution}} \times 100 \%
\]  

(1)

2.9. Foaming capacity (FC) and foam stability (FS)

FC and FS of samples were determined according to the method described by Hettiarachchi et al. [23]. The 20 mL aliquot (V₀) of protein solutions were homogenized using a homogenizer (T18BS25, IKA, Germany) at 10000 rpm for 1 min to obtain a foam. The foam volume was measured immediately (V₀) and after 10 min (V₁₀) to calculate FC and FS according to equations (2) and (3).

\[
FC = \frac{V₀}{V₁₀} \times 100 \%
\]  

(2)

\[
FS = \frac{V₀}{V₁₀} \times 100 \%
\]  

(3)
2.10. Statistical analysis

The values of three biological replicates were used to calculate the mean and standard deviation of the samples. Statistical analysis was conducted with one-way ANOVA at the significance level of \( p < 0.05 \) in SPSS Statistics version 22 software (IBM Corp., New York, USA). In addition, OriginPro 8.5 software (OriginLab, Massachusetts, USA) was used for drawing.

3. Results and discussion

3.1. Molecular weight distribution (MW)

As shown in Fig. 1A, B, the SDS-PAGE profiles of the native group and treated samples showed typical bands of casein, BSA, \( \alpha_\text{-CN}, \beta\text{-CN} \). Compared with the native sample, no significant difference was found in the electrophoretic patterns of any of the treated protein samples. This study showed that alkaline pH-shifting and sonication did not change the molecular weight of casein in MPC and MCC, and thus, did not result in the cleavage of individual caseins [4,24,25]. According to the Fig. 1A and C, the protein profiles of MPC and WPI showed an increase in molecular weight of whey protein at pH 10 and above. The WPI consists mainly of \( \beta\text{-Lg} \) at the 18 KDa band and \( \alpha\text{-La} \) at the 14 KDa band. After treatment, based on the location of the appearance of the bands and the change in intensity of the original bands, it can be assumed that a dimer of \( \beta\text{-Lg} \) was formed [26]. In addition, the formation of smear bands in the range 55–130 KDa indicated that the treatment resulted in the aggregation of proteins to form macromolecular structures [16]. The primary structure of the WPI was changed [27], mainly due to cavitation effects and strong alkaline pH-shifting that affects the molecular structure of the protein, and non-covalent interactions may be the driving force of aggregation [28,29]. Overall, SDS-PAGE analysis showed that ultrasound-assisted pH-shifting treatment had no significant effect on the molecular weight structure of casein, but aggregates began to appear in whey proteins as the degree of pH-shifting increased (pH \( \geq 10 \)).

3.2. Particle size and zeta potential

Fig. 2 showed the changes in particle size of the milk protein after ultrasound-assisted pH-shifting treatment. For MPC (Fig. 2A, B) and MCC (Fig. 2A, C), which contain large amounts of casein, an obvious and significant reduction in overall particle size occurred after treatment (p < 0.05). The particle sizes of MPC and MCC were reduced by 124.29 nm and 175.55 nm respectively. Generally, under alkaline conditions, with the increase of pH, casein micelles showed looser structure and larger size due to increasing electrostatic repulsion [30]. However, ultrasound is more destructive to casein with a looser casein structure [30]. Therefore, the particle size of the protein will not be as large as that of the native sample when it is refolded at neutral pH. The reduction in particle size is the result of a combination of the unfolding and refolding of pH and the cavitation and shearing effects of ultrasound. This result is consistent with the previous findings reported by Li et al. [7] that ultrasound treatment combined with alkaline pH-shifting reduced the particle size of rapeseed proteins. For WPI (Fig. 2A, D), the pH-shifting and ultrasound treatment did not result in particle fragmentation, but aggregation occurred at pH above 10, in consistent with results of SDS-PAGE.

The solution with higher absolute value of zeta potential indicates a strong repulsion between the molecules and is less prone to aggregation, and thus deemed to be moderately stable [31]. The zeta potentials of the samples were shown in Fig. 2A. The value of the zeta potential of MPC potential decreased from −24.83 to −41.20 mv and that of MCC decreased from −22.80 to −25.35 mv after ultrasound-assisted pH-shifting treatment. This combined treatment led to a strong electrostatic repulsion between proteins, resulting in an increase in the stability of the solution. This can be explained by the change in the structure and conformation of the protein, with more charges accumulating on the molecular chains [32]. Similarly, as the degree of pH-shifting treatment increased, it also led to stronger intramolecular electrostatic repulsion of whey proteins, but excessive extreme alkaline pH-shifting treatment led to large aggregates of whey proteins, which affected the stability of the solution, so WPI was in instability gradually for the treatment with pH ≥ 10.

3.3. Turbidity

Turbidity depends, to a large degree, on the size and the scattering of particles [33,34]. Bigger size and higher scattering of particles usually lead to higher turbidity of the system. The visual appearance and turbidity of the samples after ultrasound-assisted pH-shifting treatment were shown in Fig. 3B and C. Compared to the native, the turbidity of the treated protein solutions decreased significantly (p < 0.05) with the increase of pH. The presence of a large number of casein micelles in MCC and the compact casein micelle structure results in high particle scattering factor. After the ultrasound-assisted pH-shifting treatment, the casein micelle structure became looser, reducing the scattering factor and thus deemed to be moderately stable [31]. The zeta potentials of the samples were shown in Fig. 2A. The value of the zeta potential of MPC potential decreased from −24.83 to −41.20 mv and that of MCC decreased from −22.80 to −25.35 mv after ultrasound-assisted pH-shifting treatment. This combined treatment led to a strong electrostatic repulsion between proteins, resulting in an increase in the stability of the solution. This can be explained by the change in the structure and conformation of the protein, with more charges accumulating on the molecular chains [32]. Similarly, as the degree of pH-shifting treatment increased, it also led to stronger intramolecular electrostatic repulsion of whey proteins, but excessive extreme alkaline pH-shifting treatment led to large aggregates of whey proteins, which affected the stability of the solution, so WPI was in instability gradually for the treatment with pH ≥ 10.

3.4. Surface hydrophobicity (Hs)

The exposed hydrophobic sites of protein and changes in the tertiary structure can be evaluated with ANS due to its high affinity to hydrophobic surfaces [35]. As shown in Fig. 4A, pH-shifting and ultrasound treatments resulted in an increase in the surface hydrophobicity of MCC and MCC except for when the pH was 10 and 12. The surface hydrophobicity of WPI also showed a significant increase (p < 0.05). This can be attributed to the fact that alkaline pH-shifting resulted in stronger intramolecular electrostatic repulsions, and protein unfolding, which

Fig. 1. SDS-PAGE profiles of MPC (A), MCC (B), WPI (C) with ultrasound-assisted pH-shifting.
exposed the hydrophobic amino acid residues [11,36]. Previous studies have shown that alkaline pH treatment induced more conformational changes in the proteins than acidic pH treatment [37]. Similar results were observed for the pH-shifting treated peanut protein isolate [38], ginkgo seed proteins [11]. Meanwhile, ultrasound treatment also had a positive effect on the H_0 values of the proteins. Ultrasound treatment exposed proteins’ hydrophobic domains previously buried in these macromolecules [39]. The reduction in surface hydrophobicity of MPC and MCC protein solutions at pH 10 and 12 may be due to the unfolding of protein molecules which resulted in more non-covalent bond extensions that masked the hydrophobic regions [40].
Far-UV CD spectroscopy can be used to qualitatively and quantitatively analyze the secondary structure of proteins [41]. To find out the effect of ultrasound-assisted pH-shifting treatment on the secondary structure of milk proteins, their solutions were assessed with CD as shown in Fig. 4B and C. In CD spectroscopy, positive peaks at 192 nm, negative peaks at 208 and 222 nm represent α-helix, positive peaks at 195 nm and negative peaks at 216 nm represent β-sheet, positive peaks at 205 nm represent β-turn, negative peaks at 200 nm, and positive peaks at 212 nm represent random coil [10,42]. The minimum peaks of MPC (from 205 nm to 202 nm), MCC (from 205 nm to 203 nm), and WPI (from 208 nm to 206 nm) all showed blue shifts of the peak wavelength and significantly increased intensity of the negative peaks compared with the untreated proteins (Fig. 4B), which suggested a decrease in the α-helix content (p < 0.05). Obviously, the secondary structure of milk proteins is dominated by β-sheet and random coil. The three milk proteins with different compositions all showed the same changes in the secondary structures, with a decrease in the content of α-helix and β-sheet, accompanied by an increase in the content of β-turn and random coil (Fig. 4C). The ultrasound-assisted pH-shifting treatment disrupted the hydrogen bonds that maintained the secondary structure of the proteins, allowing the unfolding of the α-helix and β-sheet regions and their conversion into β-turn and random coil. Protein molecules have been found to undergo unfolding, dissociation and rearrangement after ultrasound-assisted pH-shifting treatment [43]. The proportion of β-turn and random coil increased, while the content of highly ordered β-sheet decreased, implying that the structure became looser. This suggested that the combined treatment of ultrasound-assisted pH-shifting weakened the rigid protein structure and improved the flexibility of the protein molecule [44]. At the same time, the surface hydrophobicity increases with the decrease of α-helix, in consistent with the hydrophobic results [36,45,46].

3.6. Solubility

Solubility is an important functional property of proteins. The milk protein needs to be dispersed and dissolved to be fully functional and edible as an ingredient [47]. For example, good solubility is a precondition for good foaming and emulsifying properties of proteins. As displayed in Table 1, the solubility of MPC and MCC was improved significantly (p < 0.05) after ultrasound-assisted pH-shifting treatment. In particular, MCC has poor solubility due to the high amount of casein. The solubility of MCC increased from 59.02 % to 99.50 % after treatment. The solubility of MPC increased by 7.29 % compared with the native sample. The solubility of WPI in the native group was well close to 100 %, and ultrasound and alkaline pH-shifting had little effect on its solubility. Therefore, alkaline pH-shifting and sonication was proved to be able to improve the solubility of milk proteins. It has been previously reported that proteins treated under extreme alkaline conditions expose some polar groups and further expand the protein chain, resulting in good solubility [7]. The reduction in sample particle size facilitates the interactions between protein and water [48], the increase in the absolute value of zeta potential improves the interparticle electrostatic repulsions, improving the dispersion stability of the protein, which leads to higher solubility of the protein [49]. Although many other studies have observed a positive correlation between solubility and H0 [50,51], the result in this study indicated that surface hydrophobicity is not the only determinant of protein solubility which is consistent with the conclusion reported by Zhang et al. [11]. A similar enhancement in protein solubility was also observed in alkaline pH-shifting treated soy β-conglycinin [46] and alfalfa protein [12].

3.7. Foaming properties

Fig. 5 shows the effects of ultrasound-assisted pH-shifting treatment on the foaming properties of samples. Compared with the native group, the FC of the protein solution was significantly (p < 0.05) higher after pH-shifting combined with sonication (Fig. 5A). The results of this study are in agreement with findings about trout proteins reported by Pezeshk et al. [52]. This study found that FC was positively correlated with H0, the surface hydrophobicity and FC were the highest at pH 11. Compared with MPC and MCC, WPI had the best FC and the most significant improvement after treatment, ranging from 60.06 % to 143 %. Ultrasound caused the unfolding of protein molecules at the air–liquid interface, increasing their ability of spreading on the air–water interface to encapsulate air, and thus resulting in better foaming capacity [53]. At the same time, pH-shifting also led to the exposure of hydrophobic groups of proteins. The combined treatment results in higher surface hydrophobicity, better solubility and smaller particle size [54], all of}

Table 1

| pH shifting | MPC | MCC | WPI |
|-------------|-----|-----|-----|
| Solubility index (%) | Native | 93.35 ± 0.71a | 59.02 ± 0.82a | 100.00 ± 0.78a |
| 8 | 96.21 ± 1.21b | 96.97 ± 0.78a | 98.71 ± 0.64a |
| 9 | 98.18 ± 0.34b | 97.83 ± 0.09a | 98.31 ± 1.69a |
| 10 | 98.93 ± 0.64a | 97.84 ± 0.78a | 98.76 ± 0.25a |
| 11 | 99.38 ± 0.82a | 99.23 ± 0.09a | 97.88 ± 0.15a |
| 12 | 99.72 ± 0.26a | 99.50 ± 0.78a | 99.01 ± 0.82a |

Note: Different letters (a−c) indicate significant differences at p < 0.05.
which contribute to improved FC. Compared with MPC and WPI, all the treated MCC samples showed decreases of FS (Fig. 5B), and the bubbles formed were found to be unstable, which may be attributed to the weaker interfacial elasticity of casein than that of whey protein [55].

4. Conclusions

In this study, ultrasound-assisted pH-shifting treatment was proved to be effective in modifying the physicochemical properties, solubility and foaming capacity of milk proteins consisting of casein and whey proteins. This study found that the combined method effectively reduced the particle size, zeta potential and turbidity of milk proteins, especially those containing caseins. However, excessive extreme alkaline pH-shifting treatment (pH ≥ 10) led to the formation of large molecular aggregates of whey proteins, which resulted in the decline in the stability. Ultrasound-assisted pH-shifting treatment resulted in the exposure of the hydrophobic groups of the protein, especially for samples treated at pH 11, which showed the highest surface hydrophobicity. As shown in CD measurements, the treated proteins showed an increase in β-turns and random coils, with a corresponding decrease in α-helices and β-sheets. The decrease in particle size and zeta potential, the increase in surface hydrophobicity and the change in protein conformation all contribute to the improvement in solubility of protein. At the same time, the excellent solubility further made the better foaming capacity of the protein. In summary, ultrasound-assisted pH-shifting treatment showed an excellent method for protein modification, especially for dairy components containing large amounts of poorly soluble casein.

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