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Research Article

Effects of Heat Treatment on Structure and Processing Characteristics of Donkey Milk Whey Protein

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Donkey milk is nutritionally rich and has low allergenicity; thus, whey protein derived from donkey milk is a suitable hypoallergenic alternative. However, the temperature during pasteurisation considerably influences the structure and processing characteristics of whey proteins. We found that donkey milk has poor thermal stability; the secondary structure of the whey proteins loosened and became disordered as the temperature increased. In addition, exposing tryptophan and tyrosine to an external polar environment changed the tertiary structure. High-temperature and long-duration heat treatments significantly affected the processing properties, such as emulsification, foaming, and rheological properties. However, a short treatment at approximately 85°C improved the processing properties. Therefore, we recommend that donkey milk be processed at temperatures no higher than this temperature to maintain the beneficial qualities of the whey proteins and provide a reference for processing donkey milk products.

1. Introduction

Whey protein, one of the main proteins in milk, has a high biological value, is simple to digest, and has several uses. Whey proteins are primarily composed of immunoglobulins, lactoferrin, serum albumin, α-lactalbumin, and β-lactalbumin, which are essential for preventing the growth of various pathogens and inflammatory disorders [1]. Whey protein powder is a common dairy product that provides essential protein nutrition to the body, particularly in special populations. Whey protein consumption accelerates body repair and maintains normal tissues, organs, and skin functions by delivering oxygen and nutrients to cells, delaying human ageing. In addition, the immune system produces antibodies against bacteria and infections. Additionally, infants with cow milk protein allergy (CMPA) are also intolerant to cow milk [2]. Therefore, developing whey protein powder from other milk sources or dairy products is necessary.

Donkey milk (DM), a minor milk source with excellent nutritional content and low allergenicity, is one of the best hypoallergenic substitutes for human milk. The chemical composition and nutritional value of DM have been extensively studied, confirming that its nutritional components resemble those of human milk, especially the lactose and protein contents [3]. The structure and composition of DM and bovine milk proteins vary. Milk proteins include whey protein and casein (CN), the latter of which mainly consists of αs1, αs2, β-CN, and κ-CN, and is the major allergen in milk. Cunsolo et al. reported significant differences in the amino acid sequences of αs1-CN between donkeys and bovines [4]. Another study employed sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to analyse bovine and donkey CN proteins, revealing the presence of four CN fractions in DM, and that DM proteins exhibited lower mobility than bovine milk proteins [5]. Thus, CN is low in DM [6], and the casein/whey protein ratio is closer to that of human milk than cow milk [7]. Further, DM contains 36.96% and 47.28% whey and CN proteins, respectively, whereas bovine milk contains 17.54% whey and 77.23% CN proteins [8]. The allergenic properties of DM are related to the protein fraction composition, which is much closer to human milk than cow milk. In general, it is

the low casein content and appropriate whey-to-casein ratio that reduce the risks and possibilities for DM to cause allergies. Therefore, replacing cow milk with DM is a valid option for people with CMPA. The main components of DM whey proteins include β-lactoglobulin (β-Lg; 29.9% of total whey proteins), α-lactalbumin (α-LA; 22.6%), lysozyme (Lys; 21%), and immunoglobulins (Igs; 11.5%) [9]. A previous study reported that β-lactoglobulin was not detected in human milk but was present in bovine and DM (approximately 0.32 and 0.27 g/100 mL, respectively) [10]. Another study reported that the grand average hydropathy (GRAVY) scores of β-Lgs from bovines and donkeys were significantly different [11]. The GRAVY scores reflect the levels of hydrophobic and hydrophilic amino acids in the proteins, providing insights into the amino acid composition of bovine milk and DM. In addition, DM contains 35–50% whey protein compared to the 20% in bovine milk, indicating that DM is more suitable as the main raw material for whey protein [12].

Heat treatment is essential in the production and processing of dairy products. The heating process for dairy products reduces the number of spoilage bacteria to avoid safety incidents caused by microorganismal growth and to extend the shelf life [13]. Although heat-treating raw milk ensures microbiological safety, temperature and processing time variations can cause irreversible changes in liquid milk and dairy products that affect the hydrolysis of milk proteins, the gastric clot structure, and other properties during digestion, thereby altering the functional properties and nutritional value of milk proteins [14]. In contrast to whey proteins, which are prone to severe denaturation and aggregation upon heating, CN has high thermal stability [15]. Figure 1 illustrates the mechanism of heat-induced denaturation of β-Lg in whey proteins [16]. β-Lg in whey proteins often exists as a dimer, which reversibly dissociates with increasing temperature during heat treatment to form two monomers, each with a free sulfhydryl group at the Cys121 and Cys119 sites. At 78°C, the content of monomers increases with increasing temperature [17]. As the temperature further increases to 85°C, the disulfide bonds break, and the protein unfolds, completely exposing the hydrophobic groups within the molecule. As the temperature increases further, an intramolecular reaction occurs, creating new disulfide-bonded polymers from the free sulfhydryl groups [18], aiding in the formation of aggregates with CN micelles [19]. Luo et al., aiming to investigate the structural characteristics and processing properties of whey proteins in DM after heating, studied CN micelles in donkey milk and found that CN was thermally unstable [15].

Given the nutritional benefits of DM and the detrimental effects of improper heat treatment, this study assessed the thermal stability of DM whey proteins to provide a reference for the optimal thermal processing of DM dairy products and enhance the use of DM whey protein in the food industry.

2. Materials and Methods

2.1. Sample Collection. The DM used in this study was purchased from Dong-E-E-Jiao Co., Ltd. (DeZhou, China). DM was collected from 86 donkeys from DeZhou, China, 30 days postpartum, using a vacuum pump automatic milker (XD025; Zibo Leniu Milking Equipment Co., Ltd, China). To minimise the effect of individual differences, we divided the 86 donkeys into 3 groups (28, 29, and 29 donkeys in each group) and sampled them separately for mixing, which allowed for three biological replicates. Each sample of DM was collected in three batches during the morning, midday, and evening using a vacuum-pumped automatic milker machine. The samples were subsequently mixed to eliminate the effect of sampling time points. The samples were placed in dry ice, transported to the laboratory at the ranch, and stored in a cryogenic refrigerator at −80°C. Once all samples were collected, they were shipped to Shenyang (Liaoning, China) in an insulated box with ice packs for further processing and analysis.

2.2. Sample Preparation. After sampling, the raw DM samples were pasteurised in a thermostatic water bath at 62–65°C for 30 min to kill most of the growing pathogenic bacteria [20]. The pasteurised DM was centrifuged for 60 min in a high-speed centrifuge (CR21N, Hitachi, Japan) at 4°C with 10,000 rpm to remove cellular impurities and fat from the DM [7], referred to as defatted DM.

Whey proteins were separated by isoelectric point precipitation, as reported by Criscione et al., with slight modifications [21]. The defatted DM was titrated with 1 M HCl to achieve a pH of 4.6 after 20 min reaction, the solution was centrifuged at 8000 rpm, and the supernatant was collected. The whey proteins were then subjected to heating treatments using an Intelligent Thermostatic Milk Mixer (HBM-H118E; Haier, Qingdao, China). Parameters were set at 75°C, 85°C, and 95°C, and once when the centre temperature met the specified requirement, heating was conducted for 15 s and 3 min, respectively, followed by cooling in an ice bath. The whey proteins extracted after pasteurisation were used as a blank control. Finally, the whey protein solution was placed in a vacuum condensing chamber at −56°C for 48 h with a maximum pressure of 0.3 mbar [21] to obtain freeze-dried DM whey proteins (more than 95% of the water in the samples was excluded). The protein content of the freeze-dried samples without heat treatment was determined by the Folin–Lowry method [22] and found that the protein content was 85.15%. Its standard curve is $y = 0.0621x + 0.0026$ ($r^2 = 0.9993$).

2.3. SDS-PAGE Analysis. SDS-PAGE, which separates proteins based on the molecular weights allowing for whey protein identification, was performed as described previously [23, 24]. Acrylamide stacking gel (pH 8.8) and acrylamide running gel (pH 6.8) concentrations were 5% and 12%, respectively. Whey protein samples (10 μL, 10 mg/mL) were mixed with buffers (10 μL; SDS, DTT, Tris-HCl, bromophenol blue, and glycerol). The mixture was analysed using a dual vertical electrophoresis system (BG-verMIDI, Beijing Baygene Biotech Co., Ltd., China). The initial voltage was set at 80 V, followed by continuous operation at 120 V for 2 h. Images of the gel were analysed using Image Lab (Gel Doc XR; Bio-Rad Laboratories, USA).

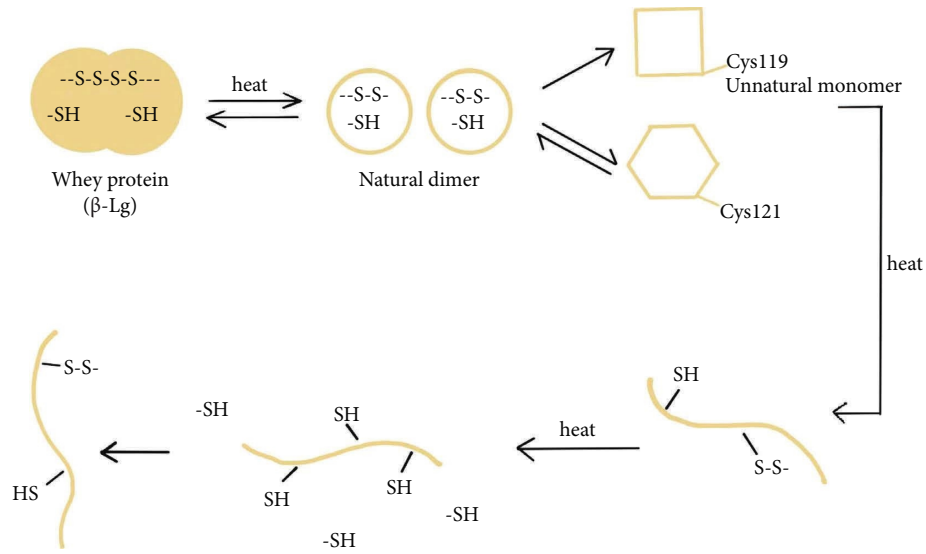


FIGURE 1: β-Lg heat denature process referred from [16–18]. The β-lactoglobulin dissociates to form two monomers, and protein structure is altered during heating.

2.4. Sulfhydryl Bonds. Whey protein solution (0.5 mL of a 10 mg/mL solution) was added to 2.0 mL of Tris-Gly buffer and 50 μL of 4 mg/mL 5,5′-dithiocarbamate (2-nitrobenzoic acid) (DTNB) (Shenyang Lab Science and Trade Co., Ltd., Liaoning, China). The absorbance at 412 nm was measured to quantify the molar concentration of the sulfhydryl groups [25].

$$C_{-\mathrm{SH}}\left(\frac{\mu\mathrm{mol}}{g}\right) = \frac{10^6 \times A_{-\mathrm{SH}} \times D}{13600 \times C}, \quad (1)$$

where $C_{-\mathrm{SH}}$ is the sulfhydryl (μM/g) content; $A_{-\mathrm{SH}}$ is the absorbance at 412 nm; C is the sample concentration (mg/mL); D is the dilution factor (5.02 for this study); and 13600 M^{-1} cm^{-1} is the extinction coefficient.

2.5. Circular Dichroism (CD) Spectrum. The CD of the proteins was determined using a CD spectrometer. Whey protein was prepared in phosphate-buffered saline (PBS; 0.1 M, pH 7.0) to form a 0.5 mg/mL whey protein solution. Using a 1 cm path length cell, the far-ultraviolet (UV) CD spectra at 190–260 nm (25°C) were recorded. The CD spectrum was collected and adjusted to the baseline buffer [26].

2.6. Fourier Transform Infrared (FTIR) Spectroscopy. The whey proteins were combined with KBr powder (Shenyang Lab Science and Trade Co., Ltd., Liaoning, China) (1 : 100 w/w). The homogeneous samples were compacted into a 13 mm disc for the FTIR spectroscopic analysis. The FTIR spectra between 4000 and 400 cm^{-1} were recorded (25°C) and scanned 32 times at a rate of 4 cm^{-1} [27]. Using curve-fitting, second-order derivatives, and Fourier self-deconvolution techniques, the secondary structures of whey proteins with amide I bands were quantified using PeakFit 4.12 software (Systat Software, San Jose, CA, USA).

2.7. Ultraviolet-Visible (UV-Vis) Absorption Spectra Measurement. The whey protein solution (0.2 mg/mL) was prepared with 0.1 M PBS buffer (pH 7.0) and incubated for 10 min. A UV-2550 spectrophotometer was used to scan the UV-vis absorption spectra of the diluent samples at a rate of 2 nm/s at room temperature in the 200 to 400 nm range [28].

2.8. Fluorescence Spectra Measurement. Fluorescence spectra were detected in a 1 cm wide quartz cuvette using an F-7100 fluorescence spectrophotometer. The whey protein samples were diluted to 0.2 mg/mL with PBS buffer (pH 7.0, 0.01 M) for measurement. The fluorescence photometer excitation and emission wavelengths were 290 nm and 300–460 nm, respectively. The working voltage was 750 V, and the slit widths were set to 5 nm with a 5 nm/s scan rate [27].

2.9. Three-Dimensional (3D) Fluorescence Spectroscopy. 3D fluorescence spectroscopy of samples (1 mg/mL) was obtained by a fluorescence spectrophotometer (F-4600, Hitachi, Japan). The excitation and emission wavelengths were set at 200–500 nm and 200–600 nm with an increment of 5 nm.

2.10. Surface Hydrophobicity. Various whey protein samples were prepared in PBS (pH 7.0), and then, 4.0 mL of the solution was mixed with 4 μL 8-anilion-1-naphthalene-suifonic acid (ANS, 8 mM) (Shenyang Lab Science and Trade Co., Ltd., Liaoning, China) solution [29]. The solution was stored in the dark for 15 min, and then, fluorescence emission spectra were acquired at 400–690 nm using an F-2700 fluoresce spectrophotometer (Hitachi, High-Tech, Tokyo, Japan) at an excitation wavelength of 385 nm. The initial slopes of the protein concentration curves and fluorescence intensities were obtained based on linear fitting [27], and the area formed by the scanned and horizontal coordinates was the surface hydrophobicity of the protein sample.

2.11. Water and Oil Retention. First, 0.5 g of whey protein (100 mg/mL) was weighed and placed into a 10 mL dry centrifuge tube (m_1). Then, 5 mL of water or oil was added to the tube and immediately shaken to mix using a vortex mixer (vortex-1; Shanghai Huxi Industrial Co., Ltd., China). The tube was incubated at 25°C for 0.5 h. Subsequently, the protein samples were centrifuged at 4000 rpm for 30 min, and the supernatant was discarded [30]. Finally, the total masses of the tube and residual material were recorded (m_2) [31]. The water (or oil)-holding capacity (WHC or OHC, respectively) of the whey protein was calculated as the ratio of the total mass of residual and centrifuge tube centrifugation minus the weight of whey protein and centrifuge tube to the weight of samples.

2.12. Foaming Capacity (FC) and Foaming Stability (FS). First, 2 g of whey protein was weighed and dissolved in 100 mL of distilled water. Then, 20 mL of this solution (V) was homogenised at 10,000 rpm for 2 min using a high shear homogeniser (XHF-DY; NingBo Scientz Biotechnology Co., Ltd., China). After homogenisation, the foam volume was recorded at 0 min (V_0) and 30 min (V_{30}) of standing [28]. FC (%) and FS (%) were calculated as follows:

$$\begin{aligned} \text{FC}\ (\%) &= \frac{V_0 - V}{V_0} \times 100, \\ \text{FS}\ (\%) &= \frac{V_{30}}{V_0} \times 100, \end{aligned} \tag{2}$$

where V, V_0, and V_{30} are the initial sample, foam at 0 min, and foam at 30 min volumes, respectively.

2.13. Preparation of the Emulsions. Whey protein samples obtained by different heat treatments (300 mg) were completely dissolved with 10 mL of distilled water using a magnetic stirrer to obtain whey protein solutions (30 mg/mL). Then, soybean oil (2.5 mL) was added into the solution and the mixture was homogenised for 3 min in a high-speed homogeniser at 12,000 rpm to obtain the emulsion.

2.13.1. Emulsion Microstructure. A microscope equipped with a camera was used to assess the microstructure of the whey protein emulsion, prepared as described above, and images were captured [32, 33].

2.13.2. Emulsifying Activity (EAI) and Emulsifying Stability (ESI). The emulsification properties of the samples were measured as previously reported with slight modifications [34]. The emulsion was pipetted out of the tube at 0 min and immediately diluted 100-fold with SDS (0.1% (w/v)). The absorbance was measured at 500 nm. A blank control was created using a 0.1% SDS solution. The EAI (m^2/g) and ESI (%) were calculated as follows [35]:

$$\text{EAI}\left(\frac{m^2}{g}\right) = \frac{2 \times 2.303 \times \text{DF} \times A_0}{\varphi \times c \times L \times 10000}, \tag{3}$$

where c is the whey protein concentration (1 mg/mL), DF is the dilution factor (100), φ is the oil volume fraction (0.25), L is the optical path width (1 cm), and A_0 is the absorbance of the emulsion after homogenisation at 0 min.

After the emulsion was allowed to stand for 15 min, the absorbance was measured as previously described, and the ESI values were determined based on the following equation:

$$\text{ESI (min)} = \frac{A_{15} \times \Delta \text{t}}{\Delta A}, \tag{4}$$

where A_{15} is the absorbance of the emulsion after homogenisation at 15 min, ΔA is the difference in absorbance of the emulsion after 15 min, and Δt is the time of 15 min.

2.13.3. Creaming Index (CI). First, 10 mL of emulsion was stored in a sealed glass vial, and the emulsion phase was observed after incubation. The heights of the lower layer and emulsion were recorded at regular intervals. The CI calculation is as follows [36]:

$$\text{CI}\ (\%) = \frac{H_S}{H_T} \times 100, \tag{5}$$

where H_S and H_T are the heights of the supernatant after emulsion layering and the emulsion sample, respectively.

2.14. Rheological Properties. The apparent viscosity of donkey whey protein (10 mg/mL) was determined using a rheometer to characterise their rheological properties [37]. The shear rate range was set to 0.1 s^{-1}–200 s^{-1}, and the entire test was conducted at 25°C. Curves were plotted and fitted by recording the shear rate and apparent viscosity values.

2.15. Statistical Analyses. All experiments were repeated three times; mean values were obtained using Excel (Microsoft Corporation, Redmond, WA, USA). Experimental data were analysed by one-way analysis of variance and multiple Duncan comparisons ($p < 0.05$) using SPSS version 17 (IBM Inc., Armonk, NY, USA). Data are presented as means ± standard deviations. The results were plotted using Origin 2017 software (OriginLab Corporation, Northampton, MA, USA), and PeakFit 4.12 was used to fit the infrared spectra data.

3. Results and Discussion

3.1. Whey Protein Composition. The DM whey protein composition in this study (analysed by SDS-PAGE) was similar to that reported previously [20]. The extracted material was a high-purity whey protein that contained very low levels of nonwhey protein components, such as CN (Figure 2(a)). The staple components of whey protein included α-lactalbumin, β-lactoglobulin, serum albumin, immunoglobulin, and lactoferrin. In each lane, the bands from top to bottom (i.e., relative molecular mass from largest to smallest) were lactoferrin, serum albumin, immunoglobulin, αs1-casein, β-lactoglobulin, and α-lactalbumin [38]. The clearest protein bands were α-lactalbumin

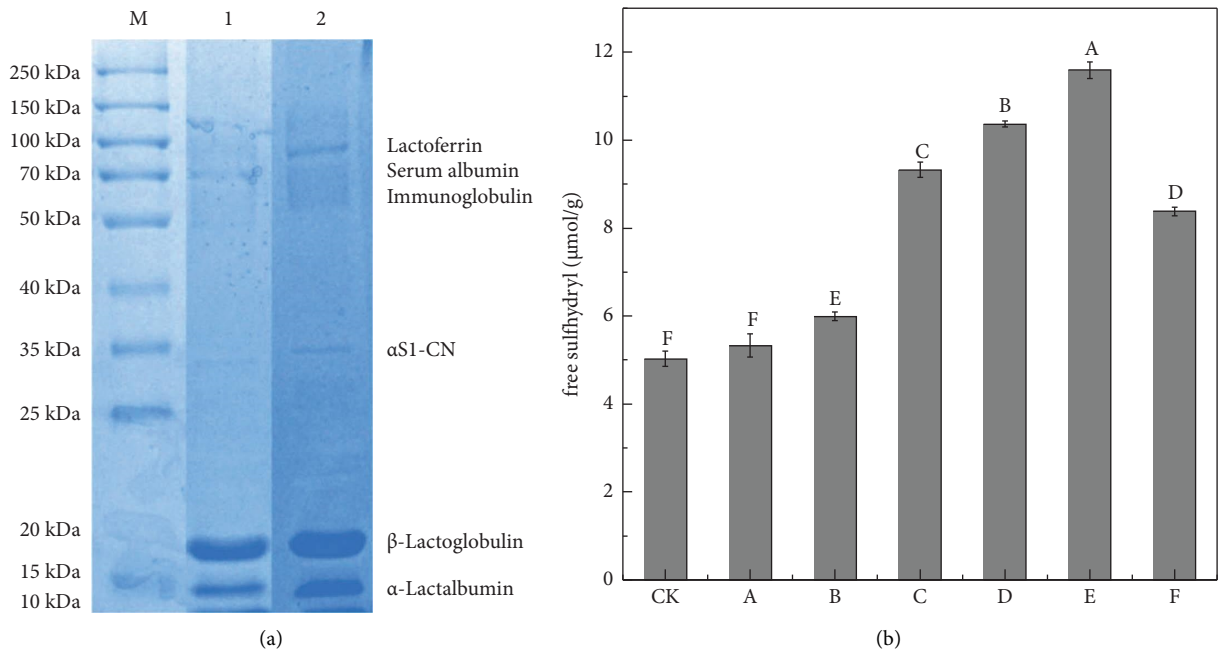


Figure 2: Donkey milk whey protein extracted by different concentrations analysed by SDS-PAGE (a). Lanes: (M) molecular weight marker (MWM), with sizes in kDa indicated on the left, (1) 8 mg/mL, and (2) 10 mg/mL. The content of free sulfhydryl group of donkey whey protein with different heat treatments (b). CK: control group (pasteurised donkey milk whey protein), A: 75°C/15 s, B: 75°C/3 min, C: 85°C/15 s, D: 85°C/3 min, E: 95°C/15 s, and F: 95°C/3 min. Error bars indicate the standard deviation from triplicate tests. Different letters indicate significant differences ($p < 0.05$).

(14.2 kDa) and β-lactoglobulin (18.4 kDa), indicating that they constituted the greatest portion. The composition of donkey and bovine milk is relatively different, with DM whey proteins being rich in lysozymes (21.03%), whereas only traces of lysozyme are present in bovine milk [9]. El-Hatmi et al. compared five milk samples using SDS-PAGE analysis, including bovine milk and DM, and found that lysozymes are present only in DM and that the α-lactalbumin content was considerably higher in DM (1.43 g/L) than in bovine milk (0.5 g/L) [39]. Moreover, Zhang et al. compared the protein composition of various milk sources using SDS-PAGE and reported that DM contained significantly higher levels of β-lactoglobulin and α-lactalbumin than other milk [40]. Ozturkoglu-Budak investigated the effects of freezing and heat treatments at different temperatures on the stability of DM, reporting that heat treatment influenced the content of the components in DM whey proteins and that heating reduced their concentrations [38]. The main components of the whey protein did not differ between the 8 and 10 mg/mL concentrations, but the 10 mg/mL bands were more heavily coloured. Furthermore, all samples retained a CN component, probably because of the short extraction time or insufficient separation time and speed, resulting in incomplete separation.

3.2. Sulfhydryl Bonds. Changes in the sulfhydryl content indicate variations in -S-S- bonds and play an important role in partially denatured proteins [41]. We found that heating denatured the whey protein samples to various degrees depending on the amount of free sulfhydryl groups. The level of sulfhydryl bonds considerably increased (from 5.02 to 11.59 μM/g) as the temperature increased. Furthermore, the free sulfhydryl content decreased as the temperature increased but was still higher than the control group after 3 min of heat treatment at 90°C (Figure 2(b)). Presumably, the breaking of the interchain -S-S- bonds increased the number of sulfhydryl bonds after heating. However, the decrease in free sulfhydryl content was caused by the oxidation of sulfhydryl groups from overheating and their synthesis into new disulfide bonds through recombination [42]. The sulfhydryl group and other bonds (such as hydrogen bonds) of whey protein are extensively exposed to oxygen and converted to disulfide bonds, which decrease the total sulfhydryl groups and enhance the oxidation of whey protein [43, 44]. Thus, heat treatment at 90°C for 3 min changed the conformation of DM.

3.3. CD. CD spectroscopy is a crucial tool for evaluating protein folding and unfolding caused by temperature variations or chemical denaturation and, thus, investigating the secondary structure of proteins [45]. The CD spectra of native proteins contain a positive peak at 190 nm and a negative peak at 205–235 nm, which affects the conformation of the main chain [25]. For our protein samples, the CD spectra had positive peaks at approximately 192, 200, and 218 nm and negative peaks at approximately 208 and 221 nm, indicating that the α-helix of the heated whey proteins was still present. In addition, as shown in Figure 3(a), negative shoulder bands were observed at 207,

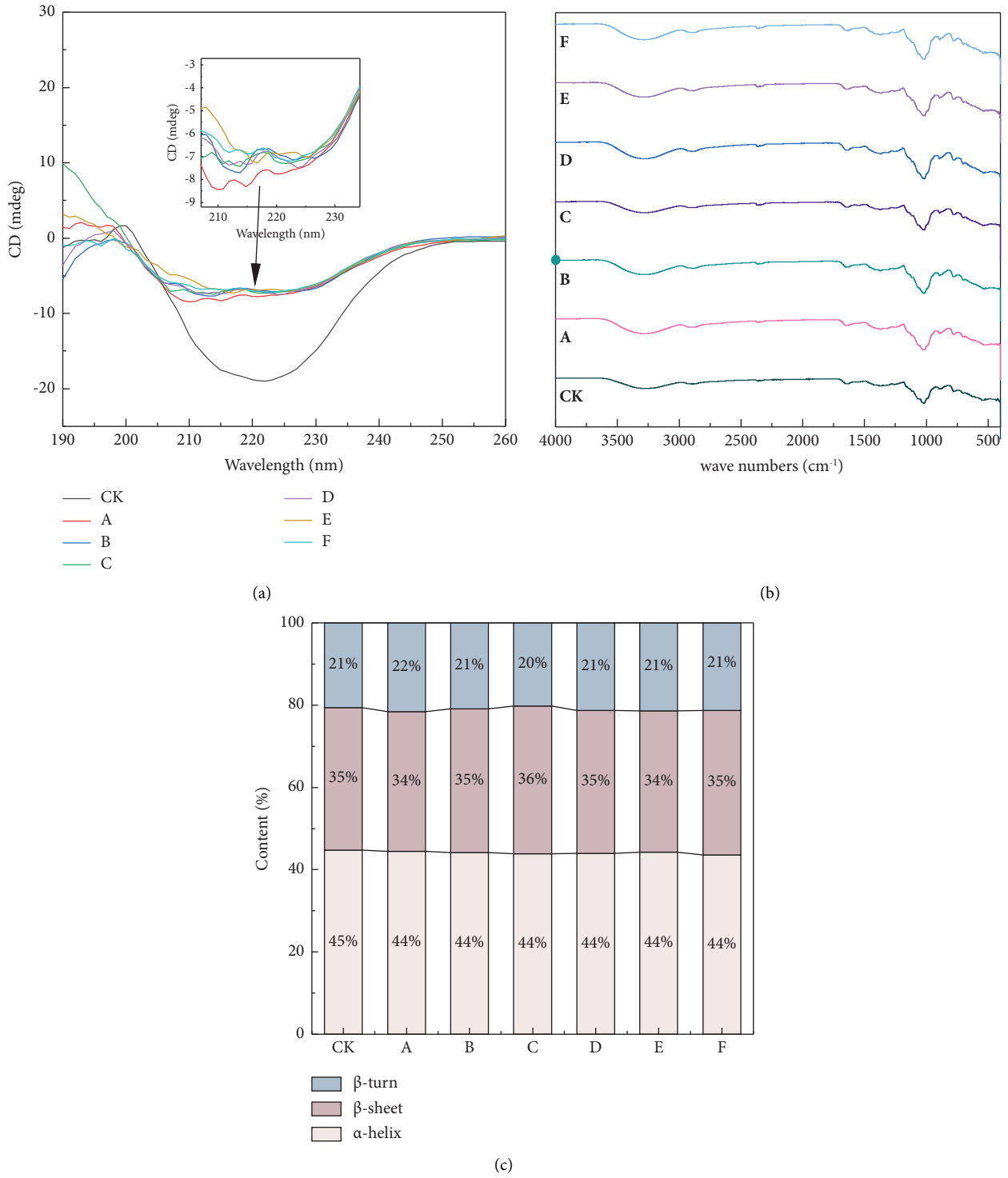


Figure 3: CD spectra of donkey milk whey protein with different heat treatments (a). Fourier transform infrared spectra of whey proteins with different heat treatments (b). Secondary structure content of whey protein under different heat treatments (c). CK: control group (pasteurised donkey milk whey protein), A: 75°C/15 s, B: 75°C/3 min, C: 85°C/15 s, D: 85°C/3 min, E: 95°C/15 s, and F: 95°C/3 min.

216, and 225 nm, suggesting the presence of β-strands. All samples had a negative peak between 207 and 210 nm and positive bands at approximately 192 nm [26], which is typical for the α-helix structure in whey proteins. Usually, the peak intensity at 222 nm directly affects the helical content of a protein [35]. The intensity of the control group's negative peak at 222 nm was noticeably higher than that of the heated protein, indicating that a decrease in the α-helix content of heated proteins, likely due to the disruption of the secondary structures of the proteins and loosening of the tightly bound whey proteins caused by heating, making the stable α-helix structure less. In particular, the intensity of the

samples exhibited a tendency to weaken with increasing temperature and time. At 75°C/15 s, a higher peak intensity was observed, indicating that the structural content was more affected by temperature. This conclusion was consistent with a previous study comparing the effects of four heat treatments on whey proteins, which reported that ultrahigh-temperature treatment disrupted the structure of whey proteins [46]. In general, positive and negative absorption peaks for the β-sheet and β-turn were observed at 185 nm and 216 nm, respectively; a positive absorption peak for the β-turn was observed at 206 nm. Additionally, one positive band and one negative band were observed at approximately 196 nm and 215 nm, respectively, indicating the protein contained β-strands (Figure 3(a)). Overall, the spectra indicated that the secondary structure of DM whey proteins comprised α-helices and β-strands, which was similar to a study by Zhang et al. [47], and that heating could affect the secondary structure of whey proteins, thereby reducing the α-helix content.

3.4. FTIR. A strong absorption was observed at wave numbers between 3440 and 3070 cm^{-1}, which are typically associated with the vibrations of the hydroxyl group and the N-H bond vibration (Figure 3(b)). Protein repeat units exhibit several characteristic infrared absorption bands, including amide A, B, and I-VII. A characteristic absorption peak in the FTIR spectra is the amide I band (1600–1700 cm^{-1}), which is associated with C=O, N-H stretching vibrations, and C-H. In addition, the absorbance bands at 1642 cm^{-1} were associated with a C=C stretching vibration [48].

FTIR spectra were analysed using PeakFit 4.12, and the amide I bands were selected for quantitative analysis of the secondary structural alterations in DM whey proteins (Figure 3(c)). The α-helix, β-sheet, β-turn, and random coil secondary structures corresponded to positional peaks at 1650–1660 cm^{-1}, 1600–1640 cm^{-1}, 1660–1700 cm^{-1}, and 1640–1650 cm^{-1}, respectively [49]. Figure 3(c) shows that the α-helix structure content of the heat-treated whey proteins was reduced compared to that of the CK group, indicating that the heat treatment loosened the structure of the proteins, which affected the structural stability of the proteins. Wang et al. investigated the effect of heat treatment on rice glutenin and phosphorylated rice glutenin by analysing the secondary structure content via FTIR [50]. They reported that the α-helix content of the proteins was decreased by heating for 15 min, which is consistent with our findings. This result may be attributed to heating likely causing the disruption of the hydrogen bonds between the peptide chains within the whey protein molecule, resulting in a reduction of the tightest α-helix structure. As the temperature rose from 75 to 95°C, the β-sheet content first decreased and then increased. Low-intensity heat treatment caused the β-sheet structure to transform from a rigid α-helical and β-sheet structure to a flexible β-sheet structure, indicating that the protein structure became loose and disordered, typical of protein denaturation. However, when the temperature reached 85°C, the structural disruption of the protein was more severe, potentially transforming the β-sheet structure into a more unstable random coil structure. Decreased β-sheet content indicates the exposure of hydrophobic groups within the whey protein molecule and enhanced surface hydrophobicity. Thus, heat treatment affected the stability of the secondary structure of whey proteins, and heat treatments higher than 85°C/15 s were more likely to alter their structure.

3.5. UV-Vis Absorption Spectra. UV-vis absorption spectra reflect changes in protein conformation via chromophores (aromatic amino acid side chains) [51]. The absorption peak at 280 nm fluctuated, suggesting that it corresponded to aromatic amino acid residues, such as phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr) [52]. Figure 4(a) presents the UV-vis spectra of whey proteins exposed to various temperatures, all with identical characteristic absorption peaks (UV regions) [53]. The absorbance values of the heated whey protein groups were all greater than that of the control group. Moreover, the absorbance of the 3 min group was higher than that of the 15 s group, indicating that prolonged heating at the same temperature can increase the exposure of tyrosine and tryptophan groups. However, the absorbances of the 95°C/15 s and 95°C/3 min groups trended downward compared to the 75°C/3 min group but were still higher than that of the control group. The absorption of aromatic amino acid residues at 280 nm reached the maximum level for the 75°C/3 min group, potentially owing to the exposure of aromatic amino acid residues to heated conditions, which altered the chromophore microenvironment [23]. However, when the temperature exceeded 85°C, the excessive temperature caused complete denaturation of the whey protein structure and aggregation, which resulted in reduced absorption and the appearance of some nonpolar aromatic amino acid residues that are partially masked [52]. This conclusion was consistent with the findings of Wang et al. who explored the mechanism of heat-induced gluten gel formation in wheat [23]. Therefore, 85°C is the recommended critical temperature for DM whey protein in processing.

3.6. Intrinsic Fluorescence Spectra. Fluorescence spectroscopy is used to obtain information on protein structure by determining the tertiary structure of proteins through the degree of exposure of Trp and Tyr residues with endogenous fluorescence properties in protein molecules. After heat treatment, the fluorescence intensity of DM whey proteins increased, causing a weak redshift in the absorption peak (Figure 4(b)). As the temperature increased from 75 to 95°C, the fluorescence intensity of the whey proteins reached a maximum value (681.7) at 85°C for 3 min. However, at 95°C, the absorption peak decreased. This result might be due to a less stable ANS-protein combination brought on by a severe thermal treatment that altered the structure of the surface hydrophobic binding sites [54]. These findings suggested that heating causes protein unfolding, fully exposing the Trp and Tyr residues hidden inside the protein

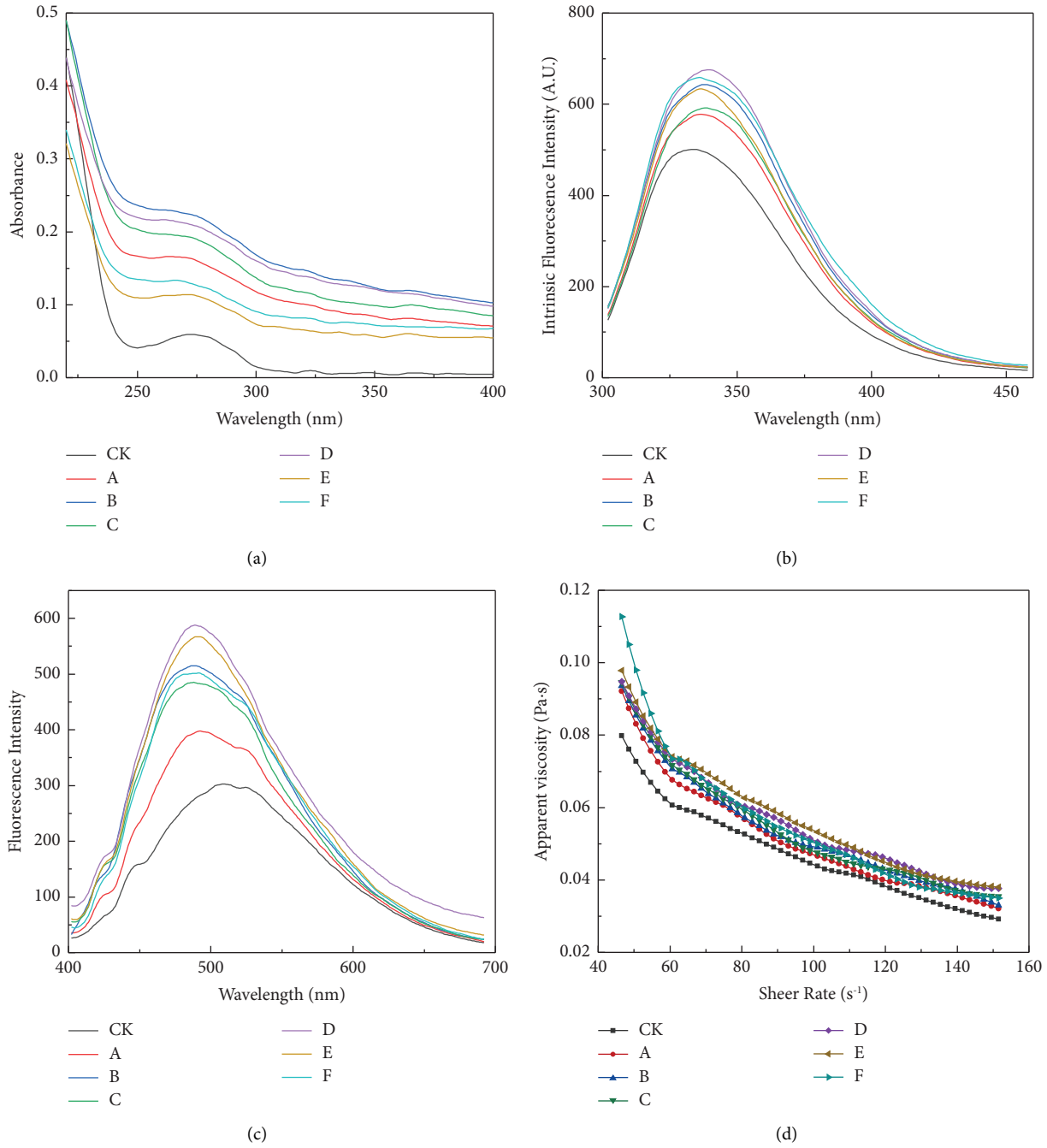


Figure 4: Ultraviolet absorption spectra of whey protein with different heat treatments (a). Intrinsic fluorescence spectra of whey protein with different heat treatments (b). Surface hydrophobicity of whey protein (c). Emulsion viscosity of whey protein (d). CK: control group (pasteurised donkey milk whey protein), A: 75°C/15 s, B: 75°C/3 min, C: 85°C/15 s, D: 85°C/3 min, E: 95°C/15 s, and F: 95°C/3 min.

molecule, increasing the fluorescence intensity, and causing a redshift [23]. This result was consistent with the findings of Wang et al. who compared the intrinsic fluorescence of wheat gluten proteins by heating and found that heating red-shifted the λ_{max} of the proteins and altered the fluorescence intensity with different heat treatment temperatures [55]. As changes in the heat-induced protein structure bury Trp residues, a "masking" effect is observed for whey proteins in fluorescence analyses [56].

3.7. 3D Fluorescence Spectroscopy. 3D fluorescence spectroscopy provides more detailed information on protein conformational changes [57]. Allahdad et al. reported that the two peaks observed in the 3D fluorescence pattern of the protein are caused by the $\pi \longrightarrow \pi^*$ leap of the aromatic heterocyclic for Tyr and Trp residues and the $n \longrightarrow \pi^*$ leap of the polypeptide backbone, respectively [57]. The contours of the 3D fluorescence spectra of the whey proteins are presented in Figure 5, and the peak intensities and

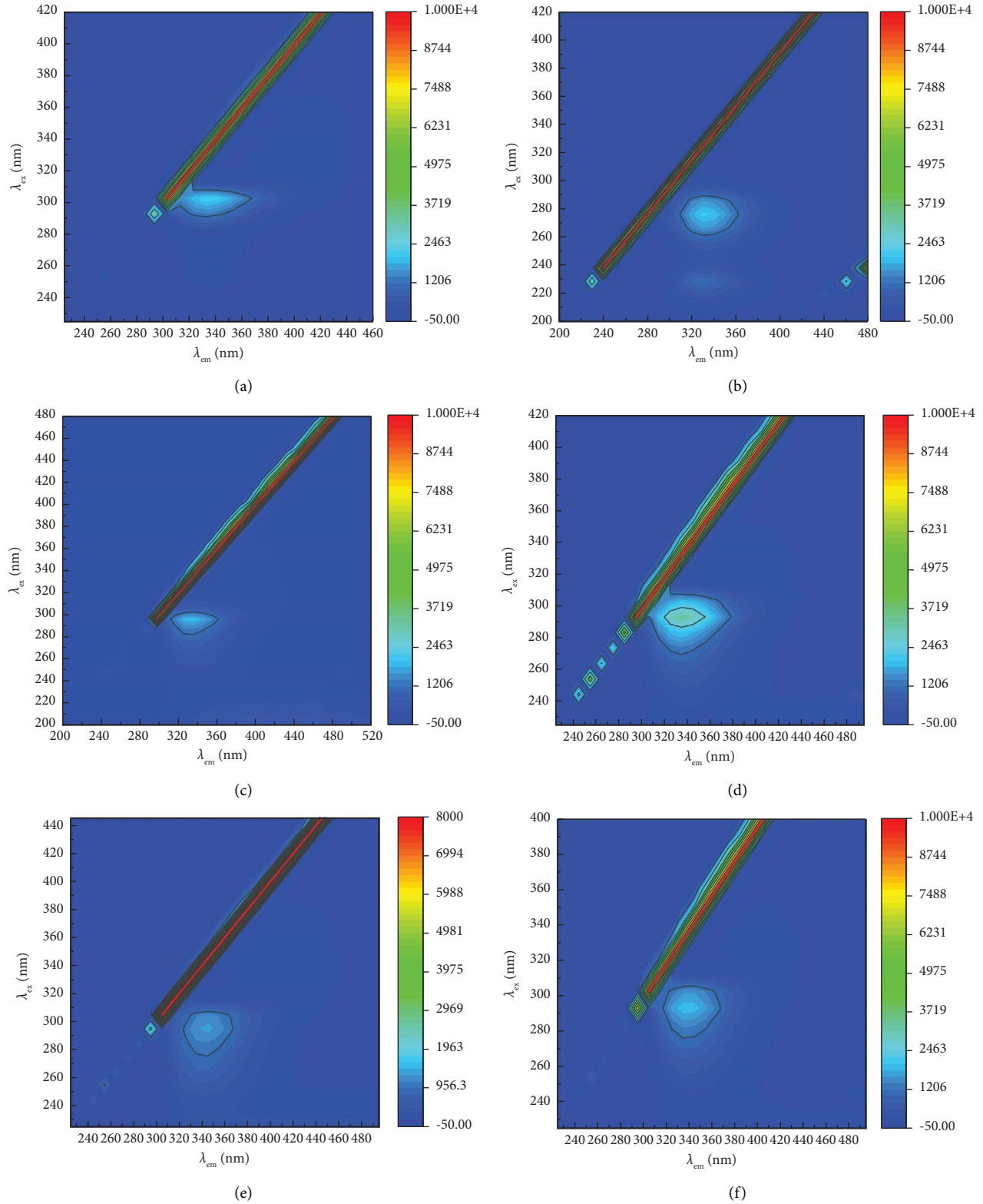
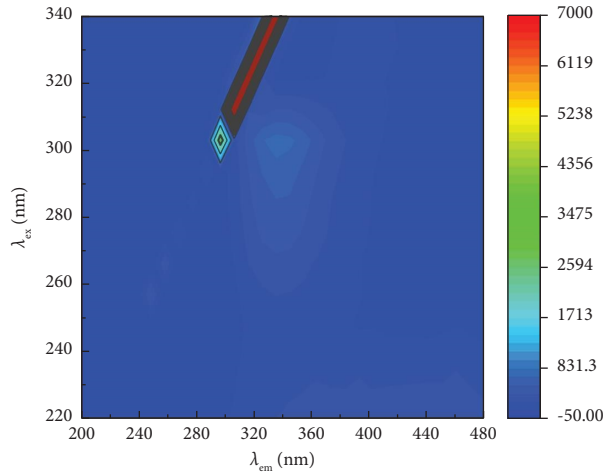


Figure 5: Continued.



(g)

Figure 5: Three-dimensional fluorescence map of heat-treated whey protein. (a) CK: control group (pasteurised donkey milk whey protein), (b) 75°C/15 s, (c) 75°C/3 min, (d) 85°C/15 s, (e) 85°C/3 min, (f) 95°C/15 s, and (g) 95°C/3 min.

positions are summarised in Table 1, providing insights into the microenvironmental and conformational changes in heated whey proteins. The peak ($\lambda_{ex} = 280$ nm) corresponds to the fluorescence property of Tyr and Trp residues. The maximum fluorescence intensity and peak positions differed based on the heat treatment intensities, demonstrating that heat treatment changed the tertiary structure of whey proteins (Table 1). The intensities of whey proteins heated for 15 s were all increased, whereas excessive heating resulted in samples with lower intensity than that of the control (Table 1). Moreover, as the heat treatment intensity increased, the peak positions trended towards a redshift and then a blueshift. At the same treatment temperature, the peak positions differed with time. Specifically, the peak positions of all heat treatment groups, except the 95°C/3 min group, red-shifted as the temperature increased. The peaks in the fluorescence reflect the characteristic bands of the Trp and Tyr residues in the protein [58]. Heat treatment exposes the aromatic amino acids inside the protein, increasing the polarity of the environment in which the chromogenic group is located. However, for all the 3 min heat treatment groups, prolonged heating caused conformational changes in the protein molecules to varying degrees, and in some cases, protein molecules aggregated, resulting in a decrease in peak intensity. The 95°C/3 min group exhibited a blueshift due to aggregation between proteins and a decrease in the ambient polarity of the colour-forming groups.

Under different heat treatment times at the same temperature, the fluorescence intensity decreased with increasing time. Specifically, the fluorescence intensity of the three groups decreased with increasing temperature after 15 seconds of heat treatment; however, the intensities in these groups increased and then decreased with increasing temperature. In other words, heat treatment exposed the internal aromatic amino acids of the proteins, increasing fluorescence intensity increased. The maximum

Table 1: Peak position and peak intensity of three-dimensional fluorescence spectrum of donkey milk whey protein.

Samples	Position ($\lambda_{ex}/\lambda_{em}$) (nm)	Intensity	Change (%)
CK	280/330	2120	—
75°C/15 s	300/335	2795	24.11
75°C/3 min	300/330	2005	5.42
85°C/15 s	295/335	3277	63.44
85°C/3 min	300/345	1406	57.09
95°C/15 s	300/340	2143	52.42
95°C/3 min	300/340	753.1	64.86

fluorescence intensity was 3277 at 85°C/15 s. The fluorescence intensity of the 3 min heat-treated group was lower than that of the control group, perhaps because of the continued polar environment of the chromogenic group due to prolonged heating, which caused fluorescence quenching of the chromogenic group of the whey protein. Zhu et al. investigated the mechanism underlying the whey protein-ligand interaction, revealing that this interaction could lead to significant loosening and unfolding of the protein backbone, leading to a reduction in the 3D fluorescence peak [59]. In the present study, whey proteins were subjected to excessive heat treatment and aggregation occurred by structural disruption, resulting in a similar reduction in 3D fluorescence peaks. Moreover, the aggregation of protein molecules reduces exposure to hydrophobic amino acids, such as Trp, altering the protein's tertiary structure. Therefore, when whey protein was heat-treated at 85°C for 15 s, the 3D fluorescence intensity reached a maximum, and a further increase in temperature was rather detrimental to the structural integrity of the protein.

3.8. Surface Hydrophobicity. Surface hydrophobicity is used to identify structural changes that characterise the surface exposure of hydrophobic sites [58]. Surface hydrophobicity is important for the biological function of proteins, related to

the antigenicity and immunogenicity of proteins, and it affects solubility and other functional properties, such as emulsification and foaming [35]. The whey protein surface hydrophobicity first increased and then decreased when heated at different temperatures (Figure 4(c)) [25]. The samples with the lowest surface hydrophobicity were the pasteurised samples. The proteins became more hydrophobic after being heated, and surface hydrophobicity was maximised (587.8) when they were heated at 85°C/3 min. Heating causes the overall structure of whey proteins to change from folded to unfolded, exposing originally buried hydrophobic residues to the surface and increasing the surface hydrophobicity of heated whey proteins [60, 61]. The surface hydrophobicity exhibited a downward trend after 85°C/3 min, and the effect of prolonged heating was more pronounced on the whey protein structure, which was fully unfolded by heat and repolymerised into a macromolecular group, altering the original hydrophobicity. This process requires high hydrophobicity and a certain thermal input, in which the polymerisation of subunits in hydrophobic interactions forms soluble or insoluble aggregates. Thus, prolonged or high-temperature heat treatment above 85°C is detrimental to the structure of whey proteins.

3.9. Water and Oil Retention. The WHC determines a protein's functional properties and its ability to bind water [62]. The WHC and OHC values significantly increased as the temperature increased (Figures 6(a), 6(b). Furthermore, the heated samples had a higher WHC value than the control group ($14 \pm 0.29\%$). When whey protein underwent heating, the WHC increased significantly. Among the treatment groups, the WHC was the highest in the 95°C/15 s group ($56 \pm 0.33\%$), followed by the 95°C/3 min group ($43.6 \pm 1.34\%$) ($p < 0.05$). An inflection point in WHC occurred at 85°C for 15 s of heat treatment in whey protein, and WHC increased again with a continued rise in temperature. This phenomenon aligns with the findings of Jinpeng Li et al. that low temperatures contribute to the unfolding of the protein structure, stretching the protein molecular chain, and exposing various groups to facilitate binding to water molecules [41]. However, our structural analysis suggests that when the temperature exceeds 85°C, macromolecular aggregation of whey proteins occurs and intermolecular interactions increase the adsorption capacity of water molecules. While this phenomenon can increase the WHC, it is not conducive to the digestion and absorption of proteins. Therefore, 85°C is the ideal condition for heat treatment.

Figure 6(b) presents the effects of heat treatment on the oil-holding properties of whey protein, which significantly differed from the control group ($p < 0.05$). The OHC of DM whey proteins differed based on the heat treatments. The OHC of the control group was 146%. Among the treatment groups, the highest OHC was 191% in the 95°C/3 min group. Heat treatment of whey proteins improved the OHC, which was similar to the finding of Mesfin et al. that roasting chickpea proteins at 150°C improved the OHC [63]. Whey protein is a tightly packed spherical structure. Thus, heat treatment stretches and dissociates the whey protein structure, exposing the hydrophilic side chain groups buried within the whey protein molecule to the surface of the molecule, altering the hydration capacity of the protein and affecting its WHC and OHC.

3.10. Foaming Properties. The foaming properties of proteins reflect changes in the structure and characteristics of the protein molecules, such as solubility and interactions. Figure 6(c) presents the FC and FS results. Overall, heating reduced the protein's ability to foam. The foam volumes gradually declined for whey proteins heated to 75–95°C, although FS improved [64]. Furthermore, FC increased by 11.6% when heated to 75°C, but as the temperature increased, the FC decreased. However, the foam was essentially unstable. FS generally reflects the ability of a foam to maintain its properties over time. Compared to the control group, heat-treated whey proteins had a relatively higher FS, and the proper heating temperature positively affected the FS enhancement of whey proteins, specifically at 85°C/15 s. The FS of the 85°C/3 min and 95°C/15 s groups did not differ, but the FS of the 95°C/3 min treatment was significantly lower than 85°C/15 s. This could result from protein-protein intermolecular interactions, which increased the quantity of the protein necessary to form a stable protein membrane at the interface, enhancing the foam's stability. Thus, whey protein exhibited improved foaming properties, particularly in terms of foam stability, when heated at 85°C for 15 s.

3.11. Emulsifying Properties. The emulsion properties are intuitively reflected in the morphology images (Figure 7(a)). The emulsion droplets differed in size and were unevenly distributed across all samples, whereas the droplets in the control group were slightly aggregated. When the samples were heated at 85°C/15 s, the emulsion droplets were evenly dispersed, the droplet particles were small, and there was no obvious aggregation phenomenon between the droplets, illustrating that the stability of the emulsion under this condition from a microscopic perspective was well characterised. However, as the temperature increased, obvious flocculation and a significant increase in particle size between the droplets were observed, indicating that the emulsion was severely unstable under these conditions, particularly at temperatures up to 95°C where aggregation occurred.

The emulsifying abilities of dietary proteins are typically assessed using EAI and ESI. Figure 7(b) presents the emulsification properties of whey proteins [33]. At the same temperature and different heating times, emulsification increased and then decreased with time. At different temperatures, emulsification increased with increasing temperature. The emulsification of the control group was $1.67 \pm 0.06\ m^2/g$, which increased with increasing heat treatment intensity, improving to $2.84 \pm 0.13\ m^2/g$ at 95°C/3 min. The 95°C/15 s group had the highest EAI among the seven heated protein sample groups ($p < 0.05$) [35]. Overall, moderate heat treatment was favourable for increasing emulsification; however, high temperatures and treatment

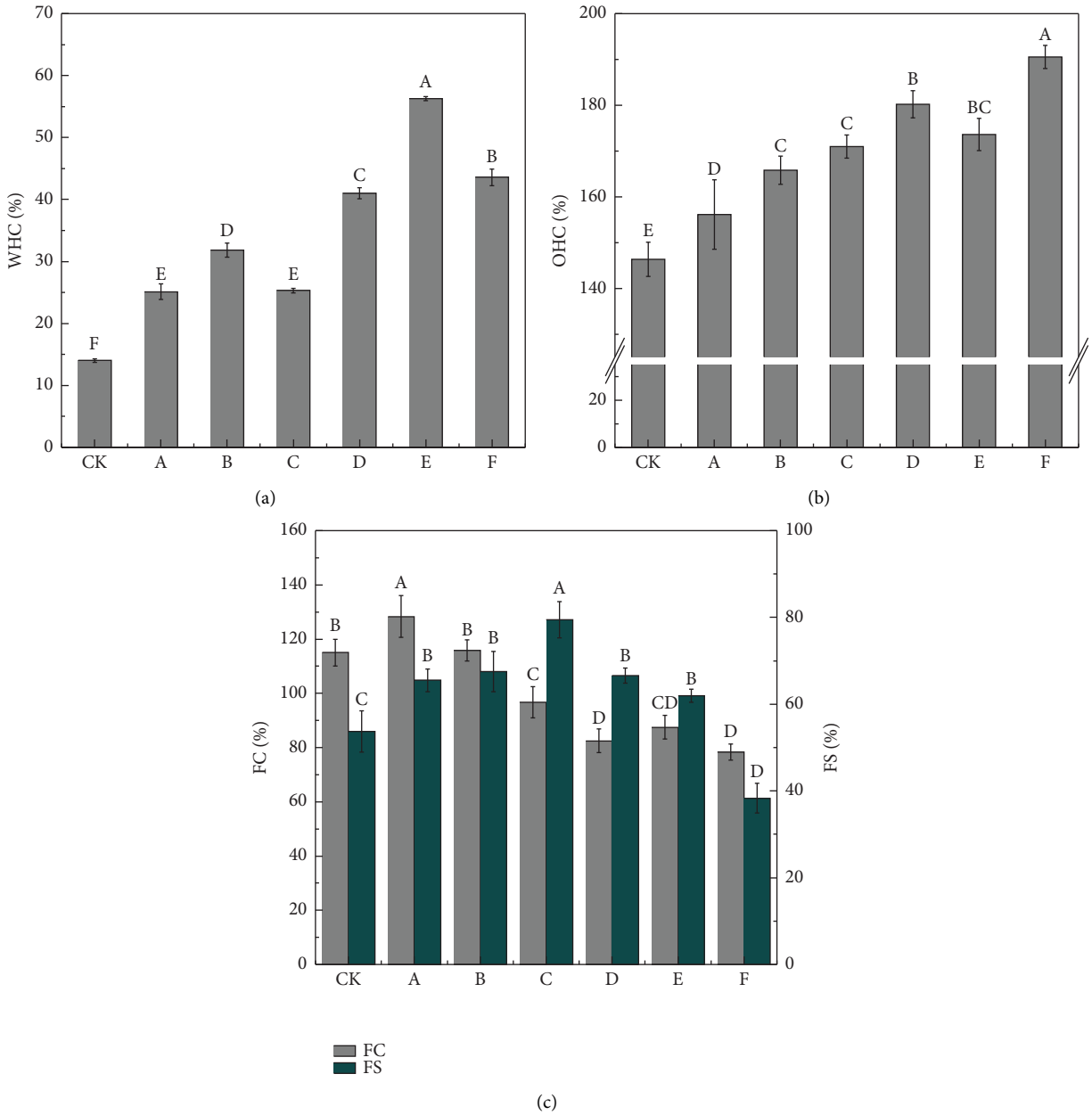


Figure 6: Water retention (WHC) and oil retention (OHC) of whey protein under different heat treatments (a, b), respectively. Foam capacity (FC) and foam stability (FS) analysis for whey protein (c). CK: control group (pasteurised donkey milk whey protein), A: 75°C/15 s, B: 75°C/3 min, C: 85°C/15 s, D: 85°C/3 min, E: 95°C/15 s, and F: 95°C/3 min. Error bars indicate the standard deviation from triplicate tests. Different letters indicate significant differences ($p < 0.05$).

times caused a decrease in emulsification, consistent with previous findings that the EAI and ESI of whey proteins can be greatly improved if denaturation is performed at the appropriate stage [32].

The ESI was similar to the EAI in that both increased and then decreased. The ESI increased with heat treatment (the maximum value was 20.16 ± 1.29 min in the 75°C/3 min group) and then decreased with increasing temperature; the minimum ESI was 13.62 ± 0.17 min in the 95°C/15 s group. The ESI in the control group was 14.57 ± 0.14 min (Figure 7(b)). Notably, the ESI in the 75°C/3 min group was nearly 1.4-fold higher than that in the control group ($p < 0.05$).

The CI was used to determine the physicochemical stability of the emulsions. In general, the larger the emulsion index, the faster the droplets move and the more likely they are to aggregate, indicating poor physical stability of the emulsion. Figure 7(c) presents the creaming index results. The emulsion CI of the seven groups increased significantly with increasing storage time, plateauing after day four. The highest CI was observed in the emulsion heated at 95°C, indicating that the repulsive forces between the droplets

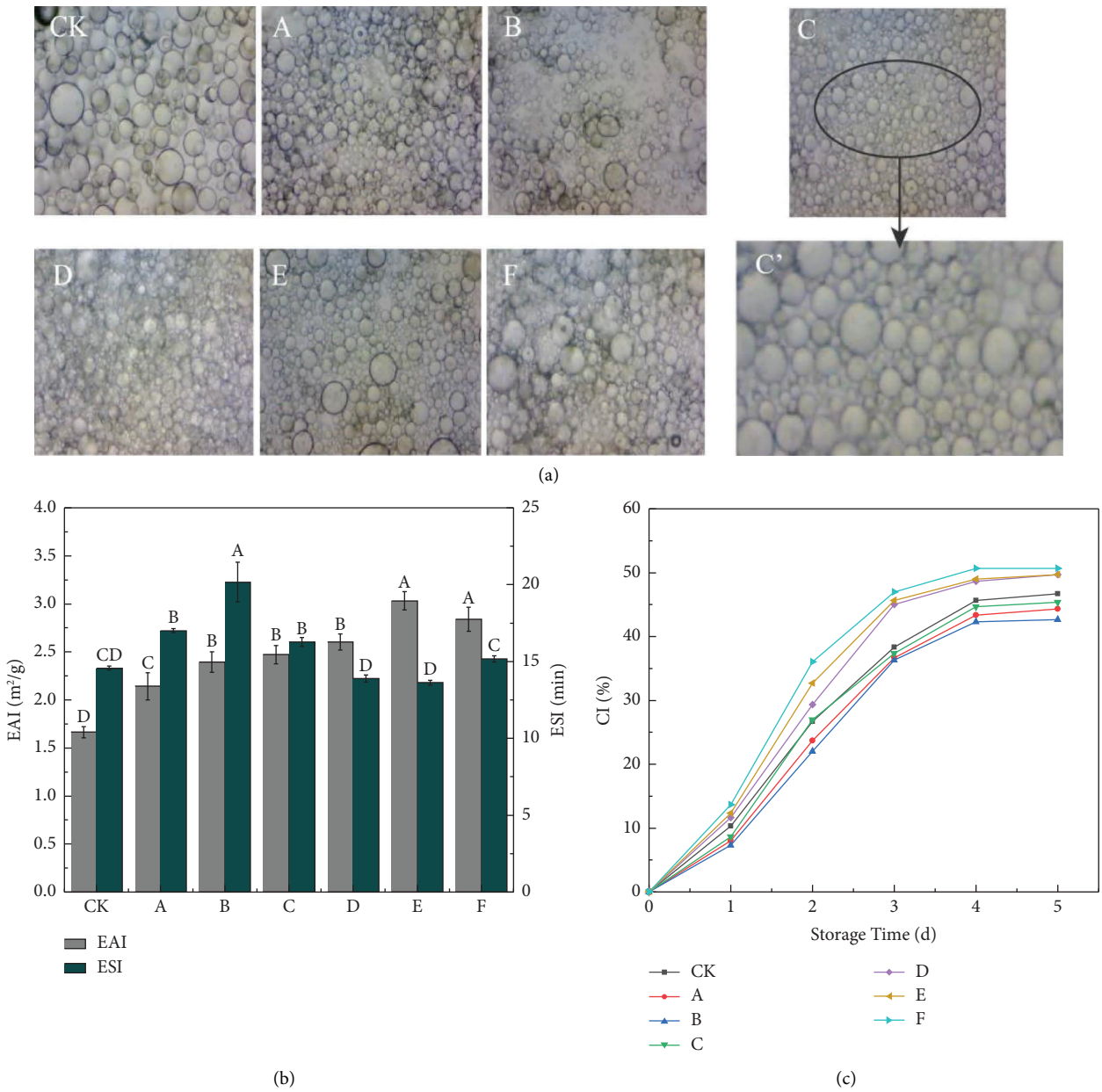


Figure 7: Images of the whey protein-stabilized emulsions obtained by optical microscope (a): CK: control group, A: 75°C/15 s, B: 75°C/3 min, C: 85°C/15 s, D: 85°C/3 min, E: 95°C/15 s, F: 95°C/3 min, and C': partial enlargement of C. Emulsification and emulsification stability of whey protein (b), and emulsion chromatography index of whey protein (c). CK: control group (pasteurised donkey milk whey protein), A: 75°C/15 s, B: 75°C/3 min, C: 85°C/15 s, D: 85°C/3 min, E: 95°C/15 s, and F: 95°C/3 min. Error bars indicate the standard deviation from triplicate tests. Different letters indicate significant differences ($p < 0.05$).

were weak and the emulsion was considerably unstable [65]. In contrast, 75°C-heated emulsions were the most stable, followed by the protein-treated group heated to 85°C, indicating that a moderate heat treatment improved their stability. Overall, high temperatures caused protein aggregation, reducing the emulsification properties of the protein, similar to previously reported conclusions [66].

3.12. Rheological Properties. Rheological properties are kinetic rather than purely physical properties of a fluid, reflecting the processing characteristics of a food product, such as its composition, internal structure, molecular form, processing stability, and changes in protein structure. Figure 4(d) presents the steady flow behaviours of whey protein samples heated at various temperatures and times. The nonlinear relationship between the shear stress and shear rate reduction for the DM whey protein solutions suggested that the samples were pseudoplastic fluids with non-Newtonian shear thinning behaviour [67, 68]. Furthermore, the shear stress decreased as the shear rate increased, indicating that all protein samples underwent structural damage due to heating and that the increasing shear rate further damaged the protein structure, reducing

the apparent viscosity [31]. Moreover, the viscosity of high-temperature-heated whey proteins was significantly higher than that of the control group, especially the 95°C/15 s group, demonstrating that temperature affected protein viscosity. The viscosity of the solution system increased with rising temperature, which might be attributed to aggregate formation or covalent cross-linking [69]. This finding was positively correlated with the WHC results, where increased temperature led to molecular aggregation and water molecule interactions, resulting in cross-linking of heated whey proteins and increasing the apparent viscosity of the solution system. Consistent with our results, Wang et al. investigated the effect of heat treatment on the rheological properties of gluten proteins and found that the increase in heat treatment time resulted in a significant increase in the apparent viscosity and consistency coefficient of the proteins, indicating that a large amount of aggregation between the protein molecules occurred [50]. Overall, the rheological properties of the whey proteins suggest that they can mimic the properties of fat and starch [67], which can be used to evaluate the thickening and gel ability of whey protein and develop potential low-calorie foods [70].

4. Conclusions

The results of this paper provide a comprehensive study of the effects of heating temperature and time on the structure and processing characteristics of DM whey protein. The results showed that when the temperature increased, the whey protein structure was loosened, resulting in a gradual increase and then a decrease of sulfhydryl content and surface hydrophobicity. Specifically, the processing properties were enhanced at 85°C, though the maximum degree of structural damage was observed when the temperature exceeded 85°C; on the other hand, the structural damage to whey protein was more pronounced with the prolongation of heating time. In particular, at the same temperature, the structural damage caused by heating for 3 min was greater than that for 15 s. Therefore, in order to maintain the optimal structure and processing characteristics of DM whey protein, it is recommended that the heat treatment temperature should not exceed 85°C, with a recommended heating time of 15 s. To ensure the optimal nutritional value and processing characteristics of DM whey protein, the effects of nonthermal processing methods such as ultrahigh pressure and pulsed electric field on DM whey protein can be further explored.

Data Availability

Some data, models, or codes generated or used during the study are available from the corresponding author upon request.

Conflicts of Interest

All authors declare no conflicts of interest.

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