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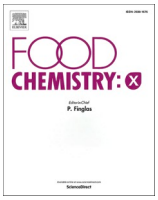

Enhacing emulsification of meat broth system mixed with myofibrillar proteins and type I collagen: The role of NaCl and heat

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ABSTRACT

A mixed system with a 5:1 ratio of beef myofibrillar protein to type I collagen was prepared to mimic meat broths. The study aimed to determine the combined effects of various NaCl concentrations (0, 0.2 M, 0.4 M, 0.6 M) and heat treatment on solubility, emulsifying properties (EAI, ESI), viscosity, and particle size of the mixed protein system. Mechanistic changes were examined through molecular interactions, intrinsic fluorescence, protein molecular weight, and Raman spectroscopy. The results showed that, without heat treatment, NaCl enhanced solubility, EAI, ESI, emulsion viscosity, and hydrogen bonding. After heating (90 °C, 30 min), elevated 0.4–0.6 M NaCl created an unstable, crowded environment, resulting in protein aggregation and reduced solubility and emulsifying performance. The results indicated that heating at 90 °C with 0.2 M NaCl was beneficial for meat emulsification, providing valuable production guidance for optimizing the formulation of meat products with low salt and high emulsifying properties.

1. Introduction

In traditional Chinese marinated meat products based on boiling technology, the heating method, as well as the heating temperature and time, significantly influence the final meat quality (Zhu et al., 2021). Salt plays a crucial role as an essential auxiliary ingredient in meat processing. The current study has focused on the changes in the emulsification properties of the primary meat protein, myofibrillar proteins (MPs). NaCl can enhance ionic strength and shield charges, thereby improving the solubility and functional properties of MPs. Decreasing NaCl concentration reduces swelling and solubility, leading to lower quality of the MPs gel (Kang et al., 2021). The emulsion activity index (EAI) and creaming index (CI) of pork MPs increased with higher NaCl concentrations (Jo et al., 2015).

Besides MPs, collagen constitutes 1 % of meat's wet weight and 5.3 % of its protein mass, primarily forming the endomysium and perimysium, which influence meat's tenderness and digestibility (Zhang et al., 2020). Type I collagen, comprising 60–80 % of all collagen in meat, is crucial for meat quality (Purslow, 2018). Collagen is sensitive to temperature changes; when heated, it denatures from its triple helix structure, reducing the α-helix content and increasing random coil formation. As the molecular structure relaxes and disorder increases, the emulsification ability of collagen with oil phases is consequently affected. Collagen loses its emulsifying properties when heated beyond its denaturation temperature (Singh et al., 2011), although thermal denaturation does not always correlate with a loss of emulsifying performance. Voutsinas et al. (1983) found that heating collagen increases its solubility, significantly enhancing its emulsifying ability. The increase in ionic strength alters the surface charge of collagen, enhances its surface hydrophobicity, and improves its solubility and extensibility, thereby enhancing emulsifying activity and stability. However, further increases in ionic strength may reduce the thickness of the diffuse double layer of salt ions, decreasing the surface potential of collagen emulsion droplets. This reduces repulsive forces within the emulsion system, leading to droplet aggregation and consequently reducing emulsification (Aider et al., 2012). Zhao et al. (2022) reported that type I collagen possesses inherent emulsifying potential, which is influenced by the presence of NaCl. Heating unfolds protein structures, increases hydrogen bonding with water, exposes hydrophobic sites, disrupts disulfide linkages, and decreases molecular order, potentially enhancing adsorption at the oil/

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water boundary and improving emulsification in relation to NaCl concentration.

While extensive research has explored the distribution of protein fractions in emulsions and aqueous phases under varying NaCl and heat conditions (Tornberg, 2005; Zhao et al., 2022; Zhu et al., 2024), less attention has been given to the interactions between myofibrillar proteins (MPs) and collagen during practical processing. The meat industry continues to face challenges in determining the optimal approach to salt addition to minimize salt content while achieving desired outcomes. This remains an issue requiring further exploration.

Therefore, in this study, mixed protein systems prepared based on the actual content of MPs and type I collagen were used as the "emulsifier" donor, and their emulsifying effect was determined under different NaCl concentrations (0, 0.2, 0.4, 0.6 M) upon heating. The effects of heat-induced mixed proteins on the emulsification system and the mechanisms underlying differences in emulsification characteristics were preliminarily discussed. This study can serve as a reference to guide the proper temperature and NaCl content in the actual production of emulsified meat products.

2. Materials and methods

2.1. Materials and chemicals

Bovine tenderloin and tendons were sourced from 3-year-old crossbred Simmental cattle produced by Henan Yisai Beef Co. (Jiaozuo, Henan, China). Post-slaughter, the carcasses were aged at 4 °C for 72 h. The samples were then sectioned, promptly vacuum-sealed, and stored at −18 °C for future use. Pepsin P7000 (enzyme activity: 1:10000) was obtained from Sigma Aodeliqi (Shanghai, China). Reagents from Solarbio Technology Co., Ltd. (Shanghai, China) included Bovine Serum Albumin (BSA), Sodium Dodecyl Sulfate (SDS), phosphate buffer, and related substances. Remaining chemicals, primarily from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), were of analytical grade.

2.2. Extraction of MPs

MPs were isolated with slight modifications based on Bai et al. (2022). A 100 g minced meat was mixed with 4 volumes (*v*/v) of isolation buffer (0.1 M KCl, 20 mM Na_2HPO_4-NaH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, pH 7.0), homogenized at 4500 rpm for 120 s in an ice bath, then centrifuged at 1000 ×*g*, 4 °C, for 10 min. The process was repeated. The precipitate was twice eluted using 4 volumes (v/v) of phosphate buffer with 1 % Triton X-100, followed by centrifugation at 1500 ×*g*. After washing with 0.1 M KCl and centrifugation at 1500 ×*g*, the final MPs were dissolved in deionized water, homogenized, centrifuged, and stored at 4 °C for 48 h. Purity and concentration were measured by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Bicinchoninic Acid (BCA) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.3. Extraction of type I collagen

Type I collagen was prepared using methods adapted from Liu et al. (2014). Beef tendons, after thawing, were stripped of excess fat and connective tissue, diced into 5 × 5 × 5 mm cubes, weighed to 100 g, and subsequently combined with warm water at 40–50 °C in a 1:5 ratio (m/V). Following this, a 5 % Na_2CO_3 solution (m/V = 1:5) was introduced into the mixture. The mixture was stirred for 10 min, filtered twice, and treated with 0.1 M NaOH solution (m/V = 1:15) for 18 h under gentle agitation. After washing with distilled water to remove residual NaOH, a degreased and partially purified sample was obtained.

The sample was swelled in 0.5 M acetic acid (1:20 m/V) with stirring for 12 h, then homogenized at 12000 rpm under 25 °C using a high-speed dispenser (T10 basic, IKA, Aachen, Germany). It was centrifuged at 4 °C and 10,000 rpm for 30 min (Allegra 64R, Beckman Coulter, Shanghai, China), and the precipitate was extracted with acetic acid-pepsin (1:25 m/V) for 48 h. After centrifugation under the same conditions for 20 min, NaCl was added until reaching 0.9 M. The flocculent was collected by centrifuging at 8000 rpm and 4 °C for 20 min, redissolved in 0.5 M acetic acid, and dialyzed against 0.1 M acetic acid for 24 h with 6-hourly changes. Type I collagen was freeze-dried at −50 °C for 48 h (LBC#7934072, Labconco, Kansas, USA), and protein concentration was measured using a BCA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4. Preparation of mixed protein solutions with different NaCl concentrations

Dissolve 0.1 g of MPs and Type I collagen separately in 100 mL of 0.1 M sodium phosphate buffer (50 mM Na_2HPO_4-NaH_2PO_4, pH 6.5) or acetic acid solution. Prepare mixed proteins by combining equal volumes of 5 $mg{\cdot}mL^{-1}$ MPs and 1 $mg{\cdot}mL^{-1}$ Type I collagen, reflecting their proportions in meat proteins. Adjust the NaCl concentration in the mixed protein solutions to 0, 0.2, 0.4, and 0.6 M. The proteins were homogenized in an ice bath using a high-speed disperser (T10 basic, IKA, Germany) for uniform dispersion, then left at 4 °C overnight for NaCl and protein to fully react. Aliquots were divided into two groups: one remained unheated, while the other was heated to 90 °C for 30 min using a thermostatic water bath (HHS-21-8, Boxun Laboratory Instrument Development Co., Shanghai, China), then rapidly cooled in ice to room temperature before measurement.

2.5. Effect of NaCl on the solubility

Collagen solubility in NaCl solution was evaluated following the method described by Takai et al. (2013). A total of 7 mL of protein solutions, each with varying NaCl concentrations, was centrifuged at 1500 ×*g* for 10 min at 4 °C. Subsequently, 1 mL of the supernatant was carefully collected. The protein concentration in the supernatant was determined using a BCA assay, with bovine serum albumin (BSA) as the standard for the calibration curve. Solubility was calculated using the following formula:

$$\text{Solubility}\Big/(\%) = \frac{\text{Supernatant protein content}/(\text{mg/mL})}{\text{Protein concentration of stock liquid}/(\text{mg/mL})} \times 100$$

2.6. Preparation of emulsion

Soybean oil was added to the mixed protein solutions at a 1:4 (*v*/v). 4 mL of protein solution, with varying NaCl concentrations, both preheated and unheated, was transferred into a 50 mL centrifuge tube placed in an ice bath. Then, 1 mL of soybean oil was added as the oil phase, and the mixture was homogenized intermittently for 1 min using a high-speed disperser to create emulsions with varying NaCl concentrations. This procedure was performed at a temperature of 0–4 °C.

2.7. Emulsifying properties (EAI and emulsion stability index (ESI))

An aliquot of 100 μL from the bottom of the prepared emulsion in a 50 mL centrifuge tube was extracted and subsequently diluted 100-fold with a 0.1 % SDS solution. Absorbance was then measured at 500 nm with a UV spectrophotometer (UV-2600 PC, Shimadzu Scientific Instruments, Inc., Shanghai, China), using a 0.1 % SDS solution as the blank (A_0). After a 10 min standing period, the procedure was repeated to obtain new sample data (A_{10}). Each experimental condition was evaluated in triplicate. EAI and ESI values were computed using the following formula (Dara et al., 2021; Zhao et al., 2022):

$$\text{EAI}/\left(\text{m}^2/\text{g}\right) = \left\{(2 \times 2.303)/\left[\text{C} \times (1-\phi) \times 10^4\right]\right\} \times \text{A}_0 \times \text{dilution}$$

$$\text{ESI}/(\%) = \text{A}_0 \times 10/(\text{A}_0 - \text{A}_{10})$$

where C was the protein concentration (g/mL) before emulsification and ϕ the oil volume fraction (*v*/v) of the emulsion (ϕ = 0.20).

2.8. Measurement of emulsion viscosity

The viscosity of the fresh emulsion was measured using a digital viscometer (NDJ—8S, Jingtian Instruments Inc., Shanghai, China). The No. 1 rotor was placed in a 50 mL centrifuge tube containing fresh emulsion, and the viscometer was set to rotate at 30 rpm. After the reading stabilized, the corresponding viscosity value was recorded. Each experimental condition was tested in triplicate.

2.9. Measurement of particle size distribution of emulsions

Emulsion particle sizes were measured with a Mastersizer instrument (RISE-2008, Runzhi Instruments Ltd., Jinan, China). The instrument settings included a medium refractive index of 1.33, with water as the dispersion medium, and particle refractive indices set to 1.53 (real) and 0.10 (imaginary). Particle sizes at 10 %, 50 %, and 90 % of the volume were recorded as D10, D50, and D90, respectively, with Dav representing the average particle size. Each sample group was tested in triplicate.

2.10. Measurement of intermolecular forces

2.10.1. Determination of hydrogen bond content

Hydrogen bond content was measured using a modified procedure adapted from Visessanguan et al. (2004). A mixture of 0.5 mL S_1 solution (20 mM Tris, 1 % SDS, pH 8.0) and S_2 solution (0.5 M NaOH) was added to 3 mL of 1 mg·mL^{-1} mixed protein solution with varying NaCl concentrations, which was then heated. After incubating at room temperature for 1 h and centrifuging at 12100 ×g for 30 min, 0.6 mL of supernatant was mixed with 50 % TCA to reach a final concentration of 10 %. The mixture was cooled at 4 °C for 15 min and then centrifuged at 2500 ×*g* for 20 min. The resulting precipitates were dissolved in S_2 solution, and protein concentration was measured using a BCA assay. Hydrogen bond content was determined by calculating the percentage of protein solubilized by S_1 relative to that solubilized by S_2。

2.10.2. Protein surface hydrophobicity

Surface hydrophobicity was measured according to a modified method of Zhang et al. (2020). A volume of 60 μL of 1 mg·mL^{-1} bromophenol blue (BPB) solution was mixed with 1 mL of 5 mg·mL^{-1} protein solution, which had been prepared at varying NaCl concentrations and heated. The mixture was centrifuged at 5000 ×*g* for 15 min at 4 °C. Supernatants were collected and their absorbance at 590 nm was measured using a UV spectrophotometer (UV-2600, Shimadzu, Japan), with phosphate buffer serving as the blank control. Surface hydrophobicity was calculated based on BPB binding (Chelh et al., 2006) using the following formula:

$$\text{surface hydrophobic content} \Big/ (\mu g) = \frac{(OD_1 - OD_2)}{OD_1} \times 60\mu g$$

where the OD_1 was the optical density value of the blank sample, and OD_2 was the optical density value of the sample.

2.10.3. Determination of disulfide bond content

The content of disulfide bonds was quantified using a method adapted from Visessanguan et al. (2004) with minor modifications. Protein solutions (3 mg·mL^{-1}, 3 mL) with varying NaCl concentrations, both pre- and post-heating, were combined with 0.5 mL of S_3 solution (20 mM Tris+1 % SDS + 8 M urea +2 % β-mercaptoethanol, pH 8.0). The mixture was heated at 95 °C for 15 min, shaken at room temperature for 1 h, and then centrifuged at 12100 ×*g* for 30 min. An aliquot of 0.6 mL of the supernatant was treated with 10 % TCA, then cooled at 4 °C for 15 min before being centrifuged at 2500 ×*g* for 20 min. The resulting precipitate was dissolved in 0.5 M NaOH (S_2 solution). Protein concentration was assessed using a BCA assay. Disulfide bond content was calculated as the percentage of protein solubilized in S_3 relative to that in S_2, with phosphate buffer serving as the blank control.

2.11. Endogenous fluorescence spectrometry

Mixed protein solutions with different NaCl concentrations were diluted to 0.1 mg·mL^{-1}. The fluorescence emission spectra of intrinsic tryptophan were recorded using a fluorescence photometer (F-4600, Shimadzu, Japan). Measurements were performed with an excitation wavelength of 280 nm, an emission range of 300–400 nm, and a voltage of 600 V (Utrera, Rodriguez-carpena, Morcuende, & Estevez, 2012). Data were acquired at 1 nm intervals.

2.12. Molecular weight determination

Protein molecular weights were determined using SDS-PAGE (5 % stacking, 12 % separating gels, pH 6.8 and 8.8). Samples were mixed with 4 × native-PAGE buffer at a 3:1 ratio and heated at 100 °C for 5 min. Gels were loaded with 10 μL of sample, run at 90 V for 30 min, and then at 120 V until the bromophenol blue disappeared. After electrophoresis, the sample was stained with Coomassie Brilliant Blue R250 for 1 h, decolorization (500 mL methanol, 100 mL acetic acid, 400 mL distilled water) to the strip clearly visible. Protein bands were identified using standard markers (11–245 KD, PR1920, Beijing Solarbio Technology Co., LTD, Shanghai, China) and imaged with a UVP-EC3–310 gel imager (Epson, Japan).

2.13. Raman spectroscopy

Raman spectra were collected using a LabRAM HR Evolution Micro-Raman spectrometer (Horiba Electronic Technology Co., Ltd., Paris, France) with a 532.8 nm laser at 15 mW maximum power. A 100 × objective was used, offering 2 cm^{-1} resolution, a 120 cm^{-1}·min^{-1} scan speed, and 60 s integration time, with data acquired every 1 cm^{-1}. Samples were analyzed, and spectra were processed using LabSpec 6.0 software for smoothing and baseline correction. The proportions of β-sheets, α-helices, β-turns, and random coils were determined following the method of Alix et al. (1988). All procedures were performed in triplicate.

2.14. Statistical analysis

All experiments were conducted in triplicate. Statistical analyses were performed using IBM SPSS Statistics 21.0 (IBM Corp., Armonk, NY, USA). Results were expressed as means ± standard error, with significance set at $p < 0.05$. Graphs, including histograms, line plots, and spectrograms, were created using Origin 19.0 (OriginLab Corp., Northampton, MA, USA) for data visualization.

3. Results and discussion

3.1. Solubility

The solubility of proteins was influenced by factors such as protein structure and interactions, reflecting denaturation. Lower solubility indicated more severe protein aggregation and greater denaturation (Estévez, 2011). Fig. 1a depicts that the solubility of mixed proteins increased continuously with increasing NaCl concentration. Specifically, the solubility of mixed proteins increased from 20.68 % to 28.75 % with NaCl concentration (0–0.2 M) when added alone ($p < 0.05$), surpassing that of MPs but remaining below that of type I collagen (Zhao et al., 2022; Zhu et al., 2024). The solubility of mixed proteins also increased from 30.67 % to 31.91 % with NaCl concentration (0.4–0.6 M), though it

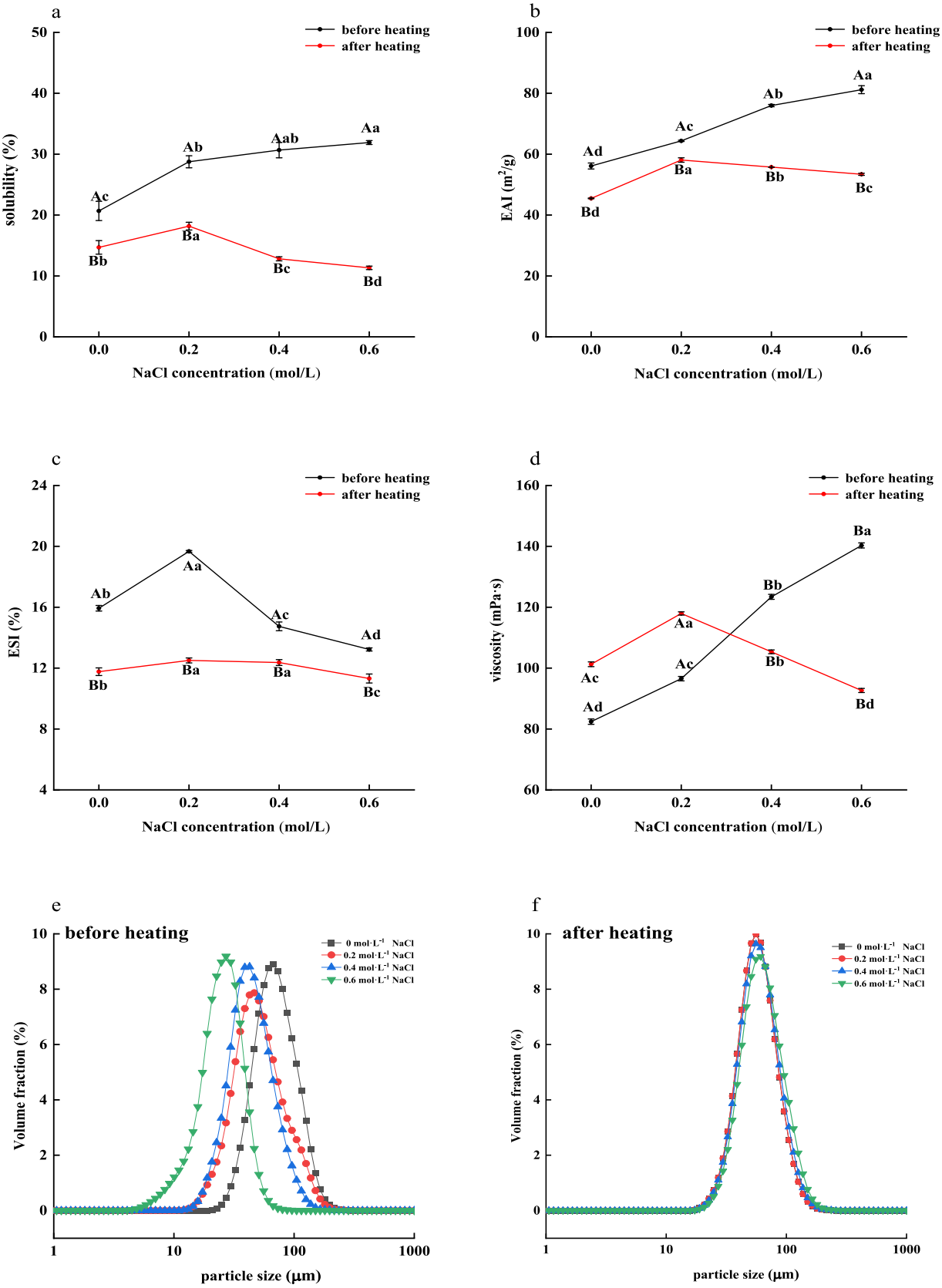

Fig. 1. Effects of NaCl concentration on the solubility (a), emulsifying activity index (EAI) (b) and emulsifying stability index (ESI) (c), emulsion viscosity (d), and emulsion particle size fraction distribution of mixed protein before (e) and after (f) heating. The data are expressed as the means ± standard deviations ($n = 3$). A, B indicate the differences of heating and without heating with significance at $p < 0.05$. a, b, c, d indicate the differences of NaCl concentration with significance at $p < 0.05$.

was lower than that of MPs and type I collagen. Generally, with increasing NaCl concentration, the solubility of the mixed protein system exhibited an upward trend. However, this increase was less pronounced than that observed for MPs and did not exhibit the decline seen in type I collagen when NaCl concentration exceeded 0.2 M. As NaCl concentration increased, the intermolecular forces of MPs were disrupted, altering their interactions. Increasing ions interacted with oppositely charged MPs molecules, leading to a double-layer effect. This caused heightened electrostatic repulsion between MPs, increased hydration, and ultimately enhanced solubility. In contrast, due to the salting-out effect, the solubility of type I collagen initially increased and then gradually decreased with increasing NaCl concentration, though it remained 40 % higher than that of the mixed protein system (Zhao et al., 2022).

Comparisons between the mixed protein system and the two individual proteins (Zhao et al., 2022; Zhu et al., 2024) indicated that, at low NaCl concentrations, collagen contributed more significantly to enhancing the solubility of the mixed protein system. The binding of anions (Cl^-) to the positively charged surface of collagen increased the electrostatic Gibbs energy of the protein, thereby preventing aggregation and inhibiting large aggregate formation between MPs and type I collagen (Zhao et al., 2022). Conversely, MPs contributed more to the increase in the solubility of the mixed protein system at high NaCl concentrations, although the rate of increase in solubility was slower, suggesting that high concentrations of NaCl promoted aggregation of both proteins (Shimada et al., 2015; Zhu et al., 2024).

When heated, the solubility of the mixed protein increased and then decreased with increasing NaCl concentration, reaching a maximum at 0.2 M and then gradually decreasing (18.18 % to 11.33 %) ($p < 0.05$). The preliminary laboratory measurements indicated that both myofibrillar and collagen proteins exhibited a similar trend (Zhao et al., 2022; Zhu et al., 2024). The solubility of both individual proteins was higher than that of the mixed protein system. These results suggested that at elevated temperatures, the solubility of MPs and type I collagen decreased. At moderate ionic concentrations, these proteins maintained equilibrium, but high temperatures combined with high ionic concentrations created an unstable and crowded environment where proteins struggled to maintain surface stability (Cox et al., 2020). Under such stressful conditions, non-covalent interactions within the proteins were disrupted, leading to the disappearance of higher-level (secondary or tertiary) structures and the formation of aggregated structures (Schramm et al., 2020). Furthermore, the results indicated that higher concentrations of NaCl promoted the cross-linking aggregation of MPs and type I collagen upon heating, which was unfavorable for the emulsification properties of mixed proteins (Marcos & Mullen, 2014).

3.2. Emulsifying properties

As shown in Fig. 1b, the EAI of the mixed protein system, both before and after heating, followed a similar trend to solubility. The EAI of the mixed protein system significantly increased (56.12–81.18 $m^2 \cdot g^{-1}$) ($p < 0.05$) with the addition of NaCl, likely due to the reduction in emulsion droplet size at lower NaCl concentrations. This reduction likely facilitated greater protein adsorption at the oil-water interface, which further reduced interfacial tension (Shen et al., 2017). After heating, the EAI of the mixed protein system increased with rising NaCl concentration, peaking at 58.08 $m^2 \cdot g^{-1}$ at 0.2 M NaCl, and then gradually decreased. The observed decrease in EAI at NaCl concentrations of 0.2–0.6 M could be attributed to the formation of insoluble aggregates between MPs and type I collagen, reducing the protein adsorption dynamics on the oil droplet surface, resulting in decreased emulsifying performance.

With increasing NaCl concentration, the ESI of the mixed protein system first rose and then declined (Fig. 1c). At a low NaCl concentration (0.2 M), the ESI reached a maximum of 20.67 %, which was higher than that of MPs under the same conditions but lower than that of type I collagen (23.04 %) ($p < 0.05$) (Zhao et al., 2022). At 0.6 M NaCl, the ESI of the mixed protein system (13.23 %) was lower than that of both MPs and type I collagen (20.79 %) ($p < 0.05$), consistent with the solubility results. It was inferred that higher NaCl concentrations did not sustain the emulsifying stability of type I collagen (Zhao et al., 2022). As the NaCl concentration increased, type I collagen played a more prominent role in cross-linking and aggregating with MPs, which at high salt concentrations, resulted in reduced emulsifying stability in the mixed protein system.

After heating, compared to the addition of NaCl alone, the ESI of the mixed protein system remained low across all NaCl concentrations, peaking at 12.51 % at 0.2 M NaCl (Fig. 1c). This result indicated that increasing NaCl concentration did not substantially enhance the emulsifying stability of the heated mixed protein system. These findings suggest that, for high-temperature meat products, salt addition provides only limited enhancement of emulsifying performance. Other factors must be addressed to improve the texture and water-holding capacity of these products. Additionally, the mixed protein system exhibited lower emulsifying stability compared to individual proteins, especially type I collagen. This phenomenon may be attributed to heat-induced denaturation of the mixed protein system and the formation of insoluble aggregates between MPs and type I collagen due to NaCl. These factors increased emulsion droplet size, leading to reduced emulsifying stability.

3.3. Viscosity of emulsions

The viscosity of the emulsion is a key indicator of its stability, as it reflects the extent of emulsion aggregation. As NaCl concentration increased, the viscosity of the mixed protein emulsion rose from 82.42 mPa·s to 140.30 mPa·s (Fig. 1d), which is consistent with the trends observed in solubility and emulsifying activity, as well as with results for chicken breast myosin (Hayakawa et al., 2012).

After heating, the viscosity of the emulsion initially increased significantly (101.28–117.96 mPa·s) ($p < 0.05$) at NaCl concentrations below 0.2 M, and then decreased to 92.66 mPa·s ($p < 0.05$) with further NaCl addition. In contrast, the viscosity of single-protein emulsions tended to increase upon heating, which suggests that these proteins rarely aggregated when heated. However, cross-linking and aggregation between MPs and type I collagen intensified, particularly at high NaCl concentrations, leading to increased emulsion droplet size and reduced stability.

3.4. Particle sizes in emulsions

With increasing NaCl concentration, the size of the emulsion particles in the mixed protein decreased from 66.87 μm to 27.14 μm (Fig. 1e). The reduction in particle size facilitated better dispersion among protein particles and between protein and fat particles, positively impacting the proteins' emulsifying properties. These results, consistent with the mixed protein EAI trend, align with findings by Shen et al. (2017), who reported that decreasing protein particle size improves emulsification performance.

There was no significant difference in the particle size of the mixed protein emulsions with increasing NaCl concentration when heated, but all of them were larger than unheated. Meanwhile, comparing the two graphs, the distribution of curves in Fig. 1e was farther and more separated than that in Fig. 1f, indicating that the effect of NaCl in reducing particle size after heating was reduced compared with that before heating.

In order to compare particle size distributions, D50 and the average particle size (Dav) can be selected as representative particle sizes. Additionally, D10 and D90 allowed the detection of changes in minimum and maximum particle diameter. As shown in Table 1, with the increase of NaCl concentration, the D10, D50, D90 and Dav of the mixed protein emulsion decreased significantly. During heating, D10, D50, D90 and Dav in the mixed protein emulsion decreased first and then

Table 1
Effects of different NaCl concentrations and heating on emulsion particle size of mixed protein.

	Content of NaCl ($mol·L^{-1}$)	D10 (μm)	D50 (μm)	D90 (μm)	Dav (μm)
Unheated	0	39.27 ± 0.65^{Aa}	65.22 ± 0.38^{Aa}	112.03 ± 0.82^{Aa}	71.24 ± 0.18^{Aa}
	0.2	26.64 ± 0.01^{Ab}	46.90 ± 0.12^{Ab}	94.06 ± 0.71^{Ab}	54.36 ± 0.25^{Ab}
	0.4	24.82 ± 0.14^{Ac}	41.20 ± 0.44^{Ac}	72.71 ± 2.08^{Ac}	45.70 ± 0.81^{Ac}
	0.6	13.36 ± 0.24^{Ad}	23.95 ± 0.06^{Ad}	37.89 ± 0.05^{Ad}	25.03 ± 0.03^{Ad}
Heated	0	34.85 ± 0.44^{Bb}	54.32 ± 0.43^{Bc}	86.67 ± 1.16^{Bc}	58.22 ± 0.49^{Bc}
	0.2	23.83 ± 1.48^{Ac}	42.99 ± 0.52^{Bd}	82.42 ± 2.79^{Bd}	48.93 ± 0.19^{Bd}
	0.4	35.15 ± 0.48^{Bab}	55.60 ± 0.33^{Bb}	91.58 ± 1.20^{Bb}	60.17 ± 0.53^{Bb}
	0.6	36.58 ± 0.55^{Ba}	59.12 ± 0.53^{Ba}	100.53 ± 1.62^{Ba}	64.55 ± 0.07^{Ba}

Note: A and B indicate significant differences between heated and unheated ($p < 0.05$). Different letters (a-c) indicate significant differences between different NaCl concentrations in the same group ($p < 0.05$).

increased, and the indicators of the emulsion reached the minimum (Dav was 48.93 μm) at 0.2 M NaCl. Continue to increase the NaCl concentration (0.4–0.6 M), the particle size of the emulsion became larger (Dav varies from 60.17 to 65.55 μm), which was larger than that without heating (Dav varies from 45.70 to 25.03 μm). The viscosity and particle size distribution data verified the change of emulsifying properties of mixed protein.

3.5. Intermolecular forces

3.5.1. The hydrogen bond content

Ionic strength has a significant effect on the tertiary structure of proteins, altering the intermolecular hydrogen bonds that bind proteins to water. As shown in Fig. 2a, the hydrogen bond content of the mixed protein gradually increased from 23.43 % to 69.80 %, which was consistent with the change trend of the hydrogen bond of MPs (Zhu et al., 2021). And for type I collagen, it increased significantly from 25.54 % to 87.81 %, and above 0.2 M NaCl concentration, it decreased slightly from to 82.85 %. Since the α-helix was stabilized by intramolecular hydrogen bonding, whereas β-sheet relies on inter-peptide chain hydrogen bonding for stabilization, a decrease in the α-helix structure of proteins as well as an increase in the β-sheet structure has been associated with the rearrangement of the protein's hydrogen bonds (Zhang et al., 2017). We inferred that in the mixed protein system, the low NaCl concentration firstly increased the β-sheet of MPs and type I collagens. Then with the increase of NaCl concentration, the β-sheet of MPs showed a continuous and sharp increase, although a small portion of the β-sheet of type I collagen was resolved, resulting in a decrease in intermolecular hydrogen bonding. Therefore, overall, the hydrogen bond content of the mixed protein system gradually increases.

The hydrogen bonding content of the NaCl thermally induced the mixed protein system peaked at 0.2 M NaCl concentration (53.37 %) and then decreased continuously. For MPs, its hydrogen bonding content increased continuously upon heating, whereas the hydrogen bonding content of type I collagen increased significantly from 24.59 % to 82.56 % (Zhao et al., 2022). Thus, we inferred that in the heat-induced mixed protein system, both MPs and type I collagen underwent a transition from α-helix to β-sheet, increasing intermolecular hydrogen bonds. At 0.2 M and higher NaCl concentrations, the system likely experienced cross-linking aggregation of MPs and type I collagen, disrupting protein structures and breaking some hydrogen bonds, thereby weakening hydrogen bond interactions.

3.5.2. The surface hydrophobicity

The hydrophobic groups of protein molecules characterize the tertiary structure of the protein. The surface hydrophobicity of the mixed protein was maintained at a low level, exhibiting a decreasing trend (Fig. 2b). At high NaCl concentrations, the net charge of MPs was elevated, which enhanced intermolecular repulsion, resulting in molecular extension and the exposure of hydrophobic groups, thereby increasing surface hydrophobicity (Zhu et al., 2024). In contrast, type I

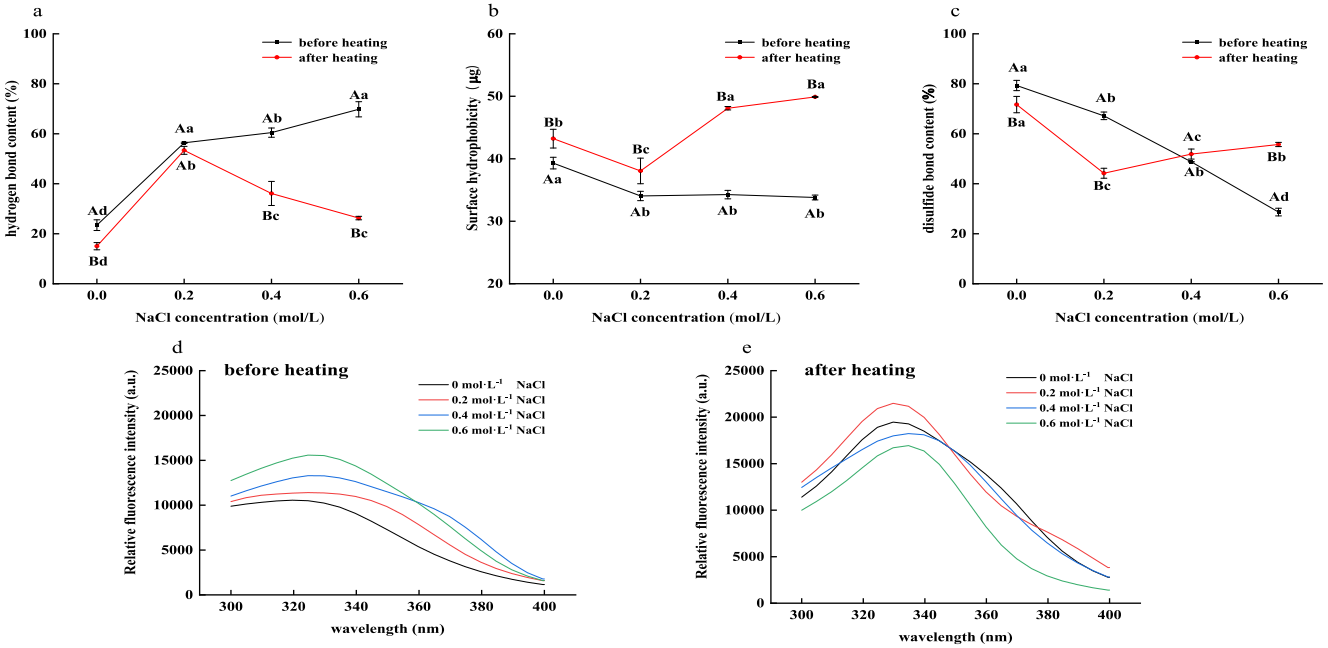

Fig. 2. Effects of NaCl concentration on the hydrogen bond content (a), surface hydrophobicity (b), disulfide bond content (c) and endogenous fluorescence spectrum of mixed protein before (d) and after (e) heating. The data are expressed as the means ± standard deviations (n = 3). A, B indicate the differences of heating and without heating with significance at $p < 0.05$. a, b, c, d indicate the differences of NaCl concentration with significance at $p < 0.05$.

collagen became more easily hydrated on surfaces with high charge density, permitting hydrophilic groups to bind water molecules and causing the burial of hydrophobic groups internally, which reduced surface hydrophobicity (Zhao et al., 2022). Therefore, it was inferred that high NaCl concentrations in the mixed protein system likely promoted the aggregation of both proteins, consistent with previous findings regarding solubility and other parameters.

During heating, the surface hydrophobicity of the mixed protein system was overall higher than that before heating, which could be attributed to thermal denaturation-induced unfolding of the tertiary structure of the proteins and the exposure of the corresponding hydrophobic core (Peng et al., 2016). The surface hydrophobicity of the mixed protein system decreased and then increased with increasing NaCl concentration, with a minimum at 0.2 M (38.05 μg) which was quite different compared to the protein alone. For NaCl heat-induced MPs, its surface hydrophobicity continued to decrease., whereas the surface hydrophobicity of NaCl heat-induced type I collagen continued to increase, especially at a rapid rate after 0.2 M NaCl concentration (Zhao et al., 2022; Zhu et al., 2024). It is shown that type I collagen plays a major role in the mixed protein system after a concentration of 0.2 M NaCl, hydrophobic side chains of type I collagen can remain more favorable in the aqueous environment under thermally induced conditions of NaCl, and the application of heat instigates a substantial disruption of the profuse hydrogen bonds and electrostatic interactions that are fundamental in maintaining the conformation of the fibrous protein, thereby resulting in the dispersion of the protein molecules and imbuing them with an increased freedom to adopt an assortment of arbitrary configurations (Tornberg, 2005). The swelling of type I collagen after heating reduces the encapsulation of hydrophobic groups by NaCl (Zhao et al., 2022). Simultaneously, NaCl had increased hydrophobic interactions during the heating process, causing the two proteins to refold and reassemble into large insoluble aggregates (Ju et al., 2023).

3.5.3. The disulfide bond content

The change trend of disulfide bond content of mixed protein heated and unheated under different NaCl concentrations is different (Fig. 2c). When not heated, the disulfide bond content of the mixed protein gradually decreased (79.33 %–28.67 %) with the increase of NaCl concentration, which was consistent with the trend of disulfide bond content in MPs.

When heated the disulfide bond content in the mixed protein system decreased sharply at 0–0.2 M NaCl concentration (71.67 %–44.20 %) and then increased slowly (44.20 %–55.72 %). The protein was denatured under heating conditions, where the forces (ionic bonds, hydrogen bonds, etc.) maintaining the protein conformation were broken, exposing internal hydrophobic groups and sulfhydryl groups to oxidize into disulfide bonds. Protein aggregation was induced, leading to the encapsulation of a large number of reactive sulfhydryl groups (Du et al., 2018), causing the disulfide bond content in the mixed protein system to increase at a slower rate. Chen (2018) found that at higher temperatures or longer heating times, additional disulfide bonds formation and interaction of hydrophobic groups could promote protein aggregate formation, consistent with the results of this study.

3.6. Endogenous fluorescence spectra

As shown in Fig. 2d and e, the emission spectrum peaked at 340 nm, and the maximum wavelength (λ_{max}) did not show a significant shift. Similar results were reported in the study by Estévez (2011), suggesting that the microenvironment of the chromophores may not affected by processing conditions. When not heated, the endogenous fluorescence intensity of the mixed protein gradually increased, and the change trend was opposite to that of surface hydrophobicity. The reason may be that the addition of NaCl caused the conformation of the mixed protein to fold, burying hydrophobic groups inside the molecular structure.

The endogenous fluorescence intensities of the mixed protein were generally elevated upon heating. Heating exacerbated the unfolding and surface hydrophobicity of the proteins, promoting hydrophobic interactions and protein aggregation (Ma & Ledward, 2004). The increase in polar located tryptophan residues in the microenvironment with increasing NaCl concentration may have contributed to this effect, suggesting that the protein chromophore was exposed to a more hydrophilic environment due to protein unfolding (Higuera-Barraza et al., 2017). This finding was consistent with previous studies reporting that heat treatment increased the fluorescence intensity of zein and β-lactoglobulin (Stănciuc et al., 2012; Sun et al., 2016).

3.7. Molecular weight distribution

As shown in Fig. 3, when not heated, at all NaCl concentrations, the two α-chain bands of the mixed protein were gradually shallow, β-chain has been degraded. When unheated, with increasing NaCl concentration, the bands of myosin heavy chain and actin gradually darkened, corresponding to changes in the solubility of the mixed protein. After heating, the bands for myosin heavy chain and actin generally become lighter and thinner, but as the NaCl concentration continues to rise, these bands gradually faded. The results indicated that during the addition of NaCl and the heating process, proteins with higher molecular weight dissolved more readily in solutions with high ionic strength, consistent with solubility study findings.

Additionally, at increasing concentrations of NaCl without heating, the appearance of some lower molecular weight protein bands within the range of 35–11 kDa on the electrophoresis gel suggested that higher concentrations of NaCl might lead to the hydrolysis and degradation of mixed proteins. Moreover, after heating, the color of these protein bands in the mixed protein system faded. During heating, type I collagen underwent denaturation with the unwinding of its helix and breakage of its α-chains, leading to a more disordered secondary structure. This denaturation enhanced the emulsifying properties of collagen. It was reported that the cross-linking of myosin made the protein molecules too large to maintain their flexibility when adsorbed on the surface of oil droplets (Xiong et al., 2008), thus insoluble protein aggregates were also formed in the mixed protein. The improvement of NaCl on the emulsifying properties of mixed proteins was inhibited.

3.8. Secondary structure

The secondary structure distribution of the mixed protein system was evaluated by inverse fold product and double integration of the amide I band (1600–1700 cm^{-1}) in Raman spectra (Fig. 4). The amide I bands in the wave number range of 1600–1700 cm^{-1} were mainly associated with stretching vibrations of C═O, bending vibrations of N—H groups, stretching of CN groups, and characteristic changes in CCN (Herrero et al., 2008), and the frequencies of the amide I bands were shown to

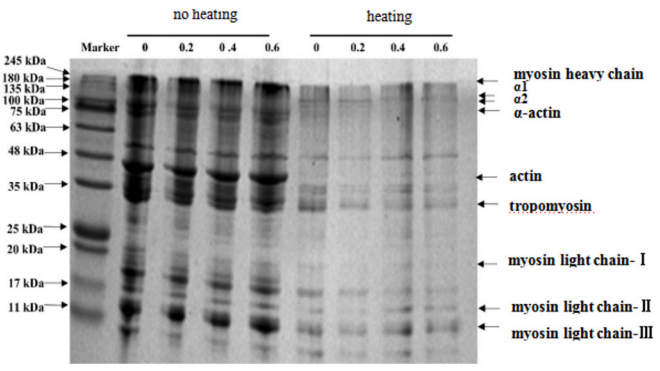

Fig. 3. Effects of different NaCl concentrations on the electrophoretic bands of mixed protein before and after heating.

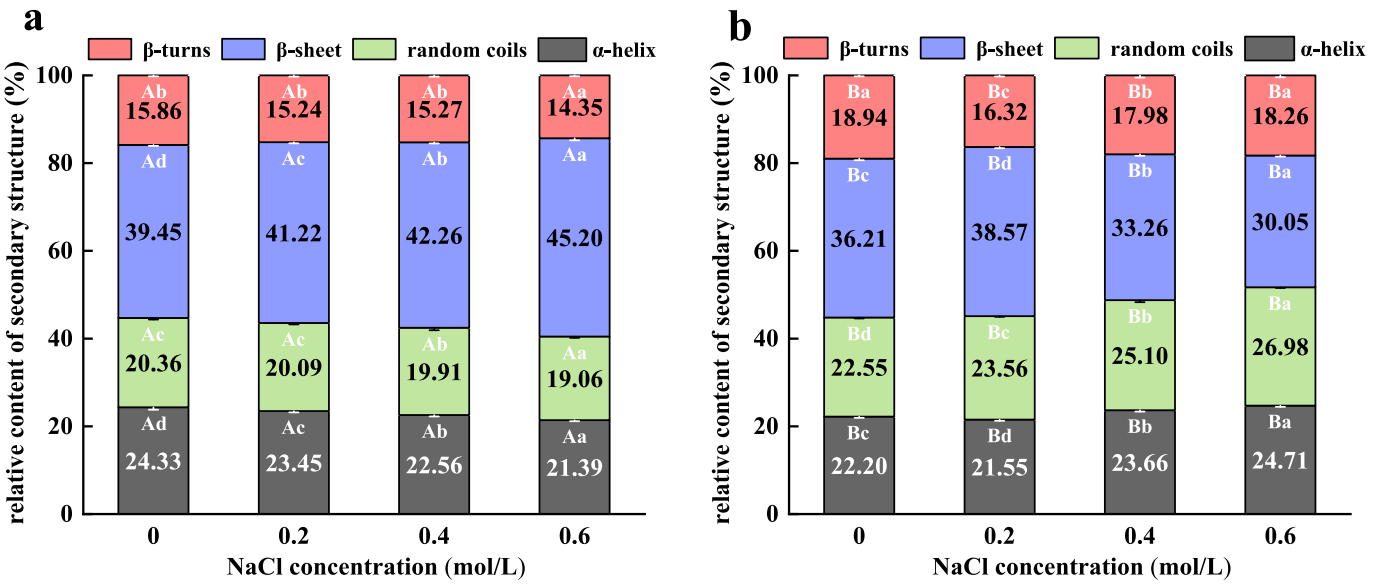

Fig. 4. Secondary structure content of mixed protein at different NaCl concentrations (0, 0.2 M, 0.4 M, and 0.6 M) before (a) and after (b) heating. The data are expressed as the means ± standard deviations (n = 3). A, B indicate the differences of heating and without heating with significance at $p < 0.05$. a, b, c, d indicate the differences of NaCl concentration with significance at $p < 0.05$.

correlate with the composition and type of the main chain conformation (Alix et al., 1988). The secondary structure content of both unheated and heat-induced mixed protein system was dominated by β-sheet. As shown in Fig. 4a, with the increase of NaCl concentration, the content of α-helix decreased from 24.33 % to 21.39 %, while the content of β-sheet increased from 39.45 % to 45.20 %. The α-helix structure was mainly maintained by the hydrogen bonds between the carbonyl (-CO) and amino (NH-) groups on the polypeptide chain. When the α-helix structure was lost or destroyed, the stability of the hydrogen bonds that maintains the protein structure was affected (Ju et al., 2023). The continuous decrease in α-helix content and the corresponding increase in β-sheet content in the mixed protein system indicated that the increase in NaCl concentration was unfavorable for the mixed protein. This finding was aligned with reports from researchers that concluded excessively high NaCl concentrations had caused protein denaturation, with α-helix structures gradually unwound and converted into β-sheet and β-turn structures, resulting in disordered conformations and aggregation (Herrero et al., 2011; Zhou et al., 2019; Zhu et al., 2022).

The β-sheet content in the mixed protein system increased with increasing NaCl concentration after heating, reaching a maximum value (38.57 %) at 0.2 M, and then decreased to 30.05 % ($p < 0.05$) (Fig. 4b). Conversely, the α-helix content initially decreased and then increased ($p < 0.05$), reaching its minimum of 21.55 % at 0.2 M. The content of random coils gradually increased from 23.56 % to 26.98 % ($p < 0.05$), which indicated a shift of the α-helix to the β-sheet at 0–0.2 M, and a transition to β-sheet at 0.4–0.6 M when β-sheet to random coils occurred to a greater extent, and the mixed protein system gradually changed from an ordered to a disordered state. Fig. 5 showed the mechanism of the effects of NaCl on emulsification properties of heat-induced mixed protein. This may be due to the unfolding of the triple helix structure

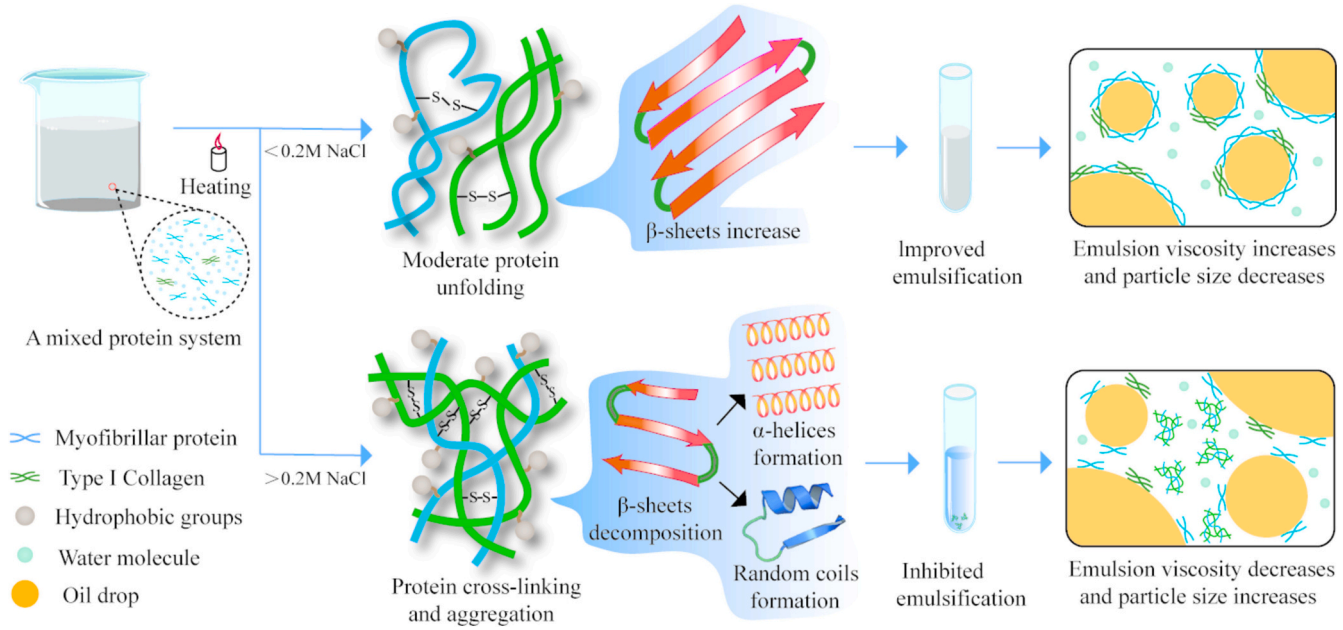

Fig. 5. Mechanism of action of the effects of NaCl on emulsification properties of heat-induced mixed protein.

during heating of type I collagen at 0–0.2 M increased the interaction of the protein structure with the oil phase, type I collagen swelled, exposing more CN and NH groups on the surface, with a gradual decrease in the α-helix content, and a gradual increase in the content of random coils and β-sheet ($p < 0.05$). The emulsification properties of the mixed protein system with low NaCl concentration were also further enhanced. At 0.2 M and higher NaCl concentrations, the mixed protein system showed an increase in α-helix during heating as the protein structure gradually stretched leading to unfolding of the protein and breaking of hydrogen bonds between neighbouring peptide chains, leading to decomposition of the β-sheet structure and formation of α-helix through intermolecular hydrophobic interactions (Ju et al., 2023). Kato and Nakai (1980) found a significant negative correlation between surface hydrophobicity and α-helix content of proteins by investigating the correlation between surface hydrophobicity and interfacial properties of proteins, which was confirmed by the results of surface hydrophobicity in this paper.

4. Conclusion

Although relatively high concentrations of NaCl can improve the texture and flavor of meat products, excessive sodium intake poses health risks. Directly reducing the amount of added NaCl lowers the emulsification capacity of proteins, resulting in poor texture and structure in the final product. In this study, the key roles of NaCl concentration and heat treatment in the mixed protein system simulating meat broth were investigated, aiming to identify optimal conditions for enhancing protein emulsification under low-sodium environments. This work provides a novel strategy for optimizing meat processing.

The results demonstrated that, prior to heating, increased NaCl concentrations resulted in high surface charge density on the proteins, which induced strong electrostatic repulsion. This repulsion, along with hydrophobic interactions, promoted the formation of large molecular aggregates between the two proteins in the mixed system, reducing emulsification performance. Meanwhile, at higher NaCl concentrations (> 0.2 M), significant structural damage was observed in type I collagen, causing myofibrillar protein to dominate the emulsification properties of the system. After heating (90 °C, 30 min), the effect of NaCl on the mixed system became less pronounced compared to pre-heating conditions. NaCl facilitated protein stretching, and heating induced moderate unfolding of the proteins. At moderate ionic concentrations, the two proteins reached a balanced state, with solubility, EAI, ESI, emulsion viscosity, intermolecular hydrogen bonding, and intrinsic fluorescence intensity all peaking at 0.2 M NaCl, which then decreased. Elevated temperatures combined with high ionic concentrations resulted in an unstable, crowded environment, preventing the proteins from maintaining a stable surface. Significant transitions from β-sheets to random coils occurred, leading to a gradual shift from an ordered to a disordered state. Hydrogen bonds broke, and additional disulfide bonds and hydrophobic interactions contributed to protein aggregation, further reducing emulsification performance.

These findings suggest that heating at 90 °C with 0.2 M NaCl is favorable for emulsification. This study provides new insights into optimizing emulsification conditions for a mixed system of myofibrillar protein and type I collagen, offering practical guidance for producing low-salt, highly emulsified meat products in the meat industry.

CRediT authorship contribution statement

Chaozhi ZHU: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Data curation, Conceptualization. **Yangyi ZHENG:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Data curation. **Guiyan ZHANG:** Validation, Supervision, Conceptualization. **Y.U. Xiaoling:** Validation, Supervision. **Qiuhui ZHANG:** Validation, Supervision. **Gaiming ZHAO:** Validation, Supervision, Funding acquisition. **L.I. Fuqiang:** Validation, Supervision.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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