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The effect of $CaCl_2$ on water migration, rheological properties, aggregation behavior and protein structure in rapidly salted separated egg yolk plasma and granules

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ABSTRACT This research investigated the effects of $CaCl_2$ on the aggregation behavior and protein structure of egg yolk plasma and granules after fast salting. The addition of $CaCl_2$ to the salt solution decreased T_{23}, D [4,3] and the absolute value of the zeta potential by 6.71%, 3.66%, and 3.15%, respectively, while increasing T_{22} by 15.85% in egg yolk plasma. Moreover, adding $CaCl_2$ also increased the apparent viscosity and G' value of egg yolk plasma. On the other hand, the addition of $CaCl_2$ decreased the T_{22}, T_{23}, D [4,3], and absolute value of the zeta potential of egg yolk granules by 56.53%, 6.71%, 6.02%, and 34.27%, respectively. Furthermore, the addition of $CaCl_2$ increased the number of β-turns by 51.22%, whereas it decreased the number of β-sheets by 26.55% in egg yolk plasma protein. The α-helices, β-turns, and β-sheets of egg yolk granule protein decreased by 6.58%, 3.58%, and 6.96%, respectively. Additionally, the addition of $CaCl_2$ can increase the degree of λ_{max} redshift in egg yolk plasma and decrease the degree of λ_{max} redshift in egg yolk granules. Overall, the addition of $CaCl_2$ can change the aggregation mode of proteins in egg yolk plasma and granules, improving the quality of salted egg yolk products.

Key words: hen egg yolk, LF-NMR, rheology, SDS–PAGE

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INTRODUCTION

Duck eggs are commonly utilized as the primary ingredient for the manufacturing of salted eggs. Moreover, poultry egg production began in China, with eggs accounting for 85% of the total egg production. The nutritional composition of hen eggs is similar to that of duck eggs (FAOSTST, 2021). The most nutritious part of an egg is the yolk, which makes up more than 36% of its weight. The protein digestion coefficient of this substance is similar to that of milk, and its biological value is even better than that of milk egg white (Huopalahti et al., 2007). The level of conversion in egg processing remains relatively low. To increase the flavor and quality of egg products, individuals have developed methods of salting to transform eggs into salted eggs (Liu and Tao, 2019). Salted eggs are traditional Chinese foods known for their delectable flavor, rich in oil, and slightly gritty texture. Currently, salted egg yolk is generally made via the traditional salting procedure. After the entire fresh egg was salted, the yolk was removed and cooked. The salt immersion method, straw-ash method, and salt-mud coating method are common methods of traditional egg salting processes. The traditional pickling method yields egg yolks with a rounder shape and greater flavor, sand, and oil yield, but it has several disadvantages, including a long manufacturing process and high salt content in the egg whites (Anton, 2013). Moreover, the processing methods for the straw-ash method and salt-mud coating are relatively complex and require high mortar and mud humidities. Mud also needs to consider the degree of soil pollution, while mortar is difficult to reuse because of its high salt content, which can cause environmental pollution (Wang, 2015). The separation and salting of egg yolks can successfully address the limitations of conventional salting techniques, but the procedure is still in its developmental stage (Liu et al., 2022b). The wet-salting method is currently the main investigative technique for separating salting egg yolks because it provides uniform seasoning and convenient regulation. To mitigate the phenomenon of egg yolk hydration, 1 may add a specific quantity of metal salts to the salting solution (Fan, et al., 2011; Liu et al., 2023c).

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Following the centrifugation process, egg yolks can be separated into 2 distinct components: the egg yolk plasma (supernatant) and the egg yolk granule (precipitate). The primary constituents of egg yolk plasma are 85% low-density lipoprotein (**LDL**) and 15% livetin. The primary constituents of egg yolk granules are 70% high-density lipoprotein (**HDL**), 16% phosvitin (**Pv**), and 12% LDL (Anton, 2013; Gandhi and Alshehri, 2021; Zhang and Ma, 2024). Egg yolk plasma has good solubility and can be dissolved within the pH range and any ion strength commonly involved in food. Egg yolk granules are biodegradable, harmless, and functional emulsifiers. In addition, Pv in egg yolk granules exists in the form of acidic peptides in solution, which have significant metal ion chelation ability and may easily bind to multivalent metal cations such as Ca^{2+}, Fe^{3+}, Fe^{2+}, and Mg^{2+} (Anton, Denmat, and Gandemer, 2010). The structure of the egg yolk granule is disrupted, and fat is separated from lipoproteins as Ca^{2+} in the egg yolk granule is progressively replaced by Na^{+} during salting (Ai et al., 2018). Furthermore, when the oil extraction rates of plasma and granules throughout salting were compared, egg yolk plasma presented a substantially greater oil extraction rate than egg yolk granules did, indicating that NaCl primarily destroyed the structure of LDL and released free lipids (Denmat, Anton, and Beaumal, 2000).

Previous investigations have demonstrated that salted egg yolks with high oil yields can be produced within 24 h at room temperature (Liu and Chi, 2011). Owing to the appearance of water filling, we improved the quality of the salted egg yolk by modifying the salt solution composition. The addition of carboxymethylcellulose sodium (**CMC**) to the salting solution can increase the viscosity after salting, simulate egg whites and adjust the penetration rate of salt; the addition of citric acid can inhibit the phenomenon of water filling; and the addition of vitamin C can inhibit the excessive oxidation of lipids in egg yolks and prevent odor (Fan, 2011). On this basis, we added $CaCl_2$ to the salting solution to regulate and improve the quality of salted egg yolks. Finally, the optimum compositions of 20% (w/v) NaCl, 1.5% (w/v) $CaCl_2$, 1% (w/v) CMC, 0.3% (w/v) vitamin C and 0.3% (w/v) citric acid were obtained via orthogonal experiments. Nonetheless, egg yolks can be separated into plasma and granules after centrifugation. Therefore, to better understand the role of egg yolk plasma and granules components in improving egg yolk quality, the effects of $CaCl_2$ on the aggregation characteristics and protein structure changes of egg yolk plasma and granules during salting were investigated, aiming to reveal the intrinsic molecular mechanism of $CaCl_2$ in improving egg yolk quality.

MATERIALS AND METHODS

Materials and Reagents

The local supermarket was contacted to obtain fresh hen eggs. The salt was obtained from Longyan Salt Industry Co., Ltd. The food-grade substances $CaCl_2$, CMC, vitamin C, and citric acid were acquired from Shengfa Biotechnology Co., Ltd. Sigma–Aldrich was the supplier of 8-anilino-1-naphthalene sulfonate (**ANS**). Unless otherwise specified, the compounds used were of analytical grade and were utilized in their original form.

Preparation of Samples

The technique for manufacturing egg yolk plasma and granules was slightly modified according to the methods of Ulrichs (Ulrichs and Ternes, 2010). An equivalent volume of deionized water was added to the freshly prepared egg yolk mixture, which was subsequently centrifuged at 10,000 × *g* for 20 min at room temperature. The supernatant consisted of egg yolk plasma and was precipitated into egg yolk granules. The egg yolk plasma and granules were then divided into separate dialysis bags and salted at ambient temperature for 24 h, after which samples were collected at 6, 12, 18, and 24 h. The salting solution of the experimental group contained 20% salt, 1% CMC, 1.5% $CaCl_2$, 0.3% citric acid and 0.3% vitamin C (**CSEY group**), whereas the salting solution of the control group did not contain $CaCl_2$ (**SEY group**).

Spin Relaxation Time Measurements

Changes in the spin relaxation time of the egg yolk were quantified via low-field nuclear magnetic resonance (**LF-NMR**) spectroscopy (MesoMR23−060H-I, Suzhou Niumag Co., Ltd., Suzhou, China) according to the methods of Liu et al. (Liu et al., 2023b). The egg yolk plasma and granules were placed separately in the sample tank for measurement.

Rheological Property Measurements

The egg yolk plasma and granules apparent viscosity curves were determined with a HAAKE MARS40 rheometer (Thermo Fisher Scientific, MA). The samples were positioned amidst parallel plates of the rheometer, and any extra material was carefully removed. The dimensions of the parallel plates were 20 mm in size, and there was a 0.3 mm gap between them. The temperature was 25°C, and the shear rate was varied between 0.1 and 100 s^{-1}.

A HAAKE MARS40 rheometer was used to measure the changes in the dynamic viscoelasticity of both the egg yolk plasma and the granules. The sample was positioned on a parallel plate with a diameter of 35 mm and a spacing of 0.3 mm while maintaining a temperature of 25°C. Vibration dynamic scanning was conducted using a consistent strain force of 1% as the frequency varied from 0.1 to 15 Hz. The storage modulus (**G'**) and loss modulus (**G''**) were measured and recorded.

Particle Size Measurements

The sample was diluted with water (1:500, w/v) and stirred via magnetic force until the sample was uniformly blended. The particle size distribution of the egg yolk plasma and granules mixture was analyzed via a Malvern Mastersizer particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK). The test parameters included a sample refractive index of 1.42 and a water RI of 1.33.

Optical Microscope Observation

The staining emulsion was prepared by sequentially combining methyl red with Coomassie Brilliant Blue following the principles of Coomassie Brilliant Blue staining for protein and Coomassie Brilliant Blue staining for fat. The microstructure was examined via an optical microscope (BX53, Olympus Corporation, Tokyo, Japan) with a 10× objective.

SDS–PAGE

The SDS–PAGE technique developed by Laemmli (Laemmli, 1970) was employed. The samples were dissolved in 0.5% SDS (w/v, 1:10). The supernatant was retained by centrifuging the mixture at 8,000 × g for 15 min after homogenization at 8,000 × g for 1 min. The protein concentration in the supernatant was measured by the BCA assay kit, and the sample was evenly diluted to a concentration of 2 mg/mL. Afterward, the sample was completely blended with 4x loading buffer and then subjected to boiling in a water bath for 5 min. Following cooling to ambient temperature, SDS–PAGE was conducted via a 5% concentration gel and 12% separation gel, with 8 μL of sample loaded per well.

Zeta Potential Measurements

The zeta potential of each sample was evaluated by the Zetasizer nanolaser, after which the sample was diluted 1000 times with deionized water and stirred with magnetic force until fully mixed (NANO ZS90, Malvern Panalytical, Malvern, UK).

Raman Spectroscopic Measurements

The sample was placed in a tiled arrangement on glass surfaces in preparation for Raman spectroscopy (Renishaw Co., London, UK). The excitation wavelength was 785 nm, the scanning range was 400 to 4,000 cm^{-1}, the exposure time was 10 s, the measurement power was 10%, and 10 scans were collected. The phenylalanine peak at 1003 ± 1 cm^{-1} was utilized as an internal reference for normalization, and the analysis was conducted via Peakfit software.

Fluorescence Spectroscopy Measurements

A suitable quantity of the sample was measured and dissolved in 0.01 mol/L, pH 7.0, PBS. Following vortexing and centrifugation at 8,000 × g for 15 min, the protein content of the supernatant was quantified by the BCA reagent kit and then diluted to a protein concentration of 0.5 mg/mL. The precise measurement conditions were as follows: the excitation wavelength was 280 nm, the emission wavelength ranged from 300 to 420 nm, the slit width was 2.5 nm, and the scanning speed was 1200 nm/min.

Statistical Analysis

The experimental data were obtained by calculating the average of 3 measurements. The statistical software SPSS 16.0 was used to perform 1-way ANOVA on the data. Duncan's test was used for multiple comparisons of the means, and Student's t test was used for comparisons between 2 sets of values.

RESULTS AND DISCUSSION

Effect of $CaCl_2$ on Water Migration

The moisture level, distribution, and degree of binding with other components in a sample significantly impact the quality of the product and the final product. The relaxation time in LF-NMR data provides insights into the fluidity of different protein, water, and lipid constituents (Liu et al., 2019). Figure 1 displays the relaxation spectra of egg yolk plasma and granules, characterized by 3 main peaks: T_{21}, T_{22}, and T_{23}. The T_{21} peak, which occurs at approximately 1.04 ms, corresponds to the presence of water-bound hydrogen protons that are strongly bonded to macromolecules such as proteins and lipids. The distribution of T_{22} primarily occurred within the range of 7.35−11.90 ms, indicating the presence of hydrogen protons trapped within the network structure of egg yolks that do not readily mix with water. On the other hand, T_{23}, which appears after 100 ms, represents the proton peak of free water in egg yolks and is characterized by a significantly greater level of freedom of movement (Liu et al., 2023b). The T_{22} values of egg yolk plasma and granules in the CSEY group increased from 7.32 ms and 8.72 ms to 11.99 ms and 9.66 ms, respectively. Similarly, in the SEY group, the T_{22} values of egg yolk plasma and granules increased from 7.32 ms and 8.72 ms to 10.35 ms and 22.22 ms, respectively. The increase in T_{22} during salting is due to the destruction of the lipoprotein structure in the egg yolk, which leads to the release of lipids in the form of free fatty acids, improving the fluidity of hydrogen protons related to lipids. T_{22} in the egg yolk plasma of the CSEY group was greater than that in the SEY group, possibly because the addition of $CaCl_2$ inhibited the formation of the egg yolk plasma gel, reduced the residual hydrogen protons in the network structure, and promoted the release of lipids, thus improving fluidity and leading to an increase in T_{22} (Speroni et al., 2005). Conversely, the T_{22} of egg yolk granules in the CSEY group was lower than that in the SEY

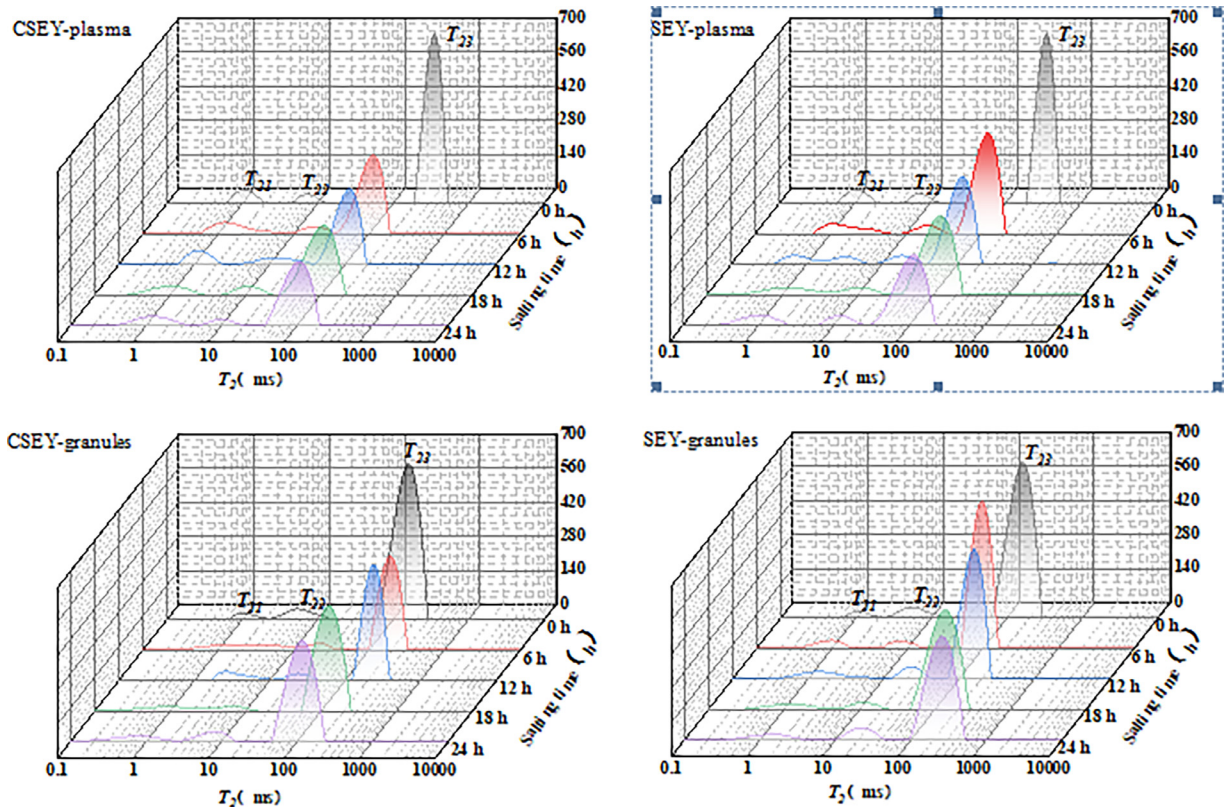

Figure 1. Changes in T2i of egg yolk plasma and granules during salting.

group. The egg yolk granules are linked together by HDL, LDL, and Pv through calcium–phosphorus bridges. Na^+ can substitute for Ca^{2+} in the calcium phosphorus bridge, which causes the rupture of the egg yolk granule, reducing the restriction on hydrogen protons and increasing their degrees of freedom, leading to an increase in T_{22} (Xu et al., 2019). These findings suggest that the inclusion of $CaCl_2$ can effectively bind HDL, Pv, and Ca^{2+} in egg yolk granules while also maintaining the structural integrity of the granules.

After the addition of $CaCl_2$ to the salting solution, Ca^{2+} and Na^+ are able to penetrate the egg yolk, which partially mitigates the detrimental effects of Na^+ on the egg yolk granule structure. Thus, the CSEY group presented relatively low levels of T_{21} and T_{22} egg yolk granules. The T_{23} of egg yolk plasma and granules in the CSEY group increased from 357.08 ms and 166.38 ms to 109.70 ms and 126.04 ms, respectively. Similarly, in the SEY group, the T_{23} of egg yolk plasma and granules increased from 357.08 ms and 166.38 ms to 117.59 ms and 135.10 ms, respectively. The egg yolk undergoes contraction and dehydration during salting, resulting in a decrease in water content, which causes the proteins in the egg yolk to bond more tightly, reducing fluidity and freedom of movement and ultimately leading to a decrease in T_{23} (Liu et al., 2022b; Xu et al., 2019). The T_{23} of the CSEY group egg yolk plasma and granules were lower than that of the SEY group, indicating that adding $CaCl_2$ to the salting solution can increase the dehydration level of egg yolk and narrow the gap between protein and protein, thus reducing the mobility of hydrogen protons associated with free water.

Effect of $CaCl_2$ on Apparent Viscosity

Figure 2 shows that the apparent viscosity of the egg yolk plasma in the SEY group and CSEY group gradually increased as the salting time increased, and both sharply decreased and tended to stabilize with increasing shear rate. The rheological curves of the CSEY group and SEY group plasma samples shifted upward as salting increased, indicating that the salting treatment had a substantial effect on the fluid properties of the egg yolk slurries. The protein conformation in egg yolk plasma changes during salting, manifested as an increased interaction between proteins as dehydration intensifies, which can improve the apparent viscosity of the material. The formation of aggregates of LDL and HDL can also increase the viscosity of egg yolks. Treatment with salt can cause the structure of LDL and HDL in egg yolk to unfold, cross-link, and aggregate with other components, resulting in an increase in egg yolk consistency (Yang et al., 2019).

The apparent viscosity of the egg yolk plasma in the CSEY group was greater than that in the SEY group, which was similar to the change in egg yolk apparent viscosity, indicating that the addition of $CaCl_2$ facilitated the dehydration of the egg yolk plasma, leading to an increased internal friction force under specific shear stress. The flow behavior of egg yolk is correlated with the length and degree of protein aggregation, indicating that the denser the protein aggregation is, the lower the shear thinning behavior it exhibits (Chen et al., 2013). Hence, adding $CaCl_2$ to the salting solution can promote the interaction between egg yolk protein molecules and shorten the distance between molecular chains. The

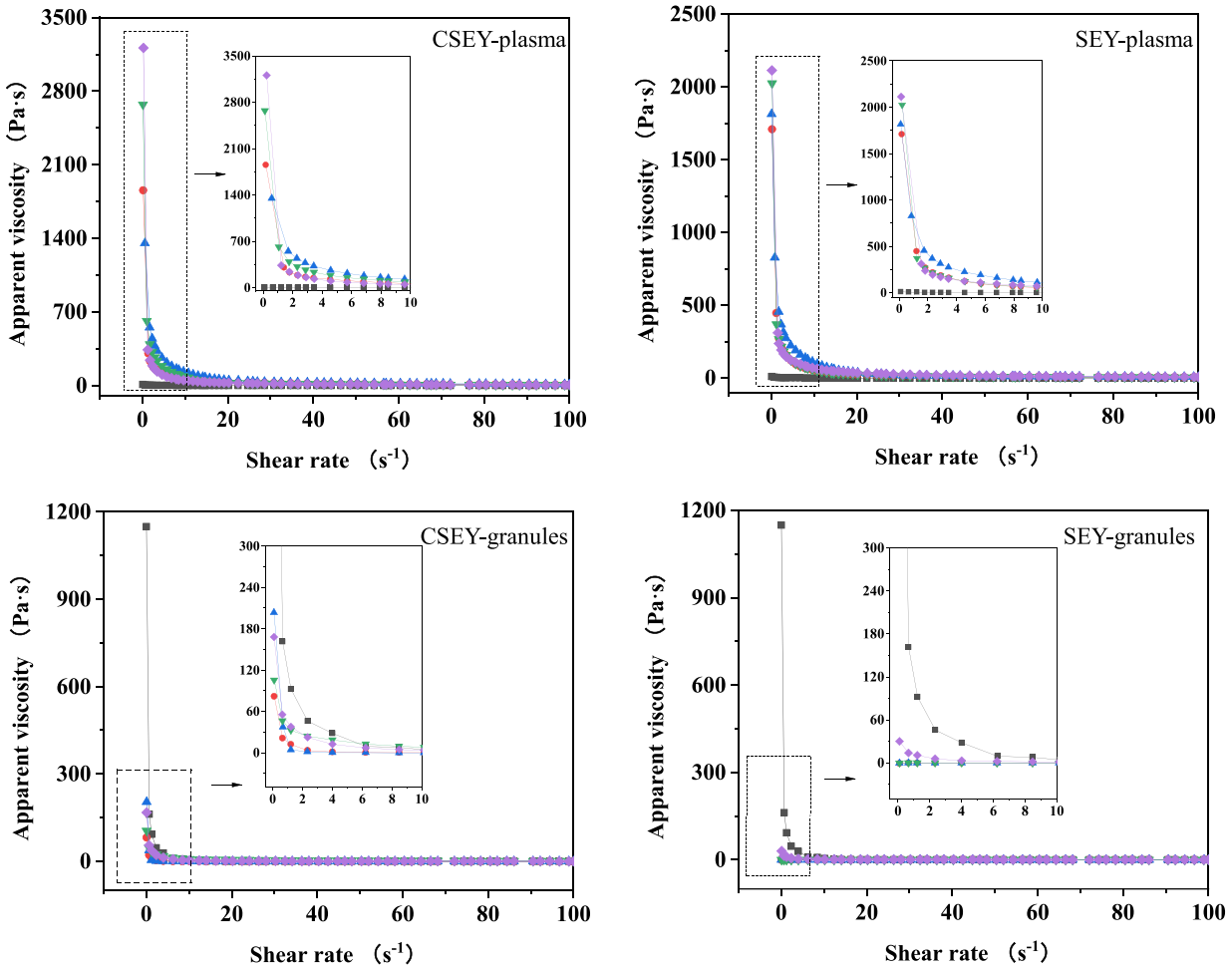

Figure 2. Changes in apparent viscosity of egg yolk plasma and granules during salting. Note: Salting time: 0 h (—■—); 6 h (—●—); 12 h (—▲—); 18 h (—▼—); 24 h (—◆—).

apparent viscosity of egg yolk granules in the SEY group and CSEY group was lower than that in the fresh egg yolk group, in contrast to the changes in the apparent viscosity of egg yolk plasma, suggesting that the intermolecular forces between egg yolk granules are greatly weakened after salting, which disrupts the chain breaks and entanglements between protein molecules, leading to a sharp decrease in their apparent viscosity (Yang et al., 2022). The egg yolk granules in the SEY group had a lower apparent viscosity than those in the CSEY group did, indicating that the addition of $CaCl_2$ can slow the depolymerization of the egg yolk granules, safeguarding their intermolecular forces against harm. Consequently, the change in apparent viscosity of egg yolks is mostly attributed to the presence of egg yolk plasma during salting. By creating a calcium–phosphorus bridge between HDL and phosvitin, Ca^{2+} can maintain the integrity of egg yolk particles. On the other hand, $CaCl_2$ promotes the dehydration of egg yolks, enhances the interactions within the structure of the egg yolk, and influences the rearrangement of the internal structure of the egg yolk (Severa, Nedomová, and Buchar, 2010).

Effect of $CaCl_2$ on Viscoelasticity

Figure 3 shows the changes in G' and G" of egg yolk plasma and granules throughout the salting procedure. Within the scanning frequency range, the G' values of the egg yolk plasma in the SEY group and CSEY group were greater than the G", indicating that the egg yolk plasma exhibited quasisolid properties. The G' and G" values of egg yolk plasma in the CSEY group and SEY group increased with frequency and were significantly greater than those of fresh egg yolk. Moreover, the G' and G" of egg yolk plasma in the SEY group and the CSEY group increased with increasing salting time, suggesting that the proteins in the egg yolk plasma interacted after salting, leading to the formation of a gel-like state and causing an increase in viscosity and a decrease in fluidity. Furthermore, the increase in G' of egg yolk plasma in the CSEY group was greater than that in the SEY group, whereas the difference in G" between the 2 groups was not significant. The higher G' in the CSEY group means that the addition of $CaCl_2$ prevents diligently bound protein molecules from changing into

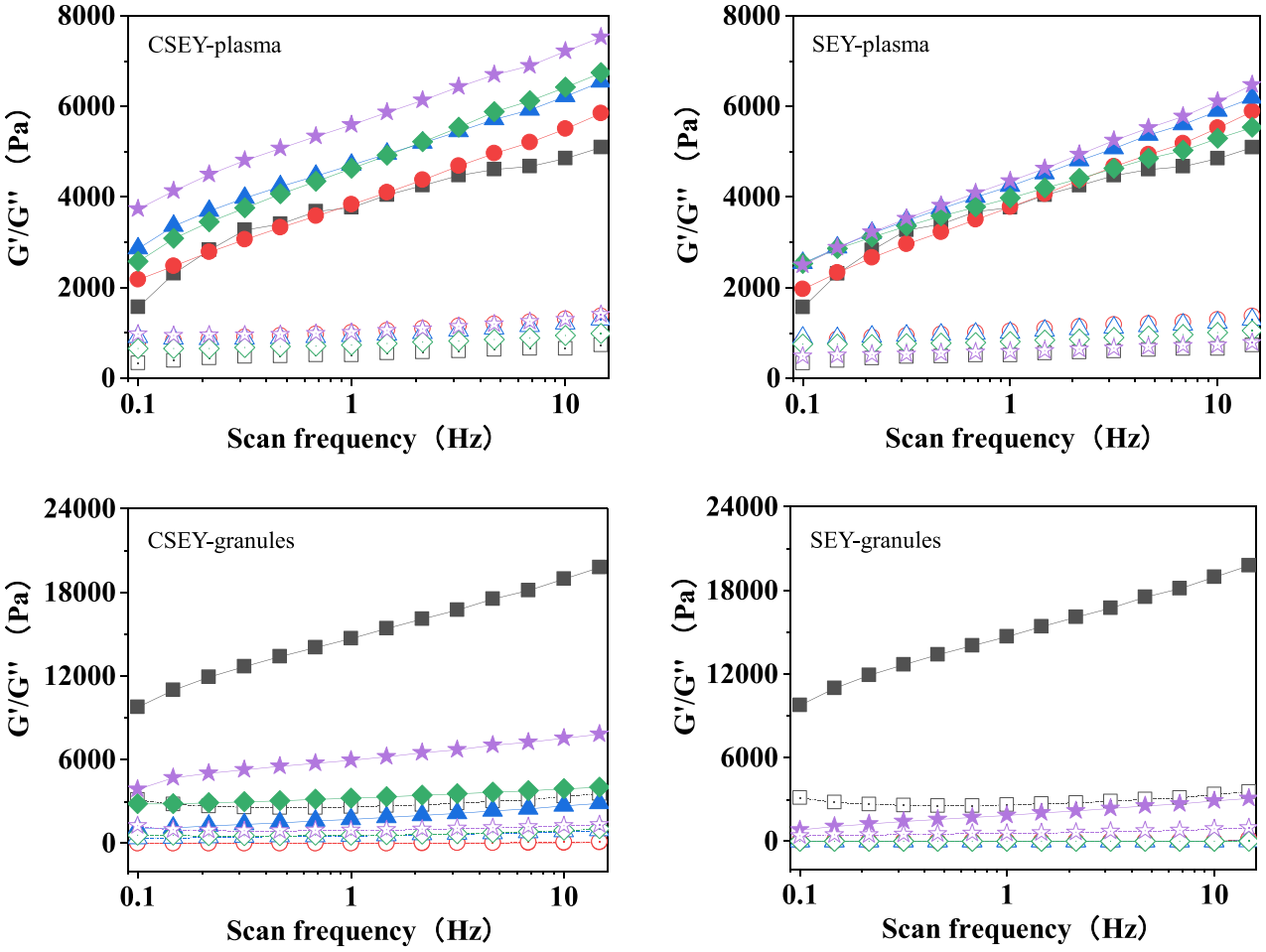

Figure 3. Changes in G' and G'' of egg yolk plasma and granules during salting. Note: Salting time: 0 h (G′: —■—; G″: --□--), 6 h (G′: —●—; G″: --○--), 12 h(G′: —▲—; G″: --△--), 18 h(G′: —◆—; G″: --◇--), 24 h (G′: —★—; G″: --☆--).

loosely structured or flexible molecules (Tu et al., 2012). In contrast to egg yolk plasma, which shows solid-like features when salted, the G'' of egg yolk granules in the SEY group and CSEY groups remained higher than G' during the whole salting process, indicating fluid characteristics. The G' of the salted egg yolk granules is lower than that of the fresh egg yolk granules because of the dissociation of "HDL-Pv-LDL" in the egg yolk granule caused by NaCl (Anton, 2013; Anton and Gandemer, 1997). The increase in G' in the CSEY group surpassed that in the SEY group, and the difference in G' between the 2 groups was not significant, indicating that the SEY group displayed a greater degree of egg yolk granules disintegration and the disruption of weak protein linkages (Gao, 2021). Causeret et al. hypothesized that Ca^{2+} can promote the oligomerization of egg yolk granules and that the addition of $CaCl_2$ can maintain the unbroken structure of egg yolk granules (Causeret, Matringe, and Lorient, 2010). When the egg yolk is salted, the salt penetrates the particle structure, causing LDL and HDL to separate. More Ca^{2+} will bond to the exposed carboxyl group as a result of the addition of $CaCl_2$ to the salting solution, which helps maintain the integrity of the egg yolk granule structure (Bryant and Mcclements, 1998; Yang et al., 2019). Bivalent metal ions, unlike univalent metal ions, can establish cross-links between specific amino acid residues of adjacent polypeptides, interact with the unfolding protein's surface negative charge, and form a "salt bridge," which strengthens protein aggregation (Remondetto and Subirade, 2003; Ai et al., 2018). Therefore, adding $CaCl_2$ to the salting solution changes the protein molecular conformation and interaction between molecules, which affects lipoprotein aggregation, and the results demonstrated that egg yolk plasma plays a major role in the dehydration and coagulation of salted egg yolks.

Effect of $CaCl_2$ on the Particle Size Distribution

The particle size distributions of the egg yolk plasma and granules in the CSEY and SEY groups are shown in Figure 4. The particle size distribution curve of fresh egg yolk plasma exhibited a solitary peak, with particles ranging from 3.27 to 352 μm in size. The distribution of the peak area and peak form changed significantly for egg yolk plasma after salting, ranging from 22 to 200 μm. When the particle size distribution of fresh egg yolk plasma was compared, the maximum peak points of the SEY group and CSEY group showed a transition toward larger particle sizes, demonstrating that proteins

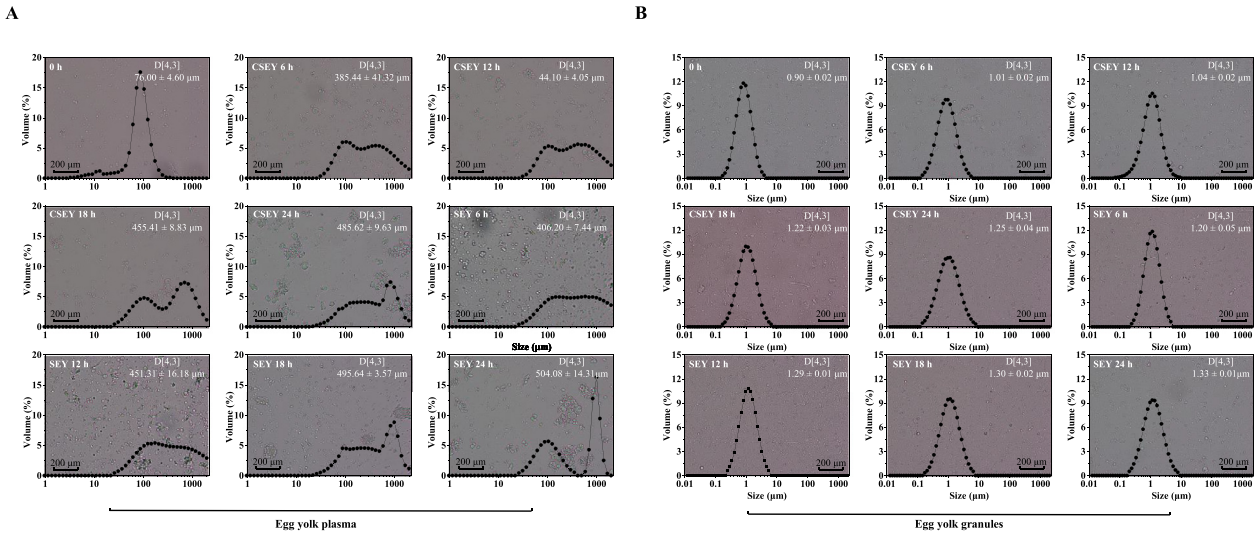

Figure 4. Optical microstructure and volume mean particle size of egg yolk plasma and granules during salting.

in the egg yolk plasma formed aggregates and that the degree of aggregation increased gradually. Dou et al. reported that reducing the pH of egg yolk increases the charge density of the LDL surface, enhances the interaction between protein and protein and between protein and solvent, and facilitates the development of LDL gels (Dou et al., 2016). Research has demonstrated that the pH of egg yolks gradually decreases during salting (Liu et al., 2023b). Consequently, LDL, as the main protein in egg yolk plasma, undergoes rearrangement and aggregation after salting, resulting in an enlargement of the granules. Anton et al. also demonstrated that LDL is the protein that is involved mainly in gel behavior in egg yolks (Anton et al., 2001). Nevertheless, the peak shape of the egg yolk granules did not change after salting, and all the samples exhibited a single peak distribution, ranging from 0.072 to 9.25 μm.

The D [4,3] of fresh egg yolk plasma is 76.00 ± 4.60 μm. Salting increased the D [4,3] of the egg yolk plasma in the SEY and CSEY groups to 504.08 ± 14.31 μm and 485.62 ± 9.63 μm, respectively. These findings suggest that the protein molecules in the egg yolk plasma aggregated and increased the average particle size. Egg yolk plasma in the CSEY group had a lower D [4,3] than that in the SEY group ($P < 0.05$), suggesting that the aggregates of egg yolk plasma in the CSEY group had smaller particle sizes. Research shows that adding $CaCl_2$ competes with NaCl for water, reducing the salt content of egg yolks, hindering the formation of LDL−NaCl complexes and inhibiting the formation of LDL gels (Naderi et al., 2017). Nevertheless, the D [4,3] of egg yolk granules exhibited a smaller increase than the degree of aggregation observed in egg yolk plasma. The D [4,3] of the egg yolk granules in the CSEY group and SEY group increased as the salting time increased. Additionally, the D [4,3] of the CSEY group was lower than that of the SEY group ($P < 0.05$). Although the D [4,3] of egg yolk granules tended to increase, it was significantly lower than that of egg yolk plasma. Yang discovered that the presence of NaCl causes the salt-soluble proteins in egg yolk granules to dissolve, disrupting the structure of the granules, resulting in a decrease in the interface stability and promoting protein aggregation to form larger granules (Yang, 2018). Consequently, the greater D [4,3] in the SEY group was partly due to the inhibitory effect of $CaCl_2$ on the breakdown of egg yolk granules, which reduced the aggregation of proteins between egg yolk granules. In addition, divalent metal ions can significantly improve the connections between protein molecules, which is beneficial for reducing the hydrophobic clustering caused by negative charges between protein molecules and maintaining the stability of egg yolk particles HDL-Pv-LDL (Liu et al., 2022a).

SDS–PAGE Analysis

Figure 5 illustrates the alterations in the composition of egg yolk plasma and granules proteins in the SEY group and CSEY group during salting. The proteins in egg yolk plasma are predominantly apo-LDL and $\alpha/\beta/\gamma$-livetin, whereas the proteins in egg yolk granules are mainly apo-HDL, γ-livetin and photosvitin (Wang et al., 2020). Previous research has indicated that in egg yolk plasma, a molecular weight less than 25 kDa represents apo-LDL, 33 kDa represents β-livetin and apo-LDL, 39 kDa represents α/β-photosivitin, 42 kDa represents β-livetin, 65 kDa represents γ-livetin, 68 kDa and 110 kDa represent apovitellin, 70 kDa, 94 kDa and 130 kDa represent apo-LDL, and 85 kDa represents apo-LDL α-livetin. In egg yolk granules, 25−35 kDa represents β-Livetin; 39 kDa, 55 kDa, 68 kDa, and 110 kDa represent apovitellin; 47 kDa represents apo HDL; 79 kDa represents apo HDL; $\alpha/\beta/\gamma$-Livetin and apo-HDL; and 130 kDa represents γ-Livetin and apo-HDL/LDL. The high-molecular-weight cross-linked proteins γ-Livetin and apo-LDL are present in bands with molecular weights exceeding 180 kDa in egg yolk plasma and granules. Figure 5A shows that the abundance of bands near 33, 110, and 130 kDa in the salted egg yolk plasma decreased, indicating a relative reduction in the quantity of apo-LDL and apovitellin in the egg yolk plasma.

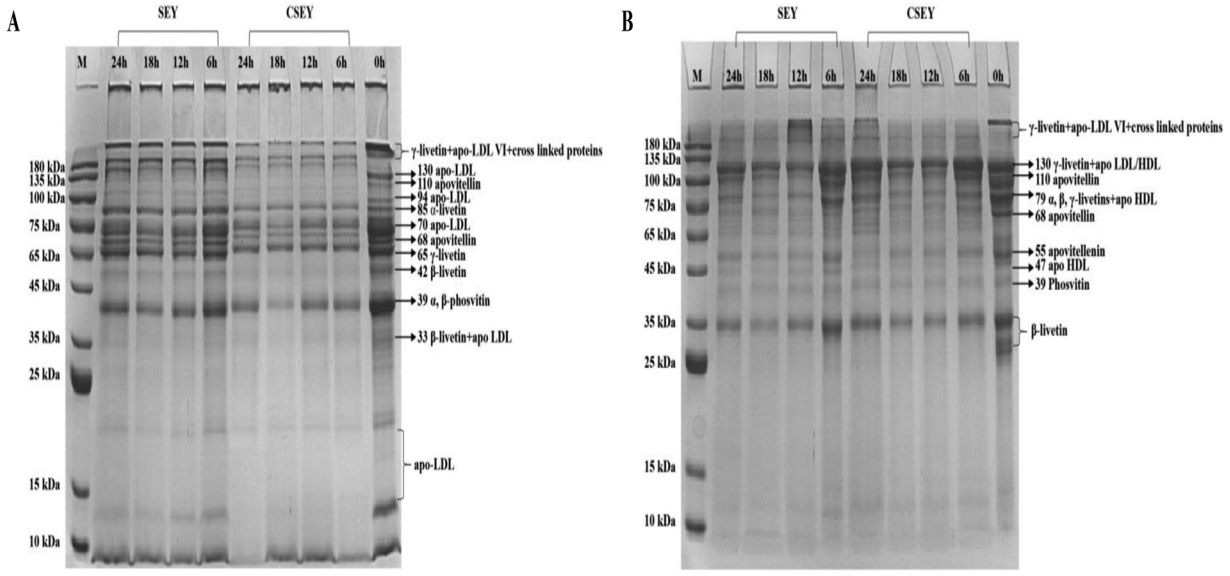

Figure 5. Changes in SDS-PAGE of egg yolk plasma (A) and granules (B) during salting.

These 2 proteins are suggested to be the primary proteins involved in protein–protein interactions in egg yolk plasma. Moreover, compared with the SEY group, the CSEY group presented a notable decrease in the quantity of protein bands exceeding 180 kDa, indicating the formation of larger aggregates in the SEY group egg yolk plasma. Figure 5B shows that the abundance of protein bands near 68 and 110 kDa in the salted egg yolk granules decreased, indicating a structural change in the HDL–Pv complex within the egg yolk granules.

Effect of $CaCl_2$ on the Zeta Potential

Figure 6A shows that the zeta potential of egg yolk plasma is positively charged. The absolute increase in the zeta potential of egg yolk plasma indicates that salting promotes the unfolding of protein structures, causing the transfer of buried charged amino acids outward and increasing the net charge on the surface of protein molecules, which is beneficial for enhancing the electrostatic interactions between proteins. The absolute value of the zeta potential in CSEY egg yolk plasma is lower than that in the SEY group because Ca^{2+} can effectively shield the electrostatic interactions between proteins and form ion bridges, leading to a decrease in the absolute value of the zeta potential (Liu et al., 2022b; Liu et al., 2023b). Figure 6B shows that the zeta potential of the egg yolk granules was negative. The absolute value of the zeta potential of egg yolk granules in the SEY group increased and subsequently decreased, whereas the absolute value of the total zeta potential of egg yolk granules in the CSEY group gradually decreased. Within 0–12 h of salting, the rupture of the egg yolk granules gradually exposed charged amino acid residues,

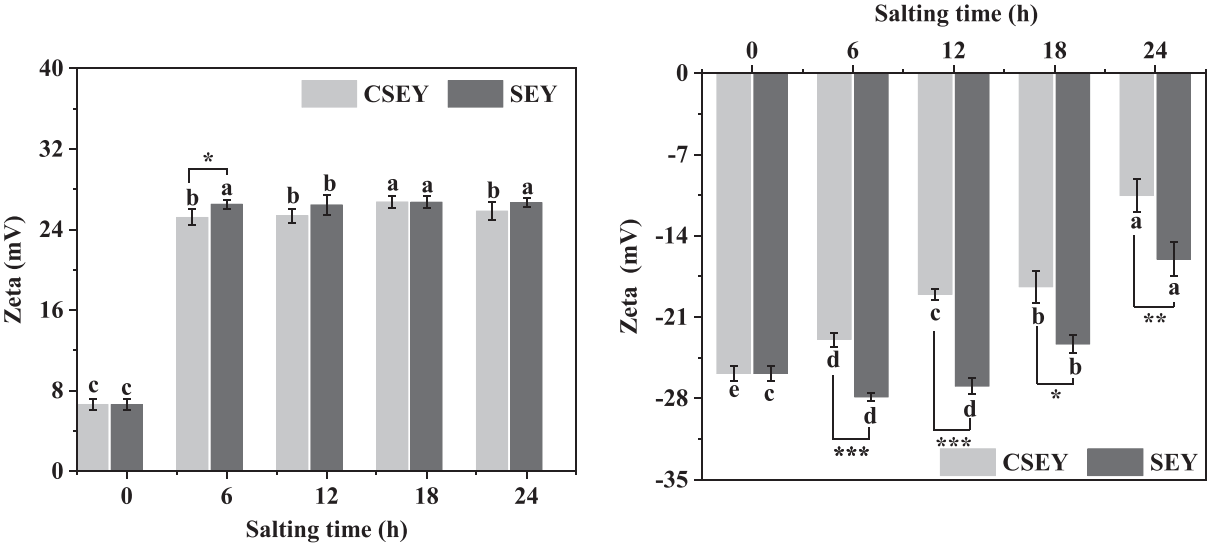

Figure 6. Changes in Zeta potential of egg yolk plasma (A) and granules (B) during salting. Note: the letters represent the difference analysis under different salting times within the same treatment group; *represents the difference analysis between different treatment groups at the same salting time.

increasing the absolute zeta potential of the egg yolk granules in the SEY group. By extending the salting duration to 24 h, the protein structures contracted and interacted with each other, causing the charged amino acids to progressively become embedded, reducing the absolute value of the zeta potential (Xu et al., 2019). The absolute zeta potential of the egg yolk granules in the CSEY group was significantly lower than that in the SEY group ($P < 0.05$) because of the reaction between Ca^{2+} and COO^{-}, which dissociates acidic amino acids in the protein chain and reduces the negative charge carried by the emulsion (Hongsprabhas and Barbut, 1997).

Raman Spectroscopic Analysis

Figure 7 displays the Raman spectra of the egg yolk plasma and granules in the SEY group and CSEY group. The spectrum of egg yolk plasma changed significantly within the range of 1250 to 1400 cm^{-1} during salting, whereas the spectrum of egg yolk granules remained unchanged between 550 and 2250 cm^{-1}. The amide I band, which is represented by the absorption peak at 1,600 to 1,700 cm^{-1}, is attributed primarily to C = O stretching vibrations, C−N stretching vibrations, and N −H bending vibrations. The presence of a C=C stretching vibration is responsible for the absorption peak observed at approximately 1,520 cm^{-1}. The lengthening vibration of the C–N bond, the stretching vibration of the C–C bond, and the bending vibration of the N–H bond are responsible for the amide III band, which is observed at the absorption peak between 1,220 to 1,350 cm^{-1}. The absorption peak at approximately 936 cm^{-1} is a result of the stretching vibration of the C–C bonds in the protein's main chain. The absorption peak at approximately 760 cm^{-1} is attributed to the presence of the indole ring in tryptophan (Wang et al., 2020). The amide I band, which ranges from 1,600 to 1,700 cm^{-1}, provides the most significant information regarding the secondary structure of proteins.

Figure 7 shows that the predominant components of the protein secondary structure in fresh egg yolk plasma are β-turns, followed by β-sheets, α-helices, and irregular curls. Compared with those in fresh egg yolk plasma, the relative proportions of β-turns and irregular curls in salted egg yolk plasma decreased, whereas the relative proportions of β-sheets and α-helices increased, indicating that the proteins in the egg yolk plasma gradually tended toward an ordered structure. Yang et al. reported that during the salting process, the structural change in egg yolk protein was significant intermolecular β-sheets, accompanied by a decrease in intramolecular β-sheets, indicating that the protein expanded after aggregation (Yang et al., 2019). NaCl can increase the order of protein molecules in egg yolk plasma, increasing the proportion of β-sheets to α-helices (Liu et al., 2023a). The SEY group presented a greater concentration of β-sheets, indicating that interactions between proteins and lipids, between proteins and proteins or hydrophobic interactions play a role in promoting the aggregation of nonpolar proteins, which is conducive to the formation of gel structures. Hence, the addition of $CaCl_2$ can inhibit the development of egg yolk plasma gel. Proteins in fresh egg yolk granules exhibit a primary structure that is mostly composed of β-turns, followed by α-helices, irregular curls, and β-sheets. Following salting, the proportions of β-turns and irregular curls in the CSEY group and SEY group decreased, whereas the proportions of β-sheets and α-helices increased. The relative contents of β-turns, β-sheets, and α-helices in the egg yolk granules of the CSEY group were lower than those in the SEY group, reflecting a reduction in protein aggregation and a relatively looser protein structure. $CaCl_2$ has the ability to prevent HDL-Pv-LDL from disintegrating and to reduce the amount of particles that are released, including LDL, phosvitin, and HDL, thereby maintaining the integrity of the egg yolk granules structure, increasing the content of the intramolecular β-sheet structure (Xu et al., 2019). Egg yolk plasmas and granules aggregate in the process of egg yolk gel, in

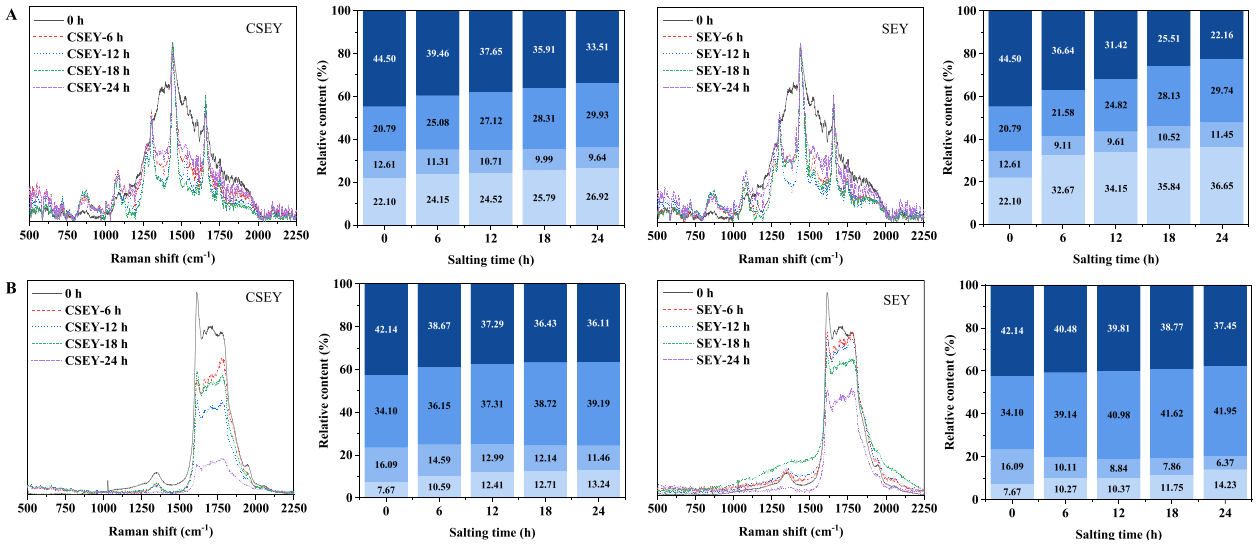

Figure 7. Changes in Raman spectroscopy and the protein secondary structures of egg yolk plasma (A) and granules (B) during salting. Note: β-sheet ( ), irregular curl ( ), α-helix ( ), β-turn ( ).

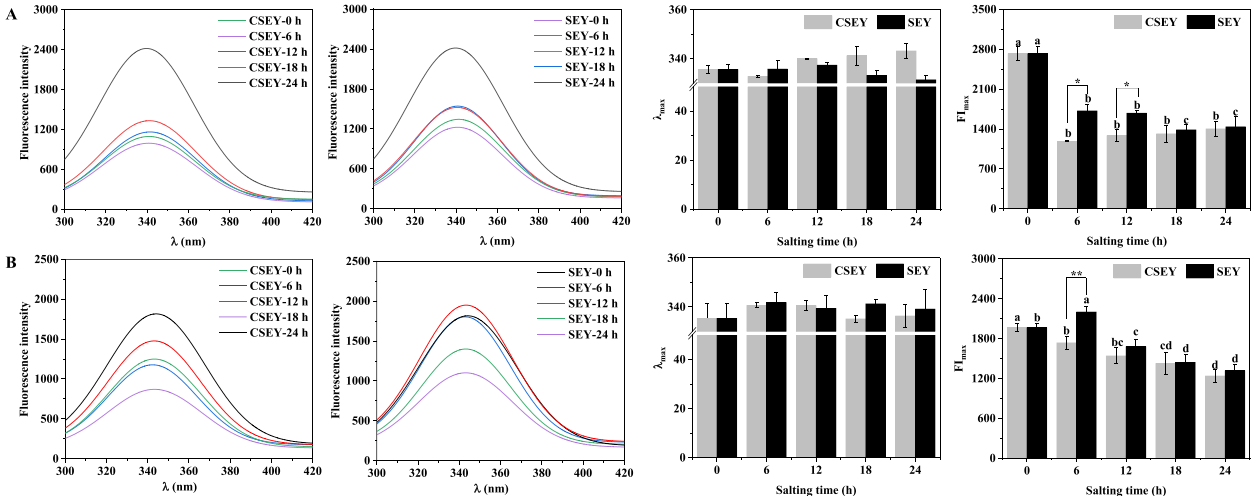

Figure 8. Changes in fluorescence spectroscopy, λmax and FImax of egg yolk plasma (A) and granules (B) during salting. Note: the letters represent the difference analysis under different salting times within the same treatment group; *represents the difference analysis between different treatment groups at the same salting time.

which yolk granules may undergo extreme unfolding and then accumulate with each other and be linked by certain types of chemical bonds (Yang et al., 2020). Hence, the changes in protein content during the process of egg yolk salting are caused by the combination of egg yolk plasmas and granules.

Fluorescence Spectroscopy Analysis

By investigating fluorescent chromophores, protein intrinsic fluorescence spectroscopy can provide valuable insights into the structural and functional characteristics of proteins. Protein intrinsic fluorescence primarily characterizes changes in tertiary structure by reflecting tryptophan, tyrosine, and phenylalanine (Jiang et al., 2014). Figure 8 shows that the fluorescence spectra of proteins in egg yolk plasma and granules remained largely consistent across various salting times. However, compared with those in fresh egg yolk, the maximum fluorescence intensity (FI_{max}) and maximum fluorescence wavelength (λ_{max}) in the SEY and CSEY groups significantly differed, revealing that salting caused changes in the tertiary structure of proteins in egg yolk plasma and granules. Compared with that of the fresh egg yolk plasma group, the λ_{max} of the CSEY group increased from 335.6 to 343.2 nm. The λ_{max} of the SEY group increased from 335.6 to 337.4 nm and subsequently decreased to 331.4 nm. Throughout the entire salting process, the trend of changes in the λ_{max} of the salted egg yolk granules was generally consistent with that of the egg yolk plasma. The λ_{max} of the egg yolk granules in the CSEY group increased from 335.4 to 336.4 nm, and the λ_{max} of the egg yolk granules in the SEY group increased from 335.4 to 339.2 nm. The fluorescence emission wavelength of amino acid residues is determined by the microenvironment, with a peak redshift occurring when the environmental polarity increases and a peak blueshift occurring when the hydrophobicity increases. A redshift in λ_{max} reveals that protein unfolding causes a minor alteration in protein structure, leading to a progressively more significant movement of the hydrophobic regions of tryptophan residues to the polar environment (Lechevalier et al., 2017). Nevertheless, the λ_{max} of egg yolk plasma in the SEY group exhibited a blueshift between 12 and 24 h after salting, which may be attributed to protein aggregation and cross-linking in the egg yolk plasma following salting, blocking tryptophan residues in the formed network gel structure (Sponton et al., 2015). Moreover, the redshift of λ_{max} in the CSEY group was greater than that in the SEY group, indicating a greater level of tryptophan exposure on the protein surface and a lower degree of aggregation between proteins. However, the redshift of λ_{max} in the egg yolk granules in the CSEY group was smaller than that in the SEY group, indicating that there were more tryptophan residues on the protein surface in the egg yolk granules of the SEY group and that the egg yolk granules structure tended to expand. The tryptophan residues in the egg yolk granules in the CSEY group were mostly in a hydrophobic environment, which means that the degree of granules disintegration was low and that the number of exposed tryptophan residues was reduced.

Following salting, the FI_{max} of egg yolk plasma in the CSEY group decreased from 2722 to1404 and from 2722 to 1439 in the SEY group. The FI_{max} of egg yolk granules in the CSEY group decreased from 1965 to 1239 and from 1965 to 1316 in the SEY group ($P < 0.05$). The FI_{max} of egg yolk plasma increased and then decreased after salting, revealing that the conformation of LDL changed during the early stage of salting, with Tyr and Trp residues originally buried inside the protein exposed to the outside, indicating that LDL transitioned from a tight state to a more extended structure. When the salting time was extended to 24 h, the decrease in FI_{max} indicated that the microenvironments of Trp and Tyr changed and that the formation of LDL aggregates was enhanced through hydrophobic interactions (Liu et al., 2023c). In contrast, the FI_{max} of egg yolk granules

significantly decreased ($P < 0.05$), further indicating that after salting, the structure of egg yolk granules unfolded, and the conformation of the lipoprotein molecular structure changed. The residues of the Tyr and Trp amino acids, which were originally hydrophobic and located inside the molecule, were exposed to the surface of the protein molecule, and the tertiary structure of the protein changed from a compact state to a more extended structure (Sheng et al., 2019). Throughout the entire salting process, the FI_{max} values of egg yolk plasma and granules in the CSEY group were generally lower than those in the SEY group, indicating a looser structural state of LDL and HDL in the CSEY group. Therefore, the addition of $CaCl_2$ can affect the hydrophobic interaction between HDL and LDL, reduce their degree of polymerization, inhibit the formation of gel, and help improve the sandiness of salted egg yolk. Consequently, the results suggest that the SEY group and CSEY group may have different aggregation modes for egg yolk proteins. After unfolding, the proteins in the SEY group cross-linked to form a 3-dimensional network structure, whereas the proteins in the CSEY group primarily collided and aggregated with one another.

CONCLUSIONS

The aggregation behavior of salted egg yolks is mostly determined by the composition of the egg yolk plasma. The addition of $CaCl_2$ enhances the relaxation duration of T_{22}, and the mobility and freedom of hydrogen protons decreases the quantity of hydrogen protons incorporated in the network structure, facilitates the release of free lipids, and prevents the development of egg yolk plasma gel. Moreover, $CaCl_2$ can maintain the structural integrity of egg yolk granules, limiting the degree of Na^+ damage to egg yolk granules. $CaCl_2$ may also hinder the increase in protein flexibility, increase intermolecular interactions between proteins, and improve the apparent viscosity and G' of egg yolk plasma and granules. Additionally, $CaCl_2$ can maintain the integrity of the egg yolk granules structure, reduce the disintegration of egg yolk granules, prevent HDL, Pv and LDL in egg yolk granules components from participating in the formation of gel structures, increase the degree of looseness between proteins, and increase the sandiness of the salted egg yolk after baking. However, the changes in egg yolk throughout the salting process are quite complicated and impacted by numerous factors, necessitating additional examination of the change patterns between lipids and HDL, LDL, livetin, and Pv using more biological methodologies and contemporary instruments.

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DISCLOSURES

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "The effect of $CaCl_2$ on water migration, rheological properties, aggregation behavior and protein structure in rapidly salted separated egg yolk plasma and granules".

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