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Research Article

Insight into the cryoprotective effect of egg yolk peptides on frozen egg yolk: functional, structural and physicochemical properties

Jiajia Zhang[1], Yuchang Jin[1], Yaru Meng[1], Yaotong Liu[1], Xiaoxue Yang[1], Ying Cao[1], Yanqiu Ma[1] ✉, Xin Gao[1] ✉, Peng Yu[2]

[1] *College of Food Science, Northeast Agricultural University, Harbin 150030, China*
[2] *Department of Grain Engineering, Heilongjiang Communications Polytechnic, Harbin 150030, China*
✉Address correspondence to Yanqiu Ma, yqma2019@neau.edu.cn; Xin Gao, Gaoxin0427@163.com
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Abstract: Egg yolk peptides were prepared by enzymatic hydrolysis of defatted egg yolk powder. The cryoprotective effects of these peptides were assessed by examining the functional, structural and physicochemical properties of egg yolk. Results indicated that egg yolk peptides could reduce viscosity and prevent the accumulation of egg yolk proteins during freezing, thereby mitigating the deterioration of its functional characteristics. The peptide hydrolyzed for 3 h demonstrated the lowest fluidity in thawed egg yolk. Additionally, Fourier transform infrared (FTIR) spectra and intrinsic fluorescence spectra revealed that egg yolk peptides could reduce damage to the secondary and tertiary structures of proteins, enhancing the stability of the protein network. Changes in the content of secondary structures showed that egg yolk peptides increased β-turns, decreased β-sheets, and maintained overall structural stability and strength. This study provides new insights for egg yolk peptides to inhibit frozen egg yolk gelation.

Keywords: frozen egg yolk; gelation; egg yolk peptides; cryoprotective effect; protein properties

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1 Introduction

Eggs contain protein, fat, vitamins, and minerals, making them a high-quality protein source favored by consumers. The yolk is the most intricate component of the egg and consists of about 48% dry matter, mainly composed of protein (31%) and fat (65%–70%)[1]. This complex lipid-protein system not only provides high nutritional value but also endows the egg yolk with excellent solubility and other functional properties[2], making it widely used in products like cakes, creams, salad dressings, and mayonnaise.

Freezing is a common and effective method for preserving food that addresses microbial contamination and quality deterioration during transportation, thus prolonging the shelf life of liquid eggs. However, egg yolk is susceptible to damage from ice crystal growth and recrystallization during freezing and frozen storage. Gelation occurs through the interaction between components after freezing at –18 °C for more than 6 h[3], causing irreversible damage to both macro-properties and micro-structure of the protein. Mechanical treatment can cause the egg yolk to scatter in spots, which makes it challenging to blend with other food ingredients[4], significantly impacting its subsequent applications. Since Moran[5] studied frozen egg yolk gelation, numerous approaches have been explored to inhibit this gelation. To date, physical methods (homogenization, colloid grinding, etc.), chemical methods (adding sugar, alcohol, salt, and antifreeze peptides, etc.), and enzymatic methods have shown some success in protecting egg yolk from freezing[6]. However, their limitations, such as health concerns from high salt or sugar and flavor issues from protein hydrolysis, restrict their broader application. Some protein hydrolysates could protect organisms from damage in freezing or sub-freezing conditions[7]. These peptides with antifreeze activity have been applied to frozen dough, frozen meat, and other sectors of the food industry, demonstrating effective freezing protection[8].

The defatted egg yolk powder is a by-product of phospholipid extraction, which contains a variety of proteins[9]. Residual organic solvents could cause the functional features of defatted egg yolk powder to be lost, resulting in the discarding of a substantial portion of the product and a significant waste of egg yolk protein[10–11]. It can be fractionated and used in various food processing or further developed into functional peptides to increase the utilization value of defatted egg yolk[12]. Recent studies have focused on the use of enzymatic hydrolysis to produce peptides, which have biological activities including anti-inflammatory, antioxidant, antihypertensive, and antidiabetic, etc.[13]. The present research suggests that egg yolk peptides are successful in averting the gelation of frozen egg yolk when used at the addition level of 5%[14].

However, there remains a scarcity of information regarding how egg yolk peptides influence protein properties in egg yolk before and after freezing. Since various food applications demand specific qualities in egg yolk proteins, making it essential to understand how freezing affects their properties. In this study, the cryoprotective effect of egg yolk peptides prepared from defatted egg powder at different enzymatic hydrolysis times on egg yolk was explored. The functional properties (viscosity, solubility, and emulsifying properties), structural properties (secondary and tertiary structure) and physicochemical properties (turbidity, particle size, and free sulfhydryl content) of unfrozen and frozen-thawed egg yolk were studied. This study aims to provide a reference for the further

processing and use of frozen egg yolk. Additionally, it lays the foundation for developing protein hydrolysates from egg yolk by-products as potential new antifreeze agents.

2 Materials and methods

2.1 Materials

Fresh eggs were sourced from the local supermarket located in Harbin City, Heilongjiang Province, China. Bromelain (300 U/mg, CAS: 37189-34-7) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Defatted egg yolk powder (protein, 77.5%) was supplied by Yibin Wild Resource Phytochem Co., Ltd. (Yibin, China). Bovine serum albumin (BSA) was obtained from BioFroxx (purity > 98%, Germany). All other reagents utilized in this study were of analytical grade.

2.2 Preparation of samples

2.2.1 Egg yolk peptides

Egg yolk peptides were prepared following by Li et al.[15] with some modifications. A 5 g/100 mL solution of defatted egg yolk powder in deionized water was prepared. After adding bromelain at 10 000 U, the hydrolysis reaction was conducted at 55 °C for 1, 3, or 5 h. The enzymatic hydrolysis temperature of the water bath was raised to 95 °C and boiled for 10 min to achieve the inactivation of bromelain. Following natural cooling, the hydrolysates were centrifuged at 6 000 r/min for 15 min. The supernatant obtained was subsequently freeze-dried for two days, ground in a mortar, and stored at −20 °C.

2.2.2 Liquid egg yolk

Liquid egg yolk was prepared as follows. The shell eggs were manually broke and the collected yolks were mixed with a glass rod. The egg yolk peptides were added to yolks at 4% (*m*/*m*) and mixed well slowly with a power mixer (JJ-1, Kanghua, Changzhou, China). The mixed liquid yolks were kept at a temperature of (4 ± 2) °C until they were needed. The samples without egg yolk peptides were designated as FEY, whereas those containing egg yolk peptides were marked as Y(B)0h, Y(B)1h, Y(B)3h, and Y(B)5h, respectively, among which 0 h was defatted egg powder without enzymatic hydrolysis.

2.2.3 Frozen egg yolk

Frozen egg yolk was conducted as follows. The 30 mL of the previously prepared liquid egg yolk sample was placed into a 50 mL centrifuge tube and then frozen at (−18 ± 2) °C for 24 h. After freezing, the egg yolk samples were left to thaw at room temperature for more than 4 h before being analyzed.

2.3 Analysis of functional properties of egg yolk

2.3.1 Viscosity and visual images

The egg yolk samples were inverted in front of a contrasting background and the flow state was observed during the thawing process and an image was taken. The apparent viscosity was measured by a digital viscometer (DVS+, Brookfield, USA) with rotor S64 at 5 r/min.

2.3.2 Solubility

Solubility was evaluated by the ratio of protein concentration before and after centrifugation following by the method of Xu et al.[16]. The egg yolk sample was stirred in deionized water until the yolk was completely dissolved. Each sample was then split into two portions. One portion was centrifuged at 8 000 r/min for 15 min at 4 °C, and the other portion was used as egg yolk solution. Both the supernatant (1 mL) and the egg yolk solution were combined with 4 mL of biuret reagent and reacted for 30 min. Absorbance was measured at 540 nm, and the protein concentration was determined using a standard curve generated with BSA as the reference. The equation of the standard curve was $y = 0.034\ 2x + 0.071\ 9$ ($R^2 = 0.996$).

2.3.3 Emulsivity

Emulsivity included the emulsifying activity index (EAI) and emulsion stability index (ESI), was determined based on the method described by Lu et al.[17] with some modifications. The yolk sample was diluted with deionized water to a concentration of 5 mg/mL. Such protein solutions and soybean oil were mixed in a 4:1 (*V*/*V*) ratio and emulsified with a high-speed disperser (XHF-DY, Scientz Biotechnology Co., Ltd., Ningbo, China) at 10 000 r/min for 2 min. Samples were drawn from the bottom of the emulsion at 0 min and after 30 min, then added thoroughly with 0.1 g/100 mL sodium dodecyl sulfate (SDS) in a 1:100 (*V*/*V*) ratio. The absorbance was recorded at a wavelength of 500 nm. The EAI and ESI were calculated by equations (1) and (2), respectively:

$$\mathrm{EAI}\ (\mathrm{m^2/g}) = \frac{4.606 \times A_0 \times D}{\rho \times (1-\varphi) \times 10} \tag{1}$$

$$\mathrm{ESI}\ (\mathrm{min}) = \frac{A_0 \times \Delta t}{A_0 - A_{30}} \tag{2}$$

Where A_0 and A_{30} were the absorbance obtained at 0 and 30 min, respectively; D was the dilution factor (100); ρ was the concentration of the protein solution (5 mg/mL); φ was the proportion of oil in emulsion (0.2); Δt was the emulsion rest time (30 min).

2.4 Analysis of structural properties of egg yolk

2.4.1 Secondary structure

The yolk samples were lyophilized and ground into powder, and the Fourier transform infrared (FTIR) spectra were determined by FTIR spectrometer (ALPHA-T, Bruker, Germany) with a scanning resolution of 4 $\mathrm{cm^{-1}}$ and a spectral range of 400–4 000 $\mathrm{cm^{-1}}$[18]. The spectra were then processed using PeakFit V4.12 software for deconvolution and second derivative analysis to ascertain the secondary structure content of the egg yolks.

2.4.2 Tertiary structure

The changes in protein tertiary structure were determined by intrinsic fluorescence spectra, which were obtained following the method of Shen et al.[19]. The yolk samples were dispersed in 0.01 mol/L phosphate-buffered saline (PBS) at pH 7.0 and centrifuged at 8 000 r/min for 15 min. The protein supernatant concentration was then adjusted to 0.5 mg/mL and examined using a fluorescence photometer (F-7100, Hitachi, Japan), with PBS as the blank. The settings for the analysis were as follows: excitation wavelength 280 nm; emission wavelength 300–420 nm; slit width 2.5 nm; voltage 500 V; scan speed 240 nm/min.

2.5 Analysis of physicochemical properties of egg yolk

2.5.1 Turbidity and aggregation rate

The turbidity of egg yolk sample was assessed following a method adapted from previous[20]. The egg yolks were diluted with 10 g/100 mL NaCl solution at the ratio of 1:100 (g/mL), and stirred until completely dissolved. The absorbance of a measurement solution at 660 nm was defined as turbidity through a ultraviolet and visible (UV/Vis) spectrophotometer (L5S, INESA, Shanghai, China). The aggregation rate was calculated following the approach outlined by Ma et al.[21].

2.5.2 Particle size

The particle size distribution of egg yolk was determined using a laser particle size analyzer (Mastersizer 2000, Malvern, UK), following the method described by Li et al.[22]. The egg yolk samples were diluted 100 times with deionized water and mixed until uniform. Measurements were taken to obtain $D_{3,2}$, $D_{4,3}$, $d_{0.1}$, $d_{0.5}$, $d_{0.9}$, and the particle size distribution curves. The refractive indices applied were 1.33 for water and 1.46 for the yolk, and the detection range of particle size was 0.1–1 000 μm. The dispersion index or span was calculated using equation (3)[23]:

$$\text{Span} = \frac{d_{0.9} - d_{0.1}}{d_{0.5}} \quad (3)$$

2.5.3 Free sulfhydryl content

The free sulfhydryl content was assessed using Ellman's assay. A 4 mg/mL 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) solution, also called Ellman's reagent, was typically prepared as described by Chen et al.[24]. For the egg yolk analysis, 1.0 g of yolk was diluted to 100 mL with Tris-Gly buffer containing the same components along with 480 g of urea. Free sulfhydryl content was measured as follows: 2 mL of the supernatant was added with 0.08 mL of Ellman's reagent and reacted at room temperature for 1 h after centrifugation at 5 000 r/min for 10 min. The absorbance at 412 nm was measured by a UV/Vis spectrophotometer (L5S, INESA, Shanghai, China). The content of the free sulfhydryl was calculated using equation (4):

$$\text{Free sulfhydryl content } (\mu\text{mol/g}) = \frac{73.53 \times A_{412\text{ nm}} \times D}{\rho} \quad (4)$$

Where $A_{412\text{ nm}}$ represented the absorbance of the sample; D was dilution multiple (1); ρ was sample concentration (mg/mL).

2.6 Statistical analysis

The experimental data were visualized using Origin 2021 (Origin Lab Co., Massachusetts, USA) and analyzed with IBM SPSS Statistics 26 (SPSS Inc., Chicago, USA). Duncan's multiple-range test was applied following one-way analysis of variance (ANOVA), with results reported as mean ± standard deviation and significance set at $P < 0.05$. All experiments were conducted a minimum of three times, except for those assessing structural properties.

3 Results and discussions

3.1 Functional properties of egg yolk

3.1.1 Viscosity and visual images analysis

Due to the gelation phenomenon induced by freezing, the viscosity of egg yolk increased[25]. Thus, viscosity and the physical state of the yolk are the most intuitive indicators of egg yolk gelatinization, with the results presented in Figures 1A–B. Values for gelatinized egg yolk samples post-freezing (FEY and Y(B)0h) were not shown due to the loss of fluidity, which exceeded the measurement range of the viscometer. It was worth noting that the same phenomenon was

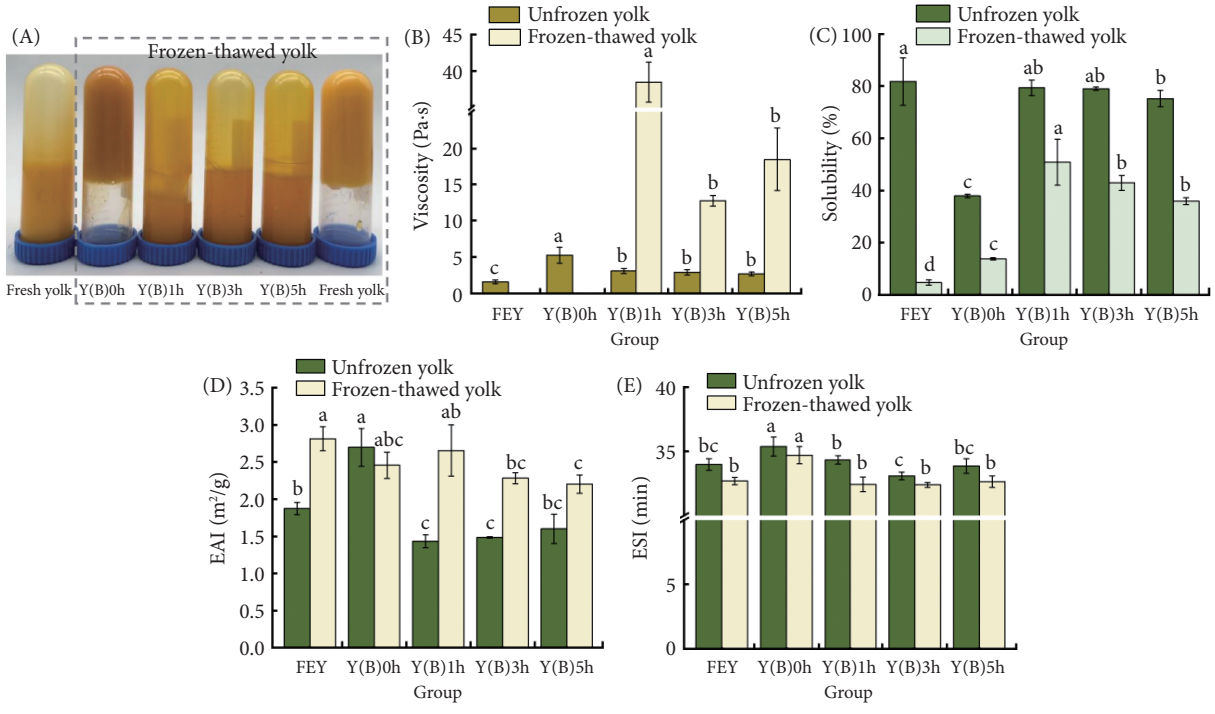

Figure 1 Changes in (A) status, (B) viscosity, (C) solubility and (D–E) emulsifying properties of unfrozen and frozen-thawed egg yolks added peptides. Different lowercase letters indicate significant differences between groups of the same indicator ($P < 0.05$).

observed at Y(B)0h after freezing, suggesting that raw materials without enzymatic hydrolysis have no cryoprotective effect and the increase in viscosity before freezing may be attributed to the introduction of additional proteins into the system, as studies have shown that an increase in protein content enhances protein-protein interactions and strengthens disulfide bonds, leading to a denser and more stable protein network structure, making the sample more viscous[26].

While Y(B)1h, Y(B)3h, and Y(B)5h exhibited a slight increase in viscosity compared with FEY before freezing, which can be attributed to enhanced interactions between peptides and yolk proteins that provide structural support[14]. On the other hand, it is similar to the addition of sugars and polyols, where the third component increases the solution viscosity through the exclusion volume effect[27]. The viscosity of egg yolk increased dramatically post-freezing, which may be due to ice crystals disrupting the interactions between water and proteins strengthened by hydrogen bonds. This disruption strengthens protein-protein interactions, thereby increasing viscosity[28]. The antifreeze proteins adsorb onto the ice crystal surfaces or interact at the ice-water boundary, regulating the size, shape, and aggregation of ice crystals. This action protects proteins during freezing and thawing[29–30]. Consequently, the peptides help restore the fluidity of egg yolk to some extent, indicating a significant cryoprotective effect. After thawing, the viscosity of Y(B)3h was the lowest at 12.720 Pa·s, while the viscosity of Y(B)5h was slightly higher than that of Y(B)3h, but there was no significant difference in statistics ($P > 0.05$).

3.1.2 Solubility analysis

Protein solubility has historically been the primary criterion for classification systems and holds significant importance in practical applications, forming the basis for various food production types and processing operations[31–32]. Protein solubility is quantitatively measured rather than directly defined as the maximum mass of protein that can be dissolved in a specific solvent[33]. As illustrated in Figure 1C, fresh egg yolk exhibited high solubility at 81.7%, primarily because yolk plasma contains approximately 85% low-density lipoprotein (LDL) and 15% livetin. These two proteins exhibit good solubility, with natural state solubility exceeding 90%[34].

Protein solubility directly reflects the degree of denaturation and aggregation of protein. The solubility of all samples slightly decreased with the addition of egg yolk peptides before freezing; however, after freezing, the solubility significantly declined. Although freezing caused substantial protein denaturation, some aggregates remained water-soluble[35]. Among them, the solubility of FEY decreased by 87.98%, primarily due to ice crystals compressing protein molecules as water molecules froze. This led to changes in the secondary structure of proteins, exposing hydrophobic groups and promoting hydrophobic interactions, ultimately forming protein aggregates with reduced solubility. Egg yolk peptides enhance protein solubility by forming hydrogen bonds with water and preventing aggregation by shielding hydrophobic regions that tend to cluster[14]. Several factors may affect protein solubility, often interacting with one another. These factors include denaturation, amino acid composition, hydrogen bond, ionic strength, aromatic/aliphatic ratio, and hydrophobicity[36]. According to Li et al.[28], the decrease in protein solubility during cryopreservation was linked to the development of hydrophobic interactions, ionic interactions, hydrogen bonds, and disulfide bonds.

3.1.3 Emulsifying property analysis

Emulsification properties reflect the capacity to create a stable emulsion between the oil and water phases that contain proteins[37]. Results were depicted in Figure 1D, the decrease of EAI with egg yolk peptides compared with FEY before freezing may be attributed to the emulsifying ability of these peptides. The amphoteric molecules with hydrophobic amino acids can adsorb at the oil-water interface, which is crucial for the enhanced emulsifying ability of the emulsion[38]. However, differences in the emulsifying properties of hydrolyzed peptides with varying molecular weights were observed, the hydrolyzed peptides with high molecular weight had lower emulsibility[39]. Meanwhile, the increase of Y(B)0h before freezing may be related to specific protein properties. Although the emulsifying functionality of proteins is concerned with solubility, it is primarily influenced by hydrophilic-lipophilic balance. In general, the rapid migration and adsorption of soluble proteins at the interface are critical for good emulsifying properties[40]. The different results of this study may be due to aggregated proteins reducing the surface tension of the oil-water interface[41]. Furthermore, freezing could expose the hydrophobic groups inside protein molecules[4], enhancing protein flexibility and facilitating adsorption and rearrangement of protein molecules at the oil-water interface. This leads to improved emulsification activity[42]. It has also been reported that freezing treatment could effectively improve the emulsifying property of egg yolk[43]. Moreover, the ESI decreased after freezing and there was no significant difference among different egg yolk samples except for Y(B)0h (Figure 1E). This may be due to the additional proteins reducing the interfacial tension between oil and water[44].

3.2 Structural properties of egg yolk proteins

3.2.1 Protein secondary structure analysis

Hydrogen bond, van der Waals bond, hydrophobicity, and ionic interactions mainly stabilize the secondary structure of proteins[45]. Freeze denaturation may induce alterations in the secondary structure. Figure 2A illustrates the changes in the infrared characteristic absorption peaks of egg yolk proteins, which are informative across the 400–4 000 cm^{-1} wave band: the band observed above 3 000 cm^{-1} is due to the stretching vibrations of the O–H and N–H bonds, indicative of the hydrogen bond structure[46]. The band in the 2 900–3 000 cm^{-1} is attributed to the stretching vibrations of C–H in CH_2. The band between 1 700–1 800 cm^{-1} is associated with C=O stretching, characterized by a higher force constant. A prominent stretching vibration with a characteristic C=C peak was observed at 1 600 cm^{-1}. As shown in Figure 2A, the peak patterns of all samples exhibited no significant changes; however, the fluctuations in wavenumbers and peak intensities above 3 000 cm^{-1} suggested alterations in the yolk protein structure post-freezing. The decreased intensity of the peak near 3 000 cm^{-1} indicated hydrogen bond interactions within the yolk, weakening the O–H stretching vibration but enhancing the interaction between protein molecules, thereby inducing gelation of the yolk. This implied that freezing increases the hydrogen bond strength in egg yolks[47]. Additionally, the introduction of peptides mitigated the magnitude of the peak intensity change and was higher than that of FEY. This suggested that egg yolk peptides could attenuate the hydrogen bond enhancement induced by freezing to some extent, likely due to the –OH group in peptides forming a stable structure with the protein and inhibit the aggregation and denaturation[48].

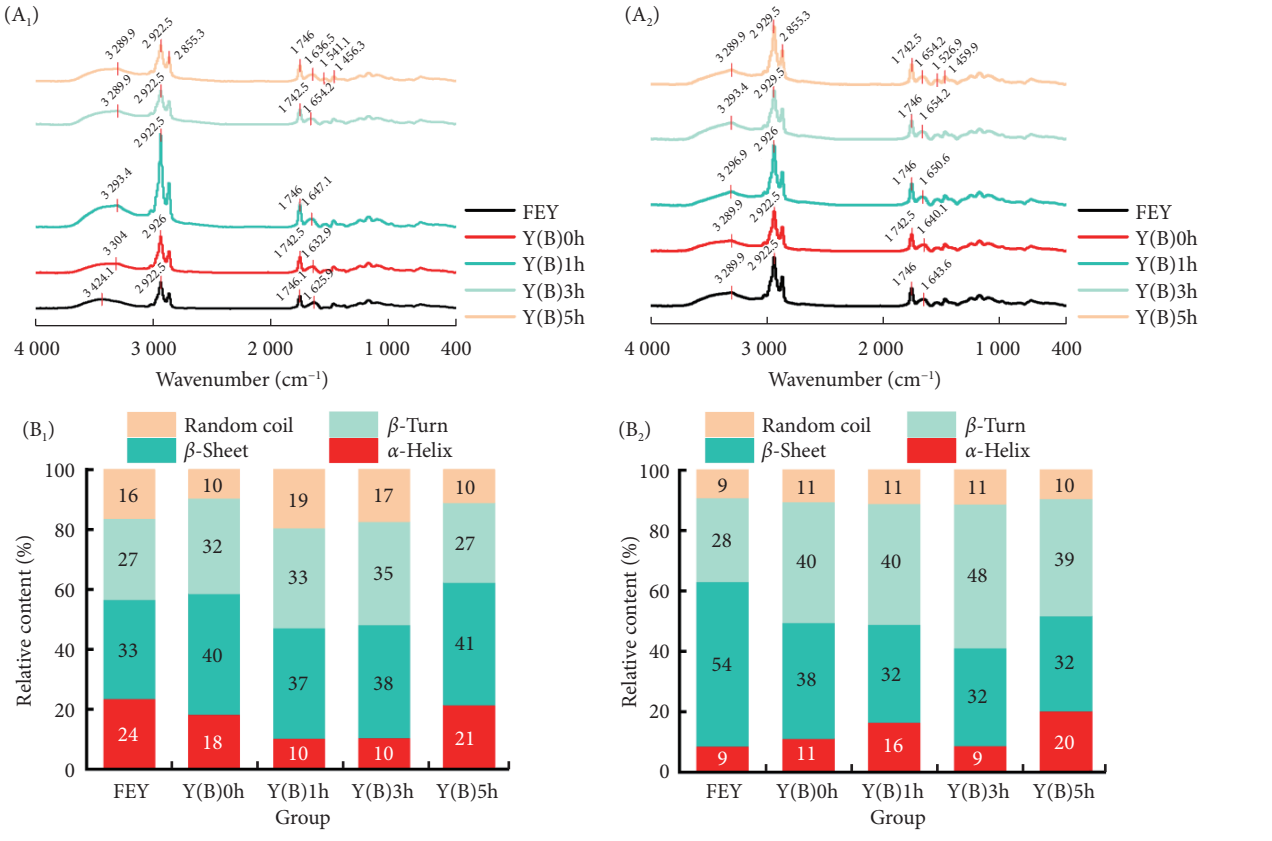

Figure 2 Changes in (A) FTIR spectra and (B) relative content of secondary structure of unfrozen and frozen-thawed egg yolks added peptides. Subscripts 1 and 2 represent unfrozen yolk and frozen-thawed yolk, respectively.

The amide I band (1 600–1 700 cm^{-1}), which primarily corresponds to the C=O stretch vibrations of peptide linkages (approximately 80%), is the most sensitive spectral region to protein secondary structural components. Changes in amide I signified disturbances in the secondary structures of α-helix and β-sheet[49]. According to the research of Jackson et al.[50], the α-helix is identified within the range of 1 651–1 660 cm^{-1}, the β-sheet within 1 600–1 639 cm^{-1}, the β-turn within 1 661–1 700 cm^{-1}, and the random coil within 1 640–1 650 cm^{-1}. The sum of β-sheet and α-helix is closely related to the total hydrogen bond interactions within the protein molecule. The relative content of the secondary structure of egg yolk is depicted in Figure 2B. During freezing, the relative contents of β-sheet and β-turn in FEY increased, while α-helix and random coil decreased. The increase of β-sheet represents the linear aggregation of proteins[51]. This indicated that the protein molecular chains unfolded, exposing hydrophobic groups, resulting in egg yolk protein aggregation after freezing. Notably, the relative content of β-sheet in the frozen-thawed egg yolks added peptides was lower than in FEY. Egg yolk peptides could inhibit this transformation and maintain the stability of the secondary structure[52].

3.2.2 *Protein tertiary structure analysis*

Aromatic amino acids like tryptophan (Trp), tyrosine (Tyr), and *L*-phenylalanine (Phe) are the primary sources of protein intrinsic fluorescence, with Trp typically dominating the fluorescence emission spectrum[53]. The absorption characteristics of these aromatic amino acids serve as reliable indicators of tertiary structure changes[54], as illustrated in Figure 3. The λ_{max} of Trp increases with the increase of the solvent polarity, typically ranging from 321 to 355 nm[55]. The λ_{max} of all samples before and after freezing was approximately 350 nm, indicating that Trp residues were fully exposed to the polar aqueous environment. However, a slight redshift was observed after freezing (354–356 nm), suggesting alterations in the protein's spatial structure. This may be attributed to the exposure of free sulfhydryl groups and the subsequent formation of additional disulfide bonds, which is consistent with the results of free sulfhydryl content measurements. Freezing can induce protein denaturation, which is reflected by the redshift in Trp fluorescence. Trp residues, typically partially buried due to their aromatic nature, become more exposed upon denaturation[56]. The magnitude of the observed redshift depends on the extent to which Trp residues are buried in the native protein structure and subsequently exposed during denaturation[57].

The fluorescence spectra of all yolk samples were alike in shape, but the addition of peptides increased the FI_{max} before freezing. This enhancement is likely attributed to the intrinsic fluorescence properties of the peptides themselves[58]. Shukla et al.[59] previously noted that self-assembled peptides can produce visible fluorescence. However, post-freezing, a significant decline in FI_{max} was observed in yolk samples. This decrease may be linked to changes in protein conformation and the exposure of Tyr and Trp in the hydrophobic region to the polar environment with increased protein oxidation and aggregation. Such protein aggregates can quench exposed chromophores, leading to reduced fluorescence intensity. This indicates that freezing alters the tertiary structure of proteins, exposing hydrophobic groups and promoting aggregation through hydrophobic interactions[60–61], thus increasing the particle size of the protein and decreasing its solubility. Furthermore, the relative fluorescence intensity in the groups with added egg yolk peptides

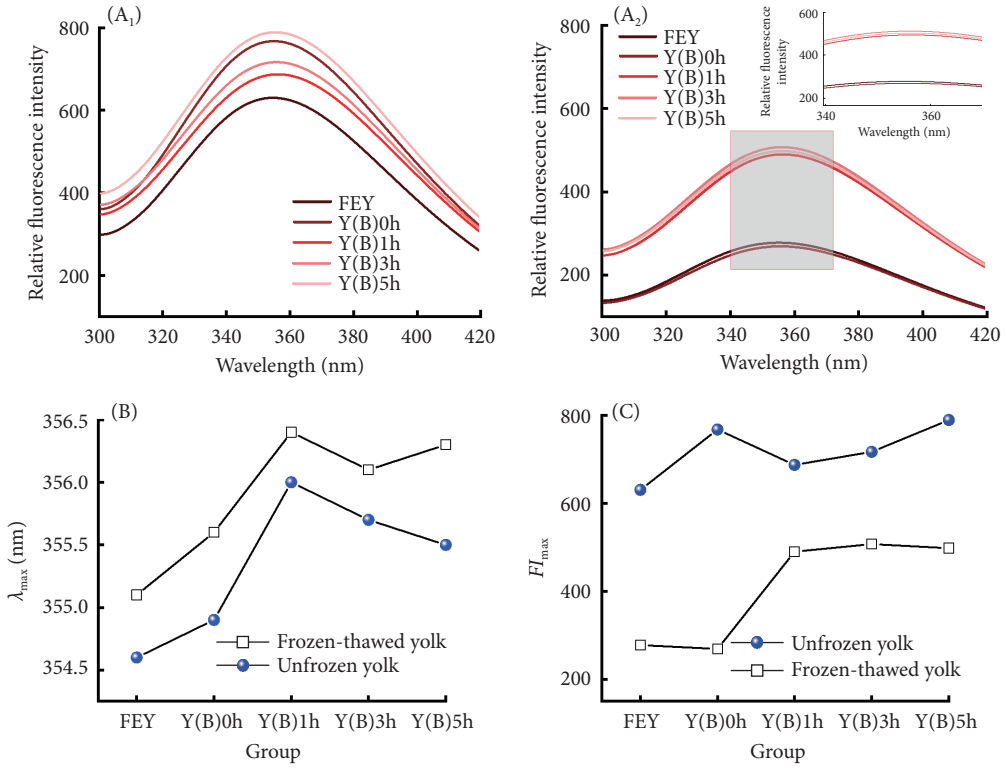

Figure 3 Changes in (A) fluorescence spectra, (B) maximum wavelength (λ_{max}), and (C) maximum fluorescence intensity (FI_{max}) of unfrozen and frozen-thawed egg yolks added peptides. Subscripts 1 and 2 represent unfrozen yolk and frozen-thawed yolk, respectively.

were higher than those of FEY and Y(B)0h, which indicated that the peptides mitigated the exposure of Trp residues and subsequent denaturation. Therefore, egg yolk peptides appear to preserve the tertiary structure of proteins against freeze-induced changes.

3.3 Physicochemical properties of egg yolk

3.3.1 Turbidity analysis

Turbidity reflects the aggregation state of the protein, with low values representing dispersed proteins and high values indicating aggregated proteins, the results are shown in Figure 4A. All samples exhibited turbidity values below 0.3 before freezing, with the turbidity value of FEY being 0.162, suggesting good dispersion in solvents[62]. The significantly higher turbidity observed in the Y(B)0h group compared to other groups may be attributed to the poor solubility of the raw materials because turbidity reflects the extent of protein aggregation and cross-linking, where solubility is negatively correlated with turbidity[63]. However, the turbidity of both FEY and Y(B)0h increased rapidly post-freezing, likely due to interactions between LDL and other protein components forming large insoluble aggregates during freezing. Conversely, the egg yolk peptide groups maintained lower turbidity values (0.155–0.170), indicating an effective inhibition of yolk particle aggregation caused by freezing so that values were comparable to that of fresh yolk.

Meanwhile, enhanced aggregation correlated with more prominent gelation behavior[21]. The fluctuation in the turbidity curve thus reflected differences in cryoprotection among samples, with the peptide groups showing a smaller rate of change post-freezing. This is consistent with the observation that egg yolk peptides effectively prevent yolk particle aggregation and improve protein solution dispersity, particularly peptides hydrolyzed for 3 and 5 h.

3.3.2 Particle size analysis

The aggregation state of proteins directly affects their particle size, which subsequently influences their solubility and processing properties[24]. $D_{3,2}$ and $D_{4,3}$ denote the surface-weighted mean diameter and volume-weighted mean diameter, respectively. Closer values of $D_{3,2}$ and $D_{4,3}$ indicate a more uniform particle shape and size distribution[64]. Moreover, $d_{0.9}$, $d_{0.5}$, and $d_{0.1}$ represent the particle diameters at the 90th, 50th, and 10th percentiles of undersized particles, respectively[65]. The span measures the width of the size distribution, where a smaller span indicates a narrower distribution[66]. The particle size distribution and corresponding values are presented in Figure 4C and Table 1. The particle size distribution of unfrozen egg yolks was unimodal and concentrated within the range of 10–100 μm, indicating a uniform distribution. However, frozen egg yolks showed multiple peaks and a shift towards larger particle sizes, suggesting an increase in particle size and the formation of more insoluble macro aggregates. Table 1 also shows that after freezing, $D_{4,3}$ of all samples increased due to protein denaturation and aggregation. Specifically, $d_{0.9}$ of FEY increased from 73.155 to 184.516 μm, and $d_{0.1}$ decreased from 5.996 to 3.649 μm after freezing, which showed that smaller molecules in the yolk formed larger particles under the action of freezing. Although the values of $d_{0.9}$ increased in groups supplemented with peptides, they remained lower than FEY, demonstrating that egg yolk peptides could inhibit large particle aggregation, reduce particle size, and enhance solubility simultaneously. Notably, the span of all samples ranged from 2 to 4 before freezing, indicating a uniform volume distribution consistent with the particle size distribution results. After freezing, the span of FEY increased from 2.860 to 9.170, whereas the peptide groups maintained a span around 3, resulting in a much narrower particle size distribution. It has been reported that smaller and more uniform particle sizes could increase solubility and contribute to the formation of stable emulsions[67].

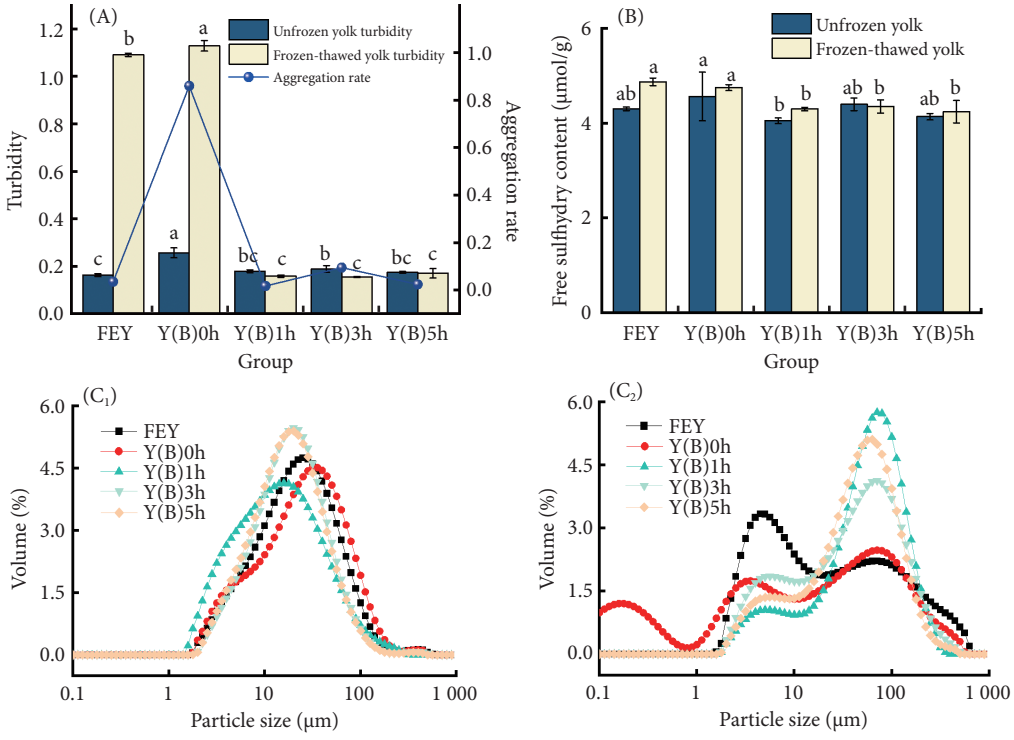

Figure 4 Changes in (A) turbidity, (B) free sulfhydryl content, and (C) particle size distribution of unfrozen and frozen-thawed egg yolks added peptides. Subscripts 1 and 2 represent unfrozen yolk and frozen-thawed yolk, respectively. Different lowercase letters indicate significant differences between groups of the same indicator ($P < 0.05$).

Table 1 Changes in particle size of unfrozen and frozen-thawed egg yolks added peptides.

Sample	Group	$D_{4,3}$ (μm)	$D_{3,2}$ (μm)	$d_{0.1}$ (μm)	$d_{0.5}$ (μm)	$d_{0.9}$ (μm)	Span
Unfrozen yolk	FEY	34.402 ± 1.637^{e}	14.221 ± 0.217^{e}	5.996 ± 0.120^{c}	23.479 ± 0.276^{e}	73.155 ± 1.847^{e}	2.860 ± 0.063^{de}
	Y(B)0h	40.916 ± 0.401^{d}	14.730 ± 0.337^{d}	5.720 ± 0.245^{d}	27.766 ± 0.093^{d}	87.843 ± 0.587^{d}	2.985 ± 0.036^{d}
	Y(B)1h	26.533 ± 1.839^{f}	9.612 ± 0.245^{g}	3.966 ± 0.125^{f}	15.296 ± 0.344^{g}	57.746 ± 1.809^{f}	3.517 ± 0.105^{c}
	Y(B)3h	28.341 ± 0.140^{f}	13.721 ± 0.180^{e}	6.315 ± 0.142^{b}	19.853 ± 0.096^{f}	56.131 ± 0.417^{f}	2.509 ± 0.023^{ef}
	Y(B)5h	26.429 ± 0.053^{f}	12.963 ± 0.123^{f}	5.915 ± 0.116^{c}	18.684 ± 0.045^{f}	52.980 ± 0.422^{f}	2.519 ± 0.035^{ef}
Frozen-thawed yolk	FEY	63.323 ± 4.224^{b}	9.849 ± 0.123^{g}	3.649 ± 0.019^{g}	19.714 ± 0.870^{f}	184.516 ± 13.537^{a}	9.170 ± 0.467^{a}
	Y(B)0h	50.499 ± 3.717^{c}	0.736 ± 0.021^{h}	0.198 ± 0.006^{h}	17.357 ± 1.051^{fg}	142.005 ± 8.193^{b}	8.186 ± 0.557^{b}
	Y(B)1h	68.539 ± 4.164^{a}	22.168 ± 0.729^{a}	8.008 ± 0.201^{a}	57.243 ± 3.169^{a}	142.115 ± 8.603^{b}	2.342 ± 0.030^{f}
	Y(B)3h	62.250 ± 4.172^{b}	15.461 ± 0.441^{c}	5.169 ± 0.073^{e}	41.253 ± 2.623^{c}	143.974 ± 9.747^{b}	3.364 ± 0.049^{c}
	Y(B)5h	59.523 ± 2.512^{b}	18.654 ± 0.331^{b}	6.470 ± 0.058^{b}	45.461 ± 1.667^{b}	126.148 ± 5.423^{c}	2.632 ± 0.032^{def}

Note: Within the same columns, values with different superscript lowercase letters are significantly different ($P < 0.05$).

3.3.3 *Free sulfhydryl content analysis*

The free sulfhydryl group is the most reactive functional group in proteins, playing a critical role in their structural stability and functional properties, mainly influenced by non-covalent interactions between protein molecules[68]. Previous studies have attributed changes in free sulfhydryl groups to two primary conditions[69]. Firstly, sulfhydryl groups normally buried within the protein structure are exposed due to external factors. Secondly, new free sulfhydryl group is generated from the dissociation of protein subunit groups. As shown in Figure 4B, the increased free sulfhydryl groups after freezing suggested the exposure of free sulfhydryl groups or the breaking of disulfide bonds within the protein molecules, which may be due to conformational changes in the yolk protein, as instability in the tertiary structure can lead to the exposure of new free sulfhydryl groups. Additionally, protein denaturation and aggregation might reduce the number of detectable free sulfhydryl groups. The free sulfhydryl content in the samples containing egg yolk peptides was lower than that in the FEY and Y(B)0h samples after freezing, consistent with changes in solubility. Wagner et al.[35] observed a trend where solubility decreased with an increase in the number of free sulfhydryl groups. Similarly, Du et al.[63] found a positive correlation between the content of the free sulfhydryl and the concentration of soluble protein, suggesting that hydrogen bonding between free sulfhydryl groups and water molecules is one of the factors affecting protein solubility.

3.4 Correlation analysis

A correlation scatter plot was created to analyze the relationships among the functional, structural, and physicochemical properties of frozen-thawed egg yolk with added egg yolk peptides. As shown in Figure 5, viscosity showed a positive correlation with both free sulfhydryl content and turbidity, while it had a negative correlation with FI_{max}. Solubility was negatively correlated with turbidity and

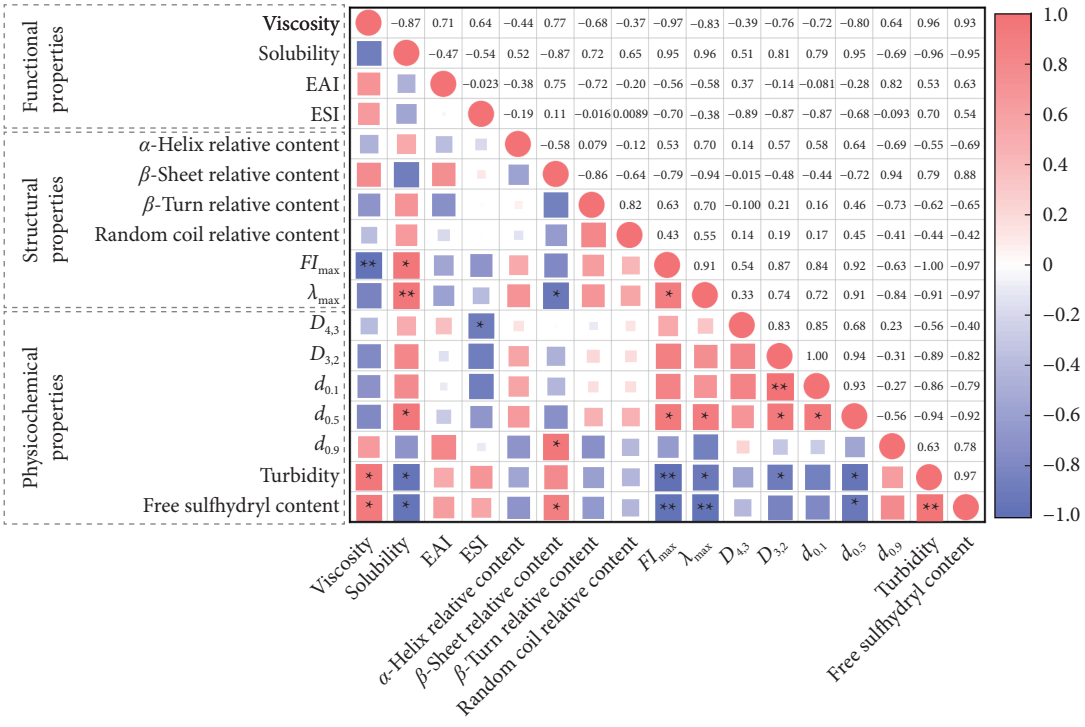

Figure 5 Correlation scatter plot of protein properties. $^{*}P < 0.05$, $^{**}P < 0.01$.

free sulfhydryl content, but positively correlated with $d_{0.5}$ and FI_{max}. Additionally, β-sheet relative content significantly influenced free sulfhydryl content and $d_{0.9}$, as well as altered the λ_{max}. Moreover, changes in the tertiary structure of proteins could affect their physicochemical properties. These findings suggested that egg yolk peptides could protect proteins during freezing, helping to maintain the stability of both secondary and tertiary structures and enhancing their functional properties.

4 Conclusion

The structural integrity of egg yolk proteins is compromised after freezing and thawing due to the formation of ice crystals and protein aggregation. This leads to gelation, which in turn increases the viscosity, emulsibility, turbidity, particle size, and free sulfhydryl content while decreasing solubility. This study demonstrated that egg yolk peptides, prepared through enzymatic hydrolysis of defatted egg yolk powder over various durations, exhibited a significant cryoprotective effect on egg yolk proteins. The addition of these peptides mitigated structural changes, inhibited protein denaturation, and minimized alterations in protein function and physicochemical properties. However, the study did not elucidate the differences and mechanisms underlying the antifreeze effects of various peptides. Future research will focus on the identification of specific amino acids and the separation of these peptides.

Conflict of interests

No conflicts of interest exist regarding the submission of this manuscript, and the manuscript has been approved for publication by all authors. I declare on behalf of my co-authors that the work described here in represents original research that has not been published previously and is not under consideration for publication elsewhere, in whole or in part. All the listed authors have approved the enclosed manuscript.

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