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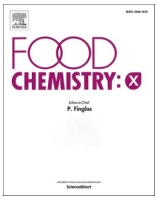

Effects of different temperatures on the physicochemical characteristics, microstructure and protein structure of preserved egg yolk

Xianlong Luo [a,b,c,1], Ji'en Tan [a,b,c,d,1], Yao Yao [a,b,c], Na Wu [a,b,c], Shuping Chen [a,b,c], Lilan Xu [a,b], , Yan Zhao [a,b,c,*], Yonggang Tu [a,b,c,*]

[a] Jiangxi Key Laboratory of Natural Products and Functional Food, Jiangxi Agricultural University, Nanchang 330045, China
[b] Agricultural Products Processing and Quality Control Engineering Laboratory of Jiangxi, Jiangxi Agricultural University, Nanchang 330045, China
[c] Nanchang Key Laboratory of Egg Safety Production and Processing Engineering, Nanchang 330045, China
[d] College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang 330045, China

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ABSTRACT

To clarify the mechanism of lower temperatures promoted the solidification of preserved egg yolk, the effects of temperature (4 °C, 10 °C and 25 °C) on the physicochemical properties, microstructure and protein structure of preserved egg yolk were studied. Results showed that the exterior egg yolk (EEY) exhibited higher pH, hardness and free sulfhydryl content at low-temperature pickling. The microstructure showed that the EEY gradually formed a denser gel network structure at lower temperatures. Electrophoresis results and Fourier transform infrared spectroscopy (FTIR) indicated that there were different degrees of protein degradation and cross-linking of proteins in the IEY (the interior egg yolk) and EEY and the decrease of β-sheets in the secondary structure was accompanied by an increase of β-turns during the formation of egg yolk gels. These results indicated that egg yolk solidification was faster and denser gel structure at 4 °C and 10 °C.

1. Introduction

Poultry eggs are rich in protein and fat and have a highly nutritional value, with varieties of essential amino acids and fatty acids that are easy to be absorbed by the human body (Xue et al., 2020). Traditional egg products such as preserved eggs, salted eggs, marinated eggs and eggs pickled in rice wine had been created on account of the different processing methods. Among the several traditional egg products, preserved eggs are distinguished by several distinctive features, such as the dark brown and transparent preserved egg white, the dark green preserved egg yolk, and various shapes "Songhua crystals" distributed on the surface and inside of the preserved egg white (Xue et al., 2022; Zhao et al., 2021). Preserved eggs are also preferred for their unique color, flavor and appearance.

The gelation process of preserved egg is that the alkali penetrating into the egg destroyed the structure of protein and promoted the unfolding, aggregation and cross-linking of protein molecules, which resulted in denatured proteins forming a highly elastic egg white gel and a low-hardness egg yolk gel (Yang et al., 2019; Zhao et al., 2016). Consequently, preserved egg was a typical example of a non-thermally induced gel that formed under strong alkali conditions. In addition, the stability of the protein gel structure was maintained by covalent bonds (ionic bonds, disulfide bonds, and so on) and non-covalent bonds (hydrogen bonds, hydrophobic interactions, and so on) (Yang et al., 2019). Relevant studies have also confirmed that preserved egg gel mainly relied on intermolecular forces including electrostatic interactions, hydrophobic interactions, and disulfide bonds to maintain the gel structure (Tan et al., 2022; Yang et al., 2019). Furthermore, the sulfhydryl groups in proteins were oxidized and converted into disulfide bonds, which could promote the formation of protein gel (Yang et al., 2020b). The formation of the egg yolk gel is complex, which involved not only protein-protein interactions but also lipids were essential for maintaining the gel network structure (Cao et al., 2021). Egg yolks, regarded as natural protein-lipid supramolecular assemblies, not only contain a protein content of 16% and a lipid content of 32% (Zhao et al., 2021). And the egg yolk proteins exist as lipoproteins, which include a variety of substances such as low-density lipoproteins (LDL), high-density lipoproteins (HDL) and phosvitin (Marcet, Sáez-Orviz,

* Corresponding authors at: Jiangxi Key Laboratory of Natural Products and Functional Food, Jiangxi Agricultural University, Nanchang 330045, China.
E-mail addresses: zhaoyan@jaxu.edu.cn (Y. Zhao), tygzy1212@jxau.edu.cn (Y. Tu).
[1] These authors are co-first authors of the article.

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Rendueles, & Díaz, 2022). Therefore, the abundance of proteins and lipids provides the basis for the gelatinization of egg yolk. The gelatinization of preserved eggs was affected by several factors, such as sodium hydroxide concentration, pickling temperature, types of metal ions and kinds of additives (Huang, Mao, Li, & Yang, 2021; Tan et al., 2022; Xue et al., 2022). The higher concentrations of sodium hydroxide resulted in faster egg yolk gel formation and higher hardness egg yolk gel (Ganesan & Benjakul, 2010). In order to investigate the effect of different cations on the degree of aggregation of preserved egg yolk, Ganesan & Benjakul, 2010 found that zinc ions and calcium ions both promoted the combination of lipids and proteins to produce high-hardness egg yolk gel. Wang et al., 2021 observed that freezing destroyed the structure of lipoprotein in egg yolk and released lipids to facilitate protein aggregation by using confocal laser scanning microscopy (CLSM). However, there are fewer reports on the gelation of preserved egg yolk at different pickling temperatures.

At present, the industrialized production of preserved eggs generally adopts the soaking method that fresh eggs are soaked in a mixed solution containing strong alkali, salt and metal ions and pickled at 25 °C for 4–6 weeks (Chen et al., 2015). Preserved eggs with semiliquid egg yok and preserved eggs with completely solidified egg yolk are the two categories into which preserved eggs can be classified based on the solidification state of the egg yolk (Cao et al., 2021). The former existed a viscous portion in the egg yolk that not has fully solidified and took around 40 days to mature at a pickling temperature of 25 °C, whereas the latter had fully formed egg yolk gel and took longer than three months to mature (Yang et al., 2019). Although excessively prolonged pickling could cause the preserved egg yolk to gradually solidify completely, it would degrade the egg white gel that has already been formed. In other words, fresh egg white first formed egg white gel, and then the gel collapsed, softened and even liquefied under the continuous treatment of higher concentration sodium hydroxide solution (Gao et al., 2020). Consequently, the length pickling period is not beneficial to maintaining the preserved egg white gel. The solidification state of the preserved egg yolk is a crucial measure of their maturity. It can shorten the pickling period of preserved eggs by promoting the solidification of their egg yolks.

In preliminary study, we found that when the pickling time was same, lowering the pickling temperature of preserved eggs could not only result in a higher elasticity egg white gel, but also accelerate the solidification of egg yolk. In addition, lowering the pickling temperature could also effectively reduce the black spots on the surface of the eggshell caused by heavy metal plugging, make the appearance of the whole preserved egg more beautiful. Therefore, we chose to lower the pickling temperature to accelerate the maturation time of preserved egg yolk. Furthermore, in order to clarify the formation mechanism of low temperature accelerated the strong alkali-induced egg yolk gel, this study systematically analyzed the changes in physicochemical properties, microstructure and protein structure of preserved egg yolk pickled at different temperatures (4 °C, 10 °C and 25 °C). This would provide a theoretical foundation for shortening the production period of preserved egg during low-temperature pickling, and to a certain extent, enrich the theoretical system of protein gelation.

2. Materials and methods

2.1. Materials and reagents

Fresh duck eggs with an average weight of 65–75 g were obtained from a farm (Jiangxi China). Sodium hydroxide (NaOH), sodium chloride (NaCl) and copper sulfate ($CuSO_4$) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai China). All reagents used in this study were of analytical grade unless otherwise indicated.

2.2. Preparation of samples of preserved egg yolk

Fresh duck eggs were graded, washed, air-dried, inspected and discarded with cracks. Subsequently, duck eggs were put into the small buckets containing a proportionally prepared mixed solution that consisted of NaOH (4.5%, m/v), NaCl (4.0%, m/v), and $CuSO_4$ (0.4%, m/v). Respectively, these were placed in constant temperature incubators at 25 °C, 10 °C, and 4 °C for 49 days. Every 7 days, six preserved eggs without cracks were chosen to be used for the determination of indicators.

Each preserved egg yolk was separated from the egg white and rolled on filter paper to get rid of the egg white residue on the surface of the preserved egg yolk gel. Preserved egg yolk was cut in half with a blade, then the viscous interior portion was scraped out with a spoon and collected with a beaker, and finally the filter paper was used to remove the viscous egg yolk residue adhering to the preserved egg yolk gel. The preserved egg yolk was separated into two portions based on their distinct states, the hardened gel portion of preserved egg yolk was referred to as the exterior egg yolk (EEY) and the viscous fluid portion was referred to as the interior egg yolk (IEY). The preserved egg yolk that was pickled for 7 days at 4 °C and 10 °C was still in the sol state and did not form egg yolk gel during the pickling process. Therefore, on days 0, 14, 21, 28, 35, 42 and 49, samples were collected for measurement.

2.3. Determination of the appearance and the hardening rate

The method of Cao et al., 2021 was used to determine the hardening rate of preserved egg yolk with slight modifications. After manually separating the egg yolk from preserved eggs and rolling them on filter paper to remove the egg white gel residue that was adhering to their surface, the weight of the preserved egg yolk was recorded as M(g). Next, the preserved egg yolk was cut in half with a blade, then the viscous portion inside the egg yolk was scooped out with a spoon and the viscous residue inside the preserved egg yolk was removed with filter paper. The remaining gel portion was recorded as m(g). Finally, the appearance of the gel portion was recorded.

The formula for the hardening rate of egg yolks is:

$$\text{Hardening rate}\ (\%) = \mathrm{m}/\mathrm{M}^{*}100$$

2.4. Determination of pH value

The pH of egg yolk samples was determined based on the method of Jin et al., 2022 with slight modifications. Each sample was weighed 2 g and homogenized using an Ultra Turrax homogenizer (IKA T18 digital, Staufen, Germany) separately with 27 mL of distilled water at 12000 rpm for 2 min. The sample was then filtered through double-layer gauze and the filtrate was collected for pH determination using a pH meter (PHS-3C, Shanghai, China).

2.5. Determination of moisture content

The moisture content was determined by the method described by Yang et al., 2019 with slight modifications. Approximately 2 g of preserved egg yolk was weighed and recorded as m_1(g); the crucible was dried to constant weight and recorded as m_2(g); the crucible containing weighed samples was placed in a blower drying oven at 102 °C to dry to constant weight, and cooled down to 25 °C in the desiccator, and finally weighed and recorded them as m_3.

The formula for calculating the moisture content is:

$$\text{Moisture content}\ (\%) = [(\mathrm{m}_1 + \mathrm{m}_2 - \mathrm{m}_3)/\mathrm{m}_1]^{*}\ 100$$

2.6. Determination of rheological properties

The rheological properties of IEY of preserved eggs were determined

according to the method of Wang et al., 2021 with slight modifications. The viscometer (R/S plus viscometer, Brookfield, USA) with a rotor-type parallel plate (PP) was used to measure the viscosity change of the internal yolk of preserved eggs. The measurement parameters: The test duration of the sample was 30 s, the shear range was 0.01–100 s^{-1}, and the number of test points was 21. The flow behaviour of IEY was studied using a power law model, power law model: $\eta = K \bullet \gamma^{n-1}$, where n is the flow behaviour index (dimensionless), K is the consistency coefficient ($Pa \bullet s^n$), η is the viscosity and γ is the shear rate (1/s).

2.7. Determination of hardness

Referring to the method of Chen et al., 2015 with slight modifications, the texture of preserved egg yolk was performed. The EEY of preserved eggs was cut into 1 cm^3 cubes. The texture analyzer (TA. XTPlus texture analyzer, Surrey, UK) equipped with a P/50 cylindrical probe was employed for hardness determination. The test parameters: pre-test speed of 2.00 mm/s, mid-test speed of 2.00 mm/s, post-test speed of 2.00 mm/s, trigger point load of 10 g, and compression twice at 70% compression ratio. The hardness (g) was averaged from five tests.

2.8. Determination of microstructure

The microstructure was determined following the method of Xin et al., 2023 with slight modifications. The preserved egg yolk gels were cut into cubic blocks of approximately 0.5 × 0.5 × 0.5 cm^3 and fixed in a glutaraldehyde solution (2.5%, v/v) at 4 °C overnight. The samples were rinsed three times with PBS butter (0.05 M, pH 7.2) for 15 min and subsequently dehydrated for 30 min in graded ethanol concentrations (60%, 80%, 90%, and 100%). Then the pretreated samples were freeze-dried thoroughly. Finally, the freeze-dried samples were sprayed with gold and then were observed by a scanning electron microscope with a magnification of 300 times at an accelerating voltage of 20 kV in low vacuum mode.

2.9. Determination of free sulfhydryl

The procedure for determining the free sulfhydryl followed the method of Jin et al., 2022 with slight modifications. The egg yolk sample of 1 g was homogenized with 9 mL of PBS (0.1 M, pH 7.3) by the homogenizer (2 min, 12,000 rpm). The supernatant was obtained by centrifugation (20 min, 8000 rpm) and was determined their protein concentration by a BCA protein quantification kit. Subsequently, 0.02 mL of Ellman's reagent (4 mg/mL, DTNB dissolved in Tris-Gly buffer) was added to a mixture solution containing 0.2 mL of supernatant and 2.8 mL of Tris-Gly buffer (0.1 M Tris, 0.1 M Glycine, 4 mM EDTA, pH 8.0). The reaction was carried out in a water bath at 40 °C for 15 min to produce color. The absorbance value of the sample solution was detected at 412 nm using an enzyme meter. The above PBS buffer was used as a blank instead of the sample solution.

The free sulfhydryl content was calculated as follows:

$$SH\ (\mu M/g\ protein) = A_{412} \times 73.53 \times D/C$$

where A_{412} represented the absorbance at 412 nm, 73.53 was the unit conversion (10^6 /1.36 × 10^4 M^{-1} cm^{-1}), D represented the dilution factor (15.1), and C represented the supernatant protein concentration (mg/mL).

2.10. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight distribution of the protein sample was analyzed utilizing 5% stacking gel and 12% separating gel via SDS-PAGE. The egg yolk sample of 1 g was homogenized (2 min, 12,000 rpm) with 9 mL of SDS solution (5%, m/v) and centrifuged (10 min, 10,000 rpm) to obtain the supernatant. The protein concentration of the supernatant was adjusted to 1 mg/mL and mixed with the sample buffer (3:1, *v*/v), and boiled for 5 min in boiling water. The prepared sample solution (10 μL) and Markers (5 μL) were loaded onto the gel by the Mini Bio-Rad vertical gel electrophoresis unit (Bio-Rad Co., Ltd., USA) and the electrophoresis was conducted until the end of electrophoresis at a constant voltage of 200 V. After be completed, the gel was stained with Coomassie Brilliant Blue G250 and then decolourized with decolorizing solution (distilled water: methanol: acetic acid = 14:5:1).

2.11. Fourier transform infrared spectroscopy (FTIR)

The determination of secondary structure was carried out by referring to the method of Xue et al., 2021 with slight modifications. Freeze-dried egg yolk samples were thoroughly mixed with spectroscopic grade potassium bromide (1:100, *w*/w) and pressed into thin slices. The samples were analyzed using an FTIR spectrometer (Thermo Scientific Nicolet iS5, USA). The egg yolk samples were scanned whole band of 4000–400 cm^{-1} in 32 scans at a resolution of 4 cm^{-1}. The data were processed using OMNIC software for baseline calibration and protein second-order derivatives in the amide I region (range 1700–1600 cm^{-1}).

2.12. Statistical analysis

All measurements were performed three times, except for hardness measurements. Statistical analyses were performed by the SPSS Statistics 25.0 software (SPSS Inc., Chicago, USA). The results were expressed as mean ± SD, and the significance between groups was performed with Duncan's multiple analysis range tests, referring to a significant difference at $P < 0.05$. The plots were drawn by employing Origin 2021 software (OriginLab Corporation, Northampton, USA).

3. Results and discussion

3.1. Effect of temperature on the appearance morphology and hardening rate of preserved egg yolk

The changes in the appearance of EEY, which had been removed the viscous interior portion, pickled at different temperatures were shown in Fig. 1a. The smaller the concave portion in the center of the egg yolk (IEY), the more solidified the egg yolk (EEY). As can be seen from Fig. 1a, with the prolongation of pickling time, the solidification portion of preserved egg yolk picked at different temperatures gradually increased and tended to be completely solidified. However, when the pickling time was the same, the solidification states of preserved egg yolk were inconsistent at different pickling temperatures. When preserved eggs were pickled for 14 days, there was no significant difference in the solidification state between preserved egg yolk at 4 °C and 10 °C and that at 25 °C. But as the picking time increased, the solidified portion of preserved egg yolk was picked at 4 °C and 10 °C was more than that of preserved egg yolk picked at 25 °C and even preserved egg yolk picked at 4 °C for 49 days was almost completely solidified. These results directly showed that duck eggs picked at 4 °C and 10 °C could effectively accelerate the solidification of preserved egg yolk.

Hardening is a process of transforming egg yolk from liquid to gel state. The hardening rate, which is the percentage of preserved egg yolk gel to the weight of the whole yolk, was used as one of the important indices to characterize the maturity of preserved egg yolk (Cao et al., 2021). The effect of different temperatures on the hardening rate of preserved egg yolk was shown in Fig. 1b. With the increase of pickling time, the hardening rate of preserved egg yolk increased, and the hardening rates of preserved egg yolk pickled at 4 °C and 10 °C were always higher than that at 25 °C (4 °C > 10 °C > 25 °C). When preserved eggs were pickled for 28 days, the hardening rates of preserved egg yolk pickled at 4 °C and 10 °C were 80.89% and 76.14%, respectively, which were significantly higher than 70.95% at 25 °C ($P < 0.05$). After 35 days of pickling at 4 °C and 10 °C, the solidification portion of egg yolks

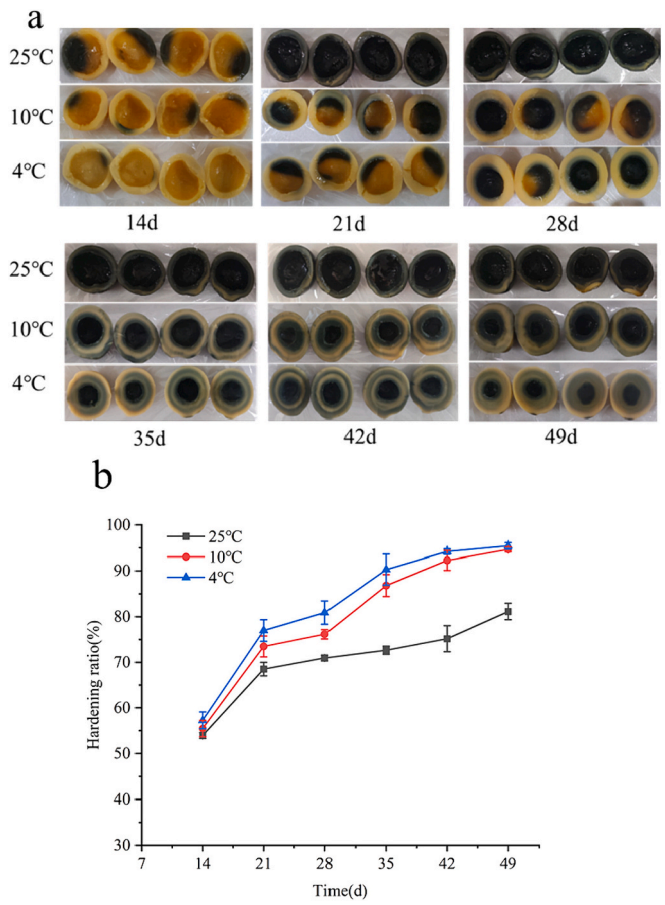

Fig. 1. Changes in the appearance and hardening rate of preserved egg yolk pickled at different temperatures (a: appearance; b: hardening rate).

(90.28% and 86.80%) even exceeded 49 days (81.13%) at 25 °C, which greatly shortened the pickling time of preserved eggs. This is consistent with the results of the appearance morphology of preserved egg yolk (Fig. 1a). Both are further indicated that low-temperature pickling promoted the solidification of preserved egg yolk.

During the pickling process of preserved eggs, the alkaline solution successively penetrated through the eggshell and egg white into the egg yolk. Proteins near the yolk membrane are the first to be induced by strong alkali to form a yolk gel (Yang et al., 2019). As pickling time increased, the continuous penetration of alkali caused the egg yolk to solidify gradually towards the center and subsequently resulted in a rising hardening rate of egg yolk (Fig. 1b). After 7 days of pickling, the egg white formed a gel at 25 °C, which hindered further alkali penetration into the egg yolk; however, the preserved egg white remained still in the sol state at 4 °C and 10 °C, resulting in more lye entering the egg yolk through the egg white (Zhao et al., 2016). The strong alkali destroyed and unfolded the structure of protein molecules, and cations combined with negatively charged protein molecules to form polymers, which not only shielded the electrostatic repulsion force between proteins but also promoted protein aggregation and accelerated gel formation (Chen et al., 2015; Zhao et al., 2021).

3.2. Effect of temperature on the pH of preserved egg yolk

The change in pH value can reflect the penetration of alkali solution to some extent. The formation of the protein gel was affected by the pH as it could change the electrostatic balance of the gel system, resulting in forming either dense or loose gel structures (Liu et al., 2022). The pH of IEY and EEY in preserved eggs pickled at different temperatures were presented in Fig. 2a. During the entire pickling period, the pH of IEY and EEY pickled at different temperatures both showed an upward trend compared to the fresh egg yolk ($P < 0.05$). The great difference in alkali penetration caused a significant difference in the pH values between IEY and EEY of preserved eggs pickled at the same temperature, which was similar to the results of Yang et al. (Yang et al., 2019). Under the action of osmotic pressure, the lye that penetrated the eggshell and the egg white into the egg yolk caused an increase in pH value (Ganasen & Benjakul, 2011; Zhao et al., 2016). The pH of EEY of preserved eggs pickled at 25 °C was lower compared to those pickled at 4 °C and 10 °C. This was because egg white gel formation in the early stage of pickling at 25 °C hindered the penetration of alkali. Conversely, preserved eggs pickled at 4 °C and 10 °C, the various types of reactions were slowed down during gelation, including oxidation of sulfhydryl groups in protein molecules and disulfide bond transformation, transformation between secondary structures and degradation of proteins and cross-linking of molecules, resulting in egg white not yet formed a gel (Chen et al., 2015; Gao et al., 2020). Consequently, during low temperature pickling, egg whites are in a sol state for a longer period of time than at 25 °C, more alkali passes through the sol-gel state of the egg white into the egg yolk, resulting in a higher concentration of OH^- in the egg yolk. It was also possible that the preserved egg yolk at 25 °C underwent a Maillard reaction, producing acidic substances that reduced the pH value of EEY (Tan et al., 2021). Interestingly, the pH values of IEY at 25 °C were higher than those at 4 °C and 10 °C (4 °C < 10 °C < 25 °C) (Fig. 2a), contrary to the result of the pH value of EEY (4 °C > 10 °C > 25 °C) (Fig. 2a). Two reasons might explain this phenomenon. On the one hand, the preserved egg pickled at 4 °C and 10 °C resulted in a dense and thick gel structure, which hindered the migration of OH^- from EEY into IEY. On the other hand, when preserved egg was pickled at 25 °C, the faster thermal motion of molecules facilitated the entry of OH^- into IEY via EEY.

In particular, EEY and IEY pickled at different temperatures both showed a trend of first decreasing and then increasing ($P < 0.05$) in the middle of pickling. This might be attributed to the decomposition of sulfur-containing amino acids from the egg white caused by strong alkali, which combined with Cu^{2+} to produce CuS and other insoluble substances that blocked the pore of the eggshell surface, slowing down the penetration rate of lye (Shao et al., 2017; Xue et al., 2022). It was the dual effect of the continuous penetration of lye from the eggshell outside and the migration of some OH^- from the egg white gel to the egg yolk that led to the increase in pH at the picking later stage (Tan et al., 2022). Comparing the changes in pH between IEY and EEY, it was evident that the egg yolks might transform from sol to gel when the pH reached 9.9 or higher in the process of strong alkali induced protein gel formation. This observation suggested the existence of a critical pH value in the gel system during the process of strong alkali induced protein gelation under the same conditions.

3.3. Effect of temperature on the moisture content of preserved egg yolk

The effects of different pickling temperatures on the moisture content of IEY and EEY were shown in Fig. 2b. Compared to fresh egg yolk, the moisture content of EEY increased sharply and then gradually decreased with the extension of pickling time ($P < 0.05$). Conversely, the moisture content of IEY was decreased compared to fresh egg yolk ($P < 0.05$), and no significant difference ($P > 0.05$) was observed in the moisture content of IEY at different pickling time and pickling temperatures. Interestingly, there was a significant difference in the moisture content between IEY and EEY ($P < 0.05$). At the beginning of pickling, the proteins near the yolk membrane broke down and then aggregated, resulting in gel formation. The gel trapped water molecules that had penetrated due to high osmotic pressure, which resulted in a significant increase in the moisture content of EEY ($P < 0.05$). The thicker EEY restricted the flow of water molecules to a certain extent, thus making it difficult for water molecules to enter IEY; additionally, the existence of sodium salt led to the water in IEY to migrate towards EEY, causing IEY

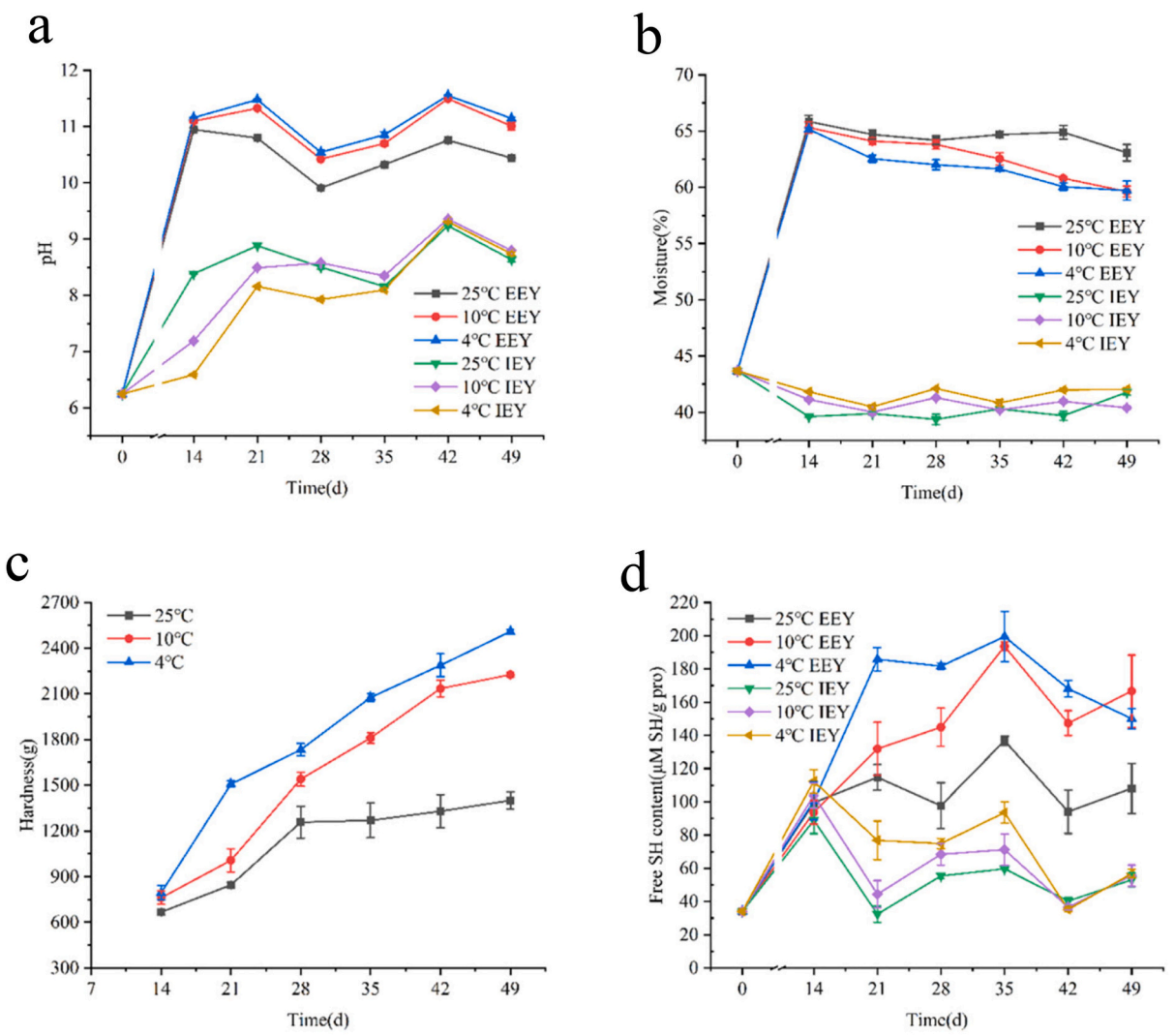

Fig. 2. Changes in pH, moisture content, hardness and free sulfhydryl content of preserved egg yolk pickled at different temperatures (a: pH; b: moisture content; c: hardness; d: free sulfhydryl content).

to gradually dehydrate and become thick (Ai, Guo, Zhou, Wu, & Jiang, 2018). Therefore, the moisture content of IEY of preserved egg yolk was lower than that of the fresh egg yolk. With the extension of pickling time, sodium salt further permeated into the egg white, the dehydration effect was strengthened, and the moisture in EEY progressively relocated to the egg white gel, causing a decrease in the moisture content of EEY ($P < 0.05$). In the pickling process of salted eggs, the moisture of egg yolk also migrated into the egg white (Xu et al., 2018).

It was noteworthy that the moisture content of EEY in preserved eggs pickled at 25 °C was higher than that of those pickled at 4 °C and 10 °C (4 °C > 10 °C > 25 °C) (Fig. 2b) while no significant difference was observed in IEY (Fig. 2b). This could be attributed to a denser gel structure of EEY, which resulting in less fixed water molecules at lower pickling temperatures. Additionally, dehydration caused by excessive osmotic alkali and salt in the process of preserved eggs pickled at 4 °C and 10 °C.

3.4. Effect of temperature on the rheological characterization of preserved egg yolk

Rheological characterization has been widely employed to study the shear effect of fluids. The IEY of semi-solid viscous conformed to the rheological characterization and its changes in viscosity could reflect the degree of protein aggregation, which was related to the protein structure and molecular properties (Sathaye et al., 2015; Xin et al., 2023). The effect of different temperatures on IEY viscosity was shown in Fig. 3. All egg yolk samples exhibited a shear thinning behaviour and the viscosity decreased with the increase in shear rate (Sathaye et al., 2015). This was due to the increase in shear rate causing a weakness in the intermolecular force of the egg yolk samples and the destruction of aggregates, resulting in a sharp decline in viscosity (Wang et al., 2021). The shear thinning behaviour of IEY of preserved eggs pickled at 4 °C and 10 °C became stronger, especially at the shear rate of 0.1–1.0 s-1. It can be seen from Fig. 3 that lowering the pickling temperature and prolonging the pickling time could both increase the viscosity of IEY. The viscosity of IEY was affected by its free moisture content (Yang et al., 2019). The moisture content of IEY was always lower than that of the fresh yolk (Fig. 2b), which corresponded to the result of viscosity. As an increase of pickling time, more alkali and salt entered the egg yolk, causing it to dehydrate, which led to an increase in viscosity of IEY. In addition, the proteins in egg yolk crosslinked with polar and nonpolar lipids that were released, forming micelles that increased the suspended particles in IEY of preserved eggs (Kiosseoglou, 2003; Souza & Fernández, 2013). At a consistent shear rate, the viscosity of IEY is higher at lower temperatures (Fig. 3). This could be due to the fact that the movement of water molecules was slowed down and protein was slowly degraded and

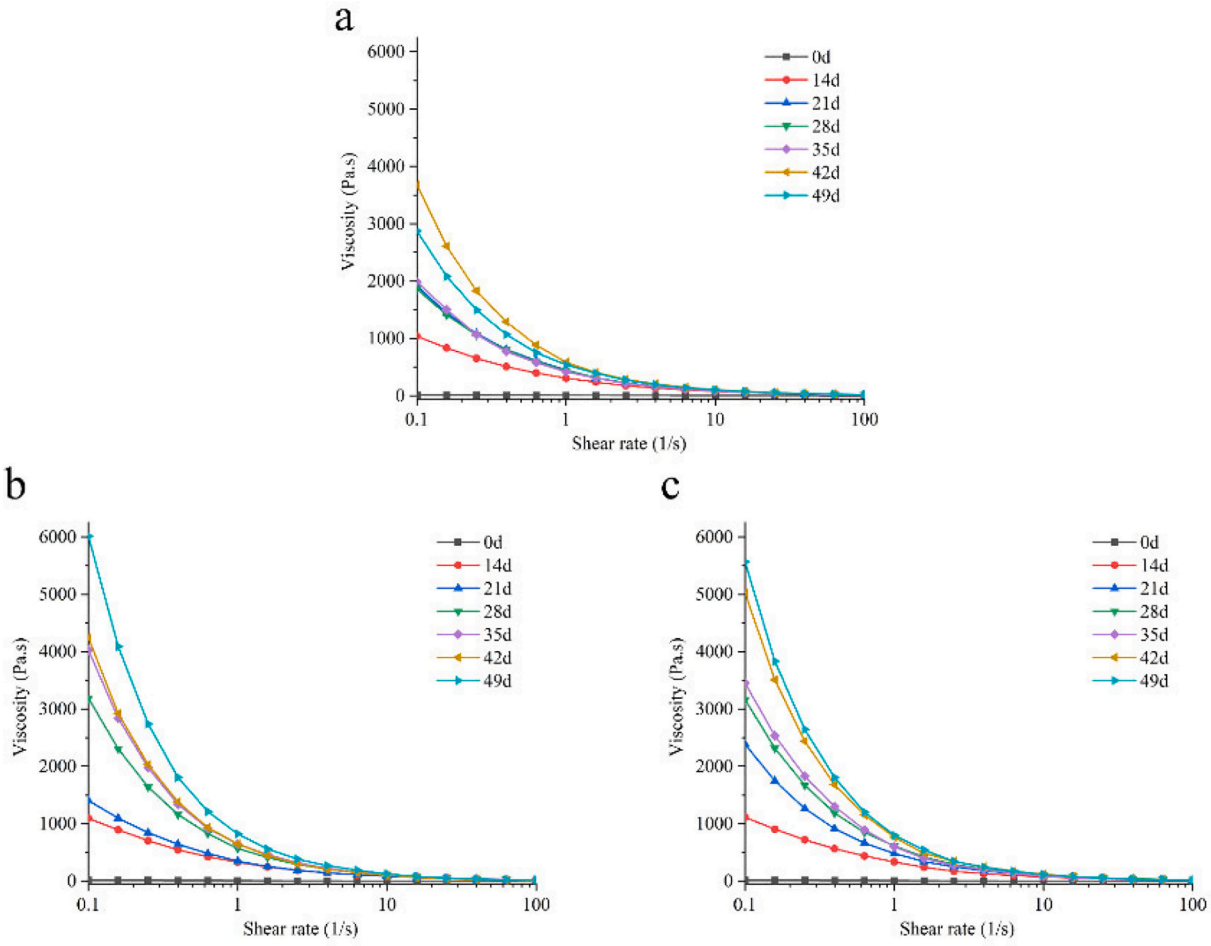

Fig. 3. Changes in viscosity of preserved egg yolk pickled at different temperatures (a-c: the viscosity of IEY at 25 °C, 10 °C and 4 °C, respectively).

formed larger aggregates in the presence of alkali under low-temperature conditions. Wang et al., 2020 found that freezing caused the egg yolks to aggregate, leading to an increase in the viscosity of the egg yolk and the formation of large aggregates.

The power-law model $\eta = K \bullet \gamma^{n-1}$ was used to fit rheological curve of viscosity and shear rate in IEY (Wang et al., 2021). The results of IEY were shown in Table 1. As anticipated, all egg yolk samples exhibited R^2 values of above 0.97, suggesting that the power-law model accorded with the flow behaviour of IEY and had a good fitting effect. The increase of n value indicated that the viscosity of egg yolk samples was decreased. The n values of all samples ranged from 0.1459 to 0.7103 and were less than 1.0 (Table 1), which indicated that they were pseudoplastic fluids (Sathaye et al., 2015). The K value indicates the interior consistency coefficient. Table 1 showed that as the pickling time is increased, the k value increased sharply and the n value decreased. ($P < 0.05$). When the pickling time was the same, the K values of IEY at 4 °C and 10 °C were always higher than that at 25 °C, whereas the n values were always less than that at 25 °C. These results suggested that the fluidity of IEY was decreased and its viscosity was increased during the pickling process. This may be due to the greater concentration of alkali and salt in the pickling liquor, causing a double dehydration effect, which led to an increase in the viscosity of IEY. During the process of freezing-induced egg yolk gel, the attraction between protein molecules (van der Waals force, hydrophobicity and dissipation force) increased, which led to an increase in viscosity of frozen egg yolk (Wang et al., 2021; Wang, Zhang, Chi, & Chi, 2022). Therefore, the formation of the egg yolk aggregates containing LDL and HDL, leading to a higher viscosity of IEY at low temperature.

3.5. *Effect of temperature on the hardness of preserved egg yolk*

Hardness in TPA mode was the maximum force required for the first

Table 1
Changes in K, n and R^2 of preserved egg yolk pickled at different temperatures.

		0d	14d	21d	28d	35d	42d	49d
25 °C	K(Pa.s^n)	7.7616	294.26	424.04	421.35	413.13	617.3	542.48
	n	0.7103	0.4064	0.3181	0.3273	0.3057	0.2345	0.2687
	R^2	0.9993	0.9931	0.998	0.9996	0.9998	0.9957	0.999
10 °C	K(Pa.s^n)	7.7616	304.99	478.28	595.88	583.25	768.31	799.86
	n	0.7103	0.3793	0.2996	0.2681	0.1954	0.1794	0.1514
	R^2	0.9993	0.9979	0.9997	0.9988	0.9834	0.9958	0.976
4 °C	K(Pa.s^n)	7.7616	310.73	345.27	582.21	665.29	641.22	843.72
	n	0.7103	0.4005	0.3561	0.261	0.2329	0.1567	0.1459
	R^2	0.9993	0.9965	0.9968	0.9994	0.9952	0.9499	0.976

compression, which was a vital parameter to characterize the texture of gel (Jin et al., 2022). As can be seen from Fig. 2c, the changes in the hardness of EEY of preserved eggs was pickled at different temperatures. The hardness of EEY of preserved eggs pickled at different temperatures increased with the prolongation of pickling time ($P < 0.05$) and the hardness of EEY of preserved eggs pickled at 4 °C and 10 °C were higher than that at 25 °C (4 °C > 10 °C > 25 °C). These indicated that lower pickling temperature resulted in the increase of preserved egg yolk hardness at the same pickling time. One reason is that water molecules were filled into non-flowing free water and bound water through protein hydration, while a higher degree of dehydration was conducive to protein aggregation to form a high-hardness egg yolk gel. (Ganesan & Benjakul, 2010; Shao et al., 2016). This is confirmed by the lower moisture content (Fig. 2b) of EEY gel at lower temperatures. The hardness of the gel was also related to the molecular forces that maintain the gel structure. The intermolecular forces maintaining the gel of EEY were mainly ionic bonds and disulfide bonds (Yang et al., 2019), where the formation of disulfide bonds was conducive to the formation of a more stable gel network structure. When pickled at 4 °C and 10 °C, more lye entered the egg yolk, resulting in more sulfhydryl groups buried in the protein inside being exposed and the portion of the exposed free sulfhydryl groups were converted into more disulfide bonds to maintain a more stable gel structure (Wang et al., 2022; Yang et al., 2019). Free lipids released from egg yolks is not beneficial for the formation of high-hardness egg yolk gels (Ganesan & Benjakul, 2010). Lowering temperature not only can reduce the release of free lipids in lipoprotein but also slow down the saponification reaction of lipids, resulting in more lipids involved in the formation of a higher hardness gels (Xue, Liu, Zhang, Tu, & Zhao, 2023). The difference in hardness of EEY of preserved eggs pickled at different temperatures reflected that the decrease of pickling temperature was conducive to the formation of high hardness egg yolk gel.

3.6. Effect of temperature on the microstructure of preserved egg yolk

To understand the influence of different temperatures on the gel structure of EEY, the egg yolk sample was observed by scanning electron microscopy. As depicted in Fig. 4, the gel network structure of egg yolk gradually became rougher and more compact with the extension of pickling time, which indicated an increase in the degree of egg yolk aggregation under the continuous action of strong alkali. When pickling for 14 days, there were few and large holes on the surface of EEY in preserved eggs; the periphery of the holes was covered with a layer of lipids. This might be due to the decomposition of lipoproteins under the action of alkali, the denatured proteins aggregated and initially formed the frame of the gel, and the released lipids were filled in the gel (Xin et al., 2023). In the middle stage of pickling, the alkali consistently broke down lipoprotein, resulting in the decomposed protein and lipid participating in the construction of gel (Xue et al., 2023). More denatured protein underwent folding, aggregation and cross-linking, resulting in a rough and loose surface of gel. On account of the differences in egg yolk composition, the larger egg yolk particles were dispersed in the continuous plasma (Marcet et al., 2022). The LDL in the plasma was first treated with strong alkali to involved in gel formation, while the granules containing a higher amount of HDL were not easy to denaturation and expansion, resulting in their embedding on the gel surface (Yang et al., 2020a, 2020b). At the later stages of pickling, the number of particles increased on the surface of the egg yolk gel, while the size of particles became smaller, and their arrangement became more orderly. This could be due to the long-term alkali treatment which led to a higher aggregation degree of EEY of preserved eggs, forming a stable gel structure after the protein degradation and cross-linking. Moreover, the dense gel structure forced water migration, resulting in the formation of smaller and more numerous pores that were gradually filled with free lipids, making the gel surface delicate and smooth (Xue et al., 2023). The change in the microstructure of the egg yolk gel was consistent with the results of hardness (Fig. 2c) and moisture content (Fig. 2b).

As can be seen from Fig. 4, when pickled for 21 days at 4 °C and 10 °C, the egg yolk gel surface became rough with large particles embedded on the surface and the particles gradually became orderly and dense with the increase of pickling time. However, the gel surface of EEY pickled at 25 °C still retained large and irregular holes. This showed that lower temperatures could accelerate the construction of gel network and make the gel structure of EEY become denser and more orderly. Macroscopically, the preserved egg yolk exhibited a higher level of hardness at lower temperatures (Fig. 2c).

3.7. Effect of temperature on free sulfhydryl groups of preserved egg yolk

The SH group, as an active functional group in proteins, was easily oxidized to disulfide bonds, which plays a crucial role in maintaining the spatial structure of proteins (Liu et al., 2022). Egg yolk contained numerous sulfur-containing amino acids, among which low-density lipoproteins and high-density lipoproteins both contained SH groups, which would significantly influence the formation of egg yolk gel (Yang et al., 2020a). The effect of different temperatures treatment on the free

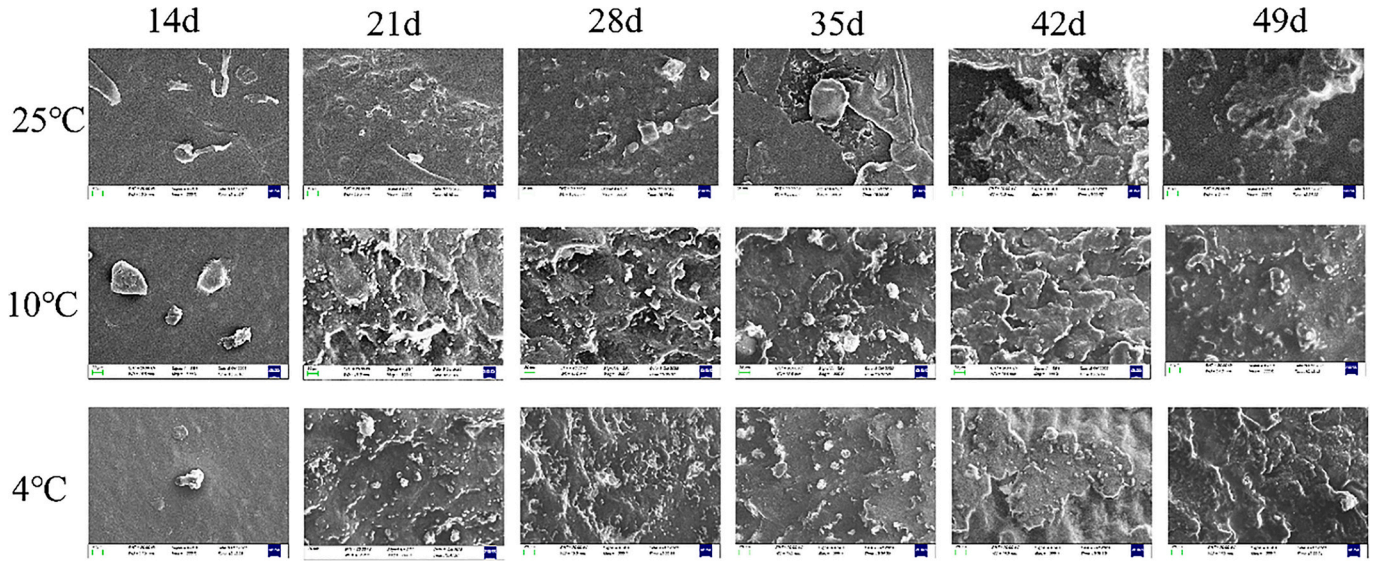

Fig. 4. Changes in microstructure of preserved egg yolk pickled at different temperatures.

SH content in the preserved egg yolk was depicted in Fig. 2d. Compared with fresh egg yolks (0 d), all yolk samples treated with strong alkali exhibited a significant increase in the free SH content ($P < 0.05$). LDL and HDL are the main proteins in egg yolk, which contain many sulfhydryl groups (Liu, Bi, Chi, & Chi, 2024). The protein spatial structure destroyed by the continuous penetration of strong alkali, which resulted in the exposure of the buried SH groups in the protein molecules and broken disulfide bonds that maintain the advanced structure of protein, thus increasing the amount of protein SH groups (Liu et al., 2024; Yu et al., 2020). The free SH content in salted egg yolk increased due to high salt treatment (Xu et al., 2018). This suggested that the sodium salt in the alkali solution also contributed to the increase of sulfhydryl content.

As depicted in Fig. 2d, the free SH content of EEY pickled at 4 °C and 10 °C was significantly higher than that at 25 °C (4 °C > 10 °C > 25 °C) ($P < 0.05$), while the SH content of IEY showed the opposite result (4 °C < 10 °C < 25 °C). This may be due to the fact that the preserved egg white did not form a gel during the early period of pickling at 4 °C and 10 °C, allowing more OH^- to enter the egg yolk, which led to a greater degree of protein denaturation and a more thorough expansion of protein molecules, exposing more SH groups of protein. Therefore, the content of free SH in EEY of preserved eggs pickled at lower temperatures was higher. The transformation between sulfhydryl and disulfide bonds was also accompanied by the formation of the protein gel (Xin et al., 2023). Furthermore, due to the thermal movement of the molecules, the conversion rate of SH into disulfide bonds and the aggregation of protein molecules both were accelerated at 25 °C, which resulted in a lower SH content in the EEY samples at 25 °C. Moreover, the penetration rate of OH^- into IEY was faster at 25 °C, causing the IEY at 25 °C to be the first affected by alkali. Consequently, the free SH content of IEY at 25 °C appeared to be higher than that of IEY at 4 °C and 10 °C.

After 35 days of pickling, the free SH in EEYs treated at different temperatures experienced a significant decrease ($P < 0.05$). The decrease in free SH in EEY samples during the pickling process could be explained by two factors: firstly, the penetration rate of alkali was slowed down due to the pore-blocking effect of heavy metal compounds in the pickling solution (Tan et al., 2022). Secondly, the denatured and unfolded protein molecules constantly underwent rearrangement and aggregation, resulting in the exposed SH groups encapsulating again during the continuous action of alkali (Chen et al., 2015). Differently, the free SH content displayed a decreasing trend after 14 days of pickling ($P < 0.05$). This was attributed to the fact that the formation of EEY prevented the continuous penetration of the lye and the unfolded proteins regrouped to form larger aggregates under the action of strong alkali. As shown in Fig. 2d, the SH content of EEY was significantly higher than that of IEY ($P < 0.05$). The difference was attributed to the disparity in the degree of lye penetration, resulting in the difference degree of protein denaturation between IEY and EEY. The disulfide bond was essential for covalent cross-linking within and between molecules; its formation promoted the aggregation of protein molecules, resulting in a more stable network structure of the gel (Wang et al., 2022). A higher content of free SH at lower temperatures increased the possibility of conversion to disulfide bonds, which meant that more disulfide bonds were generated to sustain the gel stability. This also explained the hardness of EEY at 4 °C and 10 °C was higher than that of EEY at 25 °C (Fig. 2c).

3.8. Effect of temperature on the molecular weight distribution of protein in preserved egg yolk

SDS-PAGE was an experimental technique that can separate different protein types and identify protein molecular weights (Wang et al., 2021). In order to determine the changes of egg yolk proteins induced by alkali under different temperature treatments, SDS-PAGE was used to analyze the egg yolk samples of preserved eggs. Typically, proteins in egg yolks are mainly combined with lipids and occur in fresh egg yolk as lipoproteins, including mainly LDL, HDL, phosvitin and livetin (Anton, 2013). The influence of different pickling temperatures on the protein composition of egg yolk protein in preserved eggs was showed in Fig. 5. Fresh egg yolk exhibited multiple protein bands with molecular weight ranging from 30 to 200 kDa in Fig. 5. According to previous literature, protein bands with different molecular weights were identified: the band at 35, 55, 85, and 122 kDa corresponded to apo-LDL; the band at 35, 47, and 110 kDa correspond to apo-HDL; and the band at 39 kDa, 47 kDa, and 59 kDa correspond to α-phosvitin, β-phosvitin, and phosvitin, respectively; the band at 35 kDa, 55 kDa, and 73 kDa correspond to β-livetin, and α-livetin, respectively (Wang et al., 2020; Yang et al., 2019).

As can be seen in Fig. 5, there was no significant change in IEY and EEY pickled at different temperatures with increasing pickling time. However, there were significant differences between the IEY and EEY of the preserved eggs. The number of major protein bands changed slightly in IEY pickled at different temperatures, which was comparable to that of fresh yolk (0 d); while the bands became shallow and a high molecular weight protein appeared on the top of the separating gel. This indicated that the proteins of IEY degraded slightly and formed a large protein aggregate. Moreover, the bands of IEY pickled at 4 °C and 10 °C (Fig. 5e and f) were clearer compared to that pickled at 25 °C (Fig. 5d). The quicker penetration of alkaline solution and higher reaction temperature at 25 °C might have caused some proteins in IEY to denature faster and unfold faster than at 4 °C and 10 °C, which resulted in degradation of protein and decrease of protein concentration. This was the reason that the protein bands of IEY were clearer at lower temperatures. The changes of sulfhydryl group of IEY (Fig. 2d) also confirmed the denaturation and unfolding of small amount of protein at low-temperature conditions. Interestingly, the electropherogram of EEY (Fig. 5a, b and c) displayed different results from IEY (Fig. d, e and f). In addition to standard protein band and fresh egg yolk band (0 d), EEY only exhibited some shadow of bands, while a clear and intense bands over 200 KDa were present (Yang et al., 2019). Strixner & Kulozik, 2013 suggested that the high molecular weight bands distributed between 200 kDa and 220 kDa were cross-linked proteins and γ-Livetin/apo-LDL while studying the continuous separation of egg yolks. In conclusion, the electrophoresis results indicated that during the pickling of preserved egg, on the one hand, the protein had been strongly degraded and generated some peptides with smaller molecular weight and amino acids (Chen et al., 2015). On the other hand, the process of aggregation and cross-linking occurred between proteins, resulting in the appearance on the top of the separating gel of protein bands that did not appear in fresh egg yolk (Zhao et al., 2016).

3.9. Effect of temperature on the FTIR of preserved egg yolk

The FTIR was a method for examining the structural composition of compounds based on the selective absorption of specific infrared wavelengths by molecules, which could be effectively employed to analyze the changes in molecular structure of egg yolks during the pickling process (Xue et al., 2023). The changes in FTIR spectra of IEY and EEY pickled at different temperatures were showed in Fig. 6. There was no significant change in the shape of the characteristic peaks in the FTIR spectra of IEY samples pickled at different temperatures, while the shape of the two characteristic peaks in EEY samples was smaller compared to that of the fresh yolk at the wave number of 2900–2800 cm^{-1}. According to previous literature reported, methylene asymmetric and symmetric stretching vibrations were observed at wave numbers of 2926.17 cm^{-1} and 2857.70 cm^{-1}, and the two stretching vibrations mostly existed in lipid samples (Li et al., 2023; Xu et al., 2018). This indicated that during the gelation process of preserved egg yolk, the strong alkali destroyed the chemical bonds that maintain the structure of lipid molecules, resulting in the weakening of absorption intensity (Xue et al., 2023). This could be attributed to the fact that lipoproteins were broken down by strong alkali, subsequently, the released free lipid was oxidized and saponified (Xie et al., 2020); the substances produced after

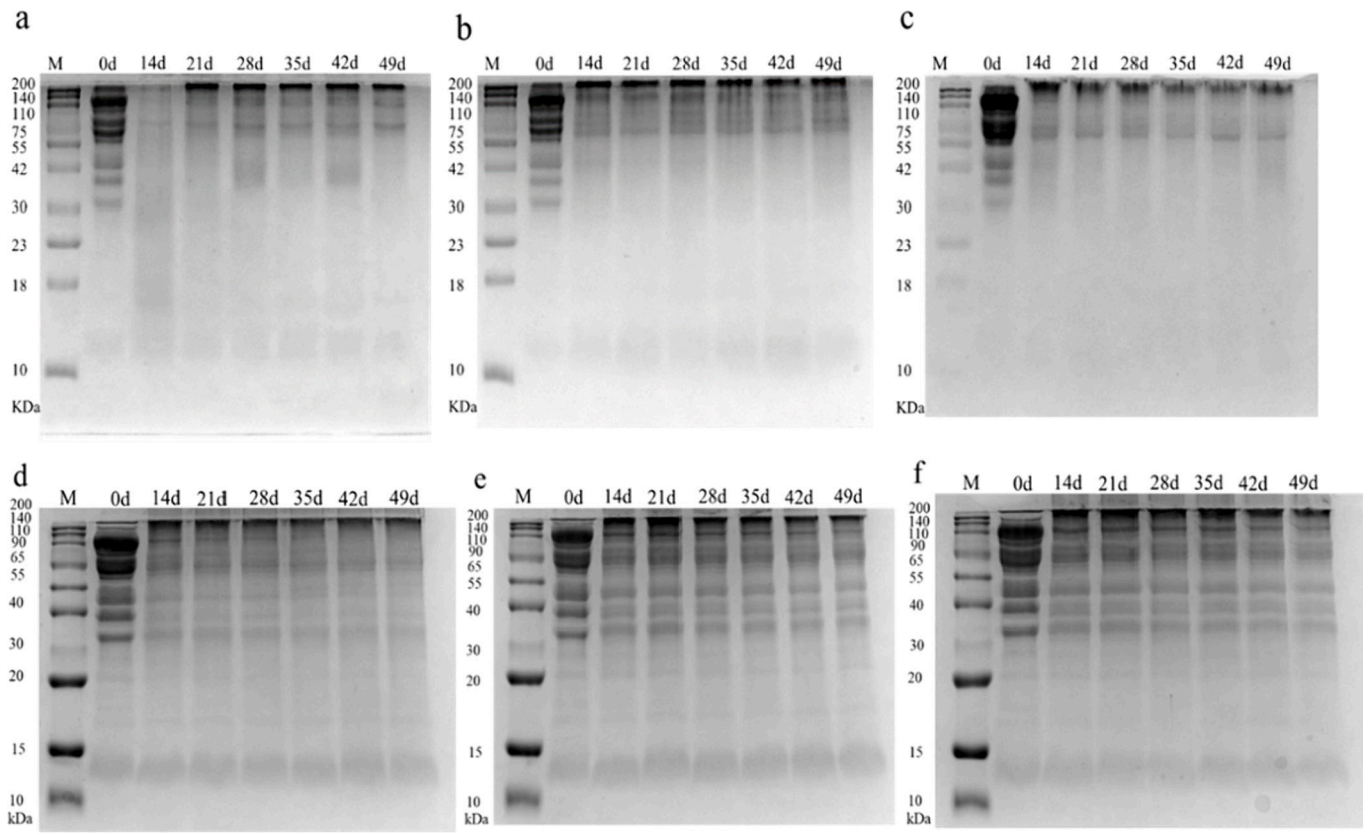

Fig. 5. Changes in SDS-PAGE of preserved egg yolk pickled at different temperatures. (M: molecular weight standard; a-c: the SDS-PAGE of EEY at 25 °C, 10 °C and 4 °C, respectively; d-f: the SDS-PAGE of IEY at 25 °C, 10 °C and 4 °C, respectively).

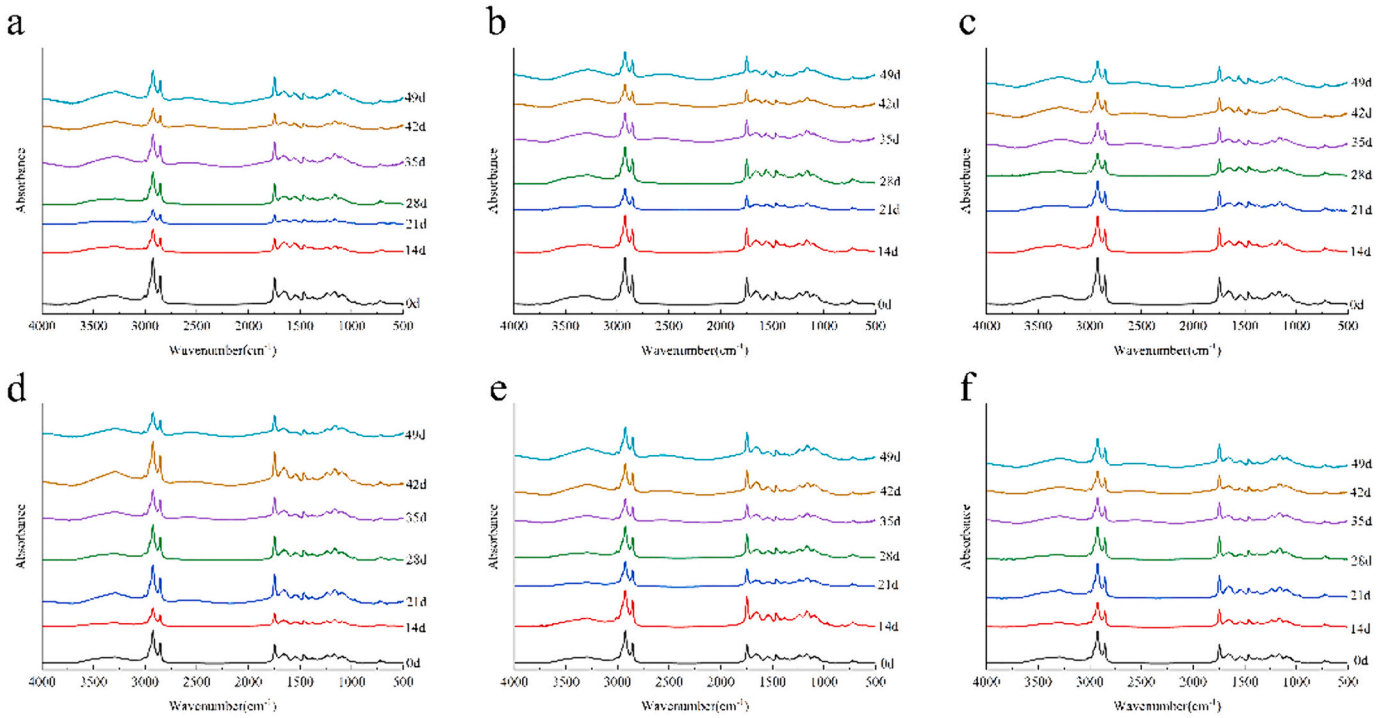

Fig. 6. Changes in FTIR spectra of preserved egg yolk pickled at different temperatures. ((M: molecular weight standard; a-c: the FTIR spectra of EEY at 25 °C, 10 °C and 4 °C, respectively; d-f: the FTIR spectra of IEY at 25 °C, 10 °C and 4 °C, respectively)).

the reactions were cross-linked with small molecular weight proteins and were involved in the forming of egg yolk gel structure (Kaewmanee, Benjakul, & Visessanguan, 2011). In contrast, the shape of the peak of IEY was basically consistent with that of the fresh egg yolk (Fig. 6d, e and f). This was due to the fact that the amount of alkali entered was less, leading to minimal alterations in the lipid structure, which remained as lipoprotein. This was consistent with the results displayed in the SDS-PAGE protein profiles of both the IEY and EEY (Fig. 5). In general, lipids presented within the egg yolk play an essential role in the formation of the gel and the stability of the gel network structure (Cao et al., 2021).

The infrared absorption region of the amide I band (1600–1700 cm^{-1}) provided the most detailed information in FTIR spectra concerning protein secondary structures (Peng, Zhu, Guo, & Zhou, 2022). Based on the results of previous related studies, the absorption peaks at different wavelengths in the amide I band were attributed as follows: the absorption peaks at 1600–1639 cm^{-1}, 1640–1650 cm^{-1}, 1651–1660 cm^{-1} and 1661–1700 cm^{-1}were assigned to β-sheets, random coils, α-helices and β-turns, respectively (Xin et al., 2023; Xue et al., 2020). The changes of different temperatures on secondary structure of IEY and EEY were shown in Table 2. The results showed that compared to fresh egg yolk, the strong alkali treatment of EEY showed a significant decrease ($P < 0.05$) in β-sheets and a significant increase in β-turns with the increase in pickling time, while there were no significant changes in α-helices and random coils. This phenomenon could be attributed to fact that the unfolding and aggregation of proteins that were induced by strong alkali, which led to the interconversions that occurred between the intramolecular and intermolecular structures of proteins (Xin et al., 2023; Yang et al., 2019). When comparing the changes in the secondary structure of EEY pickled at different temperatures, it was discovered that the content of β-sheets at 4 °C and 10 °C was lower than at 25 °C, whereas the results of β-turns content were reversed. This suggested that the formation of egg yolk gel involved a decrease in β-sheets and a rise in β-turns. Additionally, lowering pickling temperature resulted in a faster process and a higher proportion of β-turns in the secondary structure. The increase in the β-turns of EEY promoted the formation of a denser and more stable gel structure, which was observed macroscopically as greater hardness (Yang et al., 2019). Meanwhile, the IEY exhibited a similar tendency to the EEY. The α-helixes and random coils of the IEY showed slight changes, while the β-structures showed a higher degree of transformation at 4 °C and 10 °C compared to 25 °C. The IEY was a transition state from fresh yolk to gel, and the strong alkali caused the protein denaturation and unfolding, the β-sheets within the secondary structure protein molecules were unfolded, and the intermolecular aggregation increased the proportion of β-turns. In conclusion, the strong alkali treatment of the egg yolk led to the unfolding and aggregation of egg yolk proteins, meanwhile, the interconversion of secondary structures promoted the gel structure to become more stable. Moreover, lowering the pickling temperature can facilitate the increase of β-turns to maintain the stability of the three-dimensional network structure and accelerate the formation of a more stable gel.

4. Conclusion

Based on the gel formation of preserved egg yolk induced by strong alkali, this study discovered that the pickling temperature has a significant effect on the solidification of preserved egg yolk. Particularly, during the early stage of pickling, when preserved eggs were pickled at 4 °C and 10 °C, more alkali penetrated through the egg white into egg yolk due to the slower formation of egg white gel compared to 25 °C. Consequently, the excessive amount of alkali caused the protein molecules to denature and unfold the hydrophobic regions, intermolecular electrostatic interactions of proteins were weakened, forming protein aggregates and resulting in gelation of egg yolk. This also resulted in an increase in pH and free SH content of egg yolk gels pickled at 4 °C and 10 °C. In the meantime, the conversion of free sulfhydryl to disulfide bonds to stabilize the gel structure, resulting in an increase the egg yolk gel hardness and forming a denser, more stable gel network structure. Moreover, the formation of egg yolk gel was also accompanied by protein degradation and the formation of high molecular weight cross-linked proteins with a β-structure transition. In addition, the degradation of the proteins was weakened and the interconversion of β-structures was faster at lower temperatures. In summary, the rate of solidification of preserved egg yolk was faster when preserved eggs were pickled at 4 °C and 10 °C, which provides ideas and theoretical basis for the application of low-temperature pickling to shorten the pickling period of preserved eggs in practice.

CRediT authorship contribution statement

Xianlong Luo: Writing – original draft, Investigation, Formal analysis, Data curation. **Ji'en Tan:** Writing – review & editing. **Yao Yao:**

Table 2
Changes in protein secondary structure (%) of preserved egg yolk pickled at different temperatures.

	Time(d)	0d	14d	21d	28d	35d	42d	49d
25 °C EEY	β-sheets	31.89 ± 0.28^{a}	26.7 ± 0.5^{cd}	26.69 ± 0.44^{cd}	27.46 ± 1.11^{bc}	27.88 ± 0.82^{b}	27.12 ± 0.16^{bcd}	26.25 ± 0.33^{d}
	Random coils	18.54 ± 0.26^{a}	18.6 ± 0.34^{a}	17.97 ± 0.37^{a}	18.66 ± 0.37^{a}	17.54 ± 1.21^{a}	17.09 ± 1.51^{a}	17.39 ± 1.20^{a}
	α-helices	19.44 ± 0.08^{a}	18.0 ± 3.46^{a}	18.18 ± 0.96^{a}	17.42 ± 1.69^{a}	17.41 ± 3.79^{a}	19.25 ± 2.78^{a}	16.27 ± 0.90^{a}
	β-turns	30.14 ± 0.39^{a}	36.6 ± 2.83^{b}	37.16 ± 1.42^{b}	36.45 ± 2.03^{b}	37.17 ± 3.56^{b}	36.53 ± 1.32^{b}	40.10 ± 0.14^{b}
10 °C EEY	β-sheets	31.89 ± 0.28^{a}	26.0 ± 0.41^{c}	28.56 ± 1.85^{b}	26.87 ± 1.55^{bc}	25.63 ± 0.62^{c}	26.52 ± 1.27^{c}	25.45 ± 0.16^{c}
	Random coils	18.54 ± 0.26^{a}	19.2 ± 0.12^{a}	14.82 ± 2.37^{a}	19.08 ± 1.65^{a}	17.60 ± 0.23^{a}	17.98 ± 1.34^{a}	17.34 ± 0.03^{a}
	α-helices	19.44 ± 0.08^{a}	16.6 ± 0.38^{b}	16.87 ± 1.40^{b}	16.43 ± 1.15^{b}	19.03 ± 1.21^{a}	19.13 ± 1.88^{a}	19.60 ± 0.36^{a}
	β-turns	30.14 ± 0.39^{a}	38.0 ± 0.32^{bc}	39.75 ± 0.50^{c}	37.61 ± 3.82^{bc}	37.73 ± 1.45^{bc}	36.37 ± 1.85^{b}	37.62 ± 0.23^{bc}
4 °C EEY	β-sheets	31.89 ± 0.28^{a}	26.5 ± 1.02^{c}	26.87 ± 0.42^{c}	28.75 ± 0.13^{b}	23.50 ± 0.58^{d}	24.39 ± 0.42^{d}	23.18 ± 1.13^{d}
	Random coils	18.54 ± 0.26^{a}	18.5 ± 0.08^{a}	17.77 ± 0.12^{b}	14.62 ± 0.63^{c}	18.51 ± 0.44^{a}	18.74 ± 0.45^{a}	18.87 ± 0.27^{a}
	α-helices	19.44 ± 0.08^{a}	18.2 ± 2.77^{a}	19.35 ± 1.65^{a}	18.83 ± 2.28^{a}	18.36 ± 1.14^{a}	17.60 ± 0.38^{a}	17.54 ± 0.35^{a}
	β-turns	30.14 ± 0.39^{a}	36.7 ± 3.69^{bc}	36.01 ± 1.40^{b}	37.80 ± 2.10^{bcd}	39.63 ± 0.69^{cd}	39.27 ± 0.58^{bcd}	40.42 ± 1.23^{d}
	β-sheets	31.89 ± 0.28^{a}	30.09 ± 0.34^{b}	29.80 ± 0.62^{b}	29.69 ± 1.71^{b}	29.64 ± 0.85^{b}	30.17 ± 0.43^{b}	31.18 ± 0.43^{ab}
25 °C IEY	Random coils	18.54 ± 0.26^{abc}	18.2 ± 0.39^{bc}	18.37 ± 0.47^{abc}	18.87 ± 0.16^{ab}	18.96 ± 0.48^{a}	18.87 ± 0.29^{ab}	17.91 ± 0.21^{c}
	α-helices	19.44 ± 0.08^{a}	20.2 ± 1.15^{a}	19.28 ± 3.11^{a}	17.68 ± 3.50^{ab}	17.48 ± 1.62^{ab}	15.46 ± 0.40^{b}	19.90 ± 0.92^{a}
	β-turns	30.14 ± 0.39^{a}	31.4 ± 1.32^{ab}	32.55 ± 2.33^{ab}	33.76 ± 1.96^{bc}	33.92 ± 1.22^{bc}	35.49 ± 0.11^{c}	31.02 ± 0.70^{a}
	β-sheets	31.89 ± 0.28^{a}	29.6 ± 0.34^{ab}	29.97 ± 0.31^{ab}	29.14 ± 3.62^{ab}	28.29 ± 0.43^{b}	29.04 ± 0.86^{ab}	29.92 ± 1.18^{ab}
10 °C IEY	Random coils	18.54 ± 0.26^{a}	18.0 ± 0.25^{a}	18.41 ± 0.14^{a}	18.24 ± 1.76^{a}	18.72 ± 0.22^{a}	18.44 ± 0.50^{a}	18.52 ± 0.57^{a}
	α-helices	19.44 ± 0.08^{ab}	18.9 ± 0.23^{ab}	20.39 ± 0.94^{a}	18.78 ± 3.59^{ab}	16.98 ± 0.81^{bc}	19.86 ± 0.26^{a}	15.54 ± 0.23^{c}
	β-turns	30.14 ± 0.39^{a}	33.4 ± 0.38^{c}	31.24 ± 0.94^{ab}	33.85 ± 1.54^{c}	36.01 ± 0.74^{d}	32.66 ± 0.15^{bc}	36.02 ± 1.62^{d}
	β-sheets	31.89 ± 0.28^{a}	30.1 ± 0.01^{c}	29.78 ± 0.05^{c}	35.58 ± 0.18^{b}	29.59 ± 0.63^{c}	28.14 ± 0.82^{d}	28.28 ± 0.41^{d}
4 °C IEY	Random coils	18.54 ± 0.26^{a}	17.9 ± 0.07^{a}	18.06 ± 0.16^{a}	12.47 ± 0.03^{b}	18.58 ± 0.93^{a}	18.29 ± 0.31^{a}	18.69 ± 0.20^{a}
	α-helices	19.44 ± 0.08^{a}	19.0 ± 0.02^{ab}	19.30 ± 0.19^{a}	16.53 ± 0.50^{c}	17.72 ± 3.36^{abc}	16.69 ± 0.27^{bc}	17.29 ± 0.30^{abc}
	β-turns	30.14 ± 0.39^{a}	32.8 ± 0.04^{b}	32.86 ± 0.25^{b}	35.41 ± 0.58^{cd}	34.10 ± 2.51^{bc}	36.88 ± 0.59^{d}	35.74 ± 0.51^{cd}

Writing – review & editing. **Na Wu:** Writing – review & editing. **Shuping Chen:** Writing – review & editing. **Lilan Xu:** Writing – review & editing. **Yan Zhao:** Writing – review & editing, Validation, Project administration, Funding acquisition, Conceptualization. **Yonggang Tu:** Writing – review & editing, Validation, Project administration, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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