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Changes of microstructure characteristics and intermolecular interactions of preserved egg white gel during pickling

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ABSTRACT

Changes in gel microstructure characteristics and in intermolecular interactions of preserved egg whites during pickling were investigated. Spin–spin relaxation times of preserved egg whites significantly decreased in the first 8 days and remained unchanged after the 16th day. SEM images revealed a three-dimensional gel network, interwoven with a loose linear fibrous mesh structure. The protein gel mesh structure became more regular, smaller, and compacted with pickling time. Free sulfhydryl contents in the egg whites increased significantly, while total sulfhydryl contents dramatically decreased during pickling. The primary intermolecular forces in the preserved egg white gels were ionic and disulfide bonds. Secondary forces included hydrophobic interaction and relatively few hydrogen bonds. During the first 8 days, the proportion of ionic bonds sharply decreased, and that of disulfide bonds increased over the first 24 days.

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1. Introduction

Preserved eggs are traditional egg products in China that are made by preserving duck, chicken or quail eggs in a mixture of alkaline solutions, salt, black tea, and metal ions for 4–6 weeks at room temperature (Su & Lin, 1993; Tu, Zhao, Xu, Li, & Du, 2013; Wang & Fung, 1996). Preserved eggs have many distinct characteristics, including unique flavours, dark green yolks, and dark brown or transparent egg whites. Preserved eggs have many crystals that are observed throughout the egg, e.g., at the surface and inside the egg whites (Deng, 2013; Zhao, Tu, Xu, Li, & Du, 2014).

Preserved eggs are typically pickled using a strong alkaline solution. When treated with sodium hydroxide, protein molecules in the egg white are damaged and degenerate to form a highly elastic gel. This gel has a uniform, loose, and fine filamentous structure with regular voids (Zhao et al., 2014). If the concentration of sodium hydroxide in the pickling solution is too high, congealed egg white protein would hydrolyse and liquefy, and the egg yolk would become harder (Ganasen & Benjakul, 2011; Ganesan & Benjakul, 2010, 2014; Ma, 2007). An appropriate amount of metallic compounds should be added to the curing liquid to regulate the permeation of the alkaline compounds (Ganesan & Benjakul, 2010; Tu et al., 2013; Yan & Zhu, 2006) and prevent excessive alkali injury. Metallic compounds form insoluble sulfides that can plug

Abbreviations: PEW, preserved egg white; ESEM, environment scanning electron microscopy; LF-NMR, low-field NMR; β-ME, beta-mercaptoethanol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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the egg shell and membrane pores, meshes, and corrosion holes generated from the alkali processing (Ma, Xie, & Su, 2001; Zhao, Xu, & Tu, 2010) to regulate the excessive infiltration of alkaline compounds. In traditional methods of preserving eggs, PbO was used to regulate the infiltration of alkaline compounds. However, due to the harmful effects of lead (Baos et al., 2006), a large number of studies have explored lead-free techniques. Copper, zinc, iron, and other metal compounds have been used instead of lead (Yan & Zhu, 2006). Copper sulfate has been widely used in preserved egg production. Although Cu, Fe, and Zn are essential and important nutrients, excessive levels can cause significant harm to the body (Hambidge, 2007). Therefore, alternative pickling processes that do not require the addition of metal ions must be developed. To improve the pickling processes, the mechanism of forming egg white gels and the regulatory mechanisms of heavy metals should first be investigated.

Coagulation or gelation is one of the most important functional properties of proteins. A protein gel is a continuous aggregated network of denatured protein molecules (Totosaus, Montejano, Salazar, & Guerrero, 2002). Gels are obtained when proteins undergo physical or chemical processes, such as heating, pressurization, and acidic, alkaline, ion or enzymatic treatments (Totosaus et al., 2002). Protein gelling is a complex process consisting of a sequence of structural changes. In general, heating or other driving forces cause protein molecules to denature and partially unfold to expose inner hydrophobic regions. This process is followed by aggregation and gelation (Mine, 1995; Totosaus et al., 2002). Different treatments can result in protein gels with different properties. Alkaline treatments can cause protein denaturation, similar to the thermally induced protein gelation and is involved in the first stage of unfolding egg white native proteins. These native proteins, which have well-defined tertiary and secondary structures, are disrupted and denatured in strong alkaline solutions, i.e., their inner, active regions are exposed (Ji et al., 2013; Zhang, Jiang, Chen, Ockerman, & Chen, 2015). The denatured molecules may also polymerize differently in the absence of thermal treatment. At high pH, the gelation mechanism affects the protein structure and other properties. The assembly of polymer strands in gels are affected by environmental variables, such as ionic strength and pH (Heertje, 2014). Hence, preserved egg white gels have distinct physicochemical characteristics dependent on protein–protein and protein–medium interactions. Our previously published study investigated changes to egg white gel characteristics, such as the gel microstructure and intermolecular forces, when treated with strong alkaline solutions (Chen et al., 2015). However, it is different to egg white gel directly induced by NaOH and preserved egg white (PEW) gel formed after prolonged pickling.

The primary objective of this study was to investigate changes in the microstructure and intermolecular forces of PEW gels during pickling to clarify the physicochemical characteristics and formation mechanism of the protein gel induced by strong alkaline solutions.

2. Materials and methods

2.1. Chemicals

Copper sulfate ($CuSO_4$), sodium hydroxide (NaOH), and sodium chloride (NaCl) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Aldrich (Sigma-Aldrich, Co., Ltd., USA). Other analytical grade chemicals for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), including a low-molecular weight marker, were obtained from Solarbio Science & Technology Co., Ltd. (Beijing, China).

2.2. Preparation of preserved eggs

One-day old duck eggs weighing 65–75 g were obtained from a farm in Nanchang County, Jiangxi Province, China. The eggs were cleaned with tap water and checked for any cracks prior to soaking in pickling solutions, which contained NaOH (4.5%, m/v), NaCl (4%, m/v), and $CuSO_4$ (0.4%, m/v), at 25 °C for 40 days (Tu et al., 2013). During processing, six eggs were chosen at 0, 4, 8, 16, 24, 32, and 40 days. Egg white gels were carefully separated from egg yolks.

2.3. Spin–spin relaxation time (T2) measurements

Spin–spin relaxation time (T2) measurements of preserved egg white were performed on a low-field pulsed NMR Analyzer (Jiangxin Corporation, Ningbo City, China) according to the methods of He et al. (2013) with slight modifications. Cylindrical gel samples (15 mm long, 2 mm diameter) were prepared and placed into a 10-mm glass tube, which was then inserted in the NMR probe. Car–Purcell–Meiboom–Gill (CPMG) sequences were used to measure the T2 at 40 °C. The T2 measurements were made with a τ-value (i.e., the time between 90° and 180° pulses) of 100 μs. Data from 30 echoes were acquired over 10 scan repetitions.

2.4. Scanning electron microscopy (SEM)

The PEW gel microstructure was examined using an environmental SEM (ESEM, Quanta-200F, FEI, Ltd., The Netherlands) according to a previously described method (Chen et al., 2015) with some modifications. An approximately 0.5 cm^3 sample cut from a PEW gel was fixed in 2.5% (v/v) glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for approximately 24 h at room temperature. Subsequently, the sample was rinsed with 0.1 M phosphate buffer (pH 7.2) thrice for approximately 15 min. Afterwards, the fixed samples were freeze-dried in a lyophiliser (Alpha1-2, Martin Christ, Germany) and observed by ESEM at an acceleration voltage of 10 kV in low vacuum mode.

2.5. Determination of free sulfhydryl (SH) group and total sulfhydryl group contents

The concentration of SH groups in the PEW was determined using Ellman's reagent DTNB according to the method of Beveridge and Arntfield (1979), Beveridge, Toma, and Nakai (1974). Chopped PEW gel samples (approximately 3 g) were homogenised (Ultra Turrax homogeniser, IKA T18 digital, IKA Works Guangzhou Co., Ltd., China) with 27 ml of phosphate buffer (pH 8.0) for 2 min at 10,000 rpm. The homogenate was centrifuged at 10,000×g (Anke, Model TGL-20B, Shanghai, China) for 15 min.

To determine the free SH content, a 0.2 ml aliquot of the supernatant was allowed to stand and was then added to 2.8 ml Tris-glycine buffer (0.1 M Tris, 0.1 M glycine, 4 mM EDTA, pH 8.0) and 0.02 ml Ellman's reagent (4 mg/mL DTNB in Tris-glycine buffer). The mixture was incubated at 40 °C for 15 min. Subsequently, the absorbance of the supernatant was recorded using a T6 spectrophotometer (Persee Co., Ltd., Beijing, China) at 412 nm. The SH content was calculated using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}. A blank was analysed using the treatment buffer.

For the total SH content, a 0.2 ml aliquot of the supernatant was allowed to stand and was then added to 2.8 ml 0.5% SDS-8M urea-Tris-glycine buffer [0.1 M Tris, 0.1 M glycine, 4 mM EDTA, 0.5% SDS (w/v), 8 M urea, pH 8.0] and 0.02 ml Ellman's reagent. The resulting mixture was then spectrophotometrically analysed according to the same method as that for measuring the free SH content. The SH residues were calculated as follows: μM SH/g protein = $73.53 \times A_{412} \times D/C$, where A_{412} is the absorbance at 412 nm, C is the sample concentration in mg/ml, and D is the dilution factor, 15.01.

2.6. Selective protein solubility

The PEW gel protein solubility was analysed according to the method of Pérez-Mateos, Lourenço, Montero, and Borderías (1997) with some modifications. PEW gels were successively solubilised in four solvents: 0.6 M sodium chloride (S1); 0.6 M sodium chloride + 1.5 M urea (S2); 0.6 M sodium chloride + 8 M urea (S3); and 0.6 M sodium chloride + 8 M urea + 0.5 M β-ME (S4). Approximately 3 g of chopped gel was homogenised with 27 ml of S1 for 1 min at 10,000 rpm. The homogenate was centrifuged at 23,000×g (Anke, Model TGL-20B, Shanghai, China) for 20 min. The precipitate obtained from S1 was homogenised in 27 ml of S2 through the same process. The same procedure was also successively performed using S3 and S4. The protein concentrations in the supernatants were determined according to the biuret method. Bovine serum albumin was used as standard treatment, and specific standard curves of each solvent were prepared. Each S4 fraction was dialysed against solution S1 for 24 h at room temperature to prevent interference from β-ME. The results were derived from the average of three determinations and expressed as a percentage of each fraction with respect to total protein.

2.7. Electrophoresis

The total protein and all fractions extracted from the four solvents used in 2.6 were analysed by SDS–PAGE in a vertical electrophoresis unit (Bio-Rad, Richmond, CA, USA) according to previously described methods (Laemmli, 1970) to identify changes in the proteins during pickling and the involvement of proteins in the formation of certain types of bonds. Soluble proteins were mixed (1:1, v/v) with SDS–PAGE sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-ME, and 0.2% bromophenol blue) and boiled for 5 min. An approximately 12-μl aliquot and 10 μl standard protein marker were injected into each well. Electrophoresis was conducted at a constant voltage of 120 V in the resolving gel. After the electrophoresis was complete, the gels were stained with 0.125% Coomassie Brilliant Blue R-250 in 25% methanol and 10% acetic acid. Destaining was performed using 25% ethanol and 8% acetic acid.

2.8. Statistical analysis

The experimental design was a completely random design with three replications. Data are presented as mean values with standard deviations. Statistical analyses were performed with the statistical program SPSS19.1 for Windows (SPSS Inc., Chicago, IL, U. S.A.). One-way analysis of variance (ANOVA) was carried out, and means comparisons were analysed by Duncan's multiple range tests. Significant differences were determined at $P < 0.05$.

3. Results and discussion

3.1. Change of T2 in low-field nuclear magnetic resonance (LF-NMR) during pickling

The state of water in the gel sample can be determined by LF-NMR technology. By measuring the T2 using LF-NMR, the water molecules states and their various binding states, such as free water, monolayer, and multilayer bound water, can be evaluated (Yasui, Ishioroshi, Nakano, & Samejima, 1979). The interactions between the water and protein molecules play important roles in the protein–water system where water molecules exist as bound water and free water (Langton & Hermansson, 1992; Liu et al., 1995). Therefore, the combination of water molecules and protein molecules can be understood by measuring the degree of freedom or mobility of the former.

As shown in Fig. 1, the proton density corresponding to the T2 represented the total proton density in the PEW gel. During the curing process, the T2 significantly decreased in the first 8 days before levelling off ($P < 0.05$) within the range of 15–300 ms. The T2 at day 0, i.e., the fresh egg white solution, was at a maximum and subsequently declined by the 4th day of curing. A few gelatinoids in the egg white sample were observed on the 4th day, indicating gel formation. A relatively integrated PEW gel was observed on the 8th day, when the T2 was much lower when compared with the control group. However, the T2 remained essentially unchanged since the 16th day due to the stability of the protein gel. A T2 greater than 100 ms indicated free water with high degrees of freedom, while a T2 between 10 and 100 ms indicated multilayers of bound water with low mobility. A decline of the T2 as shown by ^{1}H NMR indicated that the water molecules contained in the preserved egg white were more firmly bound and that the water mobility was weakened or the amount of free water decreased. This observation may be attributed to two factors. First, the few amounts of water present in the preserved egg moved outwardly from the eggshell membrane due to an osmotic pressure gradient formed by the alkaline solution (Wang & Fung, 1996; Zhao et al., 2014). Second, the formed egg white gel may have immobilised water molecules, e.g., ions and the ionogens at the end of polypeptide chains may have formed "water-ionic bonds" with water molecules and thereby, weakened the mobility of the water molecules (Totosaus et al., 2002). The final T2 was approximately 30 ms, which indicated low-mobility multilayer bound water. The bound water likely contained a monolayer binding state (T2 < 10 ms), i.e., hydrogen bonds. The loss and weakened mobility of water may have resulted in a more stable PEW gel at later and mature stages.

3.2. Microstructural change of PEW gel during pickling

SEM can be used to observe and investigate the features of food matrix microstructures, such as 3D network structures and spatial arrangements (Heertje, 2014). The microstructure characteristics of the PEW gel observed using ESEM are shown in Fig. 2. During the curing process, a 3D network was observed for the PEW gel interwoven with a loose, linear, fibrous mesh structure. Past studies have shown that a few animal proteins (e.g., β-lactoglobulin, whey protein, ovalbumin, and bovine serum albumin) may assemble and aggregate by noncovalent bonds to form these

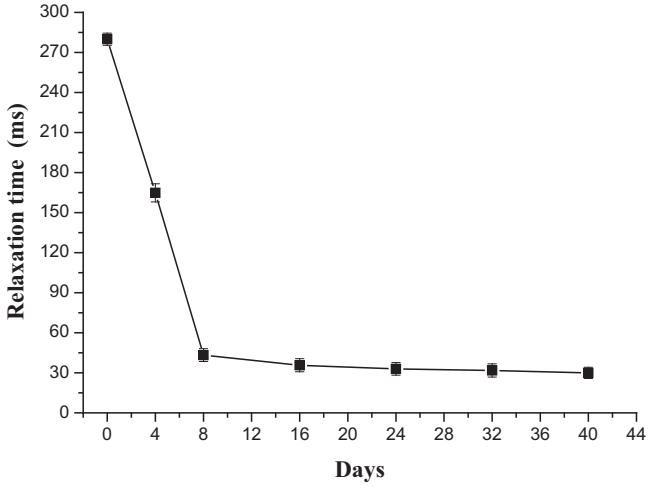

Fig. 1. NMR proton relaxation (T2) of PEW gels during pickling.

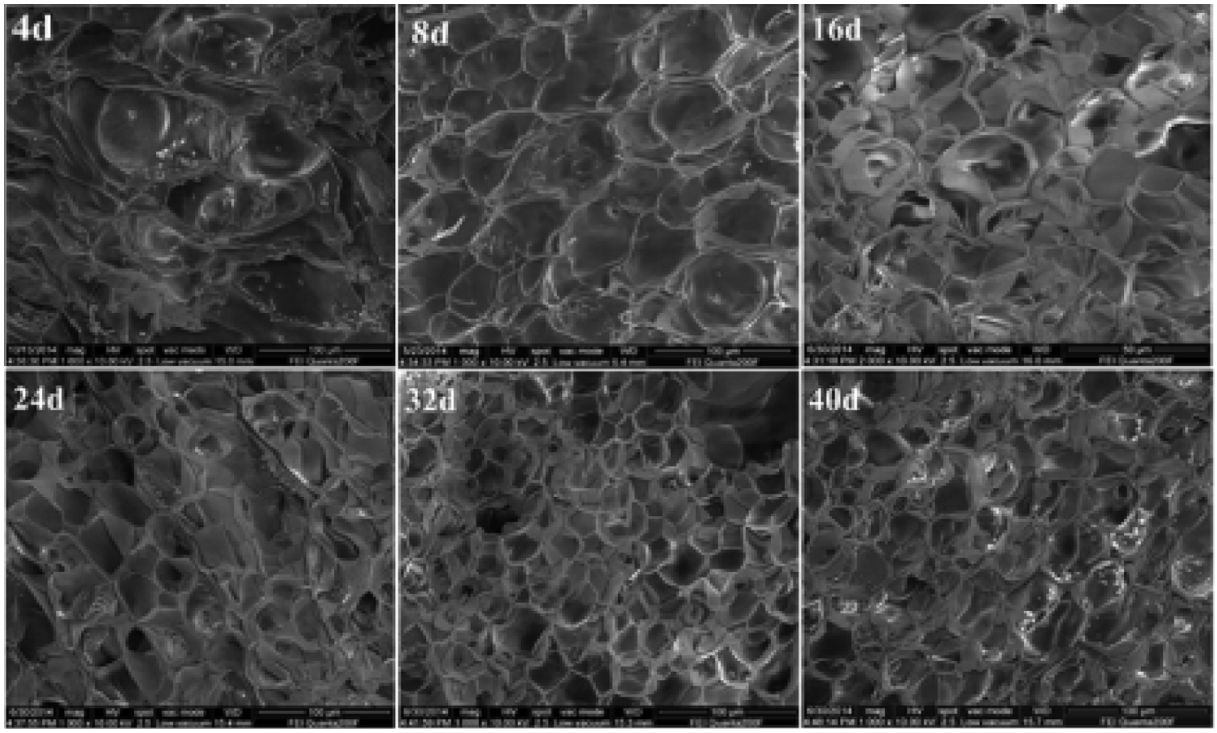

Fig. 2. Changes in the microstructure of PEW gels during pickling.

fibrous structures (Sagis et al., 2002; Veerman, Sagis, Heck, & van der Linden, 2003; Weijers, van de Velde, Stijnman, van de Pijpekamp, & Visschers, 2006). Under equilibrium conditions, protein molecules may spontaneously form aggregates or supramolecular structures through noncovalent interactions. This formation leads to a self-assembly-based fibrotic process. Proteins denatured by heat may produce a network structure of fine strands as described above under low ionic strength conditions or at pH levels much higher or lower than the isoelectric point of the proteins. However, other studies have indicated that proteins aggregate to form a network structure with particle strands under high ionic strength conditions or at a pH value close to the isoelectric point (Handa, Takahashi, Kuroda, & Froning, 1998; Langton & Hermansson, 1992). The pH levels of the PEW gel were maintained at a high level because of the dynamic permeability of the strong alkaline solution (Ganasen & Benjakul, 2011; Tu et al., 2012). Therefore, under this extreme alkaline environment, the network structure of the protein gel was similar to that of the former described by Handa et al. Moreover, such loose structures may be caused by the aggregation and extension of protein molecules. For example, the strong electrostatic repulsive force within a molecule caused by a net negative charge and extremely high pH will lead to a swelling and expansion of protein molecules. Although cupric ions and sodium ions were added to the curing liquid, they may have only formed low ionic strength bonds in the egg white and not contributed towards the formation of the protein gel. As hypothesised by Totosaus et al. (2002), if the aggregation rate of denatured protein or polypeptide chain is less than the protein denaturation rate, a regular and semi-transparent gel structure tends to form. Compared with the aggregation of protein molecules under thermal conditions, the aggregation rate is slower under strong alkaline solution treatments.

During the pickling process, strong alkaline solution enters the egg through the eggshell and the eggshell membrane at room temperature, causing the proteins of the egg white to undergo a series of chemical changes and subsequently, gelatinise (Wang & Fung, 1996). As shown in the SEM images, the protein gel mesh structure becomes more regular, smaller, and compact with pickling time. The mesh structures were likely left behind by the water molecules escaping during the drying process. Thus, the structures of these meshes likely changes with water content. During the curing process, water in the egg transfers to the curing liquid due to an osmotic gradient. Once the gel structure solidifies, water transfer is impeded, and the water content of the gel system is fixed. This solidification corresponds with the changes in total proton density as indicated by LF-NMR spectroscopy. Additionally, according to preliminary studies, the textural properties of the preserved egg protein gel significantly change with curing time; hardness and elasticity greatly increase (Tu et al., 2012; Zhao et al., 2014). A stable gel structure with a highly compact mesh would be able to resist external forces (Pérez-Mateos et al., 1997). Based on the microstructural changes to the protein gel, the proteins gradually denatured due to the strong alkaline solution.

3.3. Changes in the free sulfhydryl and total sulfhydryl contents in PEW gel during pickling

Numerous sulfhydryl groups and disulfide bonds exist in egg white proteins. For example, four free sulfhydryl groups are present in each ovalbumin molecule, and 15 disulfide bonds are found in ovotransferrin (Mine, 1995). The changes in the free sulfhydryl and total sulfhydryl contents in a PEW gel during pickling is shown in Fig. 3. The total sulfhydryl content in fresh duck egg white is 29.36 ± 3.07 μmol/g protein, i.e., slightly less than that of chicken egg white (Kaewmanee, Benjakul, & Visessanguan, 2011). This difference may be due to egg varieties and the foaming loss of protein and sulfhydryl groups because during homogenous dispersion. When compared with the fresh duck egg white, the free sulfhydryl content of preserved egg white significantly increased, whereas the total sulfhydryl content significantly decreased after pickling with a strong alkaline solution. However, the free and total sulfhydryl contents did not show any significant differences ($P > 0.05$) during

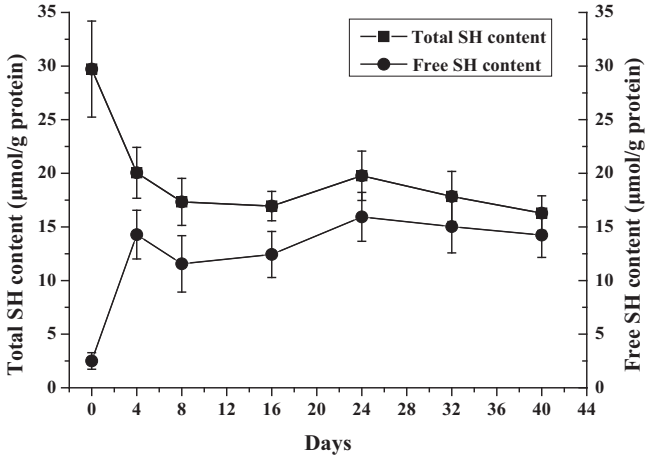

Fig. 3. Changes in free sulfhydryl and total sulfhydryl group contents of PEW gels during pickling.

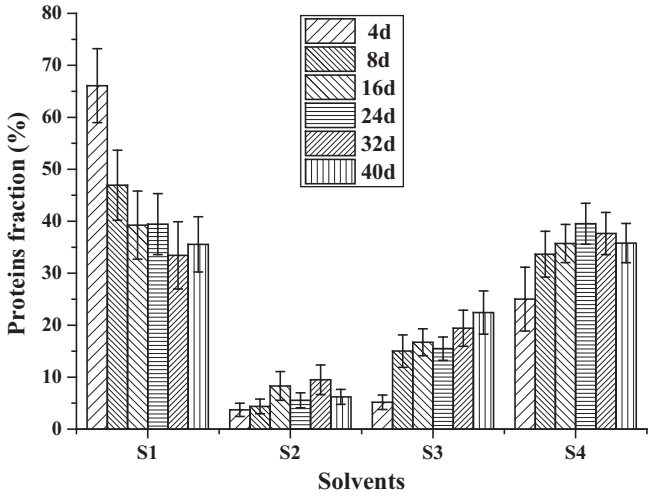

Fig. 4. Protein fraction (%) of PEW gel using different solutions: 0.6 M sodium chloride (S1); 0.6 M sodium chloride + 1.5 M urea (S2); 0.6 M sodium chloride + 8 M urea (S3); and 0.6 M sodium chloride + 8 M urea + 0.5 M β-mercaptoethanol (S4).

pickling. Intra- and intermolecular covalent crosslinking occur only by disulfide bonds in proteins. The sulfhydryl groups of two Cys residues will form disulfide bonds via an oxidation reaction with molecular oxygen (Hwang, Lai, & Hsu, 2007). Moreover, globular protein molecules with sulfhydryl groups have strong gelation capacities, as was shown by the intermolecular disulfide bonds formed by a sulfhydryl–disulfide (SH–SS) exchange reaction (He et al., 2013).

The treatment with a strong alkaline solution denatures egg white proteins and exposes active groups within the hydrophobic regions. The exposure of the active groups initially significantly increases the free sulfhydryl content. However, as the gel structure is formed, the free sulfhydryl content stabilizes at a certain level. Because the sulfhydryl oxidation and SH–SS exchange reactions are rapidly completed under extreme alkaline environments (Mine, 1996; Van der Plancken, Van Loey, & Hendrickx, 2005), the total sulfhydryl content initially significantly declines. However, as the reaction rates of the sulfhydryl oxidation and SH–SS exchange reactions slow, the decrease in total sulfhydryl content slows after gelation. The protein gelation is promoted by the formation of disulfide bonds (Totosaus et al., 2002). Additionally, other factors in the complex environment, such as ionic bonds, hydrogen bonds, hydrophobic interactions, and other interactions, affect the protein gelation in the preserved egg white.

3.4. Change of intermolecular forces in PEW gel during pickling

To measure the intermolecular forces maintaining the protein gel structure and to determine the formation mechanism of the protein gel, PEW gels that had been cured for different time periods were treated with denaturants to break the intermolecular forces. These denaturants included 0.6 mol/L NaCl (ionic bonds), 1.5 mol/L urea (hydrogen bonds), 8 mol/L urea (hydrogen bonds and hydrophobic interactions), and 0.5 mol/L β-mercaptoethanol (disulfide bonds). The different denaturants were used in various combinations to measure protein solubility to determine the roles of each type of force (i.e., ionic bonds, hydrogen bonds, hydrophobic interactions, disulfide bonds, and non-disulfide covalent bonds) in the formation of the protein gels (Pérez-Mateos et al., 1997).

As shown in Fig. 4, the protein solubility distributions in the various solvents were significantly different and dependent on the curing duration ($P < 0.05$). The highest protein solubility was observed primarily in the S1 and S4 solvents at approximately 40%. Following this distribution, 15% and 5% solubility values were observed when the S3 and S2 solvents were used, respectively. The results suggested that the main intermolecular forces in the protein gels were ionic bonds and disulfide bonds and, to lesser extents, hydrophobic interactions and relatively few hydrogen bonds. Moreover, no insolubilities were observed, indicating that no other forms of covalent bonds were present. Furthermore, the main intermolecular forces vary with the types of protein gel. For example, in fermented surimi, the gel is maintained primarily by hydrophobic interactions and non-sulphur-based covalent bonds (Hwang et al., 2007; Pérez-Mateos et al., 1997; Xu, Xia, Yang, & Nie, 2010). Thermally induced egg white protein gels are dominated by hydrophobic interactions and disulfide bonds (Croguennec, Nau, & Brule, 2002; Totosaus et al., 2002). PEW gels have numerous ionic bonds and a certain number of disulfide bonds likely due to the hydrophilic environment established in strong alkaline solution.

With curing time, the solubility of the gel in S1 remarkably decreased in the first 8 days ($P < 0.05$), showing a sharp reduction in the proportion of ionic bonds during this period. Under natural conditions, fresh egg white protein is soluble in 0.6 M NaCl. However, the strong alkaline solution induces denaturation and gelation, which in turn decreases solubility and may be closely related to the bonding and aggregation of protein molecules during gelation, i.e., involving ionic bonding interactions with other molecules. The types of ionic bonds may include electrostatic attractions with zwitterions, "salt bridges", and "water-ionic bonds". However, because of the extremely alkaline environment, the protein molecule surfaces will have net negative charges, leading to electrostatic repulsive forces between groups. This degrades the stability of the proteins and may also prevent their aggregation (Ganasen & Benjakul, 2011). However, the added cupric ions somewhat weaken the repulsive forces of the surface negative charges on the protein molecules. Furthermore, the "salt bridges" and ionic bonds may promote gel structure stability and stabilise the gel system.

The preserved egg white proteins had low solubility values in the high-concentration urea solutions of S2 and S3. This indicated that hydrogen bonds and hydrophobic interactions were not the dominant forces that stabilised the PEW gel. No obvious changes were observed in the S2 solution with curing time, whereas a gradual increase in solubility ($P < 0.05$) was observed in the S3 solution. When a considerable amount of strong alkaline solution permeates into the egg, functional groups at the ends of protein polypeptide

chains may be ionised. Thus, hydrogen bonds and hydrophobic interactions are most likely disrupted. However, an osmotic pressure gradient due to the influx of alkaline solution into the egg white will enable the gel to retain a certain degree of moisture. The retained water promotes hydrogen bonding and affects other hydrophobic interactions.

The soluble fraction dissolved in the S4 solvent indicates the proportion of disulfide bonds in the PEW gel. This fraction increased in the first 24 days of curing time. The abundant number of free sulfhydryl groups and disulfide bonds in the egg white proteins and the strongly alkaline environment provided good conditions for the sulfhydryl oxidation reactions and the SH–SS exchange reactions (Mine, 1996; Monahan, German, & Kinsella, 1995). At this initial stage, the disulfide bonds were numerous and stable. The crosslinking among proteins and polypeptide chains through these disulfide bonds increased the average molecular weight of the gel system and contributed towards gelation (Totosaus et al., 2002). The balance of crosslinking bonds between self-crosslinking proteins and protein–solvent interactions determined the degrees of gel formation and stability (Matsudomi, Nakano, Soma, & Ochi, 2002; Van der Plancken et al., 2005; Veerman et al., 2003). As the PEW gel becomes stable, the molecular interactions in the gel system reach a certain balance. However, OH^- mobility decreases due to decreased water mobility in the gel. Thus, the destructive effects of the strong alkaline environment on the protein molecules will be weakened. For example, the exposure of the hydrophobic internal groups may enhance hydrophobic effects and drive the aggregation of protein molecules via disulfide bond interactions.

3.5. Change of protein component in PEW gel during pickling

As shown in an electrophoretogram in Fig. 5, the preserved egg total protein content and the protein components changed with curing time and with different denaturants. The total protein electrophoretogram is shown in Fig. 5(A). Fresh duck egg white had five main protein bands, i.e., ovomucin, ovotransferrin, ovalbumin, ovomucoid, and lysozyme, with molecular weights of 110, 76, 44.5, 28, and 14.3 kDa, respectively (Kaewmanee et al., 2011; Mine, 1995). Over an extended curing time, significant changes were observed. The bands corresponding to ovomucin, ovotransferrin, and ovomucoid entirely disappeared from the electrophoretogram within the first 8 days. The ovalbumin bands remained throughout the pickling process, but the band density gradually reduced. Because the ovomucin, ovotransferrin, and ovomucoid contents were already low in the egg white, these proteins gradually denatured and damaged in the alkaline solution. However, the dry weight of ovalbumin accounted for 54% of the egg white protein weight and approximately 40% of the egg white weight (Mine, 1995). This indicated that ovalbumin was not completely damaged by the strong alkaline solution; hence, the ovalbumin bands were clearly observed in the electrophoretogram. However, since the 4th day, unknown bands emerged in each lane of the separating gel. These bands indicated proteins with large molecular weights and may indicate the aggregation of egg white proteins during the gelation process. Similar results have also been reported in electrophoretograms of egg white gels induced by heat and pressure (Matsudomi et al., 2002).

To study the interactions among different proteins at the molecular level, electrophoretic samples (Fig. 5(B)) were prepared using the proteins dissolved in different denaturants. The band density distributions in the S1, S2, S3, and S4 profiles corresponded with the solubility values of their corresponding proteins. In the S1 solvent, only ovalbumin bands and high molecular weight proteins bands were observed on the separating gel. This indicated that ionic bonding interactions were primarily attributed with ovalbumin protein and that some hydrophilic polymers were dissolved in 0.6 M NaCl. In the S2 solvent, given the low protein solubility, only light-coloured bands were observed. Though a strong alkaline treatment denatured the egg white proteins and exposed internal hydrophobic groups, the exposed groups were prone to damage by high OH^- concentrations, leading to decreased protein hydrophobicity and corresponding increased hydrophilicity. Furthermore, the hydrogen bonds between the hydrophilic groups tended to diminish due to the influx of massive free OH^-. In the S3 solvent, no characteristic protein bands were observed, except

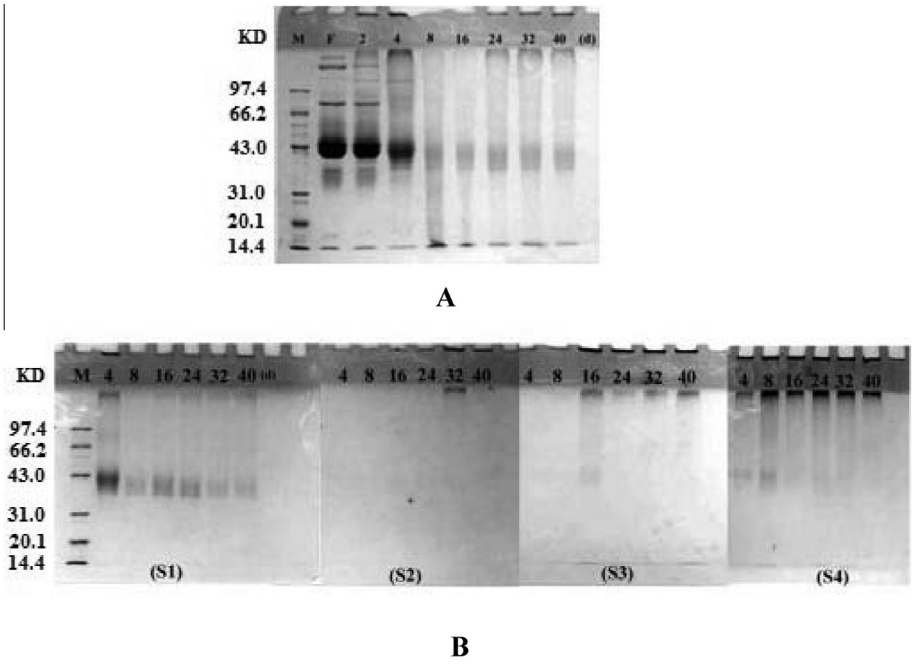

Fig. 5. SDS–PAGE patterns of PEW proteins extracted from different solutions. A: soluble fraction in Tris-HCl buffer (M: molecular weight standard; F: fresh egg white) B: PEW proteins extracted from S1, S2, S3, and S4, respectively.

for high molecular weight aggregates. Those aggregates resulted from hydrophobic interactions between protein molecules. The quantity of the aggregates increased with pickling time; this observation was consistent with the previous molecular interaction analyses. The water state in the gel system changed, and the water mobility decreased due to gelation. This further resulted in the decreased OH^- mobility and hence, prevented OH^- from disrupting hydrophobic interactions.

In the S4 electrophoretogram, the characteristic bands of ovalbumin and high molecular weight aggregates were observed. Notably, the band density of ovalbumin decreased with curing time. The density of the aggregate bands at the top of the separating gel gradually increased. This indicated that ovalbumin may be the main contributor of disulfide-linked large molecular weight aggregates. Ovalbumin provides a large number of free sulfhydryl groups and is involved in the sulfhydryl oxidation reaction and the SH–SS exchange reaction. The formation of disulfide bonds is necessary to maintain the network structure of the gel and may also promote the gelation of preserved egg white under alkaline treatments.

4. Conclusions

Preserved eggs were obtained by extended soaking in alkaline solutions. The observed egg whites were translucent, gelatinous, and had high water content. As shown by LF-NMR spectroscopy, the T2 value ranged from 15 to 300 ms. A 3D loose network structure of fine strands was observed under ESEM. A polymer gel was formed under non-thermal induction. By treating with an alkaline solution, the egg white proteins were denatured and subsequently, aggregated through crosslinking bonds. The proteins also interacted with solvent molecules, wherein ionic bonds, disulfide bonds, and hydrophobic interactions were involved. The contributions of ionic bonds and disulfide bonds accounted for approximately 40% of the protein aggregates. SDS–PAGE results showed that high molecular weight polymers were formed from egg white proteins via ionic bonds, disulfide bonds, and hydrophobic interactions. Furthermore, ovalbumin was found to be the main component of the protein aggregates. In conclusion, the characteristic gel of preserved eggs was based on multiple interactions between proteins and between proteins and solvent molecules.

Conflict of interests

The authors declare no conflicts of interest.

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