

Study on the mechanism of enhanced gel strength of heat-induced egg white by shikimic acid braising

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ABSTRACT This study evaluated the effects and mechanism of shikimic acid braising on the gelling characteristics of heat-induced egg white gel (HEWG). The results indicated that, during braising, soluble protein and hardness showed an overall increasing trend. The absolute Zeta potential value showed a decreasing trend; however, T₂ and free sulfhydryl group showed an increasing trend first and decreasing trend later, and surface hydrophobicity showed a decreasing trend. Microstructure analysis showed that protein gel aggregation increased and that holes and cracks formed first, and then the cracks decreased.

Key words: shikimic acid, egg white, braising, gelation, aggregation

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INTRODUCTION

Protein gelation is a phenomenon of protein molecule aggregation. In the process of protein aggregation, the forces of attraction and repulsion are balanced, thus forming a highly ordered three-dimensional network structure that can hold a large amount of water. Gelation is an extremely important feature of proteins and is widely used in industrial production, such as the development of new gelled foods, food preservation, and as a filling agent. Therefore, protein gelation research and development have always been a relevant topic for stakeholders. Egg white, as a food with a solid protein content of more than 90%, is one of the favorite foods of consumers. Egg white is rich in ovalbumin (54%, dry-mass basis), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), lysozyme (3.4%), and other proteins

Fourier transform infrared spectrometry showed that shikimic acid could strengthen the polarity of HEWG, and a mutual transformation occurred between intramolecular β -sheets, intermolecular β -sheets, and intermolecular antiparallel β -sheets, as well as a slight blue-shift, in the α -helices. In general, the addition of shikimic acid could alter the HEWG structure and improve its gel strength, polarity, and aggregation. Moreover, the higher the concentration of shikimic acid, the greater the influence on HEWG. Therefore, shikimic acid could be used as a new type of gel enhancer for the modification of egg white gel.

(Xue et al., 2021), providing a sufficient material basis for egg white gelation. Therefore, eggs have been seriously considered by protein gel scholars.

Research on the modification of egg white gelation mainly includes the addition of other substances or modification of physical conditions. The addition of salt or sucrose to egg white can improve the springiness and hardness of egg white gel (EWG) (after heat induction); salt decreases the water holding capacity (WHC) of EWG, while sucrose slightly improves the WHC of EWG (Khemakhem et al., 2019). The three-dimensional network structure of EWG became more regular, smaller, and denser during alkali pickling (Zhao et al., 2016). Moreover, some scholars (Zhou et al., 2019b) studied the combination of modified egg white protein with other gels to improve the gel structure. The addition of salted egg white powder to sardine surimi improved the breaking force, hardness, chewiness, and springiness, and reduced the expressible moisture content of surimi gel (Quan and Benjakul, 2019). Moreover, egg white modified with tea polyphenols was able to reduce the strength of the myosin heavy chain of surimi by crosslinking with surimi protein, thereby enhancing the gel strength of surimi (Zhou et al., 2019b).

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Marinated eggs are a type of traditional Chinese egg product that is made by boiling eggs (after removing the shell) at a high temperature for a long time (Xue et al., 2020). In our previous study (Xue et al., 2020), it was found that the gel strength of marinated egg white was enhanced after braising. Moreover, by braising an egg with star anise (*illicium verum*), one of the main raw materials in the marinade, heat-induced egg white gel (HEWG) strength could also be improved (Xue et al., 2021). Generally speaking, it is difficult to improve the gel strength of HEWG as it has low plasticity, which limits the development and utilization of HEGW. To determine which substances in *illicium verum* acted, the main components of water-soluble substances in *illicium verum* were chosen to treat HEWG. After treating HEWG with shikimic acid and *illicium verum*, the results are similar, showing the formation of a tight, highly reinforced gel structure with a frosting when touched. Shikimic acid is an active material with 1 carboxyl group and 3 hydroxyl groups. Its commercial production methods mainly include fermentation and extraction methods (the raw material is *illicium verum*) (Ramazani et al., 2021). Shikimic acid is the key ingredient in the production of the antifu drug “Tamiflu” (Ramazani et al., 2021), and was widely used in medicine because of its insecticidal, antimicrobial, sedative, antioxidant, antifu, and anticonvulsant properties (Yang et al., 2021). However, there is little research on the effects of shikimic acid on protein gelling. Therefore, the effects of shikimic acid on the gel characteristics of HEWG were studied. This study could not only support the theories behind the formation mechanism of marinated eggs but also provide a new way of protein modification using shikimic acid.

MATERIALS AND METHODS

Materials

Fresh eggs weighing approximately 50 to 60 g were purchased from a local supermarket in Nanchang, China. Shikimic acid (purity $\geq 98\%$) and potassium bromide (KBr, spectroscopic grade) were obtained from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Sodium chloride (NaCl), acetic acid, hydrochloric acid (HCl), ethanol, and sodium hydroxide (NaOH) were obtained from Tianjin Damao Reagent Company (Tianjin, China). Moreover, 5',5-Dithiobis (2-nitrobenzoic acid) (DTNB), sodium 8-Anilino-1-naphthalenesulfonate (ANS), and coomassie brilliant blue G250 were obtained from Aladdin (Shanghai, China). Glycine (Gly), ethylene diamine tetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (Tris), 2.5 % glutaraldehyde, PBS buffer (0.05 M, pH = 7.2), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample preparation kits with a low molecular weight marker (11–180 kDa), were all obtained from Solarbio (Beijing, China). The BCA assay kit was obtained from Bio-Bioengineering Co., Ltd., (Shanghai, China).

Gel Preparation

First, fresh eggs were cleaned with water and boiled (100°C) for 10 min, then the HEWG was obtained after the shell was removed. Then, according to a previous research design (Xue et al., 2021), each HEWG sample was mixed with 0.2 g (low concentration, L), 0.7 g (medium concentration, M), or 1.2 g (high concentration, H) of shikimic acid, and braised in an HH-6 water bath kettle (Shanghai Lichen Bangxi Instrument Technology Co., Ltd., Shanghai, China) at 100°C for 1.5, 2, 2.5, and 3 h, and more than three eggs were used for each time and concentration. More than 39 eggs were used per experiment, $39 = (3 \times 4 + 1) \times 3$, where 3 represents the three braising concentrations, 4 represents the four braising times, 1 is the blank control (HEWG without braising), and the last 3 indicates that the experiment was repeated 3 times; afterward. The sample was removed and equilibrated to room temperature for further use.

Determination of Physicochemical Characteristics

Determination of pH Values The pH was measured according to Chinese standard GB/T 5009.47-2003 (China, 2003), and the procedure was performed as follows: 3 boiled eggs were selected and the yolks were removed; then, the BEWGs were stirred with a blender (Joyoung Co., Ltd., Joyoung JYL-C012, Jinan, China). Each sample was homogenized with distilled water (2:1, w/w). Then, 15 g of the mixture was taken, the volume was diluted to 150 mL with distilled water, and the sample was filtered through a double-layer gauze. Finally, the pH measurement was then determined using a pH meter (Hangzhou Lei Magnetic Analysis Instrument Factory, Lei Magnetic PHS-25, China).

Determination of Zeta Potential First, 3 g of the stirred sample was dissolved in 27 mL of UPC-1-10T ultrapure water (Sichuan Ulupure Ultrapure Technology Co., Ltd., Sichuan, China), and then the mixture was homogenized using the T-18 digital homogenizer (German IKA Company, Staufen, German) at 12,000 r/min for 2 min, and the supernatant was diluted ten times after the homogenate was centrifuged in a Model TGL-20B centrifuge (Anke, Shanghai, China) at 8,000 r/min for 20 min. The following methods were used with the same centrifugation and homogenization settings, except for 2.8. Finally, the Zeta potential was determined using a Zetasizer Nano Z ZEN2600 instrument (Malvern Instruments Ltd., Malvern, UK) at 25°C.

Soluble Protein Content Measurement Soluble protein content was measured according to Xu et al. (2018) with some modifications. Nine mL of PBS buffer (0.05 M, pH = 7.2) was mixed with 1 g of the stirred sample, and then homogenized and centrifuged. The supernatant was diluted to a suitable concentration and measured with the Thermo K3 enzyme labeling instrument (Thermo Fisher Scientific, Shanghai, China) with a BCA assay kit.

Low-Field Nuclear Magnetic Resonance Measurement The relaxation time (T_2) was determined by a Niumag Low-Field (LF) pulsed Nuclear Magnetic Resonance (NMR) Analyzer (Niumag Co., Ltd., Shanghai, China) following the method described by Shao, et al. (2016) with slight modifications. The experimental conditions were as follows. Weight of the stirred sample, 2 g; sampling interval, 1000.000 ms; echo number, 220, and scanning repetitions, 4.

Environmental Scanning Electron Microscopy The microstructure was determined by the method described by Kaewmanee et al. (2013) with some modifications. Briefly, the gel samples were fixed in glutaraldehyde (2.5%) overnight. Then, the samples were repeatedly rinsed with PBS (0.05 M, pH = 7.2), and gradient dehydrated using ethanol series (60–100 %), and then the samples were completely freeze-dried using the LGJ-18B vacuum freeze dryer (Beijing Yatai Cologne Instrument Technology Co., Ltd., Beijing, China). The dried samples were observed at 2,500 × magnification on a Quanta-200F environmental scanning electron microscopy (ESEM) (FEI, Ltd., Hillsboro, OR) after being sputter-coated with gold.

Texture Analysis The texture was examined by using a BROOKFIELD CT3 instrument (Brookfield, Stoughton, MA) according to the method of Bourne (1978) with some modifications. The gel sample was cut into a cube measuring approximately $0.5 \times 1 \times 1 \text{ cm}^3$, and the experimental conditions were as follows: compression ratio, 60% at the post-recording speed; recording speed, 2.0 mm/s, and pre-recording speed, 5.0 mm/s.

Free Sulfhydryl Contents Measurement The contents of free sulfhydryl groups were determined according to methods previously described by Beveridge et al., (1974) with some modifications. Briefly, 3 g of the stirred sample was homogenized with 27 mL of PBS buffer (pH = 8.0, 0.1 M) and centrifuged. The protein concentration of the supernatant was determined by BCA methods.

The supernatant (0.2 mL) was added to 2.8 mL of the Tris-Gly buffer treatment solution (0.1 M Gly, 4 mM EDTA, 0.1 M Tris, pH = 8) and mixed with 20 ml of Ellman's reagent (4 mg/mL DTNB dissolved in Tris-Gly buffer). The mixture was then incubated at 40°C for 40 min and analyzed at 412 nm using an enzyme labeling instrument. PBS buffer was used instead of the sample as a blank. The free sulfhydryl content of the sample was calculated as follows: $\mu\text{M SH/g pro} = A_{412} \times 73.53 \times D/C$, where A_{412} is the absorbance of the sample at 412 nm, D is the dilution factor (15.1), and C is the protein concentration of the supernatant (mg/mL).

Surface Hydrophobicity Measurement The surface hydrophobicity was determined according to methods previously described by Chang et al. (2016) with some modifications. The stirred sample was diluted 10 times with PBS buffer; after homogenization and centrifugation, the supernatant was analyzed using the BCA method. Then, the protein concentration of the supernatant was diluted with PBS buffer to 0.3 mg/mL. Afterward, 40 μL of the ANS solution (dissolved with alcohol, 1 mM) was added to 2 mL of the sample. The

surface hydrophobicity was recorded with the Hitachi F-7000 fluorescence spectrofluorometer (Hitachi, Tokyo, Japan). The experimental parameters were as follows: slit width, 5 nm; emission wavelength, 485 nm, and excitation wavelength, 380 nm.

SDS-PAGE The molecular weight distributions were measured by SDS-PAGE according to the method previously described by Laemmli (1970) with slight modifications. The stirred sample was diluted 10 times with Tris-HCl (10%, pH = 8.8). After homogenization and centrifugation, the sample was mixed with loading buffer and heated in a boiling water bath for 3 to 5 min. The sample was added to a gel mixture (5% stacking gel and 10% resolving gel) and analyzed at 100 mV in the stacking gel and at 120 mV in the resolving gel with the Mini Bio-Rad vertical electrophoresis unit (Bio-Rad Co., Ltd., Hercules, CA) to completion. Finally, the electrophoresis gel was stained with Coomassie Brilliant Blue G250 and decolorized with 7.5% acetic acid and 5% ethanol.

Fourier Transform Infrared Spectrometry Analysis The stirred sample was completely frozen in an ultra-low temperature refrigerator and the freeze-dried powders of the stirred sample (1 mg) was mixed with KBr (100 mg) and compressed into thin films, according to the method of Yang et al. (2019). with some modifications. Fourier transform infrared spectrometry (FTIR) analysis of the samples was measured by using a Thermo Nicolet iS 5 FTIR (Thermo Scientific Nicolet Corporation, Waltham, MA) from 400 to 4,000 cm^{-1} at 25°C, and the automatic signals at a resolution of 4 cm^{-1} , collected in 64 scans. The secondary structure of the amide I region of the protein (1,600–1,700 cm^{-1}) was processed using the OMNIC software.

Statistical Analysis

All measurements were performed 3 times (except texture measurements, which were performed six times) and Origin 8.5 software (Originlab Corporation, Northampton, MA) was used to process the results expressed as means \pm standard deviations. Duncan's multiple range tests and one-way ANOVA were performed. Bivariate and correlation tests were chosen to analyze the relevant data using SPSS Statistics 19.0 software (IBM, Chicago, IL). Differences with a P -value of <0.05 were expressed as significant.

RESULTS AND DISCUSSION

Effects of Shikimic Acid Braising on Physicochemical Characteristics of HEWG

The physicochemical characteristics are important indices of the protein gel, which could directly or indirectly affect the protein properties, thereby affecting the quality and properties of the protein gel Figure 1.A shows the effects of shikimic acid braising on the pH of HEWG. During braising, the pH values of H showed an overall decreasing trend ($P < 0.05$), while those of both

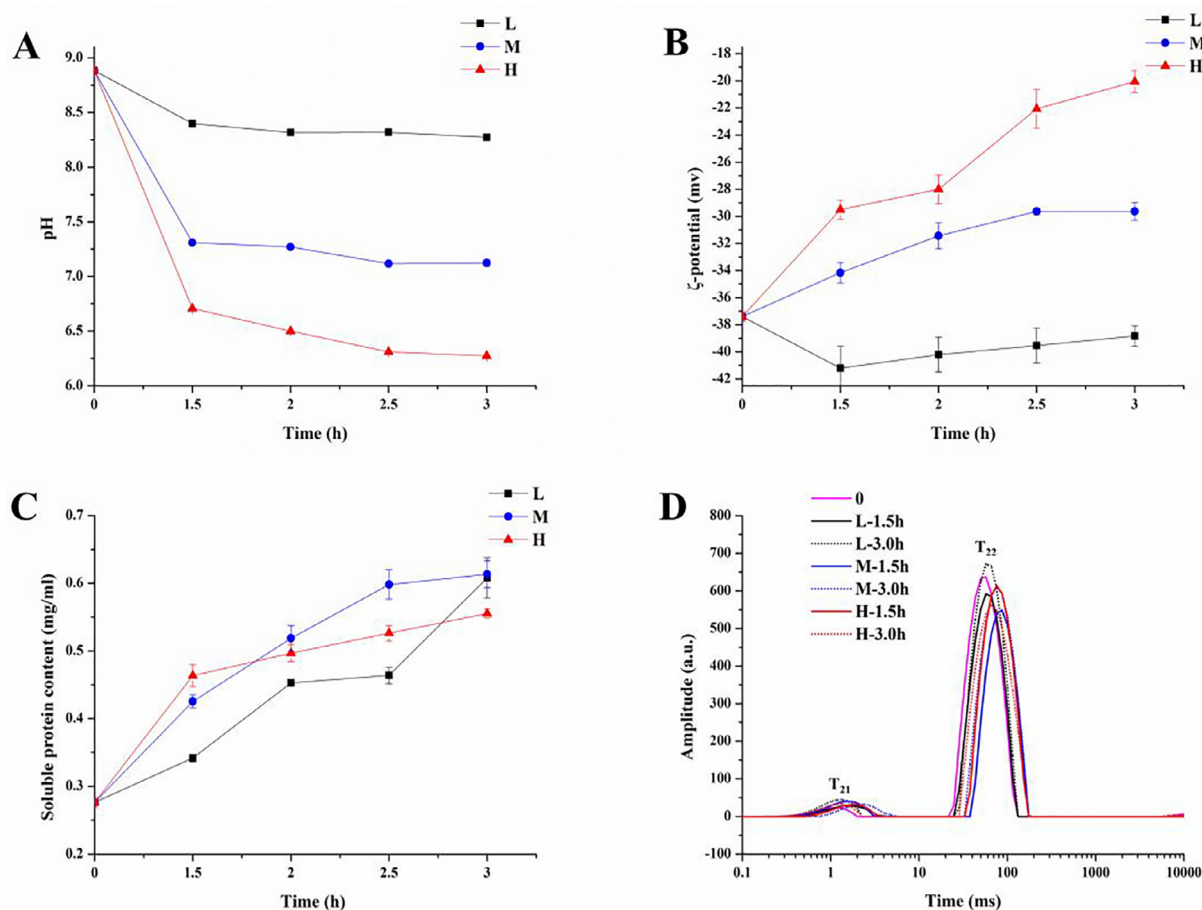


Figure 1. Effects of shikimic acid braising on physicochemical characteristics of HEWG (A, pH; B, zeta potential; C, solution protein content; D, T_2). Abbreviation: HEWG, heat-induced egg white gel.

L and M first decrease ($P < 0.05$) and then remain nearly unchanged. The reduction was due to shikimic acid entering the HEWG during braising, thus reducing the pH value of L, M, and H. The pH values of L and M remained essentially unchanged in the late processing stage, probably because the content of shikimic acid was too low, and it was consumed in the early stage. In addition, as the pH value of the sample was greater than the PI (pH = 4.5) of the main protein (ovalbumin), the protein carried a negative charge during this process.

The Zeta potential could express the forces of attraction or repulsion between particles, which is related to the stability of gel or colloidal dispersion systems, and the positive or negative symbols represent the charge of the particles. When the absolute value of the Zeta potential increases, this indicates that the gel molecules or dispersed particles are more stable. As shown in Figure 1B, the Zeta potential of the protein was negative, which could be caused by the presence of many anionic amino acid residues (aspartic acid, glutamic acid, etc.) on the protein surface (Cao et al., 2020). The consistencies of the resulting pH values are shown in Figure 1A. With the addition of shikimic acid, the absolute value of the Zeta potential showed a decreasing trend, indicating that the anionic amino acid residues in the protein were bound to the positively charged H^+ ion (water ionization of shikimic acid) in the solution. In addition, shikimic acid could change the secondary or

tertiary structures of proteins under the heat treatment and would result in the exposure of the positively charged amino acids on the protein surface, which is consistent with the results of the pH shift treatment of peanut protein isolate (Li et al., 2020). The decreased absolute value of the Zeta potential indicated that the attraction between the proteins overcame the electrostatic repulsion; therefore, the proteins tended to aggregate.

The soluble protein content is an important factor in the determination of the physicochemical properties of proteins and it is a major indicator of functional properties, such as emulsifiability, foaming and gelation (Zhong and Xiong, 2020). As shown in Figure 1C, the soluble protein content of L, M, and H shows an increasing trend, while the soluble protein content of M was higher than that of H. Through the analysis, it was found that the increase in soluble protein content was due to the interaction between shikimic acid and proteins, and the protein structure changed under high temperature, which made some proteins become water-soluble substances (Xue et al., 2021). However, according to the Zeta potential results (Figure 1B), the high concentration of shikimic acid could greatly reduce the net charge of proteins, thus inhibiting the increased soluble protein content.

LF-NMR has been widely used to evaluate the distribution and mobility of different fractions of water

molecules in a gel system (Xiao et al., 2020); the smaller the degree of freedom or the larger the binding force, the smaller the T_2 value. Figure 1.D shows the effect of shikimic acid braising on the T_2 of HEWG. There were only 2 independent peaks (0.3–6 ms and 20–200 ms) in the whole T_2 curve, named T_{21} (bound water) and T_{22} (immobilized water), meaning that the water and protein molecules were tightly bound, and the protein structure was stable. With the addition of shikimic acid, the position of T_{21} and T_{22} of the sample first shifted toward the long relaxation time direction and then shifted toward the short relaxation time direction, meaning that the water binding ability of the sample first decreased and then increased. This was probably because shikimic acid has a very strong polarity. At the beginning of the reaction, shikimic acid broke the stable structure of the protein by binding to the proteins, which changed the tertiary or secondary structures of the proteins, thus reducing the water binding ability of the protein by hydrogen bonding. After the stable combination of shikimic acid and protein, shikimic acid was able to firmly bind water molecules through extremely strong polarity. In conclusion, the addition of shikimic acid could decrease the pH and net charge, and change the water binding ability by changing the protein structure.

Effects of Shikimic Acid Braising on Microstructure of HEWG

The effects of shikimic acid braising on the microstructure of HEWG are depicted in Figure 2. At 0 h, HEWG showed a homogeneous and rigid network structure formed by the accumulation of granular materials, being similar to the results of previous research (Zhang et al., 2019). With the addition of shikimic acid, the microstructure of L, M, and H exhibited cracking (indicated by the red arrow) at 1.5 h. However, the cracks were significantly reduced after braising for 3 h. With the increase in the concentration of shikimic acid, the gel structure of the sample formed some holes along with a large aggregate, which was similar to the results of the *illicium verum* treatment with HEWG (Xue et al., 2021). Moreover, from a macro perspective, the frosted HEWG might be since the gel formed some holes along with a large aggregate. The reason for this could be that the protein bond was first broken when the shikimic acid started to react with the proteins, and the secondary or tertiary structures of the proteins were changed, forming some cracks; however, with increasing reaction time, the broken bonds re-linked and formed aggregates under the action of shikimic acid. Therefore, the cracks were significantly reduced, which was similar to the results of T_2 (Figure 1D). Conversely, the absolute value of the Zeta potential of M and H decreased rapidly ($P < 0.05$; Figure 1B), the electrostatic repulsion of proteins was significantly reduced, and protein aggregation increased, while the absolute value of the Zeta potential of L was much smaller than that of M and H. Thus, the aggregation degree of L was lower than that of M and H. The

network structure of the HEWG could have been destroyed and then some holes could have been formed under the high-temperature treatment, and it was the formation of these holes that could have caused the decrease in T_2 . Furthermore, many granular aggregates were observed in L, M, and H, which could be the result of the combination of shikimic acid with proteins.

Effects of Shikimic Acid Braising on Texture Profile Analysis of HEWG

Texture profile analysis (TPA) can directly reflect the properties and quality of protein gels. It is a comprehensive expression of the interaction between all protein bonds (including ionic bonds, hydrogen bonds, and disulfide bonds). Figure 3. shows the effects of shikimic acid braising on the HEWG TPA results. In this study, 2 typical parameters of hardness and springiness were chosen to explore the effects of shikimic acid on HEWG texture.

As shown in Figure 3A, during braising, the hardness values of L, M, and H increase ($P < 0.05$) from 1,526.12 g to 1,902.25 g, 2,214.50 g, and 2,549.69 g, respectively. Interestingly, egg white protein formed a strong and stable gel structure under high-temperature induction. If HEWG was subjected to a prolonged heat treatment, the stable structure of the protein gel would be destroyed (Xue et al., 2020). However, HEWG hardness could be increased by adding a small amount of shikimic acid to the water, indicating that shikimic acid is a potential protein gel enhancer. This increase in hardness could be attributed to 2 factors. Conversely, the carboxyl groups of shikimic acid could have reacted with the amino groups of the side chains of proteins, and the hydroxyl groups of shikimic acid could have combined the polar groups (such as hydroxyl and carboxyl groups) of different proteins via hydrogen bonding, compacting the protein gel structure and increasing the protein aggregation degree, further forming a rigid and stable gel structure. In addition, the attraction between proteins was due to the reduction of electrostatic repulsion between proteins, thus forming more macromolecular aggregates, and the aggregation of protein macromolecules was conducive to the strengthening of the gel structure (Xue et al., 2020). Conversely, shikimic acid could have broken the original protein structure and changed the secondary or tertiary structures of proteins. Probably, the molecules rearranged to adapt to environmental changes, and the protein gel was transformed into a more stable structure, increasing the hardness of HEWG..

Springiness refers to the extent to which the gel recovers its original shape after being squeezed (Zhang et al., 2019). The effects of shikimic acid braising on the springiness of HEWG are shown in Figure 3B. The springiness of L and M remained essentially constant ($P > 0.05$), but that of H decreased slightly ($P < 0.05$) in the last stage of braising. To some extent, in industrial processing, the decrease in springiness would reduce the quality of protein gels. The decreased springiness of H could be due to the formation of rough texture and uneven gel

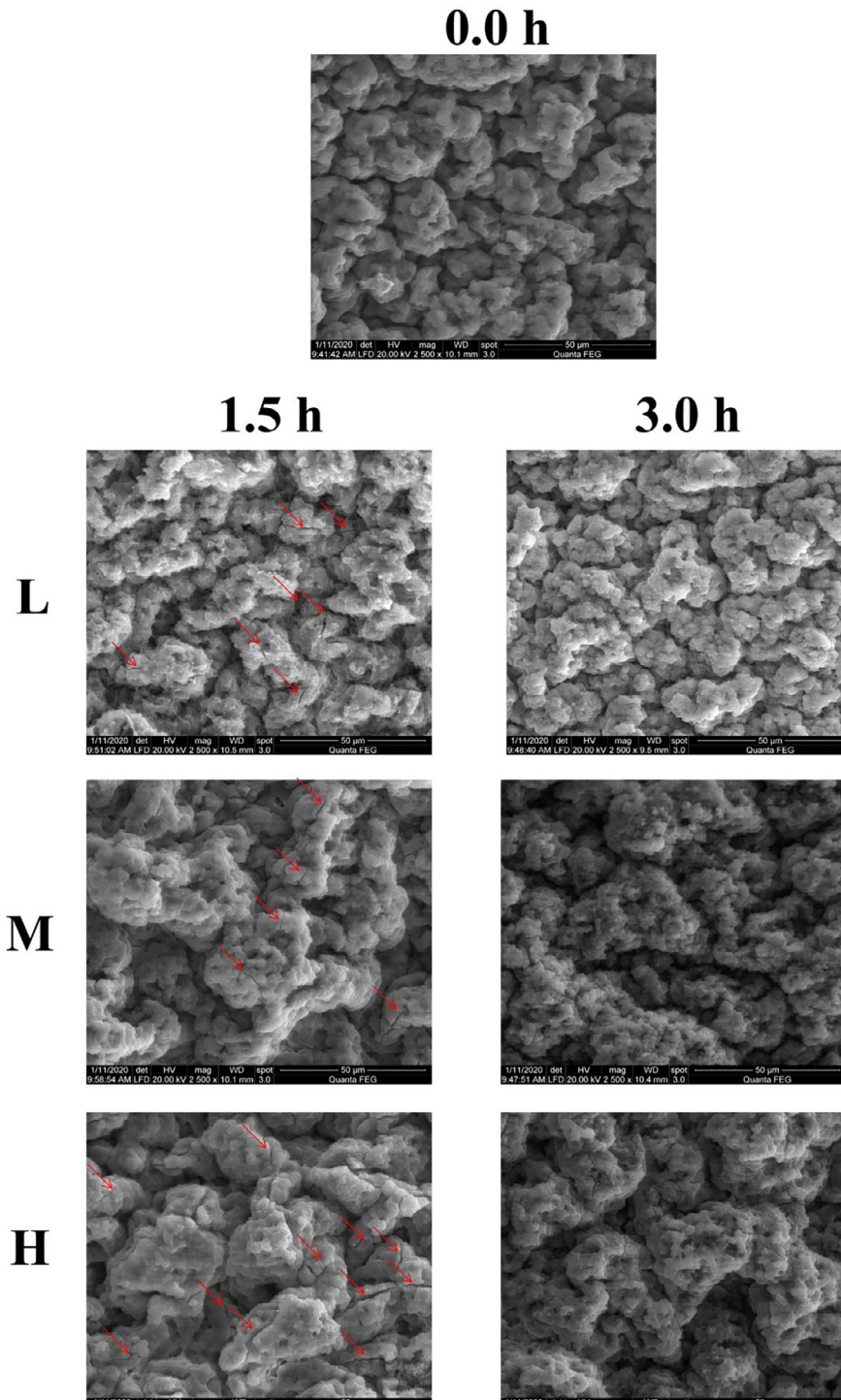


Figure 2. Effects of shikimic acid braising on microstructure of HEWG. Abbreviation: HEWG, heat-induced egg white gel.

network structure. In addition, a too tight and rigid gel structure may reduce the springiness of H. This was similar to the results of *illicium verum* treatment with HEWG (Xue et al., 2021).

Effects of Shikimic Acid Braising on Free Sulfhydryl Contents of HEWG

In protein molecules, the sulfhydryl group is one of the most active groups. Two sulfhydryl groups are converted into disulfide bonds under oxidation conditions

(Zhong et al., 2020), and disulfide bonds promote protein gelation. As shown in Figure 4, the free sulfhydryl contents of L, M, and H show an increasing trend first and then a decreasing one, with the decreasing trend in L being more significant. The increase in the number of free sulfhydryl groups could be related to the change of the protein gel structure. From ESEM (Figure 2), it can be observed that the gel structure of HEWG was destroyed at 1.5 h, forming some cracks, and leading to the exposure and release of the free sulfhydryl group. With continuous braising, on the one hand, the exposed free sulfhydryl group was reincorporated into the

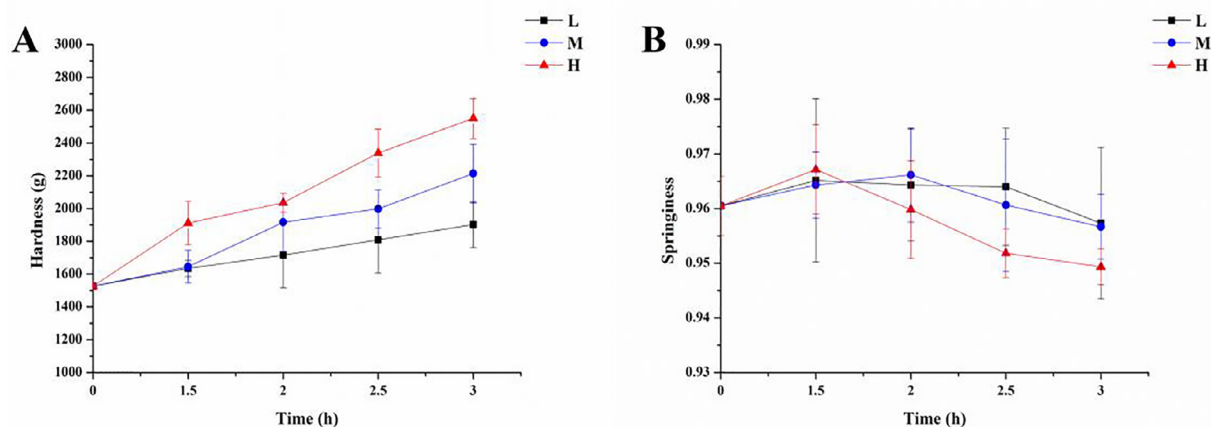


Figure 3. Effects of shikimic acid braising on TPA of HEWG (A, Hardness; B, springiness). Abbreviations: HEWG, heat-induced egg white gel; TPA, texture profile analysis.

aggregates, forming larger aggregates. On the other hand, the conformation of the protein gel structure changed during heating, and some exposed free sulfhydryl groups participated in the sulfhydryl-disulfide bond exchange reaction or sulfhydryl oxidation to form new disulfide bonds (Mine et al., 1990). The conversion of sulfhydryl to disulfide bonds played an important role in the cross-linking and aggregation of protein molecules and played an active role in the formation of stable gel structures (Wu et al., 2019). Moreover, the free sulfhydryl group of M and H slightly decreased during the later stage of braising, probably because the excessive concentration of shikimic acid could have inhibited the conversion of sulfhydryl to disulfide bonds. Therefore, it is hypothesized that shikimic acid might not improve the gel strength of HEWG mainly by generating disulfide bonds.

Effects of Shikimic Acid Braising on Surface Hydrophobicity of HEWG

Fluorescence spectroscopy was used to analyze protein structure through the reaction of exogenous fluorescent probe (ANS) and endogenous fluorescent group (tryptophan) to determine the surface hydrophobicity of proteins. The surface hydrophobicity affected the stability of proteins by affecting the cross-linking and aggregation of protein molecules (Zhou et al., 2019a). As shown in Figure 5, with the increase in braising time and shikimic acid concentration, the surface hydrophobicity shows a stepwise increase and H with a slight red-shift. Under the joint action of shikimic acid and high temperature, protein aggregation contributed to the decrease in surface hydrophobicity. The decrease in surface hydrophobicity indicated that the hydrophobic groups were

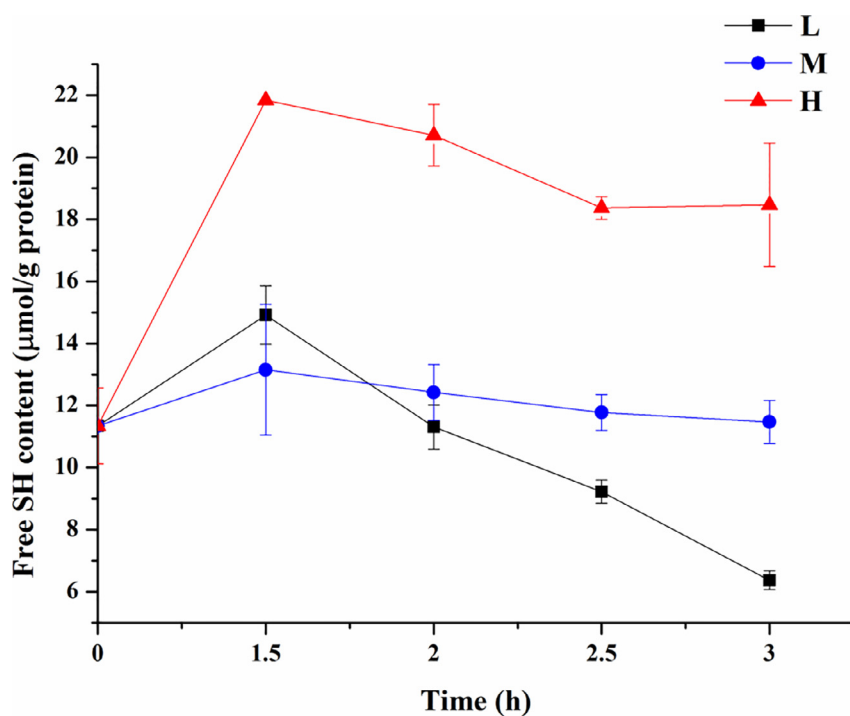


Figure 4. Effects of shikimic acid braising on free sulfhydryl content of HEWG. Abbreviation: HEWG, heat-induced egg white gel.

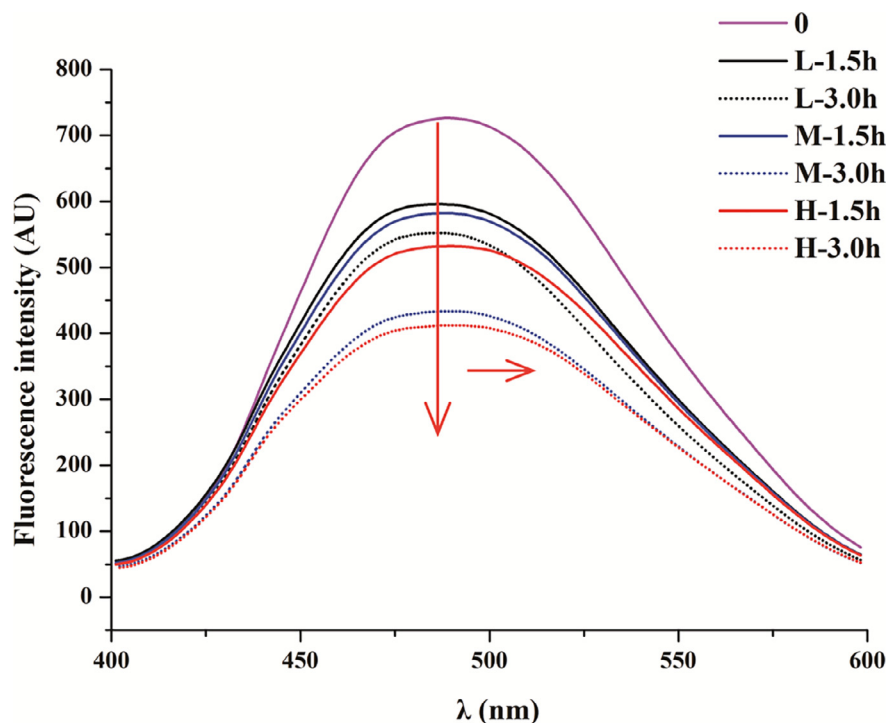


Figure 5. Effects of shikimic acid braising on surface hydrophobicity of HEWG. Abbreviation: HEWG, heat-induced egg white gel.

wrapped in the protein gel (Xue et al., 2022). From Zeta potential (Figure 1B) and ESEM (Figure 2), it was also found that the aggregation of the protein gel increased and formed a larger aggregate, which prevented the exposure of the hydrophobic groups. The red-shift in H suggested exposure of the tryptophan residues to the polar microenvironment (Zhou et al., 2019a), and this result could have been due to the dual action of shikimic acid and heat, causing HEWG unfolding and the tryptophan residues to be exposed to the external solution. However, the further exposure of tryptophan residues did not increase the fluorescence intensity of H, which may be due to the changed protein properties by the high concentration of shikimic acid, resulting in the decreased fluorescence quantum yield, which was similar to the results of the fried you-tiao (Zhou et al., 2019a). Conversely, shikimic acid itself was a strong polarity matter, and the high reaction concentration of shikimic acid with protein would increase the polarity of protein, leading to the Stokes displacement.

Effects of Shikimic Acid Braising on SDS-PAGE of HEWG

To further study the effect of shikimic acid on the gel characteristics of HEWG, the protein composition of the samples was analyzed by SDS-PAGE. As shown in an electropherogram in Figure 6, the following protein bands were divided into ovomucin (110 kDa), ovotransferrin (70 kDa), ovoglobulin (48 kDa), and ovalbumin (38 kDa) (Xue et al., 2021). Among them, ovalbumin was the main protein (Xue et al., 2021). During braising, the molecular weight of ovalbumin and ovotransferrin were lower than that of the natural state (45 kDa and 76

kDa), which was similar to our previous research reports (Xue et al., 2020, 2021). In addition, similar results were also found by Katekhong and Charoenrein (2016). It was speculated that egg white protein would reduce the molecular weight of proteins under heat-induced denaturation. With the increase in the concentration of shikimic acid, the bands of ovalbumin and ovotransferrin remained essentially unchanged, but the band of ovoglobulin was significantly deepened, indicating that shikimic acid could protect ovoglobulin from damage caused by high temperature. In addition, large amounts of cross-linked proteins appeared on the top of the electropherogram lane, and the band of cross-linked proteins deepened significantly with the increase of shikimic acid concentration, especially in H, which was caused by protein-protein or protein-shikimic acid-protein aggregations. This was similar to the results of surface hydrophobicity (Figure 5) and ESEM (Figure 2), and also proved that the addition of shikimic acid could aggravate gel aggregation.

Effects of Shikimic Acid Braising on FTIR of HEWG

Currently, although there are many analysis tools for protein conformation, FTIR remains a powerful technique for the study of protein internal interactions (Nasabi et al., 2017), and could be used to study protein groups at the molecular level and analyze protein conformational changes Figure 7. A shows the effects of shikimic acid braising on the original FTIR spectra of HEWG. The changes of the main peak of the sample were not evident, but some peaks showed an increase or a shift in trends. According to previous reports

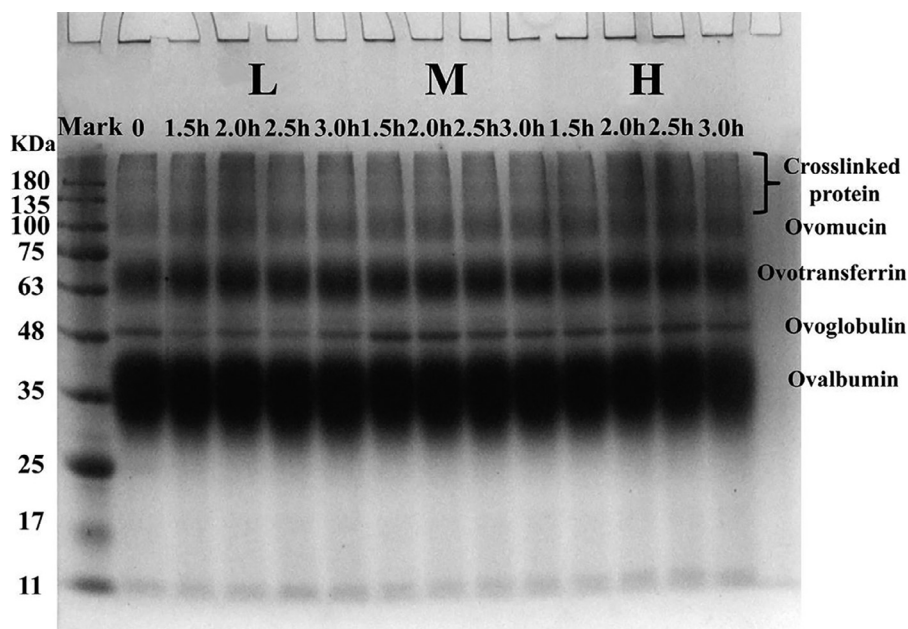


Figure 6. Effects of shikimic acid braising on SDS-PAGE of HEWG. Abbreviations: HEWG, heat-induced egg white gel; SDS-PAGE, sulfated polyacrylamide gel electrophoresis.

(Sow et al., 2019), the peak position at 3,200 to 3,400 cm^{-1} represented the O–H stretching vibration, which suggested that intramolecular and intermolecular hydrogen bonding existed in the sample. Moreover, with the addition of shikimic acid, the position of the peak slightly red-shifted, which might be due to the combination of shikimic acid and proteins to increase the polarity of the protein, because the formation of hydrogen bonds in proteins would reduce the stretching vibration frequency (Xue et al., 2022). In addition, the absorbance at 875 to 985 cm^{-1} was a band position sensitive to hydrogen bonding (Barth, 2007), and the addition of shikimic acid could significantly improve the absorbance of the absorption peak, a fact that again demonstrates that shikimic acid could improve the polarity of HEWG.

To further analyze the effects of shikimic acid braising on the gel properties of HEWG, the amide I bands (1,600–1,700 cm^{-1}) were studied to analyze the changes in the secondary structure of the protein gel

(Figure 7B). According to previous studies (Gao et al., 2020; Yang et al., 2020), the amide I bands were divided into 1,612 to 1,642 cm^{-1} (intramolecular β -sheets), 1,615 to 1,625 cm^{-1} (intermolecular β -sheets), 1,640 to 1,650 cm^{-1} (random coils), 1,651 to 1,660 cm^{-1} (α -helices), 1,661 to 1,690 cm^{-1} (β -turn), and 1690 to 1,700 cm^{-1} (intermolecular antiparallel β -sheets). When the braising time increased from 1.5 h to 3 h, the content of intermolecular β -sheets showed a decreasing trend first and then increased with the increase in the concentration of shikimic acid, while the trends of intermolecular antiparallel β -sheets and intramolecular β -sheets were the opposite. Through the previous analysis, we hypothesize that shikimic acid could break the intermolecular β -sheets of the gel structure at 1.5 h (this is the reason why many cracks were observed in ESEM) and decrease the levels of intermolecular β -sheets. With continuous braising, the protein was relinked (the number of cracks greatly decreased in ESEM), and the intermolecular

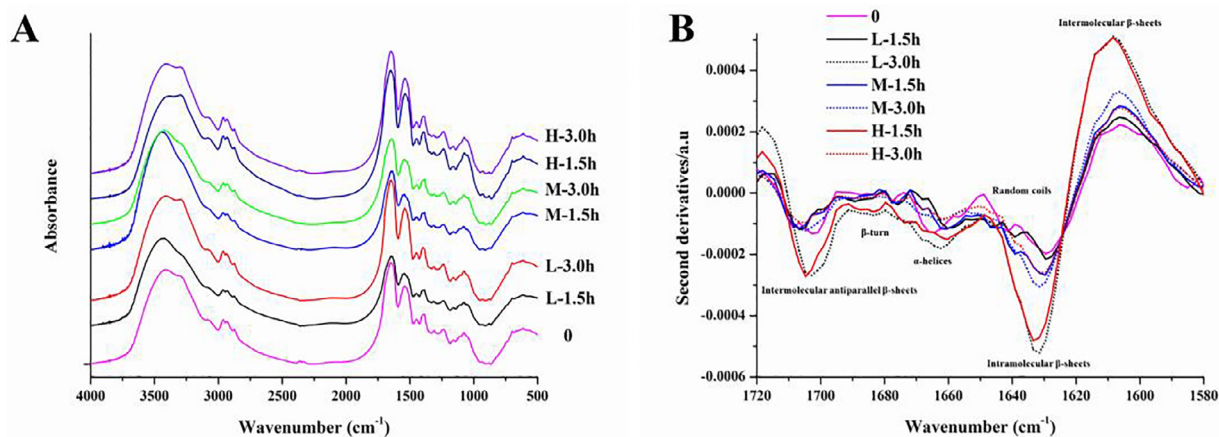


Figure 7. Effects of shikimic acid braising on FTIR of HEWG (A, Original spectra; B, second derivative spectra). Abbreviations: FTIR, Fourier transform infrared spectrometry; HEWG, heat-induced egg white gel.

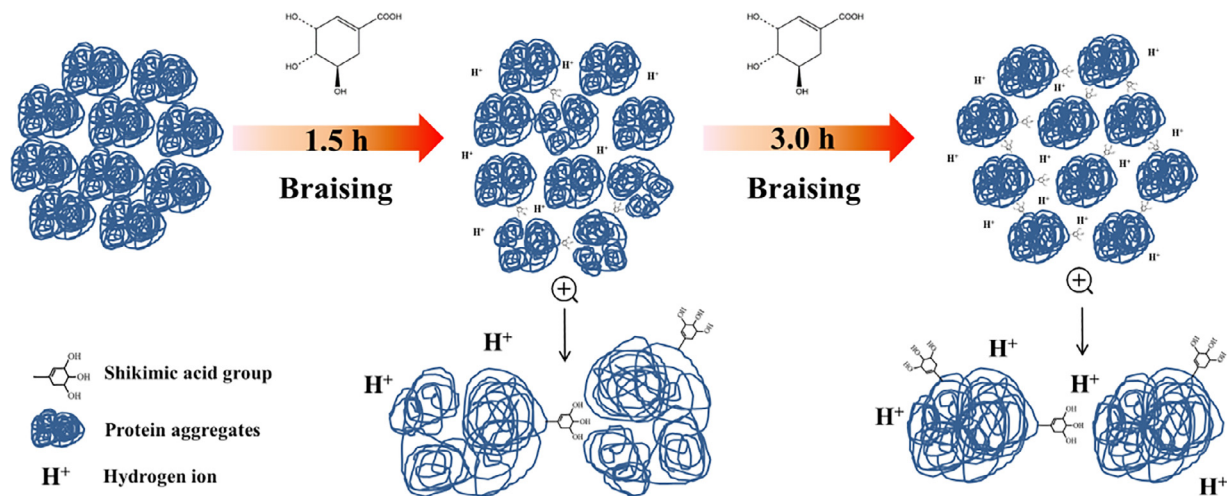


Figure 8. Schematic diagram of influence mechanism of shikimic acid on gel structure of HEWG. Abbreviation: HEWG, heat-induced egg white gel.

antiparallel β -sheets and intramolecular β -sheets transformed into intermolecular β -sheets, which could constitute a process of protein transformation into a more stable structure. Besides, they were found to remain essentially unchanged in random coils and β -turns. This could be because a stable structure formed by HEWG after treatment with shikimic acid could resist the change of the unstable structure. With the addition of shikimic acid, the position of the α -helices was slightly blue-shifted, which may be because the α -helices were stabilized by hydrogen bonds formed by the $-\text{CO}$ and NH^- groups of the polypeptide chain (Yu et al., 2019), and the increased polarity of the protein would affect the hydrogen bonding of the α -helices, thus enabling the α -helices to be blue-shifted. These results suggest that shikimic acid-treated HEWG had a relatively stable structure, and the addition of shikimic acid could change the secondary structure of proteins.

Schematic Mechanism

According to the above results, a possible mechanism for the effects of shikimic acid on HEWG was proposed, as described in Figure 8. The addition of shikimic acid made the solution acidic, the protein was surrounded by H^+ , and the net charge number of the protein decreased. After braising for 1.5 h, the shikimic acid began to react with the proteins and attracted the surrounding proteins through hydrogen bonds. The secondary and tertiary structures of the protein began to change and part of the chemical bonds were broken, thus exposing the free sulfhydryl groups. However, overall, the protein continued to aggregate, and after 3 h of braising, the aggregation of the protein gel increased and formed holes. A large quantity of shikimic acid was bound to the proteins. The polarity of the proteins increased rapidly and attracted other proteins through hydrogen bonding. This greatly enhanced the stability of the gel structure. Furthermore, the secondary and tertiary structures of the proteins gradually transformed into a more stable structure in

this process. Therefore, the gel strength of the protein increased considerably.

CONCLUSIONS

Egg white protein has good gel properties and can form a stable network gel structure after being heated. However, it is difficult to increase the gel strength of HEWG due to its poor plasticity. Interestingly, in this study, it was found that shikimic acid can greatly increase the gel strength of HEWG. With the increase in shikimic acid concentration, the pH values and the absolute value of the Zeta potential decreased gradually, thus increasing the aggregation of proteins and the hydrophobic group was re-wrapped, which was not conducive to the combination of proteins with water molecules by hydrogen bonds. However, a large amount of the shikimic acid combined with the proteins, thus increasing the polarity of the proteins and changing their conformation. The protein attracted other proteins through hydrogen bonds. The protein structure gradually changed to a more stable conformation in this process, greatly increasing the gel strength of the protein.

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DISCLOSURES

The authors declare no conflict of interest.

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