

Influence of heat treatment and egg matrix on the physicochemical and allergenic properties of egg custard

Ping Tong^{ID}, Liji Xiong, Jinyan Gao, Xin Li, Zhihua Wu, Anshu Yang, Juanli Yuan, Yong Wu, and Hongbing Chen^{ID}

Abstract: To investigate the influence of heat treatment and egg matrix on egg custard (EC) proteins, 12 different kinds of ECs with different egg/water ratios (1:1, 1:1.5, 1:2, or 1:3, v/v) and different heating temperatures (80, 90, or 100 °C) and times (10, 15, or 20 min) were prepared and evaluated for the digestibility, structure, eliciting capacity and sensitizing capacity using SDS-PAGE, fluorescence spectra, ELISA, and a BALB/c mouse model, respectively. The physicochemical properties of EC proteins were significantly affected by heat treatment and egg matrix, which showed the increased digestibility and partially unfolded structure. The eliciting capacity of EC evaluated by IgE binding to sera from egg-allergic patients was reduced after heat treatment, and the EC made by heating at 100 °C for 20 min with a whole egg/water ratio of 1:2 (v/v) was the weakest. The sensitizing capacity of EC was also reduced in the BALB/c mouse model, which showed the significantly decreased levels of specific IgE, IgG, IgG1 and IgG2a, mMCP-1 and histamine in the mouse sera, as well as cytokine secretions of IL-4, IL-5, and IL-13, compared with the raw egg (RE) group. Results demonstrate that heat treatment and egg matrix significantly reduced the eliciting and sensitizing capacity of EC by changing the tertiary structure and increasing the digestibility of EC proteins.

Keywords: allergenicity, egg allergy, egg custard, food matrix, heat treatment

Practical Application: Egg custard (EC) is one kind of savory food suitable for all ages, and is also a traditional supplementary food for infants and young children in China. However, limited information is available on the allergenicity of egg custard. In this work, we evaluated how the structure, digestibility, and allergenic potential of egg allergens in EC were altered by the degree of thermal treatment and egg matrix, and elucidated the links between the physicochemical properties and allergenic potential of EC affected by heat treatment and egg matrix. Our results demonstrate that heat treatment and egg matrix significantly reduced the eliciting and sensitizing capacity of EC by changing the tertiary structure and increasing the digestibility of EC proteins.

1. INTRODUCTION

Egg allergy is a common food allergy affecting at least 0.5 to 2.5% of infants and children (Kattan, 2016; Rona et al., 2007). The majority of relevant egg allergens have been recognized in egg white, namely, ovomucoid (OVM, Gal d 1), ovalbumin (OVA, Gal d 2), ovotransferrin (OVT, Gal d 3), and lysozyme (Lys, Gal d 4), whereas two egg yolk allergens, namely, a-livetin (Gal d 5) and yolk glycoprotein 42 (YGP42, Gal d 6), are involved in bird-egg syndrome (Chang, Lahti, Tanaka, & Nickerson, 2018). Many children with egg allergy will outgrow this condition by the age of 16 (Yawn & Fenton, 2012). However, some children have persistent egg allergy (Savage, Matsui, Skripak, & Wood, 2007), which could be life threatening (Macdougall, Cant, & Colver, 2002). To date, no effective method has been developed to manage egg allergy, and the best way for egg-allergic people is to avoid eggs

and egg-containing foods strictly (Romantsik, Tosca, Zappettini, & Calevo, 2018).

Thermal treatments, which are common and conventional during food production, potentially influence the allergenicity of food allergens by affecting the conformational changes and subsequent gastrointestinal digestibility (Verhoeckx et al., 2015). Heating-induced conformational changes, such as unfolding and aggregation, may destroy the conformational epitopes or generate new ones, and the changes may also make the allergens either further susceptible or resistant to gastrointestinal enzymes, which may affect the allergenic potential of food allergens (Bogh & Madsen, 2016; Jimenez-Saiz, Benede, Molina, & Lopez-Exposito, 2015). The effect of thermal treatment on egg allergens has also been extensively investigated, and generally, thermal treatment could decrease the allergenicity of egg allergens. However, the studies mainly focused on single allergens (Golias et al., 2012; Jimenez-Saiz, Belloque, Molina, & Lopez-Fandino, 2011; Sassi, Giugliarelli, Paolantoni, Morresi, & Onori, 2011; Tong et al., 2012) or egg white (Lemon-Mule et al., 2008; Pablos-Tanarro, Lozano-Ojalvo, Martinez-Blanco, Lopez-Fandino, & Molina, 2017; Shin, Han, & Ahn, 2013). Egg allergens exist in a complex matrix in egg, especially when egg white and yolk are mixed. The presence of other egg components on the physicochemical changes and allergenic capacity of egg allergens has not been fully understood (Benede, Lopez-Exposito, Molina, & Lopez-Fandino,

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2015). It was reported that the susceptibility to hydrolysis of egg white proteins was slightly increased with the presence of egg yolk, whereas the resultant IgE binding of allergenic proteins after *in vitro* digestion is insignificantly modified (Martos, Lopez-Fandino, & Molina, 2013). However, a recent study found that egg yolk provides Th2 adjuvant stimuli and promotes sensitization to egg white allergens in BALB/c mice (Pablos-Tanarro, Lozano-Ojalvo, Martinez-Blanco, Molina, & Lopez-Fandino, 2018). Therefore, the effect of egg matrix on the allergenicity of egg allergens is necessary for an in-depth study, especially under the thermal processing.

Egg custard (EC), a traditional Chinese savory food suitable for all ages, is made by whole egg and water mixture under thermal treatment. EC is also a traditional supplementary food for infants and young children in China, and 67% of families select it as food supplement after the first 6 months of infants. However, this supplementary feeding may increase the potential risk of allergy, especially for egg-allergic infants. Although thermal treatment can decrease the allergenicity of egg allergens, limited information is available on the allergenicity of EC, especially regarding the effects of heat treatment and egg matrix on the allergenicity of EC. Therefore, the present study aims to investigate the extent of heat treatment and egg matrix that affects the physicochemical changes of EC proteins and assesses the sensitizing and eliciting capacity of EC in BALB/c mouse model and human sera.

2. MATERIALS AND METHODS

2.1 Materials and chemicals

Fresh hen eggs were purchased from the local supermarket (Rainbow supermarket, Nanchang, Jiangxi, China). Prestained protein marker was obtained from MBI Fermentas (Burlington, Canada). Coomassie Brilliant Blue R250 and β -mercaptoethanol (β -ME) were purchased from Sangon Biotech (Shanghai, China). Histamine ELISA kits were purchased from Demeditec Diagnostics GmbH (Kiel, Germany). Mouse mast cell protease-1 (mMCP-1) ELISA kits were purchased from eBioscience (MA, USA). IL-4, IL-5, IL-13, and IFN- γ ELISA Ready-Set-Go! Kits were purchased from Affymetrix (CA, USA). Pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, and all other chemicals were purchased from Sigma-Aldrich (Shanghai, China), unless otherwise indicated.

2.2 Human serum collection

Sera of 15 egg-allergic patients with documented egg allergy histories and a positive skin prick test for egg white were collected with informed consent from the First Affiliated Hospital of Guangxi Medical University (Guangxi, China). The availability of the sera samples was approved by the internal ethical committee of the hospital. The 15 egg-allergic patients included six female patients and nine male patients with an average age of 4.9 years old. Their specific IgE level for egg and total IgE level were measured using an Immuno CAP 100 lab test (Phadia, Uppsala, Sweden). Afterward, a sera pool was made according to our previous method (Tong et al., 2018) from the 15 above sera in equal volumes and used for further analysis.

2.3 Egg custard preparation

Fresh eggs were cleaned and eggshells were removed. Egg white and egg yolk were mixed well using a whisk. After that, water was added into the egg mixture with the egg/water (v/v) ratio of 1:1, 1:1.5, 1:2, 1:3, respectively, and mixed well. The mixtures were

heated at 80, 90, or 100 °C for 10, 15, or 20 min in a steamer with boiling water, respectively, and then cooled to room temperature.

2.4 Egg protein extraction

EC proteins were extracted with four volumes of 0.02 M phosphate-buffered saline (PBS, pH 7.4) (w/v) by stirring and kept overnight at 4 °C. The sample extracts were centrifuged for 30 min at $9,000 \times g$. The precipitate was collected and suspended in four volumes of PBS. After three repeated extraction, the supernatant was collected and the protein concentrations were measured by Bradford protein assay (Bradford, 1976). The protein patterns were identified by SDS-PAGE analysis.

2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described by Laemmli (1970) with a 4% stacking gel and 12% separating gel in Mini-protein II system Electrophoresis Cell (Bio-Rad, CA, USA). Protein samples were preheated at 100 °C for 5 min and 15 μ g of each sample was loaded into each well. The gel was stained with Coomassie Brilliant Blue R250. Quantity One software (Bio-Rad, CA, USA) was used to analyze the protein patterns.

2.6 Fluorescence spectrum

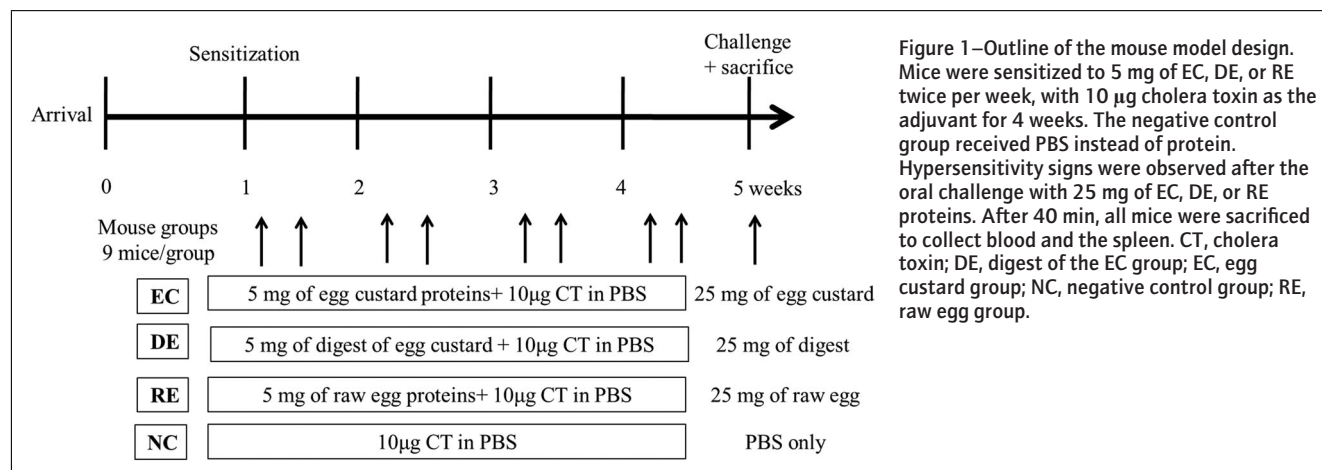
Hydrophobicity of EC proteins was determined by measuring the fluorescence intensity of EC proteins in the presence of hydrophobic fluorescence probes, 8-anilino-1-naphthalenesulfonate (ANS) as previously described (Liu, Chen, Chen, Tong, & Gao, 2018). A total of 0.1 mg/mL of each sample was mixed with 20 μ L of 5.0 mM ANS, respectively, and incubated at 25 °C in the dark for 1 hr. After that, the relative fluorescence was measured using F-4500 fluorescence photometer (HITACHI, Japan) with the excitation wavelength at 390 nm, and the emission wavelength from 400 to 650 nm at a scan speed of 1,200 nm/min.

2.7 *In vitro* gastric and duodenal digestion

Simulated gastrointestinal digestion of EC was performed as previously described (Tong et al., 2018) with some modifications. Briefly, ECs were prepared in simulated gastric fluid (SGF, 35 mmol/L NaCl at pH 2.0) at a concentration of 2 mg/mL. The SGF was preheated at 37 °C for 15 min before incubation with porcine pepsin at an enzyme/protein ratio of 1:20 (w/w). The reactions were stopped after 120 min by raising the pH to 7.0 with NaHCO_3 . Duodenal digestions were performed by using the 120-min gastric digestion as the starting substrate. Adjusting the pH to 7.0 with the addition of 1 mol/L CaCl_2 , 0.25 mol/l Bis-Tris (pH 6.5), and a 0.125 mol/L bile salts mixture containing equimolar quantities of sodium taurocholate and glycodeoxycholic acid. After preheating at 37 °C for 15 min, pancreatin was added to the mixture. The reactions were stopped by boiling for 10 min at 100 °C in a water bath. The samples were collected and analyzed by SDS-PAGE.

2.8 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to determine the allergenicity of EC protein followed by Jimenez-Saiz, Martos, Carrillo, Lopez-Fandino, & Molina (2011). Briefly, 96-well microplate was coated overnight at 4 °C with 100 μ L (2 μ g/mL) of raw whole egg protein extracts. After washing three times with PBST (PBS containing 0.05% Tween-20 (v/v)), the plate was blocked with 3% gelatin in PBS for 1 hr at 37 °C and washed three times. Another plate was also



blocked with 3% gelatin in PBS for 1 hr at 37 °C and washed three times. A total of 55 µL of EC protein extracts were added into the uncoated plate with 55 µL of pooled sera from egg-allergic patients (diluted 1:10 in PBS) and incubated at 37 °C for 1 hr. Then the mixtures were transferred to the coated plate. After 1 hr of incubation at 37 °C, the coated plate was further washed three times with PBST and incubated for 1 hr at 37 °C with 100 µL anti-human IgE-conjugated biotin (diluted 1:5000 in PBS). The plate was further washed three times with PBST followed by incubating at 37 °C for 1 hr with streptavidin-HRP (diluted 1:60 in PBS). After washing three times with PBST, the reaction was developed with o-phenylenediamine (OPD) as substrate at 37 °C for 15 min. The reaction was stopped by 2 mol/L H₂SO₄, and the optical density (OD) was measured at 490 nm. Each sample was analyzed in triplicate.

2.9 Sensitization in a mouse model

The mouse model used in this study was similar to the previously described work with some modification (Tong et al., 2017). A total of 36 female BALB/c mice (6 to 8 weeks of age) were purchased from the Laboratory Animal Center of Nanchang University (Nanchang, China, permission number "SYXK (Gan) 2015-0001"), and bred under an egg-free diet and had access to water and food ad libitum. One week later, they were randomly divided into four groups of nine mice per group ($n = \text{nine/group}$), which were EC group, digest of EC (DE) group, RE group, and negative control (NC) group. Mice were sensitized to 5 mg of EC, DE, or RE proteins twice per week, with 10 µg cholera toxin (CT) as the adjuvant for 4 weeks, as outlined in Figure 1. The NC group received PBS instead of protein. Hypersensitivity signs were observed after the oral challenge with 25 mg of EC, DE, or RE proteins. After 40 min, all mice were sacrificed by cervical dislocation, and the blood samples and the spleens were collected for the measurement of various biomarkers. All animal use protocols were approved by the Animal Care Committee of Nanchang University under the guidelines of the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1996).

2.10 Measurement of levels of serum histamine and mouse mast cell protease-1

At the end point, whole blood from each mouse was collected, and histamine concentrations in sera were determined by ELISA assay using a commercial ELISA kit (Histamine ELISA kit, Demeditec Diagnostics GmbH, Germany). Mouse mast cell

protease-1 (mMCP-1) in sera was detected by a commercial ELISA kit according to manufacturer's instructions (eBioscience, MA, USA).

2.11 Determination of specific antibodies levels

The specific IgE, IgG, IgG1, and IgG2a levels were determined by ELISA as previously described (Jimenez-Saiz, Rupa, & Mine, 2011) using a coating of 5 µg/well RE proteins in 96-well microtiter plates. Mouse sera were diluted in PBS (1:10 for egg-specific IgE and 1:200 for egg-specific IgG, IgG1, and IgG2a) and assayed in triplicate wells. HRP-conjugated goat anti-mouse IgE, IgG, IgG1, and IgG2a antibodies (diluted to 1: 5,000) were applied. The results were reported as optical density (OD).

2.12 Allergen-induced cytokine secretion in spleen cell cultures

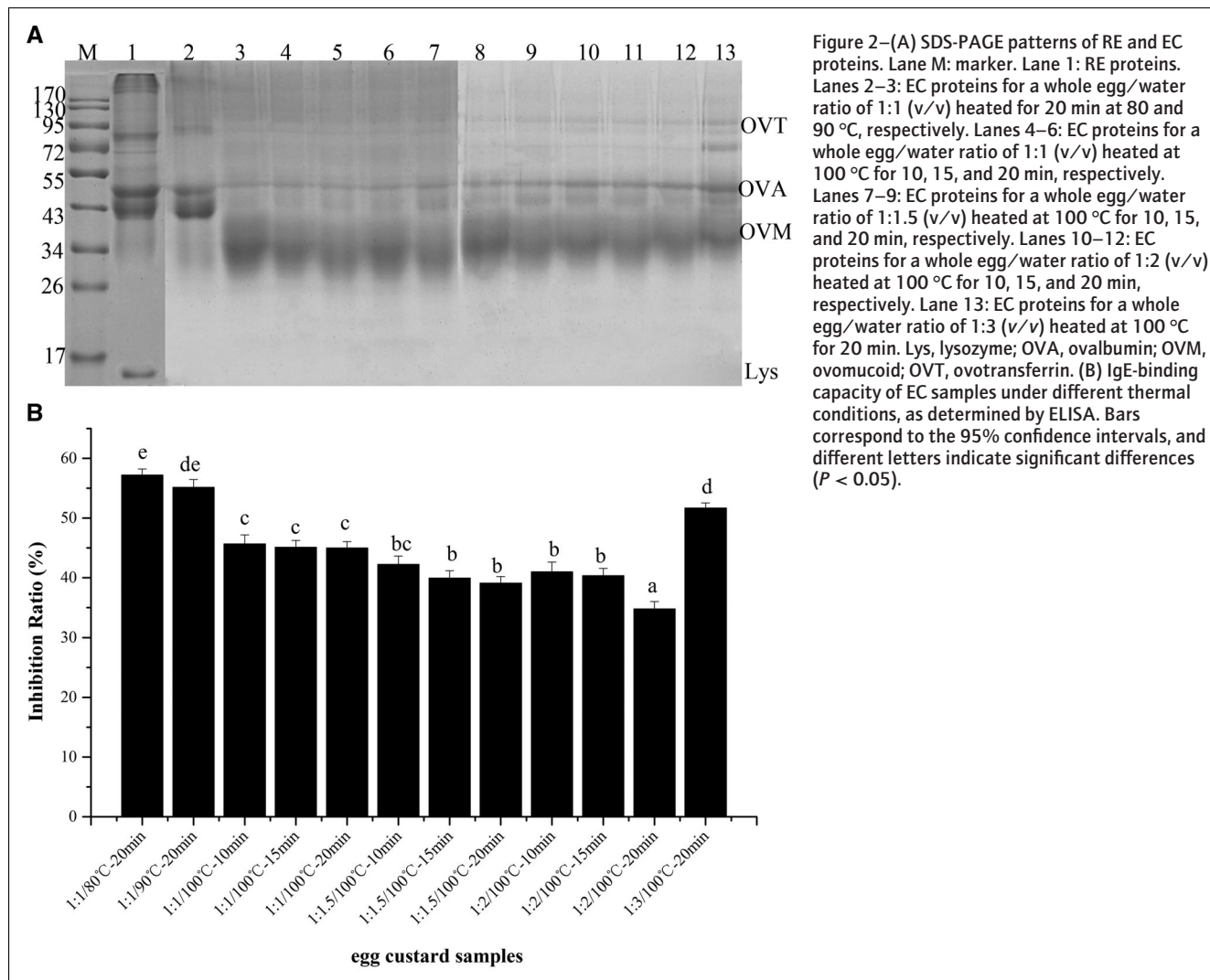
Murine splenocyte culture was performed as described previously (Rupa, Nakamura, & Mine, 2007). In brief, individual spleen from each mouse was collected aseptically and single-cell suspensions were prepared. Cells were cultured in 24-well plates (Corning, NY, USA) at a density of $1 \times 10^6/\text{mL}$ in the absence (NC wells) or presence of EC, DE, or RE proteins (100 µg). Supernatants were collected after 72 hr of culture in a 5% CO₂ humidified incubator and stored at -20 °C until analysis. IL-4, IL-5, IL-13, and IFN- γ were determined by commercial ELISA Ready-Set-Go! Kits (Affymetrix, eBioscience, San Diego, CA, USA).

2.13 Pathological examination of the intestine

Regarding the assessment of pathologic alterations, duodenum samples were collected and fixed in neutral-buffered formaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin, and then examined histologically at $\times 10$ magnification by using a light microscope on the basis of the method of Yang, Gao, Yang, Lu, and Chen (2015).

2.14 Statistical analysis

The normality of the data was tested by Kolmogorov Smirnov and Shapiro-Wilk tests using SPSS 13.0. Data are presented as a mean $\pm$ standard error of the mean (SEM) and analyzed using SPSS 13.0, while significant differences were evaluated by one-way ANOVA. Differences were considered significant if the P -value was less than 0.05.



3. RESULTS AND DISCUSSION

3.1 Structural changes of egg white proteins resulting from heat treatment and egg matrix

After the ECs were prepared, EC proteins were extracted with PBS. Figure 2A shows the soluble protein patterns of RE and ECs, which are different. EC made with a whole egg/water ratio of 1:1 (v/v) and heated at 80 °C for 20 min showed similar patterns to RE, but no band corresponding to Lys was found. Other ECs made under different conditions presented similar profiles to each other, and most of the proteins in ECs were located at approximately 30 to 50 kDa fraction. The band corresponding to OVT in ECs nearly disappeared, and Lys completely disappeared through thermal processing. The band corresponding to OVA and OVM, especially to OVM, remained stable after heating, which indicated that they were heat resistant during EC production.

To reveal further structural differences after thermal treatment, fluorescence spectra were collected and compared. Figure 3 shows that the fluorescence intensities of EC proteins induced by ANS were lower than those of unheated RE proteins. Moreover, different heating temperatures significantly affected the fluorescence intensities, which decreased when heated at a low temperature (80 °C) but increased with the increase in temperature (90 and

100 °C). The fluorescence intensities of ECs can also be affected by the egg/water ratio, which decreased with increasing water (1:1 and 1:1.5) but increased again at high water ratio (1:2 and 1:3, data of 1:3 was not shown). The decreased fluorescence intensities of EC proteins compared with those of RE indicated that the structure of EC proteins was partially unfolded, thereby, breaking the hydrophobic bond and decreasing the surface hydrophobicity. Accordingly, the tertiary structure of egg proteins was changed as a result of heat treatment and egg matrix.

3.2 Digestibility of EC proteins

To evaluate the effect of different heating conditions and egg/water ratios on the digestibility of EC, gastrointestinal digestion under simulated gastric and intestinal fluids was performed, and the products were analyzed by SDS-PAGE (Figure 4 and 5). The OVM could be easily degraded by pepsin into fragments with molecular masses of approximately 25, 18, 14, and <10 kDa, especially at a low concentration of the egg matrix. However, this degradation occurred as soon as pepsin was added to the mixture, and the major bands of the digests had no significant change even after gastric digestion for 120 min (Figure 4). The samples obtained after 120 min of simulated gastric digestion

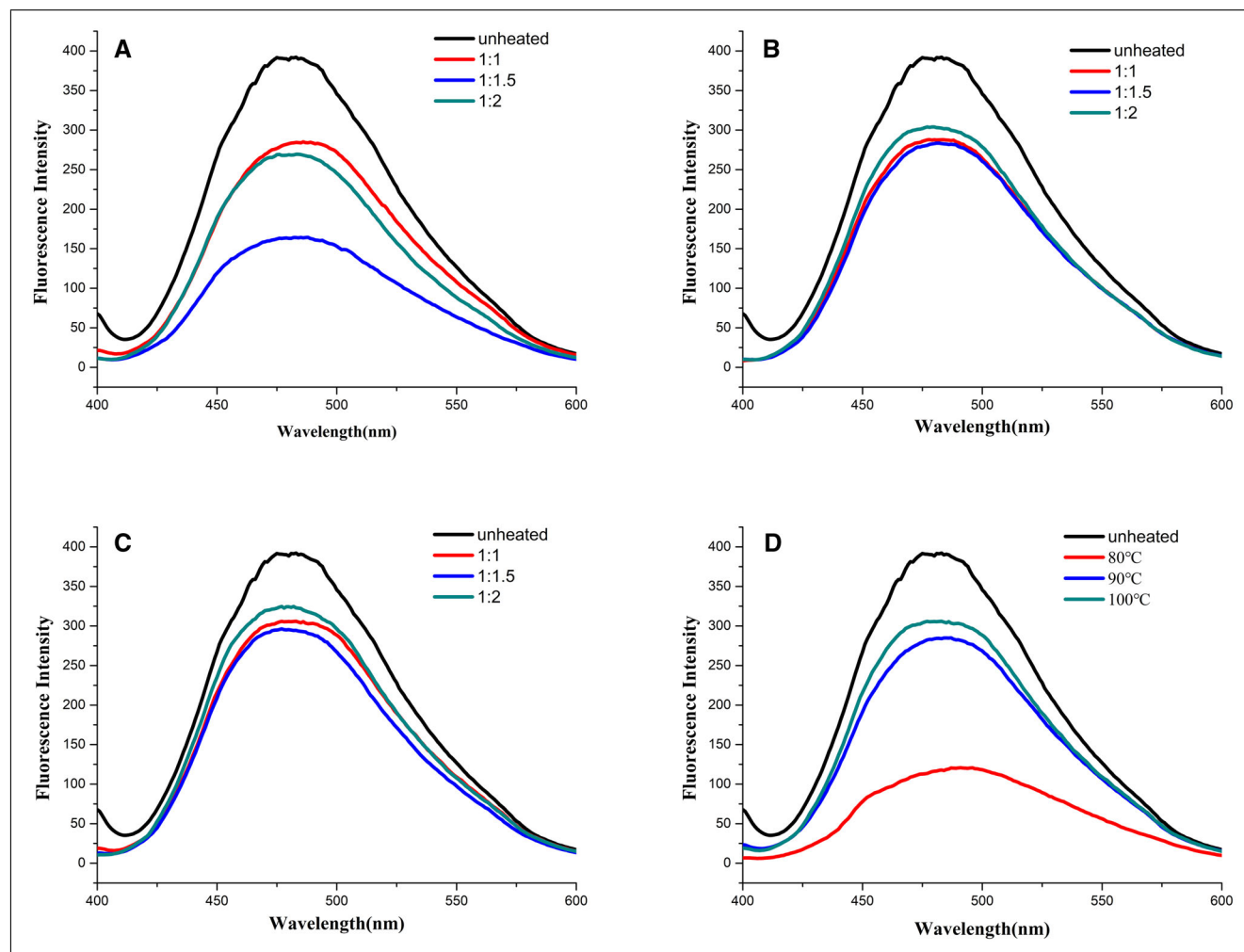


Figure 3—Extrinsic fluorescence of EC proteins with different egg/water ratios and heating temperatures. (A) Extrinsic fluorescence of EC proteins heated at 100 °C for 10 min at different egg/water ratios. (B) Extrinsic fluorescence of EC proteins heated at 100 °C for 15 min at different egg/water ratios. (C) Extrinsic fluorescence of EC proteins heated at 100 °C for 20 min at different egg/water ratios. (D) Extrinsic fluorescence of EC proteins for an egg/water ratio of 1:1 (v/v) heated for 20 min at different temperatures.

were further subjected to a mimicking duodenal digestion. The SDS-PAGE profiles indicated that the simulated intestinal fluid could digest the EC proteins, which were stable in gastric liquid. Some parts of EC proteins were rapidly digested into small molecular fragments, but the proteins could not be completely broken down into peptides. Some proteins remained detectable even after 120 min of incubation (Figure 5). This finding suggests that after heat treatment, EC proteins were only partially degraded, following subsequent incubation with pancreatin.

3.3 *In vitro* IgE-binding capacity of EC

The *in vitro* IgE-binding capacity of EC under different conditions was assessed through ELISA by using a pool of sera from 15 egg-allergic patients (Figure 2B). The IgE-binding capacity of EC was significantly different with various egg/water ratios and heating times. The EC treated at 100 °C for 20 min with an egg/water ratio of 1:2 showed the lowest IgE-binding potency.

3.4 Allergic responses to EC and the digest of EC (DE) in the BALB/c mouse model

The immunogenicity and allergenicity of EC and DE were assessed in the BALB/c mouse model, and the allergic response

changes were defined efficiently. Firstly, in comparison with RE, the EC and DE significantly decreased the levels of specific IgE, IgG, IgG1, and IgG2a in the mouse sera (Figure 6A–D, $P < 0.001$). Secondly, the mMCP-1 levels in mice sensitized by EC and DE were significantly decreased compared with those by RE (Figure 6E, $P < 0.01$ for EC, $P < 0.001$ for DE). Similarly, the histamine concentrations in the mouse plasma of the EC and DE groups were significantly lower than that of the RE group (Figure 6F, $P < 0.001$). In addition, although the Th1-related cytokine IFN- γ was undetectable based on the ELISA kit sensibility in the sera of all groups (data not shown), EC and DE significantly decreased the production of Th2-related cytokines IL-4, IL-5, and IL-13 in the stimulated splenocyte compared with RE (Figure 6G–I, $P < 0.01$ for EC, $P < 0.001$ for DE). However, the levels of specific IgE, IgG, IgG1, and IgG2a and the concentrations of mMCP-1, histamine and the cytokines of IL-4, IL-5, and IL-13 in the EC and DE groups remained significantly higher than those in the control group (Figure 6E–G, $P < 0.05$; Figure 6I, $P < 0.01$; Figure 6A–D and 6H, $P < 0.001$). Those in the EC group remained significantly higher than those in the DE group (Figure 6E and 6I, $P < 0.05$; Figure 6G and 6H, $P < 0.01$; Figure 6A–D and 6F, $P < 0.001$).

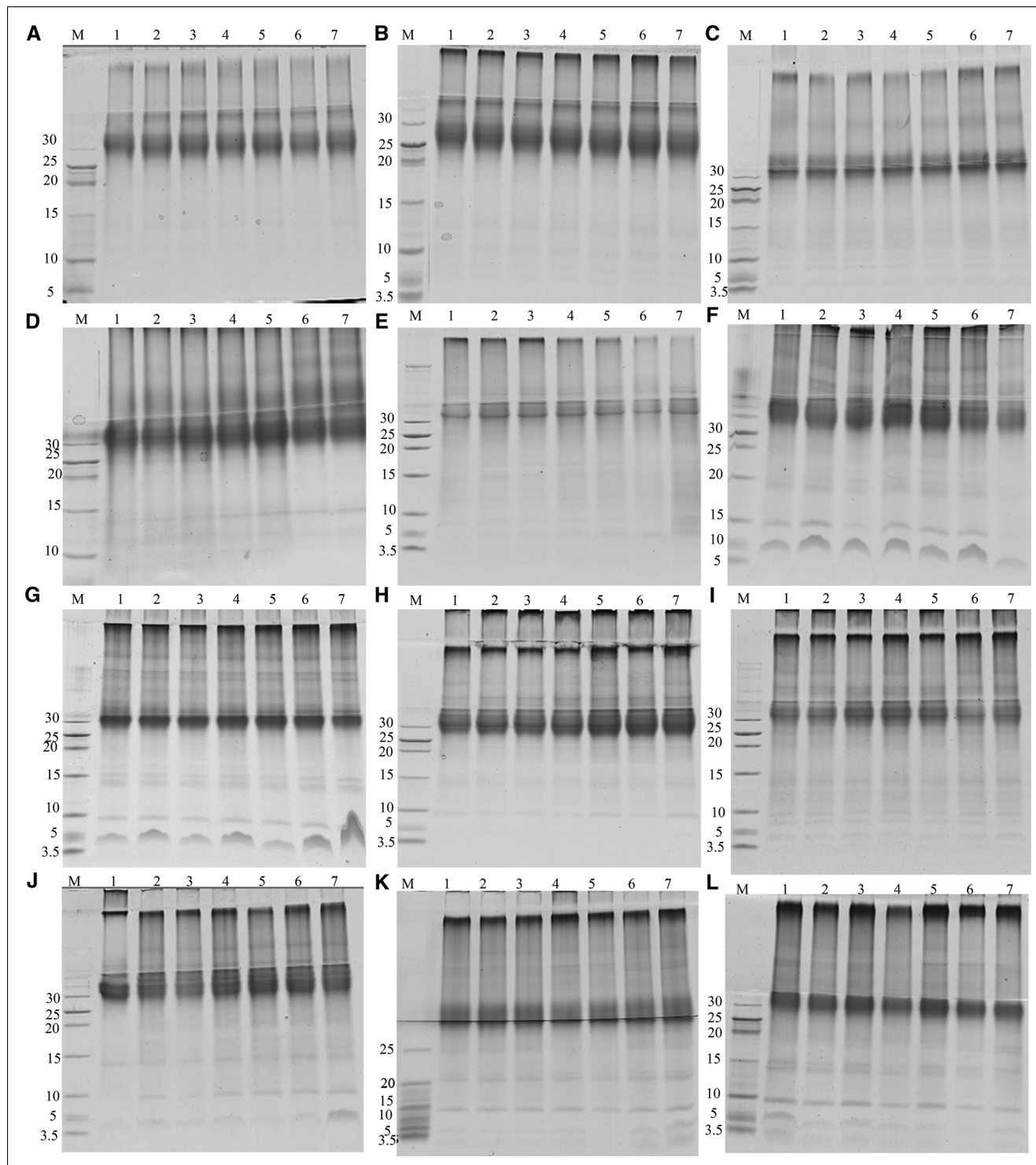


Figure 4—Tricine-SDS-PAGE patterns of ECs under different heating conditions digested by simulated gastric fluid *in vitro*. Lane M: marker. Lanes 1–7: digested at 0, 2, 5, 15, 30, 60, and 120 min, respectively. (A), 1:1/80 °C for 20 min; (B), 1:1/90 °C for 20 min; (C), 1:1/100 °C for 10 min; (D), 1:1/100 °C for 15 min; (E), 1:1/100 °C for 20 min; (F), 1:1.5/100 °C for 10 min; (G), 1:1.5/100 °C for 15 min; (H), 1:1.5/100 °C for 20 min; (I), 1:2/100 °C for 10 min; (J), 1:2/100 °C for 15 min; (K), 1:2/100 °C for 20 min; (L), 1:3/100 °C for 20 min.

3.5 Systemic anaphylactic symptoms in the challenged mice

The capability of EC and DE to induce systemic anaphylactic reactions, compared with that of RE, was estimated in the mouse model. Four weeks after initial sensitization, the mice were challenged orally. A slight diarrhea was observed in the RE group,

but no evident systemic anaphylactic symptoms were observed in the other three groups. A histological examination of the small intestines revealed pathological changes in the RE, EC, and DE groups (Figure 7). In the control group, the villi structure and crypt of the small intestine were tidy and intact (Figure 7D). In the RE group (Figure 7A), the villi structure of the small intestine

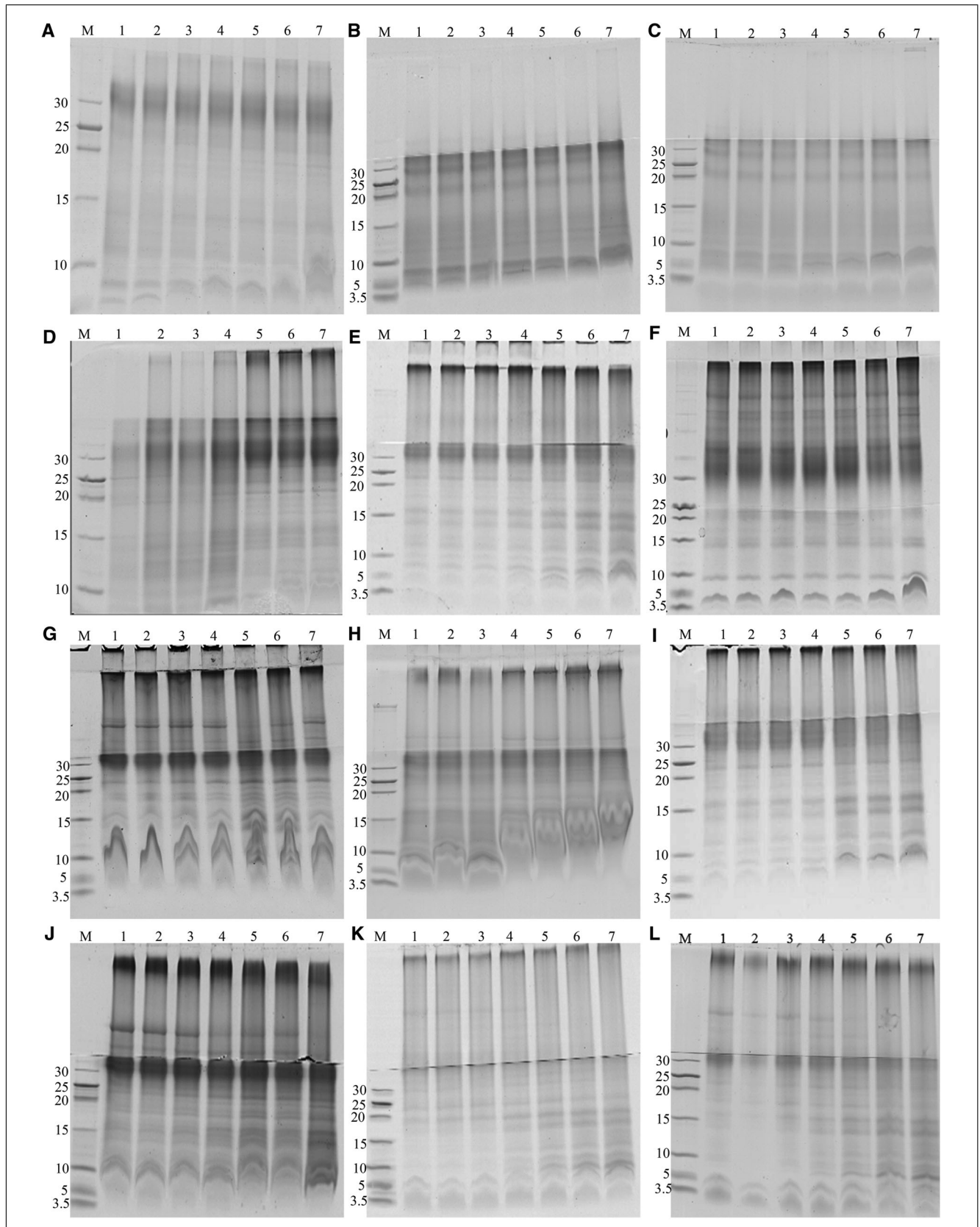


Figure 5—Tricine-SDS-PAGE patterns of ECs under different heating conditions digested by simulated intestinal fluid *in vitro*. Lane M: marker. Lanes 1–7: digested at 0, 2, 5, 15, 30, 60, and 120 min, respectively. (A), 1:1/80 °C for 20 min; (B), 1:1/90 °C for 20 min; (C), 1:1/100 °C for 10 min; (D), 1:1/100 °C for 15 min; (E), 1:1/100 °C for 20 min; (F), 1:1.5/100 °C for 10 min; (G), 1:1.5/100 °C for 15 min; (H), 1:1.5/100 °C for 20 min; (I), 1:2/100 °C for 10 min; (J), 1:2/100 °C for 15 min; (K), 1:2/100 °C for 20 min; (L), 1:3/100 °C for 20 min.

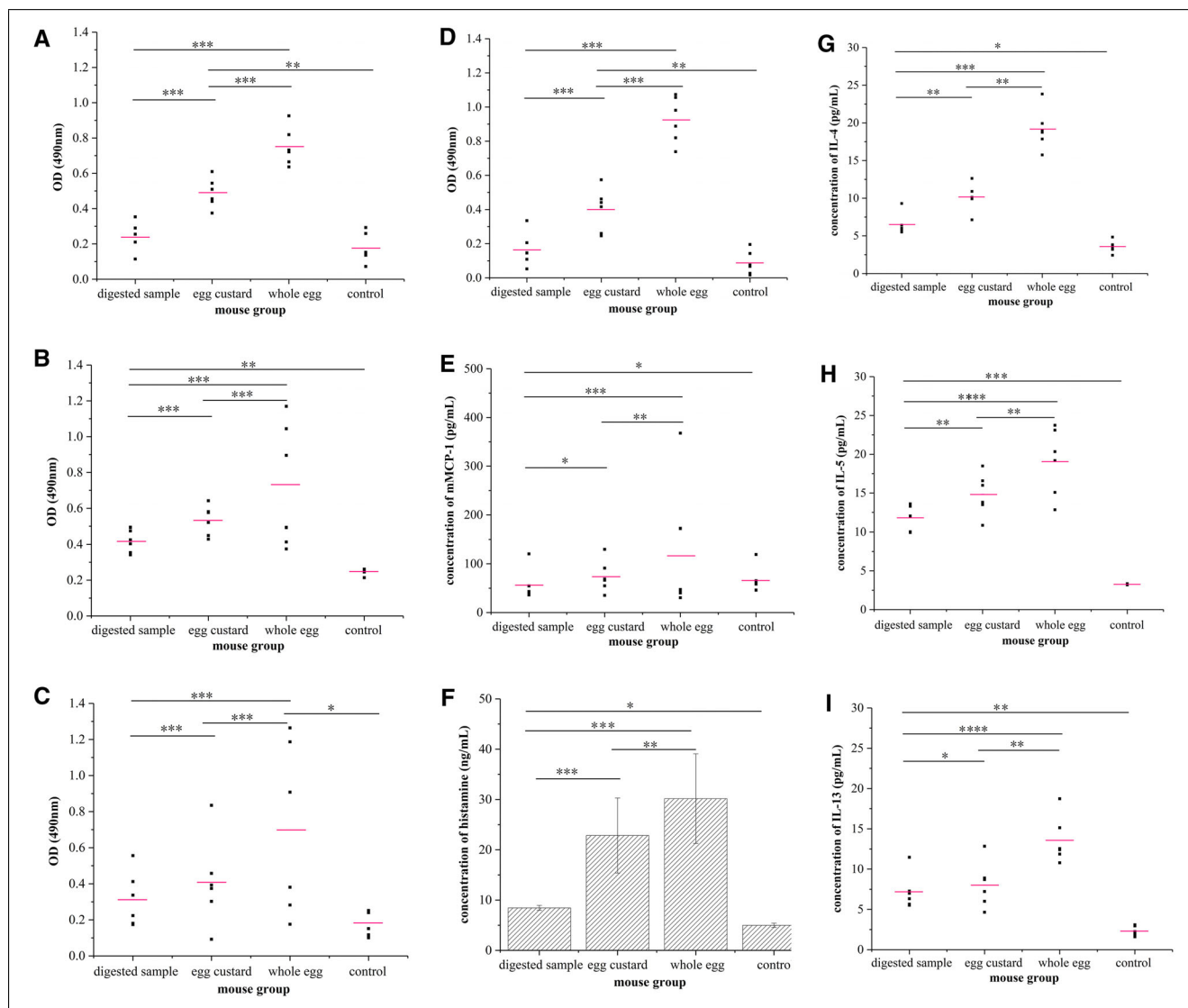


Figure 6—Allergen-induced specific IgE (A), IgG (B), IgG1 (C), IgG2a (D), mMCP-1 (E), and histamine (F) in mice sera and cytokine secretion of IL-4 (G), IL-5 (H), and IL-13 (I) in splenocyte in the BALB/c mouse model. Specific antibody levels were measured by ELISA. The concentrations of mMCP-1, histamine, and cytokines were determined using the commercial ELISA kits in accordance with the manufacturer's instructions. Significance was considered at $P < 0.05$. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

was destroyed and disappeared, and the absence of the crypt and crypt destruction was evident. In the EC group (Figure 7C), some of the villi were swollen, broken and shed, and the crypt numbers were decreased. In the DE group, the villi structure of the small intestine had a slight sloughing of enterocytes at the villi tops (Figure 7B). Therefore, the structural damage was worse in the EC group than the DE group.

3.6 Discussion

In this study, we evaluated how the structure, digestibility, and allergenic potential of egg allergens in EC were altered by the degree of thermal treatment and egg matrix. The tertiary structure of egg proteins was significantly affected by thermal treatment, as determined by SDS-PAGE and fluorescence spectra, which caused the unfolding and aggregation of proteins. Our study agreed with Davis and Williams (1998), who stated that unfolding increases with the extent of heating, followed by aggregation, and finally causes the loss of the tertiary structure. The decreased fluorescence intensity of EC proteins at 80 °C, compared with

RE proteins, indicated that the structure of EC proteins was partially unfolded, thereby, breaking the hydrophobic bonds in the protein surface and decreasing the surface hydrophobicity. With the increase in temperature, the fluorescence intensities increased again, demonstrating that the structure of egg proteins in EC was further unfolded. Furthermore, the inward-oriented hydrophobic groups turned outward, which increased the fluorescence intensity (van Dijk, Hoogveen, & Abeln, 2015). Further unfolding may cause aggregation among the egg allergens and matrix, which may form insoluble aggregates. Therefore, the protein patterns of ECs in SDS-PAGE were significantly different from that of RE proteins. In addition, the different egg/water ratios, which refer to the different concentrations of the egg matrix, could also affect the structural changes of ECs. With the same heating condition, the concentration of the egg matrix affected the interactions between the egg proteins and matrix, leading to the unfolding of egg proteins in varying degrees. The lower the concentration of the egg matrix, the easier is the denaturation of egg proteins.

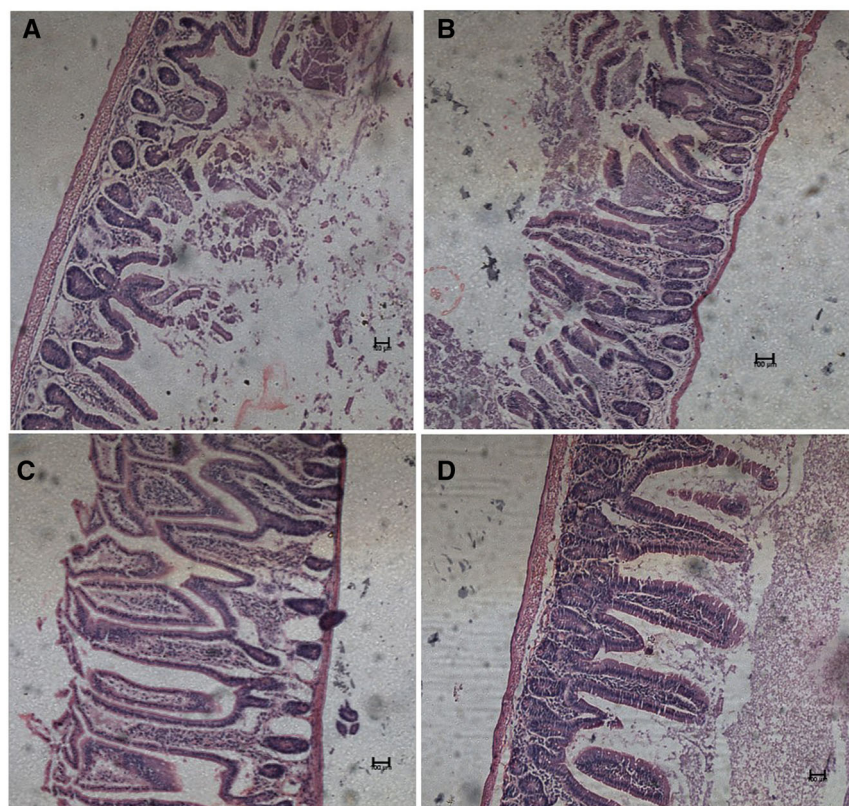


Figure 7—Typical histological alterations in the intestine of orally challenged mouse. Duodenum samples were collected and fixed in neutral-buffered formaldehyde and embedded in paraffin. Furthermore, 5 μ m sections were stained with hematoxylin and eosin and then examined histologically at $\times 10$ magnification by using a light microscope. (A), duodenum of the RE group; (B), duodenum of the DE group; (C), duodenum of the EC group; (D), duodenum of the control group.

The digestibility of food allergens is an important characteristic responsible for allergenicity, and it can also be affected by food processing and egg matrix. For example, according to Martos et al. (2013), the susceptibility of egg white proteins to hydrolysis is slightly increased with the presence of egg matrix. However, intact OVA and Lys still existed after the duodenal phase of digestion. Yoshino, Sakai, Mizuha, Shimizuike, & Yamamoto (2004) reported that heat treatment is helpful for the peptic digestibility of egg white proteins. In the current study, the heat treatment and egg matrix affected the digestibility of EC. Results showed that Lys was completely disappeared through thermal processing and OVM was easily digested by pepsin, especially at a low concentration of the egg matrix, which was rapidly degraded into fragments with molecular masses of approximately 25, 18, 14, and <10 kDa. The findings agreed with Jimenez-Saiz, Belloque, et al. 2011. It was worth mentioning that, this degradation happened as soon as pepsin was added into the mixture, and the major bands of digests had no significant change even after gastric digestion for 120 min. When the samples were further subjected to the simulated intestinal fluid, the proteins, which were stable in gastric liquid, could be rapidly digested into small molecular fragments. However, the proteins could not be completely broken down into peptides. Therefore, the digestibility of EC was increased by heat treatment and egg matrix, and the degradation of EC proteins was independent of digestion time. The increased digestibility implied that the allergenicity of EC may be lower than that of RE (Jimenez-Saiz, Ruiz-Henestrosa, Lopez-Fandino, & Molina, 2012).

The allergenicity of EC was first assessed by IgE binding to the sera from egg-allergic patients, as determined by ELISA. The

IgE-binding capacity in all types of ECs was significantly weakened compared with RE, indicating that the eliciting capacity of EC was reduced after heat treatment. Moreover, the eliciting capacity of EC made by heating at 100 °C for 20 min with a whole egg/water ratio of 1:2 (v/v) was the weakest. Afterward, the weakest EC was used to sensitize the BALB/c mice, and the sensitizing capacity of EC was investigated in a BALB/c mouse model. As we had known that, there was no naturally occurring allergic mouse. In consequence, the induction of sensitization and allergic reactions in mice would always be an artificial process. Although adjuvant-free sensitizations against egg proteins had been proposed in order to evaluate the sensitization and allergenic potential of egg proteins (Aramburo-Galvez et al., 2018; Cardenas-Torres et al., 2019; Chen, Sun, Li, & Jia, 2013; Hilton, Dearman, Sattar, Basketter, & Kimber, 1997), adjuvant-free sensitization models frequently made use of the transdermal sensitization route, which may not reflect the physiological routes of egg allergy (Gonipeta, Kim, & Gangur, 2015). To successfully establish a food allergy mouse model, the naturally occurring oral tolerance toward orally applied allergens needs to be broken. CT had been used to break oral tolerance toward coapplied allergens in many of published mouse food allergy models (Adel-Patient, Bernard, Ah-Leung, Creminon, & Wal, 2005; Li et al., 2000; Snider, Marshall, Perdue, & Liang, 1994). Therefore, we chose CT as the adjuvant to sensitize the mice. Even though CT may cause a strong allergic reaction, the focus of our work was to compare the allergenicity changes among RE, EC and DE. With the same adjuvant, the sensitizing capacity of EC may be efficiently compared with RE. The BALB/c mice were sensitized with RE, EC and DE, respectively, with CT as the adjuvant. A slight diarrhea was only

observed in the RE group, whereas no evident systemic anaphylactic symptoms were observed in the EC, DE and control groups. The histological symptom of the small intestines was considerably milder in the EC and DE groups than in the RE group, and the structural damage was worse in the EC group than in the DE group. When the levels of antigen-specific antibodies were detected, the levels of IgE, IgG, IgG1 and IgG2a were significantly decreased in the EC and DE groups compared with the RE group. Similarly, the levels of mMCP-1 and histamine in the EC and DE groups were significantly lower than those in the RE group. In addition, the production of Th2-type cytokines, such as IL-4, IL-5 and IL-13, was significantly down-regulated in the EC- and DE-treated mice, compared with the RE-treated mice. The Th1-type cytokine IFN- γ was unexpectedly undetectable in all mice (based on ELISA kit sensibility). This phenomenon was consistent with the previous study, which described that the oral treatment of neonatal pigs with *Lactococcus lactis* significantly reduced the subsequent frequency of allergy to OVM associated with reduced type-2 immune response, and IFN- γ was not detected in the control or treated animals (Rupa, Schmied, & Wilkie, 2011). Results indicated that EC relieved the allergy symptoms in mice compared with RE, especially after digestion. However, the allergenic potential of EC was not completely eliminated by the effects of heat treatment and egg matrix, as the allergy symptoms in the EC and DE groups remained significant compared with that in the control group.

The effect of heat treatment on egg allergens had been extensively investigated, and generally, heat treatment could decrease the allergenicity of egg allergens. Shin studied four types of heated egg white on the allergenicity of egg white proteins and found that the egg white boiled for 30 min significantly reduces the allergenicity of egg allergens (Shin et al., 2013). Watanabe et al. used three types of heated egg white to challenge OVA23-3 transgenic mouse and reported that heating condition at 100 °C for 5 min reduces the allergenicity of OVA and induced tolerance in OVA23-3 mice (Watanabe et al., 2014). Liu et al. investigated the effects of different traditional Chinese cooking methods on the allergenic properties of hen's egg white proteins and stated that heat processing can weaken the allergenicity of egg proteins (Liu et al., 2013). Our results supported the previous findings. The mechanism of the decreased allergenicity of EC may be explained by the conformational and digestibility changes of egg allergens induced by heat treatment and egg matrix. The unfolding and aggregation of EC proteins may destroy the conformational epitopes of egg allergens, and the increased digestibility may break down the sequential epitopes. Therefore, the allergenicity of EC was significantly lower than that of RE, especially after digestion. However, Lechevalier, Belloque, et al., 2017; Lechevalier, Martos, 2017; and Lechevalier-Datin et al., 2017 had different opinions that heat treatments applied to egg product have a rather low impact on *in vivo* allergenicity, despite significant changes in protein digestibility and antigenicity. In fact, in their studies, the allergenicity of the pasteurized liquid whole egg was investigated in 54 egg-allergic children by prick test (PT), and for 34 children, pasteurization had no impact on PT reactivity, whereas for 20 children, pasteurization significantly decreased reactivity. In addition, the heat treatments they used to the liquid whole egg or egg white powder were not extensively heated. Pasteurization and dry heating treatment may have limited impact on the allergenicity of egg white, but they couldn't represent all the heat treatments. As we had known that extensively heated egg could be tolerated by many of egg-allergic patients, and were often chosen to do food chal-

lenges in egg-allergic children (Gotesdyner et al., 2019; Lemon-Mule et al., 2008). Moreover, many studies had also proved that heat treatment could significantly decreased the allergenicity *in vivo* (Claude et al., 2016; Pablos-Tanarro et al., 2017).

Zhu, Vanga, Wang, & Raghavan (2018) and Hwang et al. (2016) had reported some novel processing techniques which could reduce the allergenicity of egg allergens, among these techniques, enzymatic hydrolysis was widely used, which was not only useful for reducing the allergic reactions of egg, but also producing some bioactive peptides. Nowadays, hydrolyzed food formula had been an attractive approach for preventing or controlling food allergy (Lozano-Ojalvo, Molina, & Lopez-Fandino, 2016; Yang et al., 2009). However, our studies suggest that the safety of hydrolyzed food formulas requires further attention.

4. CONCLUSIONS

The physicochemical properties changes and the allergenicity of EC were evaluated under the different degrees of thermal treatment and different concentrations of egg matrix, and the links between the physicochemical properties and allergenic potential of EC affected by heat treatment and egg matrix were elucidated, which demonstrate that heat treatment and egg matrix significantly reduced the eliciting and sensitizing capacity of EC by changing the tertiary structure and increasing the digestibility of EC proteins.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Ping Tong was responsible for designing the experiments and writing the manuscript. Liji Xiong made substantial contributions to most of the experiments and analysis of the data. Jinyan Gao aided to interpret the data of this work. Xin Li directed the animal experiment. Juanli Yuan aided to detect the digestibility of ECs. Yong Wu helped to structure analysis of EC proteins. Zhihua Wu and Anshu Yang aided in revising the manuscript. Hongbing Chen (corresponding author) directed the experimental design and revised the manuscript.

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