

Food-Cooking Processes Modulate Allergenic Properties of Hen's Egg White Proteins

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Key Words

Egg · Allergen · Allergenicity

Abstract

Background and Objective: Reducing the allergenicity of food allergens can suppress the clinical symptoms of food allergy. The objective of the present study was to investigate the effects of processing on the allergenic properties of hen's egg white proteins. **Methods:** Eggs were processed by traditional Chinese cooking, including steaming, water boiling, frying, spicing and tea boiling. The contents of processed egg protein were assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis; the allergenicity was evaluated by Western blotting, enzyme-linked immunosorbent assay and enzyme allergosorbent test inhibition. Circular dichroism spectrum analysis of four major egg allergens from various egg products was performed as well. A mouse model of food allergy was developed to test the allergenicity of processed egg protein in vivo. **Results:** Protein degradation was significant following tea boiling and spiced-tea boiling. The total allergenic potential of water-boiled egg and fried egg was relatively higher than that of steamed egg, spiced egg and tea-boiled egg. Challenge with proteins from

raw egg, water-boiled egg and fried egg induced skewed T-helper 2 pattern responses (Th2 responses) in the intestine of mice sensitized to egg proteins; however, when the mice sensitized to egg proteins were challenged with proteins from steamed egg, spiced egg and tea-boiled egg, respectively, only weak Th2 responses were induced in their intestine. **Conclusion:** Processing by steaming, spicing, or tea boiling can weaken the allergenicity of egg proteins.

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Introduction

Food allergy causes hypersensitivity reactions to food and has been an important nutritional problem. Its increasing prevalence may change the lifestyle of patients with the disorder. About 90% of food hypersensitivity reactions are caused by food consumption such as egg, peanut or milk [1]. A number of food allergens have been identified from food products that are known to cause food hypersensitivity. Among them, hen's egg (egg, in short) white proteins are a common cause of allergy in the world, especially among infants and young children [2]. The WHO and FAO reported that 31% of children

Up to now, no specific treatment is available for egg hypersensitivity reactions; the optimal choice is to avoid ingesting egg or egg-additive products. This avoidance may result in an imbalance in nutrients, particularly among infants and young children. Therefore, inducing tolerance to egg to reduce the reactivity to egg allergens in patients with egg allergy has been proposed [5]. In this study, with the purpose of helping to generate egg products with very low allergenic potential, the effect of several Chinese traditional food cooking processes on modulating the allergenic properties of egg proteins were studied.

TBST. Color was developed with *p*-nitrophenol phosphate in 0.1 M diethanolamine buffer (pH 9.8) for 60 min at room temperature; the reaction was stopped with a stopping solution (100 μ l, 2 M H₂SO₄) and the absorbance was measured at 450 nm.

Enzyme Allergosorbent Test Inhibition

For enzyme allergosorbent test (EAST) inhibition, diluted (1:10) sera from pooled egg-allergic patients were incubated with various dilutions of inhibitor (four major egg allergens, i.e. ovomucoid, ovalbumin, ovotransferrin and lysozyme. BSA was used as control; 1,000, 100, 10, 1, and 0.1 μ g/ml protein dissolved in incubation buffer). A total of 200 μ l of diluted pool serum was added per well and incubated for 2 h at 37°C in the dark. TBST was used as a control, after 3 washes with TBST, 50 μ l of anti-human IgE alkaline phosphatase conjugate (diluted 1:200 in incubation buffer) was added and incubated for 1.5 h at 37°C in the dark. The plate was washed again, and the bound enzyme activity was developed with 200 μ l of substrate solution (containing 3,3',5,5'-tetramethylbenzidine) for 1 h at 37°C in the dark. After addition of stopping solution (100 μ l, 2 M H₂SO₄), the absorbance was measured at 450 nm. Inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{OD}_{\text{positive without inhibitor}} - \text{OD}_{\text{with inhibitor}}}{\text{OD}_{\text{positive without inhibitor}} - \text{OD}_{\text{blank control}}} \times 100$$

Analysis of Circular Dichroism of Egg Allergens

Far UV (185–250 nm) circular dichroism (CD) spectra of four major untreated egg allergen proteins, and the four egg allergen proteins processed by tea boiling or spiced-tea boiling were obtained at room temperature with a Jasco Model J-71 spectropolarimeter. The treatment process for each egg allergen protein by tea boiling or aniseed boiling is the same as that for tea-boiled egg. Samples were dialyzed for 24 h in the Milli-Q water and immediately used in CD measurements. The CD spectrum of Milli-Q water was measured and used as a background reading. Protein concentrations were 0.15 mg/ml with Coomassie Brilliant Blue determination.

Basophil Activation Test

Twenty milliliters of heparinized peripheral blood were obtained from each subject. The peripheral mononuclear cells were separated by gradient density centrifugation. Basophils were further isolated by negative magnetic cell sorting with a commercial reagent kit (Miltenyi Biotec, Shanghai, China) with a purity of >95% (checked by flow cytometry). The basophils were resuspended in complete RPMI-1640 medium (10⁴ cells/ml). The processed egg proteins were added to the culture medium of the respective wells and incubated for 4 h. Basophils in control wells were cultured with medium alone; the cells were treated 3 times by freeze-thawing to release the histamine. The supernatants were collected and subjected to histamine assay with an ELISA kit following the manufacturer's instructions.

Mouse Model of Food Allergy

BALB/c mice (6–8 weeks old) were purchased from the Beijing Experimental Animal Center. The mice were subcutaneously injected with raw egg protein (0.05 mg/mouse) mixed in 0.3 ml alum on day 0 and day 7. The mice were challenged by gavage feeding with 1 mg egg protein (raw egg protein or cooked; saline was used

in the control group) on days 28, 30 and 32. The mice were sacrificed on day 33. Jejunal tissue protein was extracted following our published procedures [7, 8] to determine the levels of egg-specific IgE and IL-4. Pieces of jejunal segments were processed to assess the number of mast cells and eosinophils [7, 8]. Lamina propria mononuclear cells were isolated to determine egg-specific T cell proliferation using a [³H]-incorporation assay [9]. The diarrhea was recorded for 2 h after each egg antigen challenge [10]. The animal procedures were approved by the Animal Care Committee at Shenzhen University.

Statistical Analysis

All data analyses were performed with nonparametric tests (Mann-Whitney U test) by using SPSS 13 software. *p* values of less than 0.05 were considered significant.

Result

Cooking Degrades the Egg Protein Molecules

The protein fractions of egg products were separated and visualized using SDS-PAGE and Coomassie Brilliant Blue staining. As shown in figure 1, several protein bands were seen between 10 and 130 kDa; most of the proteins were located in the 30- to 50-kDa fractions. The results of the integrated density of protein bands showed that the contents of egg protein between 30 and 50 kDa were not evidently altered by steaming, water boiling and frying, but were markedly decreased by spiced-tea and tea boiling as compared to raw egg (fig. 1a). Although the protein stain between 10 and 30 kDa could be clearly seen in egg treated with spicing or tea boiling, those of eggs treated with steaming, water boiling and frying were hardly visible. Based on the molecular weight fractioning data, the results indicate that when treated by spicing or tea boiling, the egg protein molecules are significantly degraded whereas they are not changed much after treatment with water boiling and frying.

The Allergenicity of Egg Proteins Can Be Altered by Cooking

The allergenicity of egg proteins was reflected by their capacity to bind specific antibodies. The cooked egg proteins were incubated with pooled sera from 50 patients with egg allergy. As shown by figure 1b, two bands were generated by raw egg, and eggs treated by steaming, water boiling and frying, one at 30 kDa, the other at 15 kDa. No bands were generated by the proteins of the spiced egg and tea-boiled egg. However, as shown by the band at 30 kDa in figure 1b, water boiling and frying markedly increased the allergenicity while steaming also evidently reduced the integral density of the band. The

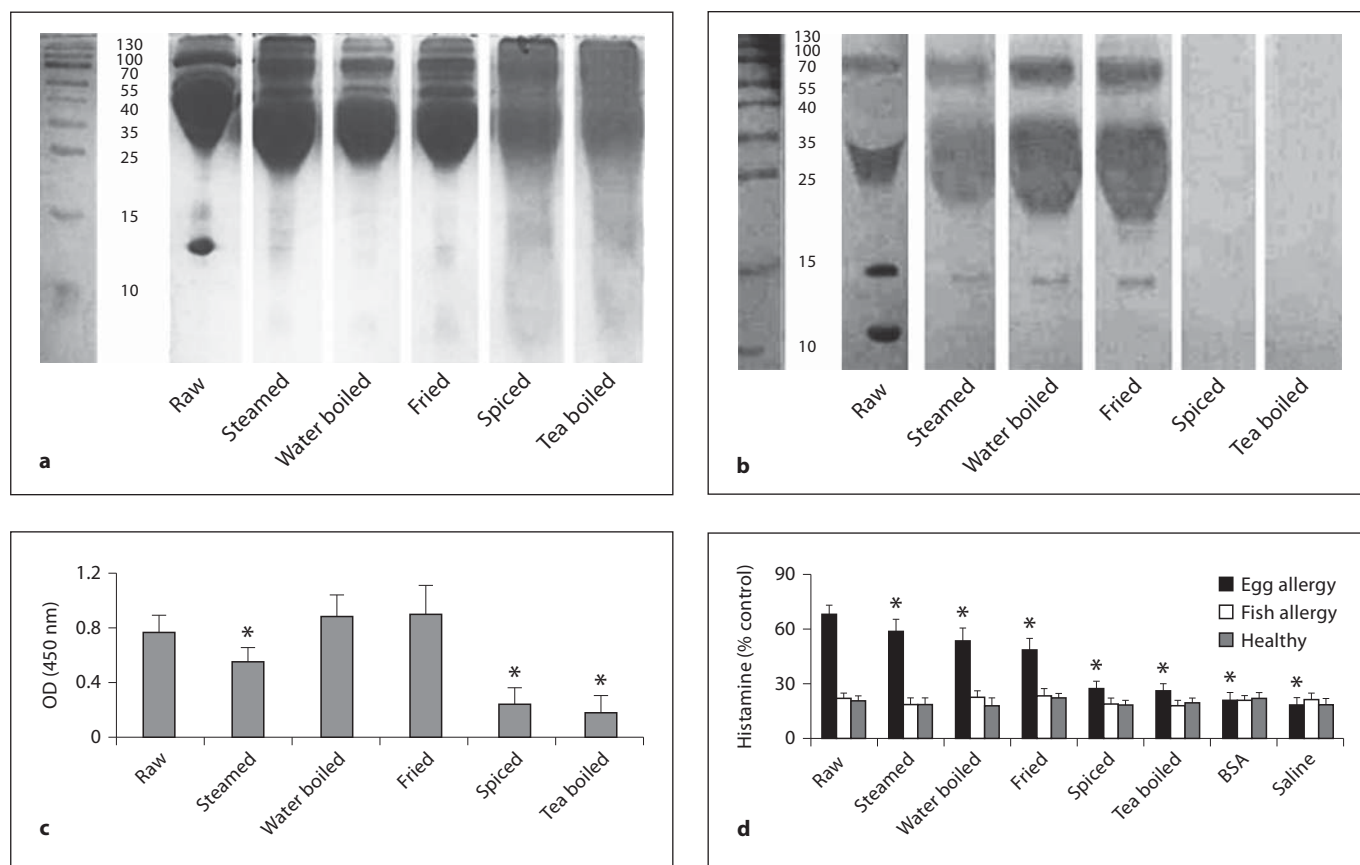


Fig. 1. Protein fractions of processed egg. **a** The egg proteins were processed as described in the text. The proteins (0.1 mg/well) were separated by SDS-PAGE. The gels were stained with Coomassie Brilliant Blue. The gel graph shows egg protein fractions. **b** The fractionated proteins on the gels in **a** were transferred onto nitrocellulose membrane and incubated in the presence of pooled human allergic sera for 1 h. The membrane was incubated with biotin-

conjugated anti-human IgE, and treated by streptavidin-conjugated peroxidase. Immune blots show processed egg proteins binding human IgE. **c** Egg proteins were processed in the same way as in figure 2 and analyzed by ELISA. Bars show the OD value at 450 nm. **d** The bars show the histamine release from basophils. Data are presented as means $\pm$ SD of 3–6 experiments. * $p < 0.05$, compared to raw egg.

data indicate that water boiling and frying do not alter much of the allergenicity of egg protein at 30 kDa; steaming, spicing or tea boiling can effectively reduce, at least partially, the allergenicity of egg protein at both 15 and 30 kDa. These results were confirmed by ELISA data (fig. 1c).

Eggs Processed by Different Cooking Approaches Have a Different Effect on Histamine Release from Basophils

Histamine release from basophils is shown as multiples (times) of the control. The addition of egg proteins from raw eggs or steamed, water-boiled or fried eggs significantly induced the histamine release from basophils of patients with egg allergy, but not from healthy subjects

or patients sensitized to fish. The protein extracts from spiced eggs and tea-boiled eggs induced much less histamine release as compared with above four types of egg extracts ($p < 0.01$) (fig. 1d).

EAST Inhibition

EAST inhibition was performed to determine the allergenicity of the processed egg proteins. A serum pool of 50 individuals with egg allergy was used in the EAST inhibition. The binding curves for competitive inhibition are shown in figure 2. The inhibitory effect of IgE-binding activities enhanced with increasing concentrations of competitive proteins.

As shown by table 1, the higher the C_{50} value, the lower the allergenic potential of the egg protein extract. The

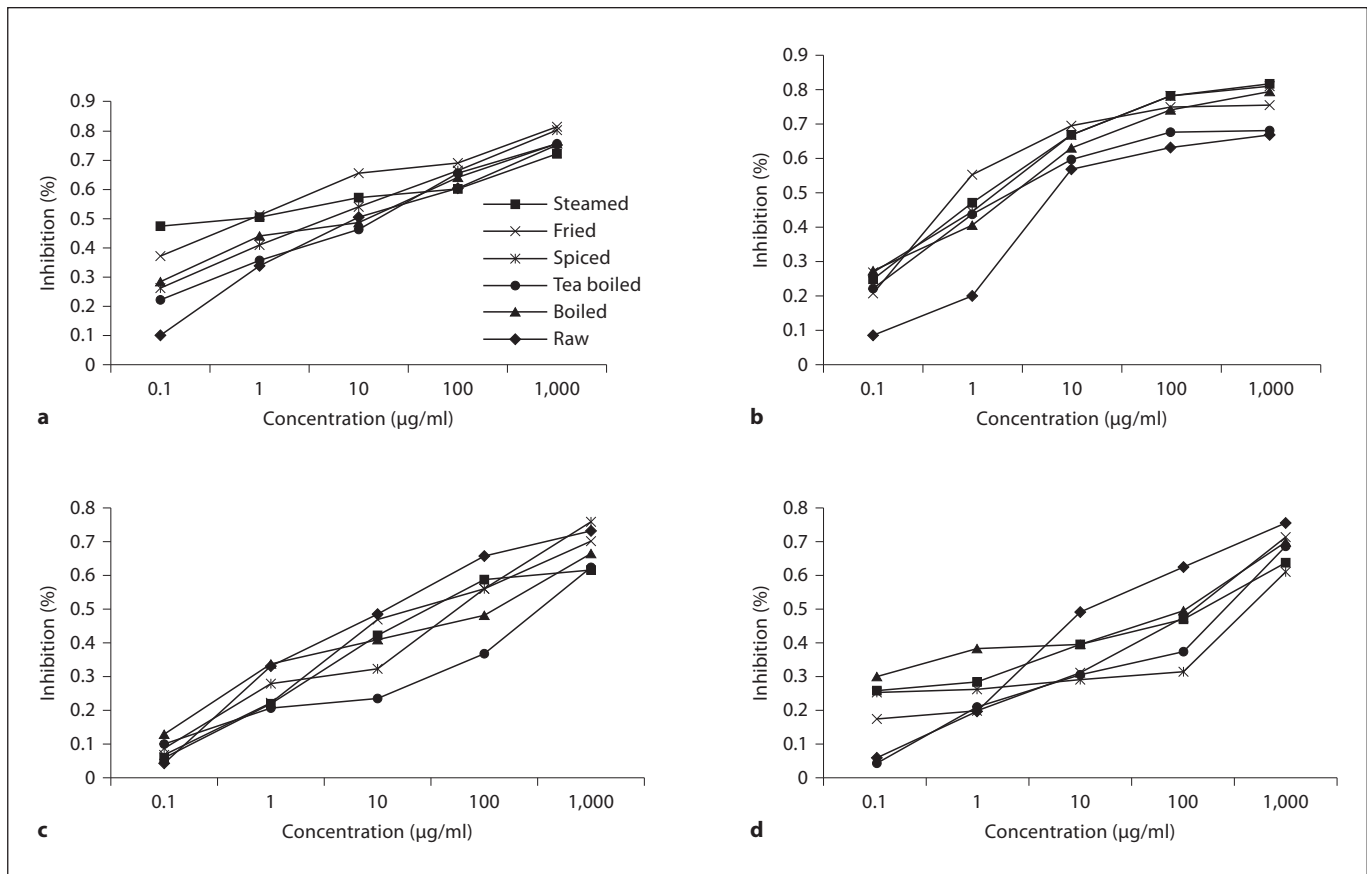


Fig. 2. EAST results. EAST was performed as described in the Material and Methods section. The curves indicate the inhibitory efficiency for graded allergen concentrations. The data represent 3 separate experiments. **a** Ovomucoid. **b** Ovalbumin. **c** Ovotransferrin. **d** Lysozyme.

Table 1. C_{50} values of the EAST inhibition experiments

Products processed	C_{50} value, µg/ml			
	ovomucoid	ovalbumin	ovotransferrin	lysozyme
Raw egg	3.26	3.43	3.30	3.40
Steamed egg	1.81	2.34	3.81	4.00
Boiled egg	2.82	2.60	3.79	3.52
Fried egg	1.99	2.26	3.54	3.92
Spiced egg	2.73	2.37	3.59	5.03
Tea-boiled egg	3.09	2.80	4.59	4.24

C_{50} values of boiled, spiced and tea-boiled eggs were higher than those of steamed and fried eggs. The C_{50} value of ovalbumin and ovomucoid was the lowest in the four major egg proteins of fried egg and steamed egg, indicating the highest binding capacity to human IgE. The C_{50} val-

ues of ovotransferrin and lysozyme were quite low among the four major egg proteins of raw egg, which is in line with the results of the Western blot in figure 1b and ELISA in figure 1c.

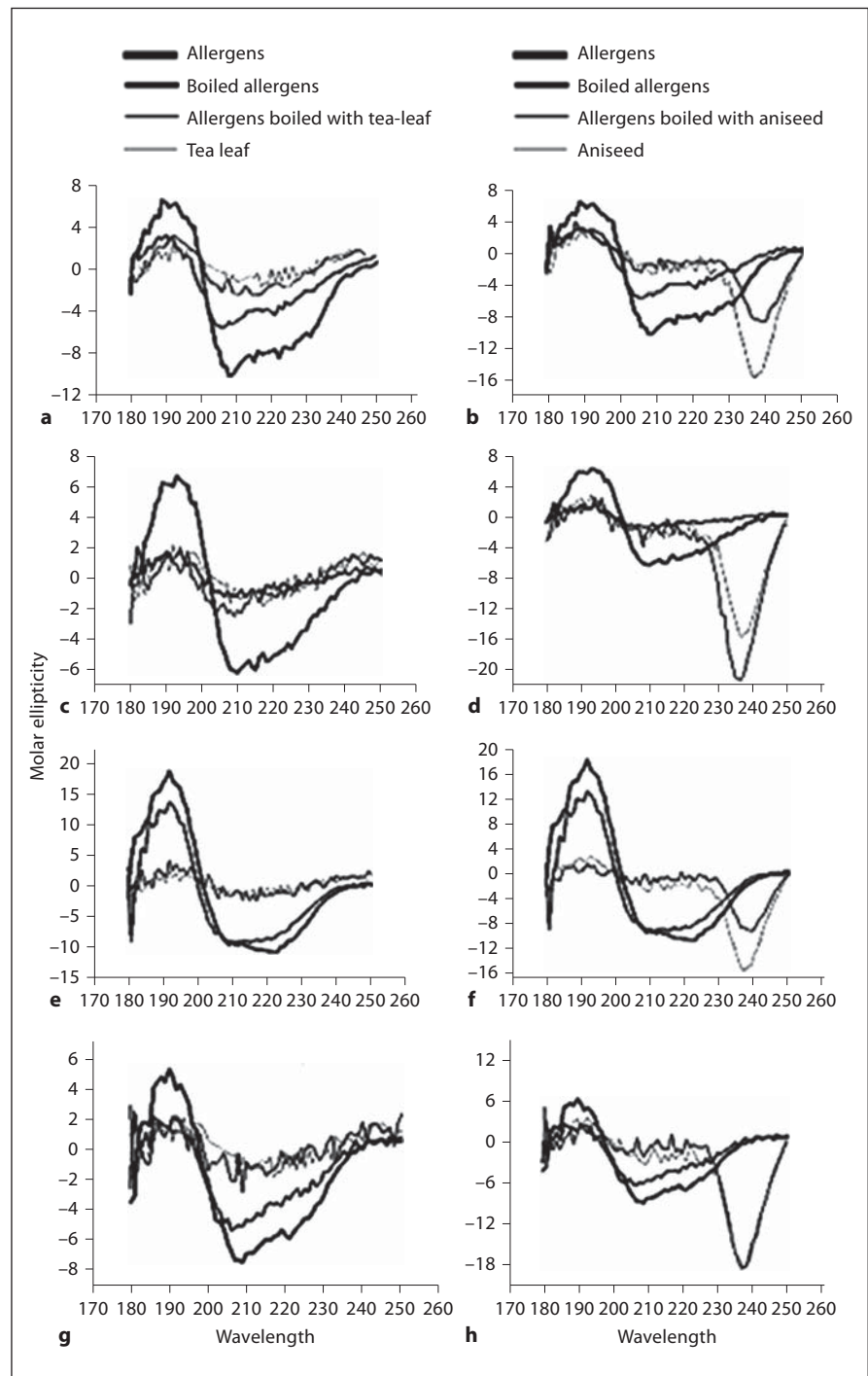


Fig. 3. CD spectra of allergens from cooked egg proteins. **a, b** Lysozyme. **c, d** Ovotransferrin. **e, f** Ovalbumin. **g, h** Ovomucoid.

We then examined the C_{50} value of the inhibition of the antigenicity of the allergen components. The results indicated an inhibitory effect on the four major egg proteins. The C_{50} values of ovalbumin and ovomucoid were relatively low in the processed egg products, which dis-

play higher levels of allergenicity of human IgE than for ovotransferrin and lysozyme; the latter showed relatively high C_{50} values. The results imply that ovotransferrin and lysozyme are more sensitive to heat denaturation than ovalbumin and ovomucoid.

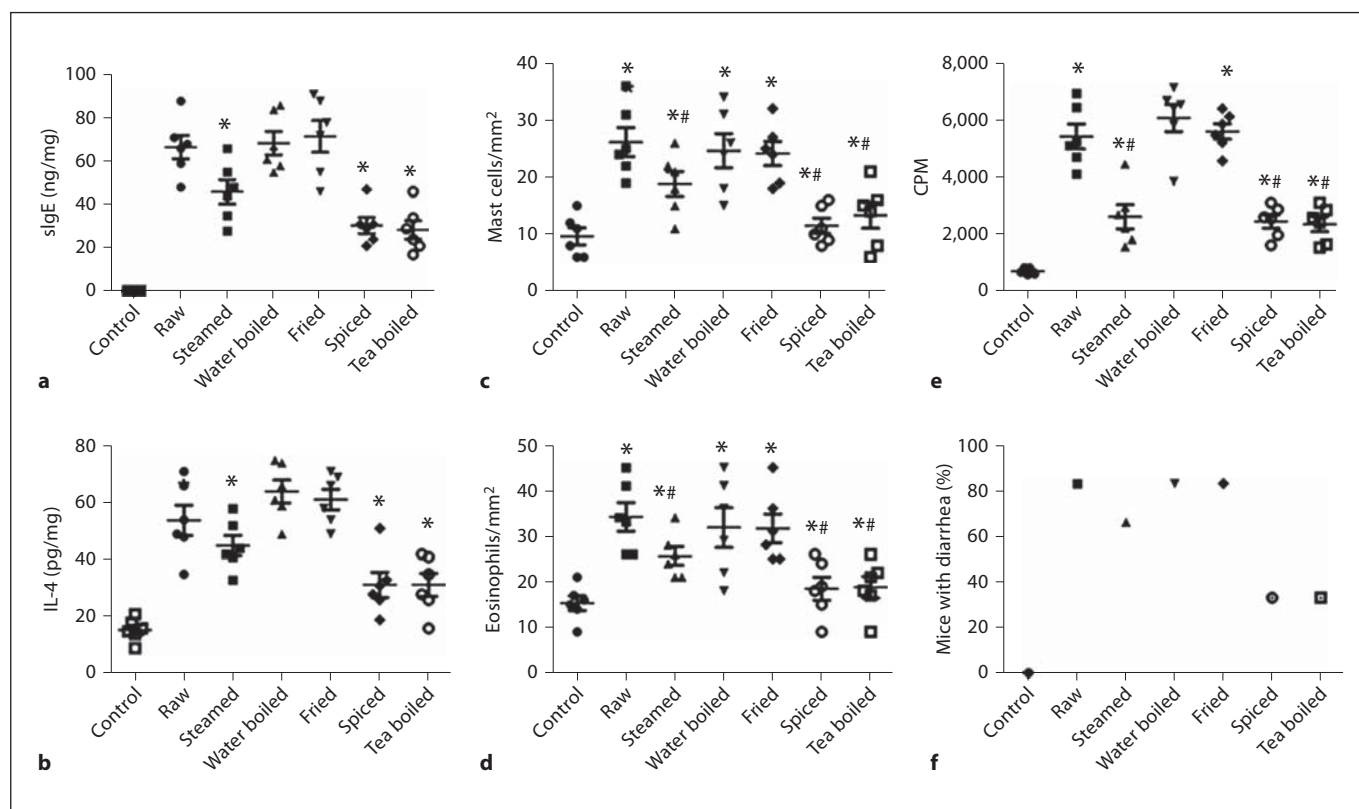


Fig. 4. Cooked egg proteins alter food allergic responses in the gut. BALB/c mice were sensitized to raw egg protein and orally challenged to cooked egg proteins. **a, b** Proteins were extracted from jejunal segments and quantified by the Radford method. The scatter dot plots show levels of specific IgE (**a**) and IL-4 (**b**) in gut extracts. **c, d** Jejunal tissue was processed for counting eosinophil and mast cell numbers. Each dot represents an average count from 20 fields of each mouse. **e** Mononuclear cells were isolated from

the small intestine and cultured in the presence of specific antigen egg protein for 4 days. ^3H tritium was added to the culture for the last 16 h. Each dot represents the average from 3 counts in each mouse. **f** Diarrhea was observed in mice after challenge with cooked egg proteins. The dots indicate the number of mice with diarrhea after the antigen challenge. * $p < 0.05$, compared with raw egg (**a**) or saline (control; **b–f**). # $p < 0.05$, compared to raw egg.

CD Analysis of the Allergen Structural Alterations

A CD spectrum, a powerful means of studying the protein structure, was used to analyze the structural changes in egg white allergens after different treatments. Figure 3 shows the results of CD spectra for the four main egg proteins from untreated, water-boiled, spiced and tea-boiled egg, respectively. As shown in figure 3, the different egg white allergens exhibit different degrees of thermal denaturation. After boiling for 30 min, thermal denaturation of ovotransferrin was complete and almost all its secondary structures were basically eliminated whereas most of the secondary structure of ovalbumin remained; its tertiary structure changed from an $\alpha + \beta$ form to an α/β form. As to lysozyme and ovomucoid, the secondary structures were partially preserved; the remaining secondary structures have a P2 left-handed heli-

cal conformation since both of them showed CD signals with a negative peak at about 206 nm [11].

As shown in figure 3, almost all secondary structures of the four egg white allergens were lost in aniseed egg or tea-boiled egg. This suggests that there may be active interactions between the four egg white allergens and aniseed or tea-leaf, leading to complete denaturation of the four egg white allergens, and that, theoretically, both aniseed and tea-leaf can totally eliminate the immunogenicity of the four egg white allergens. This could be the reason why there is an obvious decrease in the immunogenicity of tea-boiled egg and spiced egg compared with water-boiled, steamed and fried egg. On the other hand, because both tea-boiled and spiced eggs are prepared by direct boiling with tea leaf or aniseed, the immunogenicity of tea-boiled and spiced eggs still partially remained

rather than being completely eliminated, which was also supported by the results of Western blot, ELISA and EAST inhibition experiments.

Cooked Egg Proteins Show Different Effects in Allergic Reactions

The allergenicity of cooked egg proteins was further tested on an animal model of food allergy. The sensitized mice were challenged with egg proteins processed by one of the cooking processes listed in figure 1a. As depicted in figure 4, after challenge with raw egg proteins, the sensitized mice showed high levels of specific IgE (fig. 4a) and IL-4 (fig. 4b) in the extracted intestinal proteins, and a high frequency of mast cells (fig. 4c) and eosinophils (fig. 4d) in intestinal tissue; antigen-specific T cell proliferation was observed in isolated intestinal T cells (fig. 4e) and diarrhea was observed in 80–100% sensitized mice (fig. 4f). The results indicate that an egg-specific allergic hypersensitivity was induced in the intestine. When the animals were challenge with steamed, spiced and tea-boiled egg proteins, the allergic responses were significantly decreased in the intestine as compared to those of animals challenged with raw egg protein. In animals challenged with the proteins from water-boiled or fried egg, the intestinal allergic responses were similar to those induced by raw egg protein. It is worthwhile pointing out that in animals challenged with the proteins from steamed, spiced or tea-boiled egg, the clinical symptoms (diarrhea in the present study) induced only weak symptoms while more than 80% of the sensitized mice developed diarrhea in response to raw egg protein challenge.

Discussion

Eggs are used in a number of food formulations because of its excellent properties for the production of convenient food products. Therefore, eggs have become desirable ingredients in many food types, such as bakery products, meringues, meat products and cookies. Because of their marked allergenicity, a subpopulation of people suffers from egg allergy and has to avoid ingesting egg-containing foods. The present study shed light on food allergen-related disorders by showing that the allergenicity of egg allergens can be reduced by employing appropriate cooking methods.

Researchers have tried to reduce or eliminate the allergenicity of egg proteins, by treating egg foods with pasteurization (67°C). Unfortunately, most of the endeavors were unsuccessful [12]. It was reported that protein from

processed egg was significantly lower than that of raw egg [13]. It provides a safe and specific way of processing egg white. In our study, using Chinese traditional cooking processes, including steaming, water boiling, frying and tea boiling, we achieved similar results; by treating egg with tea boiling or spicing, the contents of egg protein were markedly reduced; however, they were not evidently reduced after water boiling and frying. It seems that substances such as tea used in the present study or addition of spice powder in the boiling tea play a role in degrading the egg protein.

Modifying the allergenicity of antigen also has the potential to attenuate allergic disease symptoms. A number of approaches have been tried to reduce the antigenicity of proteins. Bu et al. [14] indicated that fermentation with lactic acid bacteria could significantly reduce the antigenicity of α -LA and β -LG in skim milk; combined strains of *Lactobacillus helveticus* and *Streptococcus thermophilus* were even more effective. The influence of heat treatment and enzymatic digestion on the allergenicity of purified egg proteins either alone or in combination has been described by other investigators [12]. Our data reveal that Chinese traditional cooking approaches can also reduce the allergenicity of egg protein and thus represent a novel pathway to modulate the allergenicity of food allergens.

The results in figure 1a show that the proteins of spiced and tea-boiled egg were degraded and shifted to smaller molecular mass fragments. It can be speculated that higher temperature breaks the hydrogen bond in egg protein molecules, resulting in polypeptide cleavage. Furthermore, the stability of the protein structure was significantly reduced by spice powder or tea boiling (fig. 1a, c). Moreover, CD spectrum analysis demonstrated that the secondary structure of the 4 main egg allergens was significantly modified in eggs processed by tea and spiced tea boiling (fig. 1a, b). About 90% of the protein epitopes are conformation epitopes, while 10% are linear epitopes. Most of protein conformation epitopes are consecutive epitopes or nearly consecutive amino acid sequences. This may result in resistance to protein degradation and denaturation of the four main egg allergens.

Low IgE-binding potential with pooled sera was detected in allergens from water-boiled egg. Due to the aniseed and tea treatment of samples from spiced tea- and tea-boiled egg, protein degradation may lead to low allergenicity. The conformational structure of allergens in samples from steamed egg and fried egg may change as a result of the loss of the tertiary structure during heat treatment [15].

Eggs are used as a nutritious food for infants and young children in China. Spiced egg and tea-boiled egg are usually consumed as daily snacks. The data gathered from this study of the allergenic properties of different Chinese traditional egg foods, suggest that due to their low allergenic potential, tea-boiled egg and spiced egg may be an optimal additional food for infants and young children. On the other hand, consumption of spiced egg and tea-boiled egg may have the potential to reduce the clinical symptoms of episodes of food allergy. This inference is supported by our food allergy animal model in which the sensitized mice showed significantly lower Th2 responses and clinical symptoms when challenged with spiced or tea-boiled egg or steamed egg protein than those challenged with egg proteins from raw, water-boiled or fried egg. Others also noted that using heated egg antigens can induce intolerance in patients with egg allergy or enhance the digestive effect on egg allergens [16, 17].

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Disclosure Statement

None of the authors have a conflict of interest to disclose.

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