



Structural changes of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) treated shrimp tropomyosin decrease allergenicity

Liangtao Lv^a, Hong Lin^a, Zhenxing Li^{a,*}, Balunkeswar Nayak^b, Ishfaq Ahmed^a, Shenglan Tian^a, Guanzhi Chen^c, Hang Lin^c, Jinxia Zhao^c

^a Food Safety Laboratory, College of Food Science and Engineering, Ocean University of China, Qingdao 266003, PR China

^b Food Science and Human Nutrition, School of Food and Agriculture, University of Maine, Orono, ME 04469, USA

^c The Affiliated Hospital of Qingdao University, Qingdao 266003, PR China

ARTICLE INFO

Chemical compounds studied in this article:

1-Anilino-8-naphthalene-sulfonate (PubChem CID: 1369)

3,3',5,5'-Tetramethylbenzidine (PubChem CID: 41206)

DL-Dithiothreitol (PubChem CID: 446094)

Brilliant Blue R (PubChem CID: 61365)

Acrylamide (PubChem CID: 6579)

DL-lysine (PubChem CID: 866)

L-leucine (PubChem CID: 6106)

Keywords:

Allergenicity

Shrimp tropomyosin

AAPH

Protein oxidation

ABSTRACT

The aim of this study was to analyze the effect of AAPH on the conformational structure and allergenicity of shrimp tropomyosin (TM). The structure of AAPH-TM was evaluated by SDS-PAGE, fluorescence, circular dichroism (CD) and ultraviolet light (UV), and the allergenicity was evaluated by *in vivo* and *in vitro* methods. Results showed that AAPH can induce structural changes through TM aggregations. These aggregations can decrease the IgG/IgE binding capacity on immunoblot analysis. Further competitive inhibition ELISA (ciELISA) results showed the IC₅₀ of AAPH-TM (AAPH 0–25 mmol/l) changed from 0.086 to 46.2 µg/ml, which correlated with TM structural changes. An RBL-2H3 cell assay showed that release rate of β-hexosaminidase and histamine decreased by 51.6% and 68.1% with AAPH (5 mmol/l) treatment, respectively. A mouse model showed AAPH-TM could decrease levels of IgE/IgG1, release of histamine and mMCP-1 in sera. In conclusion, AAPH induced TM aggregation can cause structural changes and decrease the allergenicity.

1. Introduction

Shrimp is a popular seafood consumed around the world because of its delicious and ample nutritional properties. However, it belongs to one of the big 8 food allergens, which are associated with IgE-mediated hypersensitivity (Sicherer & Sampson, 2006). The clinical features of allergy are expressed as immediate symptoms involving the skin, respiratory tract and gastrointestinal tract. Thus, it can further lead to severe systemic anaphylactic reactions (Takács et al., 2014).

Many researchers have reported a change of allergenicity during food processing. For instance, it has been shown that different thermal and non-thermal methods including heating, enzymatic treatment, microwave, high pressure, and irradiation have different effects on food allergens (Ahmed et al., 2018; Zellal, Kaddouri, 2011; Nowak-Węgrzyn, Fiocchi, 2009; Long, Yang & Wang, 2015). However, the influencing factors are complex due to the large number of chemical reactions during food processing, and it is not enough to clarify the changes of food allergens. Based on the previous research, it is a good idea to

evaluate the mechanism of shrimp allergenicity effects from the perspective of chemical reactions that normally occur during food processing. Furthermore, as a kind of aquatic product, shrimp contains high contents of unsaturated fatty acids and is extremely prone to oxidation during processing and preservation (Becerra et al., 2014).

TM, a 36-kDa salt-soluble protein, has been identified as a major shrimp allergen (Ayuso, Lehrer & Reese, 2002), and was found to be involved in allergic reactions in different species of shrimp (Srinroch, Srisomsap, Chokchaichamnankit, Punyarit & Phiriyangkul, 2015; Gámez et al., 2014). In our previous study, we demonstrated that polyunsaturated fatty acid peroxidation products such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) and acrolein significantly altered the structure and IgE/IgG binding capacity of shrimp tropomyosin by binding with its amino acid residues (Song et al., 2015; Lv et al., 2016; Lv et al., 2017). Polyunsaturated fatty acid peroxidation is a free radical-mediated complex chain reaction, however, the structure and allergenicity effects of shrimp caused by peroxy radicals are still unclear. Wu, Zhang, Kong, and Hua (2009) reported that under

* Corresponding author.

E-mail address: lizhenxing@ouc.edu.cn (Z. Li).

<https://doi.org/10.1016/j.foodchem.2018.09.030>

Received 11 February 2018; Received in revised form 1 August 2018; Accepted 3 September 2018

Available online 05 September 2018

0308-8146/ © 2018 Elsevier Ltd. All rights reserved.

aerobic condition, the oxidative modification of soy protein occurs by peroxyl radicals generated in a solution containing 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). These modifications are known to further induce changes in structure and physicochemical properties. Based on this study, it was hypothesized that such oxidative modifications could affect the structure of TM and thus influence the allergenicity.

In the present study, we test the hypothesis that AAPH treated TM (AAPH-TM) of shrimp could induce structural changes and affect the allergenicity. The study of structural modification included SDS-PAGE, carbonyl group, free amine group, available lysine, surface hydrophobicity, intrinsic fluorescence, CD and UV analyses. The IgG/IgE binding activity was analyzed by immunoblot analysis and assessed by ciELISA with rabbit polyclonal antibodies and with sera from patients with shrimp allergies. The digestion stability of AAPH-TM was analyzed by simulated gastrointestinal digestion. In addition, the allergenicity of AAPH-TM was evaluated using an RBL-2H3 cell model and mouse model. This research work will help to better understand the changes in shrimp allergic properties during shrimp processing and preservation caused by lipid oxidation.

2. Materials and methods

2.1. Materials and reagents

Shrimp TM was purified in the food safety lab, Ocean University of China (Qingdao, China). AAPH, Bovine serum albumin (BSA), 1-anilino-8-naphthalene-sulfonate (ANS), 2, 4-dinitrophenylhydrazine (DNPH), 2, 4,6-trinitrobenzenesulfonic (TNBS), and 3, 3', 5, 5'-tetramethylbenzidine (TMB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The precision protein standard was purchased from Solarbio Co. (Beijing, China). The enhanced chemiluminescent (ECL) kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Secondary antibodies coupled with horseradish peroxidase (HRP) were purchased from Zhongshan Jinqiao Co. (Beijing, China). ELISA kits for histamine, cysteinyl leukotriene, prostaglandin D₂, mMCP-1 were purchased from R&D Systems (Minneapolis, MN, USA). Unless otherwise stated, all chemicals were of analytical grade.

2.2. Serum samples of shrimp-allergic patients

The serum was supplied by the Affiliated Hospital of Qingdao University (Qingdao, China). Shrimp specific IgE levels were above 0.5 kUA/l. The serum IgE levels were assessed using the ImmunoCAP system (Phadia AB, Uppsala, Sweden). The patient records showed evidence of anaphylaxis with clear exposure-related symptoms. After informed consent was acknowledged, serum samples were obtained and the sera were frozen at −80 °C until use. The protocol was approved by the local medical ethics committee (File No. QD-PF-20171126396) to fully protect the rights and interests of the human subjects.

2.3. Reaction of TM with AAPH

The reaction of TM with AAPH was prepared based on the method of Wu et al. (2009) with some modifications. Shrimp TM samples were diluted to 10 mg/ml in 10 mM phosphate buffer (pH 7.4). The samples were mixed with AAPH solution (v: v = 1:1) at 25 °C for 24 h. During the experiment, the final concentration of AAPH was 0 mmol/l (control), 0.2 mmol/l, 1 mmol/l, 5 mmol/l, and 25 mmol/l, respectively. After reaction of TM with AAPH, the resulting solution was dialyzed against PBS (pH 7.4) for 72 h at 4 °C to remove unreacted AAPH, and then lyophilized and stored at −40 °C until use. The total protein concentration in each step was determined with bovine serum albumin as a standard.

2.4. SDS-PAGE analysis

SDS-PAGE was performed with the method of Laemmli et al. (1970) with some modification. The samples were preheated for 7 min at 100 °C. Electrophoresis was performed by 5% stacking gel at 80 V for 40 min and 15% resolving gel at 120 V for 80–100 min, respectively. Separated protein bands on the gel were stained with Coomassie Brilliant Blue R-250 and then destained until the solution was clear. Protein bands were determined by using Tanon-4200SF gel imaging and analysis software (Tanon Science and Technology Co., Ltd, Shanghai, P. R. China).

2.5. Effect of IgE binding capacity of TM with AAPH treatment

2.5.1. Western blot

Protein samples were separated with SDS-PAGE and electro-phoretically transferred onto pore-sized polyvinylidene fluoride (PVDF) membrane by an iBlot2 dry transfer system (Thermo Fisher Scientific Inc. Waltham, MA, USA) for 7 min at room temperature, and the transfer efficiency was determined by prestained protein marker. The membrane was washed with iBind solution kit, and then incubated in iBind solution kit with IgG (rabbit serum, diluted 1:2000) or IgE (human serum, diluted 1:4) as the primary antibody and HRP labeled anti-IgG (diluted 1:2000) or anti-IgE (diluted 1:200) as the secondary antibody for 4 h at room temperature in total. After being washed with deionized water, the results were visualized with ECL with multiple exposure times to achieve an optimal signal by using a gel rapid documentation apparatus (Tanon-4200SF, Tanon Science & Technology Co., Ltd, Shanghai, P. R. China).

2.5.2. Competitive inhibition ELISA (ciELISA)

The IgE binding capacity of samples was evaluated by ciELISA, following an established method from our previous research (Lv et al., 2016). The inhibition rate was calculated using the following formula:

$$\text{Inhibition rate (\%)} = \frac{[OD_{\text{no inhibitor}} - OD_{\text{inhibitor}}]}{[OD_{\text{no inhibitor}}]} \times 100\%$$

2.6. Structure analysis

2.6.1. Carbonyl group

Protein carbonyl content was quantified based on the method of Huang (2006), which was formed by the interaction with DNPH. The amounts of carbonyl were expressed as nmol of carbonyl groups per milligram of soluble protein with a molar absorbance coefficient of 22,000 L/mol cm.

2.6.2. Free amine group

Free amine group content was evaluated by combining with TNBS, following previous research with some slight modifications (Blanco-Pascual et al., 2013). The content of free amine groups was determined in terms of μmol/mg, and L-leucine was used as the standard.

2.6.3. Available lysine

The method described by Ou (2004) was applied to measure the available lysine, using TNBS. The amount of available lysine in each sample was represented as g/100 g and DL-lysine monohydrochloride was used as the standard.

2.6.4. Measurement of surface hydrophobicity

The surface hydrophobicity of TM and AAPH-TM was determined using ANS as a fluorescence probe, as described by Kato and Nakai (1980).

2.6.5. Intrinsic fluorescence measurement

Based on the method of Wu and co-workers (2010), the intrinsic fluorescence of TM and AAPH-TM in phosphate buffer (0.01 mol/l, pH 7.4) was determined at 25 °C with an RF-5301PC fluorescence spectrometer (Shimadzu, Kyoto, Japan).

2.6.6. Ultraviolet (UV) absorption spectral analysis

The UV absorption spectral analysis was performed with the UV-1101 system (Techcomp Shanghai, China). The UV absorption spectra were scanned from 230 to 330 nm with all the samples at the concentration of 0.5 mg/ml.

2.6.7. Secondary structure analysis using circular dichroism (CD) spectroscopy

In order to define the secondary structure of TM and AAPH-TM, CD spectroscopy was performed in the spectral range of 190–260 nm using a JASCO J-815CD spectropolarimeter (Japan Spectroscopic Co. Inc., Japan) at room temperature (Kelly, Jess & Price, 2005) with TM concentration adjusted to 1 mg/ml. The operating parameters were as follows: scan rate, interval, and bandwidth were set to 100 nm/min, 0.25 s, and 1.0 nm, respectively.

2.7. In vitro digestion assay

2.7.1. Simulated gastric fluid (SGF) digestion stability assay

The digestibility of AAPH-TM (AAPH 5 mmol/l) was determined following the protocol described by Huang (2010) with minor modifications. The SGF was prepared accordingly as described in the United States Pharmacopoeia (1995) and was composed of porcine pepsin in 25 mM NaCl at pH 1.2. The final samples were analyzed by SDS-PAGE and western blot. IgG (rabbit serum) and IgE (human serum) were used as the primary antibodies, and HRP labeled anti-IgG and anti-IgE were used as the secondary antibodies respectively.

2.7.2. Simulated intestinal fluid (SIF) digestion stability assay

The SIF for intestinal digestion was also prepared according to the United States Pharmacopoeia (1995) and was composed of trypsin in 0.05 M KH_2PO_4 at pH 7.5. The samples were analyzed by SDS-PAGE and western blot. IgG (rabbit serum) and IgE (human serum) were used as the primary antibodies, and HRP labeled anti-IgG and anti-IgE were used as the secondary antibodies, respectively.

2.8. Allergenicity in RBL-2H3 cells

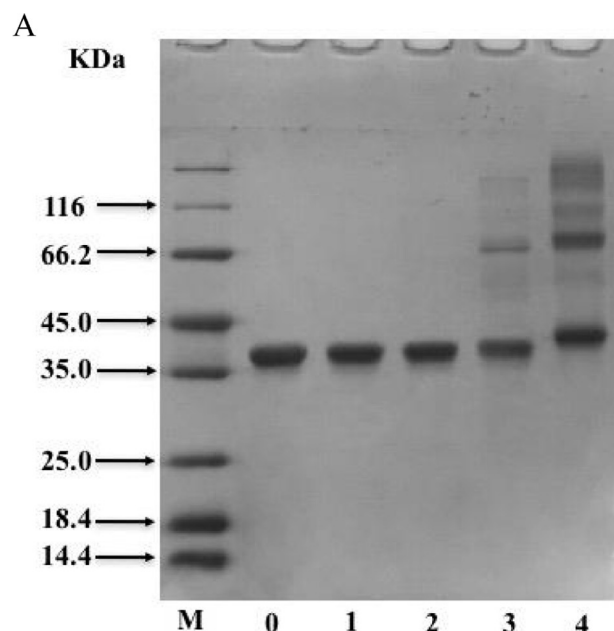
The rat mast cell line RBL-2H3 were obtained from the Chinese Academy of Sciences and grown in minimum essential medium with Eagle's salts (EMEM) (Hyclone, Logan, Utah, USA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, New York, USA) at 37 °C in a humidified incubator with a 5% CO_2 /95% air atmosphere (Thermo, Waltham, MA, USA) adopting a previously established method by Hoffmann et al. (1999).

RBL-2H3 cells were harvested at the stationary phase, seeded in 96-wells plates with 5×10^5 cells, passively sensitized with human serum IgE from shrimp-allergic children for 15 h and further washed three times with Tyrode's buffer. For the release of β -hexosaminidase described by Kuehn et al (2010), RBL-2H3 cells were stimulated with 500 ng/ml either TM or AAPH-TM (AAPH 5 mmol/l) in Tyrode's buffer at 37 °C. Tyrode's buffer only treated cells were employed as a negative control. The release of histamine, cysteinyl leukotriene and prostaglandin D2 was required for 10 min and analyzed by ELISA assay kits, following the manufacturer's instructions.

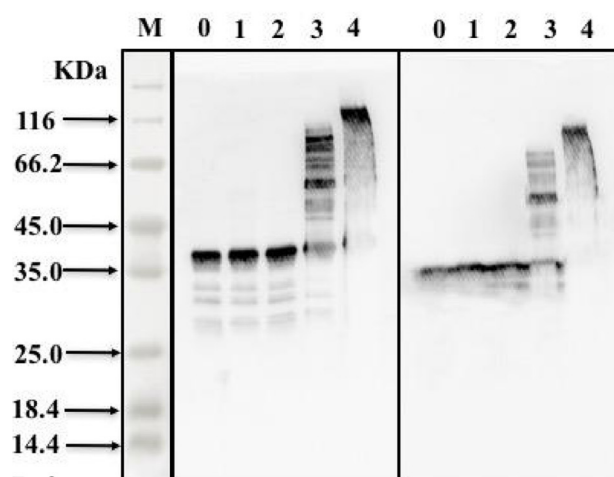
2.9. In vivo studies

2.9.1. Animals and sensitization

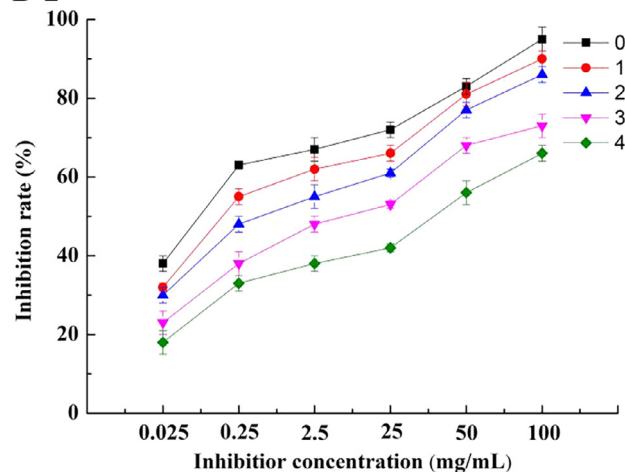
Specific pathogen-free (SPF) female BALB/c mice (five weeks old,



B-1



B-2



(caption on next page)

Fig. 1. (A) SDS-PAGE analysis of AAPH-TM. An equal amount of TM (5 μ g) was loaded into each lane; (B) Analysis of the potential allergenicity of AAPH-TM. (B-1) IgG/IgE binding capacity of AAPH-TM in immunoblot analysis, using rabbit sera (IgG) and pooled sera (IgE) from patients allergic to shrimp. (B-2) cELISA for detecting the IgE binding capacity of AAPH-TM, using the pooled sera of the patients in (B-1). The molecular mass of the standard protein marker ranged from 14.4 to 116 kDa. Lane M: Protein marker; Lanes 0–4 represent the AAPH concentrations 0, 0.2, 1, 5, and 25 mmol/L, respectively.

Table 1

Protein carbonyl, free amine, available lysine, and surface hydrophobicity of shrimp tropomyosin incubated with increasing concentration of AAPH at 25 °C for 24 h.

AAPH (mmol/L)	Protein carbonyl (nmol/mg)	Free amine (μ mol/mg)	Available lysine (g/100 g)	Surface hydrophobicity
0	1.66 (0.13) A	4.28 (0.1) A	4.26 (0.14) A	6.28 (1.24) A
0.2	2.63 (0.12) B	3.78 (0.09) B	4.18 (0.12) A	18.8 (1.16) B
1	3.26 (0.13) C	3.51 (0.08) C	3.86 (0.1) B	26.3 (1.32) C
5	6.48 (0.16) D	2.86 (0.11) D	2.89 (0.12) C	47.6 (1.25) D
25	18.26 (0.13) E	2.46(0.09) E	2.23 (0.09) D	88.6 (1.03) E

weight 20 ± 2 g) were acquired from Jinan Laboratory of Animal Center (Jinan, China). The animals were housed under SPF conditions in an air-conditioned room (23 ± 2 °C, 12/12 h light/dark cycle, relative humidity of $55 \pm 5\%$) and allowed open access to food (without shrimp protein in the diet) and tap water. All experiments involving animals were performed following established guidelines and the experimental protocols were in accordance with the Ethical Committee of Experimental Animal Care of the Ocean University of China.

2.9.2. Sensitization to TM by intraperitoneal injection

Each mouse was sensitized with 200 μ g of TM or AAPH-TM (AAPH 5 mmol/L) ($n = 10$) adsorbed in 2 mg/ml Alum adjuvant by intraperitoneal (i.p.) injection on days 7, 14, 21 and 28 as described in Fig. 1A. In parallel, the normal group was administered with an i.p. injection of a 0.9% saline solution on days 7, 14, 21, and 28 of the experiment. To measure the level of specific antibodies, blood was collected on day 31. The TM-specific IgE and IgG1 serum level were evaluated using ELISA as described previously (Ma, Lozano-Ojalvo, Chen, Lopez-Fandiño, & Molina, 2015). The concentrations of histamine and mouse mast-cell protease-1 (mMCP-1) in serum obtained 1 h after i.p. injection on day 28, was detected using commercially available ELISA assay kits.

2.10. Statistical analysis

The obtained data were expressed as the mean $\pm$ standard deviation (SD) of triplicate determinations. Differences among experimental data were analyzed by an analysis of variance (ANOVA) using the SPSS Statistics 18 software (SPSS Inc., Chicago, USA). Statistical significance was defined at $p < 0.05$.

3. Results

3.1. SDS-PAGE

The TM aggregation was observed after treatment with AAPH (Fig. 1A). When AAPH concentration was 5 mmol/L, higher molecular bands were observed at approximately 72 kDa, 118 kDa and above (Fig. 1A, lane 3). The aggregation bands were much heavier with increasing AAPH concentration, while the intensity of the TM band at 36 kDa became lighter (Fig. 1A, lane 4) at the same time. Moreover, no polymers were observed after treatment with AAPH concentrations below 1 mM (Fig. 1A, lanes 1 and 2).

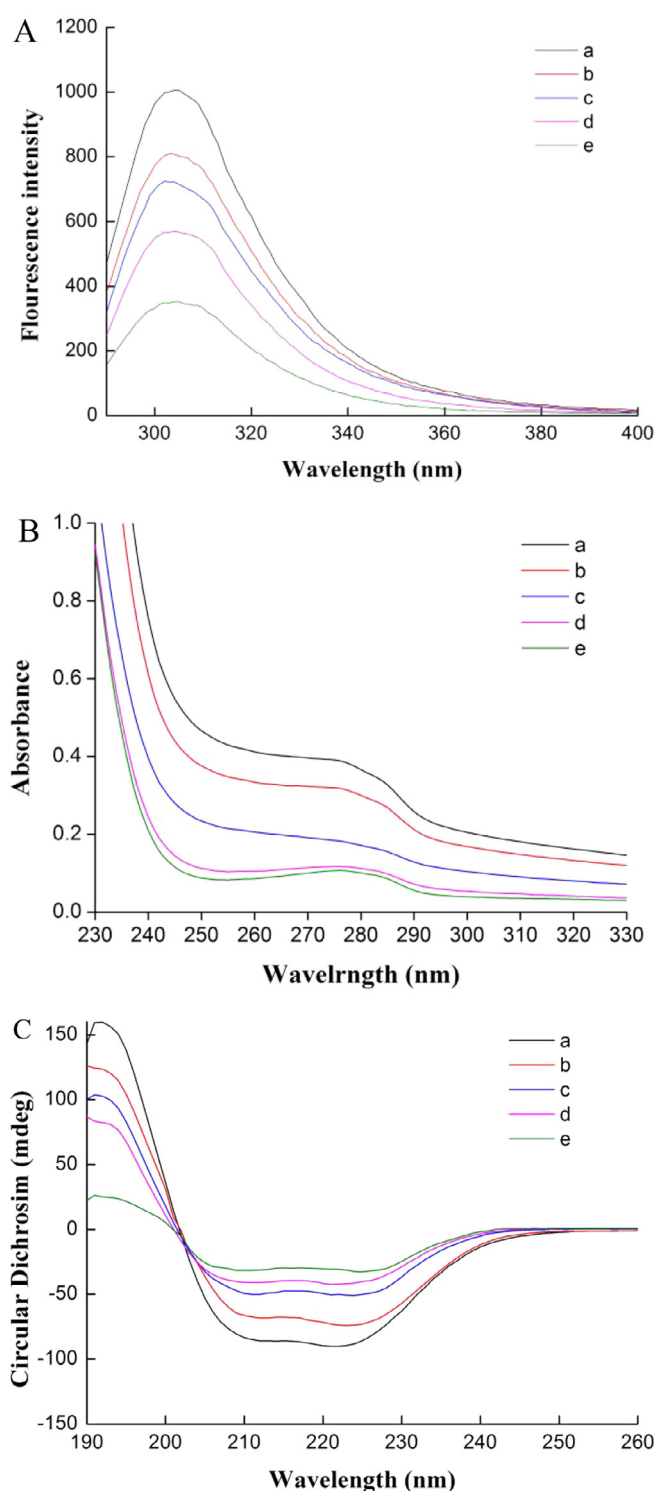


Fig. 2. Effect of increasing concentrations of AAPH treatment on the (A) intrinsic fluorescence, (B) UV absorption and (C) CD spectroscopy. 0: Control (untreated); 1: 0.2 mmol/L AAPH; 2: 1 mmol/L AAPH; 3: 5 mmol/L AAPH; 4: 25 mmol/L AAPH.

3.2. Analysis of IgG/IgE binding capacity

In order to estimate the effect of AAPH on potential allergenicity, western blot was performed to determine IgG and IgE binding capacity using rabbit polyclonal antibodies against shrimp TM and human sera from shrimp-allergic patients, respectively (Fig. 1B-1). The IgG/IgE binding capacity both decreased significantly following treatment with

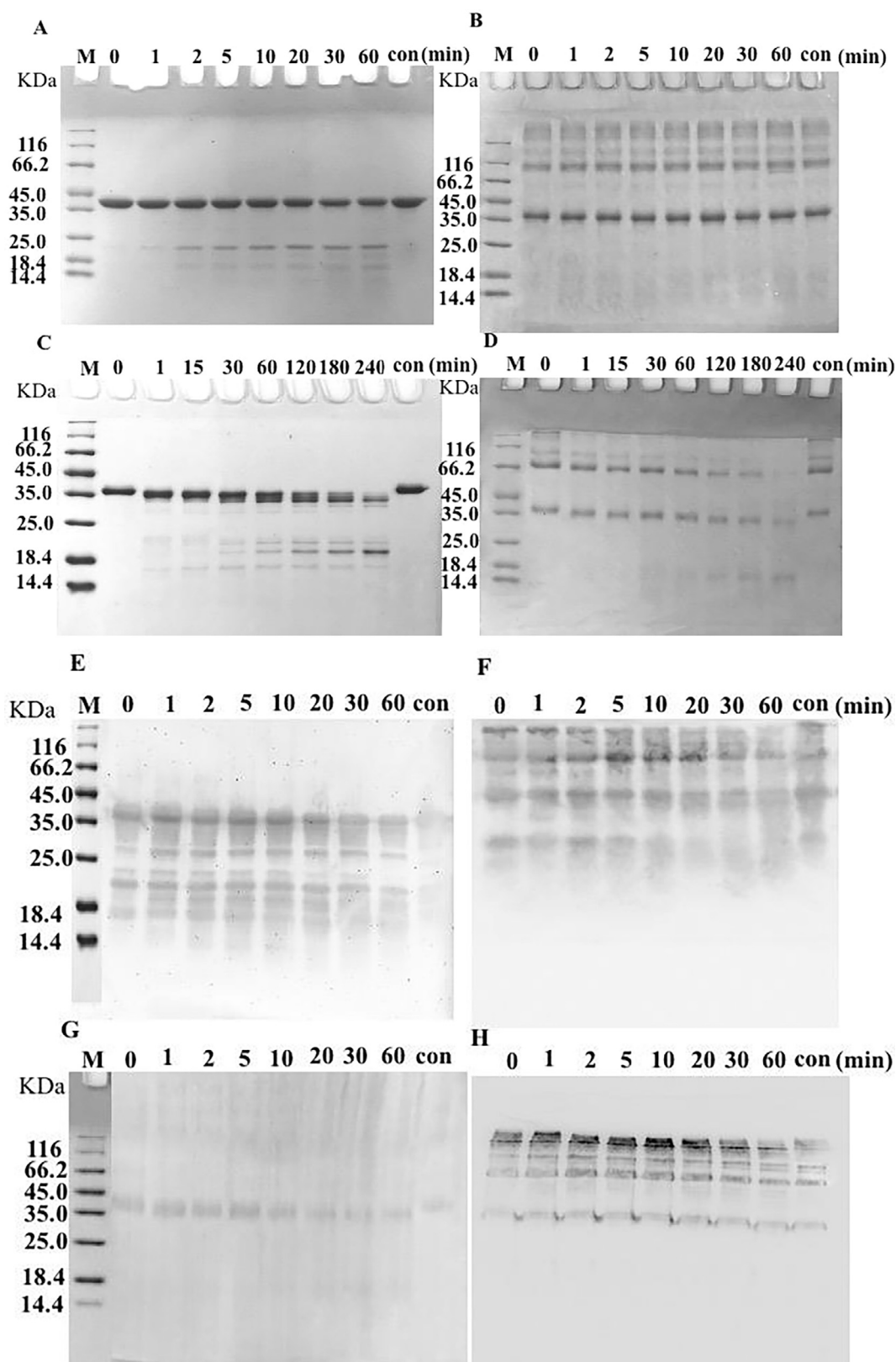


Fig. 3. Effect of simulated gastrointestinal fluid digestion on the purified shrimp TM treated with 5 mmol/L AAPH. Enzymatic digestion was performed, followed by SDS-PAGE. (A) The shrimp TM by pepsin; (B) AAPH-TM by pepsin; (C) The shrimp TM by trypsin; (D) AAPH-TM by trypsin. Enzymatic digestion was performed, followed by western blot analyses. The purified shrimp TM (E) and AAPH-TM (F) were detected using polyclonal antisera against shrimp TM with pepsin. The purified shrimp TM (G) and AAPH-TM (H) were detected using pooled sera from patients allergic to shrimp with pepsin. The purified shrimp TM (I) and AAPH-TM (J) were detected using polyclonal antisera against shrimp TM with trypsin. The purified shrimp TM (K) and AAPH-TM (L) were detected using pooled sera from patients allergic to shrimp with trypsin. In the controls (con), protease was replaced with 25 mmol/L PBS (pH 7.5). Molecular masses of the protein markers (M) are shown on the left edges.

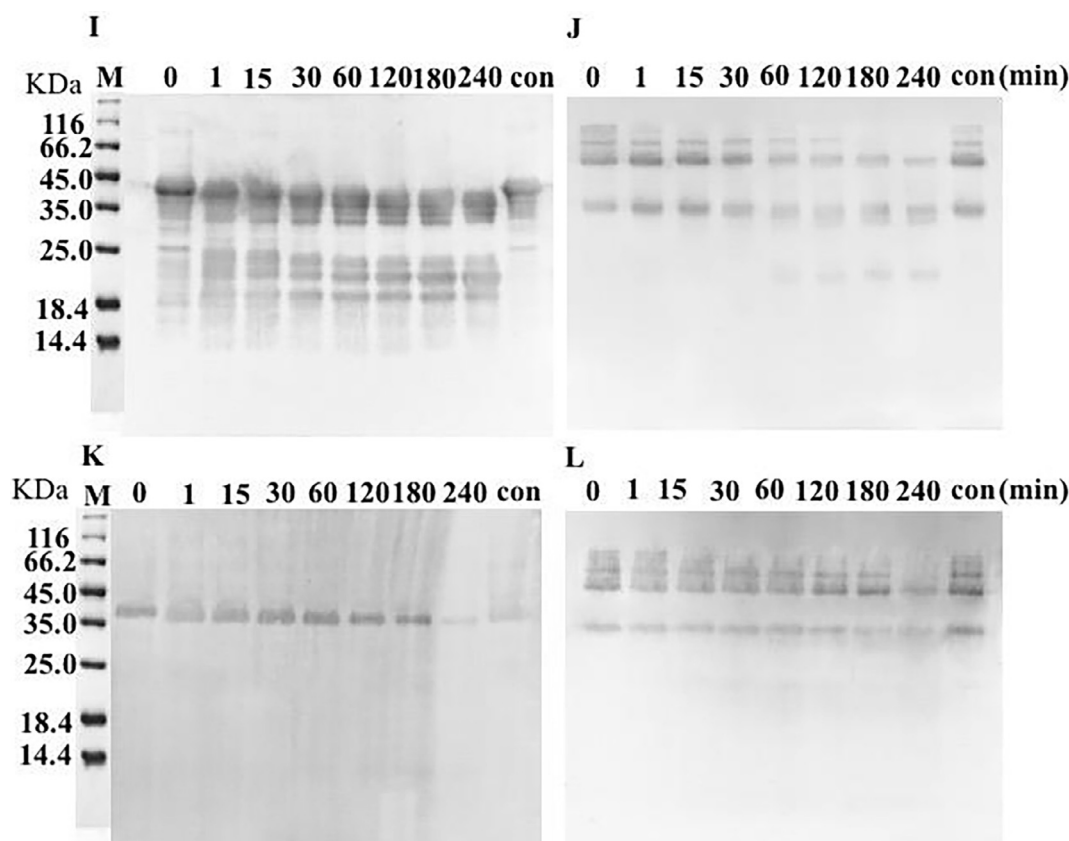


Fig. 3. (continued)

high concentrations of AAPH. New bands were observed when TM was treated with 5 mM and 25 mM AAPH, indicating cross-linking of TM. The IgG/IgE binding capacity of TM was in accordance with the SDS-PAGE results.

To determine whether AAPH affects the potential allergenicity of TM further, ciELISA was performed to evaluate IgE binding capacity of TM using human sera from shrimp-allergic patients, as shown in Fig. 1 B-2. IgE binding capacity was reduced following treatment with 0.2 and 1 mM AAPH but more significantly with 5 and 25 mM. Compared with the native TM, the IC_{50} value increased from 0.086 $\mu\text{g}/\text{ml}$ to 0.18, 1.06, 21.8, and 46.2 $\mu\text{g}/\text{ml}$ at the respective AAPH concentrations of 0.2, 1, 5, and 25 mM. The results indicated that AAPH treatment had an obvious influence on the IgE binding capacity.

3.3. Effect of AAPH treatment on the structure of shrimp TM

Protein carbonyl is a general marker of protein oxidation (Uchida et al., 1998). The effects of AAPH on TM carbonyl contents are given in Table 1. The results indicated that carbonyl contents of TM increased noticeably ($p < 0.05$) with increasing the concentration of AAPH (Table 1). AAPH-TM showed an 11-fold increase in carbonyl groups compared to the control when the concentration of AAPH was 25 mM.

The free amine group content and available lysine of AAPH-TM significantly ($p < 0.05$) decreased following AAPH treatment. The free amine group content reduced to 42.5% when TM was treated with 25 mM AAPH, and the available lysine content was reduced to 47.7% (Table 1). These data meant that the free amine group content and available lysine were liberated and then reacted with AAPH to form further adducts.

Surface hydrophobicity was employed as a probe to assess the change of conformational structure of the protein. The surface hydrophobicity of TM increased obviously ($p < 0.05$) with increasing the concentration of AAPH, as shown in Table 1. When TM was treated

with 25 mM AAPH, the surface hydrophobicity rapidly increased with a 14-fold increase compared to the native TM.

The intrinsic fluorescence intensity is one of the structural characteristics that is generally used to assess conformational structural changes. The intrinsic fluorescence intensities of TM and AAPH-TM are shown in Fig. 2(A). Upon excitation at 280 nm, an emission spectrum with a maximum at 312 nm was observed. Incubation of TM with increasing concentrations of AAPH led to a gradual decrease in the maximum fluorescence intensity and a blue shift of the maximum emission wavelength (from 311 to 308 nm) was observed. The intrinsic fluorescence intensity of TM reached a minimum (about 28% of the native TM) when TM was incubated with 25 mM AAPH.

Conformational changes of proteins can also be inferred by UV absorption. The maximum UV absorption peak of shrimp TM was approximately 275 nm. The UV absorption of shrimp TM decreased, and a slight blue shift in the UV absorbance peak was obvious after AAPH treatment (Fig. 2B).

CD spectroscopy of proteins is a direct reflection of the secondary structure. The spectra of TM exhibited a positive shoulder at ~ 196 nm and two negative troughs at ~ 212 nm and ~ 223 nm (Fig. 2C). As AAPH concentration increased from 0.2 to 25 mmol/l, the intensity of the positive peaks at ~ 196 nm and the negative peaks at ~ 212 and ~ 223 nm steadily decreased.

3.4. The stability of AAPH-TM in simulated digestion in vitro

According to the SDS-PAGE results, the original band of TM was slightly digested into small fragments (approximately 22 kDa) by pepsin within 2 min in Fig. 3A, while the AAPH-TM original band persisted for up to 60 min as shown in Fig. 3B, when the concentration of AAPH was 5 mM. However, the results of SDS-PAGE showed that both TM and AAPH-TM (Fig. 3C and D) digested into small fragments (approximately 22, 20 and 18 kDa) after trypsin digestion for 1 min and 60 min,

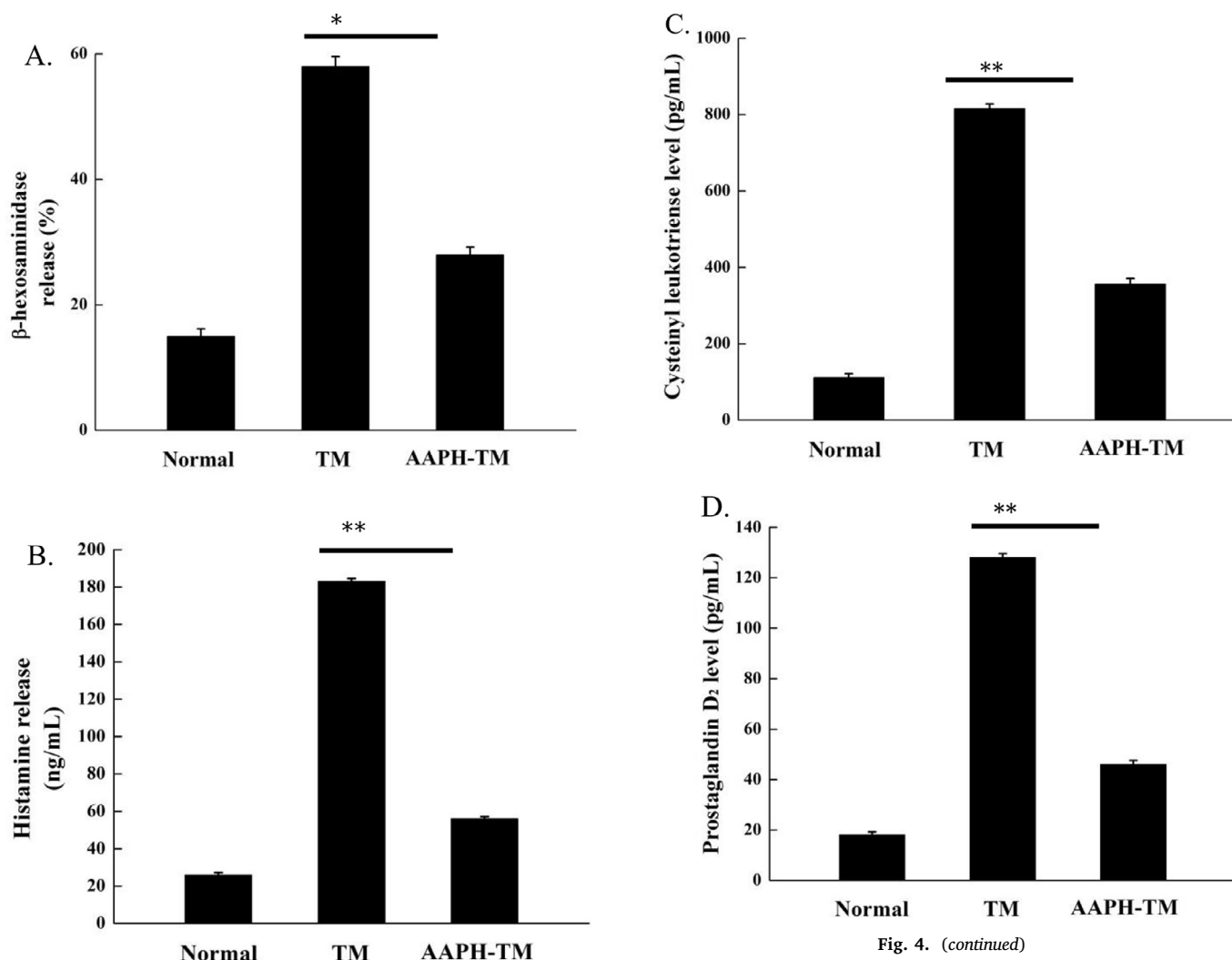


Fig. 4. (continued)

Fig. 4. Effects of AAPH-TM in IgE-mediated allergic responses in RBL-2H3 cells sensitized with sera from patients allergic to shrimp. A, The release of β -hexosaminidase; B, The release of histamine; C, The release of cysteinyl leukotriene; D, The release of prostaglandin D₂. * $p < 0.05$, ** $p < 0.01$. These data represent the mean $\pm$ SD from three independent experiments.

respectively.

The information about SGF and SIF digestion of TM and AAPH-TM that was obtained from SDS-PAGE was limited. Therefore, western blotting was used to analyze the IgG/IgE binding capacity using rabbit polyclonal antibodies (IgG) or human serum antibodies (IgE) derived from patients allergic to shrimp, at different time points during simulated gastrointestinal digestion. The intensity of the band corresponding to TM-IgG binding decreased slightly with increasing SGF digestion time (Fig. 3E), but there were little changes with 5 mM AAPH treatment (Fig. 3F). In contrast, TM and AAPH-TM showed degradation; the immune capacity gradually weakened with increasing SIF digestion time (Fig. 3I and J). The IgE binding results showed smaller TM fragments (approximately 22, 20 and 18 kDa) generated after both SGF and SIF, which had no IgE binding capacity compared to TM and AAPH-TM (Fig. 3G-H, K-L). The IgE binding capacity of TM slightly decreased with increasing digestion time. AAPH-TM exhibited more resistance than TM in SGF and SIF digestion from SDS-PAGE and western blotting results.

3.5. The impact of AAPH-TM on IgE sensitized RBL-2H3 cells

The effects of AAPH-TM on the release of β -hexosaminidase, histamine, cysteinyl leukotriene, and prostaglandin D₂ are shown in Fig. 4.

The release of β -hexosaminidase decreased (51.6%) in cells exposed to AAPH-TM (5 mM AAPH) compared to TM (Fig. 5A). In addition, AAPH-TM also inhibited histamine release, which decreased 68.1% in RBL-2H3 cells (Fig. 4B). The release of other mediators (cysteinyl leukotriene and prostaglandin D₂) followed similar trends (Fig. 4C and D).

3.6. Allergic responses after intraperitoneal injection of AAPH-TM

An animal model of allergy was used to examine the allergenicity of AAPH-TM *in vivo* following i.p. injection using the protocol shown in Fig. 5A. Exposure of mice to AAPH-TM led to a significant ($p < 0.01$) decrease in the levels of TM-specific IgE and IgG1 (Fig. 5B and C) in sera. The sera IgE/IgG1 level was $\sim$ 2-fold lower than that in the TM group. Moreover, the histamine release in sera in the AAPH-TM stimulated group (Fig. 5D) was significantly ($p < 0.01$) decreased compared with the TM-stimulated group. The mMCP-1 result between TM and AAPH-TM was similar (Fig. 5E), with the mMCP-1 release decrease from 42.36 ng/ml to 18.96 ng/ml.

4. Discussion

Lipid oxidation may induce modifications in TM structure that affect its allergenicity. In this study, SDS-PAGE was employed to monitor the structural change of TM after oxidation. Electrophoretic patterns of oxidized TM were related to AAPH concentration. TM cross-linking occurred with increasing AAPH concentration from 5 to 25 mmol l⁻¹. In this condition, β -mercaptoethanol was added to the samples to break

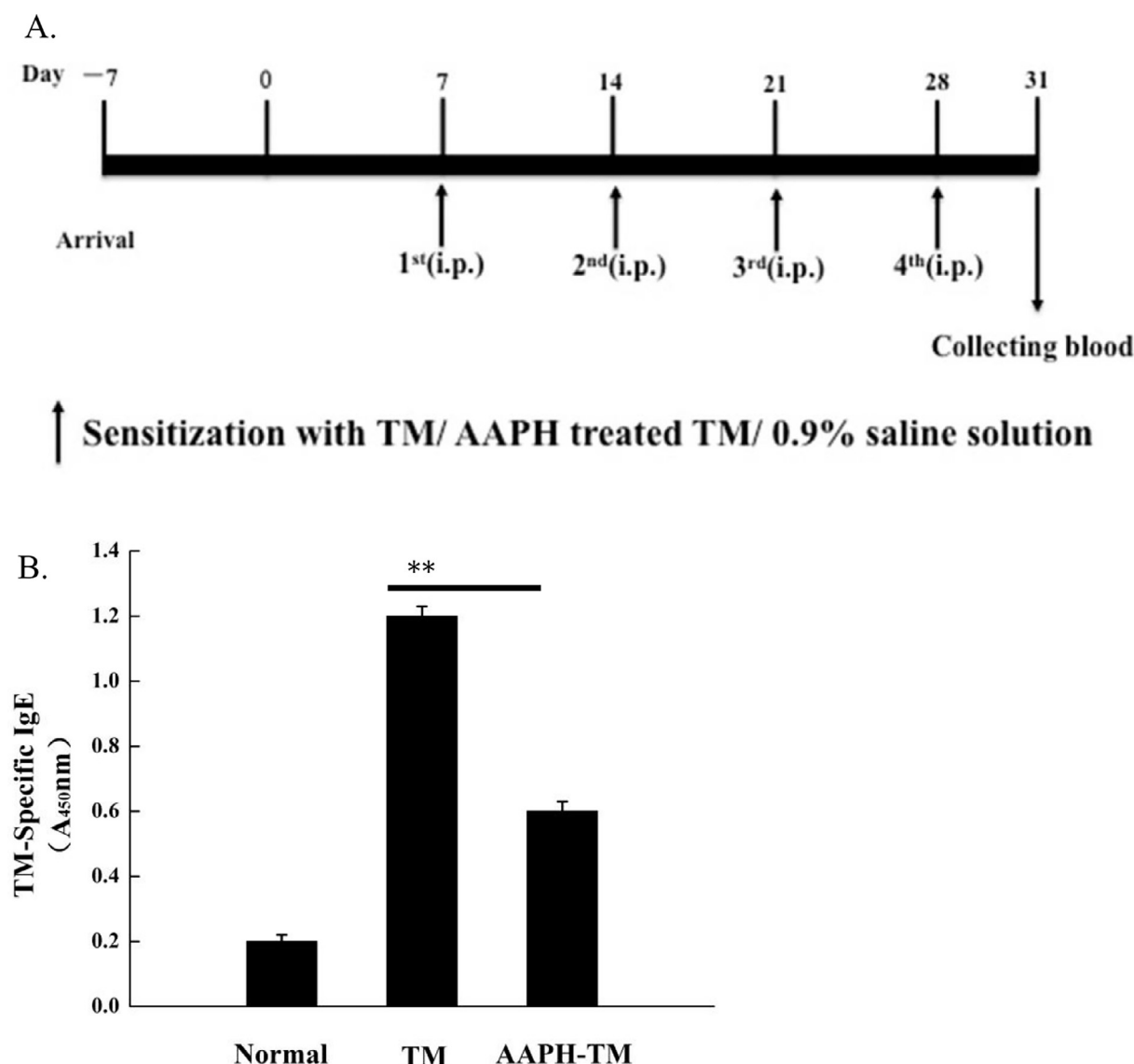


Fig. 5. Sensitization experiment. A, Sensitization protocol; B, The levels of IgE measured on day 31; C, The levels of IgG1 measured on day 31; D, The release of histamine measured in sera; E, The release of mMCP-1 measured in sera. *p < 0.05, **p < 0.01. These data represent the mean $\pm$ SD from triplicate determinations.

the disulphide bonds in TM prior to loading of protein samples into the gel. Therefore, the bands were attributed to the aggregation of TM via non-disulphide linkage. The results indicated that AAPH caused TM aggregation, where non-disulphide covalent linkages were involved in aggregate formations. Our findings are similar to Wu's research, who observed similar patterns in the case of soy protein (Wu et al., 2009). Aggregation of oxidized TM probably results from cross-linking of protein radical derivatives, induced by AAPH (Chao, Ma, & Stadtman, 1997).

The protein aggregation can affect the potential allergenicity of shrimp TM. The IgG/IgE binding capacity of TM had a more pronounced decrease after 5 mM AAPH treatment. The reduction in IgG/IgE binding capacity may be caused by structural changes due to cross-linking. The modification of amino acid side chains on the TM structure contributes to the destruction of certain epitopes and loss of IgG/IgE binding capacity. In addition, ciELISA assay showed the difference among all the samples. The IC₅₀ values revealed that the oxidation could reduce IgE binding capacity of TM. AAPH altered the molecular properties of TM.

The reduction of shrimp TM potential allergenicity is correlated

with the increase in carbonyl group content and the decrease in both free amine group content and available lysine, respectively. Protein carbonyl group content alteration is a common phenomenon of peroxyl radical-mediated protein oxidation. The formation of a carbonyl group following AAPH attack may be mediated by the nucleophilic addition of an amino acid residue or protein fragment containing highly reactive carbonyl groups. The relative research showed the exposure of glutamine synthetase to 15 mM AAPH at 37 °C for 4 h led to a 14-fold increase in the protein carbonyl content (Ma, Chao, & Stadtman, 1999). Moreover, AAPH can convert the ϵ -amino group of the lysine residue into a carbonyl group, and the newly formed carbonyl group can further react with the amino group of the protein to form a Schiff base, resulting in a decrease in free amino group content and available lysine (Liu, Xiong, & Butterfield, 2000). These changes were also apparent in the increase of surface hydrophobicity. The increase meant that hydrophobic residues, which originally should be folded and packaged to form the advanced structure of protein, are now exposed to the outside. This may better explain the reduction in IgG/IgE binding capacity following AAPH treatment.

The decrease of the potential allergenicity of TM also correlated

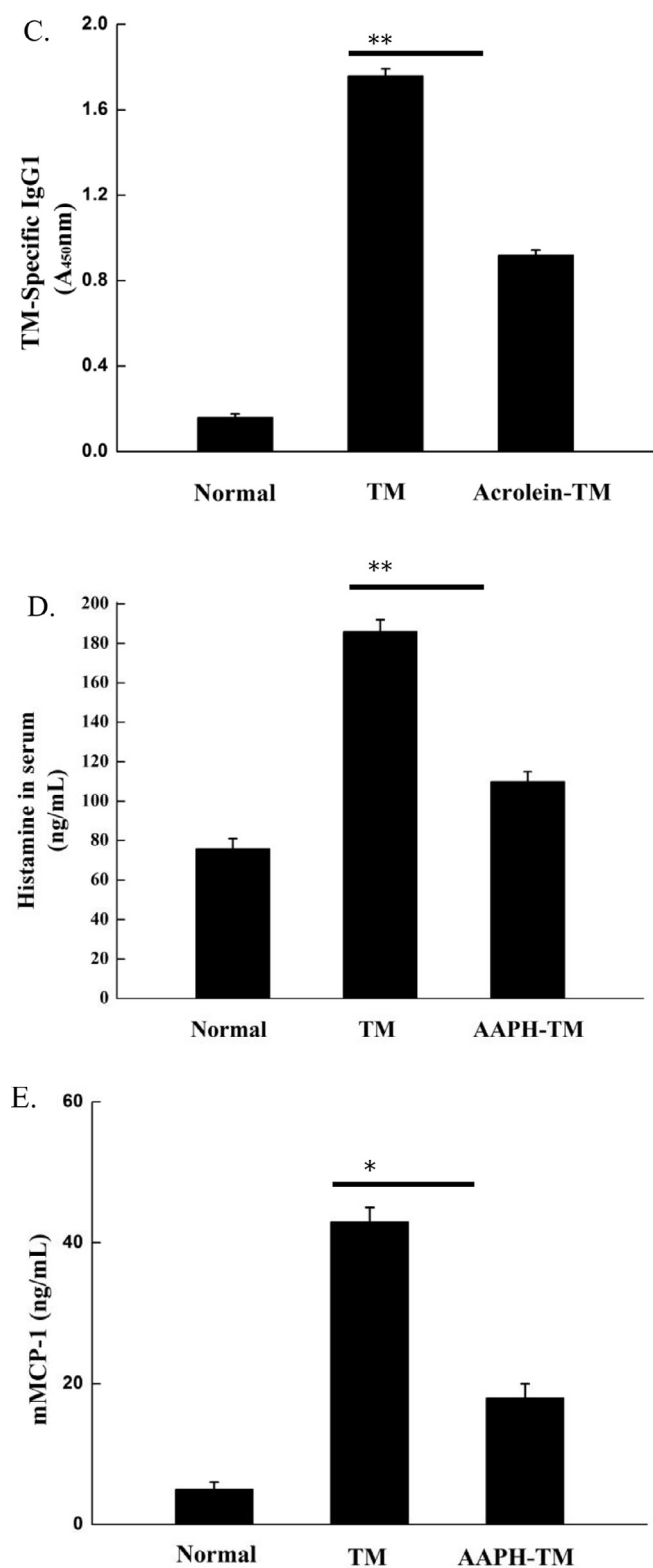


Fig. 5. (continued)

with the change of the conformational structure. The intrinsic fluorescence spectra of the protein mainly exhibited a fluorescence emission spectrum typical for tryptophan (Trp) and tyrosine (Tyr) residues. Because shrimp TM contained no Trp (Zheng, Lin, & Pawar, 2011), the Tyr content plays a critical role in the intensity of the fluorescence. AAPH treatment led to the observed decrease of maximum Tyr fluorescence intensity and a blue shifting of the maximum emission

wavelength, which indicated that the exposed Tyr residues of TM were buried in the interior of the modified TM molecule. These changes in Tyr fluorescence from AAPH-TM indicated that the treatment modified the tertiary structure of the protein, resulting in its aggregation.

The UV absorption spectrum of the protein was also mainly dependent on side-chain groups containing Tyr and Trp residues (Tong, Gao, & Chen, 2012). Tyr plays a decisive role in its UV absorption likewise. The UV absorption of shrimp TM decreased after AAPH treatment, which suggested that Tyr residues were altered on the surface of the protein molecule. Moreover, a slight blue shift illustrated that the polarity of the Tyr microenvironment changed after oxidation, probably because of broken peptide bonds. The changes in the conformational structure of AAPH-TM may elucidate the reduction in its potential IgG/IgE binding capacity.

Far-ultraviolet CD spectra showed the secondary structure of shrimp TM, comprised of four different conformations including α -helix, β -turn, β -sheet, and a random structure, in agreement with the measurement by Li, Chen, and Mo (2007). The positive peak at ~ 196 nm is indicative of the presence of β -sheet structure. Two negative peaks at ~ 212 nm and ~ 223 nm represent the presence of α -helix. The intensity steadily decreased with increasing AAPH concentration. This means that the presence of α -helix and β -sheet was reduced, which indicated that the structure became considerably disordered. According to a previous study (Mcclain, Gurnon, & Oakley, 2002), this happens mainly due to the amino acid side chains of TM combining with AAPH, disrupting the original van der Waals interactions that maintain the protein's secondary structure and hence rearranging it to a certain degree. AAPH treatment may also decrease TM IgG/IgE binding capacity by destroying its conformational epitope. The tertiary structure may also get disrupted due to denaturation of proteins, causing protein aggregation (Shin, Han, & Ahn, 2013).

In the present study, we examined the digestibility of AAPH-TM by simulated gastrointestinal fluids. Based on the time factor that ingested food normally remains in the human digestive system, the digestion time for SGF and SIF was assigned to 1 and 4 h, respectively. The results indicated that the digestive patterns of TM samples by pepsin and trypsin were very different (Fig. 3). This difference resulted from the difference in specificity of cleavage of the two proteases. Pepsin cleaves at Tyr or Phe residues and peptide bonds (Mikita & Padlan, 2007), while trypsin shows a higher specificity for cleaving at Lys and Arg residues (hydrophilic amino acids) at the P1 position (Chen, Barrett, Rawlings, & Woessner, 2004). TM was slightly degraded, but AAPH-TM did not decrease by SGF. A possible reason for this is that the available digestion sites were modified or masked by protein aggregation. We speculated that aggregation might happen at locations from the digestion sites on the protein and alter the immunogenic epitopes on the surface of TM, thereby conferring resistance to the pepsin digestion. Moreover, AAPH-TM was resistant to digestion by trypsin compared to TM. Trypsin probably damaged the lysine molecules that were exposed on the protein surface following digestion. Hence, such modifications might be responsible for the resistance of TM to trypsin digestion. The treatment with proteases, especially trypsin, is quite effective in decreasing the IgG/IgE-binding capacity of shrimp TM. The smaller (~ 23 and 20 kDa) TM proteolytic fragments generated by SGF and SIF digestion showed no binding to IgE. One reason for this might be that the binding epitopes for IgE in these fragments were obliterated after the digestion. Since the IgE binding capacity of the various digested samples decreased after treatment with proteases, we proposed that protease treatment is suitable for the elimination of IgE binding sites on TM.

In order to evaluate the ability of mediator release from stimulating cells, we conducted the IgE-mediated RBL-2H3 cell assay. RBL-2H3 cells are generally used as models for type I allergic reactions and to establish allergen-specific standards of allergenic potential. The results indicated that AAPH treatment could have significant effects on decreasing the release of mediators related to allergic symptoms. These

are in agreement with a previous study in which the treatment of TM with MDA decreased degranulation (Song, Li, Gao, Pavase, & Lin, 2016). The possible explanation was that the response of RBL-2H3 cells to the allergen by oxidation may be due to altered IgE epitopes on the surface of TM, hindering successful basophil degranulation.

We also used female BALB/c mice as an allergy animal model to evaluate the allergenicity of AAPH-TM. Allergic sensitization to TM was more prominent than AAPH-TM, as shown by a significantly higher level of TM-specific IgE/IgG1. Moreover, AAPH-TM had reduced capacity to induce the release of histamine and mMCP-1 in sera in AAPH-TM sensitized mice. Therefore, AAPH-TM decreased potential allergenicity compared to the native TM. Interestingly, some allergens achieve the same effect of reducing allergenicity due to different chemical modifications. Park, Yoon, Kim, Han, and Choi (2017) reported that the allergenicity of ovalbumin in chicken egg white decreased after treatment with N-acetylglucosaminidase. In addition, Liu et al., (2017) reported that enzymatic cross-linking of TM decreased its allergenicity in mice by decreasing the IgE/IgG1 levels in serum. One possible explanation raised for such observation is that the chemical modification covered or destroyed the IgE epitope and changed the immunological properties in vivo, thus successfully decreasing its allergen-inducing capacity. Both the *in vivo* and *in vitro* assays revealed that AAPH-TM decreased IgE binding capacity and allergenicity.

In conclusion, exposure of shrimp TM to increasing concentration of AAPH resulted in accumulation of protein carbonyls and diminution of free amine group content and available lysine. Oxidation of shrimp TM was accompanied by gradual loss of α -helix structure, backbone fragmentation, and aggregation. Non-disulphide covalent bonds were involved in aggregate formation. The interaction of AAPH and TM may alter its allergenic potential due to the changes in the structure of the allergen epitope region. The IgG/IgE binding capacity decreased with the oxidation of AAPH. In addition, we also found that TM oxidation decreased the protein stability by gastrointestinal digestion, supported by data obtained from both cell and animal studies. The study further illustrated that the AAPH treatment modified the structure, and resultant decreased the allergenicity during processing and preservation.

Acknowledgements

The authors would like to express their thanks to National Natural Science Foundation of China (31571941), and the Fundamental Research Funds for the Central Universities (201762004) for supporting this research study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.09.030>.

References

- Ahmed, I., Lv, L., Lin, H., Li, Z., Ma, J., Guanzhi, C., ... Xu, L. (2018). Effect of tyrosinase-aided crosslinking on the IgE binding potential and conformational structure of shrimp (*Metapenaeus ensis*) tropomyosin. *Food Chemistry*, 248, 287–295. <https://doi.org/10.1016/j.foodchem.2017.12.071>.
- Ayuso, R., Lehrer, S. B., & Reese, G. (2002). Identification of continuous, allergenic regions of the major shrimp allergen Pen a 1 (tropomyosin). *International Archives of Allergy and Immunology*, 127(1), 27–37. <https://doi.org/10.1159/000048166>.
- Becerra, J. A. H., Flores, A. A. O., Valerio-Alfaro, G., Soto-Rodriguez, I., Rodríguez-Estrada, M. T., & García, H. S. (2014). Cholesterol oxidation and astaxanthin degradation in shrimp during sun drying and storage. *Food Chemistry*, 145, 832–839. <https://doi.org/10.1016/j.foodchem.2013.08.098>.
- Blanco-Pascual, N., Fernández-Martín, F., & Montero, M. P. (2013). Effect of different protein extracts from *Dosidicus gigas* muscle co-products on edible films development. *Food Hydrocolloids*, 33(1), 118–131. <https://doi.org/10.1016/j.foodhyd.2013.02.019>.
- Chao, C. C., Ma, Y. S., & Stadtman, E. R. (1997). Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. *Proceedings of the National Academy of Sciences*, 94(7), 2969–2974. <https://doi.org/10.1073/pnas.94.7.2969>.

- Chen, J. M., Barrett, A. J., Rawlings, N. D., & Woessner, J. F. (2004). *Handbook of proteolytic enzymes* (second ed.). London: Elsevier.
- Gómez, C., Zafra, M. P., Boquete, M., Sanz, V., Mazzeo, C., Ibáñez, M. D., ... Pozo, V. (2014). New shrimp IgE-binding proteins involved in mite-seafood cross-reactivity. *Molecular Nutrition & Food Research*, 58(9), 1915–1925. <https://doi.org/10.1002/mnfr.201400122>.
- Hoffmann, A., Jamin, A., Foetisch, K., May, S., Aulepp, H., Hausteine, D., & Vieths, S. (1999). Determination of the allergenic activity of birch pollen and apple prick test solutions by measurement of β -hexosaminidase release from RBL-2H3 cells. Comparison with classical methods in allergen standardization. *Allergy*, 54(5), 446–454. <https://doi.org/10.1034/j.1398-9995.1999.00917.x>.
- Huang, Y., Hua, Y., & Qiu, A. (2006). Soybean protein aggregation induced by lipoxigenase catalyzed linoleic acid oxidation. *Food Research International*, 39(2), 240–249. <https://doi.org/10.1016/j.foodres.2005.07.012>.
- Huang, Y. Y., Liu, G. M., Cai, Q. F., Weng, W. Y., Maleki, S. J., Su, W. J., & Cao, M. J. (2010). Stability of major allergen tropomyosin and other food proteins of mud crab (*Scylla serrata*) by *in vitro* gastrointestinal digestion. *Food and Chemical Toxicology*, 48(5), 1196–1201. <https://doi.org/10.1016/j.fct.2010.02.010>.
- Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta (BBA)-Protein Structure*, 624(1), 13–20. [https://doi.org/10.1016/0005-2795\(80\)90220-2](https://doi.org/10.1016/0005-2795(80)90220-2).
- Kelly, S. M., Jess, T. J., & Price, N. C. (2005). How to study proteins by circular dichroism. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1751(2), 119–139. <https://doi.org/10.1016/j.bbapap.2005.06.005>.
- Kuehn, H. S., Rädinger, M., Brown, J. M., Ali, K., Vanhaesebroeck, B., Beaven, M. A., ... Gilfillan, A. M. (2010). Btk-dependent Rac activation and actin rearrangement following Fc ϵ RI aggregation promotes enhanced chemotactic responses of mast cells. *Journal of Cell Science*, 123(15), 2576–2585. <https://doi.org/10.1242/jcs.071043>.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259), 680.
- Li, Y., Chen, Z., & Mo, H. (2007). Effects of pulsed electric fields on physicochemical properties of soybean protein isolates. *LWT-Food Science and Technology*, 40(7), 1167–1175. <https://doi.org/10.1016/j.lwt.2006.08.015>.
- Liu, G., Hu, M., Sun, L. C., Han, X., Liu, Q., Alcocer, M., ... Liu, G. M. (2017). Allergenicity and oral tolerance of enzymatic cross-linked tropomyosin evaluated using cell and mouse models. *Journal of Agricultural and Food Chemistry*, 65(10), 2205–2213. <https://doi.org/10.1021/acs.jafc.6b05816>.
- Liu, G., Xiong, Y. L., & Butterfield, D. A. (2000). Chemical, physical, and gel-forming properties of oxidized myofibrils and whey- and soy-protein isolates. *Journal of Food Science*, 65(5), 811–818. <https://doi.org/10.1111/j.1365-2621.2000.tb13592.x>.
- Long, F., Yang, X., Wang, R., Hu, X., & Chen, F. (2015). Effects of combined high pressure and thermal treatments on the allergenic potential of shrimp (*Litopenaeus vannamei*) tropomyosin in a mouse model of allergy. *Innovative Food Science & Emerging Technologies*, 29, 119–124. <https://doi.org/10.1016/j.ifset.2015.03.002>.
- Lv, L., Lin, H., Li, Z., Wang, J., Ahmed, I., & Chen, H. (2017). Changes of structure and IgE binding capacity of shrimp (*Metapenaeus ensis*) tropomyosin followed by acrolein treatment. *Food & Function*, 8(3), 1028–1036. <https://doi.org/10.1039/c6fo01479h>.
- Lv, L., Lin, H., Li, Z., Yuan, F., Gao, Q., & Ma, J. (2016). Effect of 4-hydroxy-2-nonenal treatment on the IgE binding capacity and structure of shrimp (*Metapenaeus ensis*) tropomyosin. *Food Chemistry*, 212, 313–322. <https://doi.org/10.1016/j.foodchem.2016.05.152>.
- Ma, X., Lozano-Ojalvo, D., Chen, H., Lopez-Fandiño, R., & Molina, E. (2015). Effect of high pressure-assisted crosslinking of ovalbumin and egg white by transglutaminase on their potential allergenicity. *Innovative Food Science & Emerging Technologies*, 29, 143–150. <https://doi.org/10.1016/j.ifset.2015.03.017>.
- Ma, Y. S., Chao, C. C., & Stadtman, E. R. (1999). Oxidative modification of glutamine synthetase by 2, 2'-azobis (2-amidinopropane) dihydrochloride. *Archives of Biochemistry and Biophysics*, 363(1), 129–134. <https://doi.org/10.1006/abbi.1998.1076>.
- McClain, D. L., Gurnon, D. G., & Oakley, M. G. (2002). Importance of potential inter-helical salt-bridges involving interior residues for coiled-coil stability and quaternary structure. *Journal of Molecular Biology*, 324(2), 257–270. [https://doi.org/10.1016/S0022-2836\(02\)01072-0](https://doi.org/10.1016/S0022-2836(02)01072-0).
- Mikita, C. P., & Padlan, E. A. (2007). Why is there a greater incidence of allergy to the tropomyosin of certain animals than to that of others? *Medical Hypotheses*, 69(5), 1070–1073. <https://doi.org/10.1016/j.mehy.2006.12.060>.
- Nowak-Węrzyn, A., & Fioocchi, A. (2009). Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. *Current Opinion in Allergy and Clinical Immunology*, 9(3), 234–237. <https://doi.org/10.1097/ACI.0b013e3283283b8e7>.
- Ou, S., Kwok, K. C., & Kang, Y. (2004). Changes in *in vitro* digestibility and available lysine of soy protein isolate after formation of film. *Journal of Food Engineering*, 64(3), 301–305. <https://doi.org/10.1016/j.jfoodeng.2003.10.013>.
- Park, H. Y., Yoon, T. J., Kim, H. H., Han, Y. S., & Choi, H. D. (2017). Changes in the antigenicity and allergenicity of ovalbumin in chicken egg white by N-acetylglucosaminidase. *Food Chemistry*, 217, 342–345. <https://doi.org/10.1016/j.foodchem.2016.08.112>.
- Pharmacopoeia, U. S. (1995). the National Formulary. Simulated gastric fluid and simulated intestinal fluid, TS. *The United States Pharmacopoeia*, 23, 2053.
- Shin, M., Han, Y., & Ahn, K. (2013). The influence of the time and temperature of heat treatment on the allergenicity of egg white proteins. *Allergy, Asthma & Immunology Research*, 5(2), 96–101. <https://doi.org/10.4168/aaair.2013.5.2.96>.
- Sicherer, S. H., & Sampson, H. A. (2006). 9. Food allergy. *Journal of Allergy and Clinical Immunology*, 117(2), S470–S475. <https://doi.org/10.1016/j.jaci.2005.05.048>.
- Song, Y., Li, Z., Lin, H., Du, S., Hao, Z., Lin, H., & Zhu, Z. (2015). Effect of

- malondialdehyde treatment on the IgE binding capacity and conformational structure of shrimp tropomyosin. *Food Chemistry*, 175, 374–380. <https://doi.org/10.1016/j.foodchem.2014.12.001>.
- Song, Y., Li, Z., Gao, Q., Pavase, T. R., & Lin, H. (2016). Effect of malonaldehyde cross-linking on the ability of shrimp tropomyosin to elicit the release of inflammatory mediators and cytokines from activated RBL-2H3 cells. *Journal of the Science of Food and Agriculture*, 96(12), 4263–4267. <https://doi.org/10.1002/jsfa.7637>.
- Srinroch, C., Srisomsap, C., Chokchaichamnankit, D., Punyarit, P., & Phiriyangkul, P. (2015). Identification of novel allergen in edible insect, *Gryllus bimaculatus* and its cross-reactivity with *Macrobrachium* spp. allergens. *Food Chemistry*, 184, 160–166. <https://doi.org/10.1016/j.foodchem.2015.03.094>.
- Takács, K., Guillamon, E., Pedrosa, M. M., Cuadrado, C., Burbano, C., Muzquiz, M., ... Gelencsér, É. (2014). Study of the effect of instant controlled pressure drop (DIC) treatment on IgE-reactive legume-protein patterns by electrophoresis and immunoblot. *Food and Agricultural Immunology*, 25(2), 173–185. <https://doi.org/10.1080/09540105.2012.759539>.
- Tong, P., Gao, J., Chen, H., Li, X., Zhang, Y., Jian, S., ... Liu, F. (2012). Effect of heat treatment on the potential allergenicity and conformational structure of egg allergen ovomucoid. *Food Chemistry*, 131(2), 603–610. <https://doi.org/10.1016/j.foodchem.2011.08.084>.
- Uchida, K., Kanematsu, M., Sakai, K., Matsuda, T., Hattori, N., Mizuno, Y., ... Osawa, T. (1998). Protein-bound acrolein: Potential markers for oxidative stress. *Proceedings of the National Academy of Sciences*, 95(9), 4882–4887. <https://doi.org/10.1073/pnas.95.9.4882>.
- Wu, W., Wu, X., & Hua, Y. (2010). Structural modification of soy protein by the lipid peroxidation product acrolein. *LWT-Food Science and Technology*, 43(1), 133–140. <https://doi.org/10.1016/j.lwt.2009.05.006>.
- Wu, W., Zhang, C., Kong, X., & Hua, Y. (2009). Oxidative modification of soy protein by peroxy radicals. *Food Chemistry*, 116(1), 295–301. <https://doi.org/10.1016/j.foodchem.2009.02.049>.
- Zellal, D., Kaddouri, H., Grar, H., Belarbi, H., Kheroua, O., & Saidi, D. (2011). Allergenic changes in β -lactoglobulin induced by microwave irradiation under different pH conditions. *Food and Agricultural Immunology*, 22(4), 355–363. <https://doi.org/10.1080/09540105.2011.582094>.
- Zheng, L. N., Lin, H., Pawar, R., Li, Z. X., & Li, M. H. (2011). Mapping IgE binding epitopes of major shrimp (*Penaeus monodon*) allergen with immunoinformatics tools. *Food and Chemical Toxicology*, 49(11), 2954–2960. <https://doi.org/10.1016/j.fct.2011.07.043>.

Pooling of sera from patients with allergy to shrimp

Serum samples from 12 people with allergy to shrimp tropomyosin were tested in this study. Serum-specific IgE antibodies to shrimp tropomyosin were analyzed by ImmunoCAP system (Phadia AB, Uppsala, Sweden). All selected serum samples with Special-IgE values higher than or equal to 0.5 kU_A/L were pooled.

Table S1. Demographic, clinical and serologic characterization of shrimp allergic people

Patient no.	Age	Shrimp-related symptoms	TM Special-IgE (kU _A /L)
1	8 years	pneumonia	0.36
2	12 years	pneumonia	8.95
3	26 years	bronchial asthma	0.26
4	7 years	leukocytosis	0.33
5	15 years	eczema	6.87
6	23 years	cough variant asthma	0.22
7	32 years	cough variant asthma	9.38
8	18 years	asthma	6.38
9	19 years	rhinoconjunctivitis	18.6
10	26 years	pneumonia	0.18
11	54 years	rhinoconjunctivitis	0.15
12	13 years	pneumonia	9.26

TM, tropomyosin