

The Maillard Reaction Reduced the Sensitization of Tropomyosin and Arginine Kinase from *Scylla paramamosain*, Simultaneously

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ABSTRACT: The Maillard reaction was established to reduce the sensitization of tropomyosin (TM) and arginine kinase (AK) from *Scylla paramamosain*, and the mechanism of the attenuated sensitization was investigated. In the present study, the Maillard reaction conditions were optimized for heating at 100 °C for 60 min (pH 8.5) with arabinose. A low level of allergenicity in mice was shown by the levels of allergen-specific antibodies, and more Th1 and less Th2 cells cytokines produced and associated transcription factors with the Maillard reacted allergen (mAllergen). The tolerance potency in mice was demonstrated by the increased ratio of Th1/Th2 cytokines. Moreover, mass spectrometry analysis showed that some key amino acids of IgE-binding epitopes (K₁₁₂, R₁₂₅, R₁₃₃ of TM; K₃₃, K₁₁₈, R₂₀₂ of AK) were modified by the Maillard reaction. The Maillard reaction with arabinose reduced the sensitization of TM and AK, which may be due to the masked epitopes.

KEYWORDS: allergenicity reducing, arabinose, arginine kinase, Maillard reaction, tropomyosin

INTRODUCTION

An adverse immune response attributed to food is called a food allergy.¹ By reason of IgE-mediated mechanisms, food allergies are responsible for many disorders (including those of the skin and gastrointestinal and respiratory tracts).² In the last two decades, the prevalence of IgE-mediated food allergies has increased 3–7% in the European Union and the United States.^{3–5}

Shellfish is one of the “big eight” groups of allergenic foods.⁶ Tropomyosin (TM) and arginine kinase (AK) are the main causes of crab allergenic disease.^{7,8} The molecular weight of TM is 35 to 38 kDa, and TM is a type of acidic glycoprotein with good stability to protease, acid, and heating.^{9,10} AK has a complex spatial structure and a molecular weight of 40 kDa. Heating of AK can generate the dimer (a molecule made up of two simpler AK molecules) and polymer (a large molecule made of many simpler AK molecules), which also have IgG-/IgE-binding ability.⁸ With these features, the sensitization of TM and AK was difficult to be removed by conventional food processing methods.

Novel food processing technologies include ultrahigh pressure,¹¹ irradiation,¹² enzymatic cross-linking,¹³ and the Maillard reaction.¹⁴ The Maillard reaction was affected by various factors, including temperature, pH, and the types of reducing sugars. As reported, the Maillard reaction is able to influence the allergenicity of food allergens.¹⁵ Bleumink et al.¹⁶ found a considerable increase in the biological activity of β -lactoglobulin after the Maillard reaction, and Nakamura et al.¹⁷ reported that the sensitization of TM from shellfish was increased after the Maillard reaction. However, Bielikowicz et al.¹⁸ found that the sensitization of vicilin was decreased after the Maillard reaction with glucose. In addition, Nakamura et

al.¹⁹ found that the sensitization of squid TM reduced after the Maillard reaction. Thus, the actual effects of the Maillard reaction under different reaction conditions on the allergenicity were inconsistent (some increased but some decreased the allergenicity). In particular, the role of the Maillard reaction on AK has not been studied before. Further research is necessary to determine the influence on the allergenicity of TM and AK from mud crab after glycosylation.

In a previous report, an epitope was found on the surface of the antigen, which was a region recognized by a specific antibody;²⁰ therefore, the change of allergenicity is closely related to the structural changes and epitope modifications caused by the Maillard reaction. A linear epitope is a peptide (its linear sequence of amino acids or primary structure) that is specifically recognized by antibodies. Ayuso et al.²¹ calculated the linear epitope of TM from shrimp. In our previous research, Yang et al.²² calculated the linear and conformational epitope of AK from crab. Liu et al.²³ inferred that the reason enzymatic cross-linking can reduce the allergenicity of TM was due to the epitope being covered. Zhao et al.²⁴ found that the sensitization of parvalbumin after the Maillard reaction with glucose was reduced and that was due to modification of the epitope. However, it is unclear how to affect the immunoreactivity of TM and AK by the Maillard reaction.

In the current study, the changes in immunoreactivity of crab TM and AK depended on the revertose used and the reaction conditions. A cell model was adopted to examine the

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allergenicity of the Maillard reaction allergen (mAllergen), and its sensitization and oral tolerance potency were evaluated using a mouse model. The glycation sites of mAllergen were identified by mass spectrometry (MS) to explore the mechanization of the effect of the IgE binding ability by the Maillard reaction. This research showed that the Maillard reaction has great application prospects for ensuring the safety of seafood.

MATERIALS AND METHODS

Chemicals. Various reducing sugars (ribose, arabinose, galactose, glucose, and maltose) were purchased from Macklin (Shanghai, China). The goat antirabbit IgG antibody or goat antihuman IgE antibody was from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA). The goat antimouse IgE, IgG1, and IgG2a antibodies were obtained from Abcam (Cambridge, UK). An ELISA kit of histamine was purchased from IBL (Hamburg, Germany). The ELISA kits of IL-10, IL-13, IL-4, interferon (IFN)- γ , mouse mast-cell protease-1 (mMCP-1), and transforming growth factor- β (TGF- β) were purchased from R&D Systems (Minneapolis, MN, USA).

Human Sera. Sera were obtained from 20 crab-allergic patients (Table 1), following informed consent from all subjects and provided

Table 1. Specific IgE Levels in Patients in Relation to the Crab^a

No.	Serum No.	Age	Sex	Specific IgE of Crab (kUA/L)	Symptoms
1	414713	83	F	0.36	Cough
2	839758	25	M	0.47	Eczema
3	856386	38	F	0.47	Eczema
4	653594	48	M	0.47	Systemic lupus erythematosus
5	439109	30	M	0.70	Eczema
6	584173	27	M	0.80	Eczema
7	744260	38	F	1.10	Cough
8	746902	5	F	1.10	Allergic purpura
9	826985	42	F	1.10	Eczema
10	787956	12	M	1.15	Cough
11	152077	14	M	1.20	Bronchial asthma
12	722719	31	F	1.36	Eczema
13	645376	24	F	1.50	Cough
14	431984	2	F	1.50	Eczema
15	567055	6	M	1.62	Allergic purpura
16	646805	1	F	2.80	Eczema
17	787144	1	M	11.50	Eczema
18	856368	44	F	11.50	Lungs infected
19	378461	55	M	15.50	Pulmonary disease
20	680791	13	F	24.00	Eczema

^aF, female; M, male. A serum with the specific IgE > 0.35 (kU/L) is defined as positive.

by the First Affiliated Hospital of Xiamen University. This study was approved by the XFH Institutional Review Board (XFH2017-EAN0714). Sera IgE antibodies to crab were measured using the ImmunoCAP system (Pha-dia AB, Uppsala, Sweden), and the results are shown in Table 1. Positive sera were collected as a serum pool for further use. Waiting for analysis, sera were stored at $-30\text{ }^{\circ}\text{C}$.

Protein Purification and the Maillard Reaction. TM and AK were separately isolated from *Scylla paramamosain* by the methods of Liang et al.⁷ and Mao et al.²⁵ TM or AK dissolved in 10 mM PBS (pH 8.5) was mixed respectively with various reducing sugars (ribose, arabinose, galactose, glucose, and maltose), and the concentration of protein and reversion was adjusted to 5×10^{-3} mM, 4 mM, with heating at $100\text{ }^{\circ}\text{C}$ for 60 min (pH 8.5). All the samples were treated by dialysis in 10 mM PBS (pH 7.0) after reaction.

Analysis of the Immunoactivity of mAllergen *in Vitro*. SDS-PAGE, western-blot, and dot-blot were used following the method of Shen et al.⁸ The rat basophilic leukemia mast cell line (RBL-2H3) was purchased from the American Type Culture Collection (Bethesda, MD, USA) and maintained in MEM/EBSS essential medium (Hyclone, Logan, UT, USA) with 10% fetal bovine serum at $37\text{ }^{\circ}\text{C}$. The final cell count was 1×10^6 cell/mL. To evaluate the cytotoxic effect on RBL-2H3 cells, the mAllergen which was heated at 120 min under the same conditions was chosen as the control. MTT (3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to perform cytotoxicity tests.²⁶ Assessment of the β -hexosaminidase release rate followed the method reported by Ma et al.²⁷ Each of the samples (PBS, Allergen, and mAllergen) was treated with mouse sera (20 μL) overnight. The β -hexosaminidase release rate was calculated as follows: $(A1 - A2) / \{(A1 - A2) + (A3 - A4)\}$, where A1 is the absorbance of the supernatant, A2 is the absorbance of the blank supernatant, A3 is the absorbance of the cell lysate, and A4 is the absorbance of the blank cell lysate.

Mice. Four-week old female BALB/c mice (Slaccas Lab Animal Ltd. (Shanghai, China) were maintained under standard conditions (room temperature $23 \pm 2\text{ }^{\circ}\text{C}$, relative humidity 50–70%, 12/12 h light/dark cycle), and food and water were available ad libitum. All animals were used for academic research, and the institutional and national guidelines, SCXK 2012-0005, were followed by all experiments.

Sensitization to Allergen by Intraperitoneal Injection and Measurement of Allergen-Specific Antibodies and Cytokines. Mice were allocated to three groups ($n = 4$). Mice in allergen (mAllergen) groups were intraperitoneal injected with 150 μL including 150 μg of purified allergen (mAllergen on days 0 and 14).¹³ Antiallergy IgE, IgG1, and IgG2a in serum levels from blood which was collected on day 15 through cheek puncture were measured using ELISA as described previously.⁸ Commercially available ELISA kits were used to assess the concentrations of histamine and mMCP-1 in the obtained serum on day 15; the method followed the manufacturer's instructions.

On day 15, splenocyte suspensions were obtained for RNA extraction. The method was followed by Fei et al.¹³

Commercially available ELISA kits were used to measure the release of IFN- γ and cytokines IL-4 and IL-13, which were obtained from splenocyte suspensions from mice (day 15) cultured with allergen (10 $\mu\text{g}/\text{mL}$) for 72 h.

Induction of Oral Tolerance to Allergen and Measurement of Allergen-Specific Antibodies and Cytokines. Oral tolerance testing was used following the method of Fei et al.¹³ Mice were allocated to six groups ($n = 4$) and sensitized by the method described above. Through intragastric gavage 2 mg of allergen (mAllergen) was given to mice on days 21 and 28. On day 33, 10 mg of allergen (mAllergen) was given to mice as the last exposure. After 1 h of anaphylactic symptoms, rectal temperatures and diarrhea were measured. On day 34, blood was obtained, and the animals were sacrificed and allergen-specific antibody levels were determined. Histamine, mMCP-1, and TGF- β in serum were determined as described above. Using sandwich ELISA kits, levels of IL-10, IL-13, IL-4, and IFN- γ were determined, which was obtained from spleen cell and mesentery cell.

Circular Dichroism and Surface Hydrophobicity Analysis of mAllergen. Far-UV circular dichroism (CD) spectra were measured according to a method described previously.²⁸ Each spectrum represented the scan at $25\text{ }^{\circ}\text{C}$ with the allergen or mAllergen concentration adjusted to 0.5 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. The final results are shown as mdeg ($\text{deg cm}^2 \text{ dmol}^{-1}$) by a circular dichroism spectrometer (Applied Photophysics Ltd., Surrey, UK). Different secondary structures were analyzed using the CDNN program.

The surface hydrophobicity of allergen or mAllergen was determined using 8-anilino-1-naphthalenesulfonic acid (ANS) as the fluorescence probe.²⁹ Briefly, 6 μL of 2.0 mM ANS was mixed with 300 μL of samples, its excitation wavelength was measured at 370 nm, and the emission wavelength was 400–600 nm.

Identification of Glycation Sites of mAllergen. Glycation sites testing was used according to the method of Zhao et al.²⁴ mAllergen was digested with trypsin (Promega, Madison, WI, USA) for 20 h. A Thermo Scientific EASY trap column (100 μm $\times$ 25 cm, 100 Å, C18) has been used to detect the peptides obtained by enzymatic hydrolysis, which were then separated by a Thermo Scientific analytical column (75 μm $\times$ 25 cm, 5 μm , 100 Å, C18) and then with a mass spectrometer from Orbitrap-Fusion (Thermo Finnigan, San Jose, CA, USA).

Statistical Analysis. The mean values and standard deviations of the experimental data from triplicate experiments were calculated with Microsoft Office Excel 2003. All statistical analyses were performed with SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered to indicate a significant difference, and $P < 0.01$ was considered to indicate an extremely significant difference.

RESULTS

Analysis of the IgG/IgE-Binding Activity of mAllergen.

In order to analyze the immunogenic properties of mAllergen, the IgE-binding activity of mAllergen to human serum antibody was investigated (Figure 1A–B). Dot-blot analysis revealed that

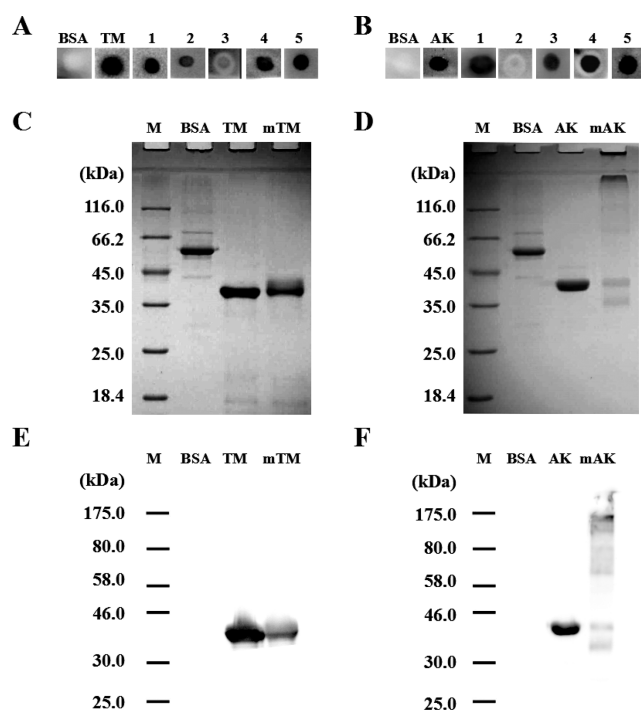


Figure 1. Analysis of mAllergen by SDS-PAGE, western-blot, and dot-blot. (A) Dot blot of BSA, TM, and TM with different revertoses in human sera. (B) Dot blot of BSA, AK, and AK with different revertoses in human sera. (1–5) Maillard reaction with ribose, arabinose, galactose, glucose, and maltose. (C) Effect on TM with arabinose by SDS-PAGE. (D) Effect on AK with arabinose by SDS-PAGE. (E) Effect on TM with arabinose by Western-blot in rabbit antibody. (F) Effect on AK with arabinose by Western-blot in rabbit antibody. M: Marker.

the allergen after reaction with different revertoses (ribose, arabinose, galactose, glucose, and maltose) had different inhibitory activities in binding crab-specific IgE. The results showed that galactose, glucose, and arabinose reduced the immunoreactivity of TM, but only arabinose reduced the sensitization of AK. Although galactose is the most effective for TM (Figure. 1A), the effect we want was to find a reducing

sugar that can reduce the sensitivities of AK and TM at the same time. Therefore, in this study, arabinose was chosen to react with TM and AK to reduce sensitization, simultaneously.

Electrophoresis that was carried out showed that Maillard reaction increased the molecular weight of TM about 2 kDa and promoted the polymerization of AK (Figure. 1D) to identify the molecular weight of mAllergen. Western-blot analysis (Figure. 1E–F) revealed that mAllergen had greater inhibitory activity, which had lower IgG-binding activity than native allergen.

Effect of mAllergen on Degranulation of RBL-2H3 Cells.

A cell viability test showed that cell viability was stable when allergen reacted with arabinose for 60 min, rather than 120 min (Figure. 2A–B). mTM of the reaction time for 60 min cannot infect cells within the increased concentrations, but mTM with reaction time of 120 min had increasing cytotoxicity during high test concentration. A similar result was also observed for mAK. Therefore, the reaction time we chose for 60 min was not poisonous.

The effect of allergen and mAllergen on degranulation of RBL-2H3 cells is shown in Figure 2C–D. Mice serum collected at 15 days was used to sensitize RBL-2H3 cells (Figure 3A). TM and AK can significantly increase the β -hexosaminidase release rate. The rate of mAllergen was almost equal to that of PBS, and differences in it were observed with the allergen, suggesting that the Maillard reaction has hypoallergenic activity on stimulated RBL-2H3 cells.

Allergic Responses after Intraperitoneal Injection of mAllergen.

A mouse model of allergenicity was used to test mAllergen *in vivo* following the protocol shown in Figure 3A. First, mAllergen exposure to mice led to the index of allergen-specificity (Figure 3B–C) in serum compared to the allergen group decreasing significantly. Second, Figure. 3D–E shows that mAllergen resulted in a higher his amine and mMCP-1 level than allergen. Third, Figure 3F shows that the level of IFN- γ was higher in mAllergen than allergen. However, the index of IL-4 and IL-13 (Figure. 3G–H) was decreased. The expression of the transcription factors (T-bet and GATA-3) was used to determine the difference in T-cells polarized between mAllergen and allergen-sensitized mice. T-bet and GATA-3 were reflected in the Th1 and Th2 cells. Figure. 3I shows that T-bet expression increased, and the relative expression of GATA-3 tended to declined. Therefore, the mAllergen group decreased cytokines secreted, down regulated the level of specific antibody, and denudated the Th2 polarized, which resulted in its lower allergenicity.

Oral Tolerance Induced by mAllergen.

The ability of mAllergen to induce systemic tolerance was investigated in a mouse model (Figure 4A). Anaphylactic symptom score, rectal temperature, and diarrhea rate are commonly used to assess anaphylaxis in mice.³⁰ Significant differences in the above index were identified among these mice groups (Figure. 4B–D). While the sensitization AK/Challenge AK (AK-AK) group can induce mice anaphylactic with low body temperature or up-regulate the diarrhea rate, sensitization mAK/Challenge AK (mAK-AK) can relieve these symptoms. In addition, it was shown that mAK cannot sensitize in mice in the group sensitized by PBS and challenged by mAK (PBS-mAK) compared with the group sensitized by PBS and challenged by AK (PBS-AK). Furthermore, the group sensitized by AK and challenged by AK (AK-AK) can give rise to severe allergic symptoms, while the group sensitized by mAK and challenged by mAK (mAK-mAK) has no significant effect on mice.

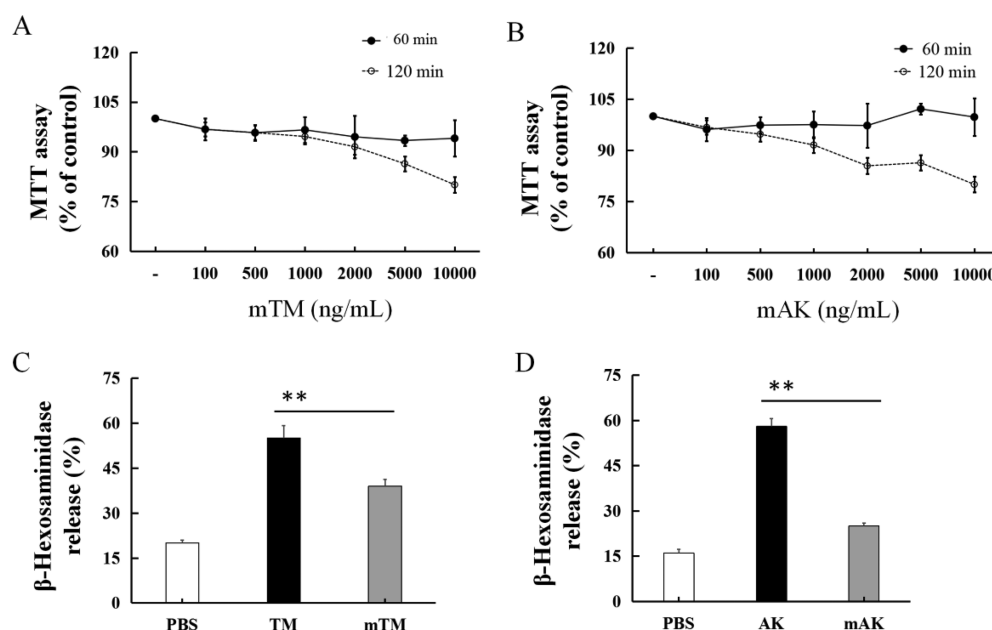


Figure 2. Effects of mAllergen in IgE-mediated allergic responses in RBL-2H3 cells. (A) The MTT assay of mTM with arabinose. (B) The MTT assay of mAK with arabinose. (C) The release of β -hexosaminidase of mTM with arabinose. (D) The release of β -hexosaminidase of mAK with arabinose. The data represent the mean $\pm$ SD of three independent experiments.

To further investigate and confirm the tolerance potency of mAK. The levels of specific antibody, histamine, and mMCP-1 in serum were assessed, which were commonly evaluated to assess the activation of mast cells in Figure 4E–G. Compared to the mAK-AK group, the AK-AK group showed higher level in AK-specific IgE, histamine, and mMCP-1. The same results also showed in the T-cell level in Figure 4H–L; that is, the mAK-AK group significantly increased the production of TGF- β and IL-10 response of Treg cells, while the IL-13 and IL-4 response of Th2 cells decreased. The IFN- γ is generally involved in the response of Th1 cells. The level of IL-4 compared to IFN- γ was regarded as the index of Th2/Th1 in Figure 4M, which showed mAK can maintain the balance of Th2/Th1 cells in mice. These results indicated the oral tolerance capacity of mAK.

Influence of Glycation on mAllergen Structure. Far-ultraviolet CD data were used to determine the secondary structures of mTM and mAK (Figure 5A–B). After the Maillard reaction, the positive (190 nm) and negative (208 and 222 nm) molar residue ellipticity peaks of the samples decreased, which indicated that the content of α -helix was decreased. Further analyses performed with CDNN software showed that the amount of α -helix in mTM was reduced to 96.6% and was reduced to 19.0% in mAK. The percentage of β -sheet was reduced to 1.3% in mTM and increased to 22.0% in mAK, the percentage of β -turn was increased to 3.7% in mTM and increased to 21.3% in mAK, and the percentage of random coils was increased to 1.1% in mTM and increased to 37.7% in mAK.

On the basis of the results of surface hydrophobicity (Figure 5C–D), mTM had higher hydrophobicity than that of TM. A similar result was shown for mAK, and the fluorescence intensity peak for mAK showed a blue shift.

Identification of Glycation Sites. LC-MS/MS was adopted to identify the glycation site of mTM and mAK. The mass spectra of mTM and mAK are shown in Table 2. A total of 19 potentially glycosylated sites were identified by comparison of

the molecular weight of the peptide with that of the native allergen peptide.

The reactive residues of mTM were K₄₉, R₉₀, R₉₁, K₁₁₂, R₁₂₅, R₁₃₃, R₁₄₀, R₁₆₀, K₁₆₁, K₁₆₇ and R₂₅₅. As shown in Figure 6A, D, the glycosylated R₁₄₀ residue located within the epitope (D₁₃₇–D₁₄₁, yellow) corresponded to the reported linear epitope (D₁₃₇–D₁₄₁, yellow) of shrimp TM,²¹ which suggested the effective destroying of IgE-binding epitopes. In addition, the reactive residues with modifications of mAK (Table 2) were M₁, K₃₃, K₁₁₈, R₁₂₄, M₁₇₃, K₁₈₀, C₂₀₁, R₂₀₂, and C₂₇₁. The K₃₃ residue was modified with acetaldehyde as shown in Figure 6B, which was the key amino acid of the conformational epitope.²² Coincidentally, as shown in Figure 6C, the Maillard reaction modified the C₂₀₁ residue with carbamidomethyl and modified the R₂₀₂ residue with carboxymethyl. As shown in Figure 6E, we found that the glycosylated R₂₀₂ residue was located within the epitope (R₂₀₂–L₁₇₀Y₂₀₃E₁₉₀P₂₀₅W₂₀₄L₁₈₇T₂₀₆Y₁₄₅, yellow), which corresponded to the reported conformational epitope of AK.²²

DISCUSSION

The Maillard reaction is commonly used in food processing to improve food quality and flavor. Optimization of the appropriate conditions for the Maillard reaction could change the immunoactivity of allergens. Various revertsos (ribose, arabinose, galactose, glucose, and maltose) were chosen to react with TM and AK, and the results showed that galactose, glucose, and arabinose reduced the allergenicity of TM, but only arabinose reduced the sensitization of AK. Therefore, in this study, arabinose was chosen to react with TM and AK to reduce sensitization, simultaneously. The effect of the Maillard reaction on allergen immunoactivity was analyzed through testing the IgG/IgE-binding activity. Depending on the surrounding pH, amadori products may be degraded into furfurals, reduction, and fragmentation derivatives in the second step.^{31,32} Thus, an increase in the extent of the reaction at pH 8.5 is due to the Maillard reaction being generated in the sample of furfural and hydroxymethyl furfural nonferrous

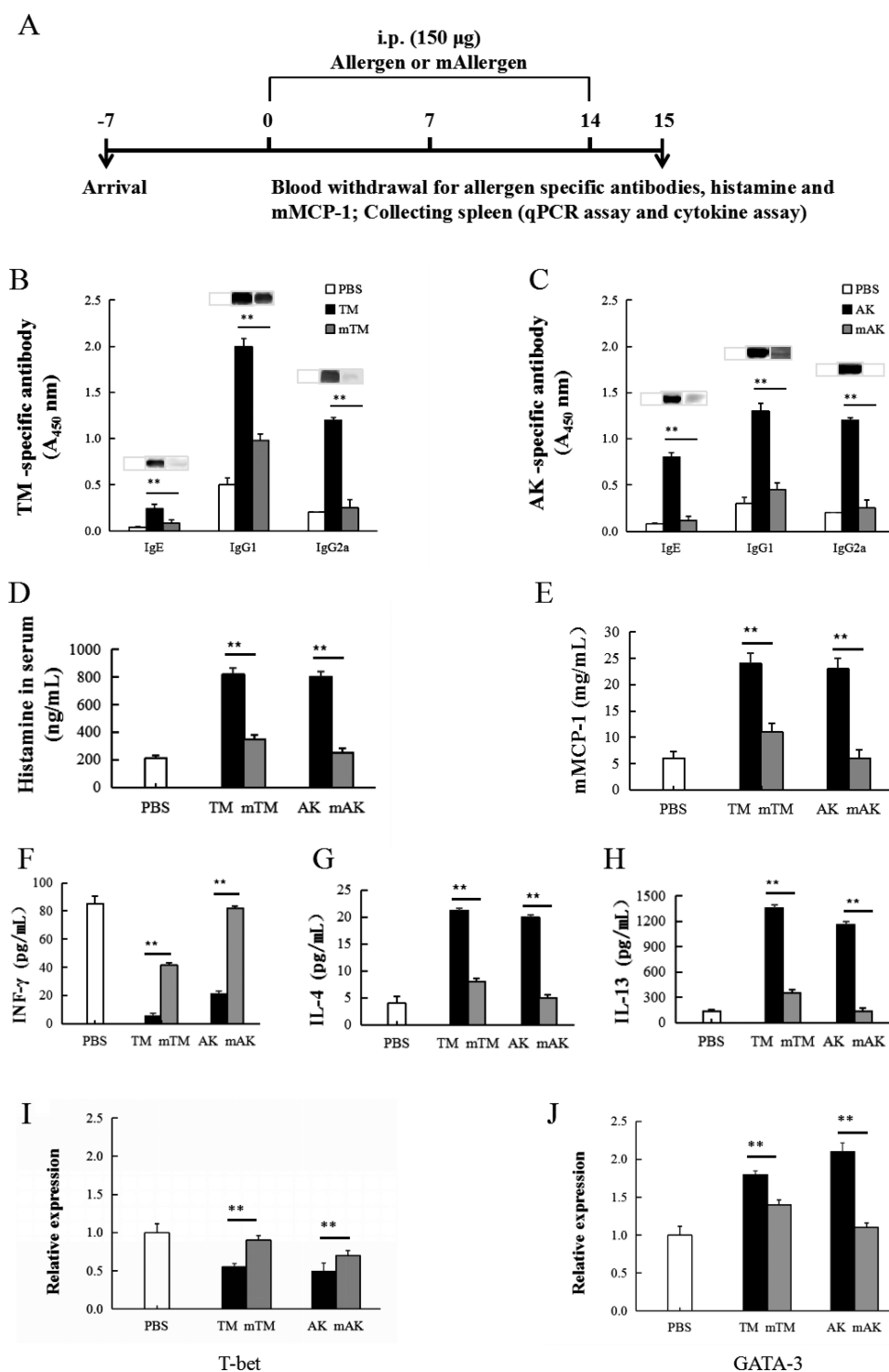


Figure 3. Sensitizing potential of mAllergen in the mice sensitization experiment. (A) Sensitization protocol. (B) The levels of IgE, IgG1, and IgG2a of mTM measured on day 15. (C) The levels of IgE, IgG1, and IgG2a of mAK measured on day 15. (D) The release of mMCP-1 of mTM and mAK measured in serum. (E) The release of histamine of mTM and mAK measured in serum. (F–H) The productions of IFN- γ , IL-4, and IL-13 were measured in a spleen cell culture. (I–J) The expression of transcription factors T-bet and GATA-3 was measured by qPCR. * $P < 0.05$, ** $P < 0.01$. The data represent the mean $\pm$ SD of triplicate determinations.

material, and anthracene copper, which was in the second stage of the Maillard reaction. Furthermore, although AK easily undergoes thermal polymerization, higher pH can promote the Maillard reaction. The Maillard products with inappropriate reaction time are poisonous;³³ thus, a cell viability test is necessary. Due to these findings, the safety of the Maillard reaction conditions, which can decrease the allergenicity of TM

and AK, was ensured, and they were verified by immunoblotting.

Optimized conditions were determined by *in vitro* experiments, and security was further authenticated by *in vivo* experiments. Gupta et al.³⁴ found that glycation of chickpea allergen attenuated its allergic immune response in Balb/c mice and was related to more Th1 and less Th2 cytokine production

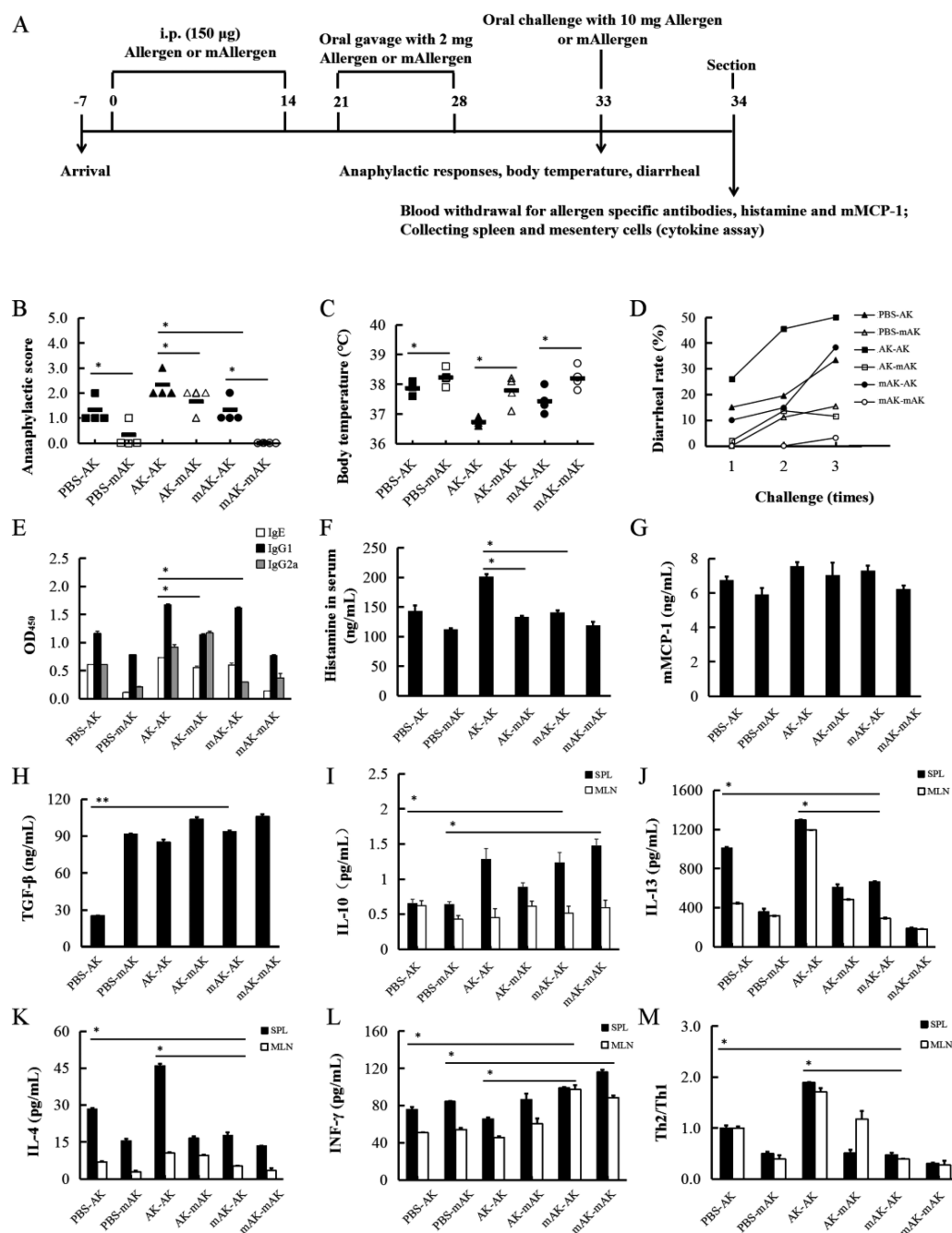


Figure 4. Allergic symptoms of mice in the tolerance induction experiment. (A) Oral tolerance induction protocol. (B) Anaphylactic symptoms were determined. (C) The body temperature was measured 1 h after intragastric gavage. (D) The diarrhea rate. (E) The levels of IgE, IgG1, and IgG2a were measured on day 34. (F) The release of histamine measured in serum. (G) The release of mMCP-1 measured in serum. (H) The release of TGF-β measured in serum. (I–L) The production of IL-10, IL-13, IL-4, and IFN-γ was measured in cultured spleen cells and mesentery cell. (M) The ratio of Th2/Th1. * $P < 0.05$, ** $P < 0.01$. The data represent the mean $\pm$ SD of triplicate determinations.

and associated transcription factors. In the studies, mice exposed to mAllergen were less prominent to allergen, which was shown by a significantly lower IgE level. And lower production of histamine in serum shows that the capacity of induced mast cell degranulation was lower in mAllergen. In the cytokines ELISA result, mice cells from mAllergen release higher IFN-γ and lower IL-4 and IL-13, results which were identical to the qPCR assay, consistent with the results reported by Gupta et al.³⁵ and Chen et al.³⁶ According to our findings, the immunological properties *in vivo* were changed by the Maillard reaction of allergen.

Rupa reported oral tolerance experiments on egg allergies after the Maillard reaction.³⁷ IFN-γ is a cytokine of Th1 cells, and IL-4 and IL-13 are cytokines of Th2 cells. It is noteworthy that mAK-AK was low compared to PBS-AK in Th2/Th1, which suggests that mAK can inhibit the body's allergic reaction to AK. IL-10 is a cytokine of Treg cells.³⁸ In food allergies, allergen-specific T cells migrate from the spleen to mesenteric lymph nodes and cause the body to produce gastrointestinal inflammation. Sakaguchi et al.³⁹ demonstrated that regulatory T cells play a central role in maintaining immune tolerance. The simultaneous elevation of TGF-β and IL-10 meant an increase

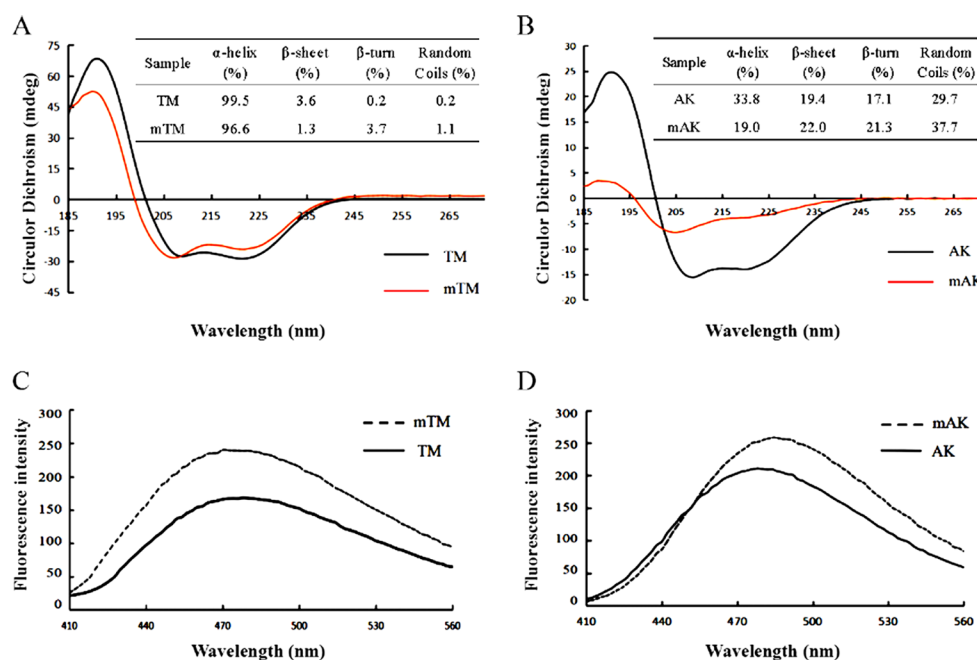


Figure 5. CD and surface hydrophobicity analysis. (A–B) Secondary structure analysis of mAllergen. (C–D) ANS assay to determine the surface hydrophobicity of mAllergen. The data represent the mean $\pm$ SD of triplicate determinations.

Table 2. Identification of Glycation Sites of mTM and mAK^a

No.	Start- Sequence	Peptide Sequence	MV obs (M+H)	Δ mass	Modification
1	49–70	KMQQVENELDQAQEQLSAANTK	2561.21	58	Carboxymethyl
2	77–91	ALQNAEGEVAALNRR	1683.87	72	Carboxyethyl
3	91–101	RIQLLEEDLER	1485.79	58	Carboxymethyl
4	92–101	IQLLEEDLER	1271.69	14	Methyl
5	106–125	LNTATTKLAEASQAADER	2200.03	116	Carboxymethyl
6	129–140	VLENRSLSDEER	1556.75	72	Carboxyethyl
7	134–149	SLSDEERMDALENQLK	1951.90	58	Carboxymethyl
8	153–167	FLAEEADRKYEVAR	1883.91	144	Carboxyethyl
9	162–178	YDEVARKLAMVEADLER	2156.05	148	Pentose
10	252–264	EVDRLEDELVNEK	1659.80	72	Carboxyethyl
11	1–16	MADAATIAKLEEGFKK	1845.92	16	Oxidation
12	30–40	YLTKSVFDQLK	1383.75	42	Acetaldehyde
13	103–124	DFGDVNQFVNVDPDGKFVISTR	2527.20	58	Carboxymethyl
14	119–129	FVISTRVRCGR	1049.61	72	Carboxyethyl
15	165–180	GTYYPLTGMTKDVQK	1845.90	16	Oxidation
16	165–180	GTYYPLTGMTK	1303.62	72	Carboxyethyl
17	190–202	EGDRFLQAANACR	1507.70	57	Carbamidomethyl
18	194–208	FLQAANACRYWPTGR	1810.88	57	Carbamidomethyl
19	194–208	FLQAANACRYWPTGR	1868.88	115	Carbamidomethyl Carboxymethyl
20	263–279	LGFLTFCPTNLGTTVR	1796.93	57	Carbamidomethyl

^a1–10, the peptide sequence of mTM; 11–20, the peptide sequence of mAK. Boldface represents the key amino acid modified.

in the regulation of Treg cells, and the reason for tolerance was the increase of Treg cells in the body thus promoting the dynamic balance of Th2 and Th1.

We found that Maillard reaction products are hypoallergenic *in vivo*, which may be related to structural changes. During the Maillard reaction, disulfide bond breakage and aggregates formation may occur by losing tertiary and secondary structure.³³ The antigenicity and potential allergenicity of TM and AK were altered by the structural changes. CD and surface hydrophobicity showed the structural changes in mTM and mAK. Compared to TM, mTM showed more β -turn structures, and compared to AK, mAK showed fewer α -helix structures,

but β -turn, β -sheet, and random coil structures increased. However, the secondary structure content of TM is only slightly modified by glycation compared to those of AK. More hydrophobic regions were generated due to the hydrophobic groupings exposed to the Maillard reaction. This reflected that glycosylation altered the tertiary and quaternary structures of TM and AK. These results partly agree with a previous report that the Maillard reaction of shrimp TM by glucosamine resulted in decreased α -helix and increased surface hydrophobicity.⁴⁰

The Maillard reaction may be effective in destroying allergen epitopes and reducing allergenicity, and the deterioration of

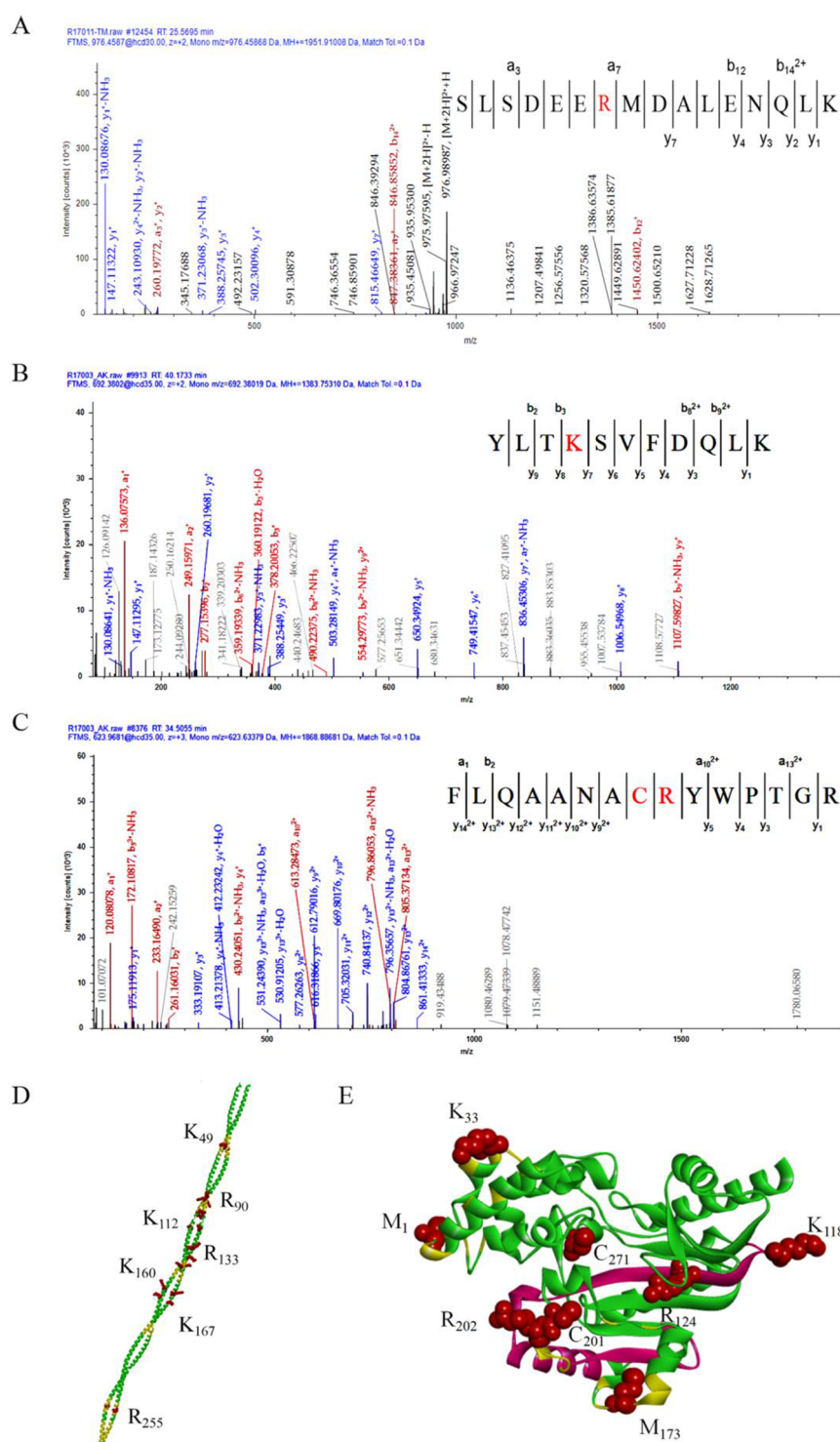


Figure 6. Identification of the Maillard reaction modified mAllergen using the LC-MS/MS. A–C, Identification of the Maillard reaction modified mTM and mAK peptide using LC-MS/MS. The fragment ion spectrum of an ion of m/z 976.4, 623.6, 692.3 is shown in the graph with the amino acid shown in the Figurer corresponding to residues 133–148 of mTM, 194–208 and 30–40 of mAK with modification sites. D–E, Structural modeling of crab TM and AK. Glycation residues (red), conformational epitopes (yellow), and linear epitopes (magenta) are shown. The key amino acid that was modified is shown.

allergies may also be caused by changes in epitopes or new allergens.^{35,41} The mass spectrum results explained the reason for IgG/IgE-binding capacity reduction was by these modifications, and they indicated that some key amino acids of IgE-binding epitopes (shown in Table 2) modified by the Maillard reaction caused epitope destruction, which was

responsible for potential allergenicity. In addition, the epitopes may be covered after forming a steric resistance, when the modified amino acids are near the epitopes. The mass spectrum results also showed that the C₂₀₁ and C₂₇₁ residues of mAK were modified by carbamidomethyl. In addition, an experiment was carried out to verify the disulfide bond has been broken by

the Maillard reaction. And the intramolecular disulfide bond at the C₂₀₁–C₂₇₁ residues was destroyed, which was essential for the allergenicity of AK.²² We think that the Maillard reaction breaks down its epitopes by modifying the key amino acids on the epitope or covering the IgE-binding epitopes even when the modified amino acids are not on the epitopes, which has also been observed by other researchers studying the immunological properties of recombinant silver carp parvalbumin.²⁴ Mueller et al.⁴² also used mass spectrum results to confirm that the receptor for glycation products combined with the glycation-modified peanut allergen which was due to the Maillard reactions modifying the allergen. In addition, the regulation of IgE production by B cells is very poorly understood.⁴³ Understanding the regulation of allergen-specific IgE at the cellular level is the key to designing therapeutics that will eliminate the production of IgE.

In summary, the Maillard reaction with arabinose can change the structure and modify the epitopes of TM and AK to reduce their sensitization, and mAllergen can induce oral tolerance potency in mice. The Maillard reaction is a useful processing method for reducing the sensitization of allergens. Therefore, it was effective in reducing the allergenicity of TM and AK in crab extract by Maillard reaction, and we designed and developed a related low-allergenic product (a processing method for instant crab legs, Chinese Patent No. 2016101825528).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AK, arginine kinase; AK-AK, sensitized by AK and challenged by AK; AK-mAK, sensitized by AK and challenged by mAK; mAK, Maillard reacted AK; mAK-AK, sensitized by mAK and challenged by AK; mAK-mAK, sensitized by mAK and challenged by mAK; mAllergen, Maillard reacted allergen; mTM, Maillard reacted TM; PBS, phosphate-buffered saline; PBS-AK, sensitized by PBS and challenged by AK; PBS-mAK, sensitized by PBS and challenged by mAK; TM, tropomyosin

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