

Reducing the Allergenicity of α -Lactalbumin after Lipid Peroxidation

Liangtao Lv,[†] Kan He,[†] Fan Sun, Xiao Lin, Liying Ye, Yansi Lyu, Lizhong Liu, Linlin Wang, Zhigang Liu, and Xuli Wu*



Cite This: <https://doi.org/10.1021/acs.jafc.1c00559>



Read Online

ACCESS |



Metrics & More



Article Recommendations



Supporting Information

ABSTRACT: This study analyzed the effect of lipid peroxidation using 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) and acrolein on the *in vitro* and *in vivo* allergenicity of α -lactalbumin (α -La). The structure of oxidized α -La was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, fluorescence spectroscopy, and circular dichroism, whereas the changes in the allergenic properties were evaluated. Lipid peroxidation induced changes to the structural properties that might destroy and/or mask α -La epitopes. In comparison to native α -La, oxidation complexes caused a decrease in the immunoglobulin E (IgE) binding capacity, as observed via immunoblotting. Moreover, the capacity to release mediators and cytokines from KU812 cells was also greatly reduced. *In vivo*, oxidation with AAPH and acrolein caused a significant reduction in IgE, IgG, IgG1, mast cell protease 1, and plasma histamine, along with the reduction of mast surface c-Kit⁺ and Fc ϵ RI⁺ expression. Therefore, these results indicate that oxidation via AAPH and acrolein can potentially reduce the allergenicity of α -La, which can help with the better understanding of the changes in allergenicity of milk allergen by lipid peroxidation.

KEYWORDS: α -lactalbumin, lipid peroxidation, allergenicity, cell model, mouse model

INTRODUCTION

Milk is rich in various nutrients, including proteins, fats, vitamins, and minerals, which are typically easily absorbed by the body. However, milk is also one of the eight major food allergens.¹ Milk allergy is a serious public health issue, affecting about 2–7.5% of the population in different countries.² Milk allergy is the most common food allergy in early childhood, which might affect childhood growth and nutrition.³ Milk allergy causes immunoglobulin E (IgE)-mediated allergic reactions, resulting in asthma, rhinitis, urticaria, and even shock and death.⁴

Milk allergenicity is related to various allergenic proteins in milk. Milk contains at least 25 different allergens that can trigger allergic reactions. The most prominent allergens in milk and milk products are caseins, β -lactoglobulin, and α -lactalbumin (α -La).² Bovine α -La is a monomeric globular Ca²⁺ binding protein, accounting for 25% of the whey proteins, causing approximately 27.6–62.8% IgE-mediated cow's milk allergy.⁵ It belongs to the lysozyme family with a molecular weight of 14.2 kDa. It shows high thermal stability and refolding capacity with a compact three-dimensional structure that contains two domains of α -helix and β -sheets.⁶ Because of its desirable techno-functional properties and high nutritional value, α -La is frequently used as ingredient or processing aids in a wide range of food products. Many studies have demonstrated that different food processing methods strongly influenced the allergenicity of milk. Typical milk processing methods include ultrahigh-temperature treatment,⁷ pasteurization,⁸ drying,⁹ dehydration,¹⁰ vacuum concentration,¹¹ and fermentation¹² that can impart various effects on the allergenicity of milk products. Moreover, milk allergens are vulnerable to oxidative damage during processing because of the abundance of unsaturated fatty acids.¹³

The oxidation of protein and lipid peroxidation is mostly interdependent. Lipid peroxidation is a complex process that involves a reaction via free radical, with lipid hydroperoxides being the main initial products, and subsequently led to the formation of reactive aldehydes.¹⁴ Lipid peroxidation products include reactive aldehyde derivative free radicals and lipid hydroperoxides, which are capable of altering the functionality of proteins.¹⁵ Wu et al.¹⁶ reported that the oxidative modification of soy protein takes place when peroxy radicals are formed in a 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH)-containing solution under aerobic conditions. Uchida¹⁷ reported that acrolein is one of the most abundant aldehydes formed from main secondary peroxidation products. These modifications can further alter the structure and physicochemical properties of milk proteins.¹⁸ However, the effect of peroxy radicals and acrolein on the allergenicity of α -La remains unclear. It is possible that such oxidative modifications impact the structural and functional properties of α -La, thus influencing the allergenicity.

Here, we tested the hypothesis that treatment of α -La with AAPH (AAPH- α -La) and acrolein (acrolein- α -La) induces structural changes that might affect the allergenic potential of milk. Structural modifications were evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), intrinsic fluorescence, and circular dichroism (CD)

Received: January 27, 2021

Revised: April 26, 2021

Accepted: April 27, 2021

analyses. The changes in the IgE binding capacity were analyzed by immunoblot analysis and enzyme-linked immunosorbent assay (ELISA) using sera of a milk-allergic patient. In addition, the allergenic potentials of α -La, AAPH- α -La, and acrolein- α -La were evaluated using KU812 cell and mouse models. The results are expected to advance the current understanding on how milk-allergic properties change during milk preservation and processing caused by lipid oxidation.

MATERIALS AND METHODS

Chemicals. α -La (purity of ≥ 85), AAPH, acrolein, 3,3',5,5'-tetramethylbenzidine (TMB), bovine serum albumin (BSA), and cholera toxin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Horseradish peroxidase (HRP)-labeled mouse anti-human IgE antibody was acquired from Kirkegaard & Perry Laboratories (Gaithersburg, MD, U.S.A.). ELISA kits for measuring histamine and mouse mast cell protease 1 (mMCP-1) were bought from IBL (Hamburg, Germany) and R&D Systems (Minneapolis, MN, U.S.A.), respectively. Enhanced chemiluminescence (ECL) reagent was purchased from GE Healthcare Co. (GE Healthcare, Buckinghamshire, U.K.). All other chemicals were of analytical grade.

Human Serum Samples. A series of sera from 12 milk-allergic children were kindly provided by the Shenzhen Children's Hospital (Guangdong, China; see Table S1 of the Support Information). The Human Ethics Committee of Shenzhen University, China (approval certificate number 202006018), approved the protocol and usage of sera. Each individual signed a written consent form. The specific IgE level to milk was measured *in vitro* using ImmunoCAP (Phadia AB, Uppsala, Sweden). All anti- α -La-specific IgE levels in sera of the patient were >1 IU/mL. All of the serum samples were stored at -30 °C until use.

Sample Preparation. The reaction of milk α -La with AAPH and acrolein was prepared as described previously, with minor changes.¹⁹ α -La proteins were dispersed fully in phosphate buffer (pH 7.4), and then AAPH and acrolein were added at various concentrations. The samples were kept for 24 h at 25 °C. The concentration of AAPH and acrolein settled from micromolar per liter to millimolar per liter based on the previous research work on protein modification with AAPH and acrolein.^{16,20} The final concentration of AAPH was set at 1, 5, and 25 mmol/L, and the final concentration of acrolein was set at 0.1, 1, and 10 mmol/L. After the reaction, samples were placed in an ice bath to reduce the temperature to below 4 °C rapidly. Finally, all samples were frozen and stored at -20 °C until use.

Characterization of Oxidation Samples. SDS-PAGE. After the reaction, SDS-PAGE was carried out to identify changes in the molecular weight of α -La. The protein solution (80 μ L, 1 mg/mL) was diluted with loading buffer (20 μ L) and preheated for 5 min at 100 °C. The samples were loaded on stacking gel (5%) and separating gel (12%). The loading volume of samples was 10 μ L. Electrophoresis was carried out at 80 and 120 V for the stacking gel and resolving gel, respectively. Coomassie Brilliant Blue R-250 (Sangon, Shanghai, China) was used to stain the gels for 30 min. The gels were destained overnight. Finally, the image was taken and analyzed using a Bio-Rad XR+ imaging system.

Western Blot Analysis. AAPH- and acrolein-treated samples were resolved with SDS-PAGE and then transferred to the polyvinylidene fluoride membranes. After blocking for 1.5 h, the membrane was incubated with milk-allergic patient sera (1:50 dilution). The membrane was then washed 3 times for 5 min with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). Subsequently, the membrane was incubated with a HRP-labeled mouse anti-human IgE antibody (1:2000 dilution) for 1 h at 37 °C. Finally, bound IgE was analyzed using ECL reagent.

Structural Analysis. Fluorescence Spectra. The protein solutions (0.2 mg/mL) were prepared in PBS (pH 7.2). Fluorescence intensity was measured on a Hitachi-850 spectrofluorometer (Hitachi Co., Tokyo, Japan) at 25 °C. Spectral analysis was performed in the range of 290–510 nm.

CD Spectra. The protein solutions (0.02 mg/mL) were prepared in PBS (pH 7.2). CD spectra of the solution were recorded using a Jasco-810 spectrophotometer (Jasco Co., Tokyo, Japan). The measurements were performed between 190 and 260 nm with a spectral resolution of 0.2 nm at 25 °C.

In Vitro Assay. ELISA. The treated α -La sample solutions (100 μ L) were added to a 96-well plate in sodium carbonate buffer (50 mmol/L, pH 9.6) and incubated at 4 °C overnight. Non-specific binding was blocked with 1% BSA in PBST for 1.5 h at 37 °C, after washing the plate 3 times with PBST. Washing was repeated after blocking, and then 100 μ L of pooled human serum (diluted 1:50) was added and incubated for 1.5 h at 37 °C. After washing again, 100 μ L of mouse immunoglobulin G (IgG) anti-human IgE antibody (diluted 1:10 000) was added per well and the plate was incubated for 1 h at 37 °C. The wells were washed, and the plate was incubated at 37 °C for 20 min after the addition of 100 μ L of TMB. Sulfuric acid (2 mol/L) was added to terminate the reaction. Finally, the absorbance at 450 nm was recorded by an ELISA plate reader.

KU812 Cell Assay. KU812 cell lines were obtained from the Chinese Academy of Sciences (Shanghai, China). The immature prebasophilic cells were maintained in a 1640 medium containing 90% L-glutamine (2 mM), 100 units/mL penicillin, 100 μ g/mL streptomycin, and 10% fetal bovine serum (FBS) following a previously reported method, with minor modifications.²¹ The cells were placed in a humidified incubator at 37 °C with 5% CO₂. KU812 cells (1×10^7 cells/mL) were pre-incubated with 100 μ L of milk-allergic serum (5:1, v/v). After 1 day, a protein sample (50 μ g) was added and incubated for 4 h. After centrifugation, the supernatant was collected to measure histamine and interleukin 13 (IL-13) using commercial ELISA kits (Elabscience Biotech, Wuhan, China), respectively.

In Vivo Assay. Five-week-old female BALB/c mice ($n = 40$) were used in this study and were approved by SPF Biotechnology (Beijing, China). All of the mice experiments were performed in accordance with the approved protocols of the Animal Care and Use Committee of Shenzhen University of China (approval certificate number 202009003). Before oral sensitization, the mice were provided *ad libitum* access to a milk-free diet for at least 1 week.

Sensitization and Challenge of Mice. All mice were separated into four groups: α -La, α -La-AAPH, α -La-acrolein, and control (no stimulation) groups. Oral sensitization of mice was employed according to the previously established method by Radosavljevic et al.²² As illustrated in Figure 4A, mice were sensitized orally with a lavage needle once a week for 4 weeks with 5 mg of native α -La or treated α -La dissolved in PBS. The samples were mixed with cholera toxin (15 μ g) as an oral sensitization. The experimental mouse groups [α -La, α -La-AAPH (5 mmol/L), and α -La-acrolein (1 mmol/L)] were orally challenged with 20 mg of α -La, α -La-AAPH, and α -La-acrolein complexes, respectively, in 500 μ L of PBS, on day 28. The control groups were sensitized with 15 μ g of cholera toxin and were then challenged with 500 μ L of PBS alone on day 28. After 1 h, validated anaphylactic scoring (Table S1 of the Support Information) was used to determine the shock severity of mice. All mice were then euthanized on day 30, and blood was collected to measure IgE, IgG, and IgG1 levels with ELISA. Blood samples were also collected 1 h after an oral challenge on day 30 to measure mMCP-1 and histamine levels.

Spleen Cell Culturing and Cytokine Analysis. Splenic lymphocytes were prepared aseptically by gently smashing spleens using a cell strainer. The suspensions were centrifuged at 500 g for 20 min. The spleen cells were then washed 2 times with RPMI-1640 medium. Subsequently, the spleen cells were resuspended and next cultured in RPMI-1640 (containing 1% penicillin-streptomycin solution and 10% FBS) with 2×10^5 cells/mL cell density. The cells were restimulated with α -La and α -La oxidation complexes for 72 h. The supernatants were collected to determine interferon γ (IFN- γ), IL-13, and interleukin 4 (IL-4) concentrations using commercial kits (Elabscience Biotech, Wuhan, China).

Detection of Peritoneal Mast Cells in Mice. The mice ($n = 6$ /group) were used for the detection of peritoneal mast cells following

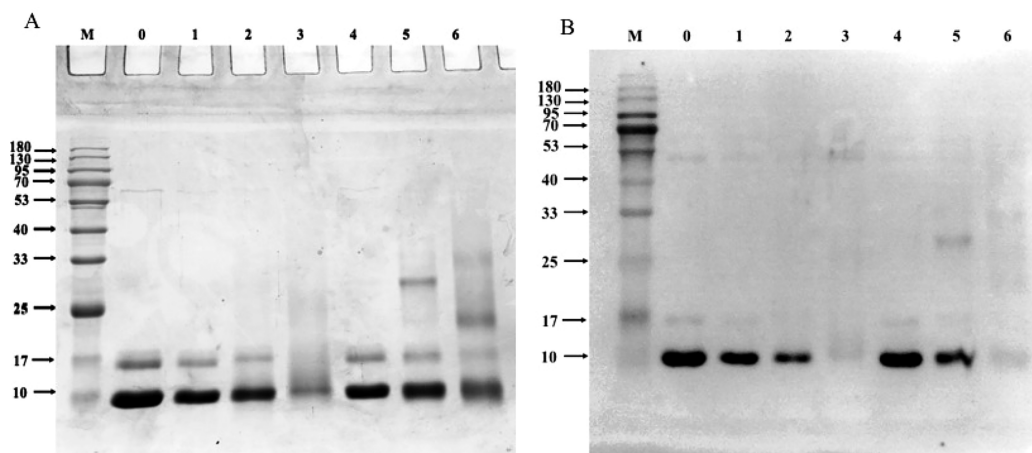


Figure 1. (A) SDS-PAGE analysis of α -La-AAPH and α -La-acrolein. An equal amount of α -La (10 μ g) was loaded on each lane. (B) IgE binding capacity of α -La-AAPH and α -La-acrolein in western blot analysis. The molecular weight of standard protein markers ranged from 10 to 180 kDa. Lane M, protein marker; lane 0, pure α -La; lanes 1–3, AAPH concentrations of 1, 5, and 25 mmol/L; and lanes 4–6, acrolein concentrations of 0.1, 1, and 10 mmol/L.

the challenge with native and treated α -La. Initially, RPMI-1640 medium (5 mL) containing 10% FBS was injected in the peritoneal cavity of each mouse. The peritoneal fluid was collected after the abdomen was gently massaged for a while. The fluid was centrifuged at 150 g for 10 min, and the cells were resuspended in flow cytometry staining buffer.

The expressions of the c-kit and IgE receptor (Fc ϵ RI) were detected in mast cells. Cells were stained with phycoerythrin (PE)-conjugated anti-c-kit antibody (0.06 μ g/test, Biolegend, San Diego, CA, U.S.A.; clone 2B8) and allophycocyanin (APC)-conjugated anti-mouse Fc ϵ RI antibody (0.25 μ g/test, Biolegend, San Diego, CA, U.S.A.; clone MAR-1) in the dark at 4 $^{\circ}$ C for 30 min. Flow cytometry analysis was carried out after washing the cells twice with flow cytometry staining buffer.

Histological Assessment of Duodenal Tissue. At 1 h after the challenge with α -La, α -La-AAPH, or α -La-acrolein, mice ($n = 6$) from each group were euthanized. The duodenum was immediately taken off immediately and fixed with formalin (10%). Standard histological techniques were employed to process the duodenum. After sectioning the duodenum at 5 μ m thickness, all of the samples were stained with eosin and hematoxylin. The morphological structures of the small intestines were observed using a light microscope (Nikon Ti microscope, Japan).

Statistical Analysis. All of the statistical results were analyzed with at least three replicates. All data are expressed as the mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) and Duncan's multiple range test were used to assess statistical significance of the differences. Results were considered significant at $p < 0.05$.

RESULTS

Characterization of Sample Oxidation. α -La aggregations were detected after treatment with AAPH and acrolein (Figure 1A). For 5 and 25 mmol/L AAPH, the α -La bands were detected in bands of about 10 and 16 kDa and above (lanes 2 and 3 in Figure 1A). At higher AAPH concentrations, the intensity of the α -La bands gradually weakened. For 1 and 10 mmol/L acrolein, the α -La bands were detected at bands of about 23 and 30 kDa and above (lanes 5 and 6 in Figure 1A), indicating that α -La was cross-linked. At higher acrolein concentrations, the intensity of the α -La bands gradually weakened. The intensity of the α -La band was most pronounced when AAPH and acrolein were absent (lane 0 in Figure 1A). To determine whether the oxidative modification of AAPH and acrolein impacted the allergenicity

of α -La, a western blot was carried out to test the IgE binding capacity of the protein using sera of a milk-allergic patient. After α -La was treated with high concentrations of AAPH and acrolein, the IgE binding capacity of α -La was dramatically reduced (Figure 1B), and these results are consistent with SDS-PAGE results.

Effect of AAPH and Acrolein Treatment on the α -La Structure. On initial excitation at 280 nm, the maximum peak was detected at 332.4 nm on the fluorescence spectrum (Figure 2A). In comparison to pure α -La, the fluorescence

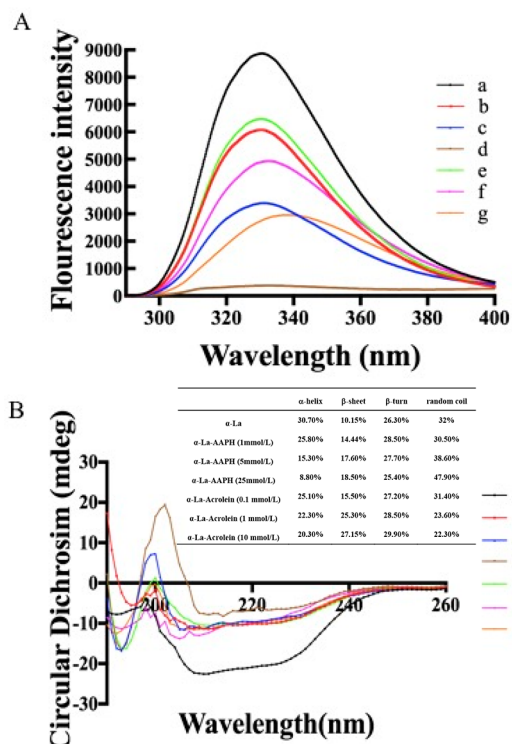


Figure 2. Effect of α -La-AAPH and α -La-acrolein on (A) fluorescence and (B) CD. a, pure α -La; b–d, AAPH concentrations of 1, 5, and 25 mmol/L; and e–g, acrolein concentrations of 0.1, 1, and 10 mmol/L.

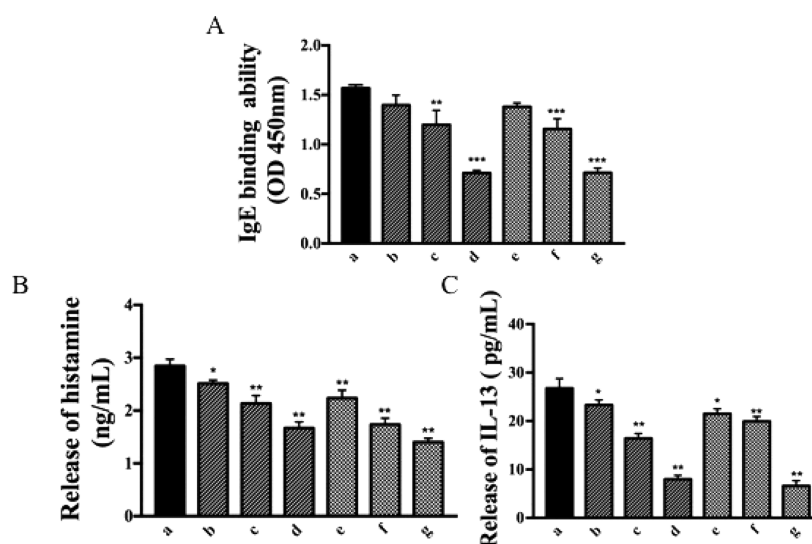


Figure 3. (A) IgE binding ability detected by ELISA, (B) basophil histamine release, and (C) IL-13 release with KU812 cell degranulation during α -La oxidation. a, pure α -La; b–d, AAPH concentrations of 1, 5, and 25 mmol/L; and e–g, acrolein concentrations of 0.1, 1, and 10 mmol/L. (*) $p < 0.05$ and (**) $p < 0.01$ represent significant differences compared to the α -La group.

intensity of α -La after reaction with AAPH and acrolein decreased. As the concentration of AAPH and acrolein increased, the fluorescence intensity significantly decreased. The increase in AAPH and acrolein concentrations caused a blue shift in the maximum emission wavelength. The fluorescence intensity of α -La was lowest at 25 mmol/L AAPH. This result might be attributed to the polymerization of α -La during the reaction, which partially masks hydrophobic amino acids, causing hydrophobicity and, hence, decreasing fluorescence intensity.

The secondary structure of α -La was determined by CD spectra. The spectra of treated α -La exhibited a negative peak at 212 nm (Figure 2B), which was characteristic of the protein α -helical structure. The CD spectra of α -La indicated that α -helical structures declined after oxidation. α -Helical contents decreased after oxidation with AAPH and acrolein, indicating that oxidation caused the protein structure to unfold, supporting the results of fluorescence spectroscopy.

In Vitro Assessment of Allergenicity. ELISA showed that the IgE binding capacity of α -La significantly declined ($p < 0.05$) following treatment with 1–25 mmol/L AAPH and 0.1–10 mmol/L acrolein (Figure 3A). AAPH and acrolein treatment significantly influenced the IgE binding capacity of α -La. However, ELISA could not be used to reliably measure the aptitude of allergenic proteins to elicit the degranulation of effector cells. Human basophilic KU812 cells were used in this study to test the ability of native and modified allergens to cause cellular degranulation. The oxidation complexes had lower levels of histamine release and IL-13 than that of native α -La (panels B and C of Figure 3). This indicates that oxidation of allergen reduces the capacity of α -La to trigger cellular degranulation, supporting the results of IgE binding capacity, as observed via the ELISA method.

In Vivo Assessment of Allergenicity. BALB/c mice of allergy induced by oral administration of α -La were employed to evaluate the *in vivo* allergenicity of α -La after oxidation with AAPH and acrolein. The anaphylactic symptoms were examined in mice following challenge with unmodified and oxidized α -La (Figure 4B). In comparison to the native α -La-

treated mice, the anaphylactic symptoms were less severe upon treatment with α -La oxidation complexes.

In comparison to α -La alone, the serum levels of α -La oxidation complex groups contained lower IgE, IgG1, IgG, mMCP-1, and histamine (panels C–G of Figure 4). The levels of IL-13 and IL-4 (Th2-related cytokines) in spleen cells were significantly lower after stimulation with α -La oxidation complexes (panels H and I of Figure 4). On the other hand, after restimulation with α -La oxidation complexes, the level of IFN- γ (Th1-related cytokine) in spleen cells was increased (Figure 4J).

Flow cytometry analysis was performed to test Fc ϵ RI⁺ and c-kit⁺ cells in peritoneal mast cells, which indicate the changes of mast cell activation following challenge with α -La and its oxidation complexes. The proportions of Fc ϵ RI⁺ and c-kit⁺ cells were greater upon challenge with α -La than that of α -La oxidation complexes (Figure 5).

The duodenum structure is presented in Figure 6, which indicated that the intestines of mice sensitized with α -La were seriously damaged compared to the α -La oxidation complexes and control groups. Moreover, the duodenum of the mice treated with native α -La showed more severe allergic symptoms compared to α -La oxidation complex groups.

DISCUSSION

This study revealed that the allergenicity of α -La is reduced when it was reacted with AAPH and acrolein. SDS–PAGE showed that cross-linked proteins formed a complicated protein complex after AAPH and acrolein treatment. The IgE binding capacity of α -La also significantly dropped above 25 mmol/L AAPH and 10 mmol/L acrolein treatment. The decrease in the IgE binding capacities might be attributed to the changes in structural properties, which are caused by lipid peroxidation. The alteration in the amino acid side chains on the protein contributed to the effect of few binding epitopes, which subsequently resulted in the reduction of IgE binding capacity. The present study confirmed that AAPH and acrolein caused changes in the molecular properties of α -La. Many previous studies have shown that cross-linking of food allergen

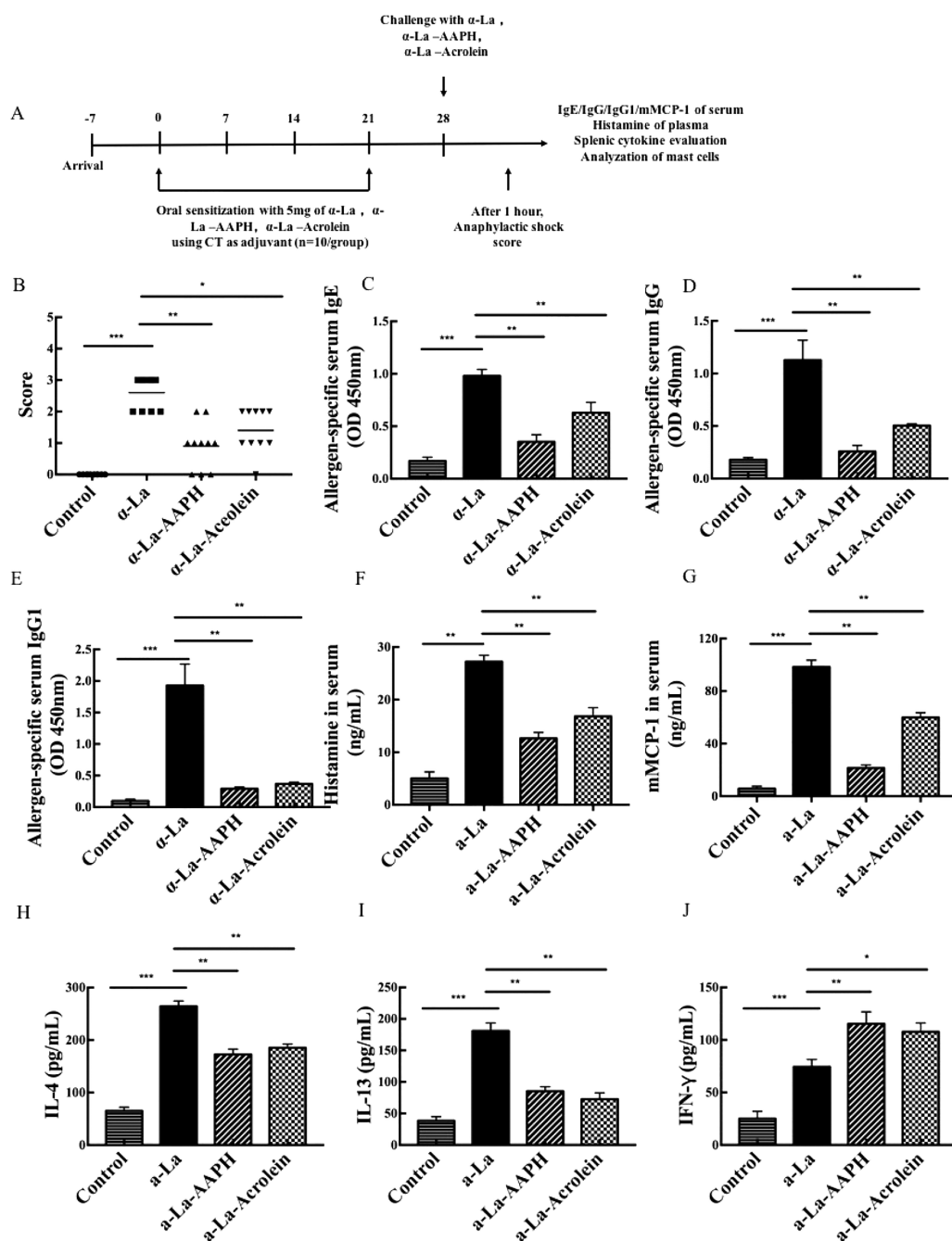


Figure 4. (A) Oral immunization of BALB/c mice with three challenges using α -La and α -La oxidation complexes. (B) Hypersensitivity symptoms were scored on a scale from 0 (no symptoms) to 5 (death), as shown in Table S1 of the Support Information. Levels of (C) IgE, (D) IgG, (E) IgG1, (F) plasma histamine, and (G) mMCP-1 in serum from mice. Results are expressed as the mean $\pm$ SD. (***) $p < 0.001$, (**) $p < 0.01$, and (*) $p < 0.05$ represent significant differences compared to the α -La group.

via various processing approaches could influence the allergenicity of food allergens.^{23,24}

The secondary structure of cross-linked α -La was altered in response to the oxidation reaction induced by AAPH and acrolein. The polymerization of α -La led to a more complex structure, with a reduction in α -helix and β -turn, while an increase in the random coil was noticed. These findings support previously reported works.²⁵ Increasing the dosage of oxidation gradually lowered the intensity of negative peaks, indicating that the proportions of α -helices declined in α -La. In addition, the reaction after oxidation might alter the fluorescence intensity of allergens. Intrinsic fluorescence

spectra are often used to observe the conformational changes of α -La. The fluorophores of proteins are comprised of tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues and are accountable for the changes in the intrinsic fluorescence spectra.²⁶ Upon oxidation, the lowering of fluorescence intensity might be caused by changes in the microenvironment around Tyr residues. This could also be caused by protein coagulation and the modification of specific amino acid residues that might lead to the lower exposure of amino acids to the aqueous environment.

Reduced IgE binding capacity by ELISA could further be explained by structural changes through oxidation. Changes in

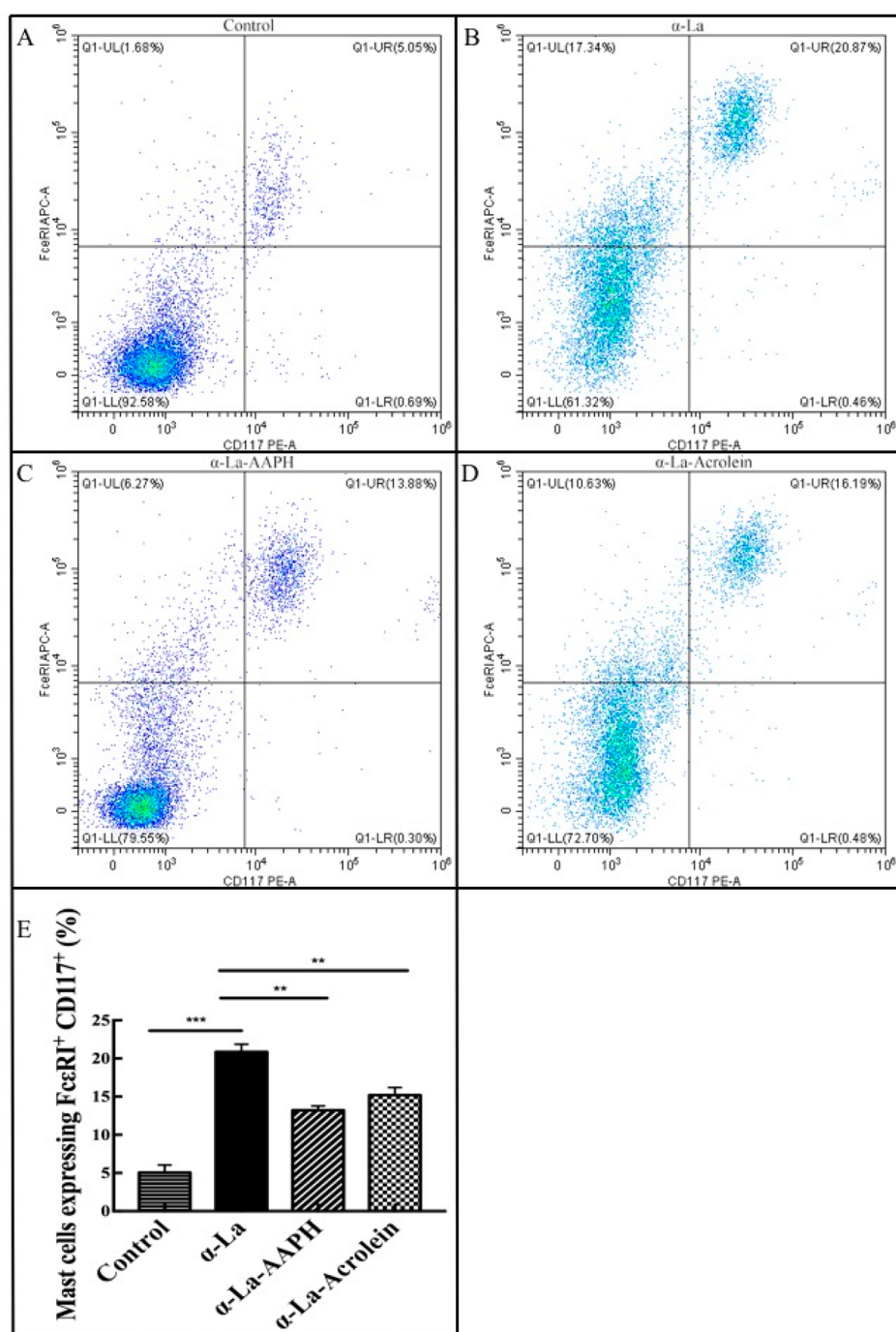


Figure 5. FcεRI and c-kit expression on peritoneal mast cells with (A) control, (B) α -La, (C) α -La-AAPH, and (D) α -La-acrolein treatment. (E) Proportion of FcεRI and c-kit cells was quantified. (***) $p < 0.001$, (**) $p < 0.01$, and (*) $p < 0.05$ represent significant differences compared to the α -La group.

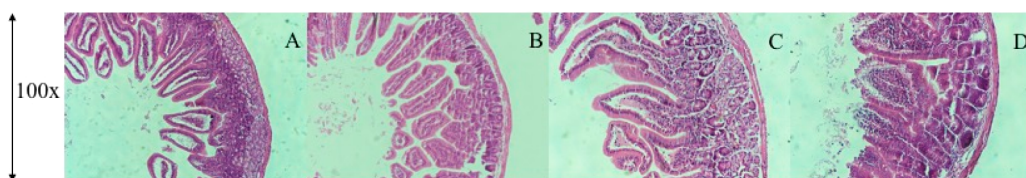


Figure 6. Effects of the (A) control, (B) α -La, (C) α -La-AAPH, and (D) α -La-acrolein treatment on the morphological structure of the duodenum in mice.

the immunostimulatory and allergenic properties of treated α -La were further demonstrated using KU812 cell models. IgE-

mediated KU812 cells are reliable models that could be used to examine the type I allergic responses. This assay is commonly

used to address variation in IgE binding and its exigency to clinical sensitization. The degree of allergic symptoms are generally dependent upon the release of cellular components; thus, such measurements are important for examining body conditions during an allergic state.²⁷ The present study indicated that α -La modification through oxidation significantly inhibited the release of histamine and IL-13 from KU812 cells. Oxidation showed substantial potential to inhibit the secretion of cytokines and mediators in KU812 cells. Zhang et al.²⁸ demonstrated that peanut allergenic proteins were less likely to trigger KU812 cell degranulation following thermal processing. Modification of milk α -La via oxidation altered the conformational structures of α -La that might alter antigenic epitopes by intra/intermolecular interactions. The response of KU812 cells could be described as that oxidation might have masked the IgE epitopes and also caused changes in the conformational structure, which subsequently hindered basophil and mast cell degranulation. The decrease in the degranulation of mast cells positively correlates with the suppression of the manifestation of allergic reactions.

The results of the *in vivo* study showed that α -La triggered allergic symptoms, whereas its oxidation complexes induced lower allergenicity compared to native α -La. After oxidation of α -La with AAPH and acrolein, the anaphylactic symptoms in mice were found to be relieved. In comparison to native α -La, the release of serum-specific IgE, IgG, and IgG1 levels was substantially reduced. Moreover, the levels of mMCP-1 and histamine were also reduced upon treatment with oxidized α -La. Oxidation with AAPH and acrolein altered the α -La structures, which might have modified the IgE binding epitopes, thus influencing the immunoreactivity and immunostimulatory potentials of α -La. Oxidation complexes on the surface of proteins might have influenced the linear IgE epitopes; therefore, oxidation with AAPH and acrolein reduced the IgE binding capacity and the histamine release, which could be responsible for the weak systemic allergic symptoms.

Th2-related cytokines (IL-13 and IL-4) could stimulate Th2 cells to induce an IgG isotype switch to IgG1 and produce IgE, whereas Th1-related cytokine (IFN- γ) could inhibit IgE production.^{29–31} The results showed that oxidation with AAPH and acrolein downregulated IL-13 and IL-4 in splenocytes while upregulated IFN- γ , thereby causing a reduction in IgE and IgG1 in mice. Therefore, oxidation with AAPH and acrolein desensitized the allergy responses by equilibrating the Th1/Th2 imbalance in response to the promotion of Th1 immune response and suppression of Th2 immune response.

Fc ϵ RI⁺ and c-kit⁺ were expressed on the surface of sensitized mast cells. The results showed a considerably higher expression of Fc ϵ RI⁺ and c-kit⁺ in α -La-challenged mice compared to α -La oxidation complexes. Histamine and mMCP-1 release were consistent with the findings of c-kit⁺ and Fc ϵ RI⁺ detection, showing that mastocyte degranulation was alleviated after oxidation with AAPH and acrolein, thus reducing the allergenicity of α -La.

In this study, we first evaluated the allergenicity of α -La after oxidative modification *in vitro* and *in vivo*. After the oxidative modification, the histidine (His), lysine (Lys), and leucine (Leu) residues on the peptide chains were easily oxidized. The peptides 1–16, 13–26, 47–58, and 93–102 are major IgE binding epitopes of α -La.³² These epitopes contain one or more His, Lys, and Leu residues. Here, oxidative modification might result in the modification of these epitopes with a

reduction in allergenicity of α -La. Furthermore, the changes in the secondary and tertiary structures of α -La after oxidative modification could significantly affect the conformational epitopes of the protein. The mechanism of the reduction in allergenicity of α -La induced by oxidative modification was similar to the other protein modification methods, such as phosphorylation, glycation, and acetylation.³³ All of these processes caused changes in the conformational epitopes and linear epitopes in α -La, leading to reduced allergenicity. However, the findings from glycation, phosphorylation, and acetylation were mostly based on IgG and IgE binding capacities *in vitro*. The alteration in the allergenicity following gastrointestinal digestion is not clear. Food allergy is related to the gastrointestinal tract *in vivo*. In addition to *in vitro* studies, we evaluated the allergenicity of digested α -La in mice and measured the serum allergen-specific antibodies, plasma histamine, splenic cytokine, peritoneal mast cell, and T cell activation. This will help to reflect the clinical and pathological characteristics of allergic reactions in allergy assessment. This study demonstrated that oxidative modification could reduce the allergenicity of α -La; however, the safety of modified α -La products still needs to be studied in the future.

In conclusion, the interaction of oxidation with AAPH and acrolein altered the allergenicity of α -La by modifying the conformation of protein and/or shielding of some allergic epitopes, thereby causing a direct effect on allergic effector cells. The oxidation complexes showed reduced allergenicity *in vitro* and *in vivo*. Oxidation of α -La with AAPH and acrolein reduced the IgE-binding ability and inhibited cellular degranulation *in vitro*. *In vivo*, specific IgE, IgG, IgG1, mMCP-1, and plasma histamine had a significant reduction. Furthermore, the mast surface Fc ϵ RI⁺ and c-kit⁺ expression was suppressed. Therefore, α -La oxidation with AAPH and acrolein reduced its allergenicity, showing the susceptibility of allergenic proteins to oxidative changes caused by lipid oxidation. This study provided new insights on the changes in the allergenic properties of milk products as a result of food preservation and processing.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c00559>.

Demographic, clinical, and serologic characterization of milk-allergic people (Table S1) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Xuli Wu – School of Public Health, Health Science Center, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China; orcid.org/0000-0003-4630-7924; Email: wxl@szu.edu.cn

Authors

Liangtao Lv – School of Public Health, Health Science Center, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China; Department of Respiratory & Allergy, Third Affiliated Hospital of Shenzhen University, Shenzhen, Guangdong 518020, People's Republic of China

Kan He – School of Public Health, Health Science Center, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China

Fan Sun — School of Public Health, Health Science Center, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China

Xiao Lin — School of Public Health, Health Science Center, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China

Liying Ye — School of Public Health, Health Science Center, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China

Yansi Lyu — School of Public Health, Health Science Center, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China; Department of Obstetricians and Gynaecologists, Shenzhen University General Hospital, Shenzhen, Guangdong 518060, People's Republic of China

Lizhong Liu — School of Public Health, Health Science Center, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China

Linlin Wang — School of Public Health, Health Science Center, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China; Department of Digestion, Shenzhen University General Hospital, Shenzhen, Guangdong 518060, People's Republic of China

Zhigang Liu — School of Public Health, Health Science Center, Shenzhen University, Shenzhen, Guangdong 518060, People's Republic of China

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acs.jafc.1c00559>

Author Contributions

[†]Liangtao Lv and Kan He contributed equally to this work.

Funding

This study was supported in part by the Key-Area Research and Development Program of Guangdong Province (2019B020213001), the Research Funding of Guangdong Province (2021A1515012413), the Research Funding for Innovation Project of Universities in Guangdong Province (2018KTSCX192), and the Research Funding of Shenzhen (JCYJ20180305163606176). The authors also thank the support from the Instrumental Analysis Center of Shenzhen University (Xili Campus).

Notes

The authors declare no competing financial interest.

REFERENCES

- (1) Skripak, J. M.; Matsui, E. C.; Mudd, K.; Wood, R. A. The natural history of IgE-mediated cow's milk allergy. *J. Allergy Clin. Immunol.* **2007**, *120* (5), 1172–1177.
- (2) Villa, C.; Costa, J.; Oliveira, M. B. P. P.; Mafra, I. Bovine Milk Allergens: A Comprehensive Review. *Compr. Rev. Food Sci. Food Saf.* **2018**, *17* (1), 137–164.
- (3) Flom, J. D.; Sicherer, S. H. Epidemiology of cow's milk allergy. *Nutrients* **2019**, *11*, 1051.
- (4) Hochwallner, H.; Schulmeister, U.; Swoboda, I.; Spitzauer, S.; Valenta, R. Cow's milk allergy: From allergens to new forms of diagnosis, therapy and prevention. *Methods* **2014**, *66*, 22–33.
- (5) Martorell-Aragonés, A.; Echeverría-Zudaire, L.; Alonso-Lebrero, E.; Boné-Calvo, J.; Martín-Muñoz, M. F.; Nevot-Falcó, S.; Piquer-Gibert, M.; Valdesoiro-Navarrete, L. Position document: IgE-mediated cow's milk allergy. *Allergol. Immunopathol.* **2015**, *43*, S07–S26.
- (6) Layman, D. K.; Lonnerdal, B.; Fernstrom, J. D. Applications for α -lactalbumin in human nutrition. *Nutr. Rev.* **2018**, *76* (6), 444–460.

(7) Ajmal, M.; Nadeem, M.; Imran, M.; Junaid, M. Lipid compositional changes and oxidation status of ultra-high temperature treated milk. *Lipids Health Dis.* **2018**, *17* (1), 227.

(8) Kellnerová, E.; Navrátilová, P.; Borkovcová, I. Effect of pasteurization on the residues of tetracyclines in milk. *Acta Vet. Brno* **2014**, *83* (10), S21–S26.

(9) Vincenzetti, S.; Cecchi, T.; Perinelli, D. R.; Pucciarelli, S.; Polzonetti, V.; Bonacucina, G.; Ariani, A.; Parrocchia, L.; Spera, D. M.; Ferretti, E.; Vallesi, P.; Polidori, P. Effects of freeze-drying and spray-drying on donkey milk volatile compounds and whey proteins stability. *LWT—Food Sci. Technol.* **2018**, *88*, 189–195.

(10) Baldwin, A. J.; Truong, G. N. T. Development of Insolubility in Dehydration of Dairy Milk Powders. *Food Bioprod. Process.* **2007**, *85* (3), 202–208.

(11) Schuck, P.; le Floch-Fouere, C.; Jeantet, R. Changes in functional properties of milk protein powders: Effects of vacuum concentration and drying. *Drying Technol.* **2013**, *31* (13–14), 1578–1591.

(12) Lordan, R.; Vidal, N. P.; Huong Pham, T.; Tsoupras, A.; Thomas, R. H.; Zabetakis, I. Yoghurt fermentation alters the composition and antiplatelet properties of milk polar lipids. *Food Chem.* **2020**, *332*, 127384.

(13) Sørensen, A. D.; Baron, C. P.; Let, M. B.; Brüggemann, D. A.; Pedersen, L. R.; Jacobsen, C. Homogenization Conditions Affect the Oxidative Stability of Fish Oil Enriched Milk Emulsions: Oxidation Linked to Changes in Protein Composition at the Oil-Water Interface. *J. Agric. Food Chem.* **2007**, *55* (5), 1781–1789.

(14) Wu, W.; Zhang, C.; Hua, Y. Structural modification of soy protein by the lipid peroxidation product malondialdehyde. *J. Sci. Food Agric.* **2009**, *89* (8), 1416–1423.

(15) Refsgaard, H. H.; Tsai, L.; Stadtman, E. R. Modifications of proteins by polyunsaturated fatty acid peroxidation products. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (2), 611–616.

(16) Wu, W.; Zhang, C.; Kong, X.; Hua, Y. Oxidative modification of soy protein by peroxy radicals. *Food Chem.* **2009**, *116* (1), 295–301.

(17) Uchida, K. Aldehyde adducts generated during lipid peroxidation modification of proteins. *Free Radical Res.* **2015**, *49* (7), 896–904.

(18) Li, X. P.; Wang, Q.; Zhou, K.; Qu, H. Y.; Li, J. R. Effects of Acrolein Oxidation on Muscle Structure and Myofibrillar Protein Structural Properties in Large Yellow Croaker (*Pseudosciaena crocea*). *Mod. Food Sci. Technol.* **2014**, *30* (10), 1–7.

(19) Lv, L.; Lin, H.; Li, Z.; Wang, J.; Ahmed, I.; Chen, H. Changes of structure and IgE binding capacity of shrimp (*Metapenaeus ensis*) tropomyosin followed by acrolein treatment. *Food Funct.* **2017**, *8* (3), 1028–1036.

(20) Wu, W.; Wu, X. J.; Hua, Y. F. Structural modification of soy protein by the lipid peroxidation product acrolein. *LWT—Food Sci. Technol.* **2010**, *43* (1), 133–140.

(21) Zhu, J.; Deng, H.; Yang, A.; Wu, Z.; Li, X.; Tong, P.; Chen, H. Effect of microbial transglutaminase cross-linking on the quality characteristics and potential allergenicity of tofu. *Food Funct.* **2019**, *10* (9), S485–S497.

(22) Radosavljevic, J.; Nordlund, E.; Mihajlovic, L.; Krstic, M.; Bohn, T.; Buchert, J.; Velickovic, T. C.; Smit, J. Sensitizing potential of enzymatic cross-linked peanut proteins in a mouse model of peanut allergy. *Mol. Nutr. Food Res.* **2014**, *58* (3), 635–646.

(23) Fei, D. X.; Liu, Q. M.; Chen, F.; Yang, Y.; Chen, Z. W.; Cao, M. J.; Liu, G. M. Assessment of the sensitizing capacity and allergenicity of enzymatic cross-linked arginine kinase, the crab allergen. *Mol. Nutr. Food Res.* **2016**, *60* (7), 1707–1718.

(24) Lv, L.; Tian, S.; Ahmed, I.; Ramesh Pavase, T.; Lin, H.; Xu, L.; Li, Z.; Liu, F. Effect of laccase-catalyzed cross-linking on the structure and allergenicity of *Paralichthys olivaceus* parvalbumin mediated by propyl gallate. *Food Chem.* **2019**, *297*, 124972.

(25) Yuan, F.; Ahmed, I.; Lv, L.; Li, Z.; Li, Z.; Lin, H.; Lin, H.; Zhao, J.; Tian, S.; Ma, J. Impacts of glycation and transglutaminase-catalyzed glycosylation with glucosamine on the conformational structure and

allergenicity of bovine β -lactoglobulin. *Food Funct.* **2018**, 9 (7), 3944–3955.

(26) Zheng, L.-N.; Lin, H.; Pawar, R.; Li, Z.-X.; Li, M.-H. Mapping IgE binding epitopes of major shrimp (*Penaeus monodon*) allergen with immunoinformatics tools. *Food Chem. Toxicol.* **2011**, 49 (11), 2954–2960.

(27) Shi, L.; Xu, H.; Wu, Y.; Li, X.; Zou, L.; Gao, J.; Chen, H. α 7-Nicotinic acetylcholine receptors involve the imidacloprid-induced inhibition of IgE-mediated rat and human mast cell activation. *RSC Adv.* **2017**, 7 (82), 51896–51906.

(28) Zhang, Y.; Wu, Z.; Li, K.; Li, X.; Yang, A.; Tong, P.; Chen, H. Allergenicity assessment on thermally processed peanut influenced by extraction and assessment methods. *Food Chem.* **2019**, 281, 130–139.

(29) Burton, O. T.; Medina Tamayo, J.; Stranks, A. J.; Miller, S.; Koleoglou, K. J.; Weinberg, E. O.; Oettgen, H. C. IgE promotes type 2 innate lymphoid cells in murine food allergy. *Clin. Exp. Allergy* **2018**, 48 (3), 288–296.

(30) Shida, K.; Takahashi, R.; Iwadate, E.; Takamizawa, K.; Yasui, H.; Sato, T.; Habu, S.; Hachimura, S.; Kaminogawa, S. *Lactobacillus casei* strain Shirota suppresses serum immunoglobulin E and immunoglobulin G1 responses and systemic anaphylaxis in a food allergy model. *Clin. Exp. Allergy* **2002**, 32 (4), 563–570.

(31) Shi, C.; Pan, T.; Cao, M.; Liu, Q.; Zhang, L.; Liu, G. Suppression of Th2 immune responses by the sulfated polysaccharide from *Porphyra haitanensis* in tropomyosin-sensitized mice. *Int. Immunopharmacol.* **2015**, 24 (2), 211–218.

(32) Järvinen, K. M.; Chatchatee, P.; Bardina, L.; Beyer, K.; Sampson, H. A. IgE and IgG binding epitopes on α -lactalbumin and β -lactoglobulin in cow's milk allergy. *Int. Arch. Allergy Immunol.* **2001**, 126 (2), 111–118.

(33) Liu, J.; Chen, W. M.; Shao, Y. H.; Zhang, J. L.; Tu, Z. C. The mechanism of the reduction in allergenic reactivity of bovine α -lactalbumin induced by glycation, phosphorylation and acetylation. *Food Chem.* **2020**, 310, 125853.

Pooled sera from milk allergic patients

Serum samples from 12 milk allergic patients were tested. Serum-specific IgE antibodies to milk α -lactalbumin were analyzed by ImmunoCAP system (Phadia AB, Uppsala, Sweden). All selected serum samples with special-IgE values higher than 1 IU/mL were pooled.

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

Patient no.	Age	Sex	Milk-related symptoms	α -La Special-IgE (IU/mL)
1	1	Female	eczema	2.27
2	3	Female	leukocytosis	3.33
3	1	Male	eczema	1.67
4	2	Female	bronchial asthma	6.62
5	15	Female	leukocytosis	2.28
6	2	Male	eczema	4.36
7	7	Male	bronchial asthma	2.32
8	4	Male	bronchial asthma	3.12
9	2	Male	eczema	1.88
10	2	Female	leukocytosis	5.83
11	3	Female	eczema	2.62
12	1	Male	bronchial asthma	3.25