

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

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Abstract

BACKGROUND: Malonaldehyde, the primary by-product of lipid peroxidation in food, modifies the structural and functional properties of proteins by cross-linking. The aim of this study was to investigate the effect of malonaldehyde on the allergenicity of shrimp tropomyosin.

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RESULTS: RBL-2H3 cells, a model of type I allergic reactions, were sensitized with sera from patients allergic to shrimp, and were stimulated with native and cross-linked tropomyosin. Release of inflammatory mediators such as β -hexosaminidase, histamine, tryptase, cysteinyl leukotriene, and prostaglandin D₂ was clearly suppressed in a manner that depended on the extent of tropomyosin cross-linking. Release of interleukin-4 (IL-4) and IL-13 was similarly decreased. Notably, cells sensitized with one patient's serum released IL-4 at comparable levels in response to native and cross-linked tropomyosin.

CONCLUSION: Cross-linking strongly modulates the ability of shrimp tropomyosin to induce release of inflammatory cytokines and mediators from activated RBL-2H3 cells.

Keywords: Allergen; Malonaldehyde; Shrimp; Rat basophil leukaemia cells;
Tropomyosin

INTRODUCTION

Shrimp is a popular ingredient that is widely consumed in the world. Despite its nutrition and delicious, it is also the most common trigger of food allergies, and may cause life-threatening events in allergic individuals.^{1,2} however, there is still no cure available and avoidance of the offending foods is essential. In order to minimize the risk to consumers, many works has been done to reduce the allergenicity of shrimp, such as heating³, irradiation⁴, Maillard reaction⁵ or some other process⁶. However, these processes can induce complex reactions in food system⁷ and the immune characters

changes on molecular basis are still limited.

As known to us, many reactions happen during food processing and preservation including oxidation, hydrolysis, cross-linking and so on⁷. Elucidate the effect of chemical reactions on shrimp allergen will be helpful to understand the allergenicity changes during food processing. Our previous work has demonstrated that malonaldehyde (MDA), a primary by-product of lipid peroxidation that accumulates during shrimp preservation, can cause cross-linking of tropomyosin and significantly reduces the affinity of tropomyosin with specific IgG and patients' IgE.⁸ These reductions do not mean the allergenicity of shrimp was decreased. Previous reports showed that the allergenicity may increase while affinity with specific IgG/IgE reduced sometimes.⁹ In order to fully understand the effect of MDA on shrimp tropomyosin, it is necessary to evaluate the allergenicity changes on effectors cells of IgE mediated allergy.

Mast cells and basophils are key triggers of IgE-mediated allergic symptoms. Accordingly, several studies demonstrated that these cells may be used in *in vitro* assays to diagnose allergies.¹⁰ However, it is impractical to collect mast cells and basophiles from allergic patients owing to ethical considerations and limited quantities. Thus, passive rat basophil leukemia (RBL-2H3) cells, which are mucosal mast cells similar to primary mast cells and normal basophils, are used as alternatives.¹¹ Previous studies have shown that these cells are rapidly and easily obtained and are reliable models of allergic reactions.¹² As a result, RBL-2H3 cells have been used to evaluate the

allergenicity changes in hazelnut (*Corylus avellana*) protein extracts.¹³ In this study, RBL-2H3 cells were used to evaluate the allergenicity changes of shrimp tropomyosin after treatment with MDA at different concentration. In addition to our previous results on IgE/IgG binding capacity, it will be helpful to understand the immune characters changes of shrimp tropomyosin after treatment with MDA.

EXPERIMENTAL

Materials and reagents

Tropomyosin from shrimp (*Metapenaeus ensis*) was purified according to method described by ZX Li (2006)¹⁴ at food safety lab of Ocean University of China, and characterized by enzyme-linked immunosorbent assay (ELISA). *p*-Nitrophenyl-N-acetyl- β -D-glucosaminide and 1,1,3,3-tetramethoxypropane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Advanced Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS; certified, heat-inactivated), and penicillin-streptomycin were purchased from Gibco Life Technologies (Grand Island, NY, USA). ELISA kits for histamine, tryptase, cysteinyl leukotriene, prostaglandin D₂, IL-4, and IL-13 were purchased from R&D Systems (Minneapolis, MN, USA). All other reagents were of analytical grade unless stated otherwise.

Patient sera

Sera were obtained from four patients allergic to shrimp. These patients had a documented clinical history of urticaria caused by shrimp, and had $> 5 \text{ kU L}^{-1}$ IgE to tropomyosin, as determined by Allergy Screen (MEDIWISS Analytic GmbH, Moers, Germany). Sera were collected at Affiliated Hospital of Medical College of Qingdao University, Qingdao, and stored at -80°C . Prior to blood draws, patients signed an informed consent form stating they were aware of the intended use of the sera. The protocol was approved by the local medical ethics committee (File No. QD-PF-20140314551) to fully protect the rights and interests of human subjects.

Cross-linking by malonaldehyde

Followed by the methods describe by Wu W(2009)¹⁵, a stock solution of malonaldehyde was prepared by hydrolysing 1,1,3,3-tetramethoxypropane in acid, then the concentration of malonaldehyde was determined and adjusted to 200 mM using an acid solution for use. Purified tropomyosin (1 mg mL^{-1}) was mixed with 0, 0.01, 0.1, 1, 10, and 100 mM malonaldehyde for 24 h in the dark in a shaking incubator at 25°C . Excess malonaldehyde was then removed by dialyzing at 4°C for 48 h against a phosphate-buffered solution. Finally, samples were freeze-dried and stored at -80°C until use.

Allergenicity in RBL-2H3 cells

Cell culture

RBL-2H3 cells were obtained from the Chinese Academy of Sciences and cultured at 37 °C in a humidified atmosphere of 5% CO₂ in DMEM supplemented with 100g L⁻¹ FBS, 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin followed by the method described by Hoffmann, A.(1999).¹⁶

Mediator and cytokine release

As described by Hoffmann, A.(1999)¹⁶, RBL-2H3 cells were harvested at the stationary phase, seeded in 96-well microtiter plates, passively sensitized with human serum IgE (P1-P4), washed three times with Tyrode's buffer, and challenged with 200 µL native and cross-linked tropomyosin. Cells treated with Tyrode's buffer were used as negative control. The concentration of tropomyosin was optimized by preliminary titrations in DMEM to induce maximum release of β-hexosaminidase. Degranulation was quenched by placing the plate on ice, washing and treating cells by DMEM supplemented with 10g L⁻¹ Triton X-100. β-hexosaminidase release was measured as described by Hoffmann, A(1999)¹⁶. Samples were then centrifuged for 10 min at 3000 *g* and 4°C to pellet debris and granules. Release of histamine, tryptase, cysteinyl leukotriene, prostaglandin D₂, IL-4, and IL-13 was measured in the resulting supernatant by ELISA, following manufacturer instructions.

Statistical analysis

All data are reported as mean $\pm$ standard deviation of three independent experiments. Differences among treatments were analysed by one-way analysis of variance in SPSS 17.0 (SPSS Inc., Chicago, IL, USA). p values < 0.05 against control treatments were considered statistically significant.

RESULTS AND DISCUSSION

Mediator release Assay

As a type of mucosal mast cells, passive rat basophil leukaemia cells are commonly used as a model of type I allergic reactions and to establish allergen-specific standards of allergenicity.^{17,18} Thus, RBL-2H3 cells sensitised with sera from patients allergic to shrimp were used to evaluate the effects of malonaldehyde cross-linking on the allergenicity of shrimp tropomyosin. The effects of cross-linking on the release of histamine, β -hexosaminidase, tryptase, cysteinyl leukotriene, and prostaglandin D₂ are shown in Fig.1. Native tropomyosin elicited 58.7%, 58.7%, 56.4%, and 58.7% release of β -hexosaminidase. Release was significantly and progressively suppressed ($p < 0.01$) as the concentration of malonaldehyde used for cross-linking increased. For instance, β -hexosaminidase release was 30 % in cells exposed to tropomyosin cross-linked with 100 mM malonaldehyde. Release of other mediators followed similar trends. Notably, release of prostaglandin D₂ decreased to zero, as shown in Fig. 1D. Although sensitisation with different sera resulted in different responses to cross-linked tropomyosin, the data generally indicate that cross-linking may significantly suppress

the release of mediators associated with allergic symptoms.

Cytokine release

RBL-2H3 is considered similar to bone-marrow derived mast cells and mast cells also produce cytokines including IL-4, IL-13, both of which play a pathologic role in the onset of various allergic including IgE-mediate diseases such as atopic dermatitis, atopic rhinitis, and asthma¹⁹. In our study, IL-4 and IL-13 were measured to illustrate the effect of MDA on shrimp tropomyosin. Result showed that IL-4 release from RBL-2H3 activated by different patients' sera to tropomyosin was different (Fig2A). With the MDA concentration increase, IL-4 release from P1 activated RBL-2H3 did not show any significant changes. While for P2, P3 and P4, IL-4 release showed significant decrease along with the MDA concentration increase. Moreover, P2 activated RBL-2H3 showed the highest IL-4 release as compared with P1, P3 and P4. The individual differences may be explained by assuming that the original material to which these individuals were sensitized differed: some people may have developed the allergy only to raw shrimp, while others may have developed shrimp allergy to partially MDA modified shrimp. For IL-13, the release patterns from RBL-2H3 activated by different patients' sera are similar (Fig 2B). With the MDA concentration increasing, IL-13 concentration decreased significantly despite the release level was different. IFN- γ , secreted from Th1 cells is a kind of cytokine which can induce T cell to Th1. It was not detectable in RBL-2H3 system due to its not included in cytokines secreted by mast cell.

These results showed that MDA modified tropomyosin has reduced capacity to secrete IL-4 and IL-13. Reduced ability to produce IL-4 and IL13 indicated that T cell clones showed T helper type 0 (Th0)- or Th1-, instead of Th2.²¹ It will be helpful to reduce allergy risk. Although the relevance of T cell differentiation to allergic reactions is not clear, it seems that MDA modification could induce suppression of IL-4 and IL-13 secretion in RBL-2H3 cell lines. Combined with changes of IgE binding capacity to tropomyosin⁸, MDA modified shrimp tropomyosin showed reduced allergenicity as compared with raw materials.

Mechanism of mediator release

Binding of allergens to high-affinity F_c receptors bound to IgE induces degranulation and release of inflammatory mediators from mast cells, which immediately resulted in allergic reactions. Hence, we used RBL-2H3 mast cells to assess the allergenicity of tropomyosin cross-linked with MDA. Due to high content of unsaturated fatty acids, lipid oxidation always happened during shrimp preservation. Previous reports showed that MDA concentration of flake-iced shrimp reached to 2.942 mg MDA/kg muscle at 8 days during chilled storage²². As a major peroxidant of lipid oxidation, MDA contains two reactive carbonyl functional groups that react with free amino groups in proteins, especially the ϵ -amino of Lys, and with the nucleophilic side chains in Cys and His.²³ Indeed, we demonstrated in previous studies that cross-linking tropomyosin with 100 mM malonaldehyde reduced free amines and available Lys to 46 % and 77 %, respectively,⁸ by modifying Lys, Gln, and Asn residues²⁴. These reactions may damage

the structure of shrimp tropomyosin and alter its allergenicity. The possible reason is that Lys is one of the most abundant amino acids in epitopes of tropomyosin²⁵. Lys modification may result in epitopes damage and affect their capacity in IgE cross-linking. This phenomenon is not alone. Previous reports showed that hazelnut proteins modified by the Maillard reaction with glucose are unable to activate basophils.^{9,26} Similarly, modification of Ara h 2 with hydroxyproline significantly reduces its allergenicity.²⁷ It seems that covalent modifications to proteins may alter allergenicity due to changes in epitopes and related protein structure.

CONCLUSION

Shrimp tropomyosin modified with MDA can reduce the ability of shrimp tropomyosin to induce mediators and cytokines release from RBL-2H3. These reductions indicated that the allergenicity of shrimp tropomyosin maybe affected during processing and preservation. When shrimp allergenicity was evaluated, the changes during food processing and preservation seem not neglectable.

COMPLIANCE WITH ETHICS REQUIREMENTS

Conflict of Interest

Zhenxing Li declares that he has no conflict of interest.

Yongna Song declares that she has no conflict of interest.

Qing Gao declares that she has no conflict of interest.

Tushar Ramesh Pavase declares that he has no conflict of interest.

Hong Lin declares that he has no conflict of interest.

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FIGURE CAPTIONS

Figure 1. (A) β -hexosaminidase, (B) histamine, (C) tryptase, (D) cysteinyl leukotriene, and (E) prostaglandin D₂ release from RBL-2H3 cells sensitized with sera from four patients (P1-P4) allergic to shrimp, and challenged with native tropomyosin or tropomyosin cross-linked with 0, 0.01, 0.1, 1, 10, and 100 mM malonaldehyde (1-6). Data are mean $\pm$ standard deviation. *, $p < 0.05$ and **, $p < 0.01$ versus native tropomyosin.

Figure 2. (A) IL-4 and (B) IL-13 release from RBL-2H3 cells sensitized with sera from four patients with shrimp allergy (P1–P4), and challenged with native tropomyosin or tropomyosin cross-linked with 0, 0.01, 0.1, 1, 10, and 100 mM malonaldehyde (1-6). Data are mean $\pm$ standard deviation. *, $p < 0.05$ and **, $p < 0.01$ versus native tropomyosin.

Figures

Figure. 1A

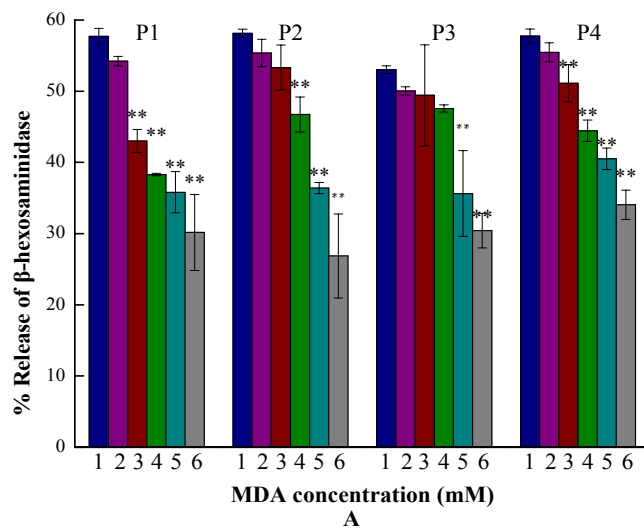


Figure. 1B

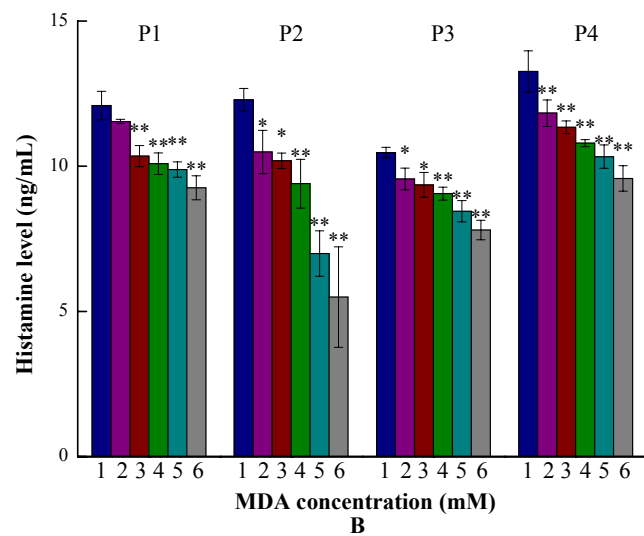


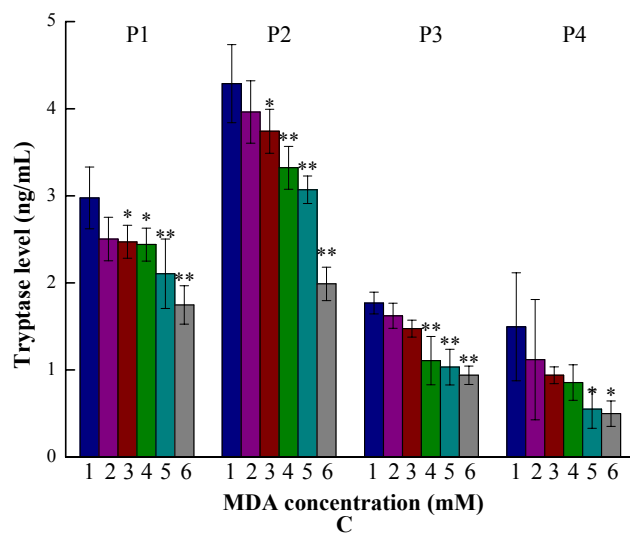
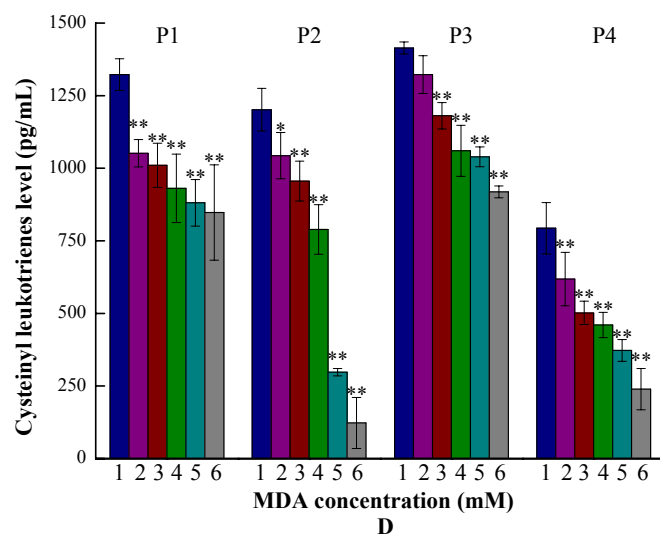
Figure. 1C**Figure. 1D**

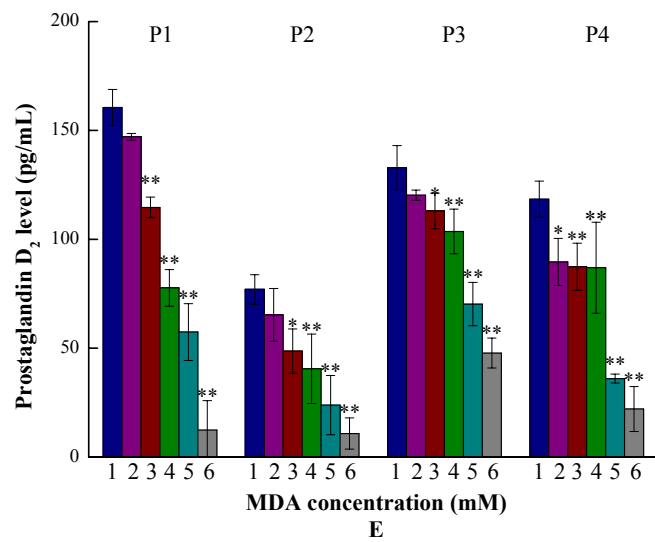
Figure. 1E

Figure. 2A

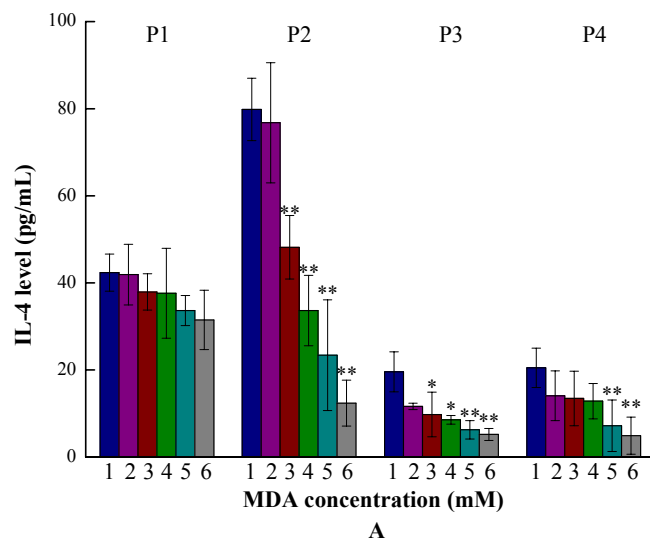


Figure. 2B

