

Allergenicity and Oral Tolerance of Enzymatic Cross-Linked Tropomyosin Evaluated Using Cell and Mouse Models

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ABSTRACT: The enzymatic cross-linking of proteins to form high-molecular-weight compounds may alter their sensitization potential. The IgG-/IgE-binding activity, digestibility, allergenicity, and oral tolerance of cross-linked tropomyosin with tyrosinase (CTC) or horseradish peroxidase (CHP) were investigated. ELISA results demonstrated CTC or CHP reduced its IgE-binding activity by 34.5 ± 1.8 and $63.5 \pm 0.6\%$, respectively. Compared with native tropomyosin or CTC, CHP was more easily digested into small fragments; CHP decreased the degranulation of RBL-2H3 cells and increased endocytosis by dendritic cells. CHP can induce oral tolerance and reduce allergenicity in mice by decreasing IgE and IgG1 levels in serum, the production of T-cell cytokines, and the percentage composition of dendritic cells. These findings demonstrate CHP has more potential of reducing the allergenicity than CTC via influencing the morphology of protein, changing the original method of antigen presentation, modulating the Th1/Th2 immunobalance, and inducing the oral tolerance of the allergen tropomyosin.

KEYWORDS: allergenicity reducing, tropomyosin, enzymatic cross-linking, horseradish peroxidase, oral tolerance potency

INTRODUCTION

Food allergy is becoming a worldwide problem and affects an estimated 6–8% of children. The perceived prevalence of food allergy is even higher, with an estimated 20% of children adhering to some form of elimination diet.¹ Shellfish allergy is the most frequent cause of food allergy, and it is responsible for the majority of emergency department visits related to severe food allergy.² Shellfish allergy in the Asia-Pacific is fairly prevalent and ranks as one of the most common foods causing allergy.³ In China, 16.7% of the rural population is sensitized to shellfish.⁴

Shellfish allergy is a type I hypersensitivity reaction, which is mediated by the binding of allergens with specific immunoglobulin E (IgE).⁵ During food processing, different methods, such as heating, pasteurization, and enzymatic cross-linking, could influence the allergenicity and immunogenicity of allergens and might be related to the conformation change of allergen on the level of food allergy sensitivity and effector phases.^{6,7} It is reported that enzymatic cross-linking of dietary proteins can increase the molecular weight of proteins and change the secondary structure.⁸ Tyrosinase is one of the most common oxidases in natural resources, which is regarded as an ideal polymerizer usually applied on cross-linking of proteins and peptides by catalyzing the oxidation of tyrosine residue.⁹ Currently, a study on milk demonstrated that cross-linked β -casein by tyrosinase can result in a significant decrease in the allergenicity of casein.¹⁰ Despite tyrosinase, peroxidases are a diverse group of oxidoreductases that use H_2O_2 as an electron acceptor to oxidize a variety of organic and inorganic substrates such as phenols, aromatic phenols, phenolic acids, and amino acids.¹¹ It has been hypothesized that peroxidases from horseradish (HRP) affect the gluten network by the cross-

linking of gluten proteins or by attaching arabinoxylans to gluten proteins via ferulic acid moieties and lysine, tyrosine, or cysteine residues.¹²

Tropomyosin (TM) is a highly conserved protein with a high degree of amino acid sequence identity among different species and has been identified as the major shellfish allergen.^{2–4,13,14} The molecular weight of TM in crab and shrimp is estimated as 38 kDa by SDS-PAGE.^{3,15} Currently, the most sufficient amino acids that have been identified in TM epitopes are tyrosine, arginine, glutamic acid, serine, and phenylalanine.¹⁶ These amino acids are extremely sensitive to oxidative stress.¹⁷ A previous study found that enzymatic cross-linking with crab arginine kinase (AK) by tyrosinase had reduced IgE-binding activity and allergenicity.⁷ However, TM exhibits strong resistance to digestive fluids and retains its IgE-binding ability even after prolonged heating due to the exceptionally stable α -helical coiled-coil secondary structure.^{18,19} Therefore, it is necessary to assess whether TM can be cross-linked by enzymes as enzymatic cross-linking can be used to influence TM stability, conformation, and digestibility and reduce allergenicity and immunogenicity.

Mouse and cellular models are usually used to evaluate allergenicity and immunogenicity in vivo and in vitro. Following exposure of allergens in vivo, inflammation can occur, which is induced by cellular components and mediated by dendritic cells (DCs), T cells, and basophils.²⁰ DCs are key effector cells in allergy, and once activated they can take in,

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process, and present antigens to T cells and release cytokines.²¹ In addition, T regulatory (Treg) cells play an important role in the inhibition of immune responses.²² Therefore, T cells, DCs, basophils, and Treg cells are important indicators in assessing allergic reactions, but the effect of cross-linked TM is unknown.

In the present study, the effect of crab TM cross-linking with horseradish peroxidase or tyrosinase on the digestibility and IgG-/IgE-binding activity was investigated with simulated digestion and ELISA in vitro. The allergenicity and cellular uptake of cross-linked TM were investigated by using cell models. The mouse model was to investigate the potential of allergenicity and oral tolerance. The aim of this study was to investigate the potential of enzymatic processing and provide valuable references for further research into the different allergens in a complex food system.

MATERIALS AND METHODS

Chemicals. The horseradish peroxidase (HRP) and tyrosinase from mushroom were purchased from Sigma-Aldrich (Seelze, Germany). The goat anti-rabbit IgG antibody or goat anti-human IgE antibody was from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA). The goat anti-mouse IgE, IgG1, and IgG2a antibodies were purchased from Abcam (Cambridge, UK). An ELISA kit of histamine was purchased from IBL (Hamburg, Germany). The ELISA kits of IL-4, IL-13, interferon (IFN)- γ , and mouse mast-cell protease-1 (mMCP-1) was purchased from R&D Systems (Minneapolis, MN, USA).

Mice. Five-week-old female BALB/c mice were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). All mice were housed under specific pathogen-free conditions. No crab protein was present in the diet. Experiments were approved by the Fisheries College of Jimei University (SCXK 2012-0005, Xiamen, China).

Human Sera. Human sera from crab-allergic and shrimp-allergic patients (no. 7543, 4768, 2389, 1426, 8734, 5396, and 4658) were provided by Xiamen Second Hospital (the human ethical approval no. is XSH2012-EAN019, Xiamen, China). The specific IgE levels to crab and shrimp were assessed in vitro using an ImmunoCAP (Phadia AB, Uppsala, Sweden). Table 1 shows the data of patients who were allergic to crab and shrimp. All sera were stored at -30°C until used.

Table 1. Specific IgE Levels in Patients in Relation to Crab and Shrimp

ID	age (years)	sex ^a	specific IgE ^b (kU/L), crab or shrimp
7543	3	F	0.93
4768	5	M	1.93
2389	2	M	2.93
1426	29	M	3.93
8734	45	F	4.93
5396	23	M	5.93
4658	3	F	6.93

^aM, male; F, female. ^bA serum with specific IgE > 0.35 (kU/L) is defined as positive.

Protein Purification and Enzymatic Cross-Linking Reaction. TM was purified from *Scylla paramamosain* according to the method of Liang et al.²³ The activity of HRP (670 nKat/mL) and tyrosinase (1900 nkat/mL) was assessed as described previously.^{8,24} Cross-linking of TM preliminarily produced a higher molecular weight protein complex.⁷ The optimal conditions for the cross-linking of TM were as follows: HRP (800 nkat/g) and tyrosinase (1000 nkat/g) at 37°C for 1 h, and the protein complexes were named CHP and CTC, respectively. In addition, CTC and CHP will be heated at 95°C for 10 min to inactivate enzymes. In the case of CTC, 0.25 mM caffeic acid was added as a mediator.

Electrophoretic and Scanning Electron Microscopy Analyses. The target protein was collected and detected by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).⁷ The protein concentrations in the eluted solutions were estimated by measuring the absorbance at 280 nm.

Cross-linked TM and TM were coated with gold, and their morphology was observed using a scanning electron microscope (SEM) (Hitachi S-4800, Zeiss, Germany).⁷ The SEM analysis was detected under the following conditions: Mag = $\times 1.00$ k, Vacc = 3.0 kV, WD = 7.2 mm, and SignalName = SE(M).

ELISA Analysis. The IgG-/IgE-binding activity of TM was measured by ELISA, which was performed as previously described, with some modifications.²⁵ An ELISA plate (P1) was coated with native purified TM (100 $\mu\text{g/mL}$) and incubated overnight at 4°C . The plate was washed and then blocked with 5% skimmed milk at 37°C for 1.5 h. In another ELISA plate (P2), cross-linked TM was incubated with a rabbit anti-crab (*S. paramamosain*) TM polyclonal antibody or crab-allergic patients' sera at 37°C for 2 h. After washing of P1, P2 solution was added to P1 at 37°C for 2 h. Then after another washing of P1, it was incubated with goat anti-rabbit IgG antibody or goat anti-human IgE antibody (diluted 1:2000) at 37°C for 1.5 h. After a final wash, tetramethylbenzidine was added and P1 was incubated at 37°C for 20 min. The reaction was terminated by sulfuric acid, and it was measured at 450 nm using an automatic microplate reader (Infinite M200 PRO, Tecan, Austria).

Secondary Structure and Surface Hydrophobicity Analysis. Far-UV CD spectra were measured with a Chirascan circular dichroism spectrometer (Applied Photophysics Ltd., Surrey, UK), according to a method described previously.²⁶ Each spectrum represents the scan at 25°C with TM 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 ($\text{deg cm}^2 \text{dmol}^{-1}$).

The surface hydrophobicity of cross-linked TM was determined using 8-anilino-1-naphthalenesulfonic acid as the fluorescence probe, according to a method described previously.²⁷

In Vitro Digestion Assay. The simulated gastric fluids (pepsin) were prepared as described previously.²⁸ The final concentration of TM and cross-linked TM was adjusted to 0.5 mg/mL. SDS-PAGE and Western blot analysis (using the goat anti-rabbit IgG antibody) were used to determine the digestibility and IgG-binding activity.

RBL-2H3 and Dendritic Cells Assay. The rat mast cell line RBL-2H3 was obtained from the American Type Culture Collection (Bethesda, MD, USA). The cells were cultured as described previously.²⁹ The release of β -hexosaminidase and histamine from RBL-2H3 cells was measured as a model of IgE-mediated mast cell allergic reaction,²⁹ using the sensitization of mouse sera and TM or cross-linked TM (500 ng/mL) for 6 h and 15 min at 37°C , respectively. The cell supernatant was used to measure the release levels of β -hexosaminidase and histamine using an ELISA kit (IBL, Hamburg, Germany).

Bone marrow cells from mice were cultured at 10^6 cells/mL in complete RPMI 1640 (Hyclone, Logan, UT, USA) with 10 ng/mL of granulocyte-macrophage colony-stimulating factor and 5 ng/mL of interleukin (IL)-4. At day 6, approximately 90% of cells expressed medium–high levels of CD11c and major histocompatibility complex (MHC) class II. To assess protein endocytosis, pHrodo Green (Invitrogen, Life Technologies, Breda, The Netherlands)-labeled TM or cross-linked TM was incubated with 10^6 cells for 0, 15, and 30 min. The pHrodo Green-positive DCs were measured by using an automatic microplate reader.

In Vivo Studies. *Oral Sensitization to TM.* Oral sensitization testing was used according to the method of Radosavljevic et al.⁸ Mice were exposed to 3 mg of TM ($n = 6$) or cross-linked TM ($n = 6$) with 7.5 μg of cholera toxin by intragastric gavage for 3 consecutive days, and this was repeated once a week for 4 weeks. The control group ($n = 6$) received phosphate-buffered saline. All mice received a challenge of 6 mg of TM or cross-linked TM intragastrically on day 28 and were sacrificed 1 day later to measure the levels of anti-TM IgG2a, IgE, and

IgG1. The blood sample used to measure the levels of mMCP-1 and histamine was collected at 30 min after oral challenge on day 28.

Splenocytes and mesenteric lymph nodes (MLN) lymphocytes were prepared by aseptic removal on day 29, and cell supernatants were measured as the levels of IL-4, IL-13 and IFN- γ after 3 days.

The spleen and MLNs cells (2×10^5 cells/well) were isolated from mice on day 29 to detect changes in the number of Treg cells. Cells were stained with anti-CD4-PerCP Cy5.5 and anti-Foxp3-Alexa 647 (BD Pharmingen, San Diego, CA, USA) fixation/permeabilization and analyzed by flow cytometry. All flow cytometry experiments were performed with the Guava easyCyte 6–2L system and GuavaSoft 3.1.1 software (Millipore, Bedford, MA, USA).

Induction of Oral Tolerance to TM. Mice ($n = 6$) received 1 mg of TM or cross-linked TM or phosphate-buffered saline via intragastric gavage for 3 consecutive days (Table 2). This was followed by

Table 2. Experimental Groups of Tolerance Induction

group name	sensitization	challenge
PBS-TM	PBS	TM
TM-TM	TM	TM
CTC-TM	CTC	TM
CHP-TM	CHP	TM

intraperitoneal immunization with 200 μ g TM/alum adjuvant (Pierce, Rockford, IL, USA) 14 and 21 days after the last exposure. Blood was obtained on day 34, and the animals were subsequently sacrificed.

The blood was collected on day 34 to measure the level of specific antibodies. Spleen and MLN single-cell suspensions (1×10^5 cells in 200 μ L of complete RPMI 1640) were incubated for 3 days. IL-4, IL-13, and IFN- γ levels in the culture supernatants of splenocytes and MLN were determined by ELISA kits.

Splenocyte and MLN cell suspensions were evaluated to determine changes in the numbers of DCs. Cells were stained with anti-CD11c-APC and anti-MHC-II-FITC (BD Pharmingen) and analyzed by flow cytometry. All flow cytometry experiments were performed with the Guava easyCyte 6–2L system and GuavaSoft 3.1.1 software.

Statistical Analysis. Data from the in vivo and in vitro studies were presented as the mean $\pm$ SD. Data were analyzed by the General Linear Model and ANOVA of Duncan's test. Differences between

groups were considered significant when p values were <0.05 . Each experiment was repeated at least three times.

RESULTS

Electrophoretic and Scanning Electron Microscopy Analysis. SDS-PAGE analysis showed that enzymatic cross-linking caused the polymerization of TM. Compared to that of TM, both CTC and CHP produced higher molecular weight (MW) proteins (Figure 1A). CTC produced proteins with MW over 66 kDa. CHP produced a more complicated protein complex, containing proteins of MW over 45 kDa and others greater than 116 kDa. In addition, SEM results showed (Figure 1B) that both CTC and CHP were built from rod proteins, which connected to form a network through the enzymatic cross-linked reaction. The extent of CTC was tighter than that with CHP, whereas the extent of CHP resulted in a loose network.

Analysis of the IgG-/IgE-Binding Activity of Cross-Linked TM. The IgG-/IgE-binding activity of CTC and CHP was investigated with regard to allergenicity (Figure 1C). ELISA analysis revealed that the IgG-binding activity of CTC and CHP decreased by 16.9 ± 0.8 and $70.8 \pm 0.5\%$ and the IgE-binding activity of CTC and CHP reduced by 34.5 ± 1.8 and $63.5 \pm 0.6\%$, respectively.

Secondary Structure and Surface Hydrophobicity of Cross-Linked TM. Far-ultraviolet CD data were used to detect the secondary structure of cross-linked TM (Figure 1D). After HRP and tyrosinase treatment, the positive and negative molar residue ellipticity peaks near 208 and 220 nm of the samples decreased, which indicated that the content of α -helix decreased. Further analyses performed with CDNN software showed that the amount of α -helix in CTC was reduced to 78.5% and that in CHP was reduced to 86.8%. The percentage of β -sheet, at around 198 nm, increased to 2.3% in CTC and to 1.5% in CHP. In addition, the percentage of β -turn in CTC was up to 10.2% and in CHP was up to 8.6%. The amount of

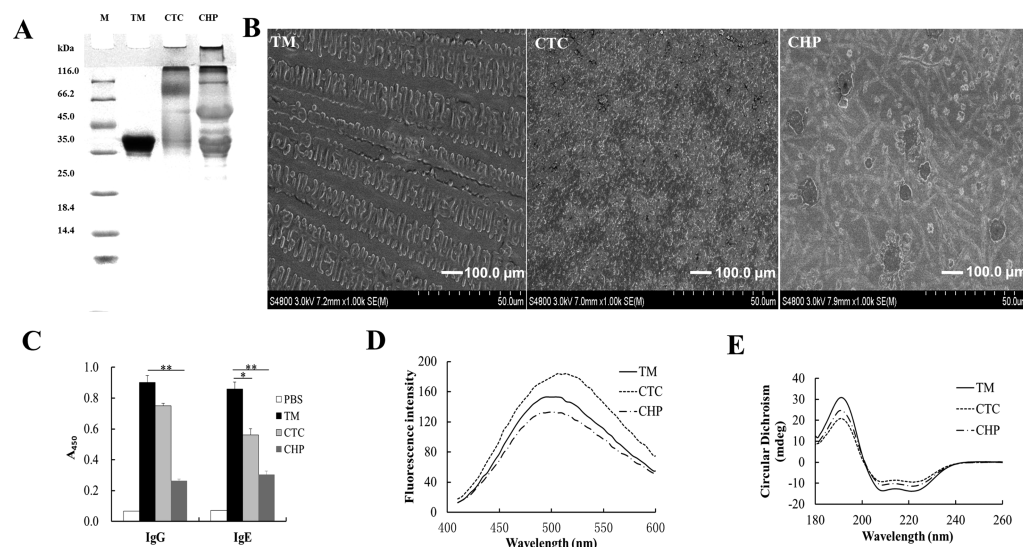


Figure 1. Polymerization and IgG-/IgE-binding activities of enzymatic cross-linked TM compared with TM: (A) cross-linked tropomyosin with tyrosinase/cafeic acid (CTC), cross-linked tropomyosin with horseradish peroxidase (CHP) resolved in 12% SDS-PAGE; (B) SEM of TM, CTC, and CHP (the scale of the photograph has been added); (C) ELISA of TM, CTC, and CHP, rabbit anti-TM IgG, or patient sera were used as the antibody; (D) secondary structure analysis of cross-linked TM; (E) surface hydrophobicity analysis of cross-linked TM. The data represent the mean $\pm$ SD from triplicate determinations.

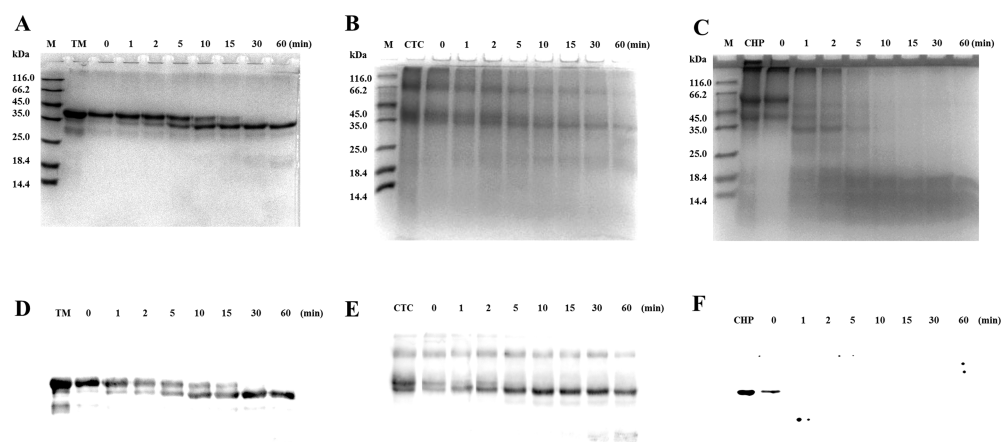


Figure 2. Stability of cross-linked TM by simulating pepsin digestion: (A–C) SDS-PAGE analysis of TM, CTC, and CHP stability to pepsin digestion; (D–F) Western blot analysis of TM, CTC, and CHP treated with pepsin.

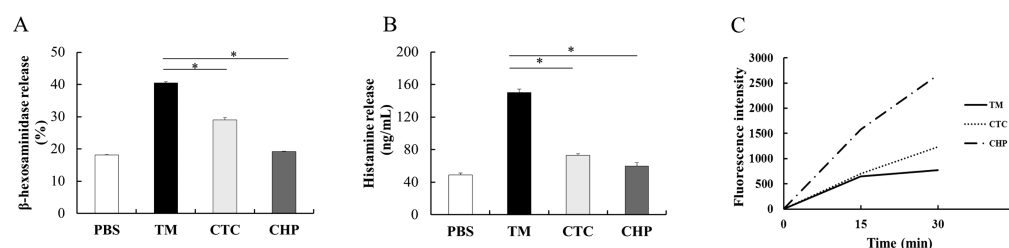


Figure 3. Impact of cross-linked TM in IgE-mediated RBL-2H3 cells and uptake by DCs. (A) release of β -hexosaminidase; (B) level of histamine; (C) uptake of pHrodo Green-labeled cross-linked TM and TM by DCs. The data represent the mean $\pm$ SD from triplicate determinations.

random coil, near 220 and 230 nm, increased to 7.9% in CTC and to 5.0% in CHP.

On the basis of the results of surface hydrophobicity (Figure 1E), CTC had high hydrophobicity compared with TM, and the fluorescence intensity peak showed a red shift, whereas CHP had low hydrophobicity compared with TM and the fluorescence intensity peak decreased.

Stability of Cross-Linked TM in a Simulated Digestion in Vitro. TM was digested to a 32 kDa fragment by pepsin within 10 min, and the 32 kDa fragment also failed to degrade until 60 min (Figure 2A). CTC remained as a macromolecular band, and the 32 kDa fragment was seen up to 60 min (Figure 2B), whereas CHP was easily digested into small fragments within 10 min (Figure 2C). CTC was more difficult to digest than TM, whereas CHP exhibited easier degradation than TM or CTC. As shown in Figure 2D–F, Western blotting revealed that the IgG-binding activity of CTC digested fragments showed increasing intensity, but CHP and its digested fragments had no IgG-binding activity. In addition, there was little change in IgG-binding activity of the TM fragments.

Impact of Cross-Linked TM in IgE-Mediated RBL-2H3 Cells and Uptake by DCs. RBL-2H3 cells are the IgE-mediated mast cell model used to release β -hexosaminidase and histamine.²⁹ TM induced significant degranulation in RBL-2H3 cells, whereas CTC and CHP induced a lower level of degranulation than that of TM (Figure 3A,B). The release of β -hexosaminidase in the CTC group reduced $11.4 \pm 1.7\%$ and in the CHP group reduced $21.3 \pm 2.3\%$. In addition, the release of histamine in the CTC group decreased $51.5 \pm 0.9\%$ and in the CHP group decreased $60.2 \pm 1.1\%$.

The pHrodo Green dye was used to label proteins as it fluoresces brightly at acidic pH with almost no fluorescence at neutral pH, which makes this dye a good indicator of the

localization of TM or cross-linked TM in the endolysosomal compartments. Endocytosis of pHrodo Green-labeled TM was internalized at a much slower rate, and at 30 min there was no obvious increase. pHrodo Green-labeled CTC was slightly increased at 30 min, and pHrodo Green-labeled CHP reached saturation at 30 min and was obviously increased compared to TM and CTC (Figure 3C).

Allergic Responses Following Intragastric Exposure to Cross-Linked TM. An animal model of allergy was used to test the allergenicity of cross-linked TM in vivo (Figure 4A). First, CTC and CHP decreased the levels of the TM-specific IgE and IgG1 in mouse serum; however, CHP led to an obvious increase in the level of the TM-specific IgG2a in serum (Figure 4B). Second, the level of histamine in sera in the CTC and CHP groups (Figure 4C) was significantly decreased compared with the TM group. In addition, it should be noted that the changes in mMCP-1 (Figure 4D) among TM, CTC, and CHP reached 28, 17, and 16 ng/mL, respectively. Third, the levels of IL-4 and IL-13 as representative Th2-related cytokines (Figure 4E,F) in spleen cells after restimulation with cross-linked TM were decreased. IFN- γ , a Th1-related cytokine in spleen cells, was increased after restimulation with cross-linked TM (Figure 4G). However, the levels of IL-4 and IL-13 (Figure 4H,I) in MLN lymphocytes after restimulation with cross-linked TM were increased. In the MLN lymphocyte, compared to the production of IFN- γ in TM, that of CTC was slightly increased and that of CHP was unchanged (Figure 4J).

To examine the change in CD4⁺ fork head box P3 (Foxp3) T cells in the four groups of mice, flow cytometry experiments were performed (Figure 4K). In splenocytes, the percentage of CD4⁺Foxp3⁺ Treg cells increased from 1.68% (TM group) to 1.91% (CTC group) and 2.42% (CHP group). No significant

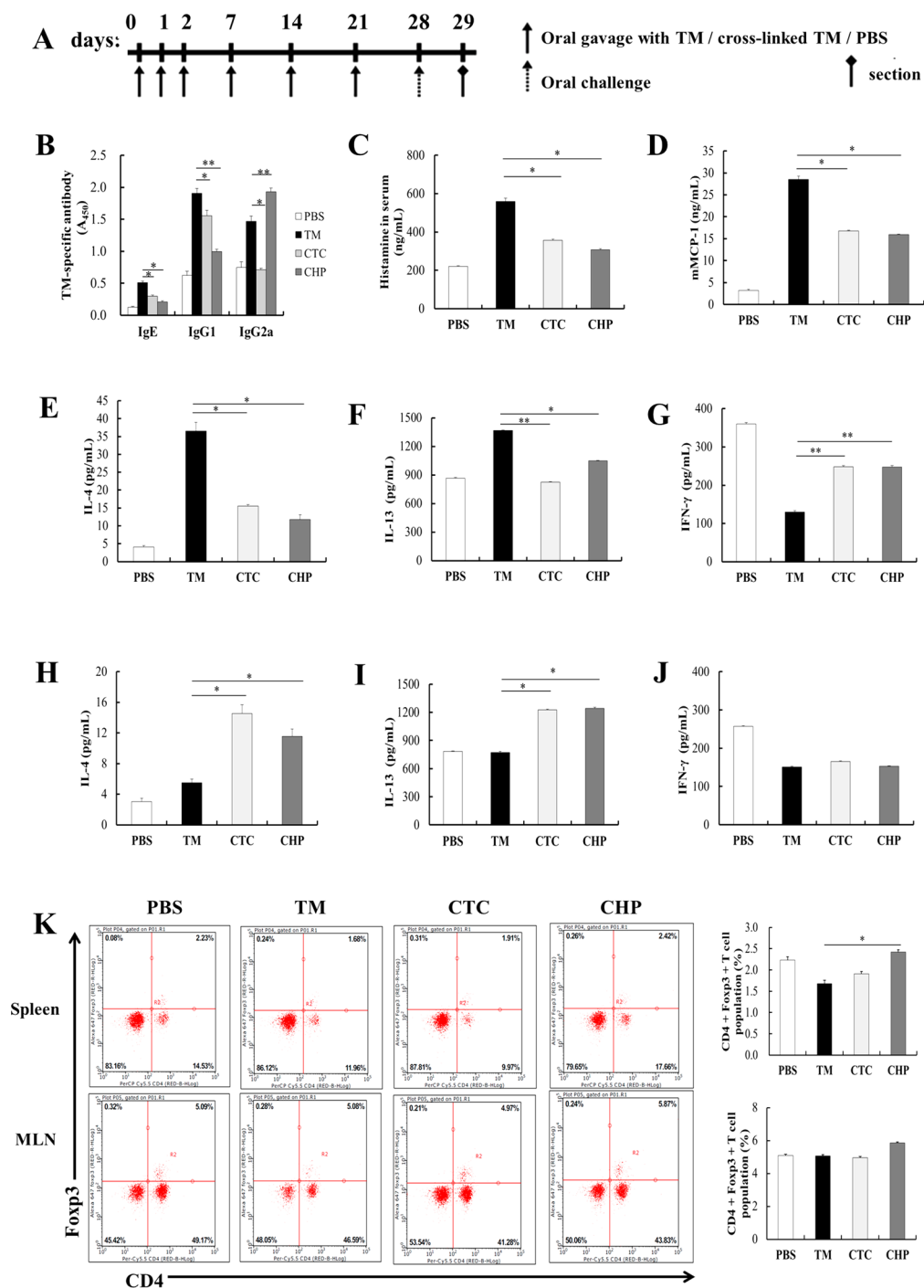


Figure 4. Sensitization experiment. Cells were isolated from spleens and MLNs and stained for CD4 and Foxp3: (A) sensitization protocol; (B) levels of IgE, IgG1, and IgG2a measured on day 29; (C, D) release of histamine/mMCP-1 measured in serum; (E–G) production of IL-4, IL-13, and IFN- γ measured in spleen cell culture; (H–J) production of IL-4, IL-13, and IFN- γ measured in MLN cell culture; (K) spleen and MLNs cells isolated from the mouse models of food allergy on day 29. Cells were cultured in the presence of 25 μ g/mL of anti-CD4-PerCP Cy5.5 and 25 μ g/mL of anti-Foxp3-Alexa 647 for 30 min. CD4⁺Foxp3⁺ T cells were analyzed by flow cytometry. *, $p < 0.05$; **, $p < 0.01$. Data represent the mean $\pm$ SD from triplicate determinations.

change in MLN lymphocytes (CTC group) was observed, whereas the CHP group slightly increased.

Oral Tolerance Induced by Cross-Linked TM. The ability of cross-linked TM to induce gut and systemic immune tolerance was investigated in oral tolerance testing (Figure 5A). Compared with mice in the TM-TM group, the CTC-TM and CHP-TM groups showed reduced levels of the TM-specific IgE, IgG1, but not IgG2a (Figure 5B). The levels of IL-4, IL-13,

and IFN- γ were measured in spleen cell and MLN lymphocyte (Figure 5C–H). Compared with TM-TM, CTC-TM and CHP-TM decreased the production of IL-4 and IL-13 and increased the production of IFN- γ in spleen cells. However, in the CTC-TM and CHP-TM groups, the production of IL-13 was increased; IFN- γ in MLN lymphocytes in CTC-TM was slightly increased, whereas CHP-TM was slightly decreased, and the production of IL-4 in MLN lymphocytes was

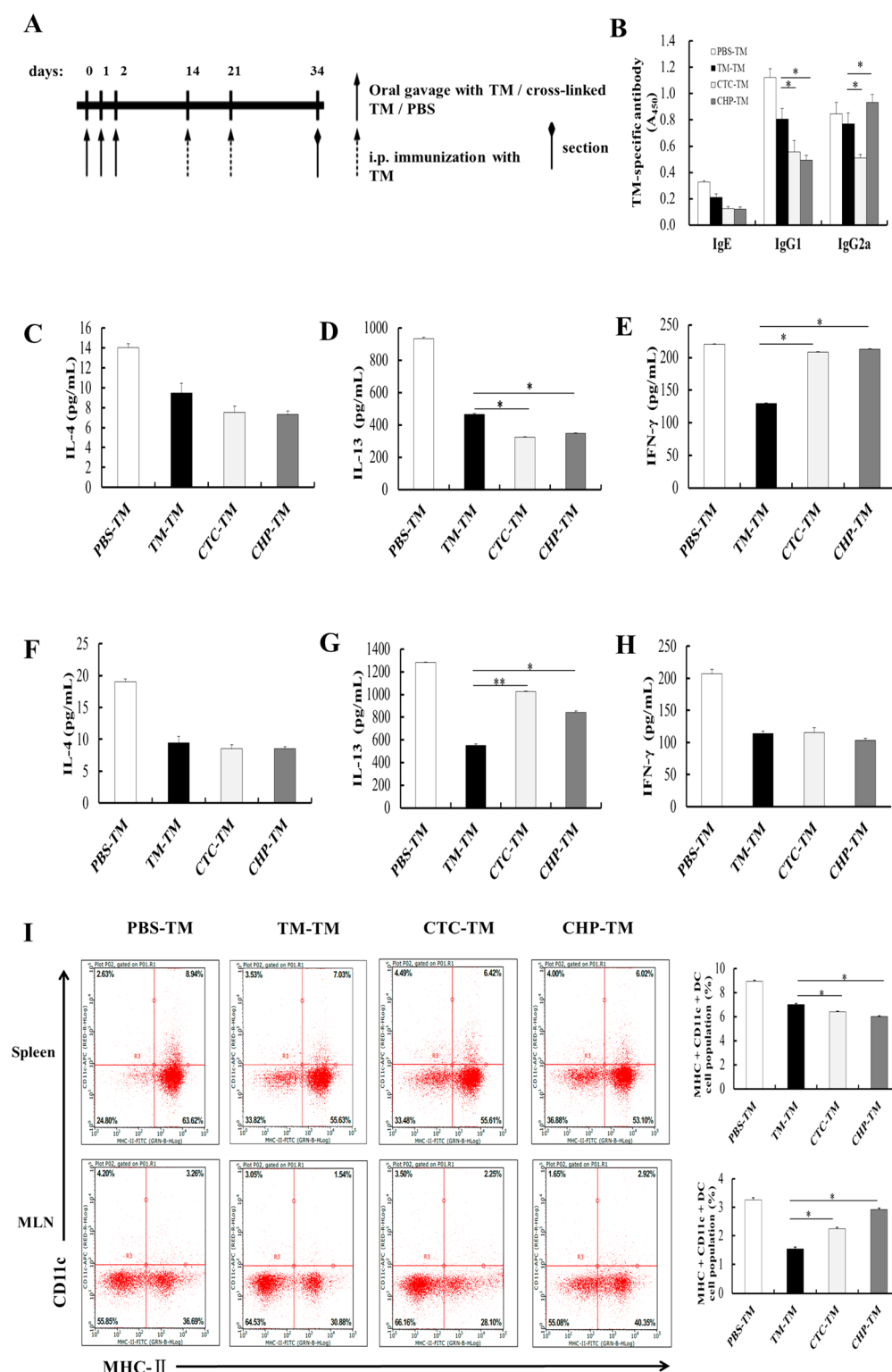


Figure 5. Sensitizing potential of cross-linked TM in the tolerance induction experiment: (A) oral tolerance induction protocol; (B) levels of IgE, IgG1, and IgG2a measured in serum; (C–E) production of IL-4, IL-13, and IFN- γ measured in the spleen cell culture; (F–H) production of IL-4, IL-13, and IFN- γ measured in the MLN cell culture; (I) spleen and MLNs cells were isolated from the mouse models of food allergy on day 34. Cells were isolated from spleens and MLNs and stained for CD11c and MHC-II. Cells were cultured in the presence of 25 μ g/mL anti-CD11c-APC and 25 μ g/mL anti-MHC-II-FITC for 30 min. CD11c⁺ MHC-II⁺ DCs were analyzed by flow cytometry. *, $p < 0.05$; **, $p < 0.01$. Data represent the mean $\pm$ SD from triplicate determinations.

unchanged. To examine changes in the numbers of DCs in the four groups of mice, flow cytometry experiments were performed (Figure 5I). In splenocytes, the percentage of CD11c⁺ MHC-II⁺ DCs decreased from 7.03% (TM-TM

group) to 6.42% (CTC-TM group) and 6.02% (CHP-TM group). However, the percentage of CD11c⁺ MHC-II⁺ DCs increased from 1.54% (TM-TM group) to 2.25% (CTC-TM group) and 2.92% (CHP-TM group) in MLN lymphocytes.

DISCUSSION

The present study shows that the allergenicity of crab TM has been reduced by the cross-linked reaction with tyrosinase or HRP. As shown in the results, cross-linked proteins CTC and CHP were formed into a complicated protein complex, which was consistent with previous studies.^{7,30,31} Moreover, many studies have shown that cross-linked dietary products further influenced their allergenicity.^{7,30–32} Tyrosinase catalyzes the oxidation of tyrosine and results in oxidative cross-linking of tyrosine side chains, so cross-linking of TM by tyrosinase catalyzed the oxidation of the amino acid residues Y₁₆₂, Y₂₂₁, and Y₂₆₇ in TM linear epitopes. Peroxidase is a diverse group of oxidoreductases, and cross-linking of TM by HRP catalyzed the oxidation of the amino acid residues of Y₁₆₂, Y₂₂₁, Y₂₆₇, and F₁₅₃ in TM linear epitopes. Therefore, TM linear epitopes were covered or destroyed to reduce the IgE-binding activity.

Due to the effect of enzymatic cross-linked reaction, the secondary structure of cross-linked TM has changed. The polymerization of TM forms more complex structures with decreases of α -helix and β -turn, associating with an increase of random coil. In addition, the enzymatic cross-linking reaction also can change the hydrophobicity of allergens. The hydrophilic amino acids of TM were covered by the cross-linking of tyrosinase, whereas the hydrophilic amino acids of TM were exposed by that of HRP. A correlation was found between hydrophobicity and digestion in two recent studies.^{31,33} CTC had high hydrophobicity compared with TM or CHP, which also explained its digestibility, as it was difficult for pepsin to find cleavage sites. Interestingly, CHP, which forms a loose network, increased digestibility by reducing its hydrophobicity. In addition, CHP resulted in more digestion of the cleavage sites, and the new small fragments did not have IgG-/IgE-binding activity. Hence, this may explain why these two treatments behaved differently in simulated digestion and surface hydrophobicity. This eventually elicited a different immunological response compared to the native TM. From the results of endocytosis of pHrodo Green-labeled TM or cross-linked TM, it is associated with digestion. pHrodo Green-labeled CHP was easily digested within 10 min; hence, it can be lead to faster accumulation and processing in DCs, and it fluoresces brightly at acidic pH in the lysosome of DCs. Therefore, the fluorescence intensity of CHP is stronger than that of CTC or TM.

Cross-linked TM influenced allergenicity and the degranulation in RBL-2H3 cells. This was in agreement with a previous study in which the treatment of AK with tyrosinase/cafeic acid decreased degranulation.⁷ The response of RBL-2H3 cells may be explained by the aggregation of cross-linked TM, which covered the IgE epitopes, thus successfully decreasing the degranulation of basophils.

Treg cells inhibited CD4⁺ T cells, thus reducing the secretion of IL-4.⁷ As a result, the percentage of CD4⁺Foxp3⁺ Treg cells (cross-linked TM group) increased in splenocytes compared to the TM group, and this was consistent with the production of cytokines. In addition, cross-linked TM relieved allergy symptoms in mice compared to TM, as shown by significantly lower levels of IgG1 and IgE, and cross-linked TM reduced the levels of mMCP-1 and the release of histamine.

The influence between spleen and MLN cells has been reported.^{34,35} Our results show that the percentage of CD4⁺Foxp3⁺ Treg cells in MLN cells was not significantly changed and that the production of cytokines was increased.

The concentration of antigen reaching the circulation may change by the nature and dose of antigen.³⁶ Cross-linked TM after gastrointestinal digestion will produce some allergic degradation segments, but this was gradually degraded into smaller nonallergenic molecular fragments while the time increased. The low concentration of allergenic degradation segments caused only local gastrointestinal lymphocyte immune responses, and smaller molecular fragments did not cause an immune response in the spleen. Thus, cross-linked TM changed the antigens presented from MLN to the spleen.

DCs as antigen-presenting cells play a central role in oral tolerance.^{37,38} In addition, DCs carry antigens from MLN to the spleen.³⁹ The number of DCs in the spleen in the CTC-TM and CHP-TM groups decreased compared with the TM-TM group. Hence, the cross-linked TM altered the method of antigen digestion and not only affected antigen presentation but also induced oral tolerance in mouse spleen cells.

Oral tolerance alleviates immune responses, and it plays an important role in immune homeostasis. The intestinal immune system includes inductive and effector sites. Inductive sites contain the gut-associated lymphoid tissues; the main effector sites contain the lamina propria (LP) and epithelium, harboring large populations of activated T cells and antibody-secreting plasma cells. The LP may also contribute to the induction of tolerance, as a site of antigen uptake and loading of migratory DCs that encounter naive T cells in the MLN.³⁷ CD103⁺ DCs from the LP and MLN induce the expression of gut-homing molecules on T cells and consequently result in the release of cytokines on T cells in the MLN. Therefore, the increase of DCs in the MLN contributes to the oral tolerance. Orally administered antigens can inhibit following immune responses in the gut and the systemic immune system; however, cross-linked TM suppresses immune responses only in the systemic immune system. Low molecular weight proteins may pass directly across the epithelium by paracellular diffusion, whereas larger molecular proteins can be taken across enterocytes. These findings showed that cross-linked TM was different from TM in the immunological response. In addition, the variation in antigen presentation from the MLN to the spleen was unknown. Therefore, further in-depth study on these issues is desirable.

In our previous study,⁷ enzymatic cross-linking was an effective way of reducing crab allergens, of which AK is the main allergen. Compared with cross-linked AK, CTC had a shorter cross-linking time and did not require heating of TM in advance. In addition, we also found that CHP reduced IgG-/IgE-binding activity more than CTC and TM and decreased the stability of pepsin digestion. Furthermore, some of the crab allergens achieved the same effect of reducing allergenicity due to enzymatic cross-linking. Therefore, the hypoallergenic crab meat was developed and submitted for a Chinese patent (no. 2015108074188) based on the enzymatic cross-linking process technique. Clinical trials are required to confirm that tyrosinase and HRP cross-linked crab allergens may reduce crab allergy.

In conclusion, enzymatic treatment of TM with tyrosine or HRP yielded high molecular weight compounds. CHP, which forms a loose network, decreased digestibility by reducing its hydrophobicity. It also has reduced IgG-/IgE-binding activity and allergenicity. The digestible CHP changed the original method of antigen-presenting and induced oral tolerance in the systemic immune system. In future investigations, it is important to examine the effects of cross-linked reaction on the different allergens in a complex food system.

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Notes

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ABBREVIATIONS USED

CHP, cross-linked tropomyosin with horseradish peroxidase; CTC, cross-linked tropomyosin with tyrosinase/caffeic acid; DCs, dendritic cells; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; mMCP-1, mouse mast-cell protease-1; MLN, mesenteric lymph nodes; TM, tropomyosin; Treg, regulatory T

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