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%±0.6, respectively. Compared with native tropomyosin or CTC, CHP was 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 decreased serum levels of IgE and IgG1, 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-presenting, modulating the Th1/Th2 immunobalance, and induce the oral tolerance of the allergen tropomyosin.

Keywords: allergenicity reducing; tropomyosin; enzymatic cross-linking; horseradish peroxidase; oral tolerance potency

1. Introduction

Food allergy is becoming a worldwide problem and it affected about 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 and specific immunoglobulin E (IgE).[5] Different methods of food processing can influence the allergenicity and immunogenicity of allergens. It may involve both the sensitivity and effector phases of food allergy by influencing allergen stability, conformation and digestibility.[6] Different processing technologies such as heating, pasteurization and enzymatic cross-linking have different effects on allergens in the complex foods.[7] Cross-linking of dietary proteins can increase the molecular weight of proteins and change the secondary structure.[8] Tyrosinase is usually applied to proteins and peptides by catalyzing the oxidation of tyrosine and results in oxidative cross-linking of tyrosine side-chains. It has also been shown that tyrosinase can polymerize peptides containing tyrosine.[9] Cross-linked β-casein by tyrosinase contributed to a decrease in allergenicity.[10] 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] The best-characterized plant peroxidase is from horseradish (HRP). It has been hypothesized that peroxidases 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] Therefore, enzymatic cross-linking is an effective approach in food processing.

Several independent biochemical and immunological studies have identified the major shellfish allergen as tropomyosin (TM).[13,14] It has been reported that the molecular weight of TM is 38 kDa in crab and shrimp.[3,15] TM is a highly conservative allergic protein with a high degree of amino acid sequence identity among different species.[2-4,13] Currently, the most sufficient amino acids in TM epitopes, which have been identified, 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 alpha 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, 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 the 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.

2. Materials and methods

2.1 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, IgG2a antibodies were purchased from Abcam (Cambridge, UK). ELISA kit of histamine was purchased from IBL (Hamburg,

Germany). The ELISA kit of IL-4, IL-13, interferon (IFN)-γ and mouse mast-cell protease-1 (mMCP-1) was purchased from R&D Systems (Minneapoils, MN, USA).

2.2 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).

2.3 Human sera

Human sera from crab-allergic and shrimp-allergic patients (No. 7543, 4768, 2389, 1426, 8734, 5396 and 4658) were provided by the Xiamen Second Hospital (the human ethical approval number 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 in -30 ℃ until used.

2.4 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 the tyrosinase (1900 nKat/mL) was assessed as described previously.[8,24] Cross-linking of TM was 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 ℃ for 1 h, and the protein complex were named CHP and CTC, respectively. In

addition, CTC and CHP will be heated at 95 ℃ for 10 min to inactivate enzymes. In the case of CTC, 0.25 mM caffeic acid was added as a mediator.

2.5 Electrophoretic and scanning electron microscopy analysis

The target protein was collected and detected on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel.[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 observed using a scanning electron microscope (SEM) (HITACHI S-4800, Zeiss, Germany). [7] The SEM analysis was detected under the following conditions: Mag=x1.00 k, Vacc=3.0 kV, WD=7.2 mm and SignalName=SE(M)

2.6 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 μg/mL) and incubated overnight at 4 ℃. The plate was washed and then blocked with 5% skimmed milk at 37 ℃ for 1.5 h. In another ELISA plate (P2), cross-linked TM was incubated with a rabbit anti-crab (*Scylla paramamosain*) TM poly-clonal antibody or crab allergic patients' sera at 37 ℃ for 2 h. After washing P1, P2 solution was added to P1 at 37 ℃ for 2 h. Then washing P1, it was incubated with goat anti-rabbit IgG antibody or goat anti-human IgE antibody (diluted 1:2000) at 37 ℃ for 1.5 h. After a final wash, tetramethylbenzidine was added and P1 was incubated at 37 ℃ for 20 min. The

153 reaction was terminated by sulfuric acid and it was measured at 450 nm using an
154 automatic microplate reader (Infinite ® M200 PRO, Tecan, Austria).

155 **2.7 Secondary structure and surface hydrophobicity analysis**

156 Far-UV CD spectra were measured with a Chirascan circular dichroism
157 spectrometer (Applied Photophysics Ltd., Surrey, UK), according to a method
158 described previously.[26] Each spectrum represents the scan at 25 ℃ with TM
159 concentration adjusted to 0.5 mg/mL. The operating parameters were as follows: scan
160 rate, interval and bandwidth were set to 100 nm/min, 0.25 s and 1.0 nm, respectively.
161 The final results are shown as mdeg (deg cm^2 $dmol^{-1}$).

162 The surface hydrophobicity of cross-linked TM was determined using
163 8-anilino-1-naphthalenesulfonic acid as the fluorescence probe, according to a method
164 described previously.[27]

165 **2.8 In vitro digestion assay**

166 The simulated gastric fluids (pepsin) were prepared as described previously.[28] The
167 final concentration of TM and cross-linked TM was adjusted to 0.5 mg/mL.
168 SDS-PAGE and western blot analysis (using the goat anti-rabbit IgG antibody) were
169 to determine the digestibility and IgG-binding activity.

170 **2.9 RBL-2H3 and dendritic cells assay**

171 The rat mast cell line RBL-2H3 was obtained from the American Type Culture
172 Collection (Bethesda, MD, USA). The cells were cultured as described previously.[29]
173 The release of β-hexosaminidase and histamine from RBL-2H3 cells was measured as
174 a model of IgE-mediated mast cell allergic reaction,[29] using the sensitization of mouse

175 sera and TM or cross-linked TM (500 ng/mL) for 6 h and 15 min at 37 ℃,
176 respectively. The cell supernatant was measured the release levels of
177 β-hexosaminidase and histamine using an ELISA kit (IBL, Hamburg, Germany).

178 Bone marrow cells from mice were cultured at 10^6 cells/mL in complete RPMI
179 1640 (Hyclone, Logan, USA) with 10 ng/mL of granulocyte-macrophage colony
180 stimulating factor and 5 ng/mL of interleukin (IL)-4. At day 6, approximately 90% of
181 cells expressed medium–high levels of CD11c and major histocompatibility complex
182 (MHC) class II. To assess protein endocytosis, pHrodo Green (Invitrogen, Life
183 Technologies, Breda, Netherlands)-labeled TM or cross-linked TM was incubated
184 with 10^6 cells for 0, 15, and 30 min. The pHrodo Green-positive DCs were measured
185 by using an automatic microplate reader.

186 **2.10 In vivo studies**

187 **2.10.1 Oral sensitization to TM**

188 The oral sensitization testing was used according to the method of Radosavljevic et
189 al.[8] Mice were exposed to 3 mg TM (n=6) or cross-linked TM (n=6) with 7.5 μg
190 cholera toxin by intragastric gavage for 3 consecutive days, and this was repeated
191 once a week for 4 weeks. The control group (n=6) received phosphate buffer saline.
192 All mice received a challenge of 6 mg TM or cross-linked TM intragastrically on day
193 28, and were sacrificed 1 day later to measure the levels of anti-TM IgG2a, IgE, and
194 IgG1. Blood sample used to measure the levels of mMCP-1 and histamine was
195 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 supernatant were measured the levels of the 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, MA, USA).

2.10.2 Induction of oral tolerance to TM

Mice (n=6) received 1 mg TM or cross-linked TM or phosphate buffer saline via intragastric gavage for 3 consecutive days. This was followed by intraperitoneal immunization with 200 μg 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 μL 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.

2.11 Statistical analysis

Data from the in vivo and in vitro studies were presented as mean ± SD. Data were analyzed by the General Linear Model and ANOVA of Duncan test. Differences between groups were considered significant when p-values were <0.05. Each experiment was repeated at least 3 times.

3. Results

3.1 Electrophoretic and scanning electron microscopy analysis

SDS-PAGE analysis showed that enzymatic cross-linked caused the polymerization of TM. Compared to that of TM, both CTC and CHP produced higher molecular weight (MW) proteins (Figure 1A). CTC produced the MW proteins over 66 kDa. CHP produced more complicated protein complex, containing proteins of MW over 45 kDa and others greater than 116kDa. 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, while the extent of CHP resulted in a loose network.

3.2 Analysis of the IgG-/IgE-binding activity of cross-linked TM

The IgG-/IgE-binding activity of CTC and CHP was investigated to the allergenicity (Figure 1C). ELISA analysis revealed that the IgG-binding activity of

CTC and CHP decreased by 16.9% and 70.8%, and the IgE-binding activity of CTC and CHP reduced by 34.5%±1.8 and 63.5%±0.6, respectively.

3.3 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 tryosinase treatment, the positive and negative molar residue ellipticity peaks at near 208 nm 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 in CHP was reduced to 86.8%. The percentage of β-sheet, at around 198 nm, increased to 2.3% in CTC and increased 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 random coil, at near 220 nm and 230 nm, increased to 7.9% in CTC and increased to 5.0% in CHP.

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

3.4 The stability of cross-linked TM in a simulated digestion in vitro

TM was digested into 32 kDa fragment by pepsin within 10 min and 32 kDa fragment also failed to degrade until 60 min (Figure 2A). CTC remained as a macromolecular band and 32kDa fragment were seen up to 60 min (Figure 2B), while CHP was easily digested into small fragments within 10 min (Figure 2C). CTC was

more difficult to digest than TM, while the CHP exhibited easier degradation than TM or CTC. As shown in Figure 2D–F, western blotting showed that the IgG-binding activity of CTC digested fragments should be in Figure 2E looks 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.

3.5 The 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, while 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% and it in CHP group reduced 21.3%. In addition, the release of histamine in the CTC group decreased 51.5% and it in CHP group decreased 60.2%.

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-labeled TM was internalized at a much slower rate, and at 30 min there was no obvious increase. pHrodo-labeled CTC was slightly increased at 30 min, and pHrodo-labeled CHP reached saturation at 30 min and was obviously increased compared to TM and CTC (Figure 3C).

3.6 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 in 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 group (Figure 4C) was significantly decreased compared with the TM group. In addition, it should be noted that the changes in mMCP-1 between TM and cross-linked TM (Figure 4D) showed that TM, CTC and CHP reached 28 ng/mL, 17 ng/mL 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 were decreased. IFN-γ, a Th1-related cytokine in spleen cells, was increased (Figure 4G). However, the levels of IL-4 and IL-13 (Figure 4H–I) in MLN lymphocytes was 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 change in MLN lymphocytes (CTC group) was observed, while CHP group slightly increased.

3.7 Oral tolerance induced by cross-linked TM

The ability of cross-linked TM to induce gut and systemic immune tolerance was investigated in the 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

303 TM-specific IgE, IgG1, but not IgG2a (Figure 5B). The levels of IL-4, IL-13 and
304 IFN-γ were measured in spleen cell and MLN lymphocyte (Figure 5C–H). Compared
305 with TM-TM, CTC-TM and CHP-TM decreased the production of IL-4 and IL-13,
306 and increased the production of IFN-γ in spleen cells. However, in the CTC-TM and
307 CHP-TM groups, the production of IL-13 was increased, IFN-y in MLN lymphocytes
308 in CTC-TM was slightly increased while CHP-TM slightly decreased, and the
309 production of IL-4 in MLN lymphocytes was unchanged. To examine changes in the
310 numbers of DCs in the four groups of mice, flow cytometry experiments were
311 performed (Figure 5I). In splenocytes, the percentage of CD11c + MHC-Ⅱ+ DCs
312 decreased from 7.03% (TM-TM group) to 6.42% (CTC-TM group) and 6.02%
313 (CHP-TM group). However, the percentage of CD11c + MHC-II+ DCs increased
314 from 1.54% (TM-TM group) to 2.25% (CTC-TM group) and 2.92% (CHP-TM group)
315 in MLN lymphocytes.
316
317 **4. Discussion**
318 The present study shows that cross-linked TM with tyrosinase or HRP possesses
319 allergenic and immunologic properties. The results that cross-linked proteins CTC and
320 CHP were formed complicated protein complex and it was consistent with previous
321 studies.[7,30,31] Moreover, many studies have shown that cross-linked dietary products
322 further influenced their allergenicity.[7,30-32] Tyrosinase catalyzes the oxidation of
323 tyrosine and results in oxidative cross-linking of tyrosine side-chains, so cross-linking
324 of TM by tyrosinase was catalyzed the oxidation of the amino acid residues of Y_{162},

Y_{221} and Y_{267} in TM linear epitopes. 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, therefore cross-linking of TM by HRP was catalyzed the oxidation of the amino acid residues of Y_{162}, Y_{221}, Y_{267} and F_{153} 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 produced the more complex structures, so the percentage of α-helix in cross-linked TM reduced and the amount of β-turn, random coil increased. In addition, from the results of SEM, the cross-linked TM generated the complex change. Enzymatic cross-linking also caused the hydrophobicity. The cross-linking of TM by tyrosinase makes the hydrophilic amino acids covered to increase the hydrophobicity, while cross-linking of TM by HRP makes the hydrophilic amino acids exposed to decrease the hydrophobicity. In addition, the 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-labeled TM or cross-linked TM, it is associated with digestion. pHrodo-labeled CHP was easily digested within 10 min, hence it can be 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 CTC or TM.

Cross-linked TM influenced allergenicity and the degranulation in RBL-2H3 cells. This was in agreement with the previous study where the treatment of AK with tyrosinase/caffeic 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 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

non-allergenic molecular fragments while the time increased. The low concentration of allergenic degradation segments only caused 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.[39,40] 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 the 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 to cause the release of cytokines on T cells in the MLN. Combined with the results of endocytosis by DCs in vitro, CHP and CTC can easily be taken up by DCs; therefore, this influenced the number of DCs in the MLN of mice. Orally administered antigens can inhibit following immune responses in the gut and the systemic immune system; however,

cross-linked TM only suppresses immune responses in the systemic immune system. Low molecular weight protein may pass directly across the epithelium by paracellular diffusion, while 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-presenting from the MLN to the spleen was unknown. A further in-depth study of these issues is planned for the future.

In our previous study,[7] enzymatic cross-linking was an effective way of reducing crab allergens, which AK is the main allergens. 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 of allergenicity due to enzymatic cross-linking. Therefore the hypoallergenic crab meat was developed and applied 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 the 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.

Conflict of interest

The authors declare that there is no conflict of interests.

Abbreviations

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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Figure legends

Figure 1. The polymerization and IgG-/IgE-binding activity of enzymatic cross-linked TM compared with TM.

A, Cross-linked tropomyosin with tyrosinase/caffeic 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 photo 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 ± SD from triplicate determinations.

Figure 2. The 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. The impact of cross-linked TM in IgE-mediated RBL-2H3 cells and uptake by DCs.

A, The release of β-hexosaminidase;

B, The level of histamine;

C, Uptake of pHrodo Green-labeled cross-linked TM and TM by DCs. The data represent the mean ± SD from triplicate determinations.

Figure 4. The sensitization experiment. Cells were isolated from spleens and MLNs and stained for CD4 and Foxp3.

A, Sensitization protocol;

B, The levels of IgE, IgG1, and IgG2a measured on day 29;

C-D, The release of histamine/mMCP-1 measured in serum;

E-G, The production of IL-4, IL-13 and IFN-γ was measured in the spleen cell culture;

H-J, The production of IL-4, IL-13 and IFN-γ was measured in the MLN cell culture;

K, The spleen and MLNs cells were isolated from the mouse models of food allergy on day 29. Cells were cultured in the presence of 25 μg/mL of anti-CD4-PerCP Cy5.5 and 25 μg/mL of anti-Foxp3-Alexa 647 for 30 min. $CD4^{+}Foxp3^{+}$T cells were analyzed by flow cytometry. *p < 0.05, **p < 0.01. The data represent the mean ± SD from triplicate determinations.

Figure 5. The sensitizing potential of cross-linked TM in the tolerance induction experiment. Cells were isolated from spleens and MLNs and stained for CD11c and MHC-Ⅱ.

A, Oral tolerance induction protocol;

B, The levels of IgE, IgG1, and IgG2a measured in serum;

C-E, The production of IL-4, IL-13 and IFN-γ was measured in the spleen cell culture;

F-H, The production of IL-4, IL-13 and IFN-γ was measured in the MLN cell culture;

I, The spleen and MLNs cells were isolated from the mouse models of food allergy on day 34. Cells were cultured in the presence of 25 μg/mL of anti-CD11c-APC and 25 μg/mL of anti-MHC-II-FITC for 30 min. CD11c+ MHC-Ⅱ+DCs were analyzed by flow cytometry. *p < 0.05, **p < 0.01. The data represent the mean ± SD from triplicate determinations.

Table 1. Specific IgE levels in patients in relation to the crab and shrimp.

ID	Age	Sex	Specific IgE (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

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

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

Figure 1

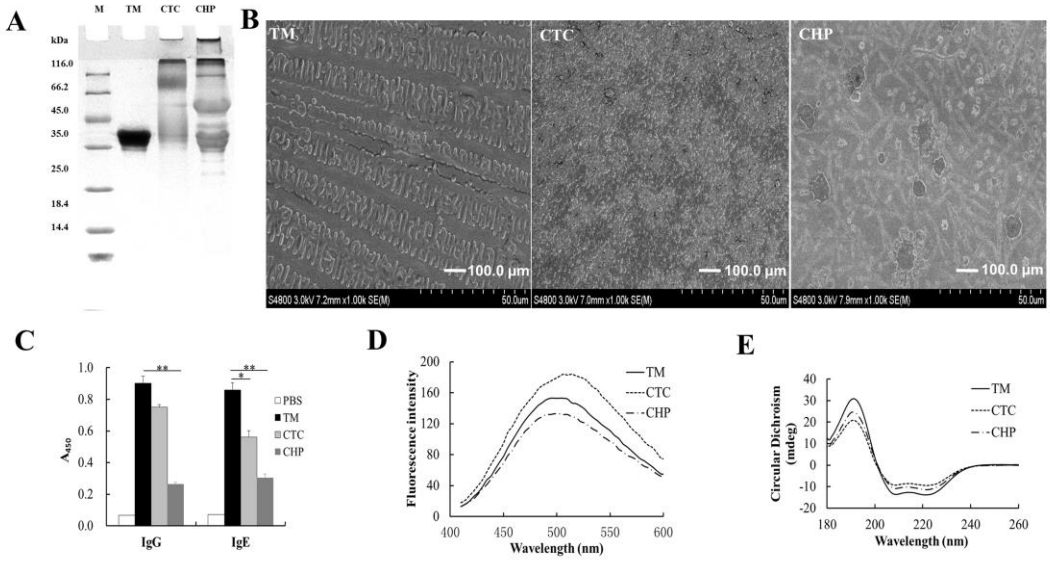
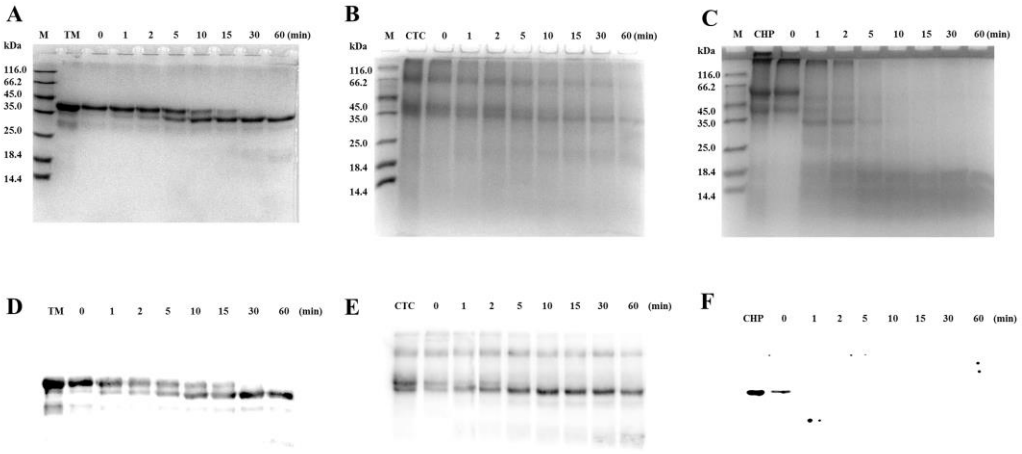

A
M
TM
CTC
CHP
kDa
116.0
66.2
45.0
35.0
25.0
18.4
14.4
B
TM
CTC
CHP
100.0 μm
C
PBS
TM
CTC
CHP
IgG
IgE
D
Fluorescence intensity
Wavelength (nm)
E
Circular Dichroism (mdeg)
Wavelength (nm)

Figure 2


A
M TM 0 1 2 5 10 15 30 60 (min)
kDa
116.0
66.2
45.0
35.0
25.0
18.4
14.4
B
M CTC 0 1 2 5 10 15 30 60 (min)
kDa
116.0
66.2
45.0
35.0
25.0
18.4
14.4
C
M CHP 0 1 2 5 10 15 30 60 (min)
kDa
116.0
66.2
45.0
35.0
25.0
18.4
14.4

D
TM 0 1 2 5 10 15 30 60 (min)
E
CTC 0 1 2 5 10 15 30 60 (min)
F
CHP 0 1 2 5 10 15 30 60 (min)

Figure 3

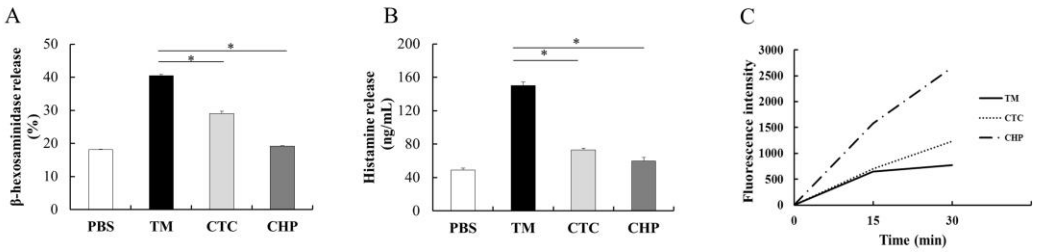

A
β-hexosaminidase release (%)
50
40
30
20
10
0
*
*
PBS
TM
CTC
CHP
B
Histamine release (ng/mL)
200
160
120
80
40
0
*
*
PBS
TM
CTC
CHP
C
Fluorescence intensity
3000
2500
2000
1500
1000
500
0
0
15
30
Time (min)
TM
CTC
CHP

Figure 4

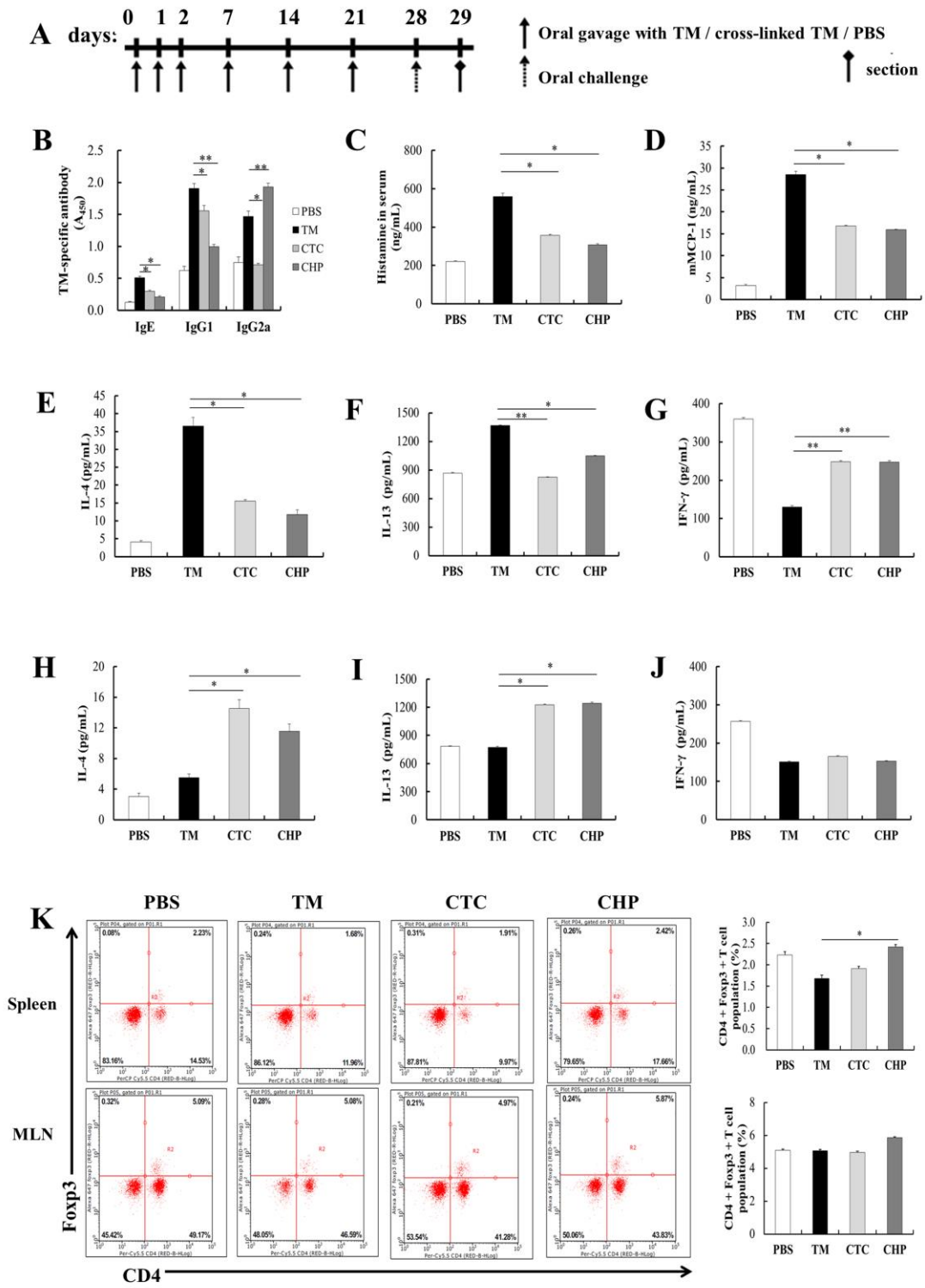
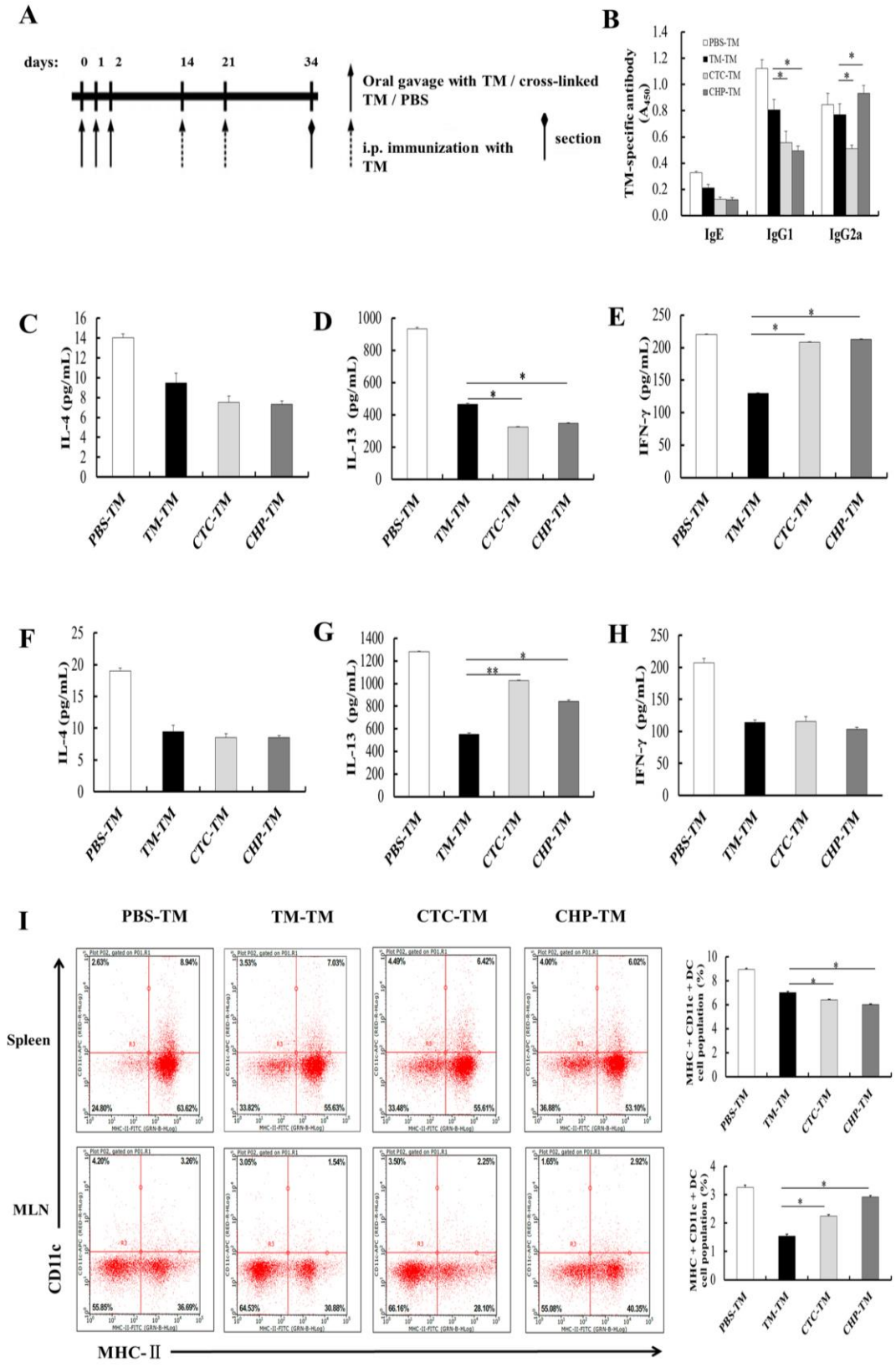

A
days: 0 1 2 7 14 21 28 29
Oral gavage with TM / cross-linked TM / PBS
Oral challenge
section
B
TM-specific antibody (A_{450})
PBS
TM
CTC
CHP
IgE
IgG1
IgG2a
C
Histamine in serum (ng/mL)
D
mMCP-1 (ng/mL)
E
IL-4 (pg/mL)
F
IL-13 (pg/mL)
G
IFN-γ (pg/mL)
H
IL-4 (pg/mL)
I
IL-13 (pg/mL)
J
IFN-γ (pg/mL)
K
PBS
TM
CTC
CHP
Spleen
MLN
Foxp3
CD4
CD4 + Foxp3 + T cell population (%)

Figure 5


A
days: 0 1 2 14 21 34
Oral gavage with TM / cross-linked TM / PBS
i.p. immunization with TM
section
B
PBS-TM
TM-TM
CTC-TM
CHP-TM
TM-specific antibody (A_{450})
IgE
IgG1
IgG2a
C
IL-4 (pg/mL)
D
IL-13 (pg/mL)
E
IFN-γ (pg/mL)
F
IL-4 (pg/mL)
G
IL-13 (pg/mL)
H
IFN-γ (pg/mL)
PBS-TM
TM-TM
CTC-TM
CHP-TM
I
PBS-TM
TM-TM
CTC-TM
CHP-TM
Spleen
MLN
CD11c
MHC-Ⅱ
MHC + CD11c + DC cell population (%)

Graphic for table of contents