

Induction of mud crab (*Scylla paramamosain*) tropomyosin and arginine kinase specific hypersensitivity in BALB/c mice

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Abstract

BACKGROUND: Shellfish hypersensitivity is among the most common food allergies. The allergens tropomyosin (TM) and arginine kinase (AK) from mud crab (*Scylla paramamosain*) were purified to homogeneity. BALB/c female mice were sensitized with TM and AK by intragastric administration. Mice treated with normal saline served as the negative control (NC) group.

RESULTS: Compared with NC group, mice that were treated with TM and AK developed reduced activity; meanwhile, their scratching behavior and specific-IgE level were increased. Specific-CD4⁺T cells were significantly elevated in the splenocyte cultures of the mice upon TM and AK stimulation. However, compared with the positive control group (ovalbumin, OVA), there was no significant difference. The expression of IL-4 in culture cells stimulated by TM, AK, and OVA group showed significant differences from the NC group, respectively.

CONCLUSION: These results indicated that a BALB/c mouse model for sensitization to TM and AK from mud crab was successfully established, and the Th2 response was observed, displaying increased immunoglobulin E levels, together with the production of interleukin 4 and allergic symptoms.

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Keywords: mud crab; allergen; tropomyosin; arginine kinase; animal model; BALB/c mice

INTRODUCTION

In recent years, seafood allergy has become one of the most important food safety issues. Seafood is one of the most frequently reported causes of allergic reactions.^{1–4} Recent studies indicate that people involved in seafood processing have a risk of food allergy.^{5,6} According to Sicherer *et al.*,⁷ the rate of allergic reaction to crustaceans was 38% of the US population. Studies have demonstrated that tropomyosin (TM) is the major allergen of crustaceans. TM, which has good heat stability and two identical subunits with molecular masses of 35–38 kDa, will cause immunoglobulin E (IgE)-mediated, type I allergy.⁸ TM derived from invertebrates is always recognized as a cross-reactive allergen, which is responsible for the cross-reactive allergies observed between various types of edible crustaceans and mollusks.⁹ Arginine kinase (AK) is mainly known as a kinase involved in energy transformation. Yu *et al.*¹⁰ used AK and serum from subjects with shrimp allergy in two-dimensional immunoblotting, and confirmed AK to be a novel and cross-reactive crustacean allergen, causing IgE-mediated allergic reactions. Later studies showed that AK is also an allergen in dust mites.¹¹

At present, allergenicity is assessed by comparison of sequence homology, IgE bindings/serum testing and digestibility/stability. However, these methods cannot directly determine the allergic sensitization of proteins. Dearman and Kimber¹² evaluated the inherent sensitizing potential of novel proteins as a function

of IgE antibody responses stimulated by parenteral exposure of mice. The mouse model provided a way for these studies to be done; therefore there is a growing interest in the development of appropriate animal models for allergen sensitization. As a result, several murine models have been established and characterized with different food allergens relevant for human sensitization, such as cow milk's allergen,¹³ ovalbumin (OVA)^{14,15} and shrimp tropomyosin.^{16,17} Part of the potential allergenicity of TM from mud crab had been studied in our laboratory;¹⁸ however, a murine model for mud crab allergen (TM or AK) has not been established.

The focus of this study was to purify TM and AK from mud crab, and establish mouse models for TM and AK that indicate the main features of human food allergy, such as the presence of allergen-specific IgE and systemic anaphylactic reactions. Moreover, verifying the allergenicity of AK through the mouse model would be persuasive.

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MATERIALS AND METHODS

Crab

Mud crab (*Scylla paramamosain*) was purchased alive at Jimei market, Xiamen, China. After washing, crabs were sacrificed by freezing at -80°C . Muscles were obtained and immediately used.

Chemicals

Protein markers for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Fermentas (Vilnius, Lithuania) and New England BioLabs (Richmond, CA, USA), respectively. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgE antibody was purchased from SouthernBiotech (Birmingham, AL, USA). HRP-labeled goat anti-rabbit IgG secondary antibodies were obtained from Dako (Glostrup, Denmark). Rabbit anti-Chinese mitten crab (*Eriocheir sinensis*) TM polyclonal antibody and rabbit anti-white shrimp (*Penaeus vannamei*) AK polyclonal antibody were prepared in our laboratory. 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was purchased from Pierce (Rockford, IL, USA). OVA, complete/incomplete Freund's adjuvant CFA/IFA and interleukin (IL)-4, IL-13 and interferon (IFN)- λ sandwich enzyme-linked immunosorbent assay (ELISA) kits were purchased from Sigma Aldrich (St Louis, MO, USA). All the chemicals used were analytical grade.

Purification of TM

All procedures were conducted at $0-4^{\circ}\text{C}$, unless stated otherwise. The purification of crab TM was carried out as described.¹⁹ Briefly, fresh crab muscle was minced in a stainless steel grinder, followed by the preparation of dried acetone powder as described by Huang and Ochiai.²⁰ The powder was then extracted using 10 volumes of buffer B containing 20 mmol L^{-1} Tris-HCl (pH 7.5), 1 mol L^{-1} KCl and 10 mmol L^{-1} 2-mercaptoethanol (2-ME). The homogenate was centrifuged at $20\,000 \times g$ for 20 min, the supernatant was precipitated isoelectrically at pH 4.5 with 3 mol L^{-1} HCl and the precipitate was then dissolved in 20 mmol L^{-1} Tris-HCl (pH 7.5) and successively salted out with 41–60% saturation of $(\text{NH}_4)_2\text{SO}_4$. After centrifugation at $20\,000 \times g$ for 20 min, the precipitate was collected and redissolved in 20 mmol L^{-1} Tris-HCl (pH 7.5). Finally, small amounts of impurities were removed by heating in a boiling water bath for 10 min, followed by immediate cooling in ice water and centrifugation at $20\,000 \times g$ for 20 min. The supernatant containing TM with high purity was used immediately in the experiments or stored at -80°C until use. The protein concentrations of purified TM were determined by measuring the absorbance at 280 nm.

Purification of AK

All procedures were conducted at $0-4^{\circ}\text{C}$, unless stated otherwise. AK was purified by a modification of a previously described protocol.²¹ Crab muscles were minced and homogenized with buffer A (containing 0.1 mol L^{-1} Tris-HCl, 10 mmol L^{-1} 2-ME, 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 5 mol L^{-1} NaN_3 and 25 mmol L^{-1} phenylmethylsulfonyl fluoride, pH 8.0). The mixture was then centrifuged at $12\,000 \times g$ for 20 min. The supernatant was adjusted until the supernatant was 70% ammonium sulfate. After centrifugation, ammonium sulfate was added to the supernatant to 90% saturation; then the precipitate was collected by centrifugation, dissolved in 20 mL buffer B (containing 10 mmol L^{-1} Tris-HCl, 10 mmol L^{-1} 2-ME and 0.1 mmol L^{-1} EDTA, pH 8.0), and dialyzed against the same buffer. The clear supernatant was then applied to a HiTrap Q Sepharose

Fast Flow column (Bio-Rad, Hercules, CA, USA) pre-equilibrated with buffer B and dialyzed against the same buffer. Fractions with AK activity were eluted in a linear gradient ranging from 0 to 1 mol L^{-1} NaCl in buffer B.

SDS-PAGE analysis and immunoblotting

SDS-PAGE was performed based on the protocol adapted from Laemmli.²² Briefly, samples were separated in 12% polyacrylamide gels with 5% stacking gel, and the gels were stained for protein with Coomassie Brilliant Blue R-250.

Immunoblotting was carried out as previously described.²³ Briefly, protein samples were subjected to SDS-PAGE, followed by electrophoresis transfer to nitrocellulose membranes. Nonspecific protein sites were blocked using 5% nonfat milk in Tris-HCl-buffered saline (TBS; 20 mmol L^{-1} Tris-HCl, pH 7.5, 0.145 mol L^{-1} NaCl). The membrane was incubated with rabbit anti-Chinese mitten crab (*Eriocheir sinensis*) TM polyclonal antibody and rabbit anti-white shrimp (*Penaeus vannamei*) AK polyclonal antibody (1:200 000 dilution) at room temperature for 2 h and washed using TBST (TBS, 0.05% Tween-20), respectively. After incubation for 1 h with HRP-labeled goat anti-rabbit IgG secondary antibodies (1:2000 dilution), the membranes were washed five times in TBST. The immunoassay was carried out using DAB as substrate.

Two-dimensional gel electrophoresis

Purified extracts were analyzed by two-dimensional gel electrophoresis as described previously.²⁴ Briefly, the first separation was carried out by applying 0.1 mg purified protein to an immobilized gel strip containing a pH range of 3–7 ampholytes, and was followed by isoelectric focusing in a Multiphor II horizontal electrophoresis system (GE Healthcare, Uppsala, Sweden). After isoelectric focusing, the strip was subjected to SDS-PAGE on 12% gels.

Induction of crab hypersensitivity in BALB/c mice

Forty female BALB/c mice, 5–6 weeks of age, $20 \pm 2\text{ g}$, were acquired from the Laboratory Animal Centre, Xiamen University (Xiamen, Fujian, China) and randomly divided into four treatment groups, with 10 mice per group. Mice were maintained on a crab-free and egg-free diet under strictly controlled conditions, at a room temperature of $20 \pm 2^{\circ}\text{C}$, relative humidity $50 \pm 10\%$, and a 12/12 light/dark cycle.

The mouse model was established based on previous studies, with few modifications.^{15–17} Briefly, mice were immunized by intraperitoneal injection with 0.1 mg purified TM, AK and OVA per mouse together with CFA in a total volume of $100\text{ }\mu\text{L}$ on days 0, 14, 21. Mice were then challenged 12 days after the last immunization (day 33) by receiving one intragastric dose of TM, AK and OVA ($600\text{ }\mu\text{g}$ per mouse) in a total volume of $500\text{ }\mu\text{L}$. The negative control (NC) group was treated with an equal volume of normal saline. The OVA group served as the positive control. Mice were bled from the tail on days 0, 14, 21, 33 and 37. Sera were collected and stored at -20°C until analysis. On days 0, 21 and 33, the fecal samples were also individually collected and immediately extracted. Fecal pellets (100 mg) were suspended in 1 mL phosphate-buffered saline–0.2% sodium azide. After complete disruption of solid material, samples were centrifuged at $7500 \times g$ for 5 min at 4°C . Supernatants were then removed and stored at -20°C until analysis. The histamine assay was done according to the instructions of the manufacturer of histamine sandwich-ELISA kits (Sigma).

Table 1. Pairs of primers used in real-time PCR analysis of cytokine gene in culture cell

	Forward	Reverse
IL-4	5'-CCCCCAGCTAGTTGTCATCC-3'	5'-TGGTGTCTTCGTTGCTGTG-3'
GAPDH	5'-TGCCAAGGCTGTGGGCAAGG-3'	5'-TCTCCAGGCGGCACGTCAGA-3'

Allergen challenge and evaluation of systemic anaphylaxis

Mice were challenged on day 33 by receiving one intragastric dose of TM, AK and OVA (600 µg per mouse). The emergence of systemic anaphylaxis was observed 30 min after challenge. Symptoms were evaluated following a scoring system reported by Li *et al.*: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, 'pilar erecti', decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and the tail; 4, no activity after prodding or tremor and convulsion; and 5, death. Fecal samples were collected individually and processed as described above.¹³

Measurement of crab allergen-specific IgE

The levels of crab allergen-specific IgE in mice were determined by indirect ELISA. Briefly, 96-well plates were coated with TM, AK and OVA (200 ng per well) in carbonate coating buffer (pH 9.6). The plates were washed with TBST and blocked with 5% nonfat milk for 2 h. After washing with TBST, diluted serum samples (1:6 dilution with 1% nonfat milk) were added to the plates and incubated overnight at 4 °C. Plates were washed with TBST and then incubated with HRP-conjugated goat anti-mouse IgE (100 µL per well, 1:5000 dilution) for 2 h. After washing, plates were incubated with TMB for 15 min at room temperature. The reaction was terminated by adding 2 mol L⁻¹ H₂SO₄ and the optical density was measured at 450 nm using a microplate reader. All assays were performed in triplicate. The data for measurement of crab allergen-specific IgE were all background subtracted.²⁵

Spleen cell cultures and cytokine level measurements

Four days after the last immunization (day 37), mice from four different groups were sacrificed. Individual spleens were aseptically removed, minced and cell suspensions were prepared. After erythrocyte lysis, spleen cells were resuspended in complete culture medium RPMI-1640 containing 10% fetal bovine serum, 1% penicillin–streptomycin and counted. For the evaluation of cytokine production, spleen cell suspensions from TM, AK and OVA-sensitized and NC mice were cultured in 24-well plates (2 × 10⁶ per well mL⁻¹) in the presence or absence of TM, AK and OVA (100 µg mL⁻¹), respectively. The supernatants were collected after 6 days of culture. Levels of cytokines IL-4, IFN-λ and IL-13 were determined by sandwich-ELISA kits (Sigma), according to the manufacturer's instructions. Sensitivity for each assay was 1 pg mL⁻¹.

Statistical analysis

The data were presented as the mean. Statistical significance of the data was determined by one-way analysis of variance (ANOVA) using SPSS.13.0. A value of *P* < 0.05 was considered significant.

Real-time polymerase chain reaction (PCR) of IL-4

Total RNA from cultured cells of immunized and naive mice were extracted using TRIzol reagent (Sigma) according to the

manufacturer's instructions. Quantity and quality of RNA were assessed by measuring the A₂₆₀/A₂₈₀ absorbance ratio and analysis on ethidium bromide-stained agarose gel. Total cDNA was obtained by reverse transcription. Briefly, the reaction mixture contained 4 µg total RNA, 2 µL oligo(dT)₁₅, 2 µL dNTPs (2.5 mmol L⁻¹ each), and 5.5 µL RNase-free double-distilled H₂O was added. The mixture was then incubated at 70 °C for 5 min and quickly cooled in ice for 2 min. After a brief centrifugation, 4 µL 5 × First-strand buffer, 0.1 mol L⁻¹ dithiothreitol 1 µL, RNasin 0.5 µL, and Transcript M-MLV 1 µL were added to the reaction mixture, followed by incubation at 42 °C for 50 min, and then the reaction was stopped by incubating at 95 °C for 5 min.

Real-time PCR was performed to quantify the gene expression levels of IL-4 using FastStart Universal SYBR Green Master (ROX) and IL-4 gene specific primers (Table 1). The housekeeping gene GAPDH was used to normalize the values obtained for transcripts under examination. The real-time PCR condition was as follows: 10 min at 95 °C, and a cycle of 15 s at 95 °C and 1 min at 60 °C, 40 cycles. The real-time PCR product was also analyzed on ethidium bromide-stained agarose gel.

RESULTS AND DISCUSSION

Recently, with the further cognition of food allergy and the discovery of many new kinds of allergens, food allergy has attracted the attention of more consumers and researchers. As one kind of crustacean, mud crab is popular around the world owing to its nutritional value and taste. However, the allergenicity of crab has affected aquaculture and its selling market. Therefore, it is meaningful to study the allergenicity of crab meat, and to develop methods for the allergenicity assessment of crab AK.

SDS-PAGE and immunoblotting analysis of TM

A single 38 kDa protein band was observed from SDS-PAGE (Fig. 1(a)) after purification from the crude protein. The purified protein displayed slight variation in molecular weight compared to shrimp TM.²⁶ The protein also had considerable thermal stability. As shown in Fig. 1(b), the purified protein reacted with the polyclonal antibody raised in rabbit against Chinese mitten crab (*Eriocheir sinensis*) TM, confirming the purified protein to be TM.

SDS-PAGE, immunoblotting and two-dimensional gel electrophoresis analysis of AK

Purification of the target protein through HiTrap Q-Sepharose Fast Flow chromatography displayed a 40 kDa protein which was thought to be AK (Fig. 1(c)). Two-dimensional electrophoresis showed that the isoelectric point of AK was 6.0 (Fig. 1(d)). The identity and purity of the purified protein as an AK were further confirmed by immunoblotting with rabbit anti-white shrimp (*Penaeus vannamei*) AK polyclonal antibody (Fig. 1(e)) as, for crustaceans, AK from shrimp and crab have a similar amino acid sequence, AK of mud crab could also display immunological cross-reactivity to rabbit anti-white shrimp (*Penaeus vannamei*)

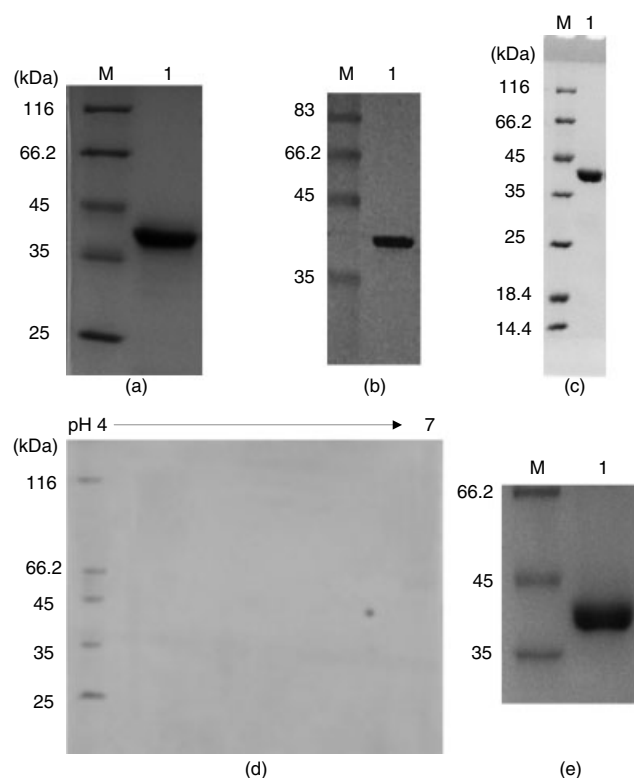


Figure 1. Purification and identification of allergen from *Scylla paramamosain*. (a) SDS-PAGE analysis of TM. Lane M, molecular mass marker; lane 1, purified protein. (b) Immunoblotting analysis of TM using rabbit anti-Chinese mitten crab (*Eriocheir sinensis*) TM polyclonal antibody. Lane M, pre-stained molecular mass standard; lane 1, purified protein. (c) SDS-PAGE analysis of AK. Lane M, molecular mass marker; lane 1, purified protein. (d) Two-dimensional gel electrophoresis analysis of AK. (e) Immunoblotting analysis of AK using rabbit anti-white shrimp (*Penaeus vannamei*) AK polyclonal antibody. Lane M, pre-stained protein marker; lane 1, purified protein.

AK polyclonal antibody. These results illustrate that the purified protein was AK.

In this study, TM and AK were purified from mud crab mainly through ammonium sulfate precipitation methods. The solubility of protein in aqueous solution was decided by the extent of the hydration shell between hydrophilic group and liquid, and the situation of protein electric charge. The addition of neutral salt such as ammonium sulfate could change the affinity of liquid and protein, and neutralize the protein electric charge, so proteins became aggregated and precipitate.²⁷

Induction of anaphylactic reaction by oral allergen challenge in sensitized mice

Animal models are affected by species of animal, sensitization methods and dosage. In our experiment, a mouse model was decided upon as its immune system is similar to that of humans. During the experiments there was little difference in body weight between the mice of treated groups. Twelve days after the third intraperitoneal injection, mice were fed with a high dose (600 µg) of TM, AK and OVA by oral gavage, respectively and the reactions were monitored for 30 min thereafter. All the sensitized mice displayed anaphylactic symptoms with severity ranging from 1 to 2 in the scoring system (Fig. 2(a)), whereas NC mice did not display any signs of anaphylaxis. Compared with the positive control group (OVA), AK and TM groups displayed similar allergic symptoms,

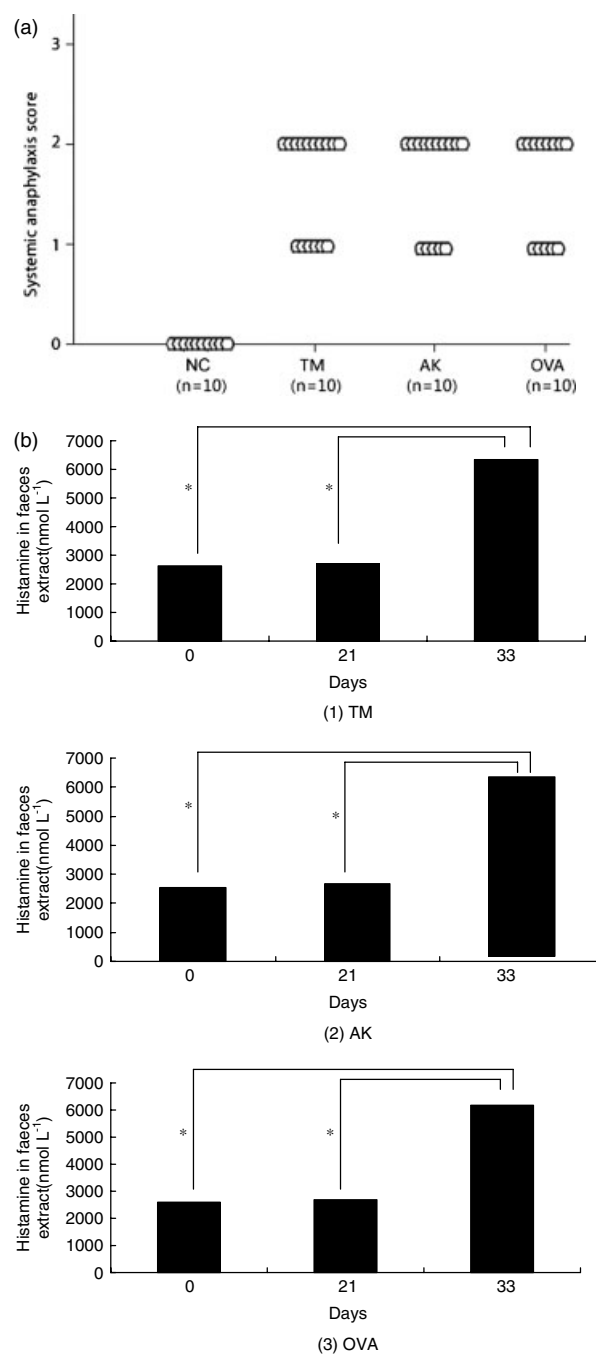


Figure 2. Allergen-induced anaphylaxis in NC and allergen-immunized mice. (a) Systemic anaphylaxis responses after intragastric challenge. Responses of the four groups of mice sensitized intragastrically with NC, TM, AK and OVA were evaluated for 30 min after oral challenge based on the scoring system. (b) Histamine levels in faecal samples collected before immunization (day 0), after immunization (day 21) and within 30 min after challenge (day 33). Data are shown as mean. (1) TM group; (2) AK group; (3) OVA group. *Mean $P < 0.05$.

although some discrepancies existed in the number of allergic symptoms in mice. As shown in Fig. 2(b), consistent anaphylactic reactions were observed in the immune mice; faecal histamine levels in TM, AK and OVA-sensitized mice were significantly increased after challenge (day 33) as compared with pre-immunization (day 0, $P < 0.05$) and post-immunization (day 21, $P < 0.05$) samples.

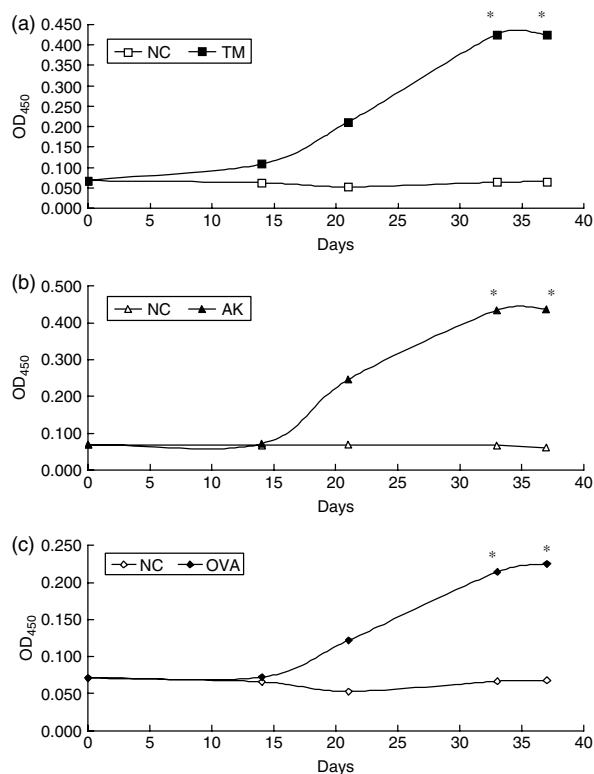


Figure 3. Specific IgE levels of TM, AK and OVA of sensitized mice. Serum were obtained from groups ($n = 10$) sensitized with individual allergens on days 0, 14, 21, 33 and 37, respectively. Data are expressed as mean. *Mean $P < 0.05$. (a) Solid squares, TM; hollow squares, NC. (b) Solid triangle, AK; hollow triangle, NC; (c) Solid rhombus, OVA; hollow rhombus, NC.

Increased level of specific IgE after intraperitoneal sensitization and challenge

Serum was obtained from mice throughout the sensitization (days 0–21) and challenge (day 33) period to post challenge (day 37). The specific IgE level increased from day 14 to day 33 in mice sensitized with TM, AK and OVA, respectively (Fig. 3). The TM, AK and OVA group-specific IgE levels were significantly higher than NC. The elevated levels of TM, AK and OVA-specific IgE persisted in the respective groups for at least 4 days after the challenge (day 37). In contrast, allergen specific IgE in the NC did not change significantly compared to day 0.

These results indicated that both TM and AK could lead to allergen-specific-IgE reactivity. In contrast to the previous study,¹⁵ the OVA-specific IgE levels were lower; this could be due to the difference in the dosage of the allergen. Strobel also demonstrated that the concentration of allergen is a major factor in determining allergenicity.²⁸ The TM and AK-specific IgE levels were also lower than previous reports,^{16,17} these could be ascribed to the different mouse strains and the immunization schedules compared to earlier reports. A number of studies have implicated the selection of mouse strains as an important factor in the development of an experimental animal model.^{29,30} Reese *et al.*³⁰ also reported that different mouse strains and different adjuvants had distinct immune responses with respect to the induction of IgE response.

Proliferation responses of T cells under *in vitro* stimulation of allergen

Systemic T cell responses were induced by sensitization with TM, AK and OVA, respectively. Spleen cells were isolated from

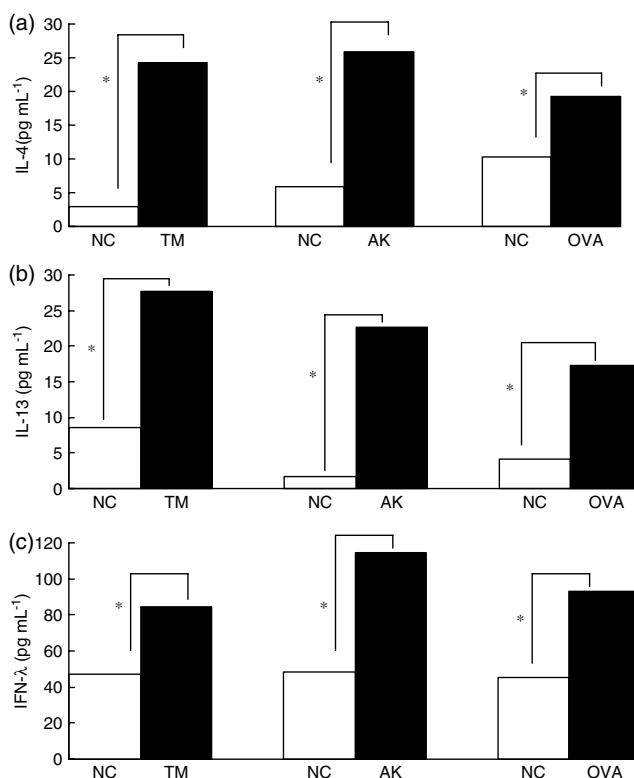


Figure 4. Cytokine secretion (pg mL⁻¹) in response to TM, AK, OVA. Data are expressed as mean. *Mean $P < 0.05$. (a) Levels of IL-4 in different groups. Black bars, TM, AK and OVA; white bar, NC. (b) Levels of IL-13 in different groups. Black bars, TM, AK and OVA; white bar, NC. (c) Levels of IFN-λ in different groups. Black bars, TM, AK and OVA; white bar, NC.

mice sensitized with 100 µg TM, AK, OVA and NC on day 37. After *in vitro* stimulation with TM, AK and OVA (100 µg mL⁻¹) for 6 days, allergen-specific proliferation was evaluated and cytokines were measured in culture supernatants by sandwich-ELISA. As shown in Fig. 4, TM-sensitized mice produced significantly higher amounts of IL-4 (24.278 ± 1.985 pg mL⁻¹), IL-13 (27.776 ± 0.0345 pg mL⁻¹), and IFN-λ (84.243 ± 0.0365 pg mL⁻¹) compared to NC ($P < 0.05$), respectively. Compared with the positive control OVA (IL-4, 19.223 ± 0.988 pg mL⁻¹; IL-13, 17.292 ± 2.831 pg mL⁻¹; IFN-λ, 92.858 ± 2.449 pg mL⁻¹), cytokine levels in the AK group (IL-4, 25.859 ± 0.072 pg mL⁻¹; IL-13, 22.716 ± 0.745 pg mL⁻¹; IFN-λ, 114.265 ± 8.220 pg mL⁻¹) showed no significant difference. However, the treatments had significant differences when compared to the NC group (IL-4, 5.915 ± 0.980 pg mL⁻¹; IL-13, 1.697 ± 0.339 pg mL⁻¹; IFN-λ, 48.379 ± 1.184 pg mL⁻¹).

Furthermore, the levels of similar cytokines showed variations between the TM group and AK group; however, no significant difference was observed, suggesting that the IgE response levels from these two proteins was similar. As to the previously established mouse model for shrimp TM,^{16,17} the cytokine levels of this present study were lower, which could be attributed to the differences in the mouse strain, adjuvant and source of allergen.

The studies have shown that Th2 cell plays an important role in food allergy. Under IL-12 and IL-14 treatment, T cells were separated into Th1 and Th2 cells. Th1 secreted IL-2 and IFN-λ, conducting cellular immune responses, while Th2 mainly produced IL-4, IL-5, IL-10 and IL-13, leading to the humoral immune responses which secreted IgG and IgE, and mediated the allergic

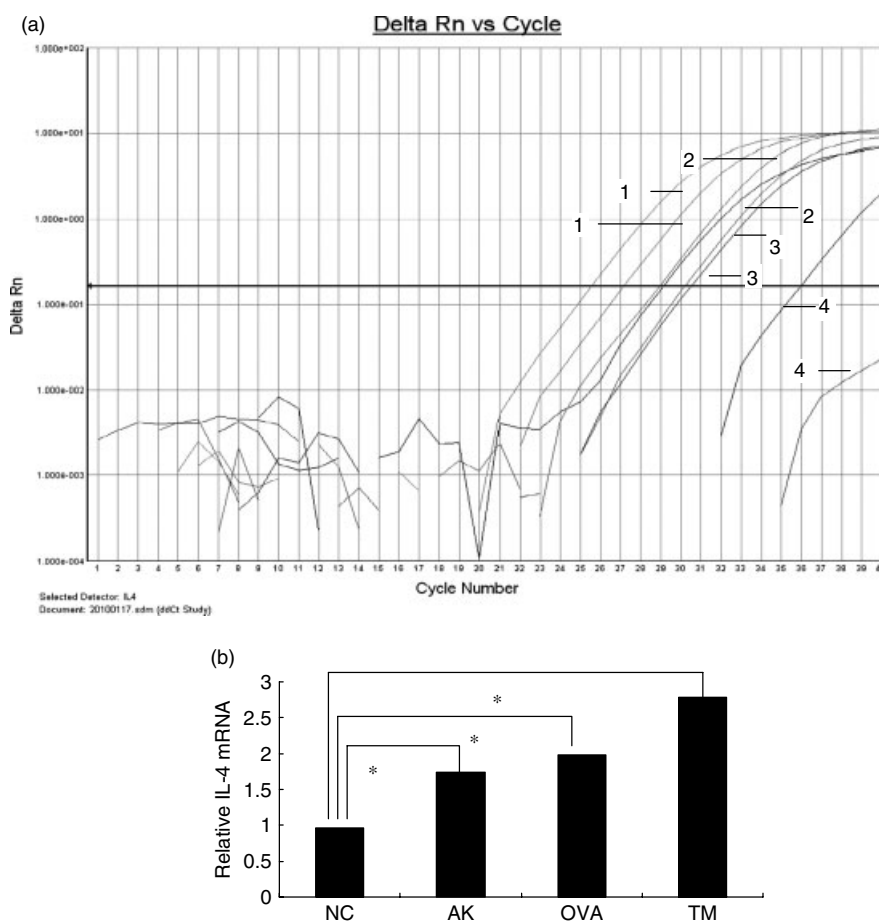


Figure 5. Relative express levels of IL-4 mRNA in culture cell stimulated by TM, AK and OVA, respectively. (a) Amplification plot of IL-4 mRNA RT-PCR. Lane 1, TM group; lane2, AK group; lane 3, AK group; lane 4, NC. (b) Relative cytokine IL-4 mRNA expression from individual mice was assessed by real-time PCR. All results were normalized against GAPDH expression. Data are expressed as mean. *Mean $P < 0.05$.

reaction. Detection of the relevant cytokines was necessary to determine the allergic reactions.¹³

Relative IL-4 mRNA cytokine expression in culture cell

Since IL-4 plays a role in the food allergy mechanism, reverse transcriptase PCR (RT-PCR) was used to analyze its mRNA quantity. The results showed that the A_{260}/A_{280} absorbance ratio of extracted RNA was about 1.9, which was suitable for reverse transcription and subsequently real-time PCR. As shown in Fig. 5(a), real-time PCR results indicated that IL-4 gene was expressed in all the groups. The products of IL-4-specific real-time PCR from different groups were then analyzed. IL-4 was expressed in the spleen lymphocyte from both immunized and NC mice (Fig. 5(b)). Compared with NC (0.963 ± 0.053), TM (2.788 ± 0.457), AK (1.739 ± 0.002) and OVA (1.980 ± 0.005) groups showed significant differences (Fig. 5b), which indicated the effectiveness of the BALB/c mouse model. Numerous studies have demonstrated that Th2 cytokines play a central role in the pathogenesis of allergic disorders including food allergy.^{31,32} This was confirmed from the elevated levels of IL-4 found in the mice fed with TM or AK from *Scylla paramamosain*.

For further study, we will try to adopt transmission electron microscopy to analyze changes in intestinal tissues of the mouse model, and detect the intestinal mucosa cytokine to analyze changes in intestinal mucosa immunology. Furthermore, an

experiment for *in vitro* detection of the allergen through the allergic mouse sera will be conducted.

CONCLUSIONS

We have purified TM and AK from mud crab, and generated a BALB/c model of TM or AK by intraperitoneal injection and oral challenge. Mice treated with TM and AK produced specific IgE levels, allergic symptoms and significantly elevated levels of IL-4, IL-13 and IFN- λ , which were connected closely with IgE level. These results indicate that the BALB/c mouse model for sensitization of TM and AK from mud crab was successfully established, and the Th2 response was observed, displaying increased IgE levels, together with the production of IL-4 and allergic symptoms. Our study also confirmed the allergenicity of AK from mud crab using the mouse model. The AK mouse model established in this study can be used in further studies for detecting the mechanism of allergic responses triggered by AK, and evaluation of future therapeutic interventions.

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