

IgE- and T-lymphocyte-Dependent Hypersensitivity Responses Induced in Mice by Exposure to Shrimp (*Fenneropenaeus chinensis*) Proteins

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Shellfish allergy is a common cause of food-induced anaphylaxis in the human, and shrimp is one of the most frequently reported causes of allergic reactions. To identify the immunoglobulin E (IgE)-binding proteins of Chinese shrimp (*Fenneropenaeus chinensis*), the crude extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. After exposure of shrimp extracts to mice, the levels of shrimp allergen-specific IgE were determined by indirect ELISA. Histological and immunohistochemical staining of intestinal tract were further performed for immunological analysis. IgE-binding components with molecular masses of 40, 37, 28, 22, and 20 kDa at a high frequency are supposed to be major allergens of *F. chinensis*. In vivo experiments showed that shrimp extracts could lead to allergen-specific IgE reactivity, and increase mast cell degranulation and numbers of T lymphocytes. IgE- and T-lymphocyte-dependent hypersensitivity plays important roles in allergic reactions triggered by *F. chinensis* allergens.

Keywords: shrimp (*Fenneropenaeus chinensis*); allergen; IgE-dependent hypersensitivity; mast cell degranulation; T lymphocyte

1. Introduction

Food allergy, one of the common allergic diseases with a substantial risk of life-threatening anaphylactic reactions, is a growing concern of human health in developed as well as developing countries. According to the epidemiological investigation from Food and Drug Administration (FDA), USA, food allergies affect less than 2% of the adults and 2–8% of children worldwide. However, the number of people with food allergies appears to be increasing, especially among children. Seafood allergy is among one of the most common food allergies, with fish and shellfish being two of the “big eight” categories of food allergens (Hajeb & Selamat, 2012; Hsieh, Hsu, Lin, Tsai, & Lin, 2003).

As consumption of seafood increases worldwide, immediate hypersensitivity reactions to seafood have become an important issue. The most recent data from Asia have highlighted seafood as a major sensitizer in up to 40% of children and 33% of adults. Particularly, shellfish can provoke serious immunoglobulin E (IgE)-mediated adverse reactions in susceptible individuals, which are typically life-long and predominantly affect the adult population. A major difficulty in managing shellfish allergy is the

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lack of reliable diagnostic assays due to limited knowledge of clinically relevant shellfish allergens (Abramovitch et al., 2013; Lopata & Lehrer, 2009). The prediction of the sensitizing potential of novel proteins based on animal models is also extremely important for evaluation of shellfish allergy. However, despite increasing research efforts in recent years, validated and widely accepted animal models or evaluation methods are not available yet (Kimber, Betts, & Dearman, 2003; Knippels & Penninks, 2005).

Shrimp is a major cause of seafood allergy in China, since a high incidence of positivity to shrimp (70.3%) was detected in a survey on allergy prevalence from China (Hajeb & Selamat, 2012). Isolation of shrimp allergens by biochemical methods was first reported in the 1980s (Haddad & Korotzer, 1972; Hoffman & Haddad, 1974). Tropomyosin (TM), a heat-stable muscle protein, is the major allergen in shrimp, with highly homologous proteins being found in the commonly edible crustaceans. These homologies are responsible for the cross-reactive allergies observed between various types of seafood, such as shrimp, lobster, crab, and squid (Reese, Ayuso, & Lehrer, 1999). In addition to TM, other allergens have been also identified and characterized in crustaceans such as the 40 kDa arginine kinase (Garcia-Orozco, Aispuro-Hernandez, Yepiz-Plascencia, Calderron-de-la-Barca, & Sotelo-Mundo, 2007), a 20-kDa myosin light chain (Ayuso et al., 2008) and the sarcoplasmatic calcium-binding protein (Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008). Despite the recent advances in the identification of novel shellfish allergens other than TM, many potential allergens still remain unidentified.

The majority of seafood allergic reactions are mainly caused by IgE-mediated hypersensitivity reactions (Leung et al., 2014). After primary exposure, seafood allergens are captured by antigen-presenting cells and presented to T cells and stimulates B cells to produce IgE antibodies (Abs) against the specific seafood allergen (Vickery, Scurlock, Jones, & Burks, 2011). Upon subsequent exposure to the same seafood allergen, a hypersensitivity reaction can happen within minutes. The allergen binds to IgE-coated cells, such as mast cells or basophils. Crosslinking of IgE to the IgE-specific receptors, Fc receptor I (FcεR I) on the cell surface, turns on the signaling transduction pathway and activates these cells to induce degranulation, during which preformed mediators (histamine, various proteases, and proteoglycan) are released into the surrounding tissue and subsequently cause clinical symptoms (Gould & Sutton, 2008; Kumar, Verma, Das, & Dwivedi, 2012). The general processes of IgE-mediated reactions are basically the same when induced by different seafood allergens; however, molecular mechanisms behind these reactions are allergen-dependent, and need a thorough examination and explanation in further studies. Studies have also demonstrated that not only can IgE-dependent reactions cause anaphylaxis, but also can immune complexes and T-lymphocyte-dependent reactions (Kemp & Lockey, 2002).

The Chinese shrimp, *Fenneropenaeus chinensis*, an economically important fished and farmed shrimp species in China, causes seafood allergy in most adults and children. The aim of this work is to characterize the IgE-binding proteins by protein profiles analysis from the muscle proteins of Chinese shrimp. In addition, the allergenicity of protein extracts was verified in vivo by systemic exposure including specific serum IgE detection, mast cell intact rate evaluation as well as estimation of T lymphocyte positive rate. Our results may have important implications for understanding the major allergens and potential allergenicity of Chinese shrimp.

2. Materials and methods

2.1. Positive sera and preparation of shrimp extracts

Sera from 30 patients with shrimp allergy were collected in the Zhejiang Provincial Center for Disease Control and Prevention (Hangzhou, China) and stored in aliquots at -80°C . The allergic response was confirmed by the clinical history, diagnosis, and IgE reactivity measuring. The initial inclusion of shrimp allergic patients was on the basis of a Pharmacia CAP score for shrimp crude extract-specific IgE Ab $>+2$. Sera from five nonallergic individuals were used as controls. Seven shrimp-allergic patients had IgE Ab detectable by immunoblotting, and all seven had a positive clinical history. Twenty-three patients with positive clinical history had negative IgE Ab detection results.

Chinese shrimp (*F. chinensis*) were purchased from a local market. The shrimp muscle was ground in a mortar filled with liquid nitrogen, then extracted at 4°C overnight with constant stirring with extract buffer (50 mM PBS, 0.2 mM DTT, 1 mM PMSF, pH 7.0). After centrifugation at 12,000 g for 10 min at 4°C , the supernatant was dialyzed for 48 h at 4°C against 10 mM sodium phosphate buffer (pH 7.0), then lyophilized to yield the crude extract.

2.2. SDS-PAGE and immunoblotting with IgE

Proteins of shrimp extracts (SE) of 15 μg per lane were separated by gradient 4–20% SDS-PAGE (Bio-Rad, Hercules, CA). For immunodetection of IgE-binding proteins, the separated proteins were electroblotted onto a polyvinylidene difluoride membrane, which was then blocked with skimmed milk and incubated for 16 h at 4°C with a 1/10 dilution of the shrimp-allergic serum. Bound IgE Abs were detected using alkaline phosphatase-labeled goat anti-human IgE Abs (BD Pharmingen, San Diego, CA) and 5'-bromo-4-chloro-3-indolyl phosphatase/nitroblue tetrazolium as the substrate system (Chow, Su, Yu, Chiang, & Shen, 1999).

2.3. Mice

Female ICR mice (6-week-old) were obtained from the Centre of Experimental Animals, Zhejiang Academy of Medical Sciences (Hangzhou, China). The mice were housed five per cage at $20 \pm 2^{\circ}\text{C}$ on a 12-h light and 12-h dark cycle, with unlimited supplies of food and water sterilized by irradiation and autoclaving. All animal procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang Provincial Experimental Animal Public Service System.

2.4. Sensitization and sample collection

Female ICR mice were randomly divided into 6 groups with 10 mice per group, and intraperitoneally injected with 0.2 mL complete Freund's adjuvanted shrimp extracts (CFA-SE) on day 1 and incomplete Freund's adjuvanted shrimp extracts (IFA-SE) on days 3 and 5. The doses of each group were 0.2, 0.4, 0.6, 0.8, and 1 mg CFA-SE/IFA-SE, respectively. Secondary exposure was performed on days 15, 22, and 29 with IFA-SE. The negative control group was treated by intraperitoneal administration with an equal volume of PBS. On days 15, 22, 29, and 36, the blood samples were collected from tail vein of each group mice and stored at -20°C until analysis. On day 36, mice were euthanized for spleen, duodenum, jejunum, and ileum sampling.

2.5. IgE ELISA

The levels of shrimp allergen-specific IgE in mice were determined by indirect ELISA. Briefly, wells of a 96-well EIA/RIA (Costar, St. Louis, MO) were coated with 100 μ L extracts (3 mg/mL) in carbonate coating buffer (pH 9.6), and incubated overnight at 4°C. The plate was washed 4 times in 0.05% Tween 20/PBS (PBS-T) between steps. Blocking was performed using 3% skim milk powder diluted in PBS-T at room temperature for 2 h. Diluted serum samples (1:20 dilution using 1% skim milk powder/PBS-T) were added to the plate and incubated at room temperature for 2 h. Plate was then incubated with Horseradish peroxidase-conjugated rat anti-mouse IgE (100 μ L per well, 1:1000 dilution; Abcam, Cambridge, UK) for 1 h. After washing, plate was incubated with o-phenylenediamine (OPD) for 15 min at room temperature. The reaction was terminated by adding 2 mol/L H_2SO_4 and the absorbance values were measured at 490 nm using a microplate reader. All assays were performed in triplicate. The data for measurement of shrimp allergen-specific IgE were all background subtracted (Ren, Zhang, Li, & Wang, 2010).

2.6. Spleen weight ratio

Body weight of mice was recorded as $m1$ before sampling, while spleen weight recorded as $m2$. The spleen weight ratio was then defined as $m1/m2$. Thus, the effect of sensitization is evaluated by comparison of spleen weight ratios between sensitized and control groups.

2.7. Histological analysis of small intestine

At necropsy, the duodenum, jejunum, and ileum were excised and gently flushed with PBS. Then, the samples were fixed in neutral-buffered 10% formaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin for tissue morphology or toluidine blue for degranulated mast cell. A degranulated mast cell was defined as a toluidine-positive cell with five or more distinct stained granules completely outside of the cell (Li, Schofield, Huang, Kleiner, & Sampson, 1999). The percentage of intact mast cells was calculated as:

$$\frac{(\text{number of total mast cells} - \text{number of degranulated mast cells})}{\text{number of total mast cells}}$$

2.8. Immunohistochemistry

The duodenum, jejunum, and ileum samples were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.2), and 5- μ m paraffin sections were used for immunohistochemistry staining of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in the intestine villi and lamina propria, respectively. Briefly, sections were first labeled with primary (Abs) (1:200 dilution of rabbit anti-mouse CD3, Abcam; 1:200 dilution of rabbit anti-mouse CD4, LSBio; 1:300 dilution of rabbit anti-mouse CD8, Abbiotec) for 60 min, then incubated with the appropriate biotinylated secondary Abs (1:400 dilution of goat anti-rabbit IgG, SouthernBiotech) for 30 min, followed by avidin-peroxidase for 30 min. Sections were then incubated for 10 min with hydrogen peroxide and diaminobenzadine before being counterstained with hematoxylin. Positive cells were identified by the presence of a brown reaction product. All incubations were at room temperature and washes were done in PBS. The ratio of stained (brown) target cells to unstained target cells was determined by differential counts. At least 150 target cells were counted per mouse. The results are expressed as the proportion of positive target cells as a percentage of the total cells.

2.9. Statistical analysis

Means and standard errors, calculated from the results of multiple experiments in most cases, are displayed in the figures. Statistical significance of the data was determined by one-way analysis of variance using SPSS.13.0. A p value of <0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Identification of IgE-binding proteins in *F. chinensis*

To identify the IgE-binding proteins of SE, the allergens were then analyzed by SDS-PAGE and Western blotting. When serum samples from the 30 shrimp-allergic patients were tested on immunoblots for IgE binding to *F. chinensis* crude extract, 7 (23.3%) showed IgE binding to protein bands with apparent molecular masses of 18–70 kDa (Figure 1). Of the prominent bands, allergens with molecular masses ranging from ~20 to 40 kDa were detected by 85.7% (6 of 7) of these sera. An apparent protein band of 40 kDa can be recognized at a high frequency of ~70%, which is probably arginine kinase, the well-known shrimp major allergen (Garcia-Orozco et al., 2007; Yu, Lin, Chiang, & Chow, 2003). IgE-binding components with molecular masses of 37, 28, 22, and 20 kDa detected at frequencies of ~43–57% are supposed to be shellfish allergens TM (32–40 kDa) (Lin, Shen, & Han, 1993; Motoyama, Suma, Ishizaki, Nagashima, & Shiomi, 2007), triosephosphate isomerase (24.4–28 kDa) (Hoppe, Stein, & Paschke, 2006), sarcoplasmic calcium-binding protein (20–22 kDa) (Ayuso et al., 2009, 2010; Shiomi et al., 2008), and myosin light chain (20 kDa) (Ayuso et al., 2008). No IgE binding was seen when serum from a healthy donor was used (Figure 1, lane 9).

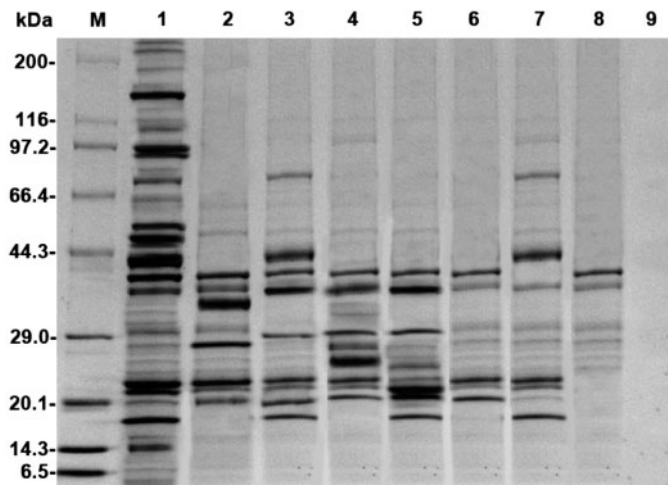


Figure 1. Immunoblots showing binding of patients' serum IgE to *F. chinensis* crude extract. Lane M, Molecular mass markers; lane 1, Coomassie blue staining of the crude extract; lanes 2–8, immunoblots showing binding of IgE from different serum samples from allergic patients; lane 9, immunoblot using serum from a nonallergic individual.

3.2. Time-course levels of shrimp allergen-specific IgE

Sera were obtained from the mice at the end of primary exposure (day 15) and secondary exposure (days 22, 29, 36). Time courses for levels of shrimp allergen-specific IgE showed that each experimental group had a tendency of a slow rise at the beginning, then accelerated to the peak (on day 29) and finally declined slowly to a relatively stable level (Figure 2). The IgE level of control group was significantly ($p < 0.05$) lower than that of

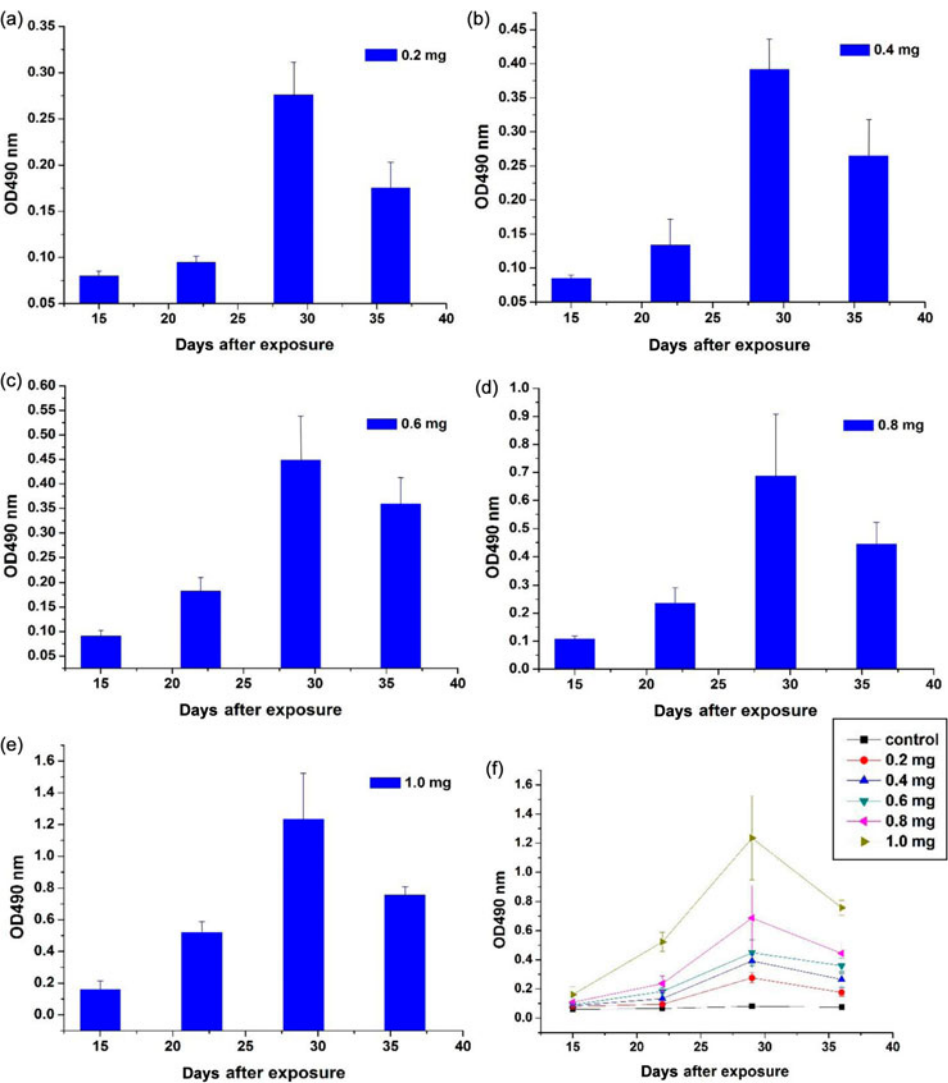


Figure 2. ELISA for serum IgE reactivity to shrimp extracts. Serum were obtained from groups ($n = 10$) sensitized with shrimp allergens on days 15, 22, 29, and 36, respectively. Serum IgE levels after exposure to 0.2 (a), 0.4 (b), 0.6 (c), 0.8 (d), and 1 mg (e) of shrimp extracts are expressed as mean (OD 490 nm) $\pm$ SD. Time-course levels of shrimp allergen-specific IgE in all of the groups are compared in (f).

each experimental group during the entire stage of exposure (Figure 2f). In addition, serum IgE reactivity to SE presents a dose-dependent manner, since the 1 mg injection group induced the highest level of shrimp allergen-specific IgE (Figure 2e).

These results indicated that the shrimp protein extracts could lead to allergen-specific IgE reactivity. Similar to the previous study (Knight et al., 2007), the amount of allergens is a major factor in determining allergenicity. However, the quantity of induced IgE varies in individual studies due to the different allergens (Knight et al., 2007; Strobel, 1986). A certain number of studies have also implied the selection of mouse strains as an important factor in the research on an experimental animal model (Morafo et al., 2003). Reese et al. (2005) pointed out that different mouse strains and adjuvants had apparently different immune responses with respect to the induction of IgE response.

3.3. Increased spleen weight and mast cell degranulation after sensitization

During necropsy at the end of the sensitization period, we noticed a striking difference in spleen size between the sensitized and control mice. Sensitization was associated with a clear and statistically significant increase in spleen weight ratios (Figure 3), indicating that mononuclear phagocyte and lymphocyte systems in spleen may react with hypersensitivity responses, thus resulting in an enlargement of the spleen.

Examination of small intestine histology indicated that severe damage of epithelium happened in the sensitized mice after exposure to SE of 1 mg dose based on inflammatory infiltration, and disruption of epithelial architecture and sub-epithelial edema (Figure 4). Moreover, histologic analysis of intestinal tissue also showed many degranulated mast cells in the SE-sensitized mice, but not in the control mice (Figure 5a and b). The percentage of intact mast cells in the control group was approximately 2 times greater than that in the SE-sensitized group (Figure 5c).

IgE-dependent mast cell activation has long been regarded as one of the essential steps in the development of active anaphylaxis (Kemp & Lockey, 2002). In response to

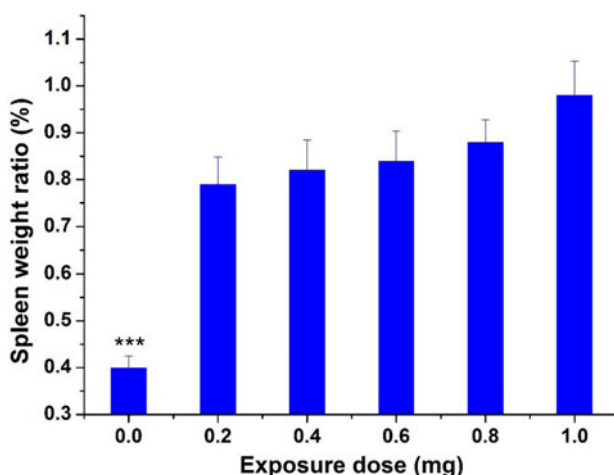


Figure 3. Comparison of spleen weight ratio in sensitized and control mice. Spleens were obtained on day 36 from groups ($n = 10$) exposure to 0.2, 0.4, 0.6, 0.8, and 1 mg of shrimp extracts. The error bars indicate standard deviation ($\pm$ SD). Statistical significance ($p < 0.001$) was marked by asterisks.

the antigen cross-linking of IgE, which is bound to receptor I (FcεR I) on the mast cells or basophils, the process of degranulation is induced and biochemical mediators and chemotactic substances, such as prostaglandins, cytokines, leukotrienes, histamine, and other mediators, are subsequently secreted by degranulated mast cells or basophils (Kumar et al., 2012). Decreased intact mast cells of the small intestine in the sensitized mice injected with SE suggested that an intraperitoneal challenge with shrimp allergens triggered the degranulation of the mast cells. Histologic observation by light microscopy also showed inflammation and disruption of epithelial architecture of the small intestine in the sensitized mice. Thus, it is indicated that the allergens of the *F. chinensis* extracts can cause IgE-dependent and mast cell-activated gastrointestinal hypersensitivity.

3.4. Role of T lymphocytes in *F. chinensis* allergen-induced hypersensitivity

Immunohistochemical staining showed that the CD3⁺, CD4⁺ and CD8⁺ T lymphocytes were widely distributed in the lamina propria of the small intestine in the sensitized mice (Figure 6a). In addition, the numbers of three types of T lymphocytes in the sensitized

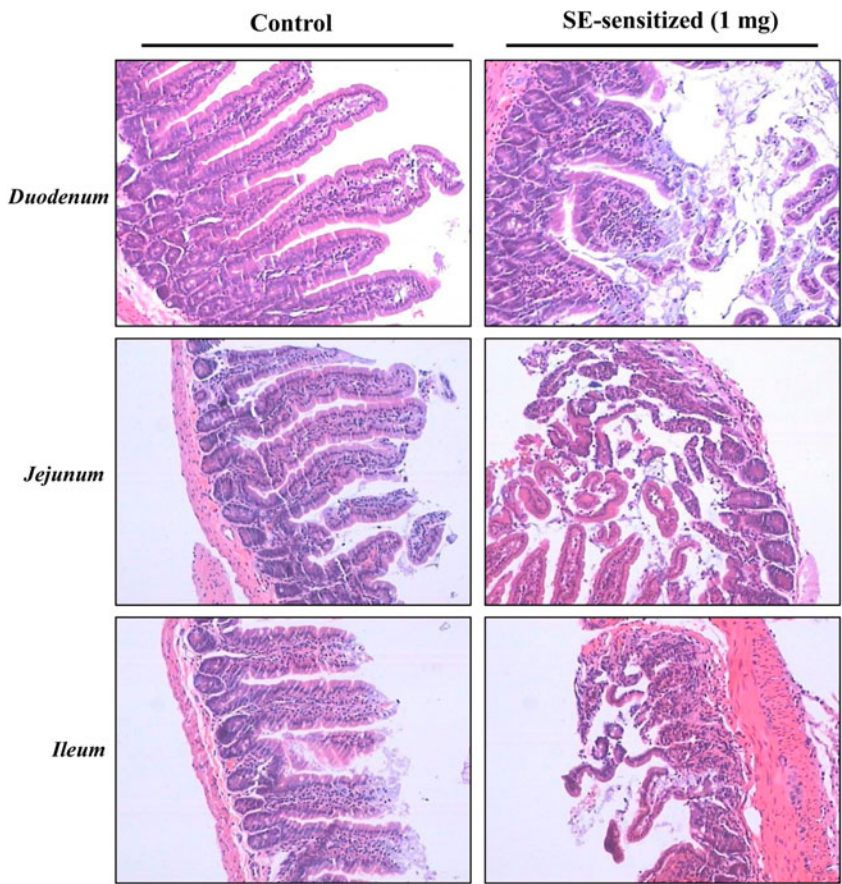


Figure 4. Histologic alterations in intestine after SE sensitization (1 mg dose). Samples were sectioned at 5 μ m thickness and stained with hematoxylin and eosin. The stained sections were photographed at $\times 10$ magnification using a light microscope.

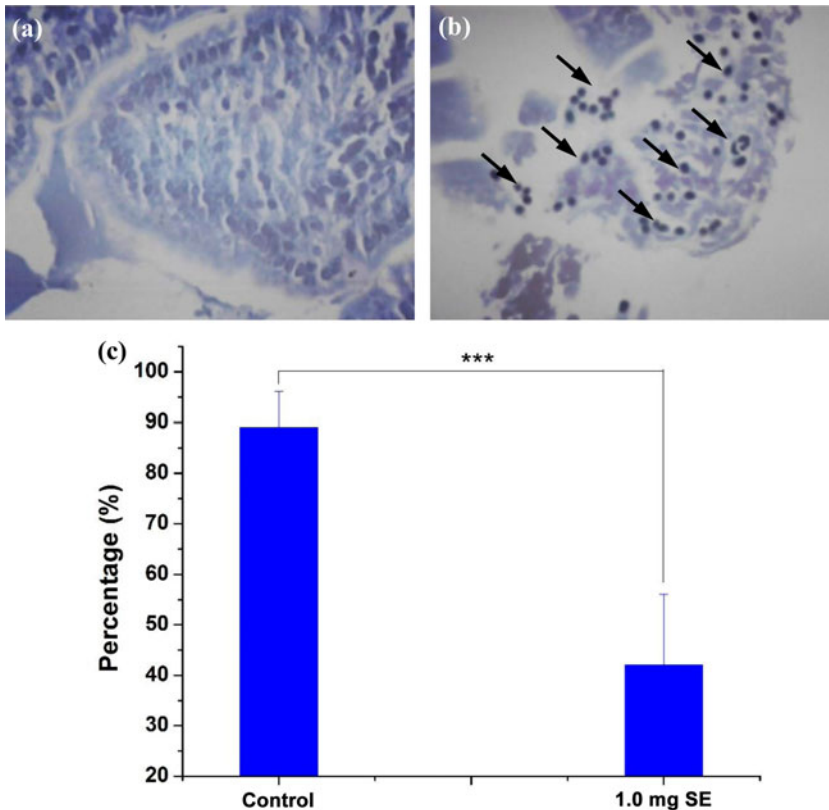


Figure 5. Degranulation of mast cells in small intestine samples after SE sensitization (1 mg dose). Samples were sectioned at 5 μ m thickness and stained with toluidine blue. Stained sections of control (a) and sensitized (b) groups were photographed at $\times 40$ magnification using a light microscope. Degranulated mast cells are indicated by arrows. (c) Percentage of intact mast cells in intestine samples of SE-sensitized mice. Values are expressed as means $\pm$ SD of 10 mice per group. Statistical significance ($p < 0.001$) was marked by asterisks.

mice were significantly higher than that in control mice (Figure 6b), which indicated that shrimp allergens can also induce T-lymphocyte-dependent hypersensitivity after intraperitoneal exposure of proteins.

Food allergic reactions are not limited to IgE-mediated reactions only as non-IgE-mediated allergic reactions, such as T-lymphocyte-dependent anaphylaxis and immune complex-mediated reactions, may also occur (Kumar et al., 2012). Studies have proposed an alternate allergic reaction system on the basis of several mechanisms on T-lymphocyte-dependent hypersensitivity. The ITAM motif of the CD3 molecule on T lymphocyte surface is important in the sensitization challenge. When the TCR recognizes the antigenic peptide – MHC complexes – the cytoplasmic domain of ITAM motif becomes tyrosine phosphorylated. After further activation of phospholipase $C\gamma$, signaling pathways could finally promote the release of mediators and cytokines (Groux, Bigler, de Vries, & Roncarolo, 1996). Moreover, it has been implicated that the initial step in the development of immediate-type allergy is the priming of allergic-specific $CD4^+$ T helper

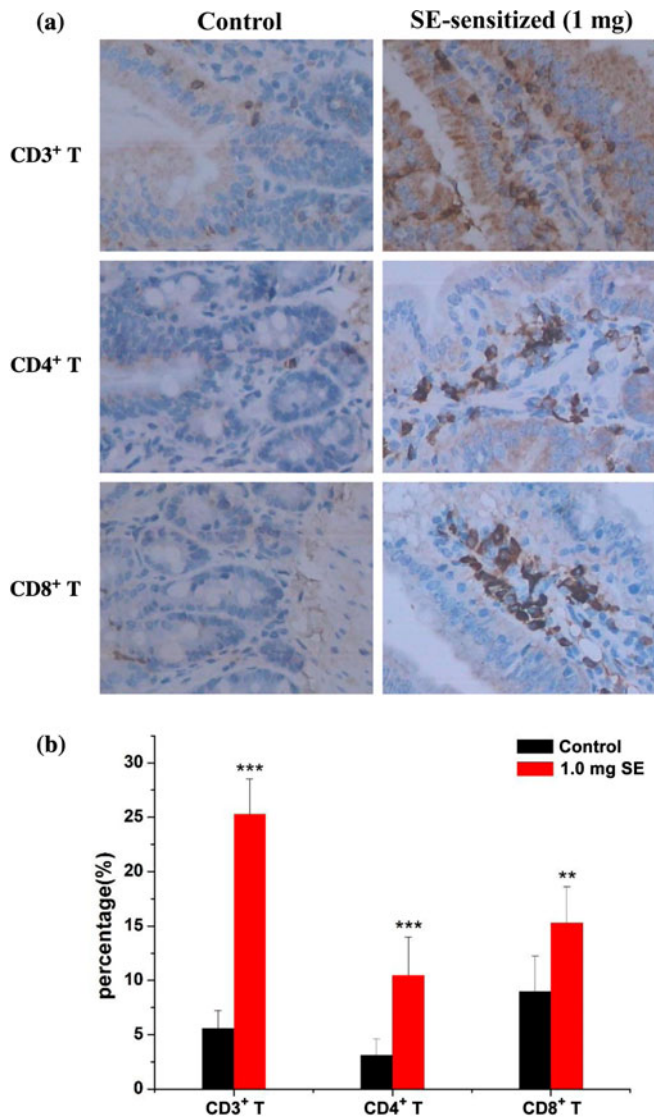


Figure 6. Immunohistochemical analysis for CD3⁺, CD4⁺, and CD8⁺ T cells of small intestinal tissues in mice sensitized by shrimp extracts. Immunohistochemical staining was performed, and the percentage of the cell subsets was determined. (a) In the control mice, only few CD3⁺, CD4⁺, and CD8⁺ T cells are visible (brown) in the lamina propria. In the sensitized mice (1 mg dose), there is a massive accumulation of positive cells at the crypt bases and in the lamina propria. Original magnification, $\times 40$. (b) Compared with control mice, sensitized mice had an increase of CD3⁺, CD4⁺, and CD8⁺ T-cell subsets. Values are presented as mean $\pm$ SD of 10 mice per group. ** represents $p < 0.01$ and *** represents $p < 0.001$.

2 (Th2) cells. The secretion of IL-4 and IL-13 by Th2 cells in the presence of allergen recognized by naïve B cells leads to the synthesis and secretion of allergen-specific IgE by plasma cells, which causes an immediate-type immune reaction (Dearman, Caddick, Basketter, & Kimber, 2000; Möbs, Slotosch, Löffler, Pfützner, & Hertl, 2008).

Furthermore, cytotoxic T lymphocytes (CD8⁺ T lymphocytes) can play an important role in T lymphocyte-mediated cytotoxicity during hypersensitivity (van Wijk & Knippels, 2007). Taken together, our results suggest that IgE-dependent hypersensitivity plays a major role in allergic reactions triggered by *F. chinensis* allergens; however, T lymphocyte-dependent activation is likely to be much less prominent and more delayed compared to IgE-dependent activation.

4. Conclusions

Shellfish are a common cause of adverse food reactions in hypersensitive individuals and shrimp is one of the most frequently reported causes of allergic reactions. In the present study, we characterized the major IgE-binding proteins by protein profiles analysis from the muscle proteins of Chinese shrimp (*F. chinensis*). In vivo experiments showed that SE could lead to allergen-specific IgE reactivity, and induce an acute IgE-dependent and mast cell-activated gastrointestinal hypersensitivity. Our results also indicate that IgE-dependent hypersensitivity plays a major role in allergic reactions triggered by *F. chinensis* allergens; however, T lymphocyte-dependent activation might be in a minor and more delayed fashion.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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