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Evaluation of electron beam irradiation to reduce the IgE binding capacity of frozen shrimp tropomyosin

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ABSTRACT

The aim was to determine the effect of electron beam (EB) irradiation on IgE binding capacity of shrimp tropomyosin (TM) at the frozen stage. Following EB irradiation, protein extracts were characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and TM degradation was observed. The degradations were further recognized by IgE from a shrimp-allergic patient by immunoblots. Specific IgE binding results showed that capacity of shrimp extracts and TM initially increased by 10% after irradiation at 3 kGy doses, and then decreased by 20% with irradiation doses increased to 10 kGy. Further inhibition enzyme linked immunosorbent assay results showed that half-maximal inhibitory concentration of shrimp extract treated with 10 kGy doses enhanced to four times as compared with raw shrimp extracts. These results suggest that EB irradiation significantly changes the IgE binding capacity of frozen shrimp at 10 kGy doses by 20% and has the potential to reduce shrimp allergy risk.

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IgE binding capacity; shrimp; electron beam irradiation; tropomyosin

1. Introduction

In coastal cities, seafood, especially shrimp, is a popular choice among consumers due to its high-protein content and abundance of polyunsaturated fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid, which are known to have health benefits. However, increased shrimp consumption has been correlated with significant hypersensitivity reaction problems. Recent epidemiological studies in Canada showed that 1.35–1.86% of the total population has shrimp allergies and this allergy rate is higher than that of tree nuts, fish, and peanuts (Ben-Shoshan et al., 2010). In China, the rate of shrimp allergy is 8.6%, which has been reported by a survey in a university (Xiangzheng, Xiumei, & Xiaoguang, 2005). Currently, there are no effective ways to completely eliminate shrimp allergies (Ayuso, 2011), hence developing methods to reduce shrimp allergenicity has become an important priority to increase food safety.

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Different processing technologies such as heat treatment (Usui et al., 2013), high-intensity ultrasonic treatment (Zhenxing, Caolimin, & Jamil, 2006), enzyme hydrolysis (Shimakura, Tonomura, Hamada, Nagashima, & Shiomi, 2005), high hydrostatic pressure (Jin et al., 2013), and irradiation (gamma and electron beam [EB]) (Zhenxing, Hong, Limin, & Jamil, 2007b) have been used to reduce shrimp allergenicity. Among these technologies, irradiation is the most attractive to the food industry because it can be performed directly on frozen foods and maintains shrimp texture better with the low cost. For example, the seafood processing industry has used ionizing radiation to improve food safety and is now legally recognized as safe in many countries (International Atomic Energy Agency, 2007). Moreover, several studies have shown that gamma irradiation effectively reduces IgE binding to shrimp extracts. It is reported that gamma irradiation combined with heat treatment almost eliminated IgE binding to shrimp extracts (Zhenxing, Hong, Limin, & Jamil, 2007a). Katial, Grier, Hazelhurst, Hershey, and Engler (2002) have reported that EB irradiation could reduce the allergenicity of glycerinated allergen extracts (Katial et al., 2002). Although irradiation could reduce food allergenicity effectively, these reductions always happened at room temperature or combined with heat treatment. As a kind of highly perishable food, shrimp is always preserved at the frozen stage (-18°C or more). Due to changes occurring in the quality of shrimp with thawing and refreezing (Sirintra, Saiwarun, Sumate, Toru, & Rikuo, 2007), it is necessary to find an effective way to reduce allergenicity of frozen shrimp to get hypoallergenic shrimp with high quality.

In recent years, EB irradiation, which was recognized as an effective way to control microorganisms in frozen seafood was frequently used by the food industry (Park et al., 2010). Compared with gamma irradiation, EB irradiation is considered as an environment-friendly, highly efficient, and high-throughput technology (Panagiota, Kyriakos, & Michael, 2011). Although few previous reports showed that it was an effective way to reduce the allergenicity of protein extracts (Katial et al., 2002), the effect of EB irradiation on reducing allergens of frozen food has not been studied in detail. The aim of this study was to investigate if various levels of EB-irradiation treatment reduce the IgE binding properties of frozen shrimp.

2. Materials and methods

2.1. Shrimp and reagents

Fresh shrimp (*Metapenaeus ensis*) was purchased from a local market (Taidong fishery market, Qingdao, China). Unless otherwise stated, all reagents were of analytical grade. Buffers used for Western blotting were phosphate buffer saline with tween 20 (PBST) (10 mM phosphate buffer [pH 7.4], 0.15 M NaCl, and 0.05% Tween 20). Buffers used for enzyme-linked immunosorbent assay (ELISA) were as follows: coating buffer (0.1 M bicarbonate buffer [pH 9.6]), blocking buffer (0.01 M phosphate buffer [pH 7.4], 0.1% bovine serum albumin [BSA], and 0.15 M NaCl) and washing buffer (PBST). Solid-phase enzyme immunoassays were performed in the 96-well microtiter plates (Nunc, Denmark) using a Multiskan MK3 ELISA reader (Thermo Labsystems).

2.2. Human sera

Human sera were collected from volunteers with shrimp allergies at the Affiliated Hospital of Medical College Qingdao University. Shrimp tropomyosin (TM)-specific IgE was detected in nine sera samples. Specific shrimp TM IgE levels and total IgE levels were determined by Allergy Screen (MEDIWISS Analytic GmbH, Moers, Germany). A serum sample from a non-allergic healthy volunteer was used as a negative control. All serum samples were stored at -80°C until use.

2.3. Treatment of peeled shrimp with EB irradiation

The EB irradiations were conducted at Qingmai High Energy Electron Irradiation Ltd, using a Linear Accelerator (LINAC) Varian Model 2100C with dichromate dosimeter system for dose measurement (uncertainty 3.7%). The samples were irradiated using the most commonly used electron energies (2.5 MeV) for irradiation at this company. Peeled shrimp was cut into slides with 2.0 mm thickness, piled separately, vacuum-packed in a sterile plastic bag (Jiangsu Jinsong Plastic Technology Co., Ltd., Dongtai, China), and stored at -18°C . The samples were put into an ice box and immediately transferred to conveyor for irradiation. During irradiation processing, each side of the sample was treated separately with doses of 0 (control), 3, 5, 7, or 10 kGy. The surface temperature of the samples was -15.6°C at the end of treatment. After irradiation, all samples were stored at -20°C until use.

2.4. Preparation of shrimp extracts

The shrimp extracts were prepared as previously described by Smillie (1982) with modifications. Briefly, 100 g of the abdominal muscle was minced and rinsed with acetone to remove lipids and pigments, followed by drying at room temperature for 10 h to remove acetone. The samples were subsequently resolved in the extraction buffer (1 M KCl, 0.2 mM 1,4-dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride) at a ratio of 1:15 (w/w) for 12 h at 4°C with constant stirring. Supernatants were collected by centrifugation (8000 g, 15 min, 4°C), dialyzed (48 h at 4°C in sterile MilliQ water), and stored at -20°C until use. Total protein concentrations of the shrimp extracts were determined using the bicinchoninic acid protein assay (Smith et al., 1985).

2.5. TM purification

TM was concentrated and purified from shrimp muscle extracts using the ammonium sulfate precipitation and sodium dodecyl sulfate (SDS) profiling method (Hong, Lina, Zhenxing, & Haider, 2010). Briefly, 5 g of shrimp extract was precipitated with a 35% saturated ammonium sulfate solution at 4°C with constant stirring. Samples were centrifuged (8000 g, 45 min, 4°C). The resulting supernatants were dialyzed against double distilled water for 48 h at 4°C . The solution was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by dyeing with 0.5 M KCl. The 35 kDa band corresponding to TM was cut out using a sharp blade, pulverized in a mortar, and

extracted in double distilled water overnight. After centrifugation at 4000 g for 15 min at 4°C, supernatants were collected and dialyzed against 0.01 M PBS (pH 7.4) and stored at -20°C until use.

2.6. SDS-PAGE and Western blotting

SDS-PAGE was performed according to the method described by Laemmli (1970). All three irradiated protein samples were mixed in a 4:1 ratio with loading buffer (2% SDS, 25% glycerol, 14.4 mM β -mercaptoethanol, and 0.1% bromphenol blue in 1 M Tris-HCl [pH 6.8]), heated at 95°C (6 min), loaded onto 5–12% Tris-glycine gels, and resolved by SDS-PAGE. Proteins were stained using Coomassie Brilliant Blue R-250. For Western blotting, proteins resolved within the SDS-PAGE gels were transferred onto a Polyvinylidene difluoride (PVDF) membrane under a constant current of 8.0 mA/cm² for 3.5 h. Western blot membranes were blocked with 5% blocking buffer (5% [w/v] dry milk powder in PBST) for 2 h at room temperature. For specific IgE binding, primary antibodies in the form of pooled sera from individuals with shrimp allergies were diluted (1:10) in 2.5% blocking buffer and incubated with the PVDF membrane overnight at room temperature. Horseradish peroxidase (HRP)-labeled goat anti-human IgE secondary antibodies were used at a 1:1000 dilution in 2.5% blocking buffer. Following the secondary antibody incubation for IgE binding, the PVDF membranes were washed extensively with PBST and incubated with electro chemiluminescence substrate according to manufacturer's instructions. Finally, immunoreactive bands were visualized using a Tanon 4200 system and the generated files were analyzed with Quantity One software.

2.7. Estimation of specific IgE

Specific IgE binding to irradiated shrimp extracts or TM was assayed by ELISA, following the method described by Singh et al. (2006). In brief, irradiated shrimp extracts or TM (1 μ g/100 μ L per well) was fixed in a microtiter plate (Nunc, USA) overnight at 4°C with carbonate buffer. After washing with PBST (0.05%), wells were filled with 1% BSA and incubated at 37°C for 2 h to block all nonspecific sites. After washing with PBS, microtiter wells were filled with test samples (1:5 [v/v] dilutions of sera from patients with shrimp allergies) and incubated overnight at 4°C. The serum from a healthy volunteer was used as the negative control. Anti-human IgE-HRP (1:1000 [v/v]) was added to microtiter wells after washing, and incubated at 37°C for 2 h. Reactions were read at 405 nm following color development with 3,3',5,5'-tetramethylbenzidine and reaction termination with 2.0 M H₂SO₄.

2.8. Inhibition ELISA

The allergenic potential of raw and EB-irradiated shrimp extracts/TM was determined by inhibition ELISA using pooled sera from hypersensitive patients. Initially, microtiter plates were coated with raw shrimp extracts/TM (1 μ g/100 μ L per well) in carbonate buffer overnight at 4°C. The patient sera (1:5 [v/v]) were simultaneously preincubated with 2, 10, 50, 250, 1000, and 10,000 ng of EB-irradiated shrimp extracts/TM overnight at 4°C. The mixture was then added to the microtiter plate coated with raw shrimp extract/TM. Serum from a healthy human volunteer was used as a control. The concentration of

protein that can inhibit 50% of IgE binding was estimated using the following formula:

$$1 - \frac{\text{OD of sample with inhibitor}}{\text{OD of sample without inhibitor}} \times 100.$$

2.9. Statistical analyses

For the specific IgE binding experiment, the A_{405} of different treatments were compared using a two-way ANOVA. For the inhibition with raw shrimp extracts/TM experiments, the absorbance data were first transformed to % inhibition. The differences in % inhibition were analyzed by two-way ANOVA. The Holm–Sidak method was used for all multiple pairwise comparison tests ($p = .05$). Statistical analyses were conducted using SigmaPlot 12.0, Systat Software Inc. (San Jose, CA).

3. Results and discussion

3.1. Changes in electrophoresis pattern

Protein profiles of shrimp muscle that was EB irradiated at various doses were analyzed by SDS-PAGE. As shown in Figure 1, no significant changes in the protein profiles were observed after treatment with different doses of EB irradiation. A protein band of 36 kDa that corresponds to the size of TM, which is considered to be the major allergen in shrimp (Daul, Slattery, Reese, & Lehrer, 2004), was observed after all EB-irradiation treatments and there was no significant change in the density of TM, even after the shrimp muscle was irradiated at 10 kGy. When comparing EB irradiation with gamma irradiation treatment, there were no significant changes in protein profiles, but new

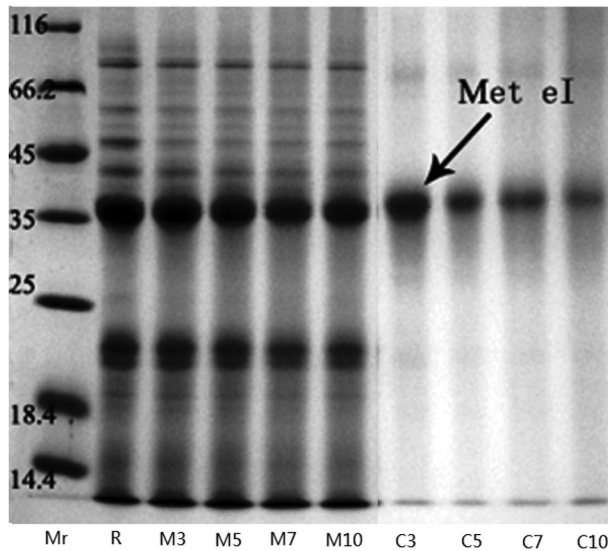


Figure 1 SDS-PAGE profiles of shrimp extracts and purified shrimp TM from irradiated samples. Met e1: *Metapenaeus ensis* major allergen; M: marker; R: raw sample without irradiation (control); M: irradiated shrimp muscle protein extracts; C: purified TM from irradiated shrimp. Numerals 3, 5, 7, 10 in the column indicate irradiation dose (kGy).

protein species were observed at 45 kDa after the shrimp muscle was gamma irradiated at 7 kGy and 10 kGy (Zhenxing, Hong, Limin, & Jamil, 2007b). Moreover, the density of TM decreased after treatment with increasing doses of gamma irradiation and following thermal treatment. This phenomenon was highly significant after gamma irradiation treatment (Zhenxing, Hong, Limin, & Jamil, 2007a).

The mechanism of protein structure changes caused by EB irradiation might be that irradiation can generate reactive oxygen species (ROS), such as hydroxyl radicals and superoxide anion radicals (Giedzinski, Rola, Fike, & Limoli, 2005). These kinds of ROS can induce amino acid modifications, such as histidine conversion to histamine or Phe conversion to Tyr or even cause peptide breakdown and result in protein–protein cross-linking (Ghanbari, Ghoochi, Shawrang, Mansouri, & Torbati-Nejad, 2012). The higher power and penetration of gamma irradiation compared with that of EB irradiation may explain why an equal dose of gamma irradiation generates a new protein band in shrimp muscle. In support, a comparative study measuring the *E. coli* killing efficiency of gamma and EB irradiation clearly showed that gamma was more powerful than EB irradiation (Lopez-González, Murano, Brennan, & Murano, 1999). As compared with TM (36 kDa band) in SDS-PAGE, it seems that the intensity decreased. The possible reason is that TM especially those modified TM which were generated during irradiation was lost during the purification process. Previous reports have shown that irradiation treatment can cause protein cross-linking through protein carbonization, which may render protein insoluble (Petrotchenko & Borchers, 2010).

3.2. Immunological reactivity of EB-irradiated shrimp allergens

To determine the allergenic potential of shrimp TM, a Western blot analysis was performed on shrimp samples that had been treated with various doses of EB irradiation. In the blotting assays, the pooled sera from individuals with shrimp allergies were used to recognize shrimp TM. The results showed that TM, the major shrimp allergen, could bind with the sera even after the shrimp was EB irradiated with a dose of 10 kGy (Figure 2). The protein extracts from EB-irradiated shrimp muscle showed significant changes especially when EB-irradiation doses increased to 7 kGy. When the TM was purified from EB-irradiation samples, the IgE binding to TM showed significant decreases too. It seems that IgE binding capacity of purified shrimp allergen decreased more obvious than shrimp protein extracts. Although EB-irradiated shrimp showed obvious decreased IgE binding capacity, the reduction rate was far less than previous reports which exposed extremely significant decline in the blot intensity (Zhenxing, Hong, Limin, & Jamil, 2007b).

This kind of phenomenon is not exceptional, as there were previous reports showing that shrimp muscle treated with low doses (<5 kGy) of gamma irradiation results in increased IgE binding (Byun et al., 2000). This may be caused by the alteration of some epitopes on protein surfaces. Recent studies have confirmed that food treated with a low dose of gamma irradiation can induce an allergic response that is higher than that of untreated raw food (Vaz, Souza, Medeiros, et al., 2013). The possible reason is that slight irradiation, which can generate low concentrations of ROS, can cause some

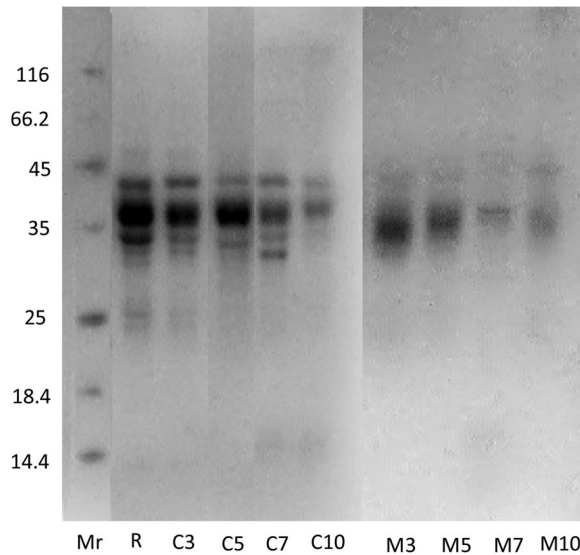


Figure 2 Western blot analysis of IgE binding in shrimp extracts and purified shrimp TM from irradiated samples with pooled sera. R: is the raw sample without irradiation (Control); M: irradiated shrimp muscle protein extracts; C: purified TM from irradiated shrimp. Numerals 3, 5, 7, and 10 in the column indicate irradiation dose (kGy).

structural changes to food allergens. A recent study demonstrated that the concentration of ROS has a linear relation to the IgE binding capacity changes (Li, Lu, Khan, Lin, & Zhang, 2014). Our results are consistent with these previous reports. The purified TM showed similar changes which can confirm the molecular structure changes during EB irradiation.

3.3. Specific IgE binding capacity of EB-irradiated shrimp allergens

ELISAs were carried out using the sera from patients with shrimp allergies to assess the IgE binding capacity of raw and EB-irradiated shrimp extracts. Approximately 10% increase in IgE binding to 3 kGy irradiated shrimp extracts was observed and then IgE binding decreased as the EB-irradiation dose increased (Figure 3(a)). As for purified shrimp TM, a similar phenomenon was observed with the average optical density (OD) was approximately 20% higher as compared with shrimp extracts (Figure 3(b)).

These results were similar to previous reports studying the effect of low gamma irradiation doses on shrimp (Zhenxing, Hong, Limin, & Jamil, 2007b). Another study also showed that low-dose gamma irradiation of food protein increased its allergenicity in a chronic oral challenge (Vaz, Souza, Medeiros, et al., 2013). These results are different from studies using gamma irradiation that showed a decrease of almost 80% in IgE binding following gamma irradiation treatment at a dose of 10 kGy (Zhenxing, Hong, Limin, & Jamil, 2007b). There are two possible reasons. One reason is related to the concentration of ROS which are created during irradiation. As known, the energy of EB irradiation is lighter than gamma irradiation. The light energy

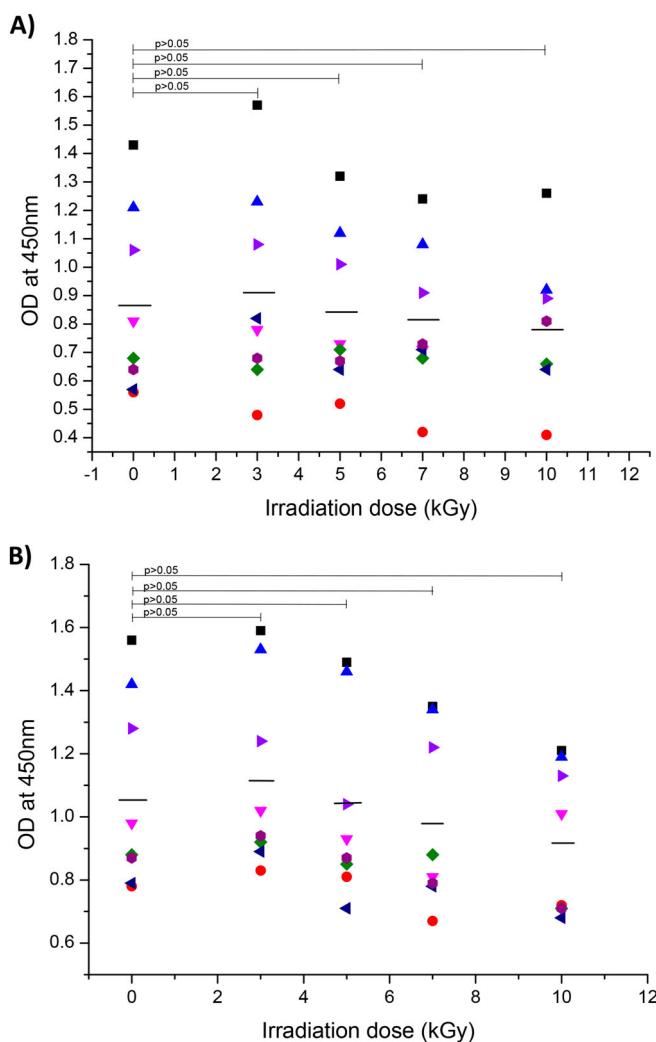


Figure 3. (a) Specific IgE binding against protein extracts from shrimps with different EB-irradiation doses. (b) Specific IgE binding against purified TM from shrimps with different EB-irradiation doses. No significant differences in specific IgE binding were observed among shrimps treated with different irradiation doses.

results in low concentration of ROS or some other active compounds, which can modify the structure of allergen (Estévez, Ventanas, & Heinonen, 2011; Liu, Xiong, & Chen, 2010). Another reason is that frozen shrimp was used in this study. At the frozen stage, it is not easy for energy to penetrate. Previous studies on disinfecting microorganism showed that the frozen stage can reduce the efficiency (Park et al., 2010).

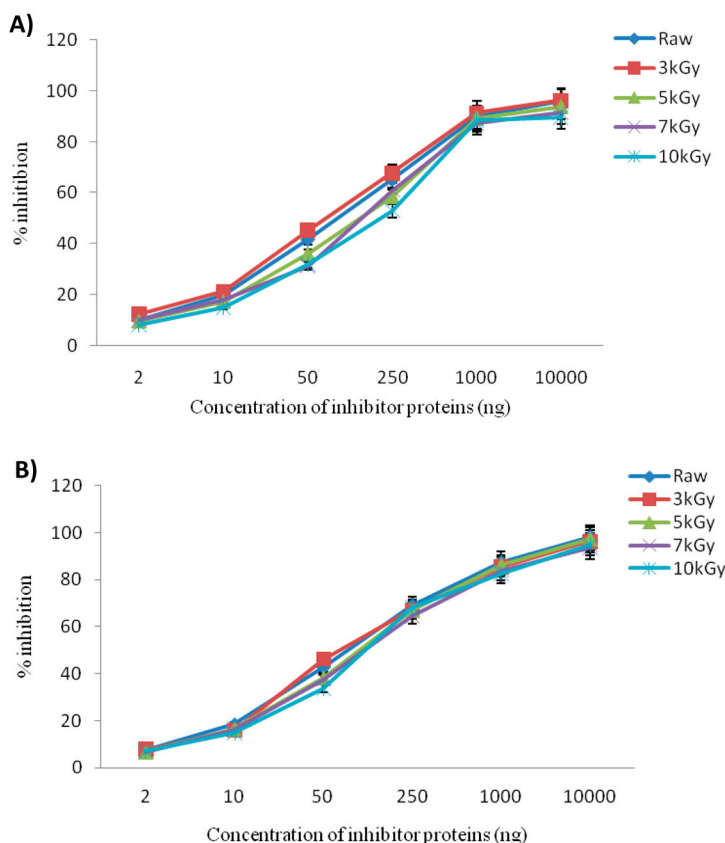


Figure 4. (a) ELISA inhibition with raw and various dose irradiated protein extracts as inhibitors. Shrimp allergy-positive patients' pooled sera (1:10 v/v) were pre-incubated separately with 2, 10, 50, 250, 1000, 10,000 ng of raw and various doses of irradiated shrimp extracts as the inhibitors. ELISA was carried out on the solid-phase coated with the raw shrimp extract (2 μ g/100 μ l/well) and pre-absorbed sera. (b) ELISA inhibition with raw and various doses of irradiated purified TM as the inhibitors. Shrimp allergy-positive patients' pooled sera (1:10 v/v) were pre-incubated separately with 2, 10, 50, 250, 1000, and 10,000 ng of raw and various doses of irradiated shrimp TM as the inhibitors. ELISA was carried out on the solid-phase coated with raw shrimp TM (2 μ g/100 μ l/well) and pre-absorbed sera.

3.4. Reduction in allergenic potency of shrimp proteins after EB irradiation

Allergenic potency was evaluated using inhibition ELISA with the pooled sera from patients with shrimp allergies. The inhibition rate of raw shrimp extracts was slightly lower following EB-irradiation treatment at a dose of 3 kGy, while increasingly higher doses ($p < .05$) (Figure 4(a)). The half-maximal inhibitory concentration of shrimp extract treated with 10 kGy enhanced to four times as compared with raw shrimp extracts. Moreover, inhibition ELISAs performed with purified TM indicated that there was a significant difference in the allergenic potential of 10 kGy EB-irradiated shrimp (Figure 4(b)) as compared with raw shrimp TM. These results indicate that EB irradiation at doses up to 10 kGy does change the IgE binding properties of shrimp allergen.

Although the trend of IgE binding capacity changes is similar, the influences of EB and gamma irradiation on shrimp allergens are different. Radiation damages allergens directly by breaking covalent bonds as a result of transfer of photon energy (Vaz et al., 2012) or indirectly by producing ROS, which can reduce IgE binding capacity by compromising structure–function relationships (Vaz, Souza, Vieira, et al., 2013). Gamma irradiation is more powerful and penetrable than EB irradiation at the same dose (Park et al., 2010). This means that EB irradiation cannot create enough energy to destroy the shrimp allergen at the same dose with gamma irradiation. Another reason is that other components in shrimp can have slight protective effects on TM; this occurs when there are unsaturated fatty acids (Li, Sinclair, & Li, 2011) that will react with ROS generated by irradiation. EB irradiation will not result in the production of high concentrations of ROS. Another difference is that our results are based on the EB irradiation of frozen shrimp, while other research was done with fresh shrimp. The ROS are likely to disperse less in frozen shrimp, which may prevent structural changes of TM, explaining why the epitope did not appear to be altered as to gamma irradiation.

In this study, EB-irradiation dose with more than 10 kGy was not involved. Here are two reasons. Firstly, for EB irradiation, 10 kGy is the maximum dosage that was recognized as not have negative effect to food properties according to the general standard for irradiated foods (General standard for irradiated foods); another reason, too high dosage can induce fatty acid oxidation and undesired smells which will affect the sensory value of the foods (Yun et al., 2012). It seems that other food processing methods such as “high hydrostatic pressure” combined with EB irradiation can result in ample reduction of shrimp allergenicity.

4. Conclusion

In summary, as our study demonstrated, the IgE binding capacity of TM reduced to approximately 20% when shrimp was irradiated with a linear accelerator at 10 kGy doses. To the best of our knowledge, this is the first time the IgE binding capacity changes of shrimp TM was evaluated when it was irradiated at the frozen stage. Although the reducing rate of IgE binding capacity observed was much less compared to the gamma irradiated one at room temperature, it can maintain shrimp freshness during processing due to the low temperature. In order to reduce the IgE binding capacity of shrimp, further studies could be done in two aspects. One is that irradiation should be combined with other thermal or nonthermal processing to reduce the IgE binding capacity efficiently. The other one is that the relationship of shrimp allergen structure and allergenicity during irradiation at the frozen stage should be done. This will be helpful to find an efficient way to reduce the allergic risk of shrimp. Although there are a lot of avenues for further research, our finding may provide one choice for shrimp allergenicity reducing at the frozen storage.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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