

The effect of electron beam irradiation on IgG binding capacity and conformation of tropomyosin in shrimp

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ABSTRACT

To evaluate the effect of electron beam (EB) irradiation on IgG binding capacity and conformation of tropomyosin in shrimp (*Solenocera melanthero*), shrimp meat was irradiated with different doses (0, 1, 3, 5, 7, 9 kGy) of EB. IgG binding capacity of TM from shrimp meat was detected by western blot and indirect enzyme-linked immunosorbent assay. The conformation of TM was analyzed with circular dichroism and Fourier transform infrared spectroscopy. Results showed that there was a significant decrease in IgG binding capacity of TM from shrimp meat treated with EB irradiation, with the most significant effect observed at 7 kGy. At this dose, the IgG binding capacity of TM decreased by 59% when compared to the control group. The structural changes were consistent with changes in IgG binding capacity of TM. It was concluded that EB irradiation can reduce the immunoreactivity of shrimp TM by altering its structure.

1. Introduction

Eight kinds of allergen source, comprising wheat, soy, peanut, tree nuts, egg, milk, fish and crustaceans, are the main cause of over 90% of all food allergies. Shrimp, a crustacean, is widely consumed for its delicious flavour and high nutritional value (Lv et al., 2016). Shrimp meat is also used as a food additive or supplement in a number of food products. However, shrimp is noted as one of main causes of allergic reactions (Long, Yang, Wang, Hu, & Chen, 2015; Lv et al., 2016). Symptoms of allergic reactions to shrimp include urticaria, angioedema, asthma, and life threatening anaphylactic shock. Accidental exposure to shrimp allergens is an important consumer health concern (Kamath, Abdel Rahman, Komoda, & Lopata, 2013). Tropomyosin (TM) is the main allergen protein in shrimp (Jeoung et al., 1997; Leung et al., 1994). Applying an effective method to eliminate or decrease TM allergenicity of shrimp in food processing is crucial for preventing accidental exposure of sensitive individuals to this allergen.

Many food technologies have been tested to reduce TM allergenicity. These include enzyme hydrolysis (Shimakura, Tonomura, Hamada, Nagashima, & Shiomi, 2005), irradiation (Kume & Matsuda, 1995), heat treatment (Kamath et al., 2013), sonication (Li, Lin, Cao, & Jameel, 2006), high pressure processing (Long et al., 2015), and malondialdehyde treatment (Song et al., 2015). Although these methods effectively decrease TM allergenicity, only a few methods can be extensively applied in the food industry.

Nowadays, non-thermal food processing techniques, including irradiation, are regarded with special interest (Li, Zhu, Zhou, & Peng, 2012) and are being increasingly used worldwide as proven and effective methods of food preservation, and for improving food safety and quality (Loaharanu & Thomas, 2001). One such method, electron beam (EB) irradiation, also known as an effective cold sterilization method, is widely used in some industrial food processing (Deng et al., 2017). Kume and Matsuda (1995) found that radiation causes denaturation, aggregation, hydrophobicity change, structural modification, and decrease in antigenicity of ovalbumin and bovine serum albumin. Irradiation may reduce allergenic capability by altering the allergen conformation. Compared to gamma irradiation which reduces IgE binding of shrimp TM at a higher dose (Li, Lin, Cao, & Jamil, 2007), KatialGrier, Hazelhurst, Hershey, and Engler (2002) found that EB irradiation significantly reduced allergenicity at a lower dose (Katial et al., 2002). Li, Lu, Khan, Lin, and Zhang (2014) reported that 10 kGy EB irradiation significantly reduced specific IgE binding to fish parvalbumin (Li et al., 2014). However, few studies have ascertained the effect of EB irradiation on immunoreactivity of shrimp TM. Furthermore, little information is available on the underlying mechanisms involved in decreasing the immunoreactivity in response to EB irradiation (Li et al., 2014). The aim of this study was to assess the changes in TM structure and immunoreactivity in response to EB irradiation and to determine the optimal dose of EB irradiation for reducing immunoreactivity of TM. For this, western blot and indirect enzyme-linked immunosorbent assay

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(ELISA) were used to assess IgG binding capacity of TM. The structural changes were analyzed by circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy. These data may provide an underlying molecular mechanism of EB irradiation in reducing shrimp allergenicity.

2. Materials and methods

2.1. Materials

Tropomyosin from shrimp (*Solenocera melanthero*) meat and rabbit anti-shrimp TM polyclonal antibodies were prepared and purified according to Liang et al. (2008). Anti-shrimp β -actin was purchased from Huaan Biotech (Hangzhou, China). Horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from Transgen Biotech (Beijing, China). Bovine serum albumin (BSA) and 3,3',5,5'-Tetramethylbenzidine (TMB) were purchased from Sigma Aldrich (St. Louis, MO, USA). Bicinchoninic acid (BCA) protein assay kit, protein marker (11–180 kDa), Coomassie brilliant blue R-250 and prestained protein standards and polyvinylidene fluoride (PVDF) transfer membranes (0.45 μ m) were purchased from Solarbio Science & Technology Co. (Beijing, China). Costar™ 96-well microtiter plates were purchased from Thermo Fisher (Waltham, MA, USA). Unless otherwise indicated, all other reagents were of analytical grade.

2.2. Sample treatment

Fresh shrimp was purchased from a local produce market. Peeled shrimp was vacuum-packed in a polyethylene bag and irradiated at different doses (0, 1, 3, 5, 7, 9 kGy) with an electron linear accelerator at a rate of 1 kGy/s. The absorption dose was determined using a cellulose triacetate dosimeter and experimental verification confirmed that the deviation of absorbed dose was within 3% of targetted dose in all cases. Shrimp meat irradiated with 0 kGy was used as control group.

2.3. Preparation of total protein extracts

Preparation of total protein extract was done as described previously with a minor modification (Shimakura et al., 2005). Briefly, 5 g of minced shrimp meat were added to 20 ml of cold 10 mM phosphate buffer (PBS), pH 7.0, containing 3% NaCl, homogenized twice and centrifuged at 8000g for 20 min. Collected supernatant was the total protein extracts. Protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit, using bovine serum albumin (BSA) as standard.

2.4. SDS-PAGE analysis

Total protein extracts (10 μ g protein/well) were run on SDS-PAGE gel (15%, w/v). Gels were stained with Coomassie brilliant blue R-250 and recorded with a GelDoc XR + Gel Documentation System (Bio-Rad, USA), using Image Lab software.

2.5. IgG binding capacity of TM

2.5.1. Western blot analysis

SDS-PAGE and western blot were done to determine the levels of TM as previously described with some modifications (Warner, Kauffman, & Greaser, 1997). β -Actin, with the molecular weight of 42 kDa, was used as standard reference for western blot. 10 μ g of protein samples were run on a SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in TBST buffer (20 mM Tris-HCl, 137 mM NaCl, pH 7.6, 0.05% Tween-20) containing 5% w/v fat-free dry milk for 1 h at 25 °C and incubated with rabbit anti-shrimp β -actin (1:2500 diluted with TBST solution containing 0.5% fat-free dry milk) or rabbit anti-shrimp TM polyclonal antibodies (1:10000 diluted with TBST solution

containing 0.5% fat-free dry milk) at 25 °C for 10 h. After rinsing with TBST 3 times (10 min/time), the membranes were incubated with goat anti-rabbit HRP-conjugated IgG secondary antibodies (1:5000 diluted with TBST solution containing 0.5% fat-free dry milk) for 40 min at 25 °C. Lastly, the membranes were rinsed extensively with TBST and the signal was detected with ECL Plus western blotting detection kit (Amersham Biosciences, UK), using a gel rapid documentation apparatus (Tanon-5200, Tanon Science & Technology Co., Shang-hai, China) with multiple exposure times to achieve an optimal signal. β -Actin was used as a loading control. Blots were quantified using Quantity One Software (Bio-Rad Laboratories, PA), and the IgG binding capacity of TM was calculated as the integrated density (intden) value ratio of TM to β -actin.

2.5.2. Indirect ELISA analysis

IgG binding capability of TM in total protein extracts was assessed by indirect ELISA using rabbit polyclonal antibodies (IgG) according to the protocol by Kamath et al. (2013) with minor modifications (Kamath et al., 2013). 50 μ l of total protein extracts (100 μ g/ml) in 50 mM sodium carbonate buffer (pH 9.6) were coated onto a 96-well microtiter plate and incubated overnight at 4 °C. After rinsing, the plate was blocked with 60 μ l of 1% bovine serum albumin (BSA) in TBST at 37 °C for 1 h. The plate was rinsed and incubated with 50 μ l of diluted rabbit antibodies (1:2500) at 37 °C for 1 h. The plate was washed with 180 ml of TBST twice and 50 μ l of HRP-conjugated goat anti-rabbit IgG secondary antibody (1:10000 dilution) were added to each well and incubated for 45 min at 37 °C. After washing with 180 μ l of TBST three times, 100 μ l of TMB were added to the plates and incubated at 37 °C for 5 min. Finally, 90 μ l 2 M sulfuric acid was added to terminate the reaction. Optical density was measured at 450 nm, using a Spectra Max i 3 multi-functional microplate reader (Molecular Devices Co., USA).

2.6. Structure analysis

2.6.1. Purification of tropomyosin

Tropomyosin was purified from shrimp meat treated with EB (0, 1, 3, 5, 7, 9 kGy) as described by Liang et al. (2008) with some modifications. Unless otherwise stated, all steps were carried out at 0–4 °C. Shrimp meat was minced and homogenized in a 10-fold (v/w) solution of 20 mM Tris-HCl (pH 7.5) containing 50 mM KCl and centrifuged at 4000g for 10 min. The pellet was washed with the same solution four times, followed by treatment with acetone three times and was dried overnight. The dried pellet was extracted overnight with a 15-fold (v/w) solution of 5 mM dithiothreitol (DTT) containing 1 M KCl. The mixture was then centrifuged at 20000g for 20 min and supernatant was precipitated with 1 N HCl (pH 4.6). After centrifugation at 20000g for 20 min, the pellet was dissolved in 0.01 M PBS (pH 7.5) and subjected to ammonium sulfate fractionation (from 30% to 40%). The final fraction was boiled for 10 min and then cooled in ice water. Slight precipitation of denatured protein was spun down. TM fraction in the solution was dialyzed against 10 mM PBS buffer, followed by freeze drying at –40 °C for 2 days.

2.6.2. CD analysis

Secondary structure of purified TM (1 mg/ml) was determined by a CD spectrophotometer (JASCO J-1000, Tokyo, Japan) at room temperature. Constant N₂ flux was employed and a jacketed cell (0.2 mm optical path length) was used. Wavelength for measurement was set from 260 to 190 nm and scan speed and bandwidth were set to 50 nm/min and 1.0 nm, respectively. The results were displayed as millidegrees (mdeg) and the content of α -helix, β -sheet, β -turn and random coil were estimated according to Yang, Wu, and Martinez (1986).

2.6.3. FTIR analysis

Freeze-dried TM purified from shrimp meat was mixed with potassium bromide (KBr) at the ratio of 1:100. The FTIR spectra of TM was

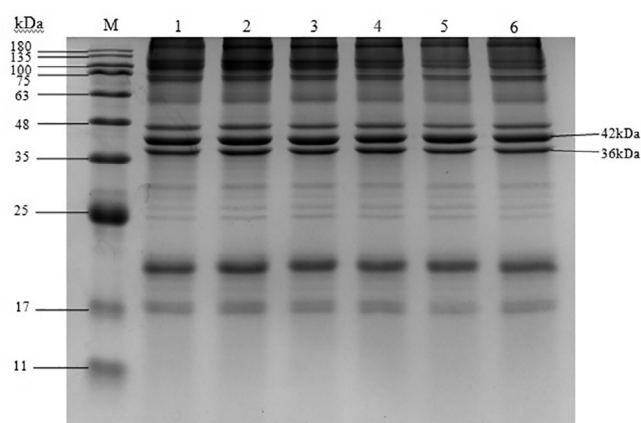


Fig. 1. SDS-PAGE profile of total protein extracts from shrimp meat. Lane M, protein marker; lane 1, 0 kGy; lane 2, 1 kGy; lane 3, 3 kGy; lane 4, 5 kGy; lane 5, 7 kGy; lane 6, 9 kGy.

detected using an infrared spectrophotometer (JASCO, Tokyo, Japan) from 4000 to 400 cm^{-1} at a data acquisition rate of 4 cm^{-1} per point.

2.7. Statistical analysis

Data are represented as mean values of three independent experiments ($n = 3$). One-way analysis of variance (ANOVA) was carried out to analyze significant differences in the data, using SPSS 19.0 and Duncan's tests. A significant difference was determined at the 0.05 probability level.

3. Results

3.1. SDS-PAGE analysis

The total protein extracts showed complex bands on SDS-PAGE (Fig. 1). The molecular weight (MW) of shrimp TM has been reported to be 36 kDa (Haddad, Kalra, & Verma, 1979; Song et al., 2015). The number and position of total protein bands did not change at different irradiation doses, which indicated that no new protein band was visualized and irradiation had no effect on the molecular weight of TM from shrimp meat. However, low levels of proteins cannot be detected by Coomassie, so it is not the ideal method for measuring changes of TM amount.

3.2. Western blot and indirect ELISA analysis

The changes in TM levels by western blot analysis are shown in Fig. 2. After quantification of the band, the intensity of TM bands in the 0, 1, 3, 5, 7, 9 kGy groups were 3.76×10^5 , 2.08×10^5 , 3.16×10^5 , 2.72×10^5 , 1.94×10^5 , 2.67×10^5 respectively, and their intensities of the β -actin band were, respectively, 1.25×10^4 , 4.58×10^4 , 10.96×10^4 , 5.48×10^4 , 8.43×10^4 , 9.01×10^4 . The corresponding intensity ratios of TM to β -actin were 30.08, 4.54, 2.89, 4.97, 2.30 and 2.96, respectively, which showed that intensity of IgG binding detected by western blot decreases dramatically after EB irradiation. Compared to irradiation doses of 1 and 5 kGy, there was a higher reduction in IgG

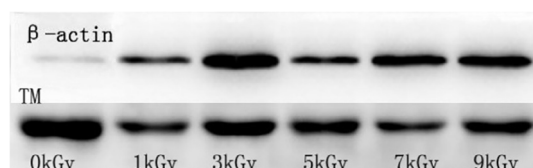


Fig. 2. Changes in TM from shrimp meat by western blot.

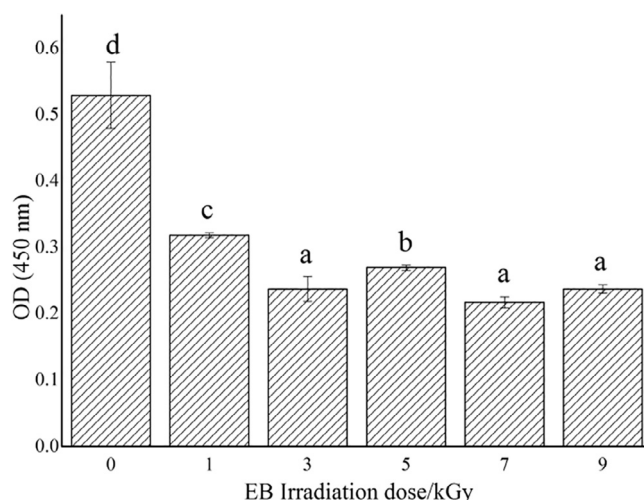


Fig. 3. Indirect ELISA analysis of TM from shrimp meat.

binding capacity after 3, 7, and 9 kGy EB irradiation. When compared to the control group, specific IgG binding capacity, detected using western blot, was reduced by 92% at 7 kGy.

Next, indirect-ELISA was carried out to quantitatively measure the binding capability of TM to TM rabbit sera (Fig. 3).

Results showed that the IgG binding capacity of TM detected by indirect ELISA has the same transition trends as that detected by western blot following different EB irradiation. In indirect ELISA, compared to the control group, the IgG binding capacity of TM to TM rabbit sera decreased by 55% and 59% at 3 kGy and 7 kGy, respectively, suggesting 7 kGy to be the optimal dose for decreasing the immunoreactivity of shrimp TM.

3.3. Secondary structural analysis of TM

The conformational changes in TM from shrimp meat irradiated with EB were detected by far-ultraviolet CD. As shown in Fig. 4, TM exhibited the characteristic α -helical structure peaks, with one positive peak near 192 nm and two negative peaks near 208 nm and 220 nm, which is consistent with a previous report (Sreerama & Woody, 2003). After EB irradiation, the molar residue ellipticity of negative peaks near 208 nm and 220 nm decreased in response to 1 kGy and 5 kGy, but increased at 3, 7 and 9 kGy. Compared with the 9 kGy group, the molar residue ellipticity of negative peaks increased more significantly at 7 kGy. Another strong positive peak was visualized near 197 nm, which

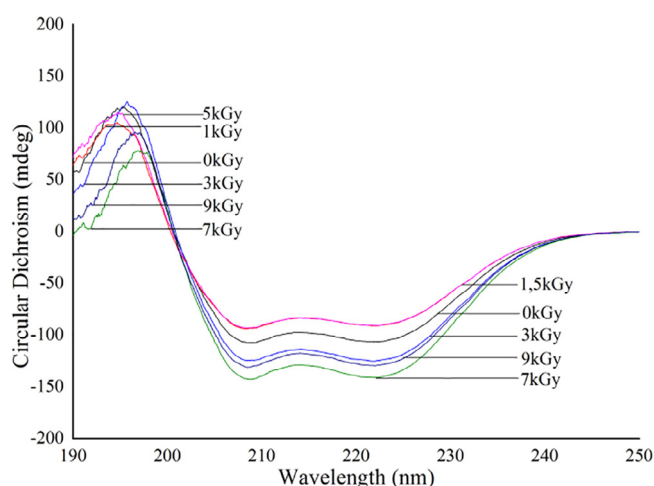


Fig. 4. The CD spectrum of TM from shrimp meat.

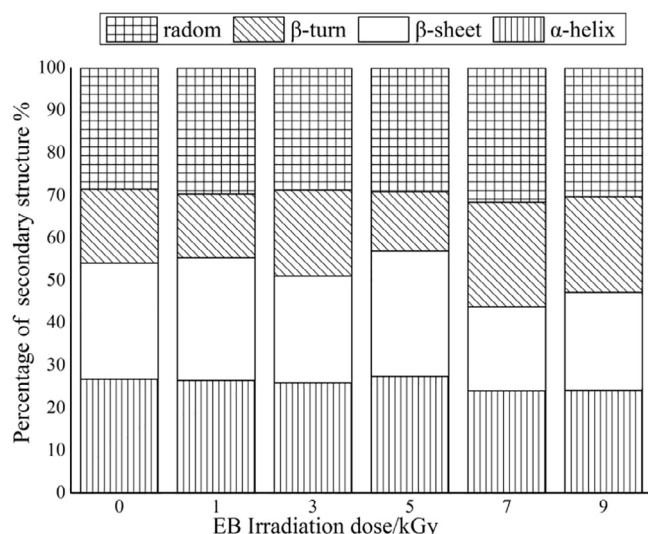


Fig. 5. The secondary structure percentage of TM from shrimp meat.

demonstrated the presence of β -sheet (Han et al., 2015). The intensity of the peak near 197 nm decreased at 7 kGy and 9 kGy and was more significant at 7 kGy.

The relative contents of α -helix, β -sheet, β -turn, and random coil of TM were estimated by JASCO secondary structure analysis software (Fig. 5). Exposure to 3, 7, and 9 kGy doses of EB irradiation caused a decrease in α -helix and β -sheet structures but increase in β -turn structure. Compared to the control, the content of random coil remained nearly unchanged, except at 7 kGy. The maximum change in relative proportion of TM structure occurred at the dose of 7 kGy where the percentages of α -helix and β -sheet decreased to 24.0% and 19.8%, respectively, when compared to the control group (26.7% α -helix and 27.4% β -sheet). In contrast, the contents of β -turn and random coil increased from 17.4%, 28.5% in the control group to 24.6%, 31.6% in the 7 kGy group. In addition, compared to 7 kGy EB irradiation, 9 kGy EB irradiation caused a more dramatic increase in β -sheet and decrease in β -turn and random coil. Furthermore, FTIR spectroscopy was employed to systematically monitor the structural changes in TM from shrimp meat treated with different doses of EB irradiation. The results are shown in Fig. 6.

FTIR spectra detected two characteristic bands at about 3300 and 2961 cm^{-1} , which corresponded to amide A and amide B regions arising from N–H and C–N stretching vibrations, respectively (Han et al., 2015). With the exception of the 5 kGy dose, EB irradiation caused movement of the amide A absorption peak of TM to a higher wave number, indicating that the stretching vibration of N–H was strengthened. That the amide A band of TM split into three bands is response to 1 kGy and 9 kGy EB irradiation as a result of a vibration coupling phenomenon. There was no significant change in the amide B band. Amides I, II and III are usually used to analyze structural transformation of protein in FTIR spectra. The amide I region results from C=O stretching vibration ranging between 1700 and 1600 cm^{-1} , the amide II region results from N–H bending vibration ranging between 1600 and 1500 cm^{-1} , and the amide III region results from C–N and N–H stretching vibration ranging between 1360 and 1200 cm^{-1} (Hosseini, Rezaei, Zandi, & Farahmandghavi, 2015). Absorption peaks of amides I, II and III of TM in the control group were found near 1655, 1540 and 1250 cm^{-1} , respectively (Fig. 6). After different doses of EB irradiation, the absorption peaks of amides I and III moved to higher wavenumbers which changed α -helix and β -sheet structures by uncoupling or interrupting the intramolecular bonding. Absorption peaks of amide I and amide III increased most significantly at 3 and 7 kGy. Wavenumbers of 600–1300 cm^{-1} are called the fingerprint region of protein. Most of the peaks in the fingerprint region disappeared at

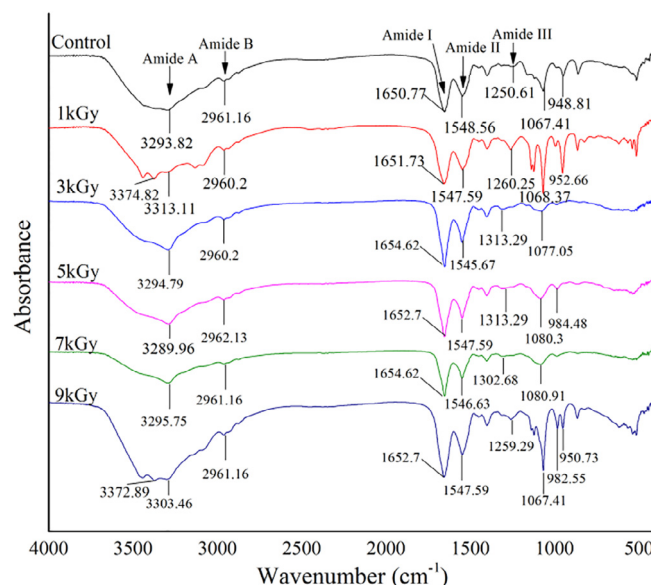


Fig. 6. FTIR spectra of TM from shrimp meat. Numbers indicate the positions of main peaks.

3 kGy and 7 kGy (Fig. 6). Moreover, after the 7 kGy EB irradiation treatment, the intensity of peaks decreased dramatically and the shifts of characteristic peaks were more significant when compared to other groups. However, in addition to the movements of TM amides I and III to lower wavenumbers, TM after 9 kGy EB irradiation showed more absorption peaks in FTIR than after 7 kGy EB irradiation.

4. Discussion

In this study, EB irradiation was found to cause conformational changes in TM and reduced its immunoreactivity. Different doses of EB irradiation showed varied influences on the structural changes, thus resulting in different degrees of change in immunoreactivity. This is in agreement with the findings of Li et al. (2014) who assessed the effect of γ -irradiation doses on the allergenicity of shrimp and found that irradiated shrimp muscle exhibited increased allergenicity in response to irradiation dose below 5 kGy while there was a decrease in allergenicity when the dose exceeded 10 kGy. Thus irradiation decreases the allergenicity of shrimp only at certain doses. In this study, the IgG binding capacity of TM decreased dramatically after EB irradiation treatment, especially at doses of 3 kGy and 7 kGy, and a similar effect should be seen in the allergenicity of TM. In comparison to a study by Song et al. (2015) who found that, after treatment with 100 mM malondialdehyde, the IgG binding capacity of shrimp tropomyosin decreased significantly to 51.1%, this study showed that IgG binding capacity of TM to TM rabbit sera decreased by 55% and 59% after 3 kGy and 7 kGy EB irradiation treatments respectively. The results suggested that EB irradiation is a safer and more effective method to reduce immunoreactivity of TM.

Irradiation had no effect on either the number of protein bands or the molecular weight of TM. This is consistent with a previous report by Yang et al. (2014) who found that the number of protein bands did not change in the irradiated sample extracts of salmon fish (Yang et al., 2014). Following 3, 7 and 9 kGy EB irradiation doses, a structural transformation from α -helix and β -sheet to β -turns and random coil occurred, especially at 7 kGy. According to the results of a study by Whitmore and Wallace (2008), an increase in disorder of protein may contribute to an increase in the intensity of peaks in the range of 200–220 nm that indicates increase in random coil content (Sreerama & Woody, 2003). Quantitative estimation of relative contents of secondary structure components produced similar results in our study. In

addition, 3, 7 and 9 kGy EB irradiation treatments reduced immunoreactivity of TM to similar extents. However, an increase in IgG binding capacity of TM at 5 kGy was observed and this may be attributed to the exposure of allergen epitopes arising from conformational changes of TM (Li et al., 2007). When the irradiation dose was increased to 7 kGy, more radicals may have been generated which disrupted allergen epitopes and further destroyed the structure into a more disordered state, observed by the CD and FTIR, resulting in the significant decrease in immunoreactivity. However, exposure to 9 kGy led to the formation of new chemical bonds which may account for increased immunoreactivity compared to 7 kGy EB irradiation. The relative contents of secondary structure components (α -helix, β -sheet, β -turn, and random coil) in TM in the control group were 26.70%, 27.40%, 17.40%, and 28.50%, respectively. These data differ from the previous CD research (Song et al., 2015) which showed that TM from *Metapenaeus* contained 61.30% α -helix, 4.90% β -sheet, 12.90% β -turn and 12.90% random coil structures. These differences may be due to different extraction methods, concentration, and source of TM or the test temperature (Huang & Ochiai, 2005).

In FTIR spectra, the left shift of the amide A band indicated that the hydrophilicity of TM was reduced (Hosseini et al., 2015). Theoretically, the amide A band is the most sensitive vibration arising from changes in hydrogen bonds. However, the C–O vibration has attracted more attention since the amide A band is too broad (Manning, 2005). Shifts of C–O and N–H vibrations of protein amide bonds can be used to monitor the formation of hydrogen bonds which is the most important force maintaining the protein secondary structure (Secundo & Guerrieri, 2005). According to FTIR spectra, hydrogen bonds of TM were severely damaged after 3 and 7 kGy EB irradiation. Amide III is associated with transformation of α -helical to random coil (Le, Maki, Takahashi, Okazaki, & Osako, 2015). The peak of amide III moved to a higher region. This demonstrated that the structure of TM from irradiated shrimp meat became more disordered, which was consistent with the data of CD. The shift in characteristic peaks resulted in conformational changes of TM. After 3 and 7 kGy EB irradiation, the breaking of hydrogen bonds brought about a more extended structure of protein, exposing hydrophobic residues, and increased hydrophobic interactions, especially at 7 kGy. This is consistent with the results of Han et al. (2015) who demonstrated similar changes in protein Z structure (Han et al., 2015). Overall, changes in TM structure suggested that immunoreactivity of TM was related to its structural changes and different doses of EB irradiation caused different changes in the protein structure.

5. Conclusions

EB irradiation caused changes in conformation and immunoreactivity of TM from *Solenocera melanthero*. The optimal effect was observed at the dose of 7 kGy. These results provide evidence for the importance of structural changes in decreasing the immunoreactivity of TM. This study also provides a basis for further exploring the possible mechanism of decreasing TM allergenicity by EB irradiation, which has great industrial potential for decreasing allergenicity of food protein.

Acknowledgments

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