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Effect of 4-hydroxy-2-nonenal treatment on the IgE binding capacity and structure of shrimp (*Metapenaeus ensis*) tropomyosin

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Abstract

Lipid peroxidation can react with free amines of proteins and induce modification of structural and functional properties. This study presents the IgE binding capacity and structural changes of shrimp tropomyosin (TM) under oxidative stress with 4-hydroxy-2-nonenal (HNE). IgE binding capacity was evaluated with the dot-blot assay and inhibition enzyme-linked immunosorbent assay. A decrease in IgE binding capacity of TM was found with 0.01 mM HNE treatment, which was more significant when the HNE concentration was increased to 0.5 mM. The conformational changes of TM, as characterized by fluorescence spectroscopy, circular dichroism spectroscopy and ultraviolet absorption spectroscopy, correlated well with IgE binding capacity changes. Further LC-ESI-MS/MS analyses showed that the side-chain groups of alanine, leucine, lysine and histidine had been modified by HNE. These results suggested that the HNE-induced conformational changes of TM significantly influenced its allergenicity and that these changes were caused by the modification of specific amino acids residues.

Keywords: Allergenicity; protein oxidation; 4-hydroxy-2-nonenal; LC-ESI-MS-MS

Chemical compounds studied in the article

Acrylamide (PubChem CID:6579); 1-anilino-8-naphthalene-sulfonate (PubChem

CID:1369); 3, 3', 5,5'-tetramethylbenzidine (PubChem CID: 19083738); Brilliant

Blue R (PubChem CID: 6333920); tween 20 (PubChem CID:443314)

1. Introduction

Shrimp, a member of the Crustacea family, is a widely consumed source of dietary protein in China, Japan and North America owing to its delicious flavour and high nutritional value. However, an increased consumption of shrimp can result in a higher frequency of hypersensitivity reactions (Lee, Gerez, Shek, & Lee, 2012), with tropomyosin (or Pen a 1) being the major allergen involved (Leung, P. S., Chu, K. H., Chow, Ansari, Bandea, Kwan & Gershwin, 1994). More than 90% of the adverse reactions to allergens are elicited by immunoglobulin E (IgE) and present as mucocutaneous (95.7%), respiratory (23.9%), gastrointestinal (16.3%), anaphylactic (11.9%), and cardiovascular symptoms (3.3%) (Takács et al, 2014).

Oxidation modification, a reaction that occurs during food processing and preservation, has been identified as a causal factor of food allergenicity. Cooke and Sampson (1997) reported a 69% reduction in IgG binding capacity to oxidized ovomucoid compared with native ovomucoid, and T cell proliferation *in vitro* was significantly greater in response to the oxidized protein. However, allergens formed by oxidation reactions during food processing and preservation have been neglected over the past decades. In 2014, our research team reported that malondialdehyde (MDA) could reduce the allergenicity of shrimp tropomyosin through structural modification, but information about the detailed mechanisms involved remains limited (Song, Li, Lin, Du, Hao, Lin, & Zhu, 2015; Lv, Lin, Li, Song, Lin & Wang, 2014). With the increasing knowledge that food allergens are both

sources and targets for reactive oxygen species and other peroxides, allergen oxidation modification is currently one of the most important research topics within the field of food allergens (Li, Lu, Khan, Lin, & Zhang, 2014).

Among the peroxides that can lead to protein oxidation in biological systems, lipid-derived radicals and hydroperoxides have attracted special attention for several years, especially in the seafood industry (Haraguchi, Silva, Neves, Dos Santos, & Pedrosa, 2011). Furthermore, the mechanism of how lipid oxidation promotes protein oxidation has been the focus of much research (Mercier, Gatellier, Viau, Remignon, & Renerre, 1998; Xiong, Park, & Ooizumi, 2008; Zhou, Zhao, Su, Cui, & Sun, 2014).

Lipid oxidation is a major cause of quality deterioration during food processing and storage (Fenaille, Guy & Tabet, 2003), which form a series of active secondary products, such as MDA, HNE and so on. These secondary products contain reactive aldehyde which can crosslink with amino acid residues and induce changes of protein structure and functional properties including allergenic characteristics. Among these secondary products, HNE is one of the major components and exist extensively in food system (Uchida, 2003; Del Rio, Stewart & Pellegrini, 2005). In order to illustrate the property changes of food allergens, it is necessary to understand the interaction among allergens and the representative secondary product - HNE.

4-Hydroxy-2-nonenal (HNE) is generated through the β -cleavage of hydroperoxides derived from n-6 polyunsaturated fatty acids, under shrimp processing conditions (Esterbauer, Schaur, & Zollner, 1991). Recent studies have shown that HNE can bind to and modify the structure of proteins (Naveena, Faustman, Tatiyaborworntham, Yin,

Ramanathan, & Mancini, 2010), resulting in functional property changes in processed seafoods (Lee, Joo, Alderton, Hill, & Faustman, 2003). As a monocarbonyl compound, HNE can bind oxidatively with the side chains of certain amino acids, resulting in conformational changes of the protein. Moreover, the strong electrophilic nature of the C-3 carbon makes HNE highly reactive with cellular nucleophiles, of which the histidine residue in proteins has been the commonly reported target (Alderton, Faustman, Liebler, & Hill, 2003; Faustman, Liebler, McClure, & Sun, 1999; Suman, Faustman, Stamer, & Liebler, 2007; Uchida, & Stadtman, 1992). It is reasonable to hypothesize that the structural transformation of proteins caused by these oxidative modifications might be the direct reason for their allergenicity.

The objective of this study was to test the hypothesis that the modification of shrimp tropomyosin, using different concentrations of HNE, could affect its allergenicity. The exact structural modification was further studied to better understand the structure–allergenicity relationship. Moreover, the site of oxidation modification was elucidated to explain the mechanism involved.

2. Materials and methods

2.1. Materials and reagents

Shrimp tropomyosin was prepared in the Food Safety Laboratory, Ocean University of China (Shandong, China). HNE was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). Bovine serum albumin (BSA), 1-anilino-8-naphthalene-sulfonate (ANS), and 3, 3', 5, 5'-tetramethylbenzidine (TMB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The precision protein

standard was purchased from Solarbio Co. (Beijing, China). The enhanced chemiluminescent (ECL) kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Secondary antibodies coupled with horseradish peroxidase (HRP) were purchased from Zhongshan Jinqiao Co. (Beijing, China). Reagents used for gel electrophoresis were obtained from Bio-Rad (Hercules, CA, USA). Unless otherwise stated, all chemicals were of analytical grade.

2.2. Patient sera

Human sera were collected from patients with a documented clinical history of shrimp allergy, and numbered as P1, P2, P3, and P4. A positive skin prick test and specific IgE levels above 5 kU/l, as determined by Allergy Screen (MEDIWISS Analytic GmbH, Moers, Germany), were used to verify the patient's allergy to shrimp. Sera stored at -80°C were provided by the Affiliated Hospital of Medical College of Qingdao University (Qingdao, China). Before sera collection, all the patients signed written consents to ensure that they were aware of the usage of their sera. The sera collection protocol was approved by the medical ethics committee (File No. QD-PF-20150814316).

2.3. Reaction of tropomyosin with HNE

Shrimp tropomyosin samples were diluted to 4 mg/ml in 10 mM phosphate buffer (pH 7.4). Different concentrations of HNE in the same buffer were mixed with the prepared samples at a 1:1 ratio and incubated at 37°C for 10 h. Based on previous research work on protein modification with HNE, the concentration of HNE arranged from micromolar per litre to millimolar per litre (Fenaille, Guy & Tabet, 2003; Yang,

Tallman, Porter, & Liebler, 2015). In this work, the final concentration of HNE was set at 0 (control), 0.01, 0.1, 0.5, and 1 mmol/l, respectively. After incubation, the temperature of the protein solution was adjusted to 0–4 °C by ice bath. Finally, the resulting solution was dialyzed against PBS (pH 7.4) for 12 h at 4 °C, and then lyophilized and stored at –40°C until use.

2.4. SDS-PAGE analysis

SDS-PAGE was performed using the method described by Laemmli (1970). Samples (8 µg/well) were mixed in a 4:1 ratio with bromophenol blue buffer and heated at 100 °C for 7 min. The acrylamide gel was prepared as a 5% stacking gel and a 12% resolving gel. Electrophoresis was performed at 80 V for the stacking gel and 120 V for the resolving gel. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Richmond, CA, USA) and destained until the solution was clear. Images were captured and analyzed using the Tanon-4200SF gel imaging and analysis software (Tanon Science & Technology Co., Ltd., Shanghai, China).

2.5. Effect of HNE-induced modifications on the antigenicity and IgE binding capacity of tropomyosin

2.5.1. Dot-blot assay

The dot-blot analysis was performed according to the method of Zheng and co-workers (2011), using IgE in the serum samples of 4 individual patients with shrimp allergies. In brief, 3 µl of phosphate-buffered saline (PBS, pH 7.0) containing the samples of control and HNE-induced tropomyosin (0, 0.01, 0.1, 0.5, and 1 mmol/l HNE) at a concentration of 0.5 mg/ml were spotted on nitrocellulose membranes

(Millipore Corporation, Bedford, MA, USA) and incubated for 2 h at 37 °C in blocking buffer (PBST (phosphate-buffered saline containing 0.1% Tween-20) containing 5% skim milk) to block the nonspecific binding sites. Then the membranes were incubated with human sera (1:10 dilution with blocking buffer, v/v) for 1.5 h at 37 °C. After incubation with HRP-labeled goat anti-human IgE (1:500 dilution with blocking buffer, v/v) for 1 h at 37 °C, IgE binding was detected using the ECL reagent, with multiple exposure times to achieve an optimal signal on the Tanon-4200SF gel imaging system (Tanon Science & Technology Co.). The intensity of the spot indicates the amount of specific IgE bound to tropomyosin.

2.5.2. Competitive inhibition ELISA analysis

The IgE binding activity of the samples was further assessed by competitive inhibition ELISA, as described by Shin and co-workers (2013), with some modifications. We performed inhibition ELISA with the pooled sera of the 4 shrimp-allergic patients. The purified tropomyosin was used as the plate-coating antigen. Samples of native (untreated) and HNE-treated tropomyosin were dissolved in deionized water (1 mg/ml final conc.). Then 100 μ l of carbonate coating buffer (0.1 M, pH 9.1), containing the purified tropomyosin at a concentration of 50 μ g/ml, was introduced into each well of a multiwell plate (Nunc-Maxisorp NO FDF). The plate was covered with plate-sealing tape and incubated overnight at 4 °C. The coated plates were washed with PBST and each well as then blocked with 200 μ l of 5% skimmed milk at 37 °C for 2 h. After being washed and blocked, 50 μ l of pooled sera from the 4 shrimp-allergic patients (diluted 1:20 with 1% skim milk in PBST) was

mixed with the same volume of the sample (native and HNE-treated tropomyosin, in the concentration range of 0.001–1000 $\mu\text{g/ml}$) in another plate. After incubation at 37 °C for 1.5 h, 50 μl of the mixture was transferred to the ELISA plate precoated with the purified tropomyosin and incubated for 1.5 h at 37 °C. As described above, after incubation with HRP-labeled goat anti-human IgE (diluted 1:500 with 1% skim milk in PBST), TMB (100 μl) was then added to each well and incubated at 37 °C for 20 min. After terminating the reaction by the addition of 50 μl of 2 M sulfuric acid, the absorbance was measured at 450 nm with an automated ELISA plate reader (Multiscan MK3; Thermo Fisher Scientific Inc.).

2.6. Structure analysis

2.6.1. Measurement of surface hydrophobicity

The surface hydrophobicity of native and HNE-treated tropomyosin was determined using ANS, as described by Kato and Nakai (1980). Briefly, 20 μl of ANS (5.0 mM) was added to 2 ml of each protein sample (diluted to 0.005–0.5 mg/ml) and allowed to react for 3 min. The resulting relative fluorescence intensity of the solutions was measured with a fluorescence spectrophotometer (F-4600; Hitachi, Japan) with an excitation wavelength of 365 nm and emission wavelength of 484 nm. Each experiment was performed at least 3 times to ensure reproducible results. Surface hydrophobicity was determined as the initial slope of the curve plot of fluorescent intensity versus protein concentration (where the protein concentration is zero).

2.6.2. Intrinsic fluorescence measurement

The intrinsic fluorescence of native and HNE-modified shrimp tropomyosin was measured with an RF-5301PC fluorescence spectrometer (Shimadzu, Kyoto, Japan), as described by Wu and co-workers (2010). The data were obtained at the excitation wavelength of 280 nm (slit width, 3 nm), emission wavelength of 300–380 nm (slit width, 3 nm), and 8 nm/s of scanning speed. Phosphate buffer (0.01 mol/l, pH 7.0) was used as the blank solution for all of the samples. Each experiment was performed at least 3 times to ensure reproductive results.

2.6.3. UV absorption spectral analysis

UV absorption spectral analysis was carried out with the UV-1101 system from Techcomp (Shanghai, China). The UV absorption spectra were scanned from 230 to 330 nm, with each of the treated tropomyosin samples at a concentration of 0.5 mg/ml. Each experiment was performed at least 3 times to ensure reproductive results.

2.6.4. Densitometry scanning calorimetry analysis

Changes in the thermal properties of tropomyosin following oxidative modification were determined using a TA Instruments DSC 200 PC for densitometry scanning calorimetry measurements (Jansson, & Swenson, 2010). In brief, samples (~15 mg) were heated from 40 to 150 °C at a heating rate of 8 °C/min and then cooled at the same rate. A sealed empty pan was used as a control. Netzsch Proteus analysis software was used to analyze the inflection point of the glass transition and the temperatures for the onset and end of the glass transition. The values reported are the mean of at least 3 measurements to ensure reproducibility of the results.

2.6.5. Secondary structure analysis using circular dichroism spectroscopy

The conformational changes of tropomyosin after oxidative modification were defined as changes in its secondary structure. Circular dichroism spectroscopy was performed in the spectral range of 190–260 nm by using a JASCO J-815CD spectropolarimeter (Japan Spectroscopic Co. Inc., Japan) at room temperature (Kelly, Jess, & Price, 2005). A scan speed of 100 nm/min, a response time of 1–3 s, and a bandwidth of 1 nm were used. A protein concentration of 1 mg/ml was used for the analysis. The results were averaged with the molar residue ellipticity and expressed as $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$. The various secondary structures were calculated using the JASCO software. All samples were measured in triplicate.

2.7. Oxidation site analysis using LC–MS/MS

LC–MS/MS was performed using the method described by Lv (2014). Analysis of the peptide samples was performed using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray ionization source. High-performance liquid chromatography (HPLC) was performed to separate the peptide samples, using an Eksigent Nanoflex cHiPLC system (Eksigent, Dublin, CA, USA). The peptide samples were separated using a reversed-phase cHiPLC column ($75\ \mu\text{m} \times 150\ \text{mm}$) at a flow rate of 300 nl/min. Mobile phase A was deionized water containing 0.1% (v/v) formic acid and mobile phase B was acetonitrile with 0.1% (v/v) formic acid. A 40-min gradient condition was applied, where mobile phase B was increased linearly from 3% to 40% within 30 min and further to 80% for 5 min, and then finally returned to 3% for re-equilibration. All the peptides eluted from the cHiPLC column at a particular elution time were analyzed by mass spectrometry at a

resolution of 60,000.

2.8. Statistical analysis

One-way analysis of variance was carried out to analyze significant differences in the data (using SPSS for Windows, version 15.0; Chicago, IL, USA). Only a significant difference was considered, unless stated otherwise.

3. Results and discussion

3.1. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze the shrimp tropomyosin protein after treatment with various concentrations of HNE (Figure 1). Using the band for the untreated protein aligned with the 36 kDa marker (Figure 1, lane 0), and based on a previous report (Leung, P. S., Chu, K. H., Chow, Ansari, Bandea, Kwan & Gershwin, 1994), it was confirmed that this dominant band was tropomyosin. Overall, the tropomyosin bands from the different HNE treatments were nearly identical, and there were no other bands associated with the treatment. In our previous study, MDA cross-linked with tropomyosin, resulted in the formation of several new bands with molecular masses greater than the native tropomyosin protein (Lv, Lin, Li, Song, Lin & Wang, 2014). The possible reason for the differences observed is that MDA possesses two aldehyde groups which can bind with two or more proteins, whereas HNE possesses only one aldehyde group which can bind with one protein. HNE cannot make the protein cross-link.

3.2. Effect of HNE treatment on the IgE binding capacity of shrimp tropomyosin

To determine whether HNE treatment can affect the IgE binding capacity of tropomyosin, the treated protein was tested by the dot-blot assay and inhibition enzyme-linked immunosorbent assay (ELISA), using the sera from 4 patients with shrimp allergy. Overall, the dot-blot results showed an increased fading of colour intensity with increasing HNE concentrations (Figure 2A), which means the IgE binding capacity decreased. However, in the case of patient 3 (P3), the 0.01 mM HNE-treated protein showed increased binding with serum IgE, whereas the binding trend was the same as the other 3 patient sera at higher HNE concentrations. This phenomenon is not unusual, Tong and co-workers (2012) also reported varying patterns of IgE binding to heated ovotransferrin in different patient sera. The possible reason is that IgE binding epitopes present at the protein surface had been modified in such a way as to create stronger binding with the patient's sera.

By applying inhibition ELISA, we confirmed the IgE binding activity of the HNE-treated tropomyosin, using pooled sera from patients allergic to shrimp. As shown in Figure 2B, the IgE binding capacity was reduced significantly when tropomyosin was treated with 0.5 and 1 mM HNE, whereas the decrease was not significant at 0.01 and 0.1 mM. Compared with the control, the IC_{50} value increased from 0.5 $\mu\text{g/ml}$ to 0.7, 0.8, 1.3 and 1.5 $\mu\text{g/ml}$ at the respective HNE concentrations of 0.01, 0.1, 0.5 and 1 mM. This indicates that the treatment had more effect on the IgE-binding activity of tropomyosin when the HNE concentration increased. And more amino acid side chains on tropomyosin were modified and epitopes likely destroyed, thus causing the protein's loss of IgE binding ability.

3.3. Effect of HNE treatment on the structure of shrimp tropomyosin

The surface hydrophobicity of proteins is one of the structural characteristics used to evaluate their conformational changes. As shown in Figure 3A, the surface hydrophobicity of tropomyosin decreased steadily ($P < 0.05$) with an increase of HNE from 0 to 1.0 mM. A previous work, that tested the effects of antioxidants on processing of soy protein isolates, reported an increase in protein oxidation accompanied by a decrease in surface hydrophobicity of the soy proteins (Boatright, & Hettiarachchy, 1995). The decrease in surface hydrophobicity was attributed to the interaction between the lipid peroxidation products and hydrophobic groups of the protein (Liang et al., 1999). The observed surface hydrophobicity could be caused by the balance between aggregation and protein partial unfolding (Dalle - Donne, Aldini, Carini, Colombo, Rossi, & Milzani, 2006). Therefore, the observed decrease in surface hydrophobicity of the HNE-modified shrimp tropomyosin could be attributed to the formation of hydrophilic groups (protein carbonyl groups), in combination with amino acid residues via hydrophobic interactions, and structural modification of the exposed hydrophobic residues of tropomyosin.

Intrinsic fluorescence spectra of native and HNE-treated tropomyosin are shown in Figure 3B. The fluorophores include tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues in proteins. Upon excitation at 280 nm, an emission spectrum with a maximum at 340 nm was observed. The fluorescence of Phe was not stimulated under the 280 nm condition, and the emission spectrum was rarely observed. Moreover, the untreated tropomyosin exhibited a fluorescence emission

spectrum at 340 nm, which is typical for Trp (Lakowicz et al, 2013). The possible reason is that the Trp content plays a decisive role in the intensity of the fluorescence. Incubation of tropomyosin with increasing concentrations of HNE led to a gradual decrease of the maximum Trp fluorescence intensity and a blue shift of the maximum emission wavelength (from 341 to 335 nm). The intrinsic fluorescence intensity of tropomyosin reached a minimum (about 24% of that of the control) under the test conditions, when tropomyosin was incubated with 1 mM HNE for 10 h. The observed decrease of maximum Trp fluorescence intensity could only be attributed to the change in the microenvironment in which the Trp residues were located, due to the binding of HNE to other sites of tropomyosin. King and Li (1999) reported that the decrease in Trp fluorescence was due to the lower exposure of this amino acid to the aqueous environment as a result of protein coagulation and specific modification of the Trp residue. In addition, the blue shifting of the maximum emission wavelength indicated that the previously exposed Trp residues of untreated tropomyosin were buried in the interior of the modified tropomyosin molecule. These changes of Trp fluorescence from the HNE-treated tropomyosin indicated that the treatment had modified the tertiary structure of the protein, resulting in its aggregation, thus supporting the results of surface hydrophobicity.

Conformational changes in proteins can also be inferred by alterations in their UV absorption. The maximum UV absorption peak of untreated shrimp tropomyosin was at approximately 275 nm, indicating that the UV absorption spectrum of the native protein was mainly dependent on side-chain groups containing Tyr and Trp

residues (Tong, Gao, Chen, Li, Zhang, Jian, & Liu, 2012). The UV absorption of shrimp tropomyosin was decreased after HNE treatment (Figure 3C), suggesting that Tyr and Trp residues were modified on the surface of the protein molecule. In addition, a slight blue shift in the UV absorbance peak was obvious in the 0.01 and 0.1 mM HNE treatments, which showed that the polarity of the microenvironment of Tyr and Trp had changed after oxidation, likely as a result of broken peptide bonds. However, no significant signals were seen at the higher concentrations of HNE, likely due to denaturation effects that could also alter the primary structure of shrimp tropomyosin.

Heat modifies protein structures and affects their functional properties. Modification of amino acid side chains can likewise affect the structure of tropomyosin and its thermal properties. Figure 4A shows the differential scanning calorimetry thermogram obtained at $8\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}\cdot\text{l}^{-1}$, showing one endothermic peak at $82.7\text{ }^{\circ}\text{C}$ for untreated tropomyosin and major peaks at 77.7 , 76.6 , 41.5 and $41.1\text{ }^{\circ}\text{C}$ for tropomyosin treated with 0.01 , 0.1 , 0.5 and 1 mM HNE, respectively. The samples differed greatly in thermal stability. The major peaks were more obvious with the 0.01 and 0.1 mM HNE treatments, and were only slightly decreased compared with that of native tropomyosin. After treatment with 0.5 and 1 mM HNE, the major peaks were faint and not as significantly obvious as those at the lower HNE concentrations. The possible reason was that the oxidation of tropomyosin had resulted in changes in thermal stability, and even caused its denaturation. Previous studies have reported that thermal denaturation of tropomyosin is characterized by alterations of its secondary and tertiary structures, leading to aggregation and intermolecular bonding, which

could modify its functional and physiochemical properties (Sarker, Rose, McDonald, Morrow, & Booth, 2010). Thus, tropomyosin appears to denature after oxidation and its thermal stability consequently decreases.

Circular dichroism spectroscopy was used to characterize the impact of HNE oxidation on the secondary structure of shrimp tropomyosin. After HNE treatment, the positive peak at 198 nm and the negative peaks at about 212 and 223 nm were slightly decreased, indicating a decrease in the amounts of α -helices in the protein (Figure 4B). The changes in secondary structure indicated that the structure of the HNE-treated tropomyosin had become more disordered, as based on the calculations performed using the JASCO secondary structure software. According to a previous study (McClain, Gurnon, & Oakley, 2002), this occurs because the amino acid side chains of tropomyosin combine with HNE, disrupting the original van der Waals interactions that maintain the protein's secondary structure and hence rearranging it to a certain degree. Generally, HNE treatment decreases a protein's allergenicity by destroying its epitope conformation. Denaturation of proteins may also disrupt their tertiary structure and lead to random coiled aggregation and insolubility (Shin, Han, & Ahn, 2013).

3.4. Identification of HNE adduction sites by LC-MS/MS

In this experiment, we used a proteomics approach to unequivocally identify and localize the oxidation sites involved in the HNE modification of shrimp tropomyosin. HNE is a readily diffusible and selective electrophile, and a key mediator of oxidative stress. Reactions between HNE and amino acid residues in peptides and proteins

mainly involve the formation of Schiff bases at the nucleophilic amino groups, and some of the amino groups react via a Michael addition. The reactive nucleophilic amino groups in the amino acid side chains that form adducts are mainly present in alanine (Ala), histidine (His), lysine (Lys), leucine (Leu), and the N-termini of the peptide chains. This was confirmed by LC–MS/MS. The protein band of HNE-treated tropomyosin from the SDS-PAGE gel was sliced and analyzed (Figure 1).

A Mascot search yielded Schiff base adducts with a mass increment of 138 Da. HNE adduction was used to identify oxidized side-chain groups of Ala, His, Lys, and Leu in the form of Schiff bases. Previous studies have shown HNE Schiff base adduction on His, Lys and Leu residues (Zhao, Chen, Zhu, & Xiong, 2012). Moreover, the methionine (Met) residues in multiple peptides were oxidized with a mass increment of 16 (as shown in Table 1), which was identified as [O] addition through a reactive oxygen species. Previous studies have reported that Met can be oxidized during LC–MS/MS analysis (Beausoleil, Jedrychowski, Schwartz, Elias, Villén, Li, & Gygi, 2004). As shown in Table 1, 41 peptides were found to react with HNE by forming Schiff bases; these were mainly side chains of Ala, His, Lys and Leu.

Typical MS/MS results for some of the HNE and [O] adducts are shown in Figures 5A–5E. These adducts were observed on peptides with 2 or more nucleophilic sites. ⁷⁷Ala-Leu-Ser-Asn-Ala-Glu-Gly-Glu-Val-Ala-Ala-Leu-Asn-Arg⁹⁰ showed HNE adduct formation on the Ala⁷⁷ residue (Figure 5A). ⁷⁷Ala-Leu-Ser-Asn-Ala-Glu-Gly-Glu-Val-Ala-Ala-Leu-Asn-Arg⁹⁰, containing Leu residues in the side-chain amino group, was oxidized via adduct formation with HNE, and the major peptide identified

in the fraction was Leu⁷⁸ (Figure 5B). ³⁶Ala-Glu-Lys-Ser-Glu-Glu-Glu-Val-His-Asn-Leu-Gln-Lys⁴⁸ showed HNE adduct formation on the Lys³⁸ residue (Figure 5C). Histidine was also altered by adduct formation with HNE (Figure 5D). Reactive oxygen species adduction occurred on the Met side-chain amino group of ⁴⁹Arg-Met-Gln-Gln-Leu-Glu-Asp-Leu-Asp-Gln-Val-Gln-Glu-Ser-Leu-Leu-Lys⁶⁶ (Figure 5E). The oxidation of certain amino acids can result in secondary or tertiary structural changes that affect the functional properties of the protein. These details on the oxidation site, combined with epitope analysis, will be helpful in understanding the changes in the immunogenicity of shrimp tropomyosin caused during food processing and preservation.

4. Conclusions

Any process that can modify the structure of a protein may influence its ability to be recognized by IgE antibodies. HNE, an endogenous molecule during the process of food processing, is easy to react with proteins. To understand the changes of allergens in the process of processing, it is necessary to understand the impact of small molecular substances. The structural changes of tropomyosin induced by lipid peroxides such as HNE during food preservation and processing may explain the reduction or abolition of the shrimp allergenicity. These conformational changes are due to modification of the side chains of certain amino acids. These results provide a new insight into the allergenicity changes that occur during food preservation and processing. Further studies should be conducted to elucidate the different chemical

modifications that occur during food processing and how such changes affect the allergenicity of shrimp.

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References

- Alderton, A. L., Faustman, C., Liebler, D. C., & Hill, D. W. (2003). Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. *Biochemistry*, 42(15), 4398-4405.
- Beausoleil, S. A., Jedrychowski, M., Schwartz, D., Elias, J. E., Villén, J., Li, J., ... & Gygi, S. P. (2004). Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proceedings of the National Academy of Sciences of the United States of America*, 101(33), 12130-12135.
- Boatright, W. L., & Hettiarachchy, N. S. (1995). Spray - Dried Soy Protein Isolate Solubility, Gelling Characteristics, and Extractable Protein as Affected by Antioxidants. *Journal of food science*, 60(4), 806-809.
- Cooke, S. K., & Sampson, H. A. (1997). Allergenic properties of ovomucoid in man. *The Journal of Immunology*, 159(4), 2026-2032.
- Dalle - Donne, I., Aldini, G., Carini, M., Colombo, R., Rossi, R., & Milzani, A. (2006). Protein carbonylation, cellular dysfunction, and disease progression. *Journal of cellular and molecular medicine*, 10(2), 389-406.
- Del Rio, D., Stewart, A. J., & Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, metabolism and cardiovascular diseases*, 15(4), 316-328.
- Esterbauer, H., Schaur, R. J., & Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free radical Biology and*

medicine, 11(1), 81-128.

Faustman, C., Liebler, D. C., McClure, T. D., & Sun, Q. (1999). α , β -Unsaturated aldehydes accelerate oxymyoglobin oxidation. *Journal of Agricultural and Food Chemistry*, 47(8), 3140-3144.

Fenaille, F., Guy, P. A., & Tabet, J. C. (2003). Study of protein modification by 4-hydroxy-2-nonenal and other short chain aldehydes analyzed by electrospray ionization tandem mass spectrometry. *Journal of the American Society for Mass Spectrometry*, 14(3), 215-226.

Haraguchi, F. K., Silva, M. E., Neves, L. X., Dos Santos, R. C., & Pedrosa, M. L. (2011). Whey protein precludes lipid and protein oxidation and improves body weight gain in resistance-exercised rats. *European journal of nutrition*, 50(5), 331-339.

Jansson, H., & Swenson, J. (2010). The protein glass transition as measured by dielectric spectroscopy and differential scanning calorimetry. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1804(1), 20-26.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta (BBA)-Protein Structure*, 624(1), 13-20.

Kelly, S. M., Jess, T. J., & Price, N. C. (2005). How to study proteins by circular dichroism. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1751(2), 119-139.

King, A. J., & Li, S. J. (1999). Association of malonaldehyde with rabbit myosin subfragment 1. In *Quality Attributes of Muscle Foods* (pp. 277-286). *Springer US*.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature*, 227(5259), 680-685.

Lakowicz, J. R. (Ed.). (2013). Principles of fluorescence spectroscopy. *Springer Science & Business Media*.

Lee, A. J., Gerez, I., Shek, L. P. C., & Lee, B. W. (2012). Shellfish allergy-an Asia-Pacific perspective. *Asian Pacific Journal of Allergy and Immunology*, 30(1), 3.

Lee, S., Joo, S. T., Alderton, A. L., Hill, D. W., & Faustman, C. (2003). Oxymyoglobin and lipid oxidation in yellowfin tuna (*Thunnus albacares*) loins. *Journal of food science*, 68(5), 1664-1668.

Leung, P. S., Chu, K. H., Chow, W. K., Ansari, A., Bandea, C. I., Kwan, H. S., ... & Gershwin, M. E. (1994). Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen. *Journal of Allergy and Clinical Immunology*, 94(5), 882-890.

Li, Z., Lu, Z., Khan, M. N., Lin, H., & Zhang, L. (2014). Protein carbonylation during electron beam irradiation may be responsible for changes in IgE binding to turbot parvalbumin. *Food and Chemical Toxicology*, 69, 32-37.

Liang, J. H. (1999). Fluorescence due to interactions of oxidizing soybean oil and soy proteins. *Food Chemistry*, 66(1), 103-108.

Lv, L., Lin, H., Li, Z., Song, Y., Lin, H., & Wang, A. (2014). Identification of oxidative modification of shrimp (*Metapenaeus ensis*) tropomyosin induced by malonaldehyde. *European Food Research and Technology*, 239(5), 847-855.

McClain, D. L., Gurnon, D. G., & Oakley, M. G. (2002). Importance of potential

interhelical salt-bridges involving interior residues for coiled-coil stability and quaternary structure. *Journal of molecular biology*, 324(2), 257-270.

Mercier, Y., Gatellier, P., Viau, M., Remignon, H., & Renerre, M. (1998). Effect of dietary fat and vitamin E on colour stability and on lipid and protein oxidation in turkey meat during storage. *Meat Science*, 48(3), 301-318.

Naveena, B. M., Faustman, C., Tatiyaborworntham, N., Yin, S., Ramanathan, R., & Mancini, R. A. (2010). Detection of 4-hydroxy-2-nonenal adducts of turkey and chicken myoglobins using mass spectrometry. *Food chemistry*, 122(3), 836-840.

Sarker, M., Rose, J., McDonald, M., Morrow, M. R., & Booth, V. (2010). Modifications to surfactant protein B structure and Lipid interactions under respiratory distress conditions: consequences of tryptophan oxidation. *Biochemistry*, 50(1), 25-36.

Shin, M., Han, Y., & Ahn, K. (2013). The influence of the time and temperature of heat treatment on the allergenicity of egg white proteins. *Allergy, asthma & immunology research*, 5(2), 96-101.

Song, Y., Li, Z., Lin, H., Du, S., Hao, Z., Lin, H., & Zhu, Z. (2015). Effect of malondialdehyde treatment on the IgE binding capacity and conformational structure of shrimp tropomyosin. *Food chemistry*, 175, 374-380.

Suman, S. P., Faustman, C., Stamer, S. L., & Liebler, D. C. (2007). Proteomics of lipid oxidation - induced oxidation of porcine and bovine oxymyoglobins. *Proteomics*, 7(4), 628-640.

Takács, K., Guillamon, E., Pedrosa, M. M., Cuadrado, C., Burbano, C., Muzquiz,

- M., ... & Gelencsér, É. (2014). Study of the effect of instant controlled pressure drop (DIC) treatment on IgE-reactive legume-protein patterns by electrophoresis and immunoblot. *Food and Agricultural Immunology*, 25(2), 173-185.
- Tong, P., Gao, J., Chen, H., Li, X., Zhang, Y., Jian, S., ... & Liu, F. (2012). Effect of heat treatment on the potential allergenicity and conformational structure of egg allergen ovotransferrin. *Food Chemistry*, 131(2), 603-610.
- Uchida, K. (2003). 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Progress in lipid research*, 42(4), 318-343.
- Uchida, K., & Stadtman, E. R. (1992). Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proceedings of the National Academy of Sciences*, 89(10), 4544-4548.
- Wu, W., Wu, X., & Hua, Y. (2010). Structural modification of soy protein by the lipid peroxidation product acrolein. *LWT-Food Science and Technology*, 43(1), 133-140.
- Xiong, Y. L., Park, D., & Ooizumi, T. (2008). Variation in the cross-linking pattern of porcine myofibrillar protein exposed to three oxidative environments. *Journal of agricultural and food chemistry*, 57(1), 153-159.
- Yang, J., Tallman, K. A., Porter, N. A., & Liebler, D. C. (2015). Quantitative chemoproteomics for site-specific analysis of protein alkylation by 4-hydroxy-2-nonenal in cells. *Analytical chemistry*, 87(5), 2535-2541.
- Zhao, J., Chen, J., Zhu, H., & Xiong, Y. L. (2012). Mass spectrometric evidence of malonaldehyde and 4-hydroxynonenal adductions to radical-scavenging soy peptides. *Journal of agricultural and food chemistry*, 60(38), 9727-9736.

Zheng, L. N., Lin, H., Pawar, R., Li, Z. X., & Li, M. H. (2011). Mapping IgE binding epitopes of major shrimp (*Penaeus monodon*) allergen with immunoinformatics tools. *Food and Chemical Toxicology*, 49(11), 2954-2960.

Zhou, F., Zhao, M., Su, G., Cui, C., & Sun, W. (2014). Gelation of salted myofibrillar protein under malondialdehyde-induced oxidative stress. *Food Hydrocolloids*, 40, 153-162

Figure captions

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of 4-hydroxy-2-nonenal (HNE)-treated shrimp tropomyosin. The molecular mass of the standard protein marker ranged from 14.4 to 116 kDa. Lane M: Protein marker; Lane 0: shrimp tropomyosin; Lane 1: 0.01 mM HNE; Lane 2: 0.1 mM HNE; Lane 3: 0.5 mM HNE; Lane 4: 1 mM HNE. An equal amount of tropomyosin (5 μ g) was loaded into each lane.

Figure 2. Analysis of the 4-hydroxy-2-nonenal (HNE)-treated tropomyosin. (A) IgE dot-blot analysis for detecting changes in the antigenic activity of HNE-treated tropomyosin, using sera of 4 patients allergic to shrimp (P1, P2, P3, and P4). (B) Inhibition enzyme-linked immunosorbent assay for changes in the antigenic activity of HNE-treated tropomyosin, using pooled sera of the 4 patients in (A). Lanes 0–4 represent the HNE concentrations 0, 0.01, 0.1, 0.5, and 1 mM, respectively.

Figure 3. Effects of increasing concentrations of 4-hydroxy-2-nonenal (HNE) treatment on the (A) surface hydrophobicity, (B) intrinsic fluorescence and (C) UV absorption of native (untreated) and treated tropomyosin in phosphate buffer. 0: control (untreated); 1: 0.01 mM HNE; 2: 0.1 mM HNE; 3: 0.5 mM HNE; 4: 1 mM HNE. In (A), columns with different letters are significantly different ($P < 0.05$). In (C), the main absorption peaks were observed between 230 and 330 nm.

Figure 4. (A) Differential scanning calorimetry heating curves (8 °C/min) and (B) far-ultraviolet circular dichroism spectroscopy of 4-hydroxy-2-nonenal (HNE)-treated tropomyosin. 0: control; 1: 0.01 mM HNE; 2: 0.1 mM HNE; 3: 0.5 mM HNE; 4: 1 mM HNE.

Figure5.(A) MS/MS spectrum for a peptide containing Ala from 4-hydroxy-2-nonenal (HNE)-treated shrimp tropomyosin. HNE Schiff base adduction (*, +138) was observed on the alanine side-chain group in Ala-Leu-Ser-Asn-Ala-Glu-Gly-Glu-Val-Ala-Ala-Leu-Asn-Arg (HNE-modified residue underlined).

Figure5.(B) MS/MS spectrum for a peptide containing Leu from 4-hydroxy-2-nonenal (HNE)-treated shrimp tropomyosin. HNE Schiff base adduction (*, +138) was observed on the leucine side-chain group in Ala-Leu-Ser-Asn-Ala-Glu-Gly-Glu-Val-Ala-Ala-Leu-Asn-Arg (HNE-modified residue underlined).

Figure5.(C) MS/MS spectrum for a peptide containing Lys from 4-hydroxy-2-nonenal (HNE)-treated shrimp tropomyosin. HNE Schiff base adduction (*, +138) was observed on the lysine side-chain group in Ala-Glu-Lys-Ser-Glu-Glu-Glu-Val-His-Asn-Leu-Gln-Lys (HNE-modified residue underlined).

Figure 5. (D) MS/MS spectrum for a peptide containing His from HNE-treated shrimp tropomyosin. HNE Schiff base adduction (*, +138) was observed on the histidine side-chain group in Ala-Glu-Lys-Ser-Glu-Glu-Glu-Val-His-Asn-Leu-Gln

-Lys (HNE-modified residue underlined).

Figure 5. (E) MS/MS spectrum for a peptide containing Met from HNE-treated shrimp tropomyosin. [O] Schiff base adduction (*, +16) was observed on the DL-methionine side-chain group in Arg-Met-Gln-Gln-Leu-Glu-Ans-Asp-Leu-Asp-Gln-Val-Gln-Glu-Ser-Leu-Leu-Lys ([O]-modified residue underlined).

FIG.1

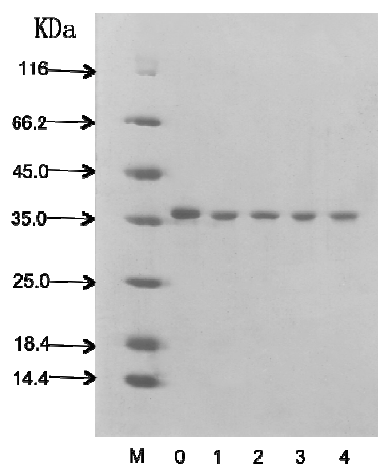
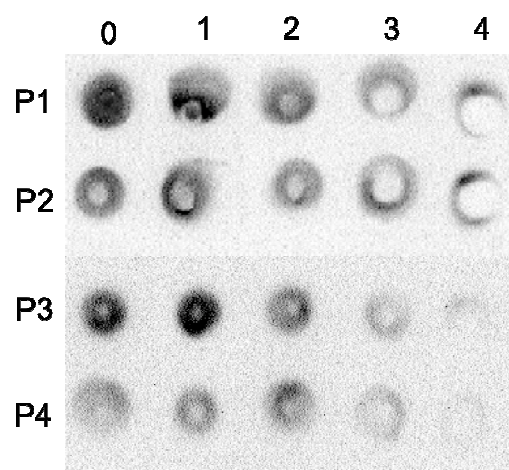


FIG2

A



B

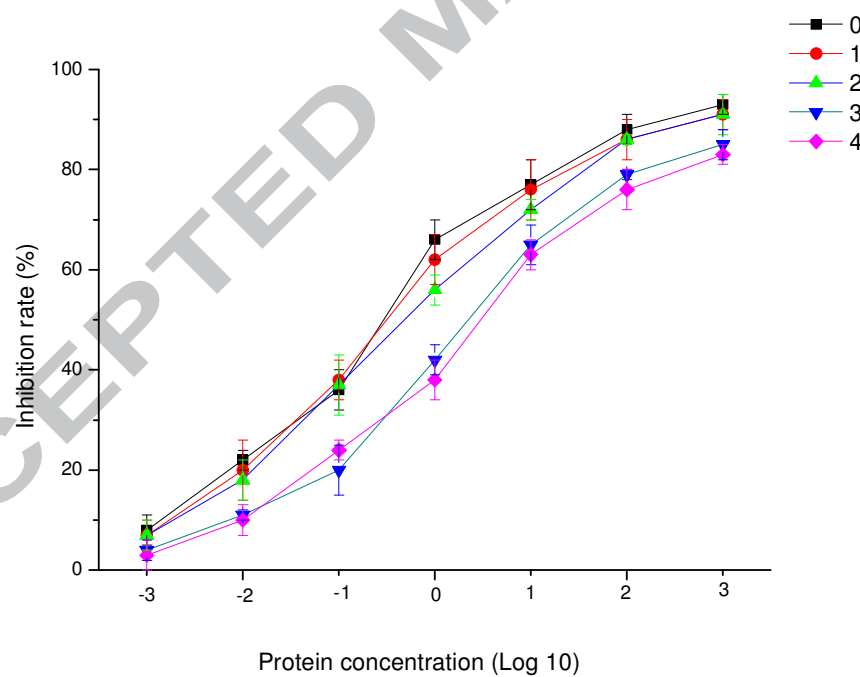
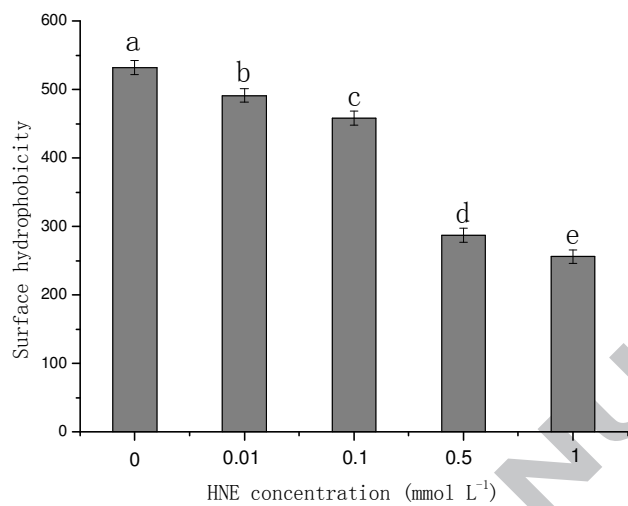
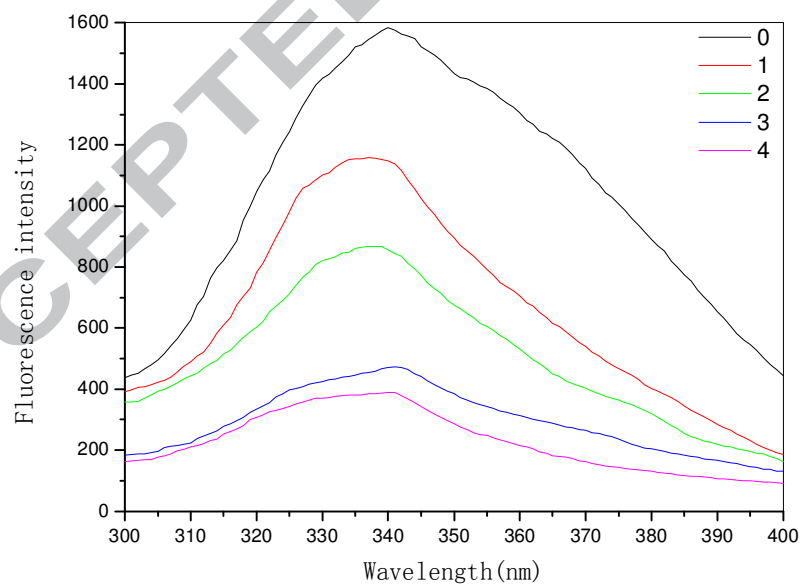


FIG.3

A



B



C

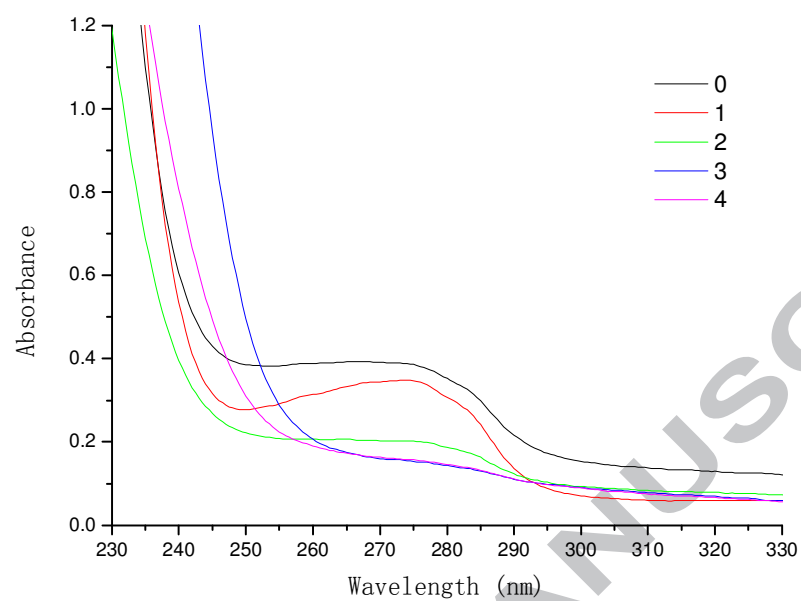
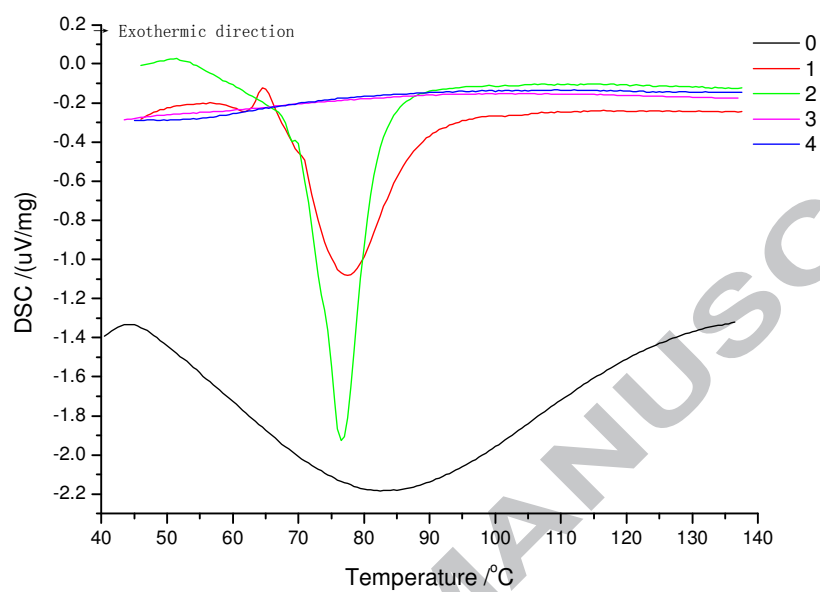


FIG.4

A



B

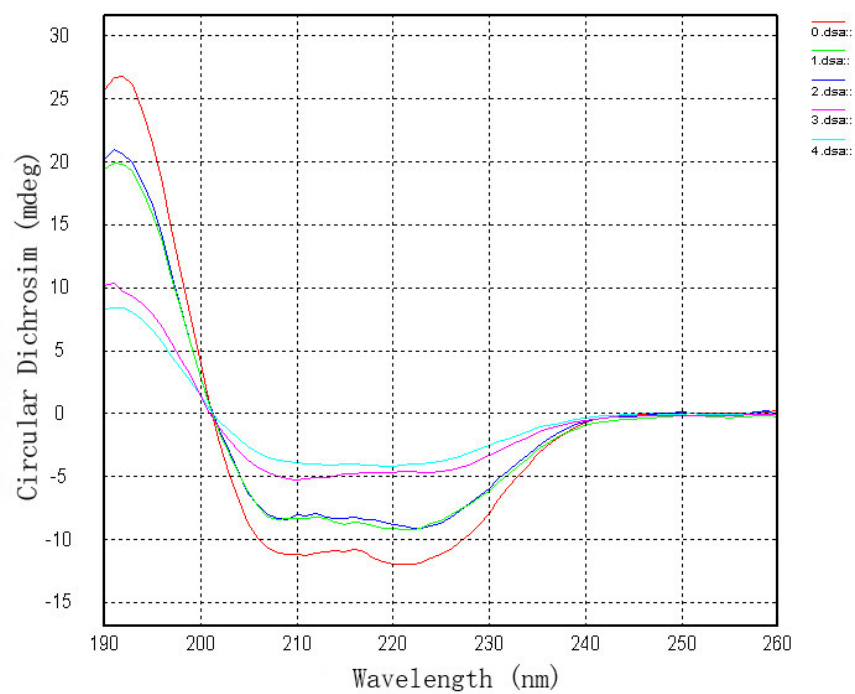


FIG5 (A)

b⁺ions: 72.04, 185.13, 272.16, 386.20, 457.24, 586.28, 643.30, 772.35, 871.42, 1098.57, 1169.60, 1282.69, 1396.73
y⁺ions: 175.11, 289.16, 402.25, 237.15(+), 700.44, 400.26(+), 928.55, 985.57, 1114.61, 593.33(+), 1299.69, 693.86(+), 1499.81

m/z: 785.918 change: +2

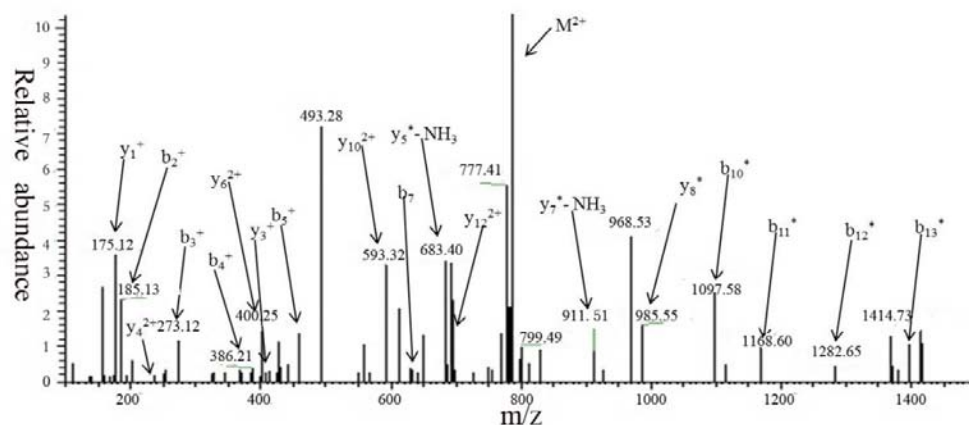
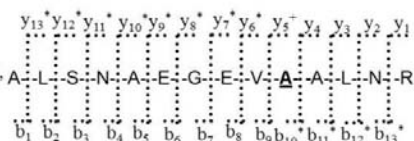


FIG5 (B)

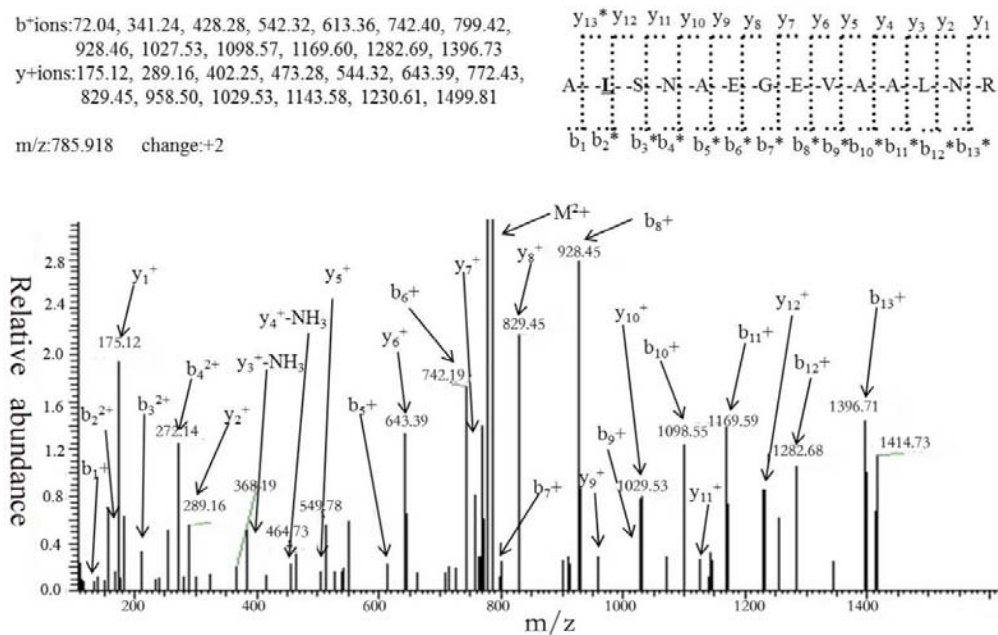


FIG.5 (C)

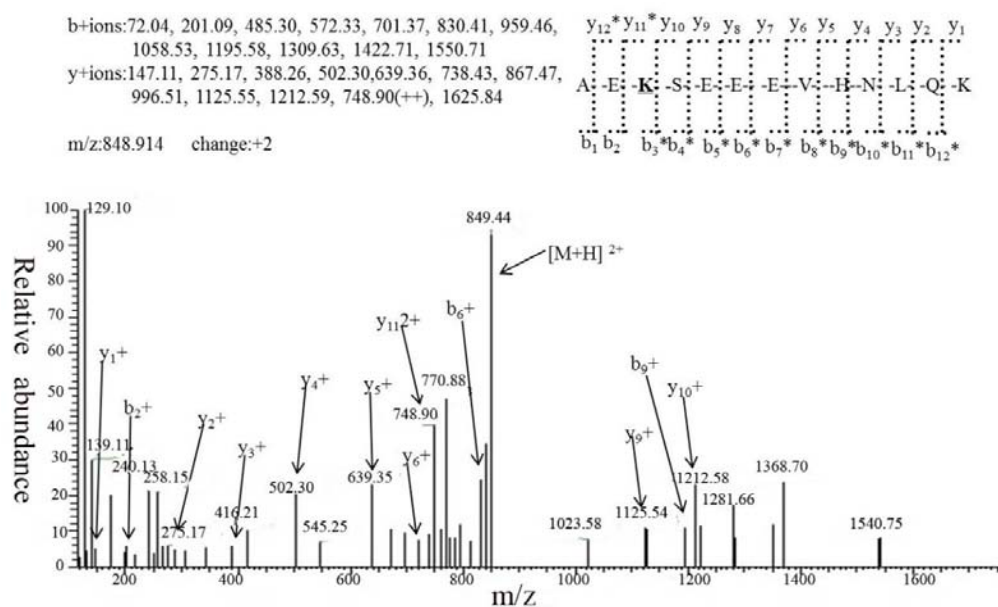


FIG.5 (D)

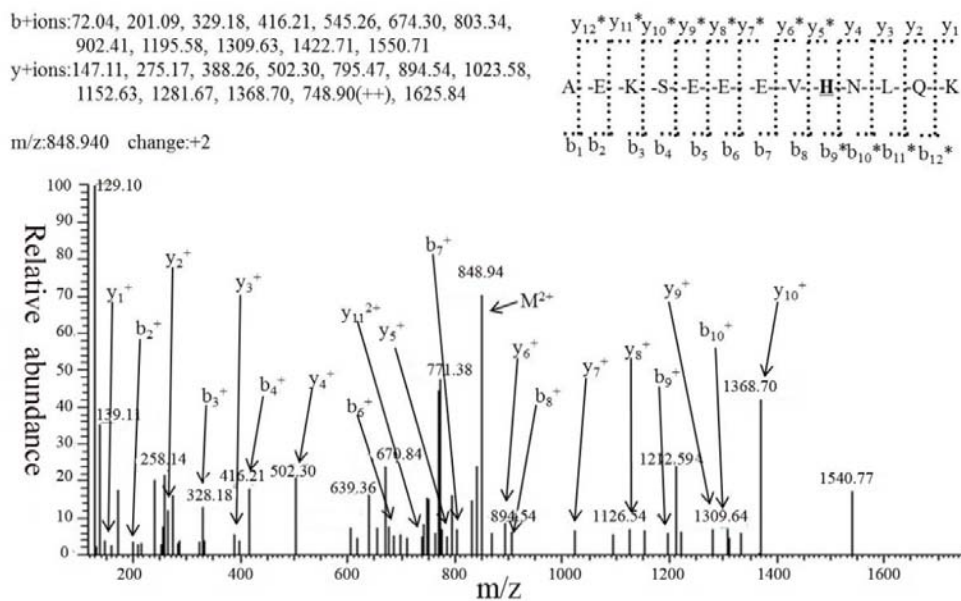


FIG.5 (E)

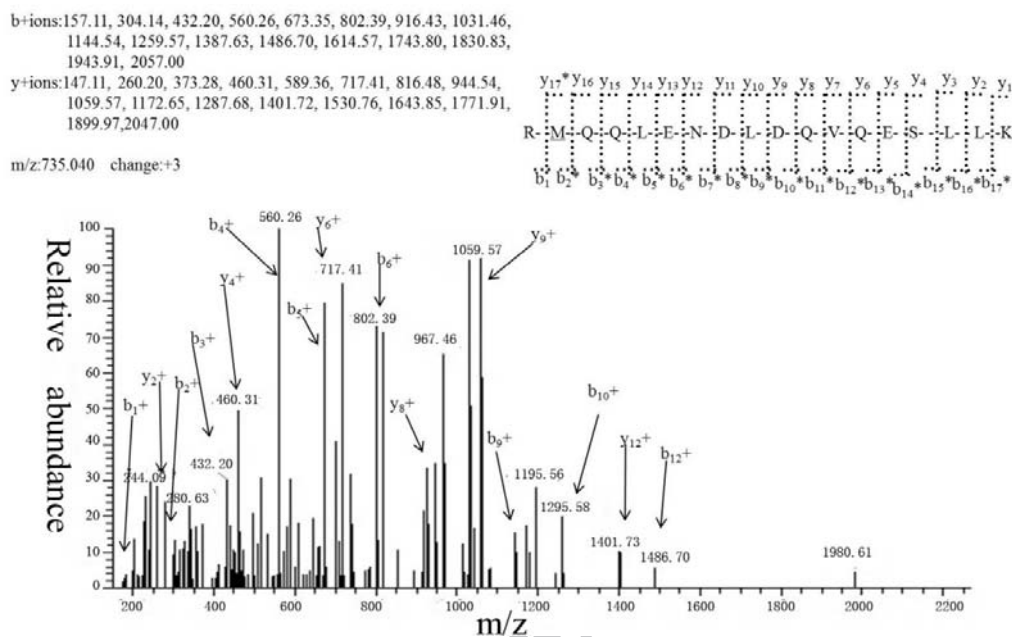


Table 1. List of 4-hydroxy-2-nonenal (HNE)-modified peptides of shrimp tropomyosin identified after date-dependent tandem mass spectrometry acquisition.

HNE-modified peptides	Modification site(s)	z	m/z	Observed mass	Theoretical
<u>A</u> EKSEEEVHNLQK(138)	36	3	566.297	1695.868	1695.868
ALSNAEGEVAALNR (138)	77	2	785.918	1569.821	1569.836
ALSNAEGEVAALNR (138)	81	2	785.918	1569.821	1569.836
ALSNAEGEVAALNR(138)	86	2	785.918	1569.821	1569.836
AEFAERSVQK(138)	242	2	660.863	1319.712	1319.709
ALSNAEGEVAALNR (138)	78	2	785.918	1569.821	1569.836
DKALSNAEGEVAALNR (138)	88	4	454.243	1812.944	1812.958
IQLLEEDLERSEER (138)	94	4	479.502	1913.980	1913.995
IQLLEEDLERSEER(138)	95	2	957.999	1913.983	1913.995
IQLLEEDLERSEER (138)	99	4	479.503	1913.980	1913.995
LNTATTKLAEASQAADSEER(138)	113	3	754.721	2261.141	2261.139
LAMVEADLERAEER (138)	169	3	596.638	1786.892	1786.914
MQQLENDLDQVQESLLK(138)	50,53	2	1102.055	2202.096	2202.109
MQQLENDLDQVQESLLK (138)	50,57	3	735.041	2202.100	2202.109
AEKSEEEVHNLQK (138)	38	2	848.941	1695.867	1695.868
AEKSEEEVHNLQK(138)	48	3	566.292	1695.858	1695.863
MQQLENDLDQVQESLLK(138)	66	4	547.532	2186.099	2186.114
MQQLENDLDQVQESLLK(138)	50,66	3	735.040	2202.097	2202.109
AEKSEEEVHNLQK (138)	44	2	848.941	1695.866	1695.868
AEKSEEEVHNLQK(138)	44,48	3	618.330	1851.967	1851.983
RMQQLENDLDQVQESLLK (16)	50	3	735.040	2202.098	2202.095
SLSDEERMDALENQLK (16)	141	3	631.968	1892.882	1892.879
LAMVEADLERAEER (16)	171	2	824.404	1646.793	1646.793

Highlights

HNE can induce structure changes of tropomyosin through amino acid modification.

HNE treatment can decrease the IgE binding capacity of tropomyosin.

The extent of allergenicity and structure changes relate to the concentration of HNE.

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