

Allergenic response to squid (*Todarodes pacificus*) tropomyosin Tod p1 structure modifications induced by high hydrostatic pressure

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ABSTRACT

The structural and allergenic modifications of tropomyosin Tod p1 (TMTp1) in fresh squids induced by high hydrostatic pressure (HHP) were investigated. The α -helix in TMTp1 decreased along with increasing pressure from 200 to 600 MPa, where almost 53% α -helix was converted into β -sheet and random coils at 600 MPa. The free sulfhydryl group dropped significantly as pressure went up, but the surface hydrophobicity increased at 200 and 400 MPa, while it slightly decreased at 600 MPa. Based on in vitro gastrointestinal digestion test, digestibility of TMTp1 was promoted by HHP treatment, in which 400 and 600 MPa were more effective in reducing the allergenicity than 200 MPa based on indirect ELISA. This study suggested that HHP can decrease allergenicity of TMTp1 by protein unfolding and secondary structure modification, thus providing potential for alleviating allergenicity of squid.

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1. Introduction

Squid is one of the most popular seafood items worldwide because of its high nutritional value and culinary properties (Deng et al., 2014). However, squid allergy is one of the most common, severe, and long lasting food allergies encountered by children and adults (Nakamura et al., 2006). Many attempts have been made to reduce its allergenicity during processing (Leung et al., 2012). Tropomyosin Tod p1 (TMTp1), a 38 kDa myofibrillar, water soluble and heat stable protein (Motoyama et al., 2007), has been identified to be a major allergen of squid *Todarodes pacificus* (Miyazawa et al., 1996), and it is also involved in allergy and cross-activity among a range of crustacean and mollusk species (Lu et al., 2007; Nakamura et al., 2006).

High hydrostatic pressure (HHP) processing is considered to be a valuable non-thermal food processing technology. Previous studies

have indicated that HHP is one of the most advantageous processing treatments for improving palatability and safety as well as for extending shelf life of seafood (Gou et al., 2010). HHP processing affects the structure of proteins resulting in the alteration of food properties. HHP-induced protein denaturation could alter allergenicity by modifying the protein conformation, such as the IgE-reactive conformational epitopes (Shriver and Yang, 2011). The β -lactoglobulin treated at 0.1–150 MPa produced only minor modification, but high pressure at 200–450 MPa induced the formation of disulfide-linked dimers and higher aggregates, while pressure at ≥ 500 MPa caused complete unfolding and the formation of soluble disulfide-linked intermolecular aggregate (Zeece et al., 2008). The reduction in IgE-specific binding activity and allergenicity of bovine γ -globulin, treated by 100–600 MPa at 5–7 °C for 5 min, were due to the changes in the tertiary structure (Yamamoto et al., 2009). Significant reductions in the allergenicity of soy protein isolate were observed by HHP treatment at 300 MPa for 15 min, in which HHP induced modification in the secondary structure and partial unfolding (Li et al., 2012). HHP was also effective to reduce the immune reactivity of allergens for other food products, such as rice and pork batter (Li et al., 2012). However, no significant effects were found on the allergen levels of almond protein extracts at 600 MPa for 5, 15, and 30 min at 4, 21, and 70 °C (Li et al., 2013). Additionally, it has been suggested that HHP at 600 MPa and 40–50 °C increases the allergen reactivity of milk due to protein unfolding and the exposure of formerly hidden epitopes (Kleber et al., 2007). Above all, these findings have shown that HHP treatment could induce a range of structural changes of proteins and allergenicity in food depending on the sources and types of proteins as well as the treatment

Abbreviations: ANS, 8-Anilino-1-naphthalenesulfonic acid; CD, circular dichroism; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HHP, high hydrostatic pressure; Ho, surface hydrophobicity; LC-Q-TOF-MS, liquid chromatography quadrupole time-of-flight mass spectrometry; PB, phosphate buffer; PSMF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SGD, simulated gastrointestinal digestion; SH, free sulfhydryl group; TBST, Tris-buffered saline and Tween 20; TMTp1, tropomyosin Tod p1; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

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conditions. However, there is currently little data on the effects of HHP treatment on the structure of TMTp1 and its allergenicity in squid. Moreover, no clear association between the structural properties and the allergenicity of squid has been established.

Therefore, the objectives of this study were to investigate the effect of HHP treatment on the structural properties of TMTp1 (secondary structure, free SH content, and surface hydrophobicity) and allergenicity (*in vitro* simulated gastrointestinal digestion and indirect ELISA), and to identify the relationship between the structure of TMTp1 and allergenicity in squid.

2. Materials and methods

2.1. Materials

Squids (*Todarodes pacificus*) were obtained from Chinese Academy of Fishery Sciences (Shanghai, China) and stored at -80°C for further study. Rabbit anti-squid TMTp1 polyclonal antibodies were prepared as described by Yu et al. (2011) in the animal experiment center of Shanghai Jiao Tong University. All procedures concerning animals were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University. The protocol was approved by the National Natural Science Foundation Commission of China (Permit Number: 31271955) and the Committee on the Ethics of Animal Experiments of School of Agriculture and Biology, Shanghai Jiao Tong University. Individual human serum samples were collected from five patients (Xinhua Hospital, Shanghai, China) who were determined to have squid allergy based on the history and the objective manifestations after ingestion of squid. The pooled sera of two non-allergic individuals from the same hospital were used as a negative control. Written informed consent was obtained from each individual before conducting the test.

2.2. Preparation of TMTp1

Squid TMTp1 was prepared according to the method of Liang et al. (2008) with some modifications, and all procedures were carried out at 4°C unless otherwise stated. In brief, the mantle muscle (100 g) of squid was minced and homogenized with buffer A (1:10, w/v) containing 50 mM KCl, 2 mM NaHCO_3 , 5 mM MgCl_2 , 1 mM DTT, and 1 mM PSMF for 1 min. The mixture was centrifuged at $8000 \times g$ for 10 min, and the precipitates were re-suspended in the above buffer. The former experimental procedures were repeated twice to remove sarcoplasmic proteins. The final residue was placed in 10-fold of acetone for 30 min and then filtered through 4 layers of gauze for 3 times. Acetone was evaporated at room temperature for 1–2 h. Dried acetone powder was extracted overnight in buffer B (1:10 w/v) containing 20 mM Tris-HCl (pH 7.5), 1 M KCl, 5 mM MgCl_2 , 1 mM DTT, and 1 mM PSMF, and centrifuged at $13,200 \times g$ for 20 min. The supernatants were collected and subjected to isoelectric precipitation at pH 4.6 with 1 M HCl followed by centrifugation at $10,000 \times g$ for 10 min. The collected residue was dissolved in 20 mM Tris-HCl (pH 7.5), and the pH was adjusted to 7.6 with 1 M NaOH. Fractionation was carried out with 40–60% ammonium sulfate solutions, and the precipitates were dialyzed against 20 mM Tris-HCl (pH 7.5) for 24 h. The obtained protein extracts were identified by SDS-PAGE and further confirmed by LC-Q-TOF-MS (Impact Q-tof ultimate3000, Bruker Co., Germany). Protein concentration of the extracts was estimated by a BCA Protein Assay Kit (Beyotime, Shanghai, China) using bovine serum albumin as the standard.

2.3. HHP treatments

HHP treatments were done in a HHP-750 unit (Kefa High Pressure Food Processing Inc., Baotou, China) with a 2.5 L of cylindrical pressure vessel and a pressure range of 0–700 MPa. The TMTp1 solution was diluted with 20 mM Tris-HCl (pH 7.5) into a final concentration of 1 mg/mL. Approximately 10 mL of the mixture was sealed in polyethylene-polyamide plastic bags. The bags were immersed in the high pressure vessel filled-up with water and treated at 200, 400 and 600 MPa at 20°C for 20 min, respectively. The pressure increase rate was 8.3 MPa/min, and the depressurization time was less than 4 s. TMTp1 without pressurization was used as control. All samples were freeze dried in a Freezone 2.5 L Triad system (Labconco Inc., USA), and dried samples were stored at -80°C until use.

2.4. Protein structure characterization

2.4.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 15% polyacrylamide slab gels as described by Deng et al. (2014), where the gels were stained with 0.1% Coomassie Brilliant Blue R-250. All samples were boiled for 5 min before electrophoresis.

2.4.2. Circular dichroism (CD) spectra

Far-UV CD spectra were recorded by a J-815 spectrometer (JASCO Inc., Japan) at 25°C , using a quartz cuvette with an optical path-length of 1 mm. Protein samples were diluted with 10 mM phosphate buffer (PB) (pH 7.0) into a concentration of 0.1 mg/mL, and then scanned from 190 to 260 nm at a scan rate of 50 nm/min with

a wavelength step of 1 nm. The spectra were reported as average of three parallel experiments. The CD data were expressed as mean residual ellipticity (θ) in $\text{deg cm}^2 \text{dmol}^{-1}$. K2D procedure (<http://dichroweb.cryst.bbk.ac.uk>) was used for the analysis of protein secondary structures.

2.4.3. Free sulfhydryl (SH) content

The free SH group levels were determined using the modified method of Cui et al. (2009). In brief, 1 mL of TMTp1 solution at a concentration of 1 mg/mL was mixed with 2 mL of 0.086 M Tris-Glycine buffer (pH 8.0) containing 0.09 M glycine, 0.004 M EDTA, and 8 M urea. A 0.02 mL of Ellman's reagent (4 mg/mL DTNB in the above Tris-Glycine buffer) was added into the mixture. After incubation at 25°C for 30 min, the absorbance was measured at 412 nm using a UV-1800 spectrophotometer (Shimadzu Co., Japan). The SH group content was calculated as: SH content ($\mu\text{M/g}$ tropomyosin) = $73.53 A_{412} \times D/C$, in which D was the dilution coefficient (3.02), C (mg/mL) was the protein concentration in the tested sample, and 73.53 was derived from the molar basis to the $\mu\text{M/mL}$ basis and from mg to g. Measurements were performed in triplicate.

2.4.4. Surface hydrophobicity (Ho)

The Ho of TMTp1 was determined using ANS as the fluorescence probe, according to the method of Puppo et al. (2004). Serial dilutions in 10 mM PB (pH 7.0) were done with TMTp1 samples to a final level of 0.05–0.2 mg/mL, and 10 μL ANS (8.0 mM) prepared in the same buffer was added to 2 mL of sample. Fluorescence intensity was recorded at wavelengths of 390 nm (excitation) and 470 nm (emission) using an F-4500 FL Spectrophotometer (Hitachi Co., Japan). The initial slope of fluorescence intensity versus protein concentration plot was used as index of Ho. Measurements were performed in triplicate.

2.5. Protein allergenicity analysis

2.5.1. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion stability assay

SGF digestion of the TMTp1 was conducted using the method from Fu et al. (2002) with some modifications. Briefly, the SGF was prepared following the United States Pharmacopoeia Standard (Anonymous, 1995) and consisted of 0.32 mg/mL pepsin (132 U/mg proteins, Sinopharm Chemical Reagent Co., Shanghai, China) in 2 mg/mL NaCl (pH was adjusted to 1.2 using HCl). The total volume of the reaction solution was 1 mL, with the ratio of pepsin to test protein at about 1:100 (w/w). Digestion was carried out at 37°C , and the SGF was incubated in a water bath at 37°C for 10 min before adding the test protein (1 mg/mL). At 0, 1, 2, 5, 15, 30, 60 and 90 min, 100 μL of the reaction solution was transferred to a 1.5 mL microcentrifuge tube, and 30 μL of 0.2 M Na_2CO_3 was added to stop the reaction.

SIF was also prepared as described in the United States Pharmacopoeia (Anonymous, 1995), containing 0.10 mg/mL of trypsin (386 U/mg proteins, Sinopharm Chemical Reagent Co., Shanghai, China) in 0.05 M KH_2PO_4 , pH 7.5. The total volume of the reaction solution was 1 mL, and the ratio of trypsin to test protein was 1:300 (w/w). Digestion was performed at 37°C with SIF preheated. At 0, 1, 2, 5, 15, 30, and 60 min, 100 μL of the reaction solution was transferred to a 1.5 mL microcentrifuge tube, and the reaction was immediately terminated by heating in the boiling water for 5 min. Samples were then analyzed by SDS-PAGE and Western blotting.

2.5.2. Western blotting

For Western blotting, digestive products of the protein samples were separated on the 15% polyacrylamide slab gels by electrophoresis and then transferred onto nitrocellulose membranes following the method of Yu et al. (2011) with some modifications. Briefly, after blocking the unbound sites using 5% non-fat milk in TBST (20 mM Tris-HCl, pH 7.5, 0.145 M NaCl, 0.05% Tween-20) for 2 h at room temperature with gentle shaking, the membranes were incubated with rabbit anti-squid TMTp1 polyclonal antibodies diluted in TBST (1:20000, v:v) at 4°C overnight. The membranes were washed five times using TBST, for 5 min each time, followed by incubation at 37°C with a goat anti-rabbit IgG-HRP (ZB-2301, Zhongshan Co., Beijing, China) diluted in TBST (1:15000, v:v) for 2 h. After washing extensively with TBST for 5 times, a DAB substrate (Sangon Co., Shanghai, China) was used for detection.

2.5.3. Indirect ELISA

Indirect enzyme linked immune sorbent assay (ELISA) was determined according to the protocol of Li et al. (2013) with some modification. The Polystyrene 96-well ELISA plates (Corning Inc., USA) were coated with TMTp1 samples, or their digestive products (5 $\mu\text{g}/100 \mu\text{L}$ per well) in the buffer containing 0.29% (w/w) NaHCO_3 and 0.16% (w/w) Na_2CO_3 , pH 9.6 and incubated at 37°C for 2 h. After washing the plates with TBST (20 mM Tris-HCl, pH 7.5, 0.145 M NaCl, 0.05% Tween-20) for 5 times, the nonspecific sites were blocked by 1% bovine serum albumin (200 μL per well) at 37°C for 2 h. The plates were washed and incubated with 100 μL of human sera (1:5 v/v diluted with TBST) or rabbit anti-squid TMTp1 polyclonal antibodies (1:100000 v/v diluted with TBST). After incubation at 37°C for 1 h, the plates were washed again and incubated with goat anti-human IgE-HRP (1:20000, v/v, ABD Serotec, Oxford, UK) or goat anti-rabbit IgG-HRP (1:15000, v/v) at 37°C for 4 h, 100 μL per well. Finally, color was developed with TMB (Boster Co., Wuhan, China). After terminating the reaction by addition of 0.5 M H_2SO_4 , the absorbance was monitored at 450 nm using

an automated ELISA plate reader (Thermo Co., USA). Three replicate measurements were carried out.

2.6. Statistical analysis

Two-way analysis of variance (ANOVA) was carried out using Statistical Packages for the Social Sciences software (SPSS 19.0) (SPSS Inc., Chicago, IL, USA). Mean differences ($p < 0.05$) were established by the Duncan's multiple range tests.

3. Results and discussion

3.1. TMTp1 identification

After purification by ammonium sulfate precipitation (60% saturation), the purified TMTp1 on SDS-PAGE presented a single band with an average molecular weight (MW) of 38 kDa (Fig. 1A),

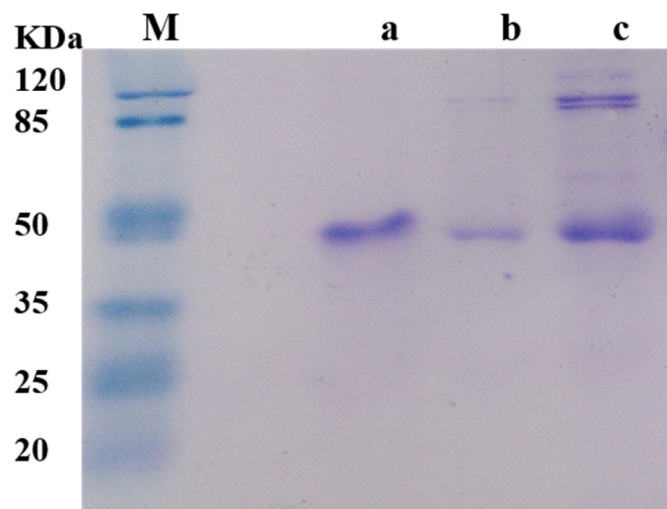
indicating that purified TMTp1 was obtained, which was further confirmed by the LC-Q-TOF-MS (data not shown).

3.2. SDS-PAGE result of TMTp1 treated by HHP

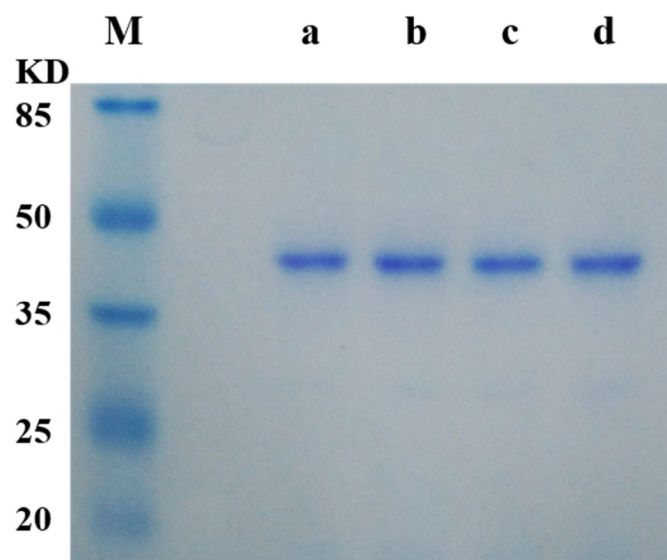
SDS-PAGE is usually performed to confirm the presence or absence of allergens (Shriver and Yang, 2011). As shown in Fig. 1B, there are no significant changes observed in the electrophoretic behaviors of TMTp1 treated with or without HHP. These results suggested that TMTp1 still existed when exposed to HHP, and no changes in molecular weights were induced by protein degradation. Similar results were observed in the case of phytoferritin and soy protein isolate, accompanied with modification in the protein tertiary structure (Li et al., 2012; Zhang et al., 2012). However, HHP at 100–300 MPa for 15 min didn't affect the electrophoretic profile of soybean but reduced the band intensity of some protein, which suggested a decrease in immunoreactivity (Peñas et al., 2011). According to Puppo et al. (2004), HHP processing at 400 and 600 MPa caused 11S subunit of soybean protein aggregation as noted by PAGE results, while the electrophoretic patterns of 7S subunit remained unchanged. However, fragments degraded or aggregated from allergens, probably because the HHP treatment was too weak for the resolution of the gel and ultimately lost in the buffer, or too large to go through the pores of the polyacrylamide gel and washed away, are invisible in the SDS-PAGE profile (Shriver and Yang, 2011).

3.3. Effects of HHP on the secondary structure of TMTp1

Far-UV CD spectroscopy was used to evaluate the secondary structure of proteins, and the effects of HHP on the secondary structure of TMTp1 are displayed in Fig. 2. All the HHP-treated samples and the control presented similar far-UV CD spectra, with a positive band at 192 nm and a strong negative band at about 216 nm and 208 nm, indicating that TMTp1 had highly ordered structure, most probably the α -helix type. As the pressure increased, the intensities of the negative peaks at 208 nm and 216 nm in the CD spectra were reduced, suggesting a decrease of α -helix. The secondary structure compositions, such as α -helix, β -sheet, and random coils were calculated by the K2D program and are listed in Table 1. The secondary structure elements in the raw TMTp1 (control) consisted of 81% α -helix and 19% random coils. With increasing pressure, the content of α -helix declined from 81% in the control to 38% in the 600 MPa treated protein, while the contents of β -sheets and random coils increased from 0 to 14% and from 19 to 48%, respectively. These results suggested that HHP induced a conversion of α -helix to β -sheet and random coils (most predominantly of the random coils type)



(A)



(B)

Fig. 1. SDS-PAGE analysis of: (A) Tropomyosin purification (M: marker; a: after treating with 60% ammonium sulfate solutions; b: after treating with 40% ammonium sulfate solutions; c: after isoelectric precipitation). (B) Untreated and HHP treated tropomyosin samples (M: protein markers; a: untreated tropomyosin; b: HHP at 200 MPa; c: HHP at 400 MPa; d: HHP at 600 MPa).

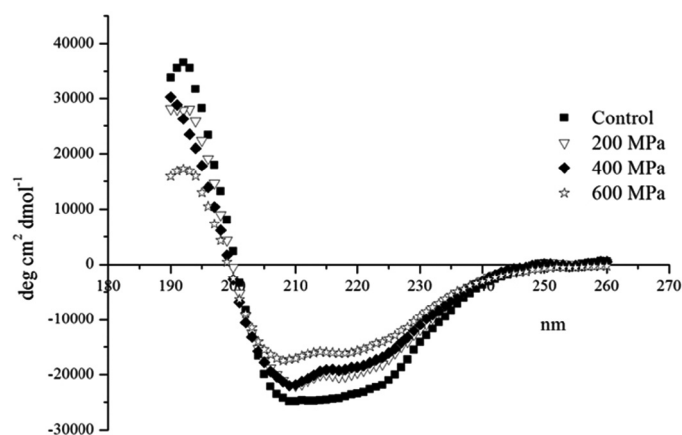


Fig. 2. Far-UV CD spectra comparison between untreated tropomyosin and HHP treated sample.

Table 1

Secondary, tertiary structure of tropomyosin under different HHP treatment conditions.

Pressure (MPa)	Secondary structure ^a			Tertiary structure ^a	
	α -helix	β -sheet	random	FSC [*] (μ mol/g protein)	Ho [#]
0.1	0.81	0	0.19	29.76 $\pm$ 1.03 ^a	316 $\pm$ 7.0 ^a
200	0.66	0.05	0.29	28.28 $\pm$ 0.78 ^a	333 $\pm$ 16 ^a
400	0.6	0.07	0.33	24.13 $\pm$ 1.61 ^b	417 $\pm$ 17 ^c
600	0.38	0.14	0.48	21.47 $\pm$ 0.51 ^c	388 $\pm$ 11 ^b

^aValues were means $\pm$ SD. Values in the same column with different letters were significantly different ($p < 0.05$).^{*} FSC: Free sulfhydryl content (μ mol/g protein).[#] Ho: Surface hydrophobicity.

in the TMTP1 protein, and the degree of this transformation was positively correlated with the level of pressure from 200 to 600 MPa. Similar results were reported by Zhang et al. (2003), who found that the random coil content of glycinin increased significantly with the decrease of the ordered structures of α -helix and β -structure when subjected to HHP treatment at 500 MPa for 10 min. Previous studies suggested that HHP treatment has slight influence on the secondary structure of bovine γ -globulin (a beef allergen) and wheat α -amylase inhibitor (Yamamoto et al., 2009, 2010). However, for Mal d 1, an apple allergen, HHP caused the decrease in α -helix and the increase in β -sheet structure, which might lead to the decline in the allergenicity (Scheibenzuber et al., 2003). These different conclusions were probably due to the different allergen protein, the level of pressure, and treatment time. The modification in the allergenicity of soybean protein isolate allergen was declared to be associated with its secondary structure (Li et al., 2012). For invertebrates, the changes in the secondary structure of tropomyosin were also related to the allergenicity variations (Ozawa et al., 2011).

3.4. Effects of HHP on the tertiary structure of TMTP1

The results of HHP induced effects on the free SH contents of TMTP1 samples are presented in Table 1. Typically, the disulfide bonds play an important role in defining protein tertiary structure (Hu et al., 2011). For rabbit tropomyosin, the presence or absence of disulfide bridges was proved to be a profound structural feature, and important to the stability and function of the molecule (Lehrer and Morris, 1982). Free sulfhydryl content of the control sample was 29.76 $\pm$ 1.03 μ mol/g protein. There was one cysteine at position 81 on each helix of TMTP1 (Motoyama et al., 2006). HHP treatment at 200 MPa did not exhibit significant differences in comparison with the control. However, when treated at 400 and 600 MPa, the free sulfhydryl contents were markedly decreased ($p < 0.05$), which might be due to the formation of disulfide bonds between the intramolecular and intermolecular protein chains through SH/S–S interchange reactions during HHP treatments. The lower free SH contents with HHP treated samples were also reported in other allergens, such as soy protein isolate samples (Puppo et al., 2004) and β -lactoglobulin (Funtenberger et al., 1997) due to the formation of S–S bonds, which was usually accompanied with hydrophobic interactions.

The surface hydrophobicity has been used as a measure of conformational difference in tertiary structure of protein (Zhang et al., 2012). As seen in Table 1, the Ho of samples processed at 400 and 600 MPa was significantly ($p < 0.05$) higher than that of control, showing a maximum value at 400 MPa. This result might be explained as HHP treatment causing the unfolding of the protein and making the hydrophobic regions exposed to the exterior of the protein molecules, leading to the increase of Ho. When treated at 600 MPa, the exposure of hydrophobic regions might increase the extent of hydrophobic interactions, promoting re-association or aggregation among the unfolded proteins. Subsequently, the total Ho

detected in the TMTP1 at 600 MPa was decreased when compared to that of 400 MPa treatment.

Similar results were obtained by Li et al. (2012) who found that HHP at 200–300 MPa results in gradual increases of Ho in the soy protein isolate allergen, but significantly decreases at higher pressure of 400–500 MPa. The Ho of bovine gamma globulin increased gradually when treated from 100 to 600 MPa compared to the control sample (Yamamoto et al., 2009). Typically, the pressure of 200 MPa is sufficient to destabilize the intermolecular interactions and the tertiary structure of proteins, leading to oligomeric proteins dissociation, and when pressure is up to about 500 MPa, which varied for different proteins from 100 MPa to 1 GPa, the unfolding of proteins started (Schay et al., 2006; Somkuti and Smeller, 2013).

Taken together, HHP treatments had significant influence on the secondary and tertiary structure of TMTP1, which were closely associated with its allergenicity. Nakamura et al. (2006) demonstrated that changes in squid tropomyosin structure induced by Maillard reaction lead to reduced allergenicity. Therefore, the effects of HHP treatments on TMTP1 allergenic responses were conducted in subsequent experiments to explore the relationship between the allergenicity and structure of TMTP1 in the squids.

3.5. Effects of HHP on the allergenicity of TMTP1

SGF and SIF digestions were carried out to assess allergenic potential of TMTP1 in the human body. Under digestion with pepsin, the SDS-PAGE showed that the band of native TMTP1 (Fig. 3A1) was gradually decreased with the formation of digested fragments, in which one major band at about 28 kDa and several minor bands of molecular mass 17 kDa were observed. Compared to the untreated sample, the TMTP1 bands after HHP treatments were digested more rapidly with the major digested fragment generated after 2 min (Fig. 3B1, C1, and D1). Moreover, the band intensity of the native TMTP1 was significantly higher than that of HHP-treated samples after 30 min, and remained visible at 90 min. Different pressure levels did not cause significant differences.

Figure 3A2 illustrates the Western blot results of native TMTP1 that correspond to Fig. 3A1, which shows similar digested fragment patterns. However, TMTP1 samples were still detectable even after 90 min for all treatments, indicating that Western blot was more sensitive than SDS-PAGE. The intensity of the native TMTP1 band at 90 min was relatively higher than that of samples treated by HHP (Fig. 3B2, C2, D2), suggesting that HHP processes accelerated the SGF digestion of TMTP1. In addition, compared to the SDS-PAGE result, 28 kDa fragments were not fully recognized by the IgG antibody, which might be caused by the partial destruction of Ig G-binding epitopes during digestion.

The limited proteolysis using trypsin was often used to confirm the structural changes of proteins (Nakamura et al., 2006). As shown in Fig. 4A1, B1, C1, D1, higher reduction rate was observed after HHP treatments. Native TMTP1 was diminished completely at 30 min, while TMTP1 samples after HHP treatments were almost

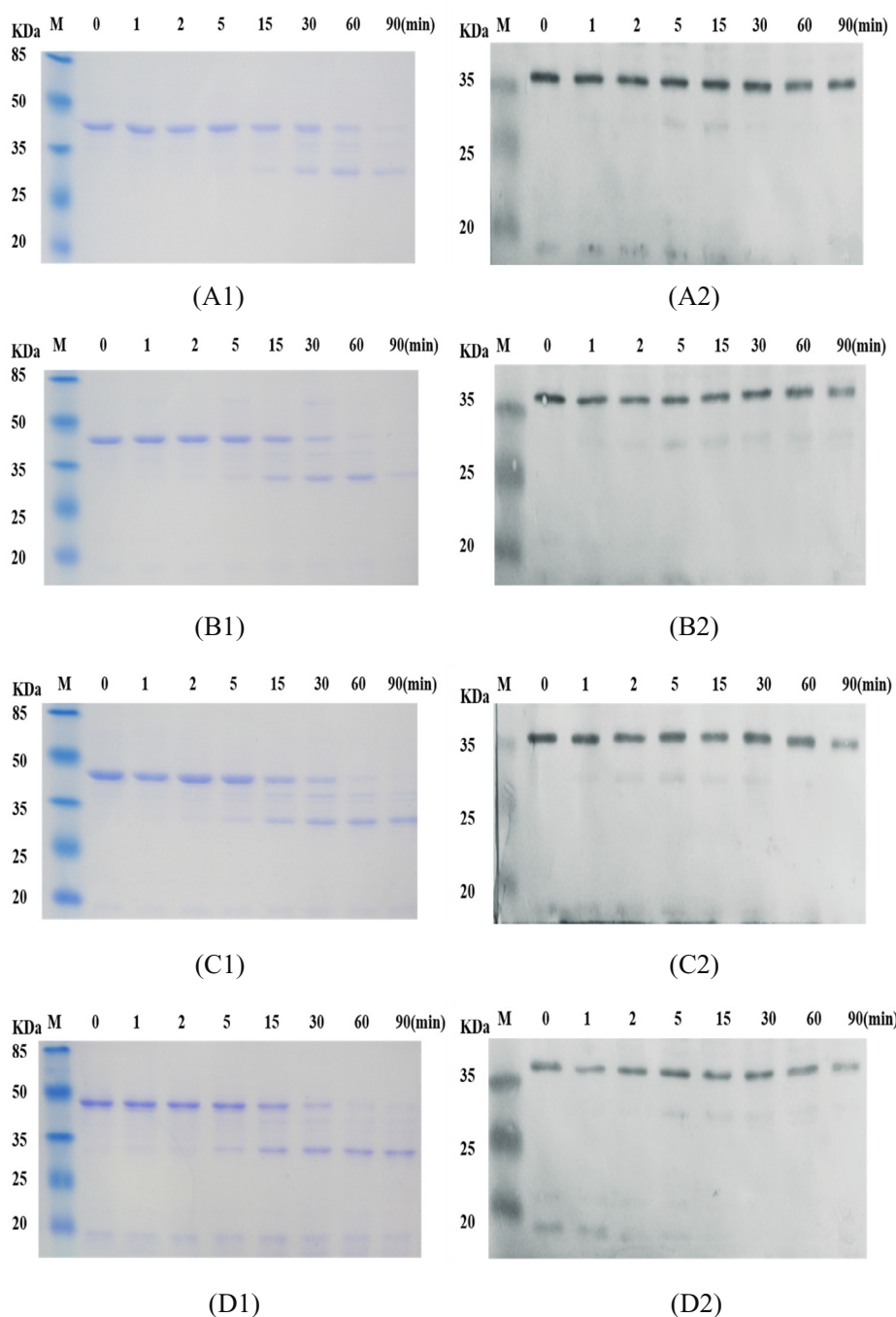


Fig. 3. Effect of simulated gastric fluid digestion (pepsin) on the tropomyosin (A1: SDS-PAGE analysis of control; B1: SDS-PAGE analysis of HHP at 200 MPa; C1: SDS-PAGE analysis of HHP at 400 MPa; D1: SDS-PAGE analysis of HHP at 600 MPa; A2: Western blot detection of control; B2: Western blot detection of HHP at 200 MPa; C2: Western blot detection of HHP at 400 MPa; D2: Western blot detection of HHP at 600 MPa).

undetectable within 15 min, suggesting that digestibility of TMTp1 was promoted by HHP. Fragments with molecular weights of about 36 kDa were mainly generated during digestion with trypsin. The different digestive patterns observed between pepsin and trypsin were due to different cleavage specificities for peptide bonds (Huang et al., 2010). Pepsin tends to cleave peptide bond next to phenylalanine or tyrosine residues, while trypsin is more likely to cleave next to lysine and arginine (Mikita and Padlan, 2007). Tropomyosin was proved to be resistant to pepsin while relatively susceptible to trypsin digestion for the high lysine content (9.2%) (Huang et al., 2010; Li et al., 2012).

Based on the corresponding Western blotting analysis of TMTp1 with the digestibility from SIF (Fig. 4A2, B2, C2, D2), all the TMTp1 samples were initially digested with one concomitant band appeared at about 36 kDa, which was in accordance with the SDS-PAGE digested fragment patterns. However, Western blotting seemed to be more sensitive to identify the 36 kDa band than the SDS-PAGE, indicating that IgG-binding sites of the digested fragments were still available. After 15 min, HHP-treated TMTp1 samples were hardly detected, whereas the native TMTp1 band still remained. Therefore, the TMTp1 was easily digested by trypsin after HHP treatments, which was in agreement with the result of SGF.

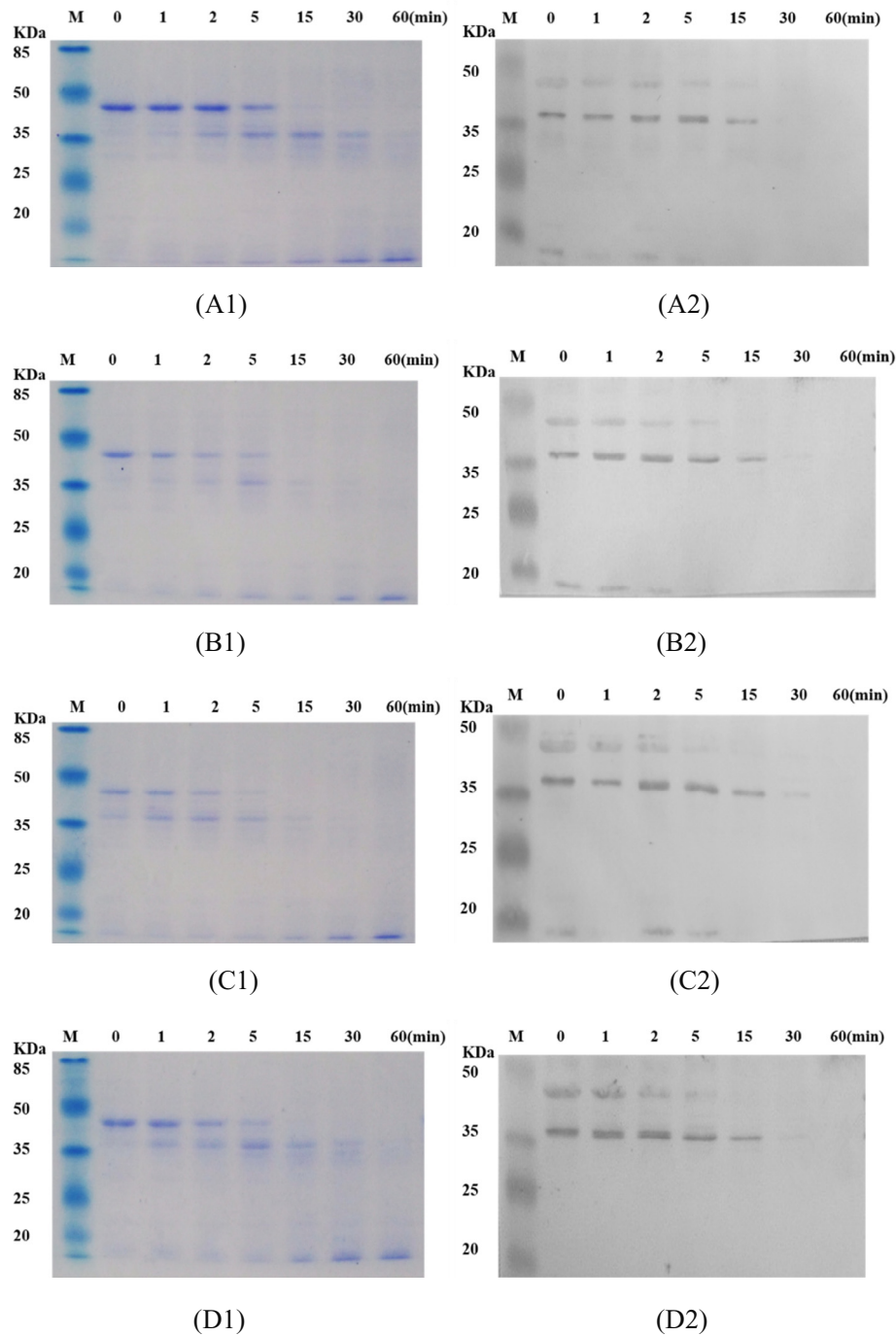


Fig. 4. Effect of simulated intestinal fluid digestion (trypsin) on the tropomyosin (A1: SDS-PAGE analysis of control; B1: SDS-PAGE analysis of HHP at 200 MPa; C1: SDS-PAGE analysis of HHP at 400 MPa; D1: SDS-PAGE analysis of HHP at 600 MPa; A2: Western blot detection of control; B2: Western blot detection of HHP at 200 MPa; C2: Western blot detection of HHP at 400 MPa; D2: Western blot detection of HHP at 600 MPa).

It is well-known that food allergens are more resistant to proteolytic digestion and more likely to be recognized by immune defenses. Hence, the digestibility test in simulated gastrointestinal fluid is an appropriate way to evaluate the allergenicity of allergen proteins (Kleber et al., 2007). Tropomyosin of crab was proved to have a lower degree of digestibility than other proteins since it was not completely digested by SGF (1:50 pepsin to protein, w/w) for 60 min when other myofibrillar proteins were undetectable (Huang et al., 2010). Yu et al. (2011) found that high pressure steam is more effective to accelerate the degradation of tropomyosin in SGF and SIF with reduction in IgE-binding reactivity than boiling and boiling

combined with ultrasound. In this study, the SDS-PAGE and the Western blotting analysis indicated that the digestibility of TMTp1 increased after high pressure treatment, and the Western blotting results also showed the decline in the IgG-binding reactivity. Similar results were found in β -lactoglobulin (Zeece et al., 2008) and ovalbumin (López-Expósito et al., 2008). Susceptibility to protein hydrolysis can be regarded as an index of structural integrity (López-Expósito et al., 2008). According to the tertiary structure results, HHP treatments could unfold TMTp1, which might expose new targets such as tyrosine residues to proteolysis and promote the digestibility. However, the effects of pressure at 200, 400 or

Table 2
ELISA response against IgE and IgG of tropomyosin under different conditions.

Pressure (MPa)	Serum IgE binding ^a				IgG binding ^a			
	S1 [*]	S2	S3	S4	S5	R [*]	R-SGF [#]	R-SIF ^Δ
0.1	0.48 ± 0.02 ^a	1.65 ± 0.04 ^a	0.79 ± 0.01 ^a	0.51 ± 0.06 ^a	0.80 ± 0.01 ^a	1.64 ± 0.03 ^a	1.35 ± 0.06 ^a	0.63 ± 0.03 ^a
200	0.37 ± 0.02 ^b	1.38 ± 0.03 ^b	0.54 ± 0.02 ^b	0.35 ± 0.03 ^{ab}	0.47 ± 0.03 ^b	1.34 ± 0.02 ^b	1.12 ± 0.01 ^b	0.46 ± 0.01 ^c
400	0.31 ± 0.01 ^c	1.23 ± 0.06 ^c	0.32 ± 0.02 ^c	0.31 ± 0.03 ^{bc}	0.31 ± 0.03 ^c	1.18 ± 0.02 ^c	0.93 ± 0.02 ^c	0.45 ± 0.02 ^c
600	0.31 ± 0.01 ^c	1.11 ± 0.07 ^d	0.32 ± 0.01 ^c	0.24 ± 0.03 ^c	0.30 ± 0.01 ^c	1.16 ± 0.01 ^c	0.93 ± 0.01 ^c	0.52 ± 0.02 ^b

^aValues were means ± SD. Values in the same column with different letters were significantly different ($p < 0.05$).

^{*} S1–S5: indirect ELISA with human sera from five allergic patients.

^{*} R: indirect ELISA with rabbit anti-squid TMTp1 polyclonal antibodies.

[#] R-SGF: indirect ELISA with rabbit anti-squid TMTp1 polyclonal antibodies for hydrolysates after Simulated Gastric Fluid digestion (SGF) digestion.

^Δ R-SIF: indirect ELISA with rabbit anti-squid TMTp1 polyclonal antibodies for hydrolysates after Simulated Intestinal Fluid (SIF) digestion.

600 MPa were undistinguishable by the SDS-PAGE and the Western blotting under current digestion conditions. Assay conditions such as the ratio of enzyme to protein was very important when trying to correlate digestibility with the allergenic potential of proteins (Leung et al., 2012).

ELISA was performed to assess the allergenicity of native and processed TMTp1 using individual patient's sera with allergy to squid or rabbit anti-squid TMTp1 polyclonal antibodies. The IgE-binding response of the native TMTp1 to the pooled sera of two non-allergic individuals was 0.20 ± 0.01 . As shown in Table 2, regardless of the variations among the individual sera, which showed different specificities toward TMTp1, a significant decrease in the IgE-binding reactivity was observed after high pressure treatment at 200 MPa. The IgE-binding reactivity was further reduced when pressure increased to 400 MPa. However, a higher pressure at 600 MPa did not show difference from that of 400 MPa among most individuals while it reduced 38%–67% compared to the native TMTp1. These results confirmed that HHP obviously reduced the allergenicity of TMTp1.

The change tendency of IgG-binding reactivity was consistent with that of the IgE-binding, which suggested that HHP led to a reduction in the allergenic potential of TMTp1, and the lowest binding capacity was obtained at 400 and 600 MPa. The tested IgG-binding properties of the hydrolysates after SGF and SIF digestion also further confirmed the increase in digestibility after HHP. For SGF and SIF hydrolysates, the allergenicity response was reduced at pressure up to 200 MPa, and the lowest IgG responses of pepsin and trypsin digestive products were corresponded to 400 and 600 MPa, 200 and 400 MPa, respectively. Besides, the hydrolysates retained a low reactivity against IgG after trypsin treatments, even if no residual protein was observed in the Western blotting, suggesting the existence of epitopes in the digested fragments. To further evaluate the sensitivity of TMTp1 after HHP treatment and digestion, *in vivo* studies including skin prick and histamine release tests are required (Yu et al., 2011).

Previous studies have shown that HHP-induced structural changes of allergens, such as denaturation, aggregation, and crosslinking, could alter allergenicity by IgE-reactive conformational epitopes modification (Shriver and Yang, 2011). For TMTp1, as detected by the structure analysis, the pressure at 400 MPa was able to significantly alter structure properties (secondary structure, SH content, and hydrophobicity) and resulted in protein unfolding, which provided direct explanation for HHP induced reduction in the allergenicity. In case of other food allergen proteins, HHP over 200 MPa reduced the IgE-specific binding activity of α -amylase inhibitor (Yamamoto et al., 2010) and bovine gamma globulin (Yamamoto et al., 2009), mainly caused by the tertiary structural changes observed since there was no significant change in the secondary structure. When applied to soy protein isolate, HHP at 300 MPa for 15 min caused the allergenicity to decrease by 48.6% due to the changes in the secondary and tertiary structures (i.e.,

increase in free SH content and hydrophobicity) (Li et al., 2012). These differences also indicated that the allergen proteins microstructure and their allergenicity to pressure are affected by HHP conditions, including pressure level, time, temperature, rate of compression and decompression, and the type of the proteins.

In conclusion, by applying HHP treatment at 200 to 600 MPa, the TMTp1 from squid (*Todarodes pacificus*) exhibited a reduction in the allergenicity as demonstrated by *in vitro* simulated gastrointestinal digestion and ELISA. The pressures at 400 and 600 MPa were more effective in reducing IgE/IgG-binding reactivity than the lower pressure level at 200 MPa. At 200 MPa, change in the secondary structure was observed. The free SH contents and hydrophobicity were significantly altered at 400 MPa, indicating unfolding of the protein. However, higher pressure at 600 MPa did not cause further unfolding as compared to 400 MPa with decrease in the hydrophobicity. These modifications in protein conformations could affect IgE-reactive conformational epitopes and alter allergenicity. HHP treatments are potentially useful for commercial application for reducing the allergenicity and extending the shelf-life of processed squid.

However, the exact reduction mechanisms of the allergenicity by different HHP treatment conditions are still not fully understood, and further studies are necessary to identify the epitope sites in TMTp1 and to study their conformational changes during HHP process. And *in vivo* studies should also be conducted to verify the validity of the current *in vitro* allergenic studies in future work.

Transparency document

The Transparency document associated with this article can be found in the online version.

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