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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.

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 × *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 × *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 × *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 deg cm^2 $dmol^{-1}$. 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 (μM/g tropomyosin) = 73.53 A_{412} × 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 $10^6/(1.36 \times 10^4)$, $1.36 \times l0^4$ was the molar absorptivity, and $l0^6$ was conversion from the molar basis to the μM/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 μg/100 μL 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