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Analytical Methods

Effect of pH shifts on IgE-binding capacity and conformational structure of tropomyosin from short-neck clam (*Ruditapes philippinarum*)

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ABSTRACT

The aim of the present study was to assess pH-induced changes in conformational structures and potential allergenicity of tropomyosin from short-neck clams. As defined with circular dichroism (CD), an unfolded structure was found at pH values ranging from 2.0 to 5.0, followed by the loss of secondary structure at pH of 1.0. Correspondingly, surface hydrophobicity was reduced by 97.7% when pH was reduced from 7.0 to 1.0. Further indirect ELISA and dot-blot results of pH shifted tropomyosin showed that potential allergenicity correlated well with structural changes, as well as with SGF digestibility. Allergenicity decreased significantly with unfolding of the protein and was stable when surface hydrophobicity recovered back to neutral conditions. These results showed that conformational changes in tropomyosin induced by pH shifting significantly influenced the allergenicity of tropomyosin, and that the resulting changes occurred predominately in the acidic pH range.

1. Introduction

Molluscan shellfish contribute substantially to the world economy and human nutrition (Wild & Lehrer, 2005). Owing to its suitability for intensive farming, the short-neck clam (*Ruditapes philippinarum*) is one of the most popular species of farmed shellfish in China (Yan, Zhang, & Yang, 2006), Southeast Asia, and Japan (Takahashi, Kuroda, & Muroga, 2008; Umasuthan et al., 2013). However, the short-neck clam has also been recognized as one of the most frequent causes of food allergies in adults (Leung et al., 1996), with an estimated incidence of 6.2% in adults and 0.15% in children (Rance, Grandmottet, & Grandjean, 2005; Sicherer, Muñoz-Furlong, & Sampson, 2004; Taylor, 2008). The only way to eliminate food allergies due to calms is to avoid eating them entirely. Nonetheless, as a common food ingredient, short-neck clams are used in many foods or flavorings, and inadvertent exposure may lead to life-threatening hypersensitivity. The development of clam-based foods that are safe for allergic consumers, therefore, has become an urgent issue.

During protein processing, pH is believed to affect functional properties of protein, such as enhancing their ability to hold water or flavors, or ensure microbiological safety (Foh, Wenshui, Amadou, & Jiang, 2012). These effects are thought to be due to modifications of secondary, tertiary, or even quaternary structures of polypeptides (Nolsøe & Undeland, 2009). Such structural modifications may increase or decrease the allergenicity of peptides, depending on the severity of the treatment. While there has been a significant amount of research on pH-induced functional and structural modifications, information on pH-induced changes in allergenicity is still very limited. Wu, Xu, Wang, and Liu (2010) reported that the immunogenicity of *Ctenopharyngodon idellus* allergens was reduced following acid treatment. However the report did not characterize the changes in allergens during acid treatment. Further research should be done to clarify how pH changes can alter allergenicity.

Tropomyosin is the major allergen of short-neck clams, as well as several other species of shellfish (EFSA, 2006; Song et al., 2015)

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and mollusks specifically (Leung et al., 1996). The effect of food processing on the allergenicity of tropomyosin has been studied. These studies focused primarily on thermal processes or non-thermal processes such as high hydro pressure and irradiation (Li, Lin, Cao, & Khalid, 2006; Lv et al., 2014). It has been reported that the conformational stability of tropomyosin, to some extent, depends on hydrogen bonds and Van der Waals forces. Some researchers have suggested that conformational changes in myofibrillar proteins during alkaline processing can expose additional functional groups for bonding (Liu et al., 2010). All of this research suggests that pH-induced structural changes can alter the functional properties of tropomyosin, including its allergenicity (Bernardi et al., 2010). However, there has been very limited information to indicate how changes in pH can alter allergenicity in tropomyosin of short-neck clams.

In the present work, the effect of pH shifts on IgE antibody binding and on the conformational structure of tropomyosin was investigated. This report suggests a possible mechanism for changes in tropomyosin. IgG and IgE binding properties were evaluated with indirect enzyme-linked immunosorbent assay (ELISA) and dot blot using rabbit polyclonal antibodies and sera from patients with clam allergies. Digestibility of allergens was assessed with a simulated gastric fluid (SGF) digestion assay using pepsin, and conformational changes were estimated by circular dichroism (CD), surface hydrophobicity, and ultraviolet (UV) absorption assays.

2. Materials and methods

2.1. Materials and reagents

Both purified tropomyosin from short-neck clam (*R. philippinarum*) and rabbit serum against tropomyosin which was evaluated by ELISA were prepared in the food safety laboratory, Ocean University of China. Bovine serum albumin (BSA), 3,3′,5,5′-tetram ethylbenzidine (TMB) and 1-anilino-8-naphthalene-sulfonate (ANS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The precision protein standard and porcine pepsin (1545 U/mg) were purchased from Solarbio Co. (Beijing, China). Enhanced chemiluminescent (ECL) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Secondary antibodies coupled with horseradish peroxidase (HRP) were from Zhongshan Jinqiao Co. (Beijing, China). Unless otherwise indicated, all chemicals and reagents were of analytical grade.

2.2. Patients' sera

Human sera were collected from patients with clam allergies. The patient had a documented clinical history of clam allergy; a positive skin prick test (SPT) to clam and the specific IgE levels to allergen were above 5 kU/L determined by Allergy Screen (MEDIWISS Analytic GmbH, Moers, Germany). Sera stored at −80 °C were provided by the Affiliated Hospital of Medical College of Qingdao University, Qingdao. Before sera collecting, all the patients signed written consents to make sure that they are aware of usage of sera. The collecting protocol has been approved by medical ethics committee to fully protect the rights and interests of the subjects (File No., QD-PF-20140314551).

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The tropomyosin treated with different pH was analyzed with SDS–PAGE according to Wang's methods (Wang, Lin, Sui, & Cao, 2013). Samples (5 μg) were reduced by heating with loading buffer and loaded into each well of the SDS–PAGE gel (12% for running gel, 5% for stacking gel). Precision protein standard was used to evaluate molecular weight of samples. After running for 1.5 h at 100 V, the gel was visualized with Coomassie Brilliant Blue and captured by a Tanon-4200SF (Tanon Science & Technology Co., Ltd., Shanghai, China).

2.4. Immunological characters

2.4.1. Indirect ELISA

IgG/IgE binding capacity was evaluated by Indirect ELISA with rabbit sera against tropomyosin or pooled sera from clam allergic patients following previously reported method (Iwan et al., 2011). Carbonate coating buffer (CBS, pH 9.6) containing 1 μg sample was introduced into each well of the plate and incubated overnight at 4 °C. The plate wells were blocked with 5% BSA in PBST for 2 h at 37 °C. Either rabbit anti-tropomyosin sera (1:100,000 dilution with blocking buffer, v/v) or pooled patients sera (1:20 dilution with blocking buffer, v/v) was loaded for 1.5 h incubation at 37 °C, followed by another 1.5 h incubation with corresponding HRP-labeled goat anti-rabbit IgG (1:5000 dilution with blocking buffer, v/v) or HRP-labeled goat anti-human IgE (1:1000 dilution with blocking buffer, v/v). After adding TMB, the optical density (OD value) was measured with a microplate reader (MultiscanMK3, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 450 nm.

2.4.2. Dot-blot assay

To further evaluate the IgG/IgE binding capacity, dot-blot assay was performed according to the Zheng with some modifications (Zheng, Lin, Pawar, Li, & Li, 2011). In brief, 3 μL of phosphate buffered saline (PBS, pH 7.0) containing samples at a concentration of 0.5 mg/mL were spotted on nitrocellulose (NC) membranes (Millipore Corporation) and incubated for 2 h at 37 °C in blocking buffer (PBST containing 5% non-fat dry milk) to block the non-specific binding sites. Then the membranes were incubated with human sera (1:10 dilution with blocking buffer, v/v) or rabbit sera (1:100,000 dilution with blocking buffer, v/v) for 1.5 h. After incubation for 40 min with HRP-labeled goat anti-rabbit IgG (1:5000 dilution with blocking buffer, v/v) or HRP-labeled goat anti-human IgE (1:500 dilution with blocking buffer, v/v), the immunoassay was photographed by a Tanon-4200SF gel imaging system with ECL. The intensity of spot indicates the amount of specific IgG/IgE bound to tropomyosin.

2.5. Digestion stability

Tropomyosin exposed to different pH was evaluated with SGF according to the method described by Shingo et al. (Fujita et al., 2012) with some modifications. Briefly, the volume of digestion system was 300 μL. A ratio of porcine pepsin (1545 U/mg) to the tropomyosin was 1:50 (w/w). SGF digestion assay was performed at 37 °C and rocked at different intervals. The reaction of rocked samples was immediately terminated by addition of 50 μL of 500 mM Na_2CO_3 at 0 °C and 50 μL loading buffer.

Samples were heated at 95 °C for 10 min and analyzed by SDS–PAGE. For 0 min digestion sample, tropomyosin was mixed with SGF which had been inactivated by 500 mM Na_2CO_3 and did the following SDS–PAGE analysis. The control samples was dissolved in reaction buffer without pepsin and treated as described above. The final concentration of the protein samples was 0.25 mg/mL. The activity of pepsin was analyzed by the described method by Cao et al. (2000).

2.6. Structure characters

2.6.1. Circular dichroism analysis

CD spectra of tropomyosin with different pH treatment were determined at a concentration of 0.5 mg/mL. Following previous