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Paramyosin of the disc abalone *Haliotis discus discus*: Identification as a new allergen and cross-reactivity with tropomyosin

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ABSTRACT

Tropomyosin, a 35–38 kDa myofibrillar protein, represents a major allergen in molluscs, as well as in crustaceans. Besides tropomyosin, a 100 kDa allergen was newly detected in the disc abalone *Haliotis discus discus*. The 100 kDa allergen was purified from the muscle of the disc abalone by salting-out and hydroxyapatite HPLC and identified as paramyosin based on the determined amino acid sequences of the peptide fragments produced by lysylendopeptidase digestion. Based on analysis by fluorescence ELISA, as many as 16 of the 18 patient sera tested, reacted to the disc abalone tropomyosin. The same patient sera also reacted to the disc abalone paramyosin, although rather less potently than to tropomyosin, suggesting that paramyosin is a major allergen. Immunoblotting data showed that IgE-reactive paramyosin is distributed in some species of molluscs other than the disc abalone. Interestingly, cross-reactivity between paramyosin and tropomyosin was demonstrated by inhibition immunoblotting and inhibition ELISA.

1. Introduction

Food allergy mediated by immunoglobulin E (IgE) is a public concern in industrialised countries. In sensitised subjects with high levels of specific IgE to a certain food, adverse reactions such as urticaria, asthma, diarrhoea and anaphylaxis are induced immediately after ingestion of the food; in severe cases, anaphylactic shock leads to even death. Of a number of allergenic foodstuffs, seafoods (fish, crustaceans and molluscs) are recognised as an important cause of food allergy especially in coastal countries, where their consumption is high. Previous studies showed that parvalbumin, a 12 kDa sarcoplasmic protein, is a major cross-reactive allergen in fish (Lehrer, Ayuso, & Reese, 2003; Van Dô, Elsayed, Florvaag, Hordvik, & Endresen, 2005; Wild & Lehrer, 2005). Besides parvalbumin, collagen has been additionally identified as a fish allergen although not major (Hamada, Nagashima, & Shiomi, 2001; Sakaguchi et al., 2000). As for crustaceans and molluscs, tropomyosin, a 35–38 kDa myofibrillar protein, is a common major allergen (Chuo, Wong, & Leung, 2000; Emoto, Ishizaki, & Shiomi, 2009; Leung et al., 1996; Motoyama, Ishizaki, Nagashima, & Shiomi, 2006; Motoyama, Suma, Ishizaki, Nagashima, & Shiomi, 2007; Reese, Ayuso, & Lehrer, 1999; Wild & Lehrer, 2005). IgE cross-reactivity of tropomyosin can be seen amongst crustaceans, amongst molluscs, between crustaceans and molluscs, and even between crustaceans and terrestrial arthropods, such as cockroaches and mites (Arlian, Morgan, Vyszenski-Moher, & Sharra, 2009; Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002). Recently, arginine kinase (García-Orozco, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, & Sotelo-Mundo, 2007; Yu, Lin, Chiang, & Chow, 2003), a sarcoplasmic calcium-binding protein (Ayuso et al., 2009; Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008) and myosin light chain (Ayuso et al., 2008) have also been confirmed to be new allergens in crustaceans.

To prevent allergic accidents, labelling systems for processed food products containing allergenic food materials have recently been established in some countries. In Japan, 25 kinds of food materials are considered to be especially allergenic, and therefore are obligated or recommended to put labels on packages or bottles of processed food products containing them as raw materials. Abalone, together with other six kinds of seafoods (salmon, mackerel, shrimp, crab, squid and salmon roe), is amongst the 25 kinds of allergenic food materials. Molecular studies with two species of abalones, the Japanese abalone *Haliotis diversicolor* (Chuo et al., 2000), and the disc abalone *Haliotis discus discus* (Emoto et al., 2009), proved that their major allergen is tropomyosin, as in the other molluscs. During our study for allergens in disc abalone by immunoblotting, however, not only tropomyosin but also a 100 kDa allergen was found to be allergenic. This finding prompted us to purify and identify the 100 kDa allergen in the disc abalone. As a result, the 100 kDa allergen was demonstrated to be paramyosin by partial amino acid sequencing of the purified preparation. Subsequent ELISA experiments showed that paramyosin, as well as tropomyosin, is a major allergen in disc abalone. Interestingly,

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the IgE cross-reactivity between paramyosin and tropomyosin was also verified by inhibition experiments. We report here the identification of the 100 kDa allergen in the disc abalone, as paramyosin, and the cross-reactivity of paramyosin with tropomyosin.

2. Materials and methods

2.1. Animal samples

Live or fresh specimens of the disc abalone, turban shell *Turbo cornutus*, Mediterranean mussel *Mytilus galloprovincialis*, Yesso scallop *Patinopecten yessoensis*, Japanese flying squid *Todarodes pacificus,* and common octopus *Octopus vulgaris,* were purchased at the Tokyo Central Whoelsale Market. Columellar muscle was collected from disc abalone, foot muscle from turban shell and Mediterranean mussel, adductor muscle from Yesso scallop, mantle muscle from Japanese flying squid, and leg muscle from common octopus. All muscle samples were stored at −20 °C until use.

2.2. Preparation of crude extracts

Each muscle sample was ground to powder in liquid nitrogen and homogenised with four volumes of 0.9 M NaCl–0.01 M phosphate buffer (pH 6.8). A part of the homogenate was centrifuged at 18,000*g* for 20 min, and the supernatant obtained was used as a non-heated extract. The remaining homogenate was heated in a boiling water bath for 10 min, similarly centrifuged, and the supernatant was regarded as a heated extract. Both non-heated and heated extracts were subjected to immunoblotting to examine whether the 100 kDa allergen under study is heat-stable or not.

2.3. Purification of tropomyosin

The disc abalone tropomyosin was purified from the muscle as reported in our previous paper (Motoyama et al., 2007). In brief, an acetone powder of myofibrillar proteins prepared from the muscle was extracted with 0.025 M Tris–HCl buffer (pH 8.0), containing 1 M KCl, 0.1 mM $CaCl_2$ and 0.1 mM dithiothreitol (DTT). The extract was subjected to salting-out with ammonium sulphate (30–60% saturation), followed by isoelectric precipitation (pH 4.6). Finally, small amounts of contaminants were removed by reverse-phase HPLC. The homogeneity of the final preparation was supported by SDS–PAGE.

2.4. Purification of a 100 kDa allergen

The muscle (5 g) of disc abalone was homogenised with 20 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.15 M NaCl and 0.5 mM cysteine. After centrifugation at 18,000*g* for 20 min, the supernatant obtained was regarded as a sarcoplasmic protein fraction. The residue was washed twice with the above solvent and shaken vigorously with 25 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.9 M NaCl and 5 mM DTT at 4 °C, for 14 h. Then, $MgCl_2$ and ATP were added to the solution until a final concentration of 1 mM for each was reached, and shaken again for 1 h. After ultracentrifugation at 117,000*g* for 1 h, the supernatant obtained was used as a myofibrillar protein fraction. To purify the 100 kDa allergen, the myofibrillar protein fraction was first subjected to salting-out using ammonium sulphate. The precipitate at a 10–20% saturation of ammonium sulphate was dissolved in 10 ml of 0.01 M phosphate buffer (pH 6.8) containing 0.9 M NaCl, 5 mM DTT, and 0.01% thimerosal, and was applied to hydroxyapatite HPLC on a Bio-Scale CHT2-I column (0.7 × 5.2 cm; Bio-Rad Laboratories, Hercules, CA, USA). Elution was achieved with a linear gradient of 0.01–0.24 M phosphate buffer (pH 6.8) containing 0.9 M NaCl at a flow rate of 1 ml/min. The proteins were monitored at 280 nm with a UV detector and the eluate containing the 100 kDa allergen was collected.

2.5. Enzymatic digestion and isolation of the peptide fragments

The purified 100 kDa allergen (150 μg) was digested with 1.5 μg of lysylendopeptidase (Wako Pure Chemicals, Osaka, Japan) in 0.5 ml of 0.025 M Tris–HCl buffer (pH 8.5) containing 1 mM EDTA, 4 M urea and 0.02 M ethanolamine at 37 °C, for 18 h. To isolate the peptide fragments produced, the digest was applied to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 × 25 cm; Tosoh, Tokyo, Japan), which was eluted at a flow rate of 1 ml/min by a linear gradient of acetonitrile (0–70% in 120 min) in 0.1% trifluoroacetic acid. Peptides were monitored at 220 nm with a UV detector.

2.6. Amino acid sequencing

Amino acid sequencing was performed by the automated Edman degradation method using a Procise 492HT protein sequencer (Applied Biosystems, Foster City, CA, USA).

2.7. Human sera

Sera were obtained from 18 crustacean-allergic patients (patients 1–18) with clinical histories of immediate hypersensitivity reactions after ingestion of crustaceans. All patients were diagnosed to be allergic to crustaceans by the capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST) performed at hospitals; the determined CAP-RAST classes were 2–6 against shrimp, or both shrimp and crab. Patients 1, 2, and 12–14 were additionally shown to have CAP-RAST classes of 3–6 against molluscs. Written informed consent was obtained from each patient. In this study, sera from 19 healthy volunteers without adverse reactions after ingestion of any foods were used as controls. All sera were stored at −20 °C until use.

2.8. Fluorescence ELISA and inhibition ELISA

Fluorescence ELISA was performed as reported previously (Hamada et al., 2004). In brief, a polystyrene microtiter plate with 96 wells (Type H (black); Sumitomo Bakelite, Tokyo, Japan) was coated with 50 μl of the 100 kDa allergen or the disc abalone tropomyosin solution (1 μg/ml) and reacted successively with the patient or control serum (diluted 1:200) and β-galactosidase-conjugated goat anti-human IgE antibody (0.25 μg/ml; American Qualex, San Clement, CA, USA). The enzyme reaction was carried out using as substrate a solution of 0.1 mg/ml 4-methylumbelliferyl-β-D-galactoside, and was stopped by addition of 0.1 M glycine-NaOH buffer (pH 10.3). Fluorescence units were measured with excitation and emission wavelengths at 367 and 453 nm, respectively. For inhibition ELISA, patient serum (diluted 1:100) was preincubated with an equal volume of inhibitor (100 kDa allergen or disc abalone tropomyosin) solution (0.002–20 μg/ml) at 37 °C for 2 h, and then used as a primary antibody. All ELISAs (including inhibition ELISAs) were performed in triplicate, and the data obtained were expressed in mean ± SD.

2.9. SDS–PAGE

SDS–PAGE was performed on a PhastSystem apparatus (GE-Healthcare, Buckinghamshire, UK) using ready-made gels (PhastGel Gradient 8-25; GE-Healthcare) and ready-made buffer strips (PhastGel SDS Buffer Strips; GE-Healthcare), according to the