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Purification and characterisation of sarcoplasmic calcium-binding protein, a novel allergen of red swamp crayfish (*Procambarus clarkii*)

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ABSTRACT

Crayfish sarcoplasmic calcium-binding protein (SCP) was purified. The physicochemical and polymorphic characterisations were also analysed. SCP was purified by column chromatography to reveal a single band with molecular mass of 22 kDa and further confirmed by mass spectrometry. The results of physicochemical characterisation showed that SCP was stable in the processes of thermal or acid/alkali treatment, and could be digested by simulate gastrointestinal fluid. Importantly, the comparison of SCP polymorphism using sera from crustacean-allergic patients demonstrated SCP-II had a weaker IgE-binding activity. The isoelectric points of SCP subunits a, b and c were 4.6, 4.7, and 4.8, respectively, as determined by two-dimensional electrophoresis and IgE immunoblotting analysis showed that patients' sera reacted to three subunits of SCP. Finally, it can be concluded that SCP is a stable polymorphic allergen in crayfish, and all of its isotypes and subunits have allergenicity.

1. Introduction

There is a high consumer demand for aquatic products, particularly in the coastal areas of China, because of their delicious taste and nutritional value. These products are, however, also well known as one of the most common causes of food allergy. Fish and crustaceans are two of the eight major sources of allergenic food proposed by the Food and Agriculture Organisation (FAO) of the United Nations and the World Health Organisation (WHO) (FAO/WHO., 2001). The increasing production and consumption of aquatic products has resulted in more hypersensitive reactions, most which are mediated by IgE antibodies and manifest as urticaria, asthma, diarrhea, and even life-threatening anaphylaxis (Lopata, O'Hehir, & Lehrer, 2010). Allergy to aquatic products has become an important public health issue.

Various worldwide studies on the allergens of aquatic products that cause IgE-mediated hypersensitivity have been reported. Parvalbumin, a 12 kDa protein, is the major allergen in fish (Elsayed & Aas, 1971; Guo, Kubota, & Shiomi, 2011). Collagen and its subunits have also been reported as potential allergens that cause allergy reactions to various fish (Hamada, Nagashima, & Shiomi, 2001; Pan et al., 2012). Tropomyosin, a thermally stable myofibrillar protein consisting of two subunits with molecular masses of 36–38 kDa, has been identified as the major allergen in molluscs and crustaceans (Leung et al., 1996; Shanti, Martin, Nagpal, Metcalfe, & Rao, 1993). Arginine kinase, which is normally involved in cell metabolism in invertebrates, is a 40 kDa water-soluble protein found in myosinogen and has been well-documented as an important cross-reactive allergen in crustaceans (Yu, Lin, Chiang, & Chow, 2003). In recent years, myosin light chain (Ayuso et al., 2008), hemocyanin (Piboonpocanun, Jirapongsananuruk, Tipayanon, Boonchoo, & Goodman, 2011), and sarcoplasmic calcium-binding protein (SCP) have been reported to be the novel allergens of shrimp.

SCP was identified as a shrimp allergen in *Penaeus monodon* (Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008) and its IgE-binding activity was demonstrated at the molecular level in *Litopenaeus vannamei* (Ayuso et al., 2009). The water-soluble SCP protein is found in myosinogen and functions as an invertebrate EF-hand calcium-binding protein. There is also some evidence to indicate that it plays a role in muscle relaxation, similar to that of parvalbumin in vertebrates (Hermann & Cox, 1995). One of the typical properties of parvalbumin and SCP is their polymorphism (Liu et al., 2010; Wnuk & Jauregui-Adell, 1983). It has been asserted that for an allergen to retain its allergenicity, it must be stable enough to survive food processing treatments and *in vivo* digestion after consumption. Although SCP is of particular interest in that it has similar characteristics to arginine kinase, collagen, and parvalbumin, it is nevertheless important to study the physicochemical and polymorphic characteristics of this protein.

Due to its worldwide popularity as a gourmet food, the red swamp crayfish (*Procambarus clarkii*), which is native to south-central United States and Northeastern Mexico, has been artificially

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propagated for a number of years. With increasing consumption of crayfish, cases of hypersensitive reactions after its ingestion have been reported. Crustacean-sensitive patients in the United States had a high prevalence of skin test positivity to crayfish extract (70%) (Daul, Morgan, & Lehrer, 1993). *P. clarkii* is one of the important economic species in China and its total production in 2010 was around 563,281 tons (Anonymous., 2011). A survey of 182 children suffering from urticaria in China indicated that 58.79% of subjects had an allergic reaction to aquatic products containing crayfish, which suggests that crayfish is an important allergenic food (Wang & Yu, 2009). It is still uncertain whether SCP is the allergen in *P. clarkii* that causes IgE-mediated hypersensitivity.

The aim of this study is to purify crayfish SCP, confirm its allergenicity, and further analyse its physicochemical and polymorphic characteristics. Then it will make the contribution to the future research of the desensitisation investigation of crayfish.

2. Materials and methods

2.1. Materials

2.1.1. Animal material

Fresh red swamp crayfish (*P. clarkii*), blue scad (*Decapterus maruadsi*), crucian carp (*Carassius auratus*), manila clam (*Ruditapes philippinarum*), Pacific white shrimp (*L. vannamei*), and mud crab (*Scylla paramamosain*), were purchased from a local market in Xiamen. Muscle samples were immediately removed and used for immediate experimentation or stored at −80 °C for later use.

2.1.2. Chemicals

Chemicals were purchased from various suppliers: Q-Sepharose and Sephacryl S-200 HR from Amersham Bioscieces (Uppsala, Sweden); protein standards for SDS–PAGE from Fermentas (Vilnius, Lithuania) or New England BioLabs (Beverly, USA); periodic acid-Schiff (PAS) staining kit from Genmed (Grand Rapids, USA); horseradish peroxidase (HRP)-conjugated goat anti-human IgE antibody from Kirkegaard and Perry Laboratories (Gaithersburg, USA); HRP-conjugated goat anti-rabbit IgG antibody and the enhanced chemiluminescent (ECL) substrate for immunoblotting from Pierce (Rockford, USA); 3,3′,5,5′-Tetramethylbenzidine (TMB) from Tiangen (Beijing, China), and porcine pancreatic juice from Sigma–Aldrich (St. Louis, USA). Rabbit anti-crayfish polymorphic SCP polyclonal antibody and porcine pepsin were prepared in our laboratory. All other reagents were of analytical grade.

2.1.3. Human sera

Sera were obtained from 17 crustacean-allergic patients (No. 475, 362, 112, 252, 303, 555, 743, 468, 354, 336, 083, 300, 384, 282, 437, 221, 347) provided by the Hospital of Jimei University. Crustacean allergy was confirmed on the basis of extensive history, physicochemical examination and objective manifestations observed after ingestion of crustacean products. Written informed consent was obtained from each patient. Sera from healthy individuals (No. 307, 776) without a history of crustacean allergy were pooled and used as a negative control. All sera were stored at –80 °C until use.

2.2. Myosinogen preparation and immunoassay

Crayfish muscle tissue was minced and homogenized with five volumes of ice-cold 20 mM Tris–HCl buffer (pH 7.0). The homogenate was then centrifuged at 7200 rpm for 20 min at 4 °C. The supernatant was the crayfish myosinogen.

SDS–PAGE was performed. Samples were separated on 15% polyacrylamide gels with 5% stacking gels. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 (CBB).

The separated sample was transferred to a nitrocellulose membrane, after which Western-blotting was carried out as described by Huang et al. (2010). Human serum (1:4 dilution) was used as the primary antibody, and HRP-labelled goat anti-human IgE antibody (1:20000 dilution) as the secondary antibody. Blot was visualized using ECL.

2.3. Protein purification and peptide analysis by matrix-assisted laser desorption ionisation mass spectrometry (MALDI MS)

The 22 kDa protein was purified according to the method of Yu et al. (2003) and Shiomi et al. (2008), with some modifications. All procedures were performed on ice or at 4 °C. The muscle tissue of crayfish was minced, then subjected to homogenization and centrifugation, and the supernatant fractionated with ammonium sulphate from 70% to 90% saturation. The resulting precipitate was dissolved and dialysed before being subjected to a Q-Sepharose column. The binding proteins were eluted with a linear gradient of 0–0.3 M NaCl and subsequently eluted with buffer (10 mM Tris–HCl, pH 8.0) containing 0.3 M NaCl at a flow rate of 1 ml/min. The fractions with SCP were concentrated and then applied to a Sephacryl S-200 HR gel column. The column was eluted at a flow rate of 0.4 ml/min and fractions with purified SCP were collected and identified. Protein concentration was estimated by measuring the absorbance of the sample solution at 280 nm.

The purified protein was migrated on 15% SDS–PAGE gel and CBB stained for mass spectrometry analysis. The target protein area was selected and sent to Shanghai Applied Protein Technology Co. Ltd. (Shanghai, China) for analysis by MALDI MS. Mass spectrometric conditions were as follows: laser source: ND; wavelength: 355 nm; accelerating voltage: 2 kV; and substrate: α-cyano-4-hydroxycinnamic acid (α-CHCA). The positive ion mode was adopted and the data were obtained automatically. Results were compared with the data base of NCBI.

2.4. Physicochemical characterisation of SCP

2.4.1. Thermal and pH stability and dot-blotting

In the study on thermal stability, purified SCP was incubated at different temperatures (30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C, and 100 °C) for 30 min. For pH stability, SCP was incubated in buffers with different pH values (pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0) for 1.5 h at room temperature. After incubation, all the samples were analysed by SDS–PAGE and dot-blotting.

For dot-blotting, the purified SCP, treated at different temperatures and different pH values, was directly blotted on nitrocellulose membrane and incubated with pooled human sera (1:4 dilution) after blocking. Following incubation with HRP-labelled goat anti-human IgE, the immunoassay was carried out using ECL.

2.4.2. Simulated digestion stability and inhibition ELISA

The digestibility of purified SCP in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was examined according to the method of Huang et al. (2010) with some modifications. The total volume of the reaction solution was 1 ml. The ratio of pepsin to purified SCP was 1:1000 (w:w). At different time intervals (0, 1, 2, 5, 10, 15, 30, and 60 min) a 100 μl aliquot of the reaction solution was removed and terminated by adding 30 μl of 200 mM Na_2CO_3. For intestinal digestion, the ratio of pancreatic juice to purified SCP was 1:1000 (w:w). The reaction interval times were 0, 1, 15, 30, 60, 120, 180, and 240 min and the reactions were immediately terminated by heating at 95 °C for 5 min. A protein sample, dissolved in