Sarcoplasmic calcium-binding protein is an EF-hand–type protein identified as a new shrimp allergen

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Background: Shellfish allergy is a long-lasting disorder usually persisting throughout life. Despite its high prevalence, there is limited information about allergenic shrimp proteins.
Objective: Characterization of shrimp allergens.
Methods: Fifty-two adults and children with a history of immediate allergic reactions to shrimp and elevated serum IgE to shrimp were selected for this study. Tryptic digests from a 20-kd IgE-binding protein were analyzed by LC-MS/MS, identifying the protein as a sarcoplasmic-calcium-binding protein. cDNA encoding sarcoplasmic calcium-binding protein (SCP) from a shrimp cDNA library (*Litopenaeus vannamei*) was amplified by PCR, cloned into an expression vector, and sequenced. Recombinant SCP was tested with patients' sera. ELISA inhibition experiments determined the fraction of total shrimp IgE recognizing SCP. A functional assay with a rat basophilic leukemia cell line was used to determine the capacity for mediator release induced by SCP.
Results: Immunoblotting demonstrated IgE binding by 31 of 52 (59.6%) of the sera to a 20-kd shrimp protein. The protein was identified as a SCP. Amplified cDNA encoding SCP was isolated and sequenced. Open reading frame translation provided the complete amino acid sequence of shrimp SCP. Recombinant SCP was recognized by serum IgE from 20 of 52 (38.4%) subjects, of whom 17 of 20 (85%) were children. ELISA inhibition of pooled sera IgE reactivity to BS extract using recombinant SCP was significant (as high as 79%). For some subjects, mediator release induced by recombinant SCP was higher than that induced by recombinant tropomyosin.
Conclusion: We have identified and cloned a new shrimp allergen, Lit v 4.0101, an SCP, which appears to be of particular importance in the pediatric population. (J Allergy Clin Immunol 2009;124:114-20.)

Key words: *Allergen, Crustacea, shellfish, shrimp, recombinant, Lit v 4, sarcoplasmic calcium-binding protein*

Shellfish allergy is a long-lasting and potentially life-threatening disorder.[1] Most shellfish species provoking allergic reactions belong to the class Crustacea, which includes shrimp, prawn, crab, lobster, and crawfish.[2] A recent survey by Sicherer et al[2] found that 1 in 50 Americans had shellfish allergy. Shellfish are the number 1 cause of food allergy in adults in the United States and are responsible for the majority of emergency department visits for food allergy, not only in adults but also in children 6 years of age and older, and a significant cause of allergic reactions in children 1 to 5 years old.[3-5]

A large variety of crustaceans are used for human consumption. Although the black tiger shrimp *(Penaeus monodon)* is the most widely cultured prawn species in the world, the Pacific white shrimp (*Litopenaeus vannamei)*, actually a prawn, is the species of choice in the shrimp farming industry in the Western hemisphere. Together these 2 species account for 80% of all farmed shrimp. Shrimp consumption has more than tripled since 1970, and it is expected that allergy to shellfish will continue to increase. Therefore, a better understanding is needed of shrimp proteins involved in the development of allergic reactions.

Until recently, the muscle protein tropomyosin was the only major cross-reactive allergen identified in different shrimp species.[6,7] In previous studies, shrimp tropomyosin inhibited 80% of patients' IgE RAST reactivity to whole-body shrimp extract, indicating that tropomyosin is responsible for most of the allergenic activity of shrimp.[6] However, we have recently described myosin light chain (MLC), Lit v 3, as a new major allergenic shrimp protein, particularly in children.[8] Although IgE recognition of MLC in boiled shrimp (BS) extract was very intense, the recombinant protein was significantly less recognized. Although this may be a result of posttranslational modifications present in the native form, the possibility of another IgE-binding protein of similar MW was considered.

In spite of the high prevalence of shellfish allergy, few options are available for treatment, and avoidance is the only therapy recommended. However, the frequency and severity of reactions after accidental exposure to shellfish make it necessary to develop improved diagnostic and therapeutic options for shellfish allergy. Recombinant allergenic proteins are now considered the basis for new diagnostic approaches and novel strategies of allergen-specific immunotherapy.[9-12] Therefore, we focused on the characterization and production of new recombinant shrimp allergens to

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Abbreviations used
BS: Boiled shrimp
MLC: Myosin light chain
LC-MS/MS: Liquid chromatography mass spectrometry of maximum sensitivity
MW: Molecular weight
r: Recombinant
RBL: Rat basophilic leukemia
SCP: Sarcoplasmic calcium-binding protein

be used as diagnostic and immunotherapeutic agents for subjects with shellfish allergy.

METHODS

Patient selection

Sera were obtained from 52 subjects with shrimp allergy, 23 children (44.2%) age 3 to 18 years (mean, 10.2 years) and 29 adults (55.7%) age 19 to 70 years (mean, 30.9 years), with immediate allergic reactions after the ingestion of shrimp, and elevated serum IgE to shrimp. Subjects 1 to 38 were characterized and used in a previous study,[8] and 14 new patients were recruited (9 children and 5 adults). Shrimp-specific IgE levels were determined by UniCAP (Phadia, Uppsala, Sweden) and considered positive if greater than 0.35 kU_A/L. Patients' sera were collected from the Hospital del Niño Jesús, Madrid, Spain; Hospital Universitario Dr Negrín, Las Palmas de Gran Canaria, Spain; and Mount Sinai Medical Center, New York. Patient characteristics did not differ significantly from those described in our previous article.[8] This study was approved by the Institutional Review Board of the Mount Sinai Medical Center.

Shellfish extract preparation

Extracts were prepared from raw and boiled tail muscle of the Pacific white shrimp (*L vannamei)* as described previously.[8] Raw crab abdominal muscle, lobster tail, squid, mussel, and scallop extracts were boiled for 5 minutes in distilled water and manually homogenized in a mortar. Protein was extracted by agitation in PBS with protease inhibitor cocktail without EDTA (Roche, Indianapolis, Ind). NaN_2 in distilled water (20% wt/vol) was added (1:400) as preservative and incubated overnight at 4°C. The mixture was centrifuged at 4°C at 3000 rpm for 10 minutes and then at 14,000 rpm for 5 minutes. The protein concentration was determined with a Coomasie Plus Protein Assay (Pierce, Rockford, Ill). Extracts were stored at –20°C. Chick pea extract was prepared as described previously.[8]

SDS-PAGE and 2-dimensional analysis

Proteins were separated by SDS-PAGE (Nupage 4% to 12% Zoom Gels; Invitrogen, Carlsbad, Calif) following the manufacturer's instructions. Protein was loaded at a concentration of 12.5 μg protein/cm gel. Two-dimensional electrophoresis was performed as described previously.[13] Gels were stained with Simply Blue SafeStain (Invitrogen), or proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, Mass).[13] Membranes were stained with 0.1% Amido Black (10% methanol, 2% acetic acid) staining solution or tested for IgE binding with patients' sera.

Protein identification was performed from 1-dimensional and 2-dimensional gels stained with Simply Blue SafeStain.[13] A 20-kd protein was excised, and in-gel digestion was performed. Sequence analysis of tryptic digests of the spot of interest (from a 2-dimensional gel) was performed at the Wistar Institute Proteomics Facility using microcapillary reverse-phase HPLC nano-spray tandem mass spectrometry on a ThermoFinnigan LTQ quadrupole ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, Mass). The mass spectrometer measures peptide masses and then fragments individual peptides to produce MS/MS spectra of fragments that reflect the peptide sequence. The MS/MS spectra are run against a nonredundant sequence database (National Center for Biotechnology Information [NCBI]) using the program SEQUEST (Thermo Fisher Scientific). If ≥3 peptide sequences in a database entry were matched by MS/MS spectra in this report, the protein identification had a high confidence level.

Immunoblot analysis with sera from subjects with shellfish allergy

Immunoblots for detection of IgE binding were performed with extracts of raw and boiled L. vannamei. Membranes were incubated with sera from patients with shrimp allergy (1:5 to 1:20 in PBS-Tween [1% BSA, 10% normal goat serum]) for 90 minutes. After rinsing with PBS, the membranes were incubated with iodine 125-labeled goat antihuman IgE (DiaMed, Windham, Me) for 1 hour, diluted as per the manufacturer's instructions, washed, and exposed to Kodak Imaging Film (Carestream Health Inc, Rochester, NY) for 1 to 12 days. As negative control, sera from 2 nonatopic subjects were used.

Molecular cloning of shrimp sarcoplasmic calcium-binding protein

The cDNA library generated from raw Pacific white shrimp in the Uni-ZAP XR vector system (Stratagene, La Jolla, Calif) was previously described.[8] cDNA encoding sarcoplasmic calcium-binding protein (SCP) was isolated from the library by means of PCR in 2 steps. First, two 5′-specific primers were designed based on sequences from *Penaeus monodon* SCP (AI253941; 5′-TATATGTACGACATTGACAAC-3′ and 5′-GATAAGAACGACTTC GAGTGC-3′) encoding the peptides YMYDIDN and DKNDFEC, respectively, identical to the homologous sequences in SCP α-B and α-A chains, and in the SCP β chain from *Penaeus species* (P02636, P02635). M13 Forward (-20) was used as the 3′-primer specific end for the cDNA library vector. PCR product was cloned into pCR2.1-TOPO vector (TOPO TA cloning kit; Invitrogen) and sequenced at the Mount Sinai Core Facility. Second, the missing 5′ end of the cDNA was amplified by PCR using the specific reverse primers (5′-TTG AACTGGTTGGCAATGAA-3′ and 5′-GTAAGCGTCATCAATCTCATTC-3′) based on the internal sequence from the cloned L. vannamei SCP cDNA obtained in the previous step, and the T3 forward primer from the cloning vector. The PCR product was ligated into TOPO vector and sequenced. The sequence analysis was performed with Vector NTI Advance 10 (Invitrogen) software.

Production of recombinant protein

Recombinant protein was obtained as described previously.[8] Briefly, the protein-coding region of SCP was amplified by PCR with specific primers, forward with Hind III restriction site (5′-AAAAAA GCT TAT GGC TTA CAG TTG GGA CA-3′) and reverse with Xho I site (5′-TAT TTC TCG AGC TGC ACC ACC TTC AGG GG-3′), and ligated into the expression vector pET24b(+) (Novagen, Madison, Wis). The vector-plasmid construct was transformed into *Escherichia coli* XL1 Blue strain according to the manufacturer's instructions (Stratagene, La Jolla, Calif). The plasmid was purified and introduced into the BL21 expression E. coli strain. Recombinant (r) SCP was expressed after induction of bacterial cultures with isopropyl β-D-thiogalactoside at 37°C for 16 hours, and detected by Western blot analyses with anti-His-Tag antibodies. Recombinant protein was purified with a His-Bind Ni2+chelating NTA-matrix resin (Novagen) under native conditions. Recombinant shrimp tropomyosin (rLit v 1) and recombinant hazelnut 11S storage protein (rCor a 9) were obtained from the Mount Sinai Food Allergen Repository.

Probing recombinant SCP with subjects' sera

Recombinant SCP was tested for IgE reactivity by immunoblotting with sera from 31 subjects with shrimp allergy that recognized a 20-kd protein. Three nonatopic adults were used as negative controls. Recombinant protein was loaded at a concentration of 2.5 μg protein/cm gel following the protocol described.

For immunoblot inhibition experiments, a serum pool was prepared from subjects 2 and 47, which recognized rSCP by immunoblot analysis with high intensity. Diluted serum pool (1/20 in PBS-Tween) was preincubated at room temperature for 2 hours with inhibitor at a concentration of 0.5 mg/mL.