# 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.

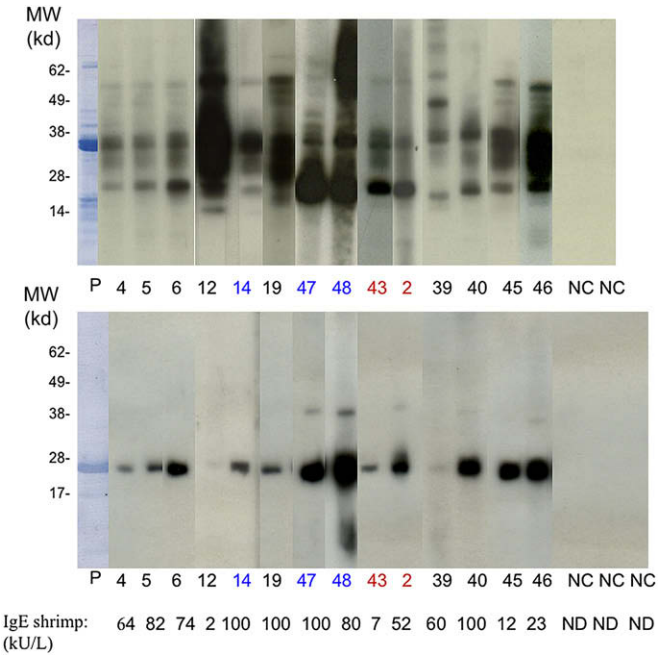


**FIG 1.** IgE Immunoblot of a representative group of 14 patients to BS extract **(A)** and rSCP **(B)**. *P,* Protein staining of total shrimp protein *(A),* rSCP *(B). Lanes,* Immunolabeling with 14 patients' sera; *NC,* negative controls (2 used in *A,* 3 in *B*). In *red* are marked subjects' sera used for ELISA experiments and in *blue* those used for the RBL assay. Levels of shrimp-specific IgE are noted in $kU_A/L$. *ND,* Not Done.

Extracts of dust mite Dermatophagoides farinae (Greer Lab Inc, Lenoir, NC), German cockroach Blatella germanica (Greer Lab Inc), BS, lobster tail muscle, crab, squid, scallop, and mussel were used as inhibitors, and chickpea extract as a negative control. The mix was then incubated with the membrane containing rSCP as described.

## ELISA inhibition

For the ELISA inhibition, 96-well plates were coated overnight at 4°C with 100 μL per well of BS extract (25 μg/mL) in carbonate-bicarbonate buffer 0.05 mol/L, pH 9.6 (Sigma-Aldrich, St Louis, Mo). Sera from subjects 2 and 43 (which recognized rSCP by immunoblotting), diluted 1:50 and 1:20 respectively in 2% BSA, 0.05% Tween Phosphate buffer saline were separately preincubated without inhibitor and with increasing concentrations (0.25 pg/mL to 25 μg/mL) of rSCP, rLit v 1, BS extract, or rCor a 9 (as negative control) for 1 hour at 37°C. Inhibition samples were added to the plate and incubated for 2 hours at room temperature. Allergen-specific IgE was detected with horseradish peroxidase–labeled goat antihuman IgE (KPL, Gaithersburg, Md) 1:2500, developed with tetramethylbenzidine peroxidase substrate (KPL), and read on a microplate reader at 650 nm. The percent inhibition values were calculated as follows: OD uninhibited – OD inhibited/OD uninhibited – OD buffer X100.

## Mediator release assay

The mediator release assay was performed as published[14,15] with rat basophilic leukemia (RBL)–2H3 cell line transfected with human Fcε receptor type 1. RBL cells were maintained in 75% Eagle's Minimum Essential Medium and 15% RPMI (Cellgro; Mediatech Inc, Herndon, Va) with 10% FCS (HyClone, Logan, Utah) and G418 sulfate (Acros Organics, Fair Lawn, NJ). RBLs were sensitized with pooled sera (1:20) of 3 patients and a nonatopic human serum in 96-well tissue-culture plates (BD Falcon, Bedford, Mass) at 37°C in 5% $CO_2$ for 18 to 20 hours. Cells were stimulated for 1 hour at 37°C in 5% $CO_2$ with 100 μL per well of the 1 μg/mL rSCP, rLit v 1, and BS extracts, followed by 10-fold serial dilutions in a release buffer containing $D_2O$ (Acros Organics). Rabbit IgG antihuman polyclonal IgE (Bethyl Laboratories Inc, Montgomery, Tex) was used as a positive control for IgE-mediated degranulation. β-Hexosaminidase was measured as a marker for mediator release. To determine β-hexosaminidase release, 30 μL supernatant was gently mixed with 50 μL P-nitrophenyl-N-acetyl-β-D-glucosaminide (pH 4.5; Sigma-Aldrich, St Louis, Mo) for 1 hour; the reaction was terminated by adding 100 μL 0.2 mol/L glycine solution (pH 10.7), and absorbance at 405 nm was measured. Total mediator release was obtained by lysing the cells with 1% Triton X-100 (Sigma). Results were expressed as percentage of release from cells sensitized with serum minus spontaneous release, divided by total release.

# RESULTS

## IgE reactivity of subjects' sera to L. vannamei extracts

Subjects with shrimp allergy showed IgE reactivity by immunoblotting to a variety of shrimp proteins ranging from 6 to 90 kd (Fig 1, *A*). A 20-kd protein was recognized in boiled extracts with particular intensity by 31 of 52 (59.6%) subjects' sera. In several cases, this protein was recognized with higher intensity than the 34-kd band corresponding to tropomyosin (Fig 1, *A*). Two control sera did not show any labeling.

## Protein identification by LC-MS/MS analysis of tryptic digests from a 2-dimensional gel

Sequence analysis of tryptic digests of the spot of interest identified the protein in question as an SCP (Table I). A number of 17 peptides matched SCP α-B and α-A chains (P02636), with sequence coverage of 59% and a spectral count of 187. Seven peptides matched SCP β chain (P02635), with sequence coverage of 31% and spectral count of 90. Results for LC-MS/MS analysis of 1-dimensional gel were similar to those from 2-dimensional gels and are not shown.

## Cloning and sequencing of shrimp SCP

The SCP gene was amplified from the shrimp cDNA library by PCR with 2 forward specific primers and a reverse primer, M13. A few clones for each PCR product were sequenced, encoding all the same SCP. Because the 5′ end of the open reading frame encoding the N-terminal part of the protein was absent, gene-specific reverse primers and T3 primer were used to obtain the missing 5′ end of the cDNA by PCR. Sequence analysis of these PCR products provided the complete cDNA encoding for SCP from *L vannamei* (GenBank accession no. FJ184279). This protein was designated Lit v 4.0101 allergen by the International Allergen Committee. The deduced amino acid sequence of Lit v 4.0101 and other SCP molecules are aligned in Fig 2, with the percentage of amino acid identity between them. The highest amino acid identity is seen with crustacean SCPs (80% to 93%), whereas identity with other arthropod and mollusk SCPs was low.

## IgE binding capacity of recombinant shrimp SCP and cross-reactivity with other arthropods and mollusks

Recombinant shrimp SCP was tested for IgE binding with individual sera from the 31 of 52 subjects (22 children and 9 adults) that recognized a 20-kd protein by immunoblot. Twenty of 31 (64.5%) subjects tested, representing 38.4% of the total (20/52), recognized the rSCP by immunoblot. Interestingly, 17 of 20 (85%) of these subjects were children. A sample of subjects with the highest IgE reactivities is shown in Fig 1, *A and B* (all the subjects are children except subject 39, who is an adult).

**TABLE I.** Protein identification by LC-MS/MS analysis of tryptic digests from a 2-dimensional gel

| Accession number P02636 | Protein name SCP α chain | Sequence coverage 59% | | Acc Nr P02635 | Protein name SCP β chain | Sequence coverage 31% | |
|---|---|---|---|---|---|---|---|
| **Peptide** | | **Position** | **Sequest score*** | **Peptide** | | **Position** | **Sequest score*** |
| YMYDIDDDGFLDK | | 14-26 | 0.92 | YMYDIDNDGFLDK | | 14-26 | 0.90 |
| YMYDIDDDGFLDKNDF ECLAVR | | 14-35 | 0.88 | YMYDIDNDGFLDKNDF ECLAVR | | 14-35 | 0.92 |
| NDFECLAVR | | 27-35 | 0.91 | NDFECLAVR | | 27-35 | 0.91 |
| GEFSAADYANNQK | | 43-55 | 0.90 | DGEVTVDEFK | | 73-82 | 0.88 |
| NLWNEIAELADFNKDG EVTVDEFK | | 59-82 | 0.86 | DGEVTVDEFKQAVQK | | 73-87 | 0.90 |
| NLWNEIAELADFNK | | 59-72 | 0.96 | EIDDAYDK | | 140-147 | 0.30 |
| DGEVTVDEFK | | 73-82 | 0.88 | YQELYAQFISNEDEK | | 164-178 | 0.99 |
| VFIANQFKAIDVNGDGK | | 103-119 | 0.87 | | | | |
| VFIANQFK | | 103-110 | 0.75 | | | | |
| AIDVNGDGK | | 110-119 | 0.53 | | | | |
| VGLDEYR | | 120-126 | 0.72 | | | | |
| SAFAEVKEIDDAYNK | | 133-147 | 0.95 | | | | |
| EIDDAYDK | | 140-147 | 0.30 | | | | |
| EIDDAYDKLTTEDDRK | | 140-155 | 0.71 | | | | |
| LTTEDDRK | | 148-155 | 0.54 | | | | |
| KAGGLTLER | | 155-163 | 0.79 | | | | |
| AGGLTLER | | 156-163 | 0.80 | | | | |

*Sequest score range, 0 to 1.

For immunoblot inhibition experiments, a pool of subjects' sera was preabsorbed with extracts from cockroach, dust mite, boiled crab, lobster, squid, scallop, and mussel as inhibitors, and chick pea as control. Inhibition with lobster and crab significantly decreased IgE binding to rSCP, suggesting cross-reacting epitopes among crustacean SCPs (Fig 3). In contrast, preincubation with dust mite (*D farinae*), cockroach (*B germanica*), or mollusks did not show significant inhibition. BS completely inhibited IgE reactivity to the rSCP, whereas chickpea extract used as negative control showed no inhibition.

## ELISA inhibition

For ELISA inhibition assays, 2 subjects (2 and 43) were chosen whose sera recognized rSCP with high intensity by immunoblot analysis (Fig 1, *B*). We compared 2 subjects, one with high IgE to shrimp (52 kU/L) and another with a low level (7 kU/L), to gauge the relative importance of SCP as an allergen, with respect to tropomyosin, Lit v 1, on an individual basis. Serum was incubated with increasing concentrations of rSCP, rLit v1, and BS extract, and rCor a 9 as a negative control. rSCP, rLit v 1, and BS were each able to inhibit the binding of the subjects' IgE antibodies to the solid phase-coated BS, whereas the negative control showed no inhibition (Fig 4, *A and B*). Maximal inhibition was obtained with rSCP at 25 μg/mL (79%) compared with 27% with rLit v 1 for subject 43 (Fig 4, *B*), and 48% compared to 44%, respectively, for subject 2, whereas 100% inhibition was seen with BS extract (Fig 4, *A*).

## rSCP causes mediator release from a RBL-2H3 cell line

Rat basophilic leukemia cells passively sensitized with shrimp-specific IgE antibodies from a pool containing sera from 3 subjects (14, 47, and 48) with high levels of shrimp-specific IgE (100 kU/L, 100 kU/L, 80 kU/L), recognizing rSCP with high intensity by immunoblot, were used to compare mediator release induced by rSCP, rLit v 1, and BS extract. The highest mediator release was obtained with BS extract (40%). Mediator release induced by rSCP was significant, approaching 30% of the total possible release (ie, release induced by Triton X-100), or 65% of the release obtained with BS extract. Interestingly, release induced by rSCP was higher than that induced by rLit v 1 (20% of total possible release, or 44% of the release induced by BS) for this particular pool of patients' sera (Fig 4, *C*).

## DISCUSSION

Crustaceans are responsible for food-induced allergic reactions in both children and adults. In spite of the high prevalence of crustacean allergy, there is limited information regarding the proteins involved in the induction of such allergic reactions. Until very recently, tropomyosin was the only major shellfish allergen identified.[6,7,16] A minor shrimp allergen, arginine kinase[17,18] (Pen m 2, Lit v 2), has been described in raw shrimp. Recently our group identified MLC as a new major shrimp allergen, Lit v 3.[8] Because some subjects who recognized a 20-kd protein by IgE immunoblotting showed little binding to rLit v3, the presence of another allergenic protein of similar molecular weight was suggested. Subsequent MS/MS analysis of a 20-kd protein from a 2-dimensional gel yielded the sequence of multiple peptides identified as belonging to a SCP. rSCP was recognized by IgE of subjects with shrimp allergy, confirming an earlier report of SCP as a shrimp allergen, based on IgE reactivity to purified native protein.[19] However, because both MLC and SCP are shrimp allergens of similar MWs and isoelectric points, it may be difficult to purify them by standard methods. Therefore, incomplete purification of SCP may have included small amounts of other proteins of similar MW, such as MLC, to which their subjects may have reacted. It is therefore of major importance to use recombinant methods to confirm the allergenic reactivity of new proposed allergens.

In this study, SCP was characterized as a new shrimp allergen, Lit v 4.0101. Lit v 4.0101 has 194 amino acids, a molecular weight of 22 kd, and a calculated isoelectric point of 4.7. High


**FIG 2.** Multiple sequence alignment of protein sequences of SCPs. Sequence identities of Lit v 4.0101 with *Penaeus* spp α chain (P02636; 93.8%), β chain (P02635; 80%), crayfish SCP (Pontascatus leptodactylus, ABB58783; Procambarus clarkii, P05946; 81-82%), scallop (Mizuhopecten yessoensis), SCP (P02637; 14%), and fruit fly SCP (Drosophila melanogaster NP_001015389 and NP_524381; 18% to 52%). Identical amino acids to *L vannamei* SCP are replaced by *dashes*. Peptides identified by MS/MS matching SCP α chain are marked in *red*, and those matching the SCP β chain in *blue*.

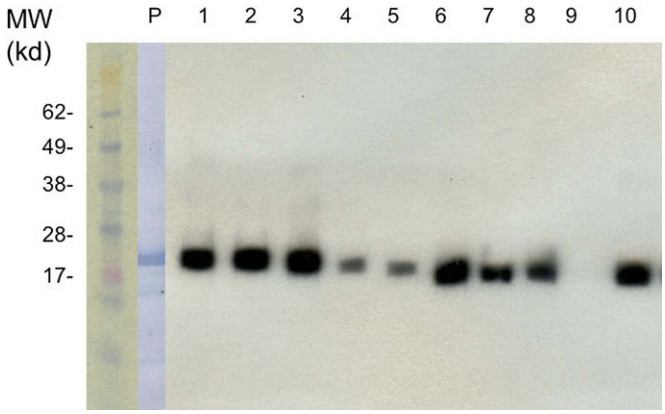


**FIG 3.** Immunoblot inhibition of IgE reactivity to rSCP with extracts from other arthropods and mollusks. *P,* Protein staining of rSCP. *Lane 1,* Pool without inhibitor. *Lanes 2-9,* pool preincubated with extract of *D farinae (2), B germanica (3),* boiled lobster *(4),* crab *(5),* squid *(6),* scallop *(7),* mussel *(8),* shrimp *(9),* and chickpea *(10)* as negative control.

sequence identity with α-B and α-A chains (93.8%) from *Penaeus* spp (P02636) and 80% with the β chain (P02635) indicate that our cloned allergen is a member of the α chain.[20,21] SCPs are acidic cytosolic EF-hand type $Ca2^+$ binding proteins (20-22 kd). In shrimp, SCPs are dimers of 2 polypeptide chains (αα, αβ, and ββ), with 3 calcium-binding sites in each chain.[22] The aligned amino acid sequences of different SCP molecules are shown in Fig 2.

Although the precise function of muscle SCP has not been determined, it has been speculated that invertebrate SCP may serve a similar function as vertebrate parvalbumins—that is, promoting rapid muscle relaxation by facilitating calcium translocation from myofibrils to the sarcoplasmic reticulum—and may protect against high calcium concentration inside the cell.[23] The amino acid composition and physicochemical characteristics of different SCPs suggest that they are not conserved proteins. Because the biological function of SCP may be carried out without interacting with other proteins, there is little need to conserve surface amino acid residues.[24] Sequence identity between shrimp and scallop SCP (P02637), for instance, is only 14%, and 18% to 52% with *Drosophila* (NP_001015389 and NP_524381;NP_524381)[20,25] (Fig 2). This is consistent with the lack of *in vitro* cross-reactivity seen by immunoblot with cockroach, dust mite, and mollusk SCPs. Although most of our 52 subjects are also sensitized to dust mites and cockroach (not shown), SCP does not appear involved in cross-reactivity among crustaceans and other arthropods. In contrast, high sequence identity with crawfish SCP (81% to 82%; ABB58783, P05946; Fig 2) helps explain cross-reactivity among crustacean SCPs, as was shown by immunoblot inhibition with lobster and crab extracts. In summary, although sensitization to tropomyosin has been implicated in cross-reactivity between crustaceans and mollusks, and also with other arthropods, sensitization to SCP appears to be involved only in cross-reactivity among crustaceans.

Similar to previously identified shrimp allergens, SCP is also a muscle protein. Interestingly, parvalbumin, troponin C, MLC, and SCP are all EF-hand–type $Ca2^+$ binding proteins.[20] EF-hand–type proteins with a variable number of EF motifs are allergenic proteins found in tree pollens (Bet v 4, Ole e 3, Ole e 8), grass pollens (Phl p7), rapeseed (Bra n 1, Bra n 2), and some vertebrates such as fish (Gad m 1, Sal s 1) and frog (Ran e 1, Ran e 2). Parvalbumins are important fish allergens.[11,26] Also among invertebrates, troponin C is a minor cockroach allergen (Bla g 6).[27] Although amino acid sequence identity of shrimp SCP with other EF-hand–type $Ca2^+$ binding proteins is low (12% sequence identity with cod parvalbumin Gad m 1, cockroach troponin C Bla g 6, cockroach MLC Bla g 8, and shrimp MLC Lit v 3), it has been suggested that they all are derived from a common ancestral

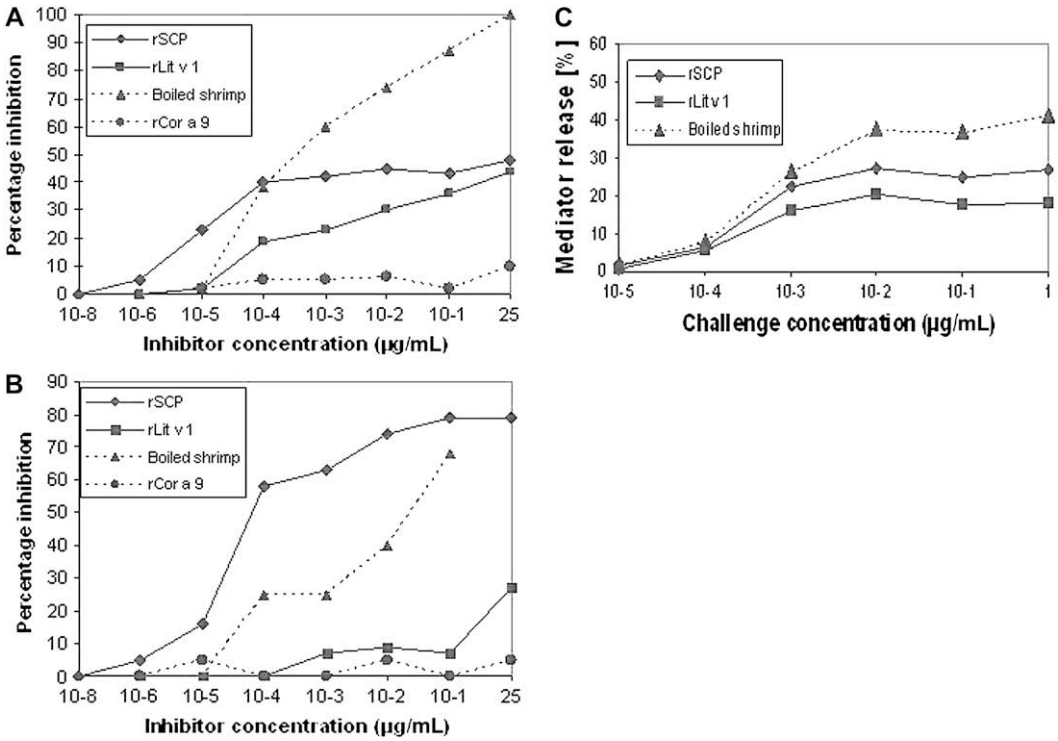


**FIG 4.** ELISA inhibition and mediator release from RBL cell line. ELISA inhibition of BS with serum from subject 2 **(A)** and subject 43 **(B)**. Inhibitors: rSCP, rLit v 1, BS, and rCor a 9. Inhibitor concentrations ranged from 25 $\times$ $10^{-8}$ μg/mL (shown as $10^{-8}$) to 25 μg/mL total protein. β-Hexosaminidase release from RBL cells induced by rSCP, rLit v 1, and BS (maximal concentration, 1 μg/mL) is expressed as percentage of total possible release induced by Triton X-100. Cells were sensitized with serum pool of subjects 14, 47, and 48 **(C)**.

protein because they possess the common structure of $Ca^{2+}$-binding sites.[28] Although sequence identity can not explain their common allergenicity, repetitive structures (calcium binding sites) may act as allergenic epitopes in all these proteins. This has been shown for cod parvalbumin, in which modification of these calcium binding sites by calcium depletion or mutagenesis can decrease IgE binding.[29] However, inhibition studies among different EF-hand proteins from pollen and vertebrates have shown limited cross-reactivity, suggesting that different families of calcium-binding allergens may posses specific epitopes involved in cross-reactivity within members of the same family.[30] Our studies also support this lack of cross-reactivity among EF-hand proteins, which appears in general limited to phylogenetically closely related species, within 1 protein type—that is, only among SCPs of crustaceans, but not with MLC, troponin, or other EF proteins of arthropods.

Although tropomyosin is the most abundant allergen in crustaceans, some of our subjects primarily recognized SCP. rSCP was recognized by 38% (20/52) of our subjects with shrimp allergy. Interestingly, 17 of 23 (74%) of children recognized rSCP compared with 3 of 29 (10%) adults, suggesting that SCP is an important allergen in the pediatric population. ELISA inhibition experiments showed that a significant proportion of some subjects' shrimp-specific IgE (as much as 78%) is inhibited by rSCP, demonstrating that for some subjects, SCP may be more important than tropomyosin as a shellfish allergen (Fig 4). Furthermore, the functional RBL-based mediator release assay confirmed that for a subset of subjects (because it has been tested only with a limited number), SCP appears to be a more potent basophil activator than tropomyosin. β-Hexosaminidase release induced by rSCP reached 30% of total maximal release, whereas release induced by recombinant tropomyosin was under 20%. Therefore, it appears critical to include SCP in future diagnostic and therapeutic strategies, particularly when children are involved. Last, as the picture of crustacean allergy becomes more complex with the recent identification of several important allergens, determining the relative importance of the different shrimp allergens, tropomyosin, arginine kinase, MLC, and SCP, remains an important goal.

In summary, we have identified shrimp SCP as a new shrimp allergen named Lit v 4.0101 that appears to be of major importance in children. Although SCP is recognized by only 38% of our subjects, for some, SCP appears to be the main shrimp allergen recognized. Because Lit v 4.0101 is the predominant shrimp allergen recognized by some subjects, identification of its IgE-binding epitopes and inclusion of SCP in the design of mutated hypoallergenic variants for use in future vaccines for individuals with shellfish allergy remains our final goal.

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**Clinical implications: Lit v 4 should be included in future diagnostic and therapeutic strategies for shrimp allergy.**

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