

Molecular cloning and functional expression of allergenic sarcoplasmic calcium-binding proteins from *Penaeus* shrimps

Hajime Mita, Aiko Koketsu, Shoichiro Ishizaki and Kazuo Shiomi*

Abstract

BACKGROUND: Sarcoplasmic calcium-binding proteins (SCPs) have recently been identified as crustacean allergens. However, information on their primary structures is very limited and no recombinant SCP (rSCP) as an alternative of natural SCP (nSCP) is available. This study was aimed to elucidate primary structures of SCPs from two species of *Penaeus* shrimp (black tiger shrimp and kuruma shrimp) by cDNA cloning and to produce a black tiger shrimp rSCP preparation that is comparable in IgE reactivity to nSCP.

RESULTS: The full-length cDNAs encoding black tiger shrimp and kuruma shrimp SCPs were successfully cloned. Both SCPs are composed of 193 amino acid residues and share more than 80% sequence identity with the known crustacean SCPs. The black tiger shrimp SCP was then expressed in *Escherichia coli* using the pFN6A (HQ) Flexi vector system. Enzyme-linked immunosorbent assay (ELISA) and inhibition ELISA experiments demonstrated that rSCP has the same IgE reactivity as nSCP.

CONCLUSION: Our results provide further evidence for the high sequence identity among crustacean SCPs. In addition, rSCP will be a useful tool in studying crustacean allergens and also in the diagnosis of crustacean allergy.

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Keywords: allergen; black tiger shrimp; kuruma shrimp; *Penaeus japonicus*; *Penaeus monodon*; sarcoplasmic calcium-binding protein

INTRODUCTION

Crustaceans, including shrimps (or prawns), crayfish, lobsters and crabs, are favorably consumed as delicious seafoods all over the world. However, they are also known as one of the most common causes of food allergy mediated by immunoglobulin E (IgE) in industrialized countries, especially in coastal countries such as Japan. Differing from hen's egg and cow's milk allergies that are primarily observed in infants and children, crustacean allergy is generally long-lasting from childhood to adulthood and hence is a particular concern in adult food allergy. In Japan, crustaceans are ranked first among various causes of adult food allergy, and shrimp and crab are among the seven mandatory items for labeling in the allergy food-labeling system.¹

The major crustacean allergen is tropomyosin, a homodimeric myofibrillar protein consisting of a 35 kDa subunit, as demonstrated with various species of crustaceans.^{2–9} Besides tropomyosin, the following four classes of proteins have recently been identified as crustacean allergens: arginine kinase,^{10–12} myosin light chain,¹³ sarcoplasmic calcium-binding protein (SCP)^{14,15} and hemocyanin.¹⁶ Of these allergens, SCP seems to be of particular importance and interest in some points. Firstly, SCP is presumably a major allergen recognized by more than 50% of crustacean-allergic patients.^{14,15} Secondly, serum IgE in crustacean-allergic patients reacts much more intensely to SCPs from shrimps of the suborder Dendrobranchiata than to other crustacean SCPs.¹⁴ Thirdly, SCP is an EF-hand Ca^{2+} -binding invertebrate-specific protein that is structurally and functionally

analogous to parvalbumin, an EF-hand Ca^{2+} -binding vertebrate-specific protein established as a major fish allergen.^{17–19}

As described above, SCP is an important and interesting allergen of crustaceans. To better understand the IgE reactivity of crustacean SCPs at a molecular level, however, much knowledge on their primary structures should be accumulated. Moreover, a recombinant SCP (rSCP) preparation with the same IgE reactivity as a natural counterpart is expected as a model antigen in future diagnosis of crustacean allergy as well as in future research on crustacean allergens. So far, Pacific white shrimp rSCP has been produced but it is unclear whether its IgE reactivity is comparable to that of natural SCP (nSCP).¹⁵ In view of these circumstances, this study was undertaken to elucidate the primary structures of SCPs from two species of *Penaeus* shrimps (black tiger shrimp *P. monodon* and kuruma shrimp *P. japonicus*) belonging to the suborder Dendrobranchiata by a cDNA cloning technique. The black tiger shrimp SCP was then expressed in *Escherichia coli* based on the cloned cDNA and the produced rSCP was evaluated for its IgE binding ability by enzyme-linked immunosorbent assay (ELISA) and inhibition ELISA, using the purified nSCP preparation as a reference.

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MATERIALS AND METHODS

Molecular cloning of black tiger shrimp SCP

The Marathon cDNA library of either black tiger shrimp or kuruma shrimp used as a template for polymerase chain reaction (PCR) was the same as previously generated from the abdominal muscle of a live specimen.⁷ In cloning experiments, PCR amplification was performed using a HotStar HiFidelity Polymerase Kit (Qiagen, Hamburg, Germany) under the following conditions: pre-incubation at 95 °C for 15 min and 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 1 min. First, a 5'-end fragment of the SCP cDNA was amplified using the AP1 adaptor primer (5'-CCATCCTAATACGACTCACTATAGGGC-3') and a degenerate reverse primer (5'-NAYRTCAGCRAAGGCRGACC-3'; refer to Fig. 1 for the primer position) designed from the reported partial amino acid sequence of the black tiger shrimp 20 kDa allergen (SCP).¹⁴ The amplified product was subcloned into the pT7Blue-2 T-vector (Novagen, Darmstadt, Germany) and sequenced using a PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit Ver. 3.1 (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 genetic analyzer (Applied Biosystems). Based on the determined

nucleotide sequence of the 5'-end fragment, a 3'-end fragment of the cDNA was then amplified by PCR using a gene-specific forward primer (5'-AACCTCTGGAACGAGATCGC-3'; refer to Fig. 1 for the primer position) and the AP1 primer. The PCR product was subcloned and sequenced as described above.

Expression and purification of black tiger shrimp recombinant SCP (rSCP)

Black tiger shrimp SCP was expressed in *E. coli* as a HisGln (HQ)-tagged protein using a pFN6A (HQ) Flexi vector (Promega, Madison, WI, USA). A cDNA encoding the mature region of black tiger shrimp SCP was amplified by PCR using the Marathon cDNA library. The nucleotide sequences of the primers used were as follows: a forward primer with *Sgf* I restriction site (underlined) 5'-AAAAGCGATCGCAGCTTACAGCTGGGACAA-3' and a reverse primer with *Pme* I restriction site (underlined) 5'-CGCGCGTTTAAACCTACTGCACCACCTTCAGGGG-3'. Amplification was performed using Ex Taq DNA polymerase (Takara Bio, Otsu, Japan) under the following conditions: 98 °C for 15 min and 30 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min. The amplified product and the pFN6A (HQ) Flexi vector

(A) Black tiger shrimp	
TTCCTCCGGTCAGGCGACGCAAGCATCTAGCAAAATGGCTTACAGCTGGGACAACCGCGTCAAGTACGTCGTCAGGTACATGTACGAC	88
M A Y S W D N R V K Y V V R Y M Y D	18
ATTGACAACAATGGCTTCTTGATAAGAAGCACTTCGAGTGCCCTTGGCGTCAGGAACACCTGATCGAGGGCGTGGCGAATTACGCGCC	178
I D N N G F L D K N D F E C L A V R N T L I E G R G E F S A	48
GATGCCTACGCCAACAACAGGATCATGAGGACCTCTGGAACGAGATCGGCGAGCTCGTGACTTCAACAAGGACGGCGAGGTAACT	268
D A Y A N N Q K I M R N L W N E I A E L A D F N K	78
GTCGACGAGTTCAAGCAGGCTGTGCAAGCATTGCCAGGCAAGAATACGGAGAATTCCCGGTGCTTCAAGGTTTTTCATTGCCAAC	358
V D E F K Q A V Q K H C Q G K K Y G E F P G A F K V F I A N	108
CAGTTCAAGGCTATTGACGTTAAGCGGACGGCAAGGTTGGCCTCGATGAGTACAGGCTTGACTGCATTACCAAGGCTGCTTTGCTGAG	448
Q F K A I D V N G D G K V G L D E Y R L D C I T R S A F A E	138
GTGAAGGAGATTGATGAAGCTTACAACAGCTCACCCTGAGGACGACAGGAAGGCGGCGGACTCACCTGGAACGCTACCGAGACCTG	538
V K E I D E A Y N K L T T E D D R K A G G L T L E R Y Q D L	168
TACGCTCAGTTTCATCTCAACCTGAAAGAGTCTTGACGCGCTGCTACCTTTTCGAGCCCTGAAGGTGGTGCAGTAGACTAGCGCATGA	628
Y A Q F I S N P E E S C S A C Y L F G P L K V V Q ***	193
GGCGGCTACATTACCTCCGCGGAGGCTCCGCGCTGTGGCGGAAACCCCGCGCATGGCGACCCCGGGAGGCGCTAGCGCCAGCCC	718
CACACACCCAAACAGTGCCGAGTTCTCACCAGCTTTTATACTCACCTCCCTGAAGCTAAGCCGCAACGACGGAACACACCCGCTG	808
CCGTAGCCGACCCGGCGCTGCTCTAGCCAGGCGCTTGGAGTGAGAGCGCGGCTCGCCCTGGCCAGCCCTCGCTCGAGGG	898
TTTCAGCCAGTTCAACACCGGAATGGTGCAGTACTGATGCGATCAACTCTTTATTATTATCAATAAACGTTGAAGATGTTAAAAAAA	988
AAAAAAAAAAAAA	1002
(B) Kuruma shrimp	
CGGCTGAGAATCAACGAACTTTAAGGTACGGCGACGCAAGCATCTAGCAAAATGGCTTACACCTGGGACAACCGCGTCAAGTACGTC	89
M A Y T W D N R V K Y V	12
GTCAGGTACATGTACGACATTGACAACAATGGCTTCTTGACAAGAAGCACTTCGAGTGCCCTTGGCGTCAGGAACACCTGATCGAGGGC	179
V R Y M Y D I D N N G F L D K N D F E C L A V R N T L I E G	42
CGCGGCAATTACGCGCGATGCCTACGCCAACAACGAGGATGAGGACCTCTGGAACGAGATCGGCGAGTTCGTCGACTTCAAC	269
R G E F S A D A Y A N N Q K T M R N L W N E I A E F A D F N	72
AAGGACGGCGAGGTAAGTGTGACGAGTTCAAGCAGGCTGTGCAAGCATTGCCAGGCAAGAATACGGCGATTTCCCGGTGCTTC	359
K D Q E V T V D E F K Q A V Q K H C Q G K K Y G D F P G A F	102
AAGTTTTTCATTGCCAACGATTTAAGGCTATTGACGTTAAGCGGACGCGATGGTTGGCCTCGATGAGTACAGGCTTGACTGCATTACC	449
K V F I A N Q F K A I D V N G D G M V G L D E Y R L D C I T	132
AGGCTGCTTTGCTGACGTTGAAGGAGATTGATGAGGCTTACAACAGCTCACCCTGAGGACGACAGGAAGGCTGGCGGACTCACTCTG	539
R S A F A D V K E I D E A Y N K L T T E D D R K A G G L T L	162
GCACGCTACGAGACCTGTACGCTCAGTTTCATCTCAACCCGACGAGTCTTGACGCGCTGTACCTTTTCGCGCCCTGAAGGTGGTG	629
A R Y Q D L Y A Q F I S N P D E S C S A C Y L F G P L K V V	192
CAGTAGACTAGCGCTTGAGGCCACCTACATTACCTCCGCGGAGGCTCCGCGCTGTGGGTGGAACCCCGCATGCGACCCCGGGG	719
Q ***	193
AGGCCCTAGCGGCTGCCCCACACCCAAAGTGGCCAGTTCTCACCAGCTTTTATACTCACCTCCCTCTGAAGCTAAGCCGCAATGC	809
AGGCCAACATCGCTGCGGTAGCCGACCCGGCGCTGCCGTCCCTAGCCAGTCCCTTTGAGTGGGGACGCGCGGACCCGCTGGC	899
GAGCCCTCGCTCGAGGGTCTTCAGCCAGTCAACACCGGAATGGTGCAGTACTGATGCGATCAACTCTTTATTATTATCAATAAACG	989
CTGAATGTAGAAAAA	1007

Figure 1. Nucleotide sequences of the cDNAs encoding black tiger shrimp SCP (A) and kuruma shrimp SCP (B). The deduced amino acid sequences are denoted below the nucleotide sequences. Nucleotide and amino acid numbers are shown at the right. In each nucleotide sequence, a stop codon (TGA) is shown by asterisks, a poly(A) signal in the 3'-untranslated region is underlined and the two regions corresponding to the primers are boxed. The two doubly underlined amino acid sequences in the black tiger shrimp SCP (A) correspond to the peptide fragments produced by lysylendopeptidase digestion of the 20 kDa allergen (SCP) previously purified from black tiger.

were individually digested with *Sgf* I and *Pme* I and ligated with each other using a LigaFast Rapid DNA Ligation System (Promega). Single-step (KRX) competent cells (*E. coli*; Promega) were transformed with the ligated product and cultured overnight at 37 °C on an LB agar plate containing 0.1 g L⁻¹ ampicillin, 0.1 g L⁻¹ X-gal and 0.5 mmol L⁻¹ isopropyl- β -D-thiogalactopyranoside (IPTG). The single colony formed was picked up and cultured overnight at 37 °C in 5 mL LB medium containing 0.1 g L⁻¹ ampicillin. This culture medium was added to 500 mL LB medium containing 0.1 g L⁻¹ ampicillin and again cultured with shaking at 37 °C until the absorbance at 600 nm reached 0.6. Then, the medium was added with rhamnose at a final concentration of 1 g L⁻¹ and IPTG at a final concentration of 1 mmol L⁻¹ and further incubated with shaking at 37 °C for 3 h. Bacteria were harvested by centrifugation, suspended in 25 mL of 20 mmol L⁻¹ phosphate buffer (pH 7.4) containing 150 mmol L⁻¹ NaCl and 1 mmol L⁻¹ phenylmethylsulfonyl fluoride and digested with 2.5 mg lysozyme at 4 °C for 1 h. After sonication and centrifugation, the supernatant containing rSCP was applied to affinity chromatography on a HisTrap Chelating HP column (GE-Healthcare Biosciences, Chalfont St Giles, UK). rSCP was adsorbed by the column and eluted with 20 mmol L⁻¹ phosphate buffer (pH 7.4) containing 150 mmol L⁻¹ NaCl and 500 mmol L⁻¹ imidazole. Finally, the HQ-tagged SCP fraction was dialyzed well against 10 mmol L⁻¹ phosphate buffer (pH 7.4) containing 150 mmol L⁻¹ NaCl to remove imidazole.

Purification of natural SCP (nSCP) from black tiger shrimp

nSCP of black tiger shrimp was purified from the abdominal muscle by extraction with 100 mmol L⁻¹ Tris-HCl buffer (pH 8.0) containing 0.025 mmol L⁻¹ phenylmethylsulfonyl fluoride, 1 mmol L⁻¹ dithiothreitol and 1 mmol L⁻¹ ethylenediaminetetraacetic acid, salting-out (70–90% saturation of ammonium sulfate) and reverse-phase high-performance liquid chromatography (HPLC) on a TSKgel ODS-120T column (0.46 $\times$ 25 cm; Tosoh, Tokyo, Japan), as reported previously.¹⁴ The homogeneity of the purified preparation was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

SDS-PAGE was performed on a PhastSystem apparatus (GE-Healthcare Biosciences) using ready-made gels (PhastGel Gradient 8-25; GE-Healthcare Biosciences), according to the manufacturer's instructions. Each sample was dissolved in 62.5 mmol L⁻¹ Tris-HCl buffer (pH 7.4) containing 20 g L⁻¹ SDS, 4 mol L⁻¹ urea and 100 mmol L⁻¹ dithiothreitol, heated at 70 °C for 10 min and subjected to electrophoresis. Precision Plus protein standards (Bio-Rad Laboratories, Hercules, CA, USA) were run as references, along with samples. After running, proteins were stained with Coomassie Brilliant Blue R-250.

Patient sera

Five of the eight crustacean-allergic patient sera, which had been confirmed to react to the black tiger shrimp SCP in our previous study,¹⁴ were used in this study as a pooled serum.

ELISA and inhibition ELISA

IgE reactivity of black tiger shrimp nSCP and rSCP was examined by fluorescence ELISA, as reported previously.²⁰ In brief, a 96-well Type H (black) microtiter plate (Sumitomo Bakelite, Tokyo, Japan) was coated with nSCP or rSCP solution (0.003–1 μ g mL⁻¹) and

reacted successively with pooled patient sera (diluted 1:250) and β -galactosidase-conjugated goat anti-human IgE antibody (0.25 μ g mL⁻¹; American Qualex, San Clement, CA, USA). Enzyme reaction was carried out using 4-methylumbelliferyl- β -D-galactoside as a substrate and fluorescence units were measured with excitation at 367 nm and emission at 453 nm. For inhibition ELISA, patient sera (diluted 1:250) were pre-incubated with nSCP or rSCP (final concentration: 0.001–10 μ g mL⁻¹) at 37 °C for 2 h and then added as a primary antibody to the microtiter plate previously coated with nSCP or rSCP solution (1 μ g mL⁻¹). The subsequent procedure was the same as that for the ELISA described above. All ELISAs (including inhibition ELISA) were performed in triplicate and the data were expressed as mean $\pm$ SD.

IgE reactivity of a peptide (MKHQHQHQAIAYSWDNRVKY) corresponding to the N-terminal region of rSCP was also estimated by fluorescence ELISA, with the only exception that a Nunc Immobilizer Amino plate for peptide (Nagel Nunc International, Rochester, NY, USA) was used for peptide coating. The peptide, composed of the HQ-tag (8 residues), restriction site (3 residues) and N-terminal portion of nSCP (10 residues), was synthesized on a PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan) by the 9-fluorenylmethyloxycarbonyl (Fmoc) strategy and purified by reverse-phase HPLC, as described elsewhere.²¹

Protein determination

Protein was determined using a Protein Assay Kit (Bio-Rad Laboratories). Bovine serum albumin was used as a standard protein.

RESULTS AND DISCUSSION

Cloning of the cDNAs encoding shrimp SCPs

In our previous paper,¹⁴ two of the peptide fragments produced by lysylendopeptidase digestion of the 20 kDa allergen (SCP) of black tiger shrimp were sequenced as follows: IMRNLWNEIAELADFNK and VGLDEYRLDCITRSAFEVK (refer to Fig. 1A for the positions). Based on these amino acid sequences, several degenerate forward and reverse primers were designed and subjected to PCR using the Marathon cDNA library as a template to amplify 5'-end, intermediate and 3'-end fragments of the SCP cDNA. As a result, only a 5'-end fragment was successfully amplified for both black tiger shrimp and kuruma shrimp SCPs with the primers described in the Materials and methods section. The determined nucleotide sequence of the 5'-end fragment made it possible to design a gene-specific forward primer, leading to the PCR amplification and nucleotide sequencing of the remaining 3'-end fragment. For each PCR product, several clones (at least three clones) obtained by subcloning were found to have the same nucleotide sequence. In both species of shrimps, therefore, isoforms of the cloned SCP (correctly the SCP subunit) are absent or trace, although three different isoforms of SCP, two homodimers ($\alpha\alpha$ and $\beta\beta$) and one heterodimer ($\alpha\beta$) have been reported for oriental shrimp^{22,23} and narrow-clawed crayfish.²⁴

The determined nucleotide sequences of the full-length cDNAs coding for the black tiger shrimp SCP (1002 bp) and kuruma shrimp SCP (1007 bp) have been deposited in the DDBJ/EMBL/GenBank databases under the accession numbers AB703461 and AB703462, respectively. An open reading frame of 579 bp (corresponding to 193 amino acid residues) is recognized at positions 35–613 for the black tiger shrimp SCP cDNA and 54–632 for the kuruma shrimp SCP cDNA (Fig. 1). In both cDNAs, a poly(A) signal (AATAAA) and

	10	20	30	40	50	60	70
Black tiger shrimp	MAYS	WDRV	KYVVR	YMD	IDNNGFLDKND	FECLAVRNTLIEGRGEFSADAYANNQKIMRNLWNEIAELAD	
Kuruma shrimp	...	T	...	...	...	...	...
Pacific white shrimp	...	...	...	...	...	...	...
Oriental shrimp (α -A chain)	...	...	...	...	...	...	...
Oriental shrimp (α -B chain)	...	...	...	...	...	...	...
Oriental shrimp (β chain)	...	...	...	...	...	...	...
Brown shrimp	...	...	...	...	...	...	...
Narrow-clawed crayfish	...	...	...	...	...	...	...
Red swamp crayfish	...	...	...	...	...	...	...
Yesso scallop	TDY-LVSKWKIWKSL	VNHD	IISIE	V	ESRNKF	DLH---	KLVG
Common orient clam	NEFLISKWKIWKSL	VNHD	TISMA	V	ESRSKFSELH---	HLD	EKKDMVM
	80	90	100	110	120	130	140
Black tiger shrimp	FNKDG	EVTVDE	FKQAVQKHCQGGK	YGEFP	GAFKVF	IANQFKAI	DVNGDGKVG
Kuruma shrimp	...	...	...	...	...	...	...
Pacific white shrimp	...	...	...	...	...	...	...
Oriental shrimp (α -A chain)	...	...	...	...	...	...	...
Oriental shrimp (α -B chain)	...	...	...	...	...	...	...
Oriental shrimp (β chain)	...	...	...	...	...	...	...
Brown shrimp	...	...	...	...	...	...	...
Narrow-clawed crayfish	...	...	...	...	...	...	...
Red swamp crayfish	...	...	...	...	...	...	...
Yesso scallop	PGA	ISE	TQ	VENLGNSFK	DKKA	LATMTACFNMI	DV
Common orient clam	---	---	---	---	---	---	---
	150	160	170	180	190		
Black tiger shrimp	EIDEAYNKLT	TEDDRKAGGLT	LERYQDL	YAQFISN	PEESCSAC	YLFGLKVVQ	
Kuruma shrimp	...	...	...	...	...	...	...
Pacific white shrimp	...	...	...	...	...	...	...
Oriental shrimp (α -A chain)	...	...	...	...	...	...	...
Oriental shrimp (α -B chain)	...	...	...	...	...	...	...
Oriental shrimp (β chain)	...	...	...	...	...	...	...
Brown shrimp	...	...	...	...	...	...	...
Narrow-clawed crayfish	...	...	...	...	...	...	...
Red swamp crayfish	...	...	...	...	...	...	...
Yesso scallop	VVRT	FAL	KPD	NN---	TVP	RTVV	AWIS
Common orient clam	LDTKFFKAYNPK	G---	LVP	KDVT	TSWVD	ATSTDA	KPSVVKTA

Figure 2. Amino acid sequences of crustacean and molluscan SCPs. Accession numbers of SCPs (GenBank or Swiss-Prot database): black tiger shrimp, AB703461; kuruma shrimp, AB703462; Pacific white shrimp *Litopenaeus vannamei*, FJ184279; oriental shrimp *Penaeus orientalis* (α -A chain), P02636; oriental shrimp (α -B chain), P02636; oriental shrimp (β chain), P02635; brown shrimp *Crangon crangon*, FJ462737; narrow-clawed crayfish *Astacus leptodactylus*, P05946; red swamp crayfish *Procambarus clarkii*, DQ256199; Yesso scallop *Patinopecten yessoensis*, AB036700; common orient clam *Meretrix lusoria*, AB012091. The residues identical to those of black tiger shrimp SCP are shown by dots and gaps by dashes. Putative Ca^{2+} -binding regions are boxed.

a poly(A) tail are contained in the 3'-untranslated region. The deduced primary structure of the black tiger shrimp SCP includes the same amino acid sequences as the two lyslendopeptidase fragments from the 20 kDa allergen at positions 57–73 and 121–140. This supports the accuracy of our cloning data and, more importantly, provides evidence that the 20 kDa allergen previously purified from black tiger shrimp is none other than SCP.

In the course of this study, the nucleotide sequence of the cDNA encoding black tiger shrimp SCP has been submitted to the DDBJ/EMBL/GenBank databases under the accession number HM034315. As compared to this nucleotide sequence, our cloned cDNA of the black tiger shrimp SCP has 10 alterations at positions 112, 154, 187, 271, 322, 325, 466, 523, 562 and 564. However, only three of the 10 alterations in the nucleotide sequence lead to the replacement of Asp by Glu at three positions (97, 144 and 177) in the amino acid sequence. We tentatively assume that the observed nucleotide alterations are ascribable to individual variations.

Amino acid sequences of SCPs

The amino acid sequences of black tiger shrimp and kuruma shrimp SCPs are shown in Fig. 2. To date, the SCPs from five species of crustaceans (Pacific white shrimp, oriental shrimp, brown shrimp, narrow-clawed crayfish and red swamp crayfish) have been elucidated for their amino acid sequences. For comparison, the amino acid sequences of these crustacean SCPs and two molluscan SCPs (from Yesso scallop and common orient clam) are also shown in Fig. 2. As analyzed by the PROSITE program,²⁵ both black tiger shrimp and kuruma shrimp SCPs were predicted to have three Ca^{2+} -binding sites at positions 18–29, 70–81 and 114–125,

similar to the other crustacean SCPs but dissimilar to the molluscan SCPs lacking the second binding site.

Amino acid sequence identities among SCPs from crustaceans and mollusks are summarized in Table 1. Prior to discussion on the amino acid sequences of SCPs, it should be noted that the order Decapoda, in which most edible crustaceans are included, is divided into two suborders: Dendrobranchiata and Pleocyemata; of the seven species of crustaceans listed in Table 1, four species of shrimps (black tiger shrimp, kuruma shrimp, Pacific white shrimp and oriental shrimp) belong to the former suborder and the remaining three species (brown shrimp, narrow-clawed crayfish and red swamp crayfish) to the latter. Although the oriental shrimp SCP is a mixture of homodimer and heterodimer comprising α (α -A and α -B) chain and/or β chain,^{22,23} such polymorphism is not observed for the SCPs in the other three species of the suborder Dendrobranchiata. As compared to the α and β chains of oriental shrimp SCP, the black tiger shrimp, kuruma shrimp and Pacific white shrimp SCPs show 90–94% identity with the α chain and obviously lower identity (about 80%) with the β chain. It is thus inferred that the dendrobranchiate SCPs are classified as the α chain family, except for the β chain of oriental shrimp SCP.

IgE cross-reactivity has been demonstrated among crustacean SCPs but not between crustacean and molluscan SCPs.^{14,15} This is readily realized by the fact that crustacean SCPs share considerably high sequence identity (mostly more than 80%) with one another, whereas the sequence identity between crustacean and molluscan SCPs is at most 20% (Table 1). On the other hand, our previous immunoblotting experiments have suggested that serum IgE

Table 1. Amino acid sequence identities among crustacean and molluscan SCPs

Species	Amino acid sequence identity (%)										
	Black tiger shrimp	Kuruma shrimp	Pacific white shrimp	Oriental shrimp			Brown shrimp	Narrow-clawed crayfish	Red swamp crayfish	Yesso scallop	Common orient clam
				α -A	α -B	β					
Black tiger shrimp (<i>Penaeus monodon</i>)	100	96	98	93	94	80	81	81	81	15	21
Kuruma shrimp (<i>Penaeus japonicus</i>)		100	96	90	91	81	82	81	81	15	18
Pacific white shrimp (<i>Litopenaeus vannamei</i>)			100	93	94	81	82	82	82	15	17
Oriental shrimp (<i>Penaeus orientalis</i>)	α -A			100	98	79	76	79	79	14	18
	α -B				100	83	77	80	80	16	20
	β					100	82	82	82	15	19
Brown shrimp (<i>Crangon crangon</i>)							100	83	85	15	18
Narrow-clawed crayfish (<i>Astacus leptodactylus</i>)								100	93	16	21
Red swamp crayfish (<i>Procambarus clarkii</i>)									100	11	21
Yesso scallop (<i>Patinopecten yessoensis</i>)										100	53
Common orient clam (<i>Meretrix lusoria</i>)											100

reacts strongly to the SCPs from two dendrobranchiate shrimps (black tiger shrimp and kuruma shrimp) and weakly to those from two pleocyemate species (pink shrimp *Pandalus eous* and American lobster *Homarus americanus*) but shows no reactivity to those from two pleocyemate crabs.¹⁴ In relation to this, it is interesting to note that the dendrobranchiate SCPs (except for the β chain of oriental shrimp SCP) share extremely high sequence identity (90–98%) with one another. Therefore, the Pacific white shrimp and oriental shrimp SCPs are reasonably considered to have strong IgE-binding ability, similar to the black tiger shrimp and kuruma shrimp SCPs. However, previous results¹⁴ with SCPs from four species of pleocyemates lead us to assume that the IgE reactivity of pleocyemate SCPs differs from species to species or from group to group. Although the suborder Pleocyemata includes a variety of members (i.e. shrimps, crayfishes, lobsters and crabs), the SCPs of only three species have been elucidated for their amino acid sequences. A number of pleocyemate SCPs should be evaluated for their IgE-binding ability and elucidated for primary structures for a molecular understanding of their allergenicity. In view of no structural information on crab SCPs, those of several species of crabs are under molecular cloning.

Expression and purification of rSCP

Black tiger shrimp SCP was successfully expressed in *E. coli* using the pFN6A (HQ) Flexi vector system producing HQ-tagged proteins. As analyzed by SDS-PAGE, a prominent 23 kDa protein band corresponding to HQ-tagged rSCP was observed in the soluble fraction of IPTG-induced bacteria (lane 3 in Fig. 3). Following affinity chromatography on a HisTrap Chelating HP column, rSCP was obtained in an electrophoretically pure state (lane 4 in Fig. 3). In a typical experiment, 500 mL of the culture medium yielded as much as 30 mg rSCP.

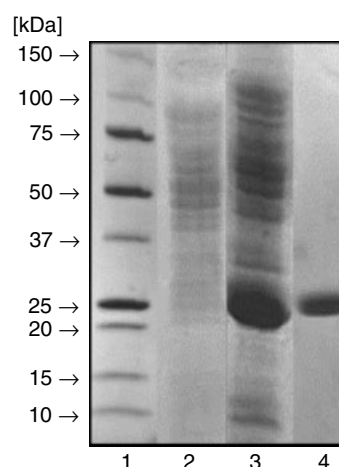


Figure 3. Analysis of expression and purification of black tiger shrimp rSCP by SDS-PAGE. Lanes: 1, reference proteins; 2, soluble fraction from non-induced *E. coli*; 3, soluble fraction from IPTG-induced *E. coli*; 4, purified rSCP.

IgE reactivity of nSCP and rSCP

Analysis by ELISA using pooled patient sera revealed that the IgE reactivity of black tiger shrimp rSCP is equivalent to that of nSCP (Fig. 4A). The synthetic 21 amino acid long peptide corresponding to the N-terminal region of rSCP exhibited no IgE reactivity even when coated at a concentration of $10 \mu\text{g mL}^{-1}$ on the ELISA plate, demonstrating that the HQ-tagged portion is not involved in the IgE reactivity observed with rSCP. When the pooled patient sera were pre-incubated with various concentrations of the inhibitor (nSCP or rSCP), their reactivity to the allergen (nSCP or rSCP) coated at a concentration of $1 \mu\text{g mL}^{-1}$ on the plate

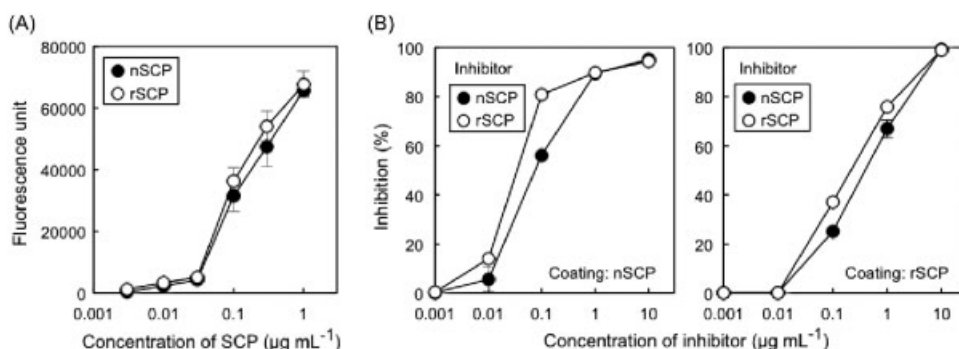


Figure 4. Comparison of IgE reactivity between nSCP and rSCP. (A) Analysis of reactivity of nSCP and rSCP with the pooled patient serum by ELISA. (B) Inhibition ELISA.

was inhibited linearly with the inhibitor concentration; regardless of the inhibitors, closely similar inhibition curves were observed (Fig. 4B).

ELISA and inhibition ELISA data demonstrate that the black tiger shrimp rSCP produced in this study has the same IgE-binding ability as nSCP. Previously, the Pacific white shrimp SCP has been successfully expressed in *E. coli* using the pET24b(+) system and its recombinant preparation shown to be IgE-reactive by immunoblotting, ELISA and mediator release assay.¹⁵ However, the Pacific white shrimp rSCP has not been assessed as to whether its IgE reactivity is comparable to that of nSCP. In this regard, the black tiger shrimp rSCP is the only crustacean rSCP that has been proved to have the same IgE reactivity as the natural counterpart. Conclusively, our black tiger shrimp rSCP is an alternative of nSCP, for use as a model antigen in future studies on crustacean allergens and also in future diagnosis of crustacean allergy.

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