

Myosin light chain is a novel shrimp allergen, Lit v 3

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Background: Shellfish allergy is a prevalent, long-lasting disorder usually persisting throughout life. Few options are available for treatment, and avoidance is the only therapy recommended.

Objective: We sought to identify relevant crustacean allergens for use as diagnostic and safe immunotherapeutic agents for subjects with shellfish allergy.

Methods: Thirty-eight patients were selected with immediate allergic reactions to shrimp and increased shrimp-specific serum IgE levels. One-dimensional and 2-dimensional electrophoresis of shrimp extracts were followed by IgE immunoblotting. Protein identification was done with matrix-assisted laser desorption/ionization–mass spectrometry and Edman sequencing. A cDNA library was generated from white pacific shrimp (*Litopenaeus vannamei*) and screened with primers designed on the basis of internal sequences obtained from 2-dimensional tryptic digests. Full-length cDNA clones were isolated from the library and sequenced. Recombinant protein was expressed and tested with sera from patients with shrimp allergy.

Results: Immunoblotting demonstrated IgE binding to a 20-kDa shrimp protein by 21 (55%) of 38 sera. Tryptic digestion of the protein followed by matrix-assisted laser desorption/ionization–mass spectrometric analysis and Edman sequencing identified it as a myosin light chain (MLC). Screening of the shrimp cDNA library resulted in isolation of a novel protein cDNA. Open reading frame translation provided the amino acid sequence of a new allergenic shrimp protein with high similarity to Bla g 8 (cockroach MLC). Recombinant protein was recognized by 17 patients, confirming the allergenicity of shrimp MLC.

Conclusions: We have identified and cloned a new major shrimp allergen, Lit v 3.0101, an MLC protein. (J Allergy Clin Immunol 2008;122:1000–1006.)

Key words: Myosin light chain, allergen, crustacean, shrimp, recombinant, Lit v 3

Shellfish allergy is a long-lasting disorder, usually persisting throughout life, that is often associated with severe reactions, including life-threatening anaphylaxis. Allergenic shellfish comprise 2 major phyla, Mollusks and Arthropods. The phylum Arthropod contains the class Crustacea, which includes shrimp, prawn, crab, lobster, and crawfish. Shrimp are distinguished from the superficially similar prawns by the structure of the gills. However, there is much confusion between the two, especially among nonspecialists, and many shrimp are called prawns and *vice versa*. The term “prawn” is also loosely used to describe any large shrimp. In European countries the word “prawns” is more commonly used on menus than the term “shrimp,” which is used more often in North America. The prevalence of immediate-type shellfish allergy is higher in areas with high shellfish intake. Among Spanish children, crustaceans and mollusks cause 3.8% and 1.6%, respectively, of the food-induced allergic reactions¹ and up to 33% of the food allergies in adults.^{2,3} About 15 years ago, it was estimated that approximately 250,000 Americans experienced allergic reactions to shellfish,⁴ including anaphylaxis.⁵ A more recent survey by Sicherer et al⁶ found that 1 in 50 Americans had shellfish allergy. Shellfish are the number one cause of food allergy in adults in the United States and are responsible for the majority of emergency department visits.⁷ Among shellfish, shrimp and prawns cause most of the allergic reactions.⁶

A large variety of crustaceans are used for human consumption. The black tiger shrimp (*Penaeus monodon*) is the most widely cultured prawn species in the world, although it is gradually losing ground to the white leg Pacific shrimp (*Litopenaeus vannamei*), another prawn. More than 900,000 tons are consumed annually. The brown shrimp (*Penaeus aztecus*) is a highly valued commercial fishery species, especially in the United States.

The muscle protein tropomyosin is the only major cross-reactive allergen identified to date in shrimp.^{8–10} Among others, it has been identified in brown shrimp (Pen a 1), black tiger shrimp (Pen m 1), and white leg pacific shrimp (Lit v 1). Pen a 1 inhibited 85% of patients' IgE RAST reactivity to whole-body shrimp extract, indicating that Pen a 1 is responsible for most of the allergenic activity of shrimp. Pen a 1 and Lit v 1 are 100% identical. Recently, 2 new shrimp allergens have been characterized: arginine kinase (Pen m 2 and Lit v 2), a minor cross-reactive crustacean allergen,^{11,12} and a sarcoplasmic calcium-binding protein.¹³ However, sera from shrimp-sensitive patients are able

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Abbreviations used

2-D: Two-dimensional
MALDI: Matrix-assisted laser desorption/ionization
MLC: Myosin light chain
MS: Mass spectrometry

to recognize qualitatively different allergens in shrimp, which deserve further evaluation.^{8,11}

In spite of the high prevalence of shellfish allergy, few options are available for treatment, and avoidance of the offending food is the only therapy recommended. However, the frequency and severity of reactions after accidental exposure to shellfish makes necessary the development of improved diagnostic and therapeutic options for shellfish allergy. Recombinant proteins are now considered the basis for new approaches to the diagnostic use of allergens and the development of novel strategies of allergen-specific immunotherapy.¹⁴⁻¹⁶ Therefore efforts should be directed at the production of recombinant food proteins for the diagnosis and treatment of individuals with food allergy. This study focused on the characterization of shrimp allergens and the recombinant production of proteins identified to be used as diagnostic and safe immunotherapeutic agents for subjects with shellfish allergy.

METHODS**Patient selection**

Sera were obtained from 38 patients with shrimp allergy (18 [47.3%] adult and 20 [52.6%] pediatric patients) with histories of immediate allergic reactions after the ingestion of shrimp and increased serum IgE levels to shrimp. Specific IgE levels to shrimp were determined by using UniCAP (Phadia, Uppsala, Sweden) and considered positive if greater than 0.35 kU/L. Levels of specific serum IgE to other arthropods, the house dust mite *Dermaphagoides pteronyssinus*, and the cockroach *Blattella germanica* were also included. Patient sera were collected from Hospital del Nino Jesus, Madrid, Spain (subjects 1-10); Hospital Universitario Dr. Negrín, Las Palmas de Gran Canaria, Spain (subjects 17-37); and Mount Sinai Medical Center, New York (subjects 11-16 and 38; Table I). This study was approved by the institutional review board of Mount Sinai Medical Center.

Shellfish extract preparation

Extracts were prepared from raw tail muscle of the white leg Pacific shrimp (*L. vannamei*). Raw peeled and deveined shrimp tail muscle was manually homogenized in a mortar until a smooth paste was achieved. Protein was extracted by means of agitation in PBS with a protease inhibitor cocktail without ethylenediamine tetra-acetic acid (Roche, Indianapolis, Ind). Na₂N₂ in distilled water (20% wt/vol) was added (1:400) as preservative and incubated overnight at 4°C. The mixture was centrifuged at 3000 rpm for 10 minutes at 4°C and then at 15,000 rpm for 5 minutes at 4°C.

Extract of boiled shrimp was prepared by boiling peeled, deveined shrimp tail muscle for 5 minutes in distilled water and homogenized according to the same protocol as above. Protein concentration was determined by means of spectrophotometry with the Coomassie Plus Protein Assay (Pierce, Rockford, Ill). Extracts were stored at -20°C.

Extract of boiled shrimp used for protein sequencing was prepared from peeled fresh market shrimp. PBS was added for protein extraction and incubated overnight at 4°C. Homogenized paste was centrifuged at 3000 rpm for 20 minutes at 4°C. The supernatant was collected and recentrifuged for 20 minutes and stored at -20°C until use.

For inhibition studies, chickpea (*Cicer arietinum*) extract was used as the control. Briefly, chickpeas purchased locally at an Indian supermarket were

boiled in water for 30 minutes. Cooked chickpeas were homogenized, and protein was extracted as described for shrimp.

SDS-PAGE and 2-dimensional analysis

For SDS-PAGE analyses, samples were heated at 70°C for 10 minutes with Nu-page sample buffer in the presence of 0.05 mol/L dithiothreitol. Proteins were then separated by means of SDS-PAGE (Nupage 4-12% Zoom Gels; Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. Protein was loaded onto the gel at a concentration of 12.5 µg/cm of gel.

For 2-dimensional (2-D) electrophoresis, protein extract was suspended in rehydration buffer.¹⁷ Then 25 µg of shrimp extract was applied to immobilized pH 4 to 7 (7-cm) gradient strips (Bio-Rad, Hercules, Calif) for rehydration and focusing, as described previously.¹⁷ Strips were equilibrated, as described by Beyer et al,¹⁷ and run with SDS-PAGE.

Gels were either stained with Simply Blue SafeStain (Invitrogen), according to the manufacturer's protocol, or proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, Mass), as described previously.¹⁷ Membranes were stained with amidoblack 0.1% (10% methanol and 2% acetic acid) staining solution or tested for IgE binding with patients' sera.

Protein identification was done from 2-D gels stained with Simply Blue SafeStain. Proteins of interest were analyzed at the Wistar Institute Protein Microchemistry/Mass spectrometry Facility, as described previously.¹⁷ Proteins of interest were excised, and "in-gel" digestion was performed. The tryptic digests were separated by means of HPLC, followed by matrix-assisted laser desorption/ionization (MALDI)/mass spectrometric (MS) analysis of selected peaks and subsequent Edman sequencing of selected peaks.

Immunoblot analysis with sera from patients with shellfish allergy

Immunoblot detection for IgE binding was done with extracts of raw and boiled *L. vannamei*. Membranes were incubated with sera from 38 patients with shrimp allergy (1:5 to 1:20 in PBS-Tween [1% BSA and 10% normal goat serum]) for 1 hour. After rinsing with PBS, the membranes were incubated with iodine 125-labeled goat anti-human IgE (DiaMed, Windham, Me) diluted as per the manufacturer's instructions, washed, and exposed to Kodak Imaging Film (Rochester, NY) for 1 to 12 days. As a negative control, serum was used from a nonatopic subject. IgE-binding proteins in 2-D immunoblots were visualized with phosphatase-labeled goat anti-human IgE, as described previously.¹⁷

For immunoblot inhibition experiments, a serum pool was prepared from 7 subjects with IgE antibodies that recognized a 20-kd shrimp protein by means of immunoblotting. Then 150 µL of the diluted serum pool (1:20 in PBS-Tween) was preincubated at room temperature for 2 hours with recombinant tropomyosin at a final concentration of 25 µg/mL. The mix was incubated with the shrimp membranes, as described above. As a control, the pool was preinhibited with chickpea extract at a final concentration of 1.5 mg/mL.

Shrimp cDNA library

A cDNA library was generated from raw pacific white shrimp, *L. vannamei*. Total RNA was extracted and mRNA purified on Oligotex mRNA Spin-column (Qiagen, Valencia, Calif). Synthesis and cloning of cDNA was done with a cDNA synthesis kit from Stratagene (La Jolla, Calif). The double-stranded cDNA was ligated into the lambda vector Uni-ZAP, packaged *in vitro* (Gigapack Gold; Stratagene), and transfected into *Escherichia coli* XL1-Blue cells.¹⁸

The library was screened with primers designed on the basis of internal sequences obtained from 2-D tryptic digests. For screening of the cDNA library, 2 degenerated oligonucleotides were used, resulting in isolation of a novel protein cDNA. Open reading frame translation of this cDNA sequenced at the Mount Sinai Core Facility provided the complete amino acid sequence of a new allergenic shrimp protein.

TABLE I. Clinical characteristics, type of reaction with shrimp, and specific serum IgE values of the 38 study subjects

Patient no.	Age (y)	Diagnosis	Shrimp reaction	Shrimp IgE (kU/L)	<i>B germanica</i> IgE (kU/L)	<i>D pteronyssinus</i> (kU/L)
1	10	A, AR, FA	U	49.5	12	9.5
2	17	A, AR, FA, AD	Inh-A, AE	52.1	12	9.7
3	10	A, AR, FA, AD	U	11.3	6.3	3.8
4	12	A, AR	U, AE	64.5	28	13.5
5	13	A, AR, FA	Inh-A, AE	82.4	43	22.4
6	12	A, AR, FA, AD	Inh-A, U	74.2	32	12.1
7	8	A, AR, FA, AD	U	0.38	0.4	0.6
8	12	AR	AE, RC	47	32	18
9	11	FA	U	5.6	0.9	<0.35
10	7	FA	U	14	5.6	5.3
11	29	AR, FA	RC, AE	15	1.8	1.9
12	6	AD, FA, A, AR	AE, U, GI, CU	1.9	3.4	4.7
13	3	AD, FA	U	100	74	>100
14	8	AD, A, AR	GI, RC, U	100	30	23
15	6	AD, FA, A	OAS	8	5.3	>100
16	8	AR, A	AE, U, A	30	42	7.1
17	38	A/AR	AE, U, CU	>100	17.5	>100
18	34	AR	AE, U	>100	<0.35	>100
19	16	A/AR/FA	ANA	>100	8.9	>100
20	27		OAS	93	4.2	>100
21	33	AR/A	AE, A	65	22.6	40.3
22	32	AR	AE, RC	48	12.1	59
23	15	AR	AE	55	23	>100
24	11	A/AR	U, GI	5	1	>100
25	40	A/AR	AE, A	14	3.2	9
26	13		U	0.8	<0.35	>100
27	24	A/AR	OAS	0.36	<0.35	77
28	34		ANA, A, AE	1	2.4	1.1
29	19		AE, RC	10	3	8.4
30	19	A/AR	AE	1	<0.35	56
31	26	A/AR	AE	3.7	3.8	>100
32	32	AR	AE, U	8	2	34.6
33	46	FA	AE, U, A	0.6	1	21
34	26	AR	OAS	2	1.5	>100
35	19	FA	U	14	8	>100
36	17	AR	AE, U	11	0.6	55
37	22	AR	AE	6	2.8	77
38	30	AR	AE	15	35	24.5

A, Asthma; AR, allergic rhinitis; FA, other food allergy; U, urticaria; AD, atopic dermatitis; Inh-A, asthma by inhalation; AE, angioedema; RC, rhinoconjunctivitis; GI, gastrointestinal symptoms; CU, contact urticaria; ANA, anaphylaxis; OAS, oral allergy syndrome.

Production of recombinant proteins

Recombinant proteins were obtained under native conditions, as described previously.¹⁸ Briefly, the protein-coding regions of tropomyosin and myosin-like protein were amplified by means of PCR with specific primers for ligation into the expression vector pET24b(+) (Novagen, Madison, Vt). The vector-plasmid construct was then transformed into *E coli* according to the manufacturer's instructions (Stratagene). The plasmids were then purified, and the nucleotide sequence was confirmed. Each individual plasmid was introduced into the BL21 expression *E coli* strain. The recombinant proteins were expressed after induction of bacterial cultures at 30°C for 16 hours and detected by means of Western blot analyses with anti-His-Tag antibodies. Finally, recombinant proteins were purified with a His-Bind Ni²⁺ chelating NTA-matrix resin (Novagen).

Probing recombinant MLC with patients' sera

Recombinant MLC was tested for IgE reactivity with individual sera from 19 patients with shrimp allergy that recognized a 20-kd protein by means of immunoblotting. A nonatopic adult was used as a negative control. Recombinant protein was loaded onto the gel at a concentration of 2.8 µg of protein/

cm of gel. The remainder of the protocol is as described above in the SDS-PAGE and immunoblotting sections. Membranes were incubated with iodine 125-labeled goat anti-human IgE and exposed to Kodak Imaging Film for 12 days.

RESULTS

IgE Reactivity of patients' sera to *L vannamei* extracts

Subjects with shrimp allergy show IgE reactivity by means of immunoblotting to multiple shrimp proteins ranging from 6 to 90 kd (Fig 1). Although boiled shrimp extracts have multiple allergenic proteins, in raw extracts IgE reactivity is mostly directed at tropomyosin and a limited number of proteins of less than 40 kd. Among all proteins, a 20-kd protein was recognized in boiled extracts with particular intensity by 21 (56.6%) of 37 sera. Most subjects recognized a protein of similar molecular weight in raw extracts but generally with lower intensity, except for a group of pediatric patients (subjects 2-8) who showed increased IgE

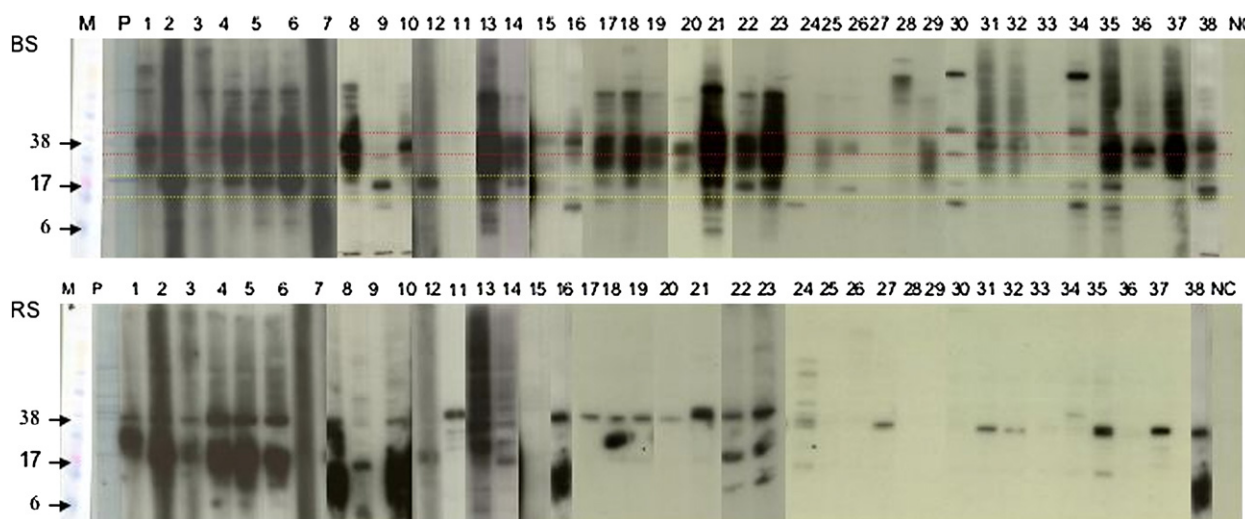


FIG 1. IgE immunoblotting of 38 patients' sera to boiled (BS, top) and raw (RS, bottom) shrimp extract. M, Molecular weight; P, total shrimp protein; lanes 1 to 38, immunolabeling with patients' sera; NC, negative control. IgE reactivity to tropomyosin is represented at 34 kD (red lines) and IgE reactivity to MLC is represented at 20 kD (yellow lines).

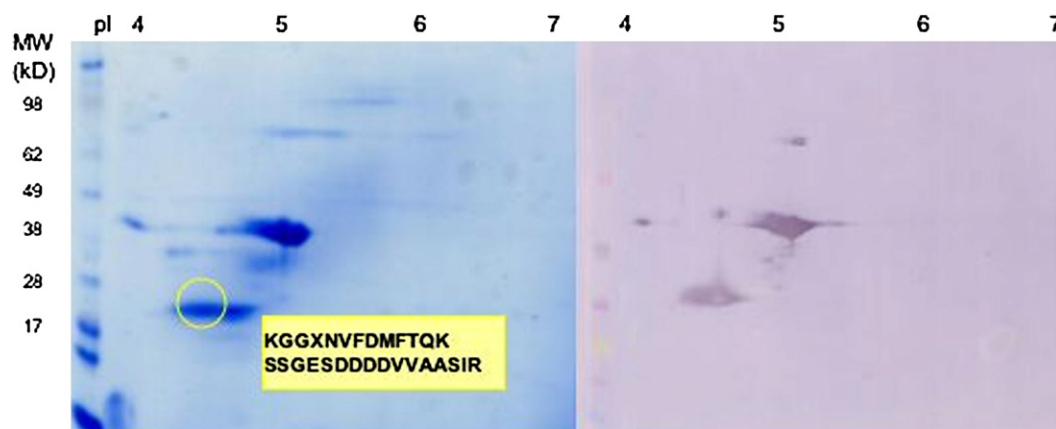


FIG 2. 2-D proteomics map and immunolabeling of boiled shrimp extract. Left, Gel stained for total protein analysis; right, IgE immunolabeling with 1 representative subject. The circle shows the spot sent for MALDI/MS analysis. The square shows 2 amino acid sequences obtained by means of Edman sequencing. MW, Molecular weight; pI, isoelectric point.

binding to the raw 20-kD protein. In several cases this protein was recognized with higher intensity than the 34-kD band corresponding to tropomyosin. Interestingly, for subjects 9 and 12, this was the only protein recognized in both raw and boiled extracts (Fig 1). Immunolabeling of 2-D membranes of boiled shrimp with serum from subject 38 demonstrated intense IgE binding to a 20-kD shrimp protein, with an isoelectric point of 4.2 (Fig 2). The control sera did not show any labeling (not shown).

Immunoblot inhibition with recombinant tropomyosin

A pool of patients' serum was preabsorbed with recombinant tropomyosin to determine whether this was a new allergenic shrimp protein. Preincubation of the serum pool with tropomyosin almost completely abolished IgE binding to most shrimp proteins. However, IgE reactivity to the 20-kD protein was not

inhibited, indicating that this is a novel shrimp allergen (Fig 3). Inhibition of IgE binding to the 34-kD protein tropomyosin was incomplete, possibly because of an insufficient amount of inhibitor or because of the presence of IgE-binding dimers of the 20-kD protein.

MALDI/MS analysis and Edman sequencing

Tryptic digestion of the 20-kD protein from a 2-D gel separated by means of microbore HPLC and MALDI/MS analysis of the peptides at the Wistar Facility, followed by Edman sequencing of 2 selected peaks, identified the protein as a myosin light chain (Fig 2). The sequences of 2 peptides were obtained: KGGXNVFDMFTQK and SSGESDDDDVVAASIR. The identified peptides showed high sequence identity with the homologous sequence of another allergenic MLC molecule, the German cockroach protein Bla g 8.

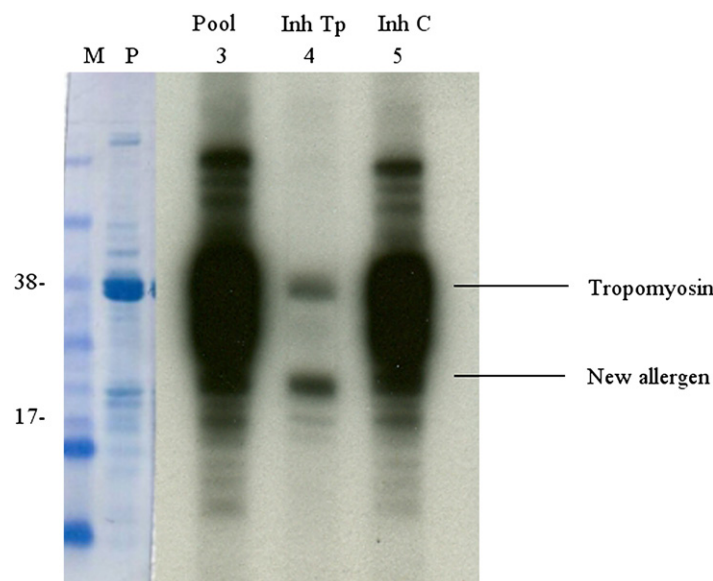


FIG 3. Immunoblot inhibition of IgE reactivity to boiled shrimp extract with recombinant tropomyosin. *M*, Molecular weight; *P*, protein staining of boiled shrimp extract; *lane 3*, IgE immunoblotting with a serum pool; *lane 4*, pool preincubated with recombinant tropomyosin (*Inh Tp*); *lane 5*, pool preincubated with chickpea extract as a control (*Inh C*).

	M	S	R	K	S	G	S	R	S	S	S	K	R	S	K	K	S	G
1	ATG	TCC	CGC	AAG	TCA	GGC	TCT	CGC	TCC	TCC	TCC	AAG	AGG	TCC	AAG	AAA	TCC	GGA
	TAC	AGG	CGC	TTC	AGT	CCG	AGA	CGC	AGG	AGG	AGG	TTC	TCC	AGG	TTC	TTT	AGG	CCT
55	G	G	S	N	V	F	D	M	F	T	Q	R	Q	V	A	E	F	K
	GGA	GGA	AGC	AAT	GTG	TTC	GAC	ATG	TTC	ACG	CAG	CGG	CAG	GTG	GCC	GAG	TTC	AAG
	CCT	CCT	TCG	TTA	CAC	AAG	CTG	TAC	AAG	TGC	GTC	GCC	GTC	CAC	CGG	CTC	AAG	TTC
109	E	G	F	Q	L	M	D	R	D	K	D	G	V	I	G	K	T	D
	GAG	GGC	TTC	CAG	CTG	ATG	GAC	CGC	GAC	AAG	GAC	GGC	GTC	ATC	GGC	AAG	ACC	GAC
	CTC	CCG	AAG	GTC	GAC	TAC	CTG	GCG	CTG	TTC	CTG	CCG	CAG	TAG	CCG	TTC	TGG	CTG
163	L	R	G	T	F	D	E	I	G	R	I	A	T	D	Q	E	L	D
	CTG	CGC	GGA	ACC	TTC	GAC	GAG	ATC	GGC	CGC	ATC	GCC	ACG	GAC	CAG	GAG	CTC	GAC
	GAC	GCG	CCT	TGG	AAG	CTG	CTC	TAG	CCG	GCG	TAG	CGG	TGC	CTG	GTC	CTC	GAG	CTG
217	E	M	L	A	D	A	P	A	P	I	N	F	T	M	L	L	N	M
	GAG	ATG	CTG	GCC	GAC	GCG	CCC	GCG	CCC	ATC	AAC	TTC	ACC	ATG	TTG	CTC	AAC	ATG
	CTC	TAC	GAC	CGG	CTG	CGC	GGG	CGC	GGG	TAG	TTG	AAG	TGG	TAC	AAC	GAG	TTG	TAC
271	F	A	E	R	Q	T	G	E	S	D	D	D	D	V	V	A	K	A
	TTC	GCG	GAA	CGG	CAG	ACG	GGC	GAG	AGT	GAC	GAC	GAC	GAC	GTG	GTG	GCG	AAG	GCC
	AAG	CGC	CTT	GCC	GTC	TGC	CCG	CTC	TCA	CTG	CTG	CTG	CTG	CAC	CAC	CGC	TTC	CGG
325	F	L	A	F	A	D	E	E	G	N	I	D	C	D	T	F	R	H
	TTC	CTG	GCG	TTC	GCC	GAC	GAG	GAA	GGC	AAC	ATC	GAC	TGC	GAC	ACC	TTC	CGC	CAC
	AAG	GAC	CGC	AAG	CGG	CTG	CTC	CTT	CCG	TTG	TAG	CTG	ACG	CTG	TGG	AAG	GCG	GTG
379	A	L	M	T	W	G	D	K	F	S	S	Q	E	A	D	D	A	L
	GCC	CTC	ATG	ACC	TGG	GGC	GAC	AAG	TTC	TCC	TCG	CAG	GAA	GCC	GAC	GAC	GCC	CTC
	CGG	GAG	TAC	TGG	ACC	CCG	CTG	TTC	AAG	AGG	AGC	GTC	CTT	CGG	CTG	CTG	CGG	GAG
433	D	Q	M	D	I	D	D	G	G	K	I	D	V	Q	G	V	I	Q
	GAC	CAG	ATG	GAC	ATC	GAC	GAC	GGA	GGC	AAG	ATC	GAC	GTC	CAA	GGC	GTC	ATC	CAG
	CTG	GTC	TAC	CTG	TAG	CTG	CTG	CCT	CCG	TTC	TAG	CTG	CAG	GTT	CCG	CAG	TAG	GTC
487	M	L	T	A	G	G	G	D	D	A	A	A	E	E	A	*		
	ATG	CTC	ACA	GCC	GGC	GGC	GGA	GAC	GAC	GCG	GCC	GCC	GAG	GAA	GCC	TAA		
	TAC	GAG	TGT	CGG	CCG	CCG	CCT	CTG	CTG	CGC	CGG	CGG	CTC	CTT	CGG	ATT		

FIG 4. cDNA sequence and translation of Lit v 3: nucleotide sequence of shrimp MLC cDNA clone and deduced amino acid sequence (Gene Bank accession no. EU449515).

Molecular cloning of the shrimp MLC

A cDNA library was prepared from fresh white pacific shrimp, *L. vannamei*. The shrimp cDNA library was screened with primers designed on the basis of the internal sequence obtained from one of the 2-D tryptic digests (KGGXNVFDMFTQK). The degenerated oligonucleotides 5' TTYGAYATGTTYACICARA3' and

5' AACGTGTTCGACATGTTACCCAGAAG3' were used as primers, resulting in isolation of a shrimp cDNA. Open reading frame translation of this cDNA provided the complete amino acid sequence of a new allergenic shrimp protein (Fig 4). The new allergenic shrimp protein was named Lit v 3.0101 by the international Allergen Nomenclature Committee.

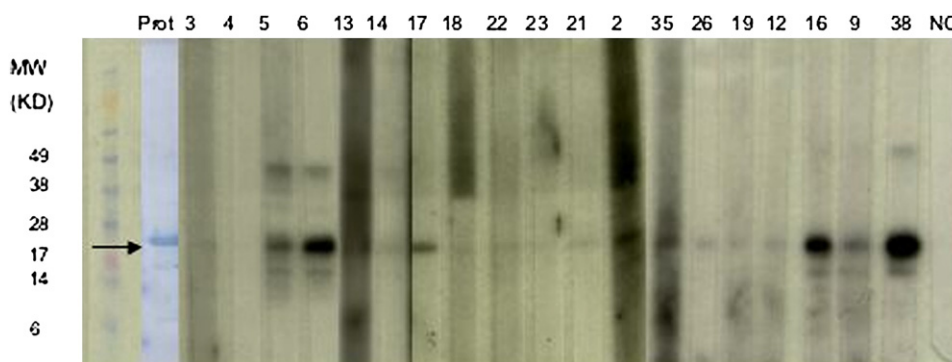


FIG 5. IgE binding to recombinant shrimp MLC. Numbered lanes, Immunoblotting with patients' sera. NC, Negative control; Prot, amidoblack staining of recombinant MLC (arrow).

IgE-binding capacity of recombinant shrimp MLC

Recombinant shrimp MLC was tested for IgE binding with individual sera from the 19 patients who recognized a 20-kDa protein by means of immunoblotting. Most subjects (17/19) tested recognized the recombinant shrimp MLC, although with weaker intensity than the native protein (Fig 5).

DISCUSSION

Crustaceans are highly allergenic foods responsible for food-induced allergic reactions in both children and adults. Among them, shrimp cause most of the allergic reactions.⁶ However, in spite of the high prevalence of crustacean allergy, there is a limited knowledge of the proteins involved in the induction of such allergic reactions. To date, only one major shrimp allergen has been identified, the muscle protein tropomyosin,⁸⁻¹⁰ termed Pen a 1, Pen m 1, Met e 1, Pen i 1, or Lit v 1, depending on the species used. In addition, a minor shrimp allergen, arginine kinase (Pen m 2 and Lit v 2),¹¹ has been reported. Most recently, a sarcoplasmic protein has been reported as a new crustacean allergen¹³; however, molecular approaches are necessary to prove its allergenic capacity. Importantly, multiple IgE-binding shrimp proteins that are recognized by individuals with shrimp allergy require further characterization.

In the present study we have identified an MLC protein as a new shrimp allergen, Lit v 3, which is recognized by more than 50% of our subjects with shrimp allergy. Although tropomyosin appears to be the most abundant allergen in crustaceans, some of our patients showed predominately binding to Lit v 3. In 2 patients Lit v 3 was almost the only allergen recognized. Therefore it appears crucial to include Lit v 3 in future diagnostic and therapeutic strategies.

Myosins are a large superfamily of motor proteins that move along actin filaments while hydrolyzing adenosine triphosphate. In muscle myosin is composed of 2 heavy chains. The globular motor domains interact with actin, whereas the tails dimerize in a coiled-coil structure. Two light chains, each of 20 kDa, wrap around the neck region of each myosin heavy chain. While resting, troponin prevents actin-myosin interaction. During sarcolemmal depolarization, cytosolic calcium increases and binds troponin C, which undergoes conformational changes, moving the attached tropomyosin away from the myosin-binding sites on actin filaments. The actin-myosin complex is influenced by phosphorylation of the MLC followed by myosin conformational

changes and translocation of attached thin filaments, causing muscle contraction.

Previously identified shrimp allergens are abundant in shrimp muscle. Tropomyosin is the best characterized, and its IgE-binding epitopes have been identified.¹⁹ Arginine kinase,^{17,12} with a molecular weight of 40 kDa, and a sarcoplasmic calcium-binding protein¹³ with a molecular weight of 20 kDa, are also found in muscle in significant quantities. The new shrimp allergen we are reporting, MLC, is also a muscle protein. MLC is recognized in both raw and boiled shrimp extracts, emphasizing the fact that the concentration of an allergen in foods and its stability to heat and other factors during processing are important factors in determining the allergenicity of the proteins.²⁰⁻²²

Lit v 3 has 177 amino acids, a molecular weight of 20 kDa, and a calculated isoelectric point of 4.2. Lit v 3 and the sarcoplasmic calcium-binding protein, recently reported as an allergen, appear to have similar molecular weights and isoelectric points.¹³ It is therefore difficult to determine which protein is recognized by a particular patient's IgE antibodies by using standard laboratory methods. In this study, however, we have demonstrated the potential allergenicity of recombinant MLC, which was recognized by 17 of 19 subjects tested. This demonstrates the utility of using recombinant allergens to correctly identify allergenic proteins and to appropriately profile allergenic proteins to which an individual patient is reactive. Identification of particular allergenic proteins with recombinant allergens in a patient is of interest because it would allow the design of immunotherapy *a la carte*. Recombinant allergens are therefore important tools for the diagnosis of shellfish allergy with the potential to be used as immunotherapeutic agents. However, it must be noted that recombinant MLC binds patients' IgE with a lower intensity than the native MLC. It is possible that the native protein contains conformational epitopes or specific epitopes because of posttranslational modifications that are not present in the recombinant form, which account for the differences in IgE binding.

IgE binding to a 20-kDa protein is detected in both raw and boiled shrimp extracts, sometimes with stronger IgE-binding intensity to the raw protein extract. This is in contrast to several other shrimp proteins that appear to have stronger IgE recognition to boiled extracts.²³ IgE binding to the boiled form of MLC appears to be greater in adults compared with children (see patients 2-6), who tend to recognize the MLC in the raw extract with higher intensity. Interestingly, some children (patients 2, 5, and

6) had asthmatic episodes when exposed to the steam of boiling shrimp, suggesting that MLC is aerosolized and might contribute to respiratory symptoms in such patients. In fact, in a previous study IgE binding to an undetermined 21- to 26-kd protein was detected in shrimp boiling water and raw shrimp by a patient with occupational asthma.²⁴ Furthermore, protein profiles of boiling water, steam, and boiled shrimp were very similar, suggesting that some proteins become aerosolized during boiling. It is possible that the 21- to 26-kd protein described by Goetz and Whisman²⁴ is the MLC. Whether the MLC is more important than other shrimp proteins in the induction of asthma symptoms remains to be investigated.

It is well known that crustaceans are highly cross-reactive, and usually avoidance of all crustaceans is recommended. Tropomyosin has been identified as the main cross-reactive molecule among crustaceans.^{25,26} Important *in vitro* cross-reactivity exists with mollusks and other invertebrates, such as dust mites or cockroach.²⁷⁻²⁹ The cross-reactivity among crustaceans, cockroach, and dust mites seems to be based on sequence similarities of tropomyosin IgE-binding epitopes.³⁰ Also, arginine kinase has been described as a cross-reacting allergen among crustaceans and between crustaceans and insects.¹¹ Interestingly, the amino acid sequence of MLC is 66% similar and 51% identical to cockroach Bla g 8, the allergenic MLC of *B germanica*. Sequence similarity between MLCs can be implicated for *in vitro* and possibly clinical cross-reactivity among shrimp and cockroach and possibly also with dust mites. In contrast, sequence identity with other invertebrate MLCs, such as *Schistosoma* (13% identity) and *Aedes* (17% identity) species, was low.

Although sensitization to *D pteronyssinus* is detected in 41% of the Spanish asthmatic population (Madrid, 12%; Canary Islands, 72%),³¹ the house dust mite sensitization rate among our subjects with shellfish allergy was much higher: 37 (97%) of 38 of our patients (90% of the Madrid patients and 100% of the Canary Island patients) were sensitized to dust mites. Also, 89% of our subjects were sensitized to cockroach (100% of the patients in Madrid, 80.9% of the patients in the Canary Islands, and 100% of the patients in New York). Although not all subjects with dust mite or cockroach allergy have crustacean allergy, it is possible that sensitization to particular dust mite or cockroach allergens, possibly tropomyosin, MLC, or others, might be responsible for the induction of allergy to crustaceans. However, to date, the initial sensitizing protein remains unknown.

Lastly, although crustacean muscle proteins are particularly allergenic, homologous vertebrate proteins are not allergens. Although many of those proteins are phylogenetically conserved throughout evolution, substantial amino acid substitutions appear to abolish IgE binding to vertebrate proteins, as has been shown for tropomyosin.³² This is of importance in the design of mutated hypoallergenic molecules for use in the treatment of individuals with shellfish allergy and can be applied for designing hypoallergenic tropomyosin and MLC variants.

In summary, we have identified shrimp MLC as a new major shrimp allergen named Lit v 3.0101. Recombinant Lit v 3 was recognized by subjects with shrimp allergy who reacted to the native shrimp protein. Because Lit v 3 appears to be a new major shrimp allergen and is recognized predominantly by some patients, identification of its IgE-binding epitopes and design of a mutated hypoallergenic form for use as a nonallergenic vaccine remains one of our goals.

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Clinical implications: Lit v 3 should be included in future diagnostic and therapeutic strategies for shrimp allergy.

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