

RESEARCH ARTICLE

Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: A comprehensive allergenomic approach

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Scope: Prawn allergy is one of the leading causes of IgE-mediated hypersensitivity to food. Alterations of IgE-antibody reactivity to prawn allergens due to thermal processing are not fully understood. The aim of this study was to analyze the impact of heating on prawn allergens using a comprehensive allergenomic approach.

Methods and results: Proteins from raw and heat-processed black tiger prawn (*Penaeus monodon*) extracts as well as recombinant tropomyosin (rPen m1) were analyzed by SDS-PAGE and immunoblotting using sera from 16 shellfish allergic patients. IgE antibody binding proteins were identified by advanced mass spectroscopy, characterized by molecular structure analysis and their IgE reactivity compared among the prepared black tiger prawn extracts. Heat processing enhanced the overall patient IgE binding to prawn extracts and increased recognition of a number of allergen variants and fragments of prawn allergens. Allergens identified were tropomyosin, myosin light chain, sarcoplasmic calcium binding protein, and putative novel allergens including triose phosphate isomerase, aldolase, and titin.

Conclusion: Seven allergenic proteins are present in prawns, which are mostly heat-stable and form dimers or oligomers. Thermal treatment enhanced antibody reactivity to prawn allergens as well as fragments and should be considered in the diagnosis of prawn allergy and detection of crustacean allergens in processed food.

Keywords:

Allergen dimers / Allergen fragments / Black tiger prawn / Heat processing / In vitro diagnostics



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Abbreviations: **AK**, arginine kinase; **FBA**, fructose 1,6 bisphosphate aldolase; **MLC**, myosin light chain; **SCBP**, sarcoplasmic

1 Introduction

Shellfish allergy is one of the leading causes of hypersensitivity reactions to food, reported to affect over 2% of the world population [1]. Unlike milk and egg allergies, allergy to shellfish typically persists into adulthood [1,2]. Despite the adverse effects of shellfish allergy, there is still no cure available and avoidance of the offending food is essential. Food allergy is

calcium binding protein; **TIM**, triose phosphate isomerase; **TM**, tropomyosin; **Ttn**, titin

based on the generation of IgE antibodies against particular proteins, i.e. allergens, which bind to FcεRI receptors on the surface of mast cells and basophils. Subsequent binding of allergenic proteins to these cell-bound IgE antibodies results in cross-linking of receptor-IgE complexes, which triggers the activation of the cells, resulting in mediator release and clinical symptoms. Current in vitro diagnostics for allergy are based on the quantification of these specific IgE antibodies against various allergen extracts. Currently, over 2700 allergenic proteins have been identified, 20% of which are derived from food sources [3]. Food allergens can be classified into 71 protein families and these comprise less than 2% of all the known protein families. Allergenic proteins have specific biological functions, however the factors that make a protein allergenic are largely unknown [4].

The major allergenic protein in crustaceans currently characterized is tropomyosin (TM), a heat-stable muscle protein that constitutes up to 20% of the total protein content [5–8]. TM has a highly conserved alpha helical coiled-coil structure and is reported to cause IgE cross-reactivity in patients to crustaceans and other arthropods such as house dust mites [9]. Recent reports suggest that IgE reactive to TM may be a good marker for detection of clinical prawn allergy [10, 11]. In addition, specific food processing techniques seem to impact IgE binding to TM [12]. The effects of food processing on the IgE binding reactivity to different shellfish allergens are poorly characterized at the molecular level. Moreover, it is unknown how food processing affects the detection of shellfish allergens using antibody-based tests, as required for food labeling regulations. Most studies on the molecular characterization of crustacean allergens along with associated allergy prevalence studies mainly focus on single allergens and not on the complete allergen repertoire affected by food processing. In addition, as prawn allergens that are clinically relevant to the Asia-Pacific region are not fully identified, we selected the black tiger prawn (*Penaeus monodon*) as a model species, due to its high consumption in this region.

The aim of this study was to analyze the impact of heat processing on a comprehensive panel of identified prawn allergens, by utilizing an allergenomic approach including patient's IgE-binding diversity and advanced mass spectroscopy.

2 Materials and methods

2.1 Prawn heat treatment and protein extract preparation

The outer shell of fresh prawns (*P. monodon*) was removed, the abdominal muscles shredded into small pieces and homogenized in 200 mL of PBS. This slurry was mixed for 3 h at 4°C, followed by centrifugation at 14 000 rpm for 15 min. The supernatant was filtered through a glass-fiber filter, followed by a 0.45 µm membrane filter (Millipore, Billerica, MA, USA) and stored at –80°C until further use, named “P-raw” extract. An aliquot of the P-raw extract was heated

at 100°C for 20 min in a water bath and the resultant extract centrifuged and filtered as above, named “P-H” extract. The third method, based on the natural exposure to crustacean allergens included heating the whole raw prawn with its outer shell in PBS at 100°C for 20 min and extracting the proteins as described above and named “P-WH.” The protein content of each prepared extract was calculated using the Bradford's protein assay (1976).

2.2 Purification of natural TM

TM was purified from the heated whole black tiger prawn using strong anion exchange chromatography as described previously [7]. Briefly, 20 mg of the P-WH extract was loaded onto a Mini Macroprep High Q column (BioRad, Hercules, CA, USA) using 30 mM Tris-HCl buffer pH 6.8 as the mobile phase. TM was eluted using a gradient salt concentration from 400 to 600 mM of sodium chloride in the same mobile phase. The resultant TM fraction was dialyzed overnight against PBS and stored at –80°C until further use.

2.3 Sequencing and expression of black tiger prawn TM

Total RNA was extracted from black tiger prawns using the RNeasy RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Single-stranded cDNA was generated by RT-PCR using a Transcriptor High Fidelity cDNA synthesis kit (Roche, Basel, Switzerland). The generated cDNA was used as a template to amplify the coding region of TM using forward (5'-GCGGATCC-GACGCCATCAAGAAGAAGATGC-3') and reverse (5'-GCGAATTC-TTAGTAGCCAGACAGTTCGCTG-3') primers and cloned into a sequencing vector, pCR 2.1 using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA).

For recombinant TM expression, the coding region for TM was subcloned into the expression vector pRSET A (Invitrogen) and transformed into BL21 *Escherichia coli* cells using electroporation. Expression was induced using 0.6 mM IPTG from a fresh overnight culture in Luria broth containing 100 µg/mL ampicillin. Recombinant TM from black tiger prawn (rPen m 1) was subsequently purified from the crude cell lysate using nickel-charged metal chelate affinity chromatography, dialyzed in PBS and stored at –80°C until further use.

2.4 Patients

Sixteen subjects with a convincing clinical history of allergic reactivity to shellfish and one nonatopic subject were recruited by The Alfred Hospital, Allergy Clinic, Melbourne,

Table 1. Demographics of 16 shellfish allergic patients and one nonatopic control donor. Total IgE and shrimp-specific IgE (f24) were quantified using ImmunoCAP®

Subject	Sex	Age	Total IgE (kU/L)	Shrimp-specific IgE (f24) (kU/L)	Shellfish exposure	Symptoms	Reactivity to other reported seafood species	Reactivity to HDM	
								slgE (kU/L)	SPT
1	M	23	179	3.27	Oral	Laryngeal angioedema	Tuna, salmon, trout	NT	7 mm
2	F	24	372	1.82	Oral	OAS	–	3.90	9 mm
3	F	17	242	1.32	Contact	Asthma, rhinitis	–	NT	7 mm
4	M	34	55	0.37	Oral	Asthma, angioedema, itch	–	7.36	15 mm
5	M	43	136	4.54	Oral	OAS, dyspnoea	Scallop	NT	NT
6	M	37	100	2.11	Oral	Asthma, anaphylaxis, urticaria	–	NT	NT
7	F	65	150	0.6	Oral	OAS, asthma, rhinitis	–	3.25	6 mm
8	M	51	164	0.4	Oral	Rhinitis, hypotension, erythema	–	NT	NT
9	F	56	440	0.5	Oral	Urticaria, OAS, pruritus, rhinitis	Crab	NT	5 mm
10	F	33	28	0.35	Contact	Urticaria, rhinitis, angioedema	Lobster	0.75	3 mm
11	M	23	158	0.85	Oral	Urticaria, collapse, dyspnoea, angioedema	–	2.27	6 mm
12	M	46	822	5.33	Oral	Urticaria, anaphylaxis	–	37.3	8 mm
13	F	20	3401	6.65	Oral	Asthma, rhinitis, urticaria, anaphylaxis	–	>100	NT
14	M	21	283	19.7	Oral	Asthma, urticaria, angioedema, rhinitis	–	51.3	NT
15	F	25	238	5.93	Oral	Anaphylaxis, angioedema	–	16.70	8 mm
16	F	23	1946	9.5	Oral	Anaphylaxis, angioedema, rhinitis	Flounder, crab	14.10	NT
17 (control)	F	45	–	–	–	Nonatopic	–	–	–

OAS, oral allergy syndrome; NT, not tested.

Victoria, Australia. Shrimp-specific IgE antibodies were quantified using the ImmunoCAP system (Thermo Scientific, USA). Skin prick testing and oral challenge with crustacean extracts were not conducted routinely in these patients, in keeping with the clinicians' preference for safer serum-specific IgE testing and use of clinical history of reactions on exposure. Demographic details of patients are given in Table 1. Ethics approval for this study was granted by James Cook University's Ethics committee (Project number H4313) in collaboration with The Alfred Hospital (Project number 192/07) and Monash University's Ethics Committees (MUHREC CF08/0225).

2.5 SDS-PAGE and Immunoblotting

SDS-PAGE was performed as described previously [13]. Each extract (10 µg/well) was mixed with reducing sample buffer containing SDS and dithiothreitol, heated at 90°C for 3 min, and loaded onto a 12% polyacrylamide gel. The protein components were resolved using a BioRad Mini-Protean Tetra cell (BioRad) and stained using Coomassie brilliant blue R-250 to visualize the separated proteins.

For immunoblotting, the separated proteins were transferred to an activated PVDF membrane (BioRad) and blocked with 5% skim milk powder in PBS with 0.05% Tween-20. For TM detection in various extracts, the proteins were exposed to monoclonal anti-TM antibody (Abcam, Cambridge, MA, USA) and subsequently rabbit anti-mouse IgG horseradish peroxidase labeled antibody (Sigma, USA). Antibody binding was visualized using the enhanced chemiluminescence.

To analyze the IgE-binding patterns to prawn extracts, the membrane was blocked with 5% skim milk powder in PBS with 0.05% Tween-20 and subsequently incubated overnight with 1:10 diluted patient sera using a slot blot apparatus (Idea Scientific, MN, USA). IgE binding was detected using horseradish peroxidase labeled rabbit anti-human IgE polyclonal antibody diluted 1:2500 (DAKO Corporation, USA) and visualized using the enhanced chemiluminescence method. Serum from a nonatopic donor was used as a negative control and a label control was included to confirm lack of reactivity by the secondary antibody to the extracts.

To characterize the IgE reactive protein band patterns, densitometric analysis was performed using the Geldoc System and Quantity One analytical software (BioRad). Molecular weights of the antibody-binding proteins were calculated against a standard curve of proteins with known molecular weight (Biorad) using the Quantity One software. Using the arbitrary density values of each patient's IgE-binding protein bands, the antibody reactivity was graded as low, medium and strong.

For the analytical representation of IgE recognition patterns among the 16 patient's to various prawn proteins, allergograms were created, based on IgE reactive bands for each patient sera and their estimated molecular weights as calculated above. Analogous IgE-reactive protein bands among

patients were grouped together with an error range of ± 0.5 kDa.

2.6 Mass spectrometric identification of IgE-binding proteins

The black tiger prawn proteins reacting with patient serum IgE antibodies were excised from an equivalent SDS-gel for mass spectrometric analysis. The bands were destained and digested with trypsin as described previously [8, 13] using the DIONEX Ulti-242 Mate3000 Nano LC System (Germering, Germany). Tryptic peptides were separated on a nanoflow analytical column (LC Packing, Sunnyvale, CA) at 180 nL/min using a gradient regimen developed previously [7]. Tandem spectra were searched using the NCBI Inr with the Matrix Science (Mascot) search engine (precursor and product ion mass tolerance set at 0.2 Da).

2.7 Three-dimensional (3D) homology modeling of identified prawn allergens

Homology models of the identified prawn allergens were created using SWISS MODEL [14]. For visualization of the allergenic protein's quaternary configuration, 3D ribbon and space filling protein models were created using UCSF's Chimera software [15]. For homology modeling, template protein structures were used for TM (PDB ID, 1C1G), arginine kinase (AK) (PDB ID, 4AM1), myosin light chain (MLC) (PDB ID, 2W4A), sarcoplasmic calcium binding protein (SCBP) (PDB ID, 2SAS), fructose bisphosphate aldolase (PDB ID, 1FBA), triose phosphate isomerase (TIM) (PDB ID, 2I9E), and titin (Ttn) (PDB ID, 1G1C).

2.8 Cellular reactivity of prawn allergens

Confirmation of biologically relevant IgE reactivity of prawn allergens was demonstrated by activation of blood-derived basophils as described previously [16]. In brief, the extracts were tested at increasing concentration (0.005–5 µg/mL) on basophils from whole blood of two prawn allergic patients, number 2 and 12. Stimulation buffer alone was used as a negative control. Results were expressed as the percentage of basophils loaded with IgE expressing high CD63 (IgE^{hi} cells) for each concentration of allergen extract with the negative control level subtracted.

3 Results

3.1 Protein separation and allergen-specific antibody-binding pattern

The heated extracts showed fewer protein bands compared to the raw extract, with major bands at about 37, 22, 18, and 16 kDa (Fig. 1A). The presence of TM in the analyzed protein

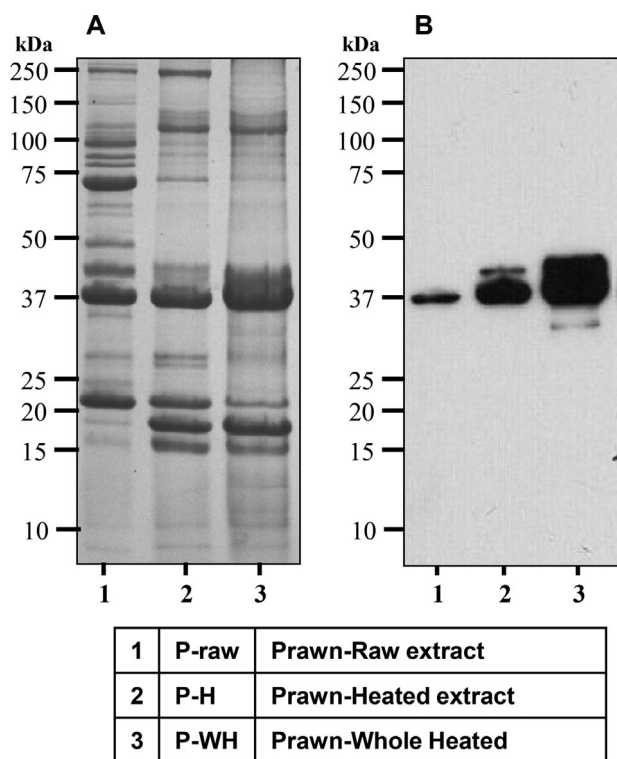


Figure 1. SDS-PAGE and immunoblot analysis: (A) 12% SDS-PAGE of black tiger prawn extracts stained with Coomassie brilliant blue and (B) immunoblotting with monoclonal anti-TM antibody. (1) Raw muscle extract (P-raw), (2) muscle extract heat treated (P-H), (3) whole animal heat treated (P-WH).

extracts was confirmed using a TM-specific mAb (Fig. 1B). The antibody-binding pattern for TM in P-H was different compared to P-WH, indicating possible TM variants.

3.2 Patient demographics

Eight female and eight male shellfish allergic patients, aged 17–65 years were recruited for the present study (Table 1). Total serum IgE levels ranged from 28 to 3401 kU/L and shrimp-specific IgE ranged from 0.35 to 19.7 kU/L.

3.3 Cloning and sequencing of black tiger prawn TM

Black tiger prawn TM was generated as a recombinant protein in *E. coli* to analyze the IgE recognition pattern in patients to unmodified TM (Fig. 2A). Immunoblot analysis using monoclonal anti-TM antibody revealed a single band, confirming the presence of TM. Seven of the 16 patients (43%) showed strong IgE binding to the rPen m 1 while 11 patients (69%) recognized the purified natural TM (Figs. 2A and B). The coding region of TM was successfully amplified and the cDNA sequence (Pen m 1.0101) published in GenBank under the accession number HM486525.1 (Fig. 2C).

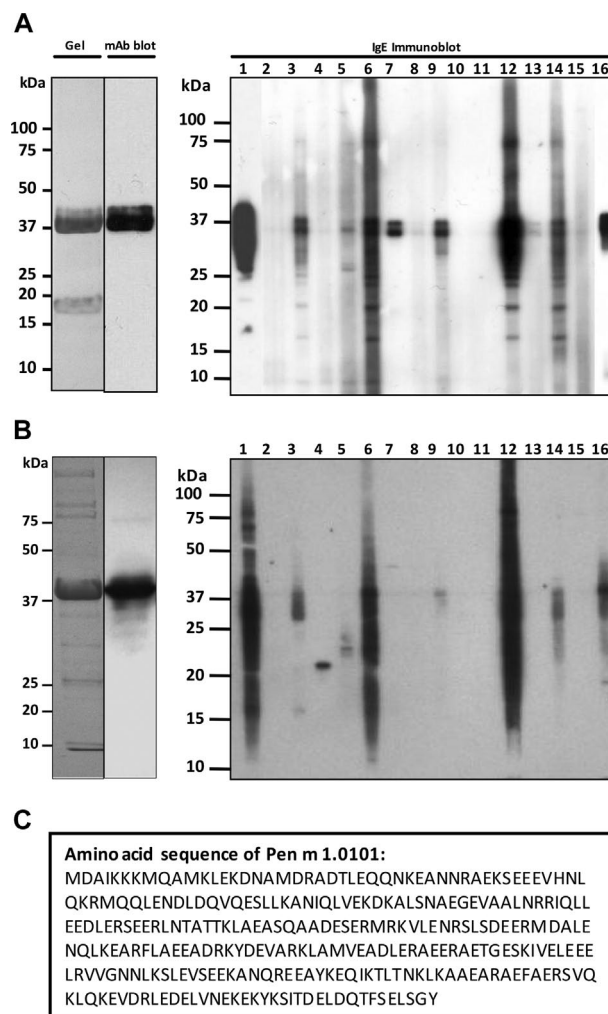


Figure 2. A total of 12% SDS-PAGE Coomassie stain, monoclonal anti-TM antibody immunoblot, and IgE immunoblot using sera from 16 shellfish allergic patients of (A) natural prawn tropomyosin, Pen m 1 and (B) recombinant prawn tropomyosin, rPen m 1. (C) Amino acid sequence of Pen m 1.0101 (Genbank accession number HM486525.1).

Circular dichroism spectroscopic analysis confirmed similar secondary structures of the natural and recombinant prawn TM (data not shown).

3.4 Patient IgE immunoblotting and allergogram analysis

Strong IgE binding was observed mainly for proteins in the range of 25–75 kDa for raw and heated prawn extracts (Fig. 3). In order to compare and analyze the IgE antibody binding data from all the subject sera, allergograms were generated for each prawn extract (Fig. 4). The molecular weight (MW) of each IgE-binding protein was calculated and IgE-binding intensity of each band graded as low, medium or strong as detailed in Section 2.

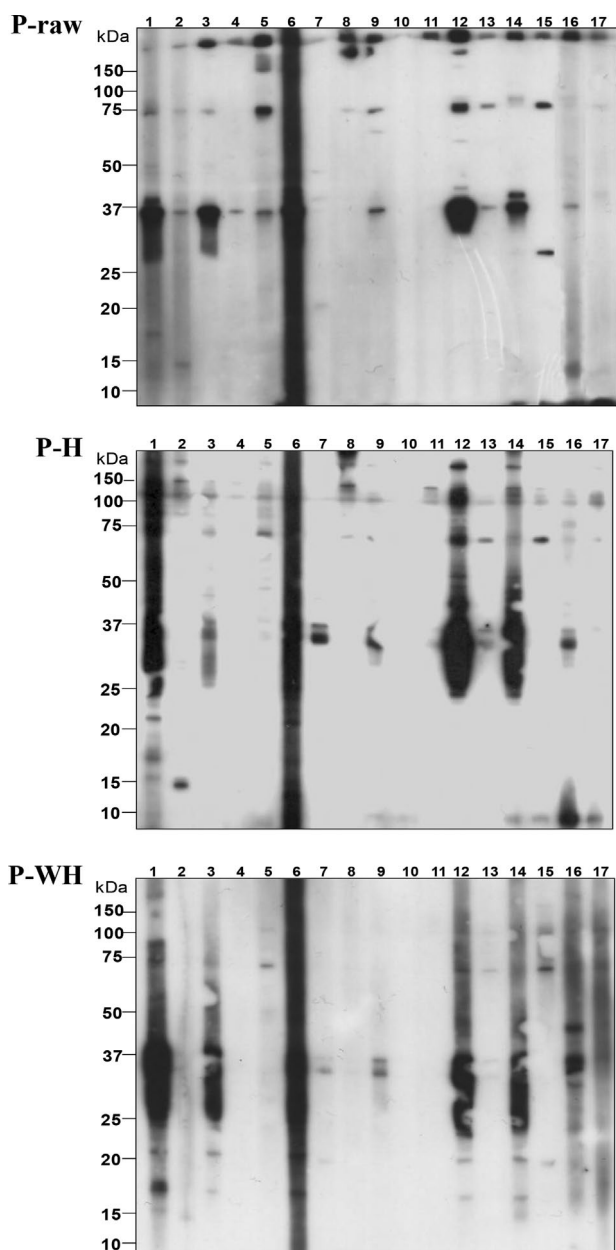


Figure 3. IgE immunoblotting: IgE antibody reactivity to black tiger prawn extracts with 16 shellfish allergic patient sera and one control nonatopic donor serum against raw muscle extract (P-raw), muscle extract heat treated (P-H), and whole animal heat treated (P-WH).

IgE binding to lower molecular weight proteins, between 10 and 30 kDa, increased considerably after heating (Fig. 4). High IgE binding was observed for a 35 kDa protein in P-raw, while heated extracts P-H and P-WH showed IgE binding to 33.5 and 36.5 kDa proteins in 50% of the patients. IgE reactivity was also noted to a 70 kDa protein. Eleven (68%) and seven (43%) patients demonstrated IgE binding to this protein in the raw and heated prawn extracts, respectively. A

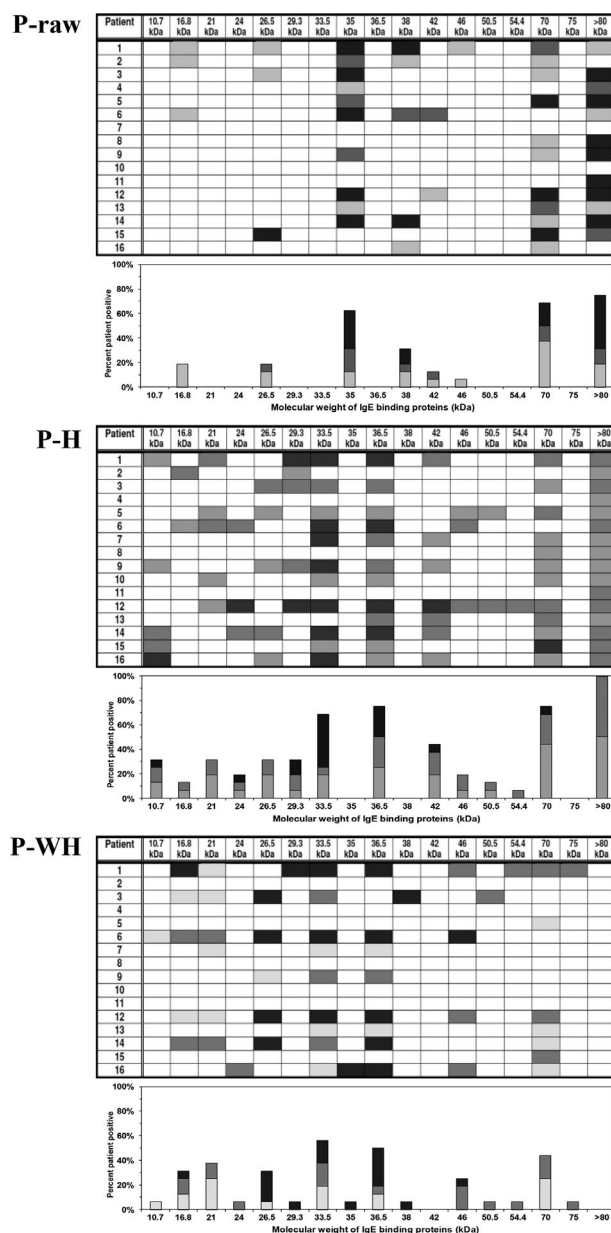


Figure 4. Allergogram analysis of patterns of IgE binding to proteins in different black tiger prawn extracts; P-raw, P-H, and P-WH. IgE binding intensities are graded as low, medium, and strong, and percentage patient reactivity toward different IgE-binding proteins is shown.

nonatopic control serum, lane 17, did not show significant binding to any of the prawn extracts or purified TM.

3.5 Identification of IgE-binding proteins by shotgun mass spectrometric analysis

Based on the IgE-binding patterns of the prawn extracts, selected bands were excised from SDS-PAGE gels of P-raw and

Table 2. IgE antibody reactive proteins identified by SDS-PAGE using LC-MS technique with sequence coverage denoted as percentage. IgE reactive proteins have been matched to known allergens using GenBank accession numbers. Number of patients tested positive with percentage shown in parentheses. See Supporting Information Table 1 for full list of peptide sequences

SDS-PAGE band (kDa)	Protein identity	Sequence coverage (%)	Accession number (GenBank)	Protein function/mass (kDa)	Known allergens	Patients tested positive (%) (n = 16)
Prawn raw (P-row)						
70	Titin fragment	2	CAD60685.2	Muscle protein, 1200	–	11 (68)
42	Arginine kinase	27	GQ246164	Phosphotransferase, 40	Pen m 2	2 (12.5)
	Putative fructose 1,6 biphosphate aldolase	12	Q9URB4	Glycolytic enzyme, 39.2	Cand a FBA	
38.0	Arginine kinase	29	GQ246164	Phosphotransferase, 40	Pen m 2	5 (31)
35	Tropomyosin	15	HM486525	Muscle protein, 37	Pen m 1	
	Arginine kinase	37	GQ246164	Phosphotransferase, 40	Pen m 2	10 (62)
26.5	Sarcoplasmic calcium binding protein	39	FJ184279	Muscle protein, 20	Lit v 4	3 (19)
16.8	Triose phosphate isomerase	8	FJ462738	Isomerase enzyme, 28	Cra c 8	3 (19)
Prawn whole heated (P-WH)						
70	Titin fragment	2	CAD60685.2	Muscle protein, 1200	–	7 (43)
38	Tropomyosin	48	HM486525	Muscle protein, 37	Pen m 1	1 (6)
36.5	Arginine kinase	11	GQ246164	Phosphotransferase, 40	Pen m 2	8 (50)
	Tropomyosin	47	HM486525	Muscle protein, 37	Pen m 1	
33.5	Tropomyosin	48	HM486525	Muscle protein, 37	Pen m 1	9 (56)
26.5	Tropomyosin fragment	10	HM486525	Muscle protein, 37	Pen m 1	5 (31)
16.8	Myosin light chain	9	ADM34185	Muscle protein, 20	Lit v 3	5 (31)
	Sarcoplasmic calcium binding protein	14	FJ184279	Muscle protein, 20	Lit v 4	

P-WH extracts for mass spectrometric identification (Table 2). TM was detected at 38.0 kDa in P-row while it was identified at 33.5 and 36.5 kDa in P-WH. TM fragments were also identified at 26.5 kDa in P-WH (Supporting Information Table 1). The 18 peptides sequenced in this protein represent a large stable fragment of TM, excluding the N- and C- terminal ends of the complete protein structure (Supporting Information Fig. 1). In addition, SCBP, a recently identified allergen in Vannamei prawn (*Litopenaeus vannamei*), was detected at 26.5 kDa in P-row as well as 16 kDa in P-WH. AK was identified in P-row at three different MWs: 42, 38, and 35 kDa. In contrast, AK was identified in P-WH at 36.5 kDa. MLC was detected only in P-WH at 16 kDa but not in P-row.

For the first time the allergen fructose 1,6-bisphosphate aldolase (FBA), previously identified in cockroach [17], was identified in black tiger prawn at 42 kDa in P-row but not in P-WH, consistent with heat sensitivity. Two additional novel

allergenic proteins were identified in this prawn species. TIM previously identified in North Sea shrimp [18] was detected at 16 kDa in P-row. Furthermore, a 70 kDa IgE-binding fragment of the invertebrate muscle protein Ttn, one of the largest known proteins was identified with IgE binding in P-row and P-WH, demonstrating heat stability.

3.6 Homology modeling of identified allergens

For better visualization of the allergen's tertiary and quaternary protein structure in their natural state, 3D homology models of the identified allergens were created (Fig. 5). TM exists as a coiled-coil dimer made up of two alpha-helical subunits, 33 kDa in size each. AK and MLC are present in a monomeric globular form of 40 and 19 kDa, respectively.

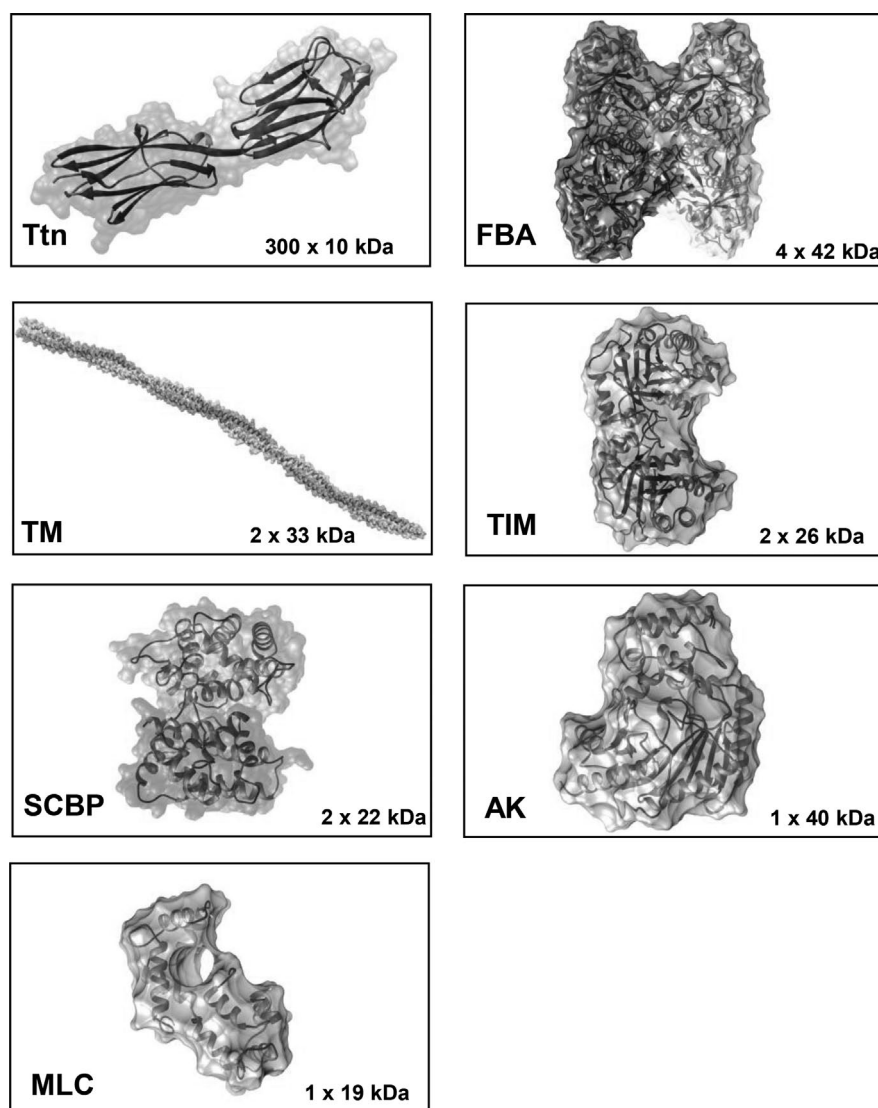


Figure 5. Three-dimensional homology structural models of allergenic proteins identified in black tiger prawn. Titin's (Ttn) molecular structure consists of 300 tandem repeats of Ig- and fibronectin-like domains. Triose phosphate isomerase (TIM) forms a tetramer; tropomyosin (TM), sarcoplasmic calcium binding protein (SCBP), and fructose 1,6 bisphosphate aldolase (FBA) form a homodimer in their physiological state; arginine kinase (AK) and myosin light chain (MLC) are functional in their monomeric forms.

Similar to TM, SCBP and TIM form a dimer with subunits of 22 and 26 kDa, respectively. FBA forms a tetrameric structure of four identical subunits, each 42 kDa in size. In contrast, Ttn exists as a super repeat of Ig-like and fibronectin-like domains with a native size of around 3000 kDa. The biochemical and biological properties and functions of the identified allergens are summarized in Table 3. Five of the seven prawn allergens have a pI below 6, while FBA and AK have very basic pIs.

3.7 Selective recognition of TM

Due to TM's major allergen status in shellfish allergy and wide usage in *in vitro* diagnosis as well as detection in consumer products for food labeling, a comparative analysis was performed between the IgE-binding patterns of the patients to natural untreated or processed TM and the recombinant

form of TM. Based on the IgE antibody binding patterns to natural and recombinant TM (rPen m 1), as well as other IgE-binding proteins by immunoblotting, subjects could be divided into group A, with "strong TM binding" ($n = 10$) and group B, with "weak/no TM binding" ($n = 6$) (Supporting Information Table 2). All subjects of the "strong TM binding" group exhibited mild or severe symptoms on exposure to shellfish, but only 70% of these patients also recognized rPen m 1. These findings highlight the possible implication of posttranslational protein modifications in the allergic reaction. In contrast, despite all six subjects in the "weak/no TM binding" group having also mild to severe clinical reactions to shellfish, only 50% demonstrated weak binding to natural TM and none recognized rPen m 1. However, 50% of the latter group demonstrated IgE binding to allergens other than TM, indicating the possible importance of non-TM allergens as the main allergen for some patients.

Table 3. Biochemical properties and physiological functions of identified black tiger prawn allergenic proteins

Allergens		Native size (kDa)	Identified size of IgE bands (kDa)	Heat stability of allergen	Native conformation	Function	Route of exposure	p/ value of allergen
Ttn	Titin	3000	70	Stable	Tandem repeats of Ig- and fibronectin-like units	Passive muscle elasticity	Ingestion	4.96–5.24
FBA	Fructose 1,6 bisphosphate aldolase	39–43	42	Labile	Tetramer	Glycolysis (energy metabolism)	Ingestion Inhalation	5.69–8.68
TM	Tropomyosin	33–38	38, 33, 26	Stable	Dimer	Muscle contraction	Ingestion Inhalation	4.57–4.76
SCBP	Sarcoplasmic calcium binding protein	22	16.8	Stable	Dimer	Muscle contraction regulation	Ingestion	4.6–5.06
TIM	Triose phosphate isomerase	26–29	16.8	Labile	Dimer	Glycolysis (energy metabolism)	Ingestion Inhalation	5.67–6.24
AK	Arginine kinase	39–40	42, 38, 35	Stable	Monomer	Energy for muscle contraction	Ingestion Inhalation	5.58–8.60
MLC	Myosin light chain	19–22	16.8	Stable	Monomer	Muscle contraction	Ingestion	4.23–4.67

3.8 Cellular reactivity of prawn allergens

To demonstrate biologically relevant IgE reactivity of the different prawn extracts, activation of isolated basophils from two patients was analyzed. Representative data are shown in Supporting Information Fig. 2. Patient 2 demonstrated higher basophil sensitivity (i.e. basophil activation by lower concentrations of extract) to natural prawn TM (P-TM; Supporting Information Fig. 2C) compared to the other black tiger prawn extracts. In contrast, patient 12 showed markedly higher basophil activation to allergens from P-raw compared to the heated prawn extracts (Supporting Information Fig. 2D).

4 Discussion

Shellfish allergy is one of the primary causes of hypersensitivity reactions to food [1]. The current in vitro diagnostics for shellfish allergy are based on the quantification of allergen-specific antibodies. Although TM is accepted to be the main shellfish allergen, crude substandardized preparations of prawns (raw or boiled) are generally employed in most of the current in vitro and in vivo diagnostics. Component resolved diagnosis for prawn allergy makes use of only prawn TM (Pen a 1) for specific IgE quantification, and other identified allergens are not taken into account. Similarly, to comply with food labeling regulations (Food Allergen Labeling and Consumer Protection Act of 2004), immunoassays commonly

used to detect shellfish allergens in processed food, often utilize antibodies raised against uncharacterized crude extracts or detect only TM as a representative shellfish allergen. Thus, other identified shellfish allergens may often be overlooked and need to be investigated in further detail. Novel crustacean allergens have been identified in recent years. However, there is still a lack of a comprehensive allergenomic analysis of the whole allergen repertoire of prawns.

In the present study, we have identified and characterized the IgE-reactive prawn proteins and further analyzed the modification of their reactivity due to the “cooking” process, using patient IgE screening and mass spectrometric analysis. Additionally, we have identified three putative novel prawn allergens. Patients showed different reactivity to these allergens, but importantly, of the patients who did react with these allergens, not all were TM reactive. This indicates that these new allergens as well as TM should be included in a more detailed component-resolved diagnostic approach for shrimp allergy. We have also shown that heating of whole prawns increases the IgE reactivity of specific allergenic proteins.

TM is a highly cross-reactive pan-allergen among crustaceans, insects, and dust mites [1, 9]. Prawn TM-specific IgE has been suggested to be the best single molecular marker for predicting clinical symptoms of crustacean allergy [10, 11]. Specificity of IgE antibody to TM has been shown to be around 70%, which suggests that it may not lead to accurate diagnosis of general crustacean allergy, and other allergens might be of importance [19].

Impact of heat processing on shellfish allergenicity has been discussed previously. Recent studies conducted with patients allergic to blue swimmer crab from the Asia-Pacific region demonstrated differential IgE reactivity to raw or cooked allergen extracts [12]. Cellular activation experiments in the current study support these findings with different antibody reactivity to heat-stable allergens, such as TM, and possible reactivity to heat-labile allergens including FBA and TIM.

Using allergogram analysis of the IgE reactivity, we observed that 30% of patients recognized TM in the raw extract. Heating of the extract caused an increase in this IgE reactivity where 87% of patients reacted to three different TM variants. The additional higher MW TMs are most likely generated during the heating process, which demonstrated increased antibody binding after heating, suggesting conformational changes that may have led to better epitope recognition [20]. The smaller 26.5 kDa TM variant was recently characterized by our group as being a stable peptide fragment of the native TM [21].

In addition to TM, yet another crustacean allergen that also showed 62% of patient recognition was identified as AK. Unlike TM, AK showed presence of three distinct antibody-binding variants in the raw extract. Interestingly, after heat treatment, AK IgE reactivity was reduced to just one AK variant at 36.5 kDa. We recently demonstrated that the heat-stable novel AK from northern shrimp can be aerosolized and cause inhalational sensitization in the workplace [22].

SCBP and MLC, two additional allergens, previously identified in other prawn species [23, 24], were both recognized by 30% of our patients. However, IgE binding to MLC was only observed in the heated prawn extract and not in raw. In contrast, a study by Ayuso et al. demonstrated stronger IgE binding to Lit v 3 in raw extract, however only in the adult population [23]. The different IgE reactivity in our study may indicate different expression levels of MLC in black tiger prawn in addition to the presence of possible isoforms and the impact of heat treatment. While MLC has not been analyzed in the literature as detailed as TM, our study confirms the heat stability of MLC also in black tiger prawn.

The current study identified three novel allergens, not previously characterized in black tiger prawns, of which Ttn could have the “major allergen” status according to the IUIS guidelines [25]. Ttn, a 3000 kDa protein is one of the largest known proteins [26] and is responsible for passive muscle elasticity. The secondary structure of Ttn consists of tandem repeats of Ig- and fibronectin-like domains. We have identified a heat-stable 70 kDa IgE-reactive fragment of this protein, which belongs to the Ig-like domain, and is highly conserved among invertebrates (e.g. crayfish, oyster, and silk worm) [27, 28]. A total of 68% of the patients showed reactivity to this protein fragment. FBA is an approximately 160 kDa enzyme, which exists in a tetrameric form and is involved in glucose energy metabolism. FBA was previously characterized as an allergen in various fish species and nematode parasites (Anisakis). We have for the first time identified FBA as an IgE reactive protein in a crustacean species, which is

a substantial find for improved diagnosis and management of shellfish allergy. TIM is a 26–29 kDa enzyme protein that exists as a dimer, and similar to FBA is involved in glucose energy metabolism. This allergen was previously identified in northern shrimp (*Crangon crangon*) and cockroach (*Blattella germanica*) [17, 18]. The identification of IgE-reactive TIM in black tiger prawn highlights its importance as a possible invertebrate pan-allergen.

Most of the identified black tiger prawn allergens (5/7) in the current study exist in their native configuration as dimers or oligomers. The formation of oligomeric protein aggregates seems to have considerable influence on the allergenic reactivity as discussed by Rosenberg [29]. Highly arrayed structures, as found on large nondenatured protein aggregates, are potent inducers of antibody responses. In the case of allergenic proteins, this might relate to the ability of multivalent allergens to extensively cross-link IgE-receptor complexes on B-cells as well as on mast cells and basophils. The formation of dimeric and oligomeric allergens seems to be more common than expected [30]. A recent study showed presence of homodimers in over 80% of the protein crystal structures of 55 different food or inhalant allergens analyzed. Also hypoallergenic variant of the major milk allergen Bos d 5 was shown to exist in its native state as a monomer [30]. Of the seven prawn allergens identified in this study, TM, TIM, and SCBP exist as dimers, FBA as a tetramer, and Ttn is essentially a super-repeat of similar structured domains. The quaternary structure of the allergenic protein may offer enhanced stability to the molecule thereby increasing its IgE reactivity by helping in efficient cross-linking by the antibody. Current diagnostics for shellfish allergy may not be able to detect IgE antibodies targeted to oligomeric allergens. Moreover, specific IgE to these oligomers might vary among different patients.

The findings of this study will help toward better standardization of allergen extracts used in in vitro diagnostics and toward improved detection of shellfish allergens in processed food products to protect sensitized consumers.

Residual IgE reactivity to allergen fragments generated by enzymatic digestion of TM has been previously demonstrated [31]. We report the presence of natural and heat-induced IgE-reactive fragments of TM, Ttn, AK, TIM, and MLC. While allergen fragments of Ttn, AK, and TIM may occur due to natural factors, our findings suggest that the TM and MLC fragments could also be generated due to thermal processing. These findings are supported by our recent analysis of a stable TM fragment, comprising of five out of eight previously characterized linear IgE-binding epitopes [32] generated during heat processing [21].

In summary, heating causes a considerable increase in IgE reactivity to prawn allergen extracts. In addition to TM, AK, MLC, and SCBP, we have identified three novel allergens Ttn, FBA, and TIM in black tiger prawns. Further studies using recombinant protein expression and screening will be required to confirm clinical reactivity of these novel prawn allergens. Allergen fragments in addition to the native

allergens are possible inducers of IgE reactivity in patients. Most of the identified prawn allergens exist in multimeric configurations that may have implications on current allergy diagnostics and allergen detection in food sources. Our findings expand the current knowledge on allergenic protein–IgE interactions and facilitate improved allergen detection and developing novel diagnostic strategies.

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The authors have declared no conflict of interest.

5 References

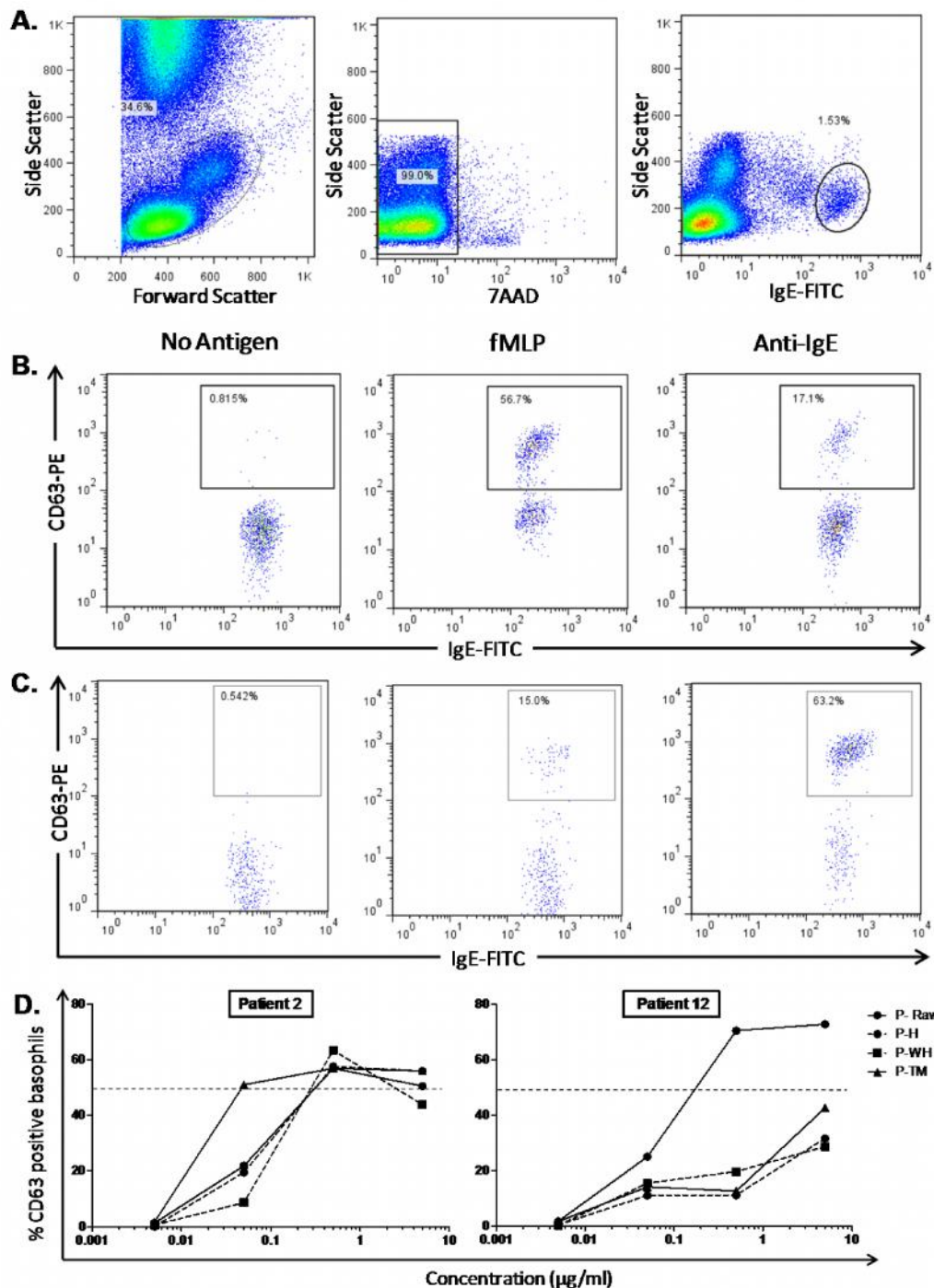
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-----10 -----20 -----30 -----40 -----50 -----60
MDAIKKKMQA MKLEK **DNAMD RADTLEQQNK** EANNRAEKSE EE **VHNLQKRM QQLEND**LDLQV
-----70 -----80 -----90 -----100 -----110 -----120
QESLLK**ANIQLVEKDK**ALSNAE**GEVAALNR** **RIQLLEEDLERSEER**LNTAT TK**LAEASQAA**
-----130 -----140 -----150 -----160 -----170 -----180
DESERMRKVL ENRSLS**DEERMDALENQLKE**AR**FLAEEADR** KYDEVARK**LA**MVEADLERAE
-----190 -----200 -----210 -----220 -----230 -----240
ERAETG**ESKIVELEEEEL**RVV GNNLKSLEVS EEKANQREEA YKEQIKTLTN KLKAAEARAE
-----250 -----260 -----270 -----280 -----284
FAERSVQKLQ **KEVDRLEDEL** **VNEKEKYKSI** TDE**LDQTFSE** LSGY

No.	Amino acid sequence of peptide fragments derived from the 26 kDa protein
1	ANIQLVEK
2	FLAEEADR
3	IVELEEEELR
4	MDALENQLK
5	ANIQLVEKDK
6	LAMVEADLER
7	LEDELVNEKEK
8	KLAMVEADLER
9	LAEASQAADSEER
10	EVDRLEDELVNEK
11	IQLEEDLERSEER
12	ALSNAEGEVAALNR
13	LAMVEADLERAEER
14	LAEASQAADSEERM
15	DNAMD RADTLEQQNK
16	SLQDEERMDALENQLK
17	SLSDEERMDALENQLK
18	DKALSNAEGEVAALNR

Supporting Information, Figure S1: Amino acid sequence of Black tiger prawn tropomyosin. The peptide fragments derived from the 26 kDa protein by tryptic digest were identified by mass spectrometric analysis and are highlighted in different colors. The proposed eight IgE antibody binding epitopes by Reese et al. 2005 are boxed and numbered from I to VIII.



Supporting Information, Figure S2: Activation of basophils was determined by up-regulation of the CD63 cell surface marker on viable high IgE binding cells (IgE^{hi} cells, basophils) by flow cytometry. Two positive controls included activation through rabbit anti-human IgE antibody to induce IgE cross-linking, and application of the peptide f-Met-Leu-Phe to induce IgE-independent activation of basophils. Representative dot plots showing gating of basophils (7AAD⁻, high IgE positive) (A) and expression of cell surface CD63 in negative and positive controls (B) and with increasing concentration of prawn extract (C). (D) Dose response to different prawn extracts for two subjects (No. 2 and 12). Basophils were stimulated with P-Raw (●), P-H (●, dotted line), P-WH (■, dotted line), P-TM, natural prawn tropomyosin (▲) at increasing concentrations (0.005–5 $\mu\text{g/mL}$), and shown as percentage of CD63 positive basophils.

Supporting Information, Table S1: Amino acid sequences of peptides identified for each IgE reactive protein using mass spectrometry, with accession numbers for identified proteins using GenBank database.

Band no.	Mass (kDa)	Peptide sequence	Database no.	Protein identity	Score	Identified allergen
Prawn-Raw (P-raw)						
1	70	AGQIINLVTR IELSVQMKK	AB055927.1	Titin	57	-
2	42	AVFDQLKEK VSSTLSSLEGELK LIDDHFLFK IISMQMGGDLGQVFR LTSAVNEIEK GTRGEHTEAEGGIYDISNK MGLTEFQAVK EMQDGILELIK	GQ246164	Arginine kinase	461	Pen m 2
		ALQASVLR IANAIVAPGK GILAADESVMGK LKDIGVENTEENR GILAADESVMGKR RIANAIVAPGK KPWALTFSYGR ETPSYLGMLENANVLAR	Q9URB4	Aldolase	268	Cand a FBA
3	38.0	FLQAANACR AVFDQLKEK LTSAVNEIEK MGLTEFQAVK LIDDHFLFK GTYYPPLTGMSK VSSTLSSLEGELK IISMQMGGDLGQVFR GEHTEAEGGIYDISNK	GQ246164	Arginine kinase	485	Pen m 2
		IVELEEELR ANIQLVEKDK IQLLEEDLER ALSNAEGEVAALNR	HM486525	Tropomyosin	253	Pen m 1
4	35	AVFDQLK FLQAANACR AVFDQLKEK LTSAVNEIEK MGLTEFQAVK LIDDHFLFK GTYYPPLTGMSK LTSAVNEIEKR EMQDGILELIK VSSTLSSLEGELK IISMQMGGDLGQVFR GEHTEAEGGIYDISNK LGFLTFCTNLGTTVR GEHTEAEGGIYDISNKR GTRGEHTEAEGGIYDISNK	GQ246164	Arginine kinase	961	Pen m2
4	26.5	AGGLTLER VGLDEYR VFIANQFK NDFECLA VR DGEVTVDEFK GEFSADAYANNQK YMYDIDNNGFLDK AIDVNGDGK VGLDEYR YMYDIDNNGFLDKNDFECLA VR	FJ184279	Sarcoplasmic calcium protein	437	Lit v 4
5	16.8	KFFVGGNWK GAFTGEISPAMVK TQEVVFAQMK AAIDGII SFMK	FJ462738	Triose-phosphate isomerase	128	Cra c 8
Prawn-Whole Heated (P-WH)						
1	70	AGQIINLVTR IELSVQMKK	AB055927.1	Titin fragment	56	-

2	38	ANIQLVEK FLAEEADR MDALENQLK IVELEELR ANIQLVEKDK LAMVEADLER IQLLEEDLER KLAMVEADLER LAEASQAADER RIQLLEEDLER ALSNAEGEVAALNR EVDRLEDELVNEK LAMVEADLERAEER IQLLEEDLERSEER DNAMDRADTLEQQNK SLSDEERMDALENQLK	HM486525	Tropomyosin	910	Pen m1
3	36.5	LIDDHFLFK TVTAVNEIEK	GQ246164	Arginine kinase	77	Pen m 2
	33.5	ANIQLVEK FLAEEADR MDALENQLK IVELEELR LAMVEADLER ANIQLVEKDK LAMVEADLER IQLLEEDLER KLAMVEADLER LEDELVNEKEK LAEASQAADER ALSNAEGEVAALNR EVDRLEDELVNEK LAMVEADLERAEER DKALSNAEGEVAALNR LAEASQAADERMR DNAMDRADTLEQQNK IQLLEEDLERSEER DNAMDRADTLEQQNK SLSDEERMDALENQLK SLQDEERMDALENQLK	HM486525	Tropomyosin	961	Pen m 1
4	26.5	ANIQLVEK FLAEEADR MDALENQLK IVELEELR LAMVEADLER ANIQLVEKDK LAMVEADLER KLAMVEADLER LEDELVNEKEK LAEASQAADER ALSNAEGEVAALNR EVDRLEDELVNEK LAMVEADLERAEER DKALSNAEGEVAALNR LAEASQAADERMR DNAMDRADTLEQQNK IQLLEEDLERSEER SLQDEERMDALENQLK SLSDEERMDALENQLK	HM486525	Tropomyosin	1187	Pen m 1
5	16.8	GTFDEIGR EGFQLMDR	ADM34185	Myosin light chain	79	Lit v 3
		VGLDEYR VFIANQFK DGEVTVDEFK GEFSADAYANNQK	FJ184279	Sarcoplasmic calcium protein	165	Lit v 4

Supporting Information, Table S2: IgE antibody binding reactivity of patient sera to natural and recombinant TM and other allergens in the raw and heated prawn extracts by immunoblotting. Symptoms have been grouped as severe or mild*.

Patient	Total IgE (kU/l)	Shrimp Specific (f24) IgE (kU/l)	IgE binding to natural Tropomyosin	IgE binding to recombinant Tropomyosin	IgE binding to other allergens		Symptoms
					Raw extract	Heated extract	
Group A: Patients with strong IgE antibody binding to tropomyosin							
1	179	3.27	Yes	Yes	Yes	Yes	Mild
3	242	1.32	Yes	Yes	Yes	Yes	Severe
5	136	4.54	Yes	No	Yes	Yes	Mild
6	100	2.11	Yes	Yes	Yes	Yes	Severe
7	150	0.16	Yes	No	No	No	Severe
9	440	0.5	Yes	Yes	Yes	Yes	Mild
12	822	5.33	Yes	Yes	Yes	Yes	Severe
13	3401	6.65	Yes	No	Yes	Yes	Severe
14	283	19.7	Yes	Yes	Yes	Yes	Severe
16	1946	9.5	Yes	Yes	Yes	Yes	Severe
Group B: Patients with weak/no IgE antibody binding to tropomyosin							
2	372	1.82	No	No	Yes	No	Mild
4	55	0.37	Low	No	No	No	Severe
8	164	0.04	Low	No	Yes	No	Mild
10	28	0.21	No	No	No	No	Mild
11	158	0.85	No	No	No	No	Severe
15	238	5.93	Low	No	Yes	Yes	Severe

*Symptoms:

Severe – Asthma, anaphylaxis, collapse

Mild – Oral allergy syndrome, angioedema, rhinitis, urticaria, dermatitis, dyspnoea, pruritus