

Analysis of the allergenic proteins in black tiger prawn (*Penaeus monodon*) and characterization of the major allergen tropomyosin using mass spectrometry

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Crustaceans are the third most prevalent cause of food-induced anaphylaxis after peanuts and tree nuts. The severity of the allergenic proteins depends mainly on the amino acid sequence that induces production of IgE antibodies. In black tiger prawn (*Penaeus monodon*), the crude protein extract was profiled and its allergenic potency was examined against patient's sera. Proteins having strong immunoreactivity with patient's IgE were characterized using peptide mass fingerprinting (PMF). Tropomyosin (TM) (33 kDa), myosin light chain (20 kDa), and arginine kinase (40 kDa) were identified as allergenic proteins. Tropomyosin, the most abundant and potent allergen, was purified using ion-exchange chromatography for *de novo* sequencing experiments. Using *bottom up* tandem mass spectrometry, the full amino acid sequence was achieved by a combination of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) tandem mass spectrometry (QqToF). Myosin light chain and arginine kinase were also characterized, and their related peptides were *de novo* sequenced using the same approach. The immunological reactivity of the crude prawn extracts and purified TM samples were analyzed using a large number of patients' sera. A signature peptide was assigned for the TM protein for future quantification work of black tiger prawn TM levels in different matrices (i.e. water, air, food) in the seafood industry. Copyright © 2010 John Wiley & Sons, Ltd.

Black tiger prawn (*Penaeus monodon*) is one of the most important commercial products of the shrimp industry in south-east Asia and is widely distributed in the Indo-West-Pacific region.¹ However, it is also recognized as one of the most common causes of type I (IgE)-mediated food hypersensitivity.² Ingesting black tiger prawn may cause hypersensitivity reactions such as urticaria, angioedema, asthma, diarrhea and anaphylaxis allergic patients.³ Immediate hypersensitivity reactions to seafood have become an important issue since consumption of seafood and subsequent processing of the shellfish has increased worldwide. The seafood processing industry has experienced tremendous growth in recent years with over 42 million workers worldwide engaged in various activities of seafood production (i.e. processing, harvesting, etc.), and these workers are being continuously exposed to seafood allergens.^{4–6}

Tropomyosin² and arginine kinase^{4,7–10} have been identified as immunologically reactive proteins from black tiger prawn (BTP) in previous studies using molecular genetics approaches. Tropomyosin, which is a well-known shellfish allergen, was first identified by Hoffman *et al.* in 1981¹¹ and recently characterized as a major allergen in other prawns.^{12,13} Tropomyosin (TM) is a water-soluble and

heat-stable protein with a molecular weight (MW) ranging between 34 and 39 kDa.¹⁴ It has a highly conserved amino acid sequence among different invertebrate organisms and is present in muscle as well as non-muscle cells.^{15,16} Furthermore, differential splicing of the pre-messenger ribonucleic acid (pre-mRNA) produce isoforms of TM.¹⁷

Previous studies have evaluated some of these seafood allergens and reported that the prevalence for occupational protein contact dermatitis ranges from 3% to 11% and for occupational asthma from 7% to 36%.⁶ Immunochemical techniques used in previous studies to identify reactive proteins in air samples include enzyme-linked immunosorbent assay (ELISA),^{16,18} a radioallergosorbent test (RAST),¹⁹ and immunoblotting.^{16,20} However, these techniques are generally not sensitive, selective, or specific enough to evaluate the levels of the individual and most harmful protein allergens in sample matrices such as air samples. These techniques only recognize the total airborne proteins, which could include both allergenic and non-allergenic from the seafood and may also include allergenic proteins from non-seafood sources (e.g. dust mites, microbes, etc.). Non-specificity of the antibody-antigen interactions may have led to uncertainty in previous characterization studies.

Quantifying the major BTP allergens to enable the correlation of severity with specific allergen load requires a highly sensitive, specific, and reproducible analytical technique. Isotopic dilution mass spectrometry has played a

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crucial role in the protein quantification in the last two decades and is the method of choice for our further study in quantification of protein allergens. The current study targets the meat of BTP as the chief source of potential airborne allergens in the workplace. The allergenicity of purified TM from BTP was analyzed using immunoblotting against specific patients' sera and specific polyclonal antibodies. A very specific and selective method has been developed in our laboratory to both characterize and quantify these allergens using mass spectrometry whereby specific allergenic proteins have been targeted rather than a group of reactive but uncharacterized proteins. This will assist in identifying the actual allergen in the environment and in quantification using isotopic dilution mass spectrometric techniques.

Black tiger prawn tropomyosin is, for the first time, *de novo* sequenced using *bottom up* tandem mass spectrometry. Multiple types of enzyme digestion, ion sources, and derivatization protocols are used to cover the whole amino acid sequence. Furthermore, the mass spectra of the peptide mass fingerprinting (PMF) and the peptide fragment fingerprinting (PFF) were uploaded to the Mascot search engine which reports the amino acid sequence of the target allergen as tropomyosin. The post-translational modification motifs of TM were evaluated theoretically using bioinformatics tools and by visually screening through the mass spectra.

EXPERIMENTAL

Chemicals and reagents

All chemicals were used without further purification. Acetonitrile, hydrochloric acid, and methanol were supplied from ACP (Montreal, Canada). RapiGest SF surfactant was purchased from Water Corporation (Milford, MA, USA) and trypsin, Asp-N and endoproteinase (Glu-C V8) sequencing-grade enzymes from Sigma-Aldrich (St. Louis, MO, USA). Ammonium bicarbonate, *O*-methylisourea hemisulfate, ammonium hydroxide, horseradish peroxidase (HRP), and α -cyano-4-hydroxycinnamic acid (HCCA) matrix were purchased from Sigma-Aldrich. The Bradford assay kit was purchased from BioRad (Hercules, CA, USA). The dialysis bags were from Fischer Scientific (Roncho Dominguez, CA, USA). For desalting purposes, ZipTip C₁₈ filters were purchased from Millipore Corporation (Bedford, MA, USA). For TM purification steps, phosphate-buffered saline (PBS) and Tris buffer saline (TBS) tablets were purchased from Amresco, USA. Acetic acid, sodium acetate, sodium chloride, and Tween-20, used for the washing steps, were purchased from Sigma-Aldrich. The Amicon spin filters used for fraction concentration were from Millipore Corporation. The tracker dye (Coomassie stain R250) and PVDF membrane for the immunoblotting were from BioRad (Hercules, CA, USA). Skimmed milk for the immunoblotting procedure was purchased from a local supermarket.

Protein extracts

Fresh black tiger prawns were purchased from a local market (Victoria, Australia) and transported to the laboratory on ice. For the preparation of raw protein extract the prawn muscles were shredded into pieces and homogenized in PBS, pH 7.2, using an Ultraturrax homogenizer (IKA). The slurry was

then centrifuged at 8000 rpm for 20 min and the supernatant was filter sterilized. This PBS protein extract was stored in aliquots at -80°C until further experiments. A cooked prawn extract was prepared by heating whole prawns at 100°C in PBS, pH 7.2, for 20 min, and then a similar procedure was followed as described for the raw prawn protein extract.

Tropomyosin purification

The tropomyosin from BTP was purified from the crude (raw and cooked) extract using a strong anion-exchange chromatographic column on a Biologic LP purification system (BioRad, USA).

Before loading the proteins onto the column, the crude extract was exchanged into the chromatographic starting buffer (30 mM acetate buffer, pH 5.5) using Amikon spin filters of 3 kDa molecular weight cutoff (MWCO). After equilibrating the column with the starting buffer, approximately 10 mg of crude proteins were loaded onto the column. The column was then washed with 5 column volumes of the starting buffer. It was then further washed with 250 mM NaCl in 30 mM acetate buffer, pH 5.5, to elute unwanted proteins. The tropomyosin (TM) eluted at approximately 47 min. The collected fraction was then concentrated using an Amikon spin filter with a 3 kDa MWCO.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The BTP crude extracts and the purified TM were profiled using 12% SDS-PAGE. A protein solution (10 μg) was added to each of the wells, and electrophoresis was run at a voltage of 170 V for 1 h, or until the tracker dye was seen at the base of the gel. One gel was stained with Coomassie brilliant blue using a standard protocol. For the second gel, the separated proteins were transferred to a nitrocellulose membrane at 100 V for 1 h. After the transfer was completed, the membrane was placed in a blocking solution (5% skimmed milk in Tris-buffered saline (TBS)) for immunoblotting.

Immunoblotting

The in-house rabbit polyclonal anti-crustacean antibody was generated by injecting a mixture of heated protein extracts from black tiger prawn (*Penaeus monodon*), king prawn (*Melicerus latisulcatus*), mud crab (*Scylla serrata*), and slipper lobster (*Thenus orientalis*) into rabbits. The final bleed was performed on week 9 and the sensitivity of the serum was analyzed using ELISA.

After separating the proteins using SDS-PAGE, samples were immobilized on a PVDF membrane using a semi-dry immunoblot apparatus (BioRad, USA). The membranes were then blocked with 5% skimmed milk solution for 1 h at room temperature following a 1-h incubation with the rabbit antibody (primary antibody) using a working dilution of 1:40000. The blot was washed three times with TBS-Tween. The secondary antibody used was polyclonal goat anti-rabbit antibody conjugated to horse radish peroxidase (HRP) (Promega, USA), with a working dilution of 1:20000. The blot was incubated for 30 min in the secondary antibody solution, washed again three times with TBS-Tween and incubated with chemiluminescent substrate (Sigma Aldrich).

The bands were then visualized using the enhanced chemiluminescent (ECL) technique.²¹

To demonstrate the allergenicity of the isolated prawn proteins, different extracts were analyzed for IgE antibody binding from allergic patients. The human sera were collected from patients with strong allergic reactivity to shellfish. Ethical approval for this study was acquired by Monash University, Melbourne, Australia. For immunoblotting, protein extracts were separated by electrophoresis (see SDS gels above), proteins transferred and incubated with human serum (diluted 1:20 in 1% skimmed milk) overnight at 4°C. Subsequently, blots were washed three times with PBS-T and the membrane incubated for 1 h with rabbit antihuman IgE antibody (DAKO, USA) (diluted 1:1000) in PBS-T containing 1% skimmed milk. After washing the membrane with PBS-T three times, it was incubated for 30 min with HRP tagged goat anti-rabbit polyclonal antibody (DAKO, USA) (diluted 1:1000) in PBS-T containing 1% skimmed milk. Finally, the membrane was washed with PBS three times, incubated with the chemiluminescent substrate, and the immunoblot membranes analyzed for IgE reactivity using the ECL technique.²¹

Enzymatic digestion and guanidation

The examined protein bands were excised from the SDS-PAGE plate. To increase the sensitivity of the lysine-containing peptides in the MALDI experiments, the *in-gel* guanidation procedure was performed on all protein samples using the protocol developed by Sergeant *et al.*²² The gel pieces were destained by washing three times with 200 mM of (NH₄)₂CO₃ in a solution of 50% acetonitrile in dH₂O, at 30°C for 30 min. The destained pieces were dried under a stream of N₂, then covered by a solution of 50 mM (NH₄)₂CO₃, pH 7.8, containing 5 ng/μL trypsin, Asp-N, and endoproteinase Glu-C V8 in ice for 30 min for rehydration. After rehydration, any excess solution was removed. The gel was then covered by a solution of 50 mM of (NH₄)₂CO₃ and incubated at 37°C overnight to enhance protein digestion. The water-soluble peptides were extracted twice with the incubation solution and other remaining peptides extracted twice with 0.15% trifluoroacetic acid (TFA) in 50% ACN after vortex mixing for 2 min. The samples were freeze-dried, and reconstituted prior to analysis with 10 μL of 0.1% TFA and desalted with C₁₈ ZipTips.

An *in-solution* digestion for the pure extract of the BTP TM was performed using RapiGest SF surfactant,²³ which enhances the digestion efficiency. Subsequently, the solution was incubated with proper buffers as in the *in-gel* digestion protocol overnight with a concentration 20 ng/mL of enzyme. The digestion was quenched and the surfactant was precipitated by 1% formic acid at room temperature.

MALDI plate preparation

The protein samples were prepared for MALDI analysis using a protocol developed by Abdel Rahman *et al.*²⁴ Two-layer sample/matrix preparation was employed for plate spotting. The first layer solution consisted of 20 mg/mL HCCA in (1:9) methanol/acetone. The second layer of solution consisted of saturated HCCA in 40% ACN. A 0.5 μL sample of the first layer of matrix solution was applied to a MALDI target. A 1 μL sample of the second layer matrix solution was mixed with 1 μL of sample. Finally, 1 μL of the

sample/matrix mixture was deposited onto the first layer and allowed to dry, followed by an on-target wash step.²⁴

MALDI- and ESI-QqToF MS

MALDI-MS and low-energy collision-induced dissociation (CID) analyses were carried out on a QSTAR XL hybrid quadrupole-quadrupole/time-of-flight tandem mass spectrometer (QqToF-MS/MS; Applied Biosystems/MDS Sciex, Foster City, USA) equipped with an o-MALDI ion source (Applied Biosystems, Foster City, CA, USA).

Peptide separation was conducted using a DIONEX UltiMate3000 Nano LC system (Germering, Germany). A 250 fmol sample of protein digest dissolved in 0.1% TFA was loaded onto a precolumn (300 μm i.d. × 5 mm, C₁₈ PepMap 100, 5 μm; LC Packing, Sunnyvale, CA, USA) for desalting and concentrating. Peptides were then eluted from the precolumn and separated on a nanoflow analytical column (75 μm i.d. × 15 cm, C₁₈ PepMap 100, 3 μm, 100 Å; LC Packing) at 180 nL/min using the following gradient. The aqueous mobile phases consisted of (A) 0.1% formic acid/0.01% TFA/2% ACN and (B) 0.08% formic acid/0.008% TFA/98% ACN. A gradient of 0% B for 10 min, 0–60% B in 55 min, 60–90% in 3 min, 90% B for 5 min was applied. Including a regeneration step one run was 106 min long.

The ESI mass spectra of the LC-eluting peptides were measured with the same hybrid QqToF-MS/MS system equipped with a nano-ESI source (Protana XYZ manipulator). The nanoelectrospray was generated from a PicoTip needle (10 μm i.d.; New Objectives, Woburn, USA) at a voltage of 2400 V. Individual target proteins were further analyzed by CID-MS/MS; the resulting peptides were *de novo* sequenced and the results confirmed by using the National Center for Biotechnology Information non-redundant database (NCBI nr) with the Matrix Science (Mascot) search engine (precursor and product ion mass tolerance set at 0.2 Da). Methionine oxidation was allowed as a variable modification and guanidinylation (K) as a fixed modification since the guanidation derivatization has been performed in MALDI experiments. Peptides were considered identified if the Mascot score was over the 95% confidence limit.

RESULTS AND DISCUSSION

The crude protein extracts were isolated from fresh BTP tissues then profiled by SDS-PAGE (Fig. 1(A)) then transferred to nitrocellulose membranes for Western blotting. Subsequently, the blots were incubated with rabbit polyclonal anti-crustacean antibody (Fig. 1(B)) or sensitized patients' sera. This permitted the examination of the immunoreactivity of the extracted proteins, before and after heat treatments (band I vs. band II). The strong IgE reactive band with a MW ~33 kDa was further characterized using peptide mass finger printing (PMF). The 33 kDa band was excised and exposed to trypsin digestion. The generated peptides were subjected to MALDI-MS and LC/ESI-MS analyses. The precursor ions spectra (Fig. 2) were uploaded to the Mascot search engine NCBI nr databases, where the sequences of the proteins are *in silico* generated. The Mascot search engine scored 112 identifying this band as a *Penaeus monodon* tropomyosin. This represents a top probability

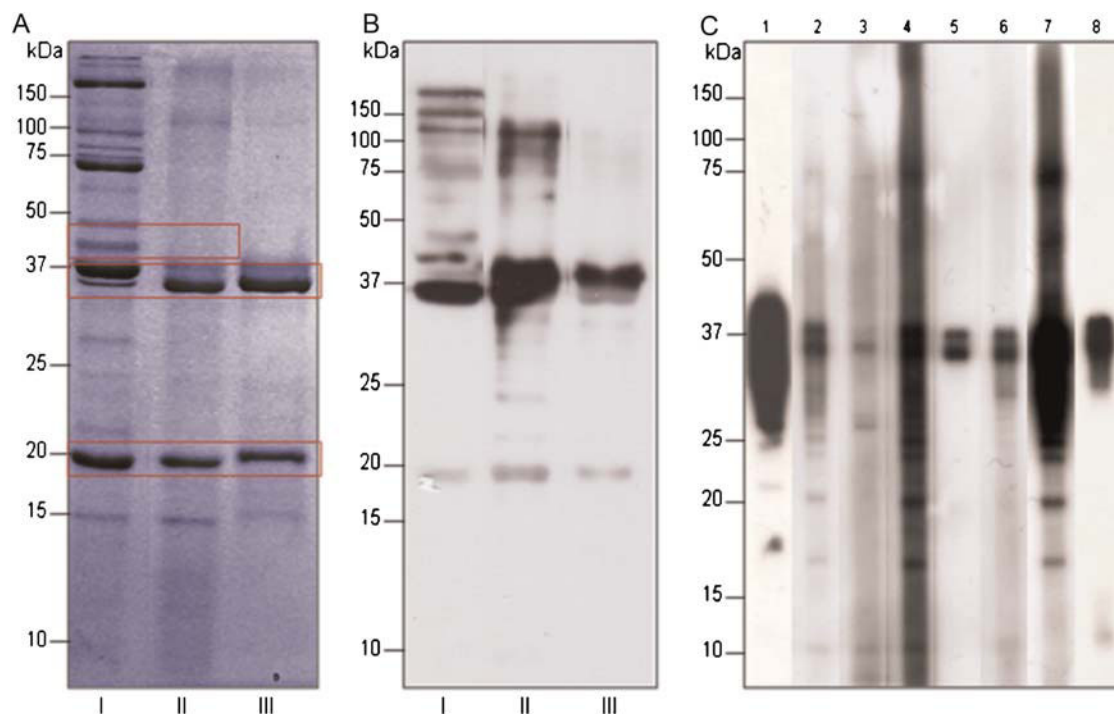


Figure 1. Immunoprofile of the various protein extracts of the Black tiger prawn: I – Raw muscle extract, II – heat-treated muscle extract, III – purified fraction of TM. (A) SDS-PAGE Coomassie profile of the prawn extracts; (B) immunoblot using rabbit polyclonal anti-crustacean antibody; (C) IgE antibody immunoblot against BTP TM using eight shellfish allergic patients' sera. The red box in (A) indicates the position of (top to bottom) arginine kinase, tropomyosin and myosin light chain, respectively.

based on Mowse scores matching with the BTP TM protein (complementary deoxyribonucleic acid (cDNA) based library (NCBIInr)). The above scores were matched with the Mascot algorithms' criterion; individual ions scores >82 indicate identity or extensive homology ($p < 0.05$).

As discussed above, the tropomyosin protein was isolated from fresh BTP and purified using an effective ion-exchange chromatography protocol (see Experimental section and

Fig. 3). The allergenicity of the pure extract was further studied using eight allergic patients' sera, as shown in Fig. 1(C). The levels of IgE in the patient's serum vary with respect to the severity of the hypersensitivity. Nevertheless, all of them have a strong reactivity with the purified BTP TM, which confirmed TM to be the major allergen in this prawn species.

Further characterization is necessary to understand the structural biochemistry of this potent allergen. The purified

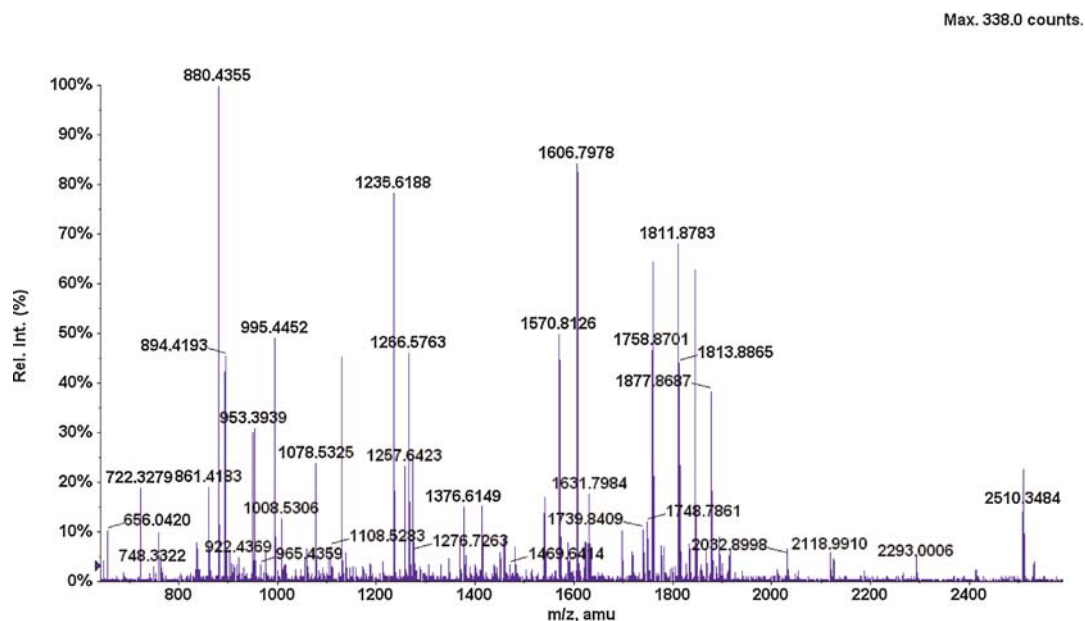


Figure 2. Precursor ion spectrum generated after tryptic digestion of BTP TM. This spectrum scored 112 in the Mascots algorithm as TM for black tiger prawn.

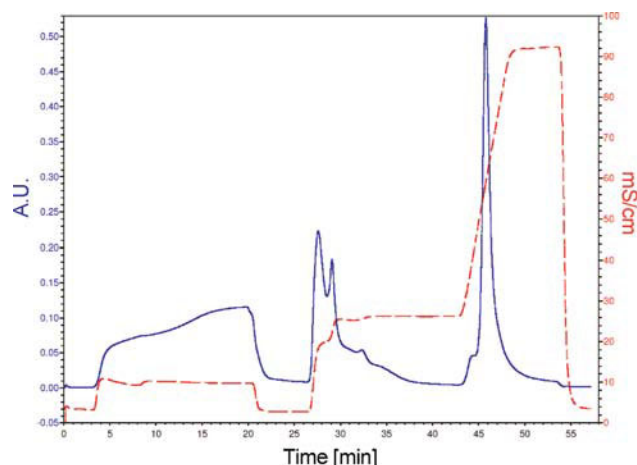


Figure 3. Ion-exchange chromatogram representing the purification of tropomyosin from BTP. The increase in the absorption (in A.U.) at 280 nm (blue or solid line) at 47 min represents the elution of the tropomyosin fraction with increasing salt concentration (red or dashed line; conductivity in mS/cm).

TM extract was *de novo* sequenced using *bottom up* approach peptide fragment fingerprinting. Various sets of enzymatic peptides were produced by trypsin, Glu-C V8, or Asp-N digestions and individually introduced into both the MALDI and ESI ion sources of a tandem mass spectrometer.

In the precursor ion spectra, the most abundant peptide ions (i.e., m/z 721.2927 $[M+H]^+$, m/z 879.3927 $[M+H]^+$, m/z 949.3927 $[M+H]^+$, m/z 1060.4927 $[M+H]^+$, m/z 1107.4927 $[M+H]^+$, m/z 1128.4927 $[M+H]^+$, m/z 1136.4927 $[M+H]^+$, m/z 1145.5927 $[M+H]^+$, m/z 1156.5927 $[M+H]^+$, m/z 1211.4927 $[M+H]^+$, etc.) were selected sequentially in the gas phase and exposed to low-energy CID. The peptide fragment ions were further separated by the TOF analyzer with respect to their mass-to-charge ratio.

In LC/ESI-QqToF analyses, the peptides were initially separated with respect to their polarity on the nano-HPLC column (C₁₈ PepMap), using LC separation and a suitable solvent to minimize ion suppression and increase the abundance of ionized peptides.²⁵ Therefore, the multiply charged precursor ions (i.e. m/z 457.7712 $[M+2H]^+$, m/z 539.7791 $[M+2H]^+$, m/z 565.3142 $[M+2H]^+$, m/z 606.7909 $[M+2H]^+$, m/z 629.3417 $[M+2H]^+$, m/z 688.8189 $[M+2H]^+$, m/z 695.3667 $[M+2H]^+$, m/z 514.2560 $[M+3H]^+$, m/z 1016.0235 $[M+2H]^+$, etc.) were selected and isolated for low-energy CID experiments. The fragments of these peptides were separated by the ToF mass analyzer.

The product spectra generated by both ionization mass spectrometry techniques were uploaded to the Mascot MS/MS search engine against the NCBI nr database. The Mowse scores for these runs were on average 1461, 481, and 187 as BPTM identification for the *in-solution* generated peptides by trypsin, Glu-C V8, and Asp-N digestion,

Table 1. A list of generated peptides of TM extracted from BTP that have been generated by this study using different enzymes and MS ion sources

Protease	Position of residues	Peptide sequence (Z)	Molecular weight		Ion source
			Calculated	Expected	
Trypsin	8–15	MQAMKLEK (2)	977.5038	977.4839	ESI
	16–30	DNAMDRADTLEQQNK (3)	1747.7737	1747.7795	
	22–35	ADTLEQQNKEANNR (2)	1747.7795	1747.7958	
	36–48	AEKSEEEVHNLQK (3)	1539.7529	1539.7462	
	39–48	SEEEVHNLQK (2)	1211.5782	1211.5673	
	39–49	SEEEVHNLQKR (2)	1367.6793	1367.6832	
	50–66	MQQLENDLDQVQESLLK (2)	2029.9990	2030.0026	
	67–76	ANIQLVEKDK (2)	1156.6452	1156.6454	
	77–90	ALSNAEGEVAALNR (2)	1413.7212	1413.7342	
	77–91	ALSNAEGEVAALNRR (2)	1569.8223	1569.8431	
	91–101	RIQLLEEDLER (2)	1412.7623	1412.7934	
	102–112	SEERLNTATTK (2)	1248.6310	1248.6226	
	106–125	LNTATTKLAESQAADER (3)	2105.0236	2105.0447	
	113–125	LAEASQAADER (2)	1375.6215	1375.6232	
	134–149	SLSDEERMDALENQLK	1876.8836	1876.8873	
	141–149	MDALENQLK (2)	1060.5223	1060.5236	
	153–160	FLAEEADR (2)	949.4505	949.4376	
	168–178	KLAMVEADLER (2)	1273.6700	1273.6709	
	169–182	LAMVEADLERAEER (2)	1630.7984	1630.8204	
	190–198	IVELEELR (2)	1128.6026	1128.6139	
	206–217	SLEVSEKANQR	1388.6895	1388.7188	
	218–226	EEAYKEQIK (2)	1136.5713	1136.5687	
	252–264	EVDRLDELVNEK (2)	1586.7787	1586.7966	
	267–284	YKSITDELDTFSELSGY (2)	2094.9633	2094.9916	
	269–284	SITDELDTFSELSGY	1803.8051	1803.8475	
Glu-C (V8)	3–14	AIKKMQAMKLE (2)	1417.8148	1417.8501	
	43–54	VHNLQKRMQQLE (2)	1522.8038	1522.8268	
	63–73	SLLKANIQLVE (2)	1226.7234	1226.7528	
	85–96	VAALNRRIQLLE (2)	1394.8357	1394.8680	

(Continues)

Table 1. (Continued)

Protease	Position of residues	Peptide sequence (Z)	Molecular weight		Ion source
			Calculated	Expected	
Trypsin	85–97	VAALNRRIQLLEE (2)	1523.8783	1523.8982	MALDI
	105–115	RLNTATTKLAE (2)	1216.6775	1216.6927	
	125–131	RMRKVLE (2)	930.5433	930.5576	
	151–157	ARFLAEE (2)	834.4235	834.4310	
	165–173	VARKLAMVE (2)	1015.5848	1015.5959	
	197–208	LRVVGNNLKSLE (2)	1340.7776	1340.8081	
	224–236	QIKTLTNKLKAAE (2)	1456.8613	1456.8909	
	244–252	RSVQKLQKE (2)	1114.6458	1114.6608	
	266–273	KYKSITDE (2)	982.4971	982.5179	
	16–30	DNAMDRADTLEQQNK	1747.7795	1747.7927	
	22–30	ADTLEQQNK	1747.7795	1747.7788	
	22–35	ADTLEQQNKEANNR	1629.7706	1629.7785	
	36–48	AEKSEEEVHNLQK	1539.7529	1539.6927	
	39–48	SEEEVHNLQK	1211.5782	1211.4927	
	39–49	SEEEVHNLQKR	1367.6793	1367.5927	
	50–66	MQQLENDLDQVQESLLK	2029.9990	2029.9927	
	67–74	ANIQLVEK	913.5232	913.4396	
	67–76	ANIQLVEKDK	1156.6452	1156.5927	
	77–90	ALSNAEGEVAALNR	1413.7212	1413.6927	
	91–101	RIQLLEEDLER	1412.7623	1412.6927	
	92–105	IQLLEEDLERSEER	1757.8795	1757.7927	
	102–112	SEERLNTATTK	1248.6310	1248.5582	
	113–125	LAEASQAADSEER	1375.6215	1375.5927	
	134–149	SLSDEERMDALENQLK	1876.8836	1876.7927	
	141–149	MDALENQLK	1060.5223	1060.4927	
	153–160	FLAEEADR	949.4505	949.3927	
	153–161	FLAEEADRK	1077.5454	1077.5252	
	162–168	YDEVARK	879.4450	879.4282	
	168–178	KLAMVEADLER	1273.6700	1273.5927	
	169–178	LAMVEADLER	1145.5750	1145.5927	
	169–182	LAMVEADLERAEER	1630.7984	1630.7927	
	190–198	IVELEELR	1128.6026	1128.4927	
	206–217	SLEVSEEEKANQR	1388.6895	1388.5927	
	214–222	ANQREEAYK	1107.5308	1107.4927	
	218–226	EEAYKEQIK	1136.5713	1136.4927	
	232–238	LKAAEAR	757.4446	757.4322	
	239–244	AEFAER	721.3395	721.2927	
ASP-N	252–264	EVDRLDELVNEK	1586.7787	1586.6927	ESI
	269–284	SITDELDTQTFSELGY	1803.8051	1803.7927	
	2–15	DAIKKKMQAMKLEK (3)	1660.9368	1660.9815	
	58–74	DQVQESLLKANIQLVEK (3)	1954.0734	1954.1163	
	103–114	EERLNTATTKLA(2)	1345.7201	1345.7476	
	121–130	DESERMVKVL (2)	1261.6448	1261.6673	
	150–158	EARFLAEEA (2)	1034.5032	1034.5286	
	163–174	DEVARKLAMVEA (2)	1330.6915	1330.7083	
	184–194	ETGESKIVELE (2)	1232.6136	1232.6384	
	195–207	EELRVVGNNLKS (2)	1469.8202	1469.8368	
	223–235	EQIKTLTNKLKAA (2)	1456.8613	1456.8808	
	243–253	ERSVQKLQKEV (2)	1342.7568	1342.7718	
	275–284	DQTFSELGY(2)	1145.4877	1145.5052	
	2–15	DAIKKKMQA MKLEK	1660.9368	1660.8927	MALDI
	58–74	DQVQESLLKANIQLVEK	1954.0735	1953.9927	
	62–74	ESLLKANIQLVEK	1483.8609	1483.7927	
	75–95	DKALSNAEGEVAALNRRIQLL	2280.2549	2280.1927	
	82–97	EGEVAALNRRIQLLEE	1838.9850	1838.8927	
	103–114	EERLNTATTKLA	1345.7201	1345.6927	
	142–149	DALENQLK	929.4818	929.3927	
	150–158	EARFLAEEA	1034.5032	1034.3927	
	163–174	DEVARKLAMVEA	1330.6915	1330.5927	
	195–207	EELRVVGNNLKS	1346.6864	1346.5927	
	223–235	EQIKTLTNKLKAA	1456.8613	1456.7927	
	236–242	EARAEFA	792.3766	792.2927	
	243–253	ERSVQKLQKEV	1342.7568	1342.6927	
	258–271	DELVNEKEKYKSIT	1694.7826	1694.7927	
	275–284	DQTFSELGY	1145.4877	145.3927	

respectively. Table 1 reports the only peptides that match the Mascot criteria for the individual ions along with expected and calculated molecular weight. Elucidated representative MS/MS spectra for two peptides are shown in Fig. 4, where the most dominant peptide fragment ions (y and b ions) are highlighted.

Multiple types of enzymatic digestions, ion sources, and derivatization protocols were applied to maximize the amino acid coverage of the TM protein.²⁶ As stated, three different sets of enzymatic peptides were generated from BTP TM by trypsin, Asp-N, and Glu-C (V8) proteases. These different peptides with different termini enlarge the amino acid sequence coverage probability of the entire protein. A list of

those peptides was organized in order as listed in Table 1. The full amino acid *de novo* sequence for BTP TM is shown in Fig. 5.

In MS studies, peptides containing the N-terminal methionine are difficult to ionize by ESI ion sources due to PTM acetylation on the amino acid. The evaluation of this PTM in shrimp TM by the *NetAcet 1.0* server scored a high value of 0.471.²⁷ Acetylation modification is quite common for eukaryotic TM, as reported for bovine, chicken (P04268) and human (P09493) cases in the UniProtKB/Swiss-Prot databases.²⁴

In another case with MS sensitivity, the MALDI ion source is highly selective for the arginine-containing peptides. Therefore, a guanidination reaction was performed to increase

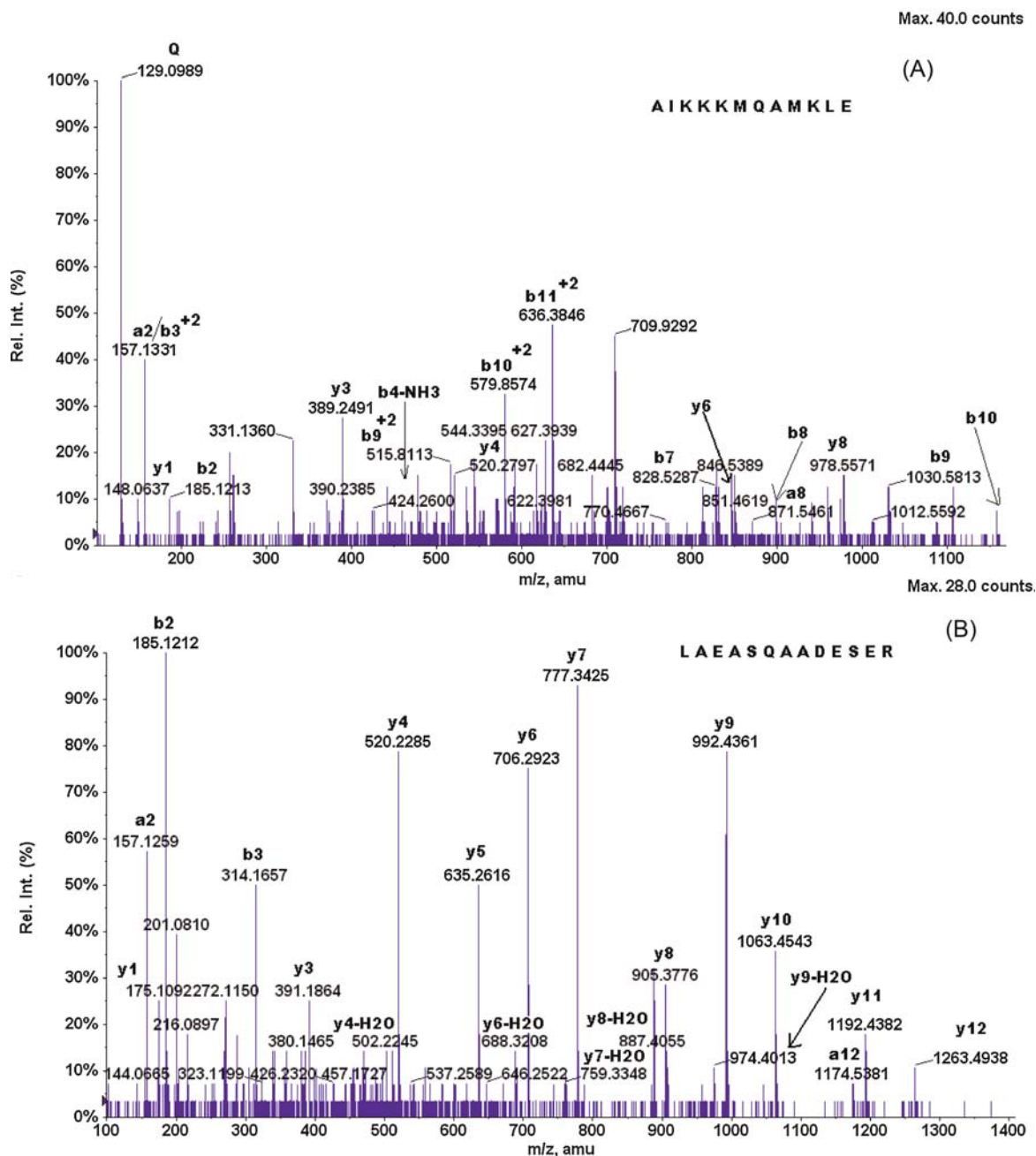


Figure 4. A representative product spectra of two selected peptides (A) generated from Glu-C V8 digestion and the ESI ion source for m/z 709.9323 $[M+2H]^{+2}$ with sequence AIKKKMQAMKLE and (B) generated from tryptic digestion and the MALDI ion source for m/z 1375.6215 $[M+H]^{+}$ with sequence LAEASQAADER.

```

1  MDAIKKKMQA MKLEKDNAMD RADTLEQQNK EANNRAEKSE EEVHNLOKRM
51  QQLENDLDQV QESLLKANIQ LVEKDKALSN AEGEVAALNR RIQLLEEDLE
101 RSEERLNTAT TKLAEASQAA DESERMRKVL ENRSLSDER MDALENQLKE
151 ARFLAEADR KYDEVARKLA MVEADLERAE ERAETGESKI VELEELRVV
201 GNNLKSLEVS EEKANQREEA YKEQIKTLTN KLKAAEARAE FAERSVQKLO
251 KEVDRLEDEL VNEKEKYKSI TDELDQTFSE LSGY

```

Figure 5. BTPTM amino acid *de novo* sequence by the *bottom up* approach.

the sensitivity of the lysine-containing peptides in MALDI work. The guanidation reaction chemically modifies the lysine side chain to homoarginine, which has a proton affinity equivalent to the arginine residues resulting in better sensitivity using the MALDI source (results are not shown).

Additional BTP proteins have been characterized in this study using mass spectrometry. A number of their enzymatic peptides were sequenced using the same MS strategy as described for TM. The 20-kDa band, as shown in Fig. 1(A), and which reacts strongly with three patients' IgE (Fig. 1(C)), was excised and characterized. Two relevant and abundant peptides of this band were *de novo* sequenced using PFF. The amino acid sequences for these peptides were EGFQLMDR and GTFDEIGR and searched against the NCBI nr databank using the Mascot search engine. These peptides matched with myosin light chain, a recently reported allergen in whiteleg shrimp (*Litopenaeus vannamei* (Lit v3)).²⁸

The BTP arginine kinase was also characterized as the 40-kDa protein which also reacted with seven patients' IgE. The band was excised, tryptic digested, and analyzed by MS. The most abundant peptides were *de novo* sequenced (i.e. AVFDQLKEK, VSSTLSSLEGELK, GTYYPLTGMSK, LIDDHFLFK, IISMQMGGDLGQVFRR, LTSAVNEIEKR, IPFSHHDR, GTRGEHTEAEGGIYDISNK).

Additional muscle proteins were profiled by mass spectrometry in the same manner as the arginine kinase and myosin light chain. These proteins are troponin C, myosin heavy chain, and calmodulin.

As stated above, another major objective of this study is to identify a suitable signature peptide as a surrogate for the

BTP TM protein in quantitative measurements of TM in the seafood workplace. A very important factor for selecting a signature peptide is the absence of PTM groups (i.e. phosphorylation and glycosylation).²⁹ Therefore, the precursor ions and intensity data generated from PMF experiments were uploaded on the ExPASy FindMod tool to check if there was any potential peptide having any PTM motif(s). The report indicated the absence of any type of modifications. Further confirmation was obtained by manual searching for the calculated molecular ions of PTM motifs in the precursor spectra. Additional assessment was performed using the *NetPhos 2.0* server.³⁰ Next, peptides that scored >0.50 in abundance rating were excluded as the signature peptide nomination for more suitable candidate sensitivity in quantification.

All resultant peptides from the tryptic digestion without any missing cleavage (Table 1) were examined for signature peptide criteria. The uniqueness of peptides was recommended by the Mascot search engine along with PTM evaluation from the *NetPhos 2.0* server.³⁰ Those selected peptides were then subjected to the protein BLAST test. The NCBI BLAST test, which is used to find regions of local similarity between sequences of the NCBI database and calculates the statistical significance of matches, reported that the peptide located at 67-74 (ANIQLVEK) would be a suitable signature peptide for black tiger prawn TM (with 100% identity, score = 28.2 bits (590), and expected = 94). The product ion spectrum of this peptide, m/z 457.7712 $[M+2H]^{+2}$, was collected using an ESI source (Fig. 6), where the most abundant y and b ions were assigned.

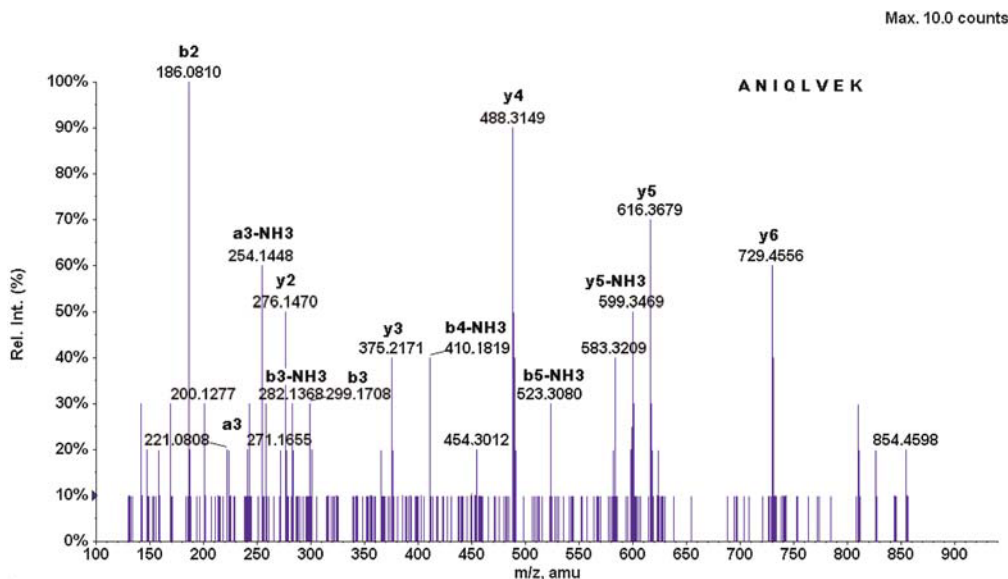


Figure 6. The product ion spectrum of m/z 457.7712 $[M+2H]^{+2}$ for the selected signature peptide with sequence ANIQLVEK.

CONCLUSIONS

A general strategy was demonstrated to characterize the important allergenic proteins present in black tiger prawn. Tropomyosin, myosin light chain, and arginine kinase were identified using PMF, and some of their highly abundant peptides were *de novo* sequenced using PFF. Tropomyosin as the major allergen was completely sequenced using different enzymatic digestion strategies, derivatization protocols, and ion sources for mass spectrometry. The allergenicity of these three proteins was confirmed in this study by the immunoblotting of these proteins with patient's sera.

For BTP TM quantification purposes, the selection rules for a recommended signature peptide have been followed. The experimental data for the resultant peptides was evaluated by bioinformatics approaches to determine the PTM-free peptides. The nominated peptides were subjected to the BLAST test to acquire the highest scoring peptide as signature peptide. This peptide (ANIQLVEK) will be chemically synthesized (labeled and unlabeled forms) and used in further studies to develop a quantification method for BTP TM in different environments in the seafood industry workplace.

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Erratum

Analysis of the allergenic proteins in black tiger prawn (*Penaeus monodon*) and characterization of the major allergen tropomyosin using mass spectrometry

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This affects the citation record of the Corresponding Author.