



Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies



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ARTICLE INFO

Article history:

Received 10 January 2013

Received in revised form 2 June 2013

Accepted 24 June 2013

Available online 3 July 2013

Keywords:

Tropomyosin

Monoclonal antibodies

Seafood

Allergen heat-treatment

IgE epitope recognition

Crustacean

Mollusc

ABSTRACT

The major heat-stable shellfish allergen, tropomyosin, demonstrates immunological cross-reactivity, making specific differentiation of crustaceans and molluscs for food labelling very difficult. The aim of this study was to evaluate the application of allergen-specific monoclonal antibodies in differential detection of shellfish-derived tropomyosin in 11 crustacean and 7 mollusc species, and to study the impact of heating on its detection. Cross-reactive tropomyosin was detected in all crustacean species, with partial detection in molluscs: mussels, scallops and snails but none in oyster, octopus and squid. Furthermore, we have demonstrated that heating of shellfish has a profound effect on tropomyosin detection. This was evident by the enhanced recognition of multiple tropomyosin variants in the analysed shellfish species. Specific monoclonal antibodies, targeting the N-terminal region of tropomyosin, must therefore be developed to differentiate tropomyosins in crustaceans and molluscs. This can help in correct food labelling practices and thus protection of consumers.

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1. Introduction

Seafood plays an important role in human nutrition and health. The growing international trade in seafood species and products has added to the popularity and frequency of consumption of a variety of seafood products across many countries. This increased production and consumption of seafood has been accompanied by more frequent reporting of allergic health problems among consumers. Allergic reactions are manifested by gastrointestinal and dermatological symptoms, as well as respiratory and anaphylactic reactions (Lopata & Lehrer, 2009; Lopata, O'Hehir, & Lehrer, 2010). The appearance of allergic symptoms results not only from ingestion of seafood; it can also be triggered by inhaling cooking vapours and handling shellfish (Jeebhay & Lopata, 2012; Jeebhay, Robins, Lehrer, & Lopata, 2001; Lopata & Jeebhay, 2013). Importantly, patients with shellfish allergy, similarly to those with peanut allergy, mostly remain clinically reactive throughout their

lives and are at increased risk of wheezing illness and hyper-reactive airways at school age (Lopata, O'Hehir, & Lehrer, 2010).

Food allergy to shellfish is on an increase, affecting approximately 2% of the general population. Several commercially important shellfish are used as food additives or supplements in a number of consumer food products (e.g. oyster sauce, krill oil). Accidental exposure to food products, cross-contaminated with shellfish allergens during processing, can occur and is an important consumer health concern.

The three most important seafood groupings causing allergic reactions include fish, crustacea and mollusc. The latter two phyla of crustaceans and molluscs are generally referred to as 'shellfish' in the context of seafood consumption. The allergic response in sensitised consumers is mediated by serum IgE antibodies directed to specific allergens, such as the major allergen tropomyosin, an abundant shellfish muscle protein (Albrecht et al., 2008). The presence of this very same allergenic protein in processed food, even at very low concentrations, can cause severe reactions in sensitised consumers. Therefore the labelling of food products containing crustaceans has already become mandatory in many countries, including the USA, Europe and Japan. Recently the European Union adapted guidelines to include molluscs as a separate food allergen, based on the limited cross-reactivity to crustacean allergens (Opinion of the Scientific Panel on Dietetic products, 2006).

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Commercially available shellfish allergen detection kits usually make use of polyclonal rabbit antibodies. However their ability to differentiate between the major allergens from crustaceans and molluscs is often not defined and, in the case of such polyvalent rabbit antibodies, it is very difficult to achieve.

The aims of this study were to evaluate the use of allergen-specific monoclonal antibodies for the detection of shellfish-derived tropomyosin in a comprehensive range of crustacean and mollusc species and to analyse the impact of heat-processing on antibody recognition for improved allergen detection in processed food.

2. Materials and methods

2.1. Shellfish samples

Fresh or frozen specimens of 11 different crustacean and 7 mollusc species were acquired from local markets and distributors across Melbourne, Australia, as listed in Table 1. The specimens were transported to the laboratory on ice and frozen at -20°C prior to further use.

2.2. Preparation of protein extracts

For the preparation of raw protein extract, the outer shell of the specimen was removed and the edible meat cut into small pieces. The abdominal or tail muscles were used from prawns, crabs and lobster specimens. For the bivalves, the shell was split open and the inner muscle parts used for extraction. About 50 g of the muscle mass was homogenised in 150 ml of phosphate buffered saline (PBS) for 10 min, using an Ultra turrax blender (IKA, Staufen, Germany). This slurry was then agitated for 3 h at 4°C , followed by centrifugation at 14,000 rpm for 15 min. The supernatant was clarified through a glass fibre filter, followed by filtration through a $0.45\text{ }\mu\text{m}$ membrane filter (Millipore, Billerica, MA, USA) and stored at -80°C prior to further use.

For the generation of heated protein extracts, a more natural way of heat treatment was utilised, instead of just heating the raw extract, to mimic the way consumers are usually exposed to food allergens. The complete shellfish specimen, in its outer shell, was heated in liquid (PBS) at 100°C for 20 min. The outer shell was removed after cooling and the proteins from these muscle tissues extracted using the same method as described above.

2.3. Protein quantification

The total protein content of each prepared extract was determined using the Quick Start Bradford Assay kit (BioRad, USA), following the manufacturer's instructions. Bovine serum albumin (BSA) was used as the protein standard.

2.4. SDS-PAGE analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to visualise the total protein repertoire in the prepared extracts, as described previously (Abdel Rahman, Kamath, Gagne, Lopata, & Helleur, 2013; Abdel Rahman et al., 2010). Twelve microgram of protein extract were briefly heated in Laemmli buffer with dithiothreitol and loaded onto a 12% bis-acrylamide gel. Electrophoretic separation was performed at 170 V until the tracker dye reached the base, using a Mini-Protein Tetra Cell electrophoresis system (BioRad, Hercules, CA, USA). The separated proteins were visualised by staining with Coomassie brilliant blue R250 (BioRad, Hercules, CA, USA).

2.5. Immunoblotting

2.5.1. Immunoblotting with monoclonal anti-tropomyosin antibody

Four microgram of the crustacean protein extract were resolved by SDS–PAGE, as detailed above. The separated proteins were transferred to an activated polyvinylidene fluoride (PVDF) membrane, using the Semi-dry TransBlot Apparatus (BioRad, Hercules, CA, USA). After blocking with 5% (w/v) skim milk powder (SMP) in PBS-T, the membrane was subsequently incubated with monoclonal anti-insect tropomyosin antibody, mac-141 (Abcam, Cambridge, MA, USA) diluted 1:6000 in 1% SMP, PBS-T and rabbit anti-mouse IgG antibody conjugated with HRP (Sigma, St. Louis, MO, USA) diluted 1:50,000. After washing three times with PBS-T, the membrane was visualised using the enhanced chemiluminescent technique, as reported previously (Abdel Rahman, Kamath, Lopata, & Helleur, 2010; Abdel Rahman, Kamath, Lopata, Robinson, & Helleur, 2011). Briefly, the blots were incubated with chemiluminescent substrate (Sigma, St. Louis, MO, USA) and exposed to photographic film (GE Healthcare Biosciences, USA) to visualise the antibody-binding protein bands.

Table 1
Common and scientific names of the eleven crustacean and seven mollusc species analysed in this study. The theoretical molecular weight and GenBank accession numbers of characterised tropomyosins are listed for each species if available.

No.	Shellfish species				Theoretical MW (kDa)	Accession numbers (GenBank)
			Common name	Scientific name		
1	Crustaceans	Prawn	Black tiger prawn	<i>Penaeus monodon</i>	32.8	HM486525
2			King prawn	<i>Melicertus latisulcatus</i>	32.6	JX171685
3			Vannamei prawn	<i>Litopenaeus vannamei</i>	32.8	EU410072
4			Banana prawn	<i>Fenneropenaeus merguensis</i>	32.8	GU369817
5			Green tiger prawn	<i>Penaeus semisulcatus</i>	–	–
6		Crab	Blueswimmer crab	<i>Portunus pelagicus</i>	32.8	JX874982
7			Sand crab	<i>Ovalipes australiensis</i>	–	–
8			Snow crab	<i>Chionoecetes opilio</i>	32.6	BAF47267
9		Lobster	Slipper lobster	<i>Thenus orientalis</i>	32.0	KC291443
10			Rock lobster	<i>Jasus edwardsii</i>	32.9	KC291442
11			Yabby	<i>Cherax destructor</i>	32.0	KC291443
12	Molluscs	Bivalve	Green mussel	<i>Perna viridis</i>	32.7	AAG08988
13			Blue mussel	<i>Mytilus edulis</i>	32.7	U40035
14			Scallop	<i>Pecten fumatus</i>	–	–
15			Oyster	<i>Crassostrea gigas</i>	33.0	BAH10152
16		Gastropod	Sea snail	<i>Turbo cornutus</i>	32.7	AB444940
17		Cephalopod	Octopus	<i>Octopus vulgaris</i>	32.8	BAE54433
18			Calamari (squid)	<i>Sepioteuthis lessoniana</i>	32.6	AB218914

2.5.2. Patient sera IgE Immunoblotting

To confirm the allergenicity of tropomyosin, IgE antibody reactivity was evaluated by immunoblotting, using a pool of sera from patients with confirmed allergy to shellfish. IgE immunoblotting was performed as described previously (Abdel Rahman et al., 2013). Briefly, the proteins on the membrane were incubated with patient sera (1:10 in 1% SMP, PBS-T) and subsequently incubated with rabbit anti-human IgE polyclonal antibody (DAKO Corporation, Carpinteria, CA, USA) and goat anti-rabbit IgG labelled with HRP (Promega, USA). IgE antibody binding was visualised as described above. A healthy donor's serum was used as a negative control. Ethical approval for this study was acquired through Monash University, Melbourne, Australia.

2.6. Purification of natural tropomyosin

Tropomyosin from black tiger prawn was purified through anion-exchange chromatography, as described previously (Abdel Rahman, Kamath, et al., 2010), using a Biologic LP fast protein liquid chromatography system (BioRad, Hercules, CA, USA). About 20 mg of the protein extract were diluted in starting buffer (30 mM acetate buffer), pH 5.5, loaded onto a Mini Macroprep High Q column (BioRad, Hercules, CA, USA) and tropomyosin eluted using an increasing NaCl concentration (0.4–0.6 M). The collected tropomyosin fraction was further purified through a Sephadex G-50 gel filtration column (Sigma, MO, USA), using PBS as medium, and subsequently concentrated, using an Amicon spin column (Merck, USA) and stored at -80°C prior to further use.

2.7. Mass spectrometric identification of tropomyosin

The IgE and monoclonal antibody-reactive prawn tropomyosin was excised from the SDS–PAGE gel for mass spectrometric analysis. The band was de-stained and digested with trypsin, as previously reported (Abdel Rahman, Gagne, & Helleur, 2012; Abdel Rahman et al., 2011). About 250 fmol of total protein were injected into a DIONEX Ulti-242 Mate3000 Nano LC System (Germering, Germany) and the tryptic peptides separated on a nanoflow analytical column (75 μm ID $\times$ 15 cm, C18 PepMap 100, 3 μm , 100 Å, (LC Packing, Sunnyvale, CA) at 180 nl/min, using a gradient regime. The resultant tandem spectra were searched, using the National centre 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 guanidination derivatisation had been performed. Peptides were considered identified if the Mascot score was over 95% confidence limit.

2.8. Cloning and cDNA sequencing of complete prawn tropomyosin

Total RNA was extracted from black tiger prawn with an RNeasy RNA extraction kit (Qiagen, Hilden, Germany), using the manufacturer's instructions. Single stranded cDNA was generated, using RT-PCR from the isolated mRNA with the Transcriptor High Fidelity cDNA synthesis kit (Roche, Basel, Switzerland). The generated cDNA was used as a template to amplify the coding region of tropomyosin, using the forward (BT-TM-F) and reverse (BT-TM-R) sets of primers. The primer sequences used were

BT-TM-F: GCGGATCCGACGCCATCAAGAAGAAGATGC

BT-TM-R: GCGAATTCTTAGTAGCCAGACAGTTCGCTG

For cDNA amplification, a PCR reaction was performed with the following setting; one cycle of denaturation at 94°C for 2 min, 35 cycles at 94°C for 30 s, annealing at 55°C for 45 s and elongation at 72°C for 1 min, and a final elongation step at 72°C for 7 min. The amplified 860 bp fragment was purified and cloned into the

sequencing vector, pCR 2.1, using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA).

2.9. Expression and purification of recombinant prawn tropomyosin

To express recombinant tropomyosin for antibody reactivity studies, the coding region for tropomyosin was cloned into the expression vector, pRSET A (Invitrogen, Carlsbad, CA, USA), using the restriction enzymes, *Bam*H1 and *Eco*R1 (Promega, Madison, Wisconsin, USA). Ligation of the coding region into the expression vector was done using T4 DNA Ligase (Invitrogen, Carlsbad, CA, USA). The cloned vector was transformed into BL21 *Escherichia coli* cells, using electroporation and incubated in SOC medium at 37°C for 1 h. The cells were grown overnight on Luria Bertani (LB) agar with 100 $\mu\text{g}/\text{ml}$ of ampicillin at 37°C . The white colonies were tested for the presence of the insert, using PCR and protein expression induced by 0.6 mM IPTG from a fresh overnight culture in LB broth containing 100 $\mu\text{g}/\text{ml}$ of ampicillin. Recombinant tropomyosin was extracted from the bacterial cells using lysis buffer (25 mM Tris–HCl, 300 mM NaCl, 1 mM imidazole, 2 mg/ml of lysozyme, pH 8), purified using a nickel-charged metal chelate affinity chromatography column (GE Healthcare, USA) and subsequently by ion-exchange chromatography, as described above, and stored at -80°C prior to further use.

2.10. Inhibition-ELISA

An inhibition-ELISA was performed to analyse the mAb cross-reactivity to tropomyosin in the various shellfish extracts. A 96-well polystyrene high binding plate (Costar, USA) was coated with 0.1 $\mu\text{g}/\text{well}$ of recombinant tropomyosin for 4 h at room temperature, using carbonate buffer, pH 9.6, and subsequently blocked using 5% SMP in PBS-T. The mAb was mixed with increasing concentrations of inhibitors; namely raw and heated protein extracts of black tiger prawn, blue swimmer crab, rock lobster, blue mussel, scallop and squid and exposed to the coated wells for 1 h at 37°C . A fish protein extract was used as a negative control. Antibody binding to the coated antigen was detected using rabbit anti-mouse IgG antibody conjugated with HRP (Sigma, St. Louis, MO, USA) and visualised using 3,3',5,5'-tetramethylbenzidine (TMB) substrate for HRP (BD Biosciences, USA). The reaction was stopped using 2 M sulphuric acid and the absorbance measured at 450 nm. Percent inhibition was calculated as $100 - [(O.D.450 \text{ nm of antibody with inhibitor} / O.D.450 \text{ nm of antibody without inhibitor}) \times 100]$.

2.11. Amino acid sequence alignment of crustacean and mollusc tropomyosin

An amino acid sequence comparison was performed, using representative tropomyosin sequences from crustaceans; namely black tiger prawn (GenBank accession number, ADM34184.1), blue swimmer crab (GenBank accession number, JX874982), rock lobster (GenBank accession number, KC291442) and molluscs; namely green mussel (Genbank accession number, AAG08988.1), oyster (Genbank accession number, BAH10152.1) and octopus (Genbank accession number, BAE54433.1). Sequences were obtained from the National Centre for Biotechnology Information (NCBI). Multiple sequence alignment was performed using the ClustalW alignment in MEGA.

3. Results

3.1. Protein profile of shellfish extracts and effect of heat treatment

Eleven crustacean and 7 mollusc samples listed in Table 1 were analysed for their protein repertoire in raw and heated extracts to

evaluate the effect of heat treatment by 1D-SDS-PAGE (Fig. 1). The raw extracts displayed a complex protein pattern with the majority of visible protein bands within the MW range of 18–75 kDa. For crustaceans, the most prominent protein bands were in the range of 18–20 kDa and 35–40 kDa. Similarities could be observed among the protein profiles of the prawn group (lanes 1–5) and crab group (lanes 6–8), while there were variations in the profiles of the three lobster species (lanes 9–11). In the case of the mollusc raw extracts, the profiles varied considerably among each other with no obvious common protein pattern.

To analyse the effects of heat processing, the shellfish protein extraction parameters, such as osmolarity, pH, extraction buffer volume, specimen weight, heating temperature and heating time were kept constant. This allowed direct comparison of the effects of heating on different shellfish species tropomyosin without any bias. The extracts of heat-treated shellfish displayed a more uniform protein-banding pattern. The heated crustacean extracts possessed major protein bands at about 18 kDa and 37 kDa, signifying the presence of heat-stable proteins. Similarly, the heated mollusc extracts displayed a single major protein band at about 37 kDa, but with very different intensities.

3.2. Purification and identification of tropomyosin and IgE reactivity

In order to confirm specific monoclonal antibody (mAb) reactivity to tropomyosin, natural tropomyosin was purified from prawn protein extract, using ion-exchange chromatography (Fig. 2A). IgE

antibody reactivity to natural tropomyosin was confirmed by immunoblotting against a pool of shellfish allergic patient sera (Fig. 2B). Mass spectrometric analysis confirmed the mAb and IgE antibody reactive protein to be tropomyosin by comparing generated peptides, using the MASCOT database (Fig. 2C). In addition, a 26 kDa fragment of tropomyosin was detected, which demonstrated IgE binding but was unable to bind to the mAb. The peptides identified in this fragment are shown in Supplementary Table 1.

Subsequently this prawn tropomyosin was fully sequenced by cDNA analysis (Fig. 2D) and demonstrated greater than 89% amino acid identity with tropomyosin from the other investigated crustacean species. In contrast, the amino acid identity to the six investigated mollusc tropomyosins was very low and ranged from 55% (green mussel) to 63% (octopus) (Supplementary Fig. 1). The generated recombinant tropomyosin (rTM) from black tiger prawn was further used as a standard for additional cross-reactivity studies (see Section 3.4).

3.3. mAb reactivity to tropomyosin in raw and heated shellfish extracts

The raw and heated protein extracts of crustaceans and molluscs were evaluated for their mAb reactivity by immunoblotting (Fig. 3). In the raw crustacean extracts, a single band was observed for each sample except for vannamei prawn. The molecular weight of these bands ranged from 31 to 36 kDa. Based on densitometric analysis of the bands (data not shown), mAb reactivity was the strongest to black tiger prawn, followed by blue swimmer crab

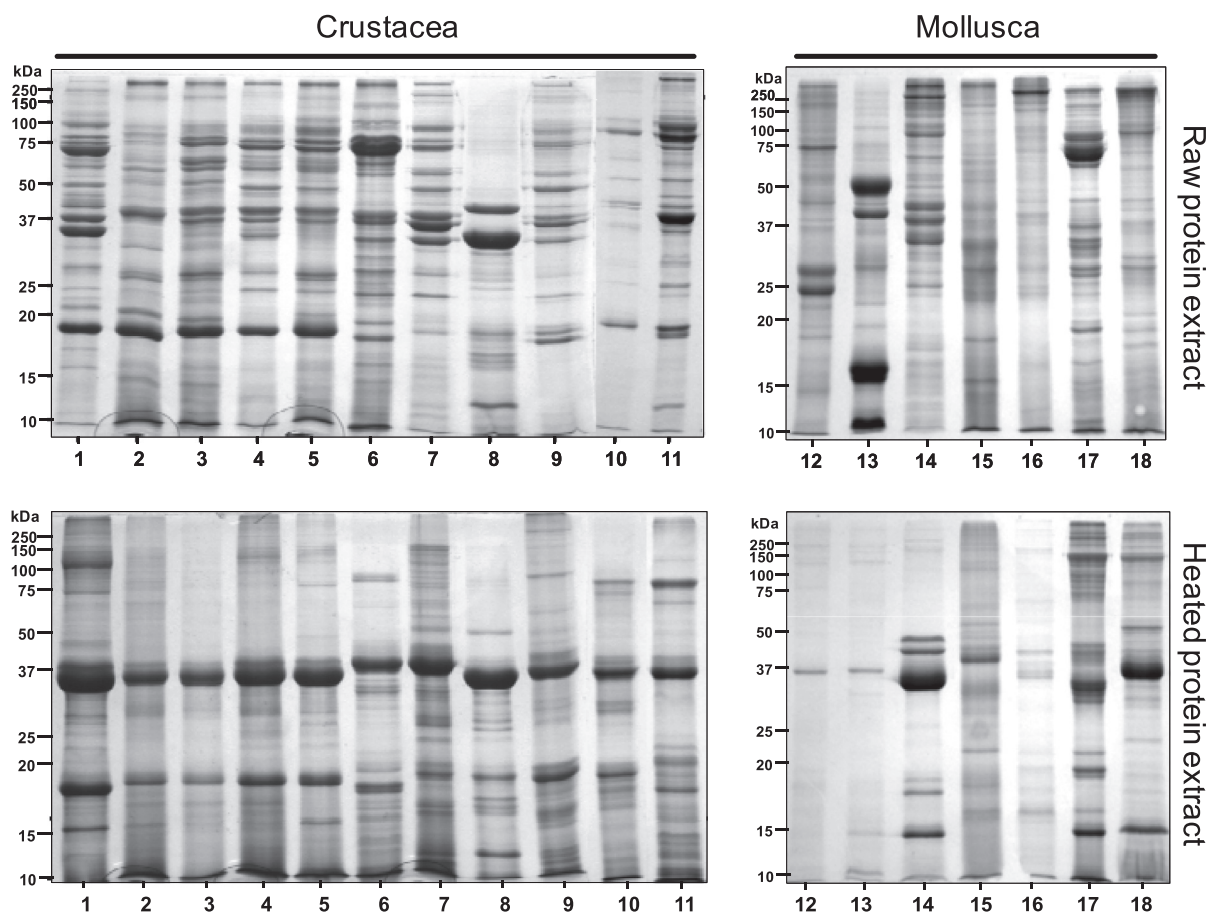
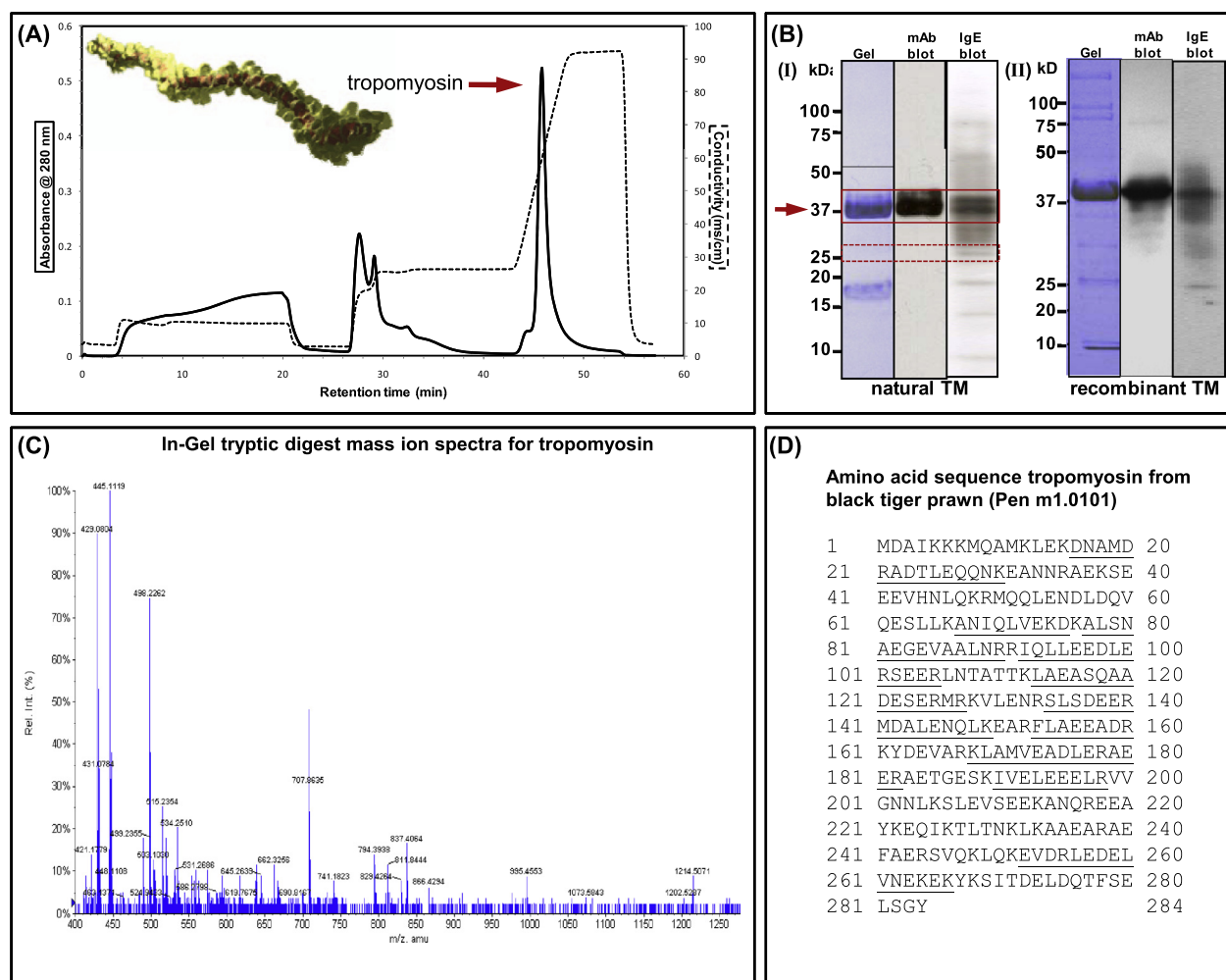


Fig. 1. SDS-PAGE analysis of raw and heated protein extracts of crustacean and mollusc species. Lanes 1–11, crustacean extracts; 1, black tiger prawn, 2, king prawn, 3, vannamei prawn, 4, banana prawn, 5, green tiger prawn, 6, blue swimmer crab, 7, sand crab, 8, snow crab, 9, slipper lobster, 10, rock lobster, 11, yabby. Lanes 12–18, mollusc extracts; 12, green mussel, 13, blue mussel, 14, scallop, 15, oyster, 16, sea snail, 17 octopus, 18, squid. See Table 1 for complete list of scientific names for all species.



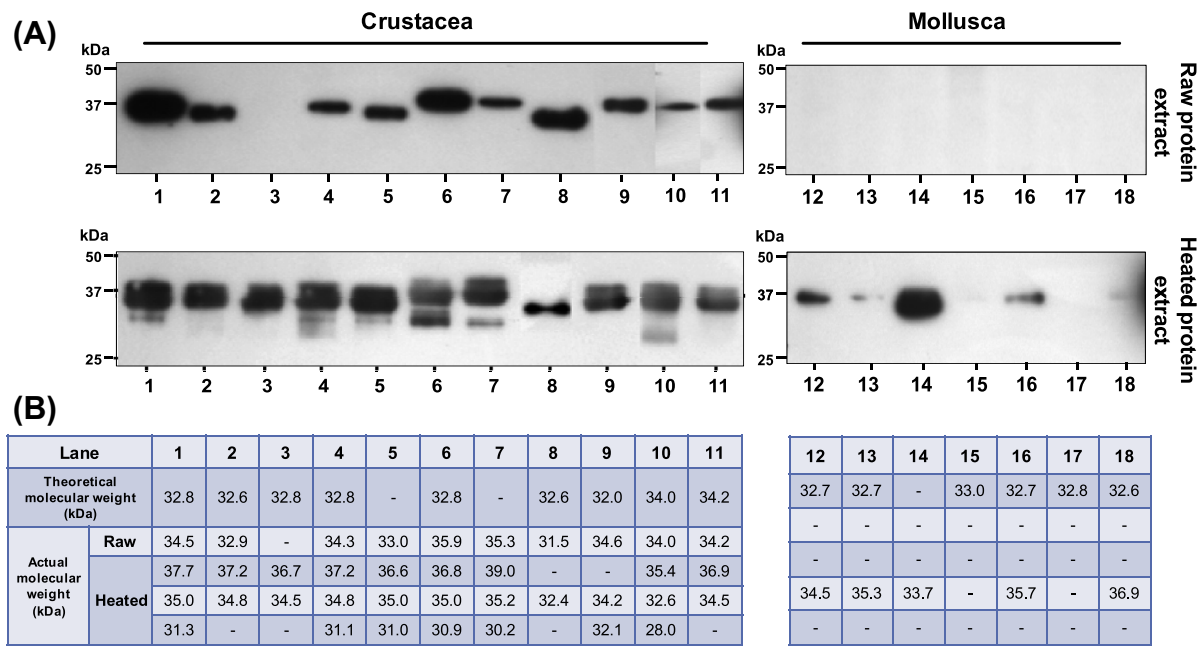


Fig. 3. Immunoblot analysis of raw and heated protein extracts of crustacean and mollusc species, using monoclonal anti-tropomyosin antibody. (A) Lanes 1–11, crustacean extracts; 1, black tiger prawn, 2, king prawn, 3, vannamei prawn, 4, banana prawn, 5, green tiger prawn, 6, blue swimmer crab, 7, sand crab, 8, snow crab, 9, slipper lobster, 10, rock lobster, 11, yabby. Lanes 12–18, mollusc extracts; 12, green mussel, 13, blue mussel, 14, scallop, 15, oyster, 16, sea snail, 17, octopus, 18, squid. See Table 1 for a complete list of scientific names. (B) Theoretical and actual molecular weights of tropomyosin variants detected in the raw and heated crustacean and mollusc extracts using the mAb.

tity, while mollusc tropomyosins (mussel, oyster and octopus) share only 63% sequence identity within the individual species.

Based on the differential mAb reactivity to tropomyosin in the immunoblotting experiments and using amino acid sequence alignment from select crustacean and mollusc species, we attempted to predict the most likely binding site of the mAb on shellfish tropomyosin. Strong mAb binding to tropomyosin was demonstrated for prawn, crab, lobster and mussel and no binding to oyster and octopus. This information was used to locate amino acid substitutions due to evolutionary changes in the primary structure along the entire protein, which could be responsible for changing the mAb reactivity. Twenty-two amino acid substitutions were identified on tropomyosin, fulfilling this criterion, as shown by marked asterisk (Fig. 5).

In addition, an approximately 26 kDa stable fragment of tropomyosin was identified using mass spectrometry in the heated prawn extract (Supplementary Table 1), which we demonstrated to elicit IgE antibody binding, but lacked the ability to bind mAb. Based on the peptide matches, this fragment has been highlighted with its N- and C-terminal marked by red arrows (Fig. 5). Therefore, it is predicted that the tropomyosin amino acid region 9–19 “QAMKLEKDNAM” is the most likely mAb-binding epitope.

4. Discussion

Tropomyosin is the major shellfish allergen. Due to its primary role in muscle function, it is present in much higher quantity than other identified shellfish allergens. Moreover, highly conserved primary structure, is responsible for its allergenic cross-reactivity, not only between crustaceans and molluscs but also other invertebrates, such as mites and insects (Arlian, Morgan, Vyszynski-Moher, & Sharra, 2009; Zhang, Matsuo, & Morita, 2006). Therefore, tropomyosin is a commonly used biomarker for the detection of shellfish allergens (Fuller, Goodwin, & Morris, 2006; Lopata, Luijck, Fenemore, Sweijd, & Cook, 2002; Seiki et al., 2007; Shibahara et al., 2007; Werner, Faeste, & Egaas, 2007).

The ability of tropomyosin to withstand heat-treatment and most known forms of food processing techniques can be attributed to its exceptionally stable alpha helical coiled-coil secondary structure (Reese, Ayuso, & Lehrer, 1999). Effect of heat treatment on the reactivity of patient IgE antibody to tropomyosin from crustacean species has previously been discussed. (Carnes et al., 2007; Martin-Garcia et al., 2007; Yu et al., 2011) Nevertheless not much information is available on the effect of heat processing for specific detection of tropomyosin from various shellfish groups, particularly molluscs. Monoclonal antibodies are preferred to conventional polyclonal antibodies as the former bind exclusively to a specific epitope, on the antigen, usually comprising of not more than ten amino acids. Using a specific monoclonal antibody, we have demonstrated that heating of shellfish increases the antibody reactivity to tropomyosin. Furthermore, we have shown that heating can also cause molecular differences between tropomyosins from the different shellfish groups investigated.

Heat-processing resulted in increased antibody detection of tropomyosin for crustacean and mollusc extracts. Multiple variants of tropomyosin were observed for crustacean extracts. Interestingly, while no antibody reactivity was seen for raw mollusc extracts, characteristic binding was observed after heating. This increase in the mAb reactivity to tropomyosin may have been caused by conformational changes in the secondary structure due to heating. (Albrecht et al., 2009). Yet another observation made was the presence of higher molecular weight tropomyosin bands. These may be attributed to the phenomenon called the “Maillard reaction” which occurs due to chemical interaction of amino acid residues with sugar moieties at elevated temperatures. This phenomenon has been previously reported for shellfish and peanut allergens (Gruber, Becker, & Hofmann, 2005; Nakamura, Watanabe, Ojima, Ahn, & Saeki, 2005; Nakamura et al., 2006). Tropomyosins, being rich in lysine residues, may readily react with reducing sugars at elevated temperatures, resulting in the Maillard reaction. Further studies, focussing on the observed tropomyosin variants in the current study, are needed to confirm the presence and impact of Maillard products after heating of shellfish.

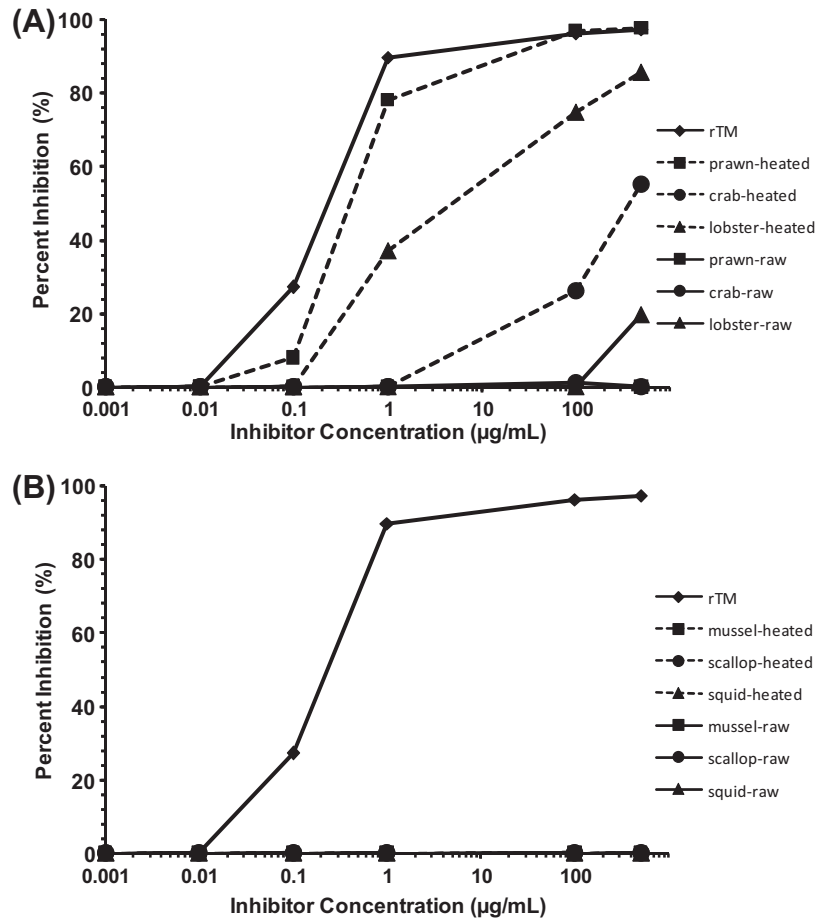


Fig. 4. Inhibition-ELISA for the quantitative analysis of cross-reactivity of the mAb between recombinant black tiger prawn tropomyosin and different crustacean (A) and mollusc (B), raw and heated extracts.

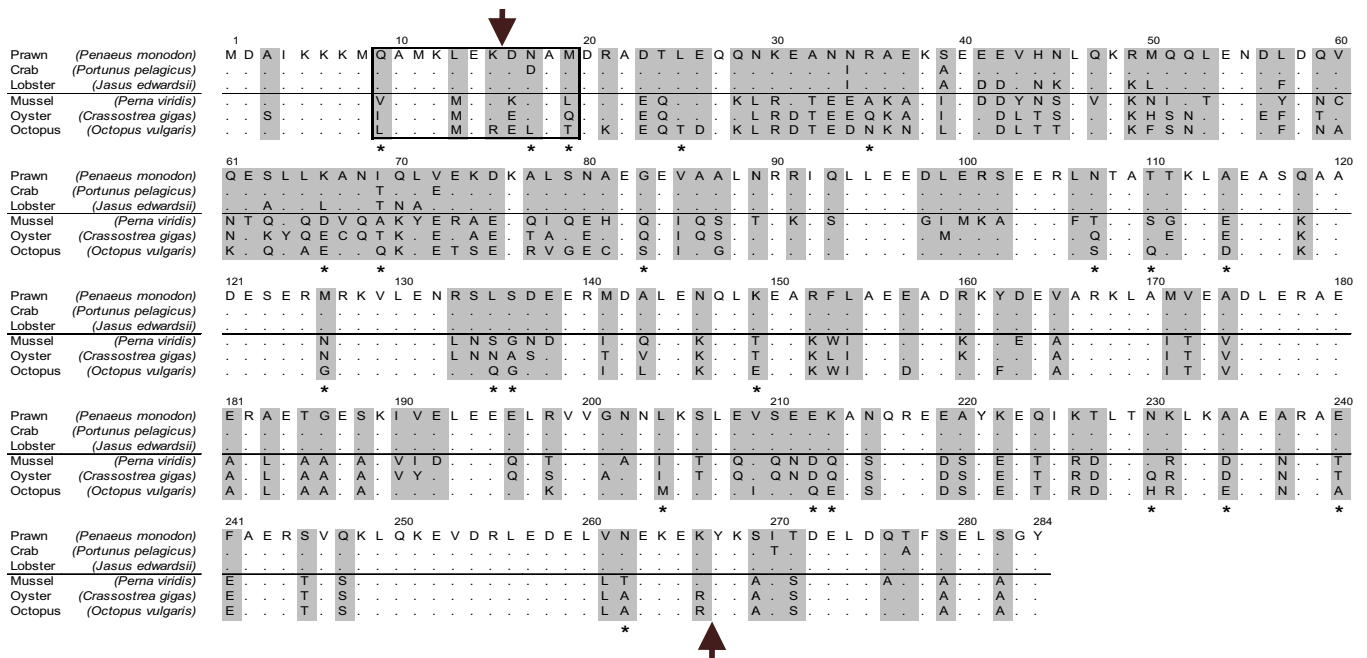


Fig. 5. Amino acid sequence alignment of representative tropomyosin sequences from crustaceans (GenBank accession numbers); black tiger prawn (ADM34184), blue swimmer crab (JX874982), rock lobster (KC291442) and molluscs; green mussel (AAG08988), oyster (BAH10152) and octopus (BAE54433). Variable amino acid regions of the proteins' primary structure are shaded in grey. The amino acid substitutions between crustacean and mollusc tropomyosins are denoted with "*". The red arrows indicate the N- and C-terminal ends of the 26 kDa fragment of tropomyosin which does not bind to the mAb. The predicted mAb binding epitope of tropomyosin is identified by a solid box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Several assays have been developed for the sensitive detection of tropomyosin in food matrices (Seiki et al., 2007; Werner et al., 2007). However, there is a lack of analytical tools that can be employed for specific detection of tropomyosin from various shellfish species. We have demonstrated for the first time the use of a commercially available, monoclonal anti-tropomyosin antibody for the detection of tropomyosin in an extensive panel of crustacean and mollusc species. While all eleven crustacean tropomyosins demonstrated reactivity, not all mollusc tropomyosins were detected using this mAb. This lack of antibody recognition of mollusc tropomyosin was further confirmed using immunoblotting and inhibition ELISA, with both raw and heated mollusc extracts failing to inhibit mAb binding to prawn tropomyosin.

One of the main objectives of this study was to characterise a commercially available anti-tropomyosin mAb, which could possibly be used for the detection of the major shellfish allergens in food products as part of international food safety regulations. For this reason, we have selected black tiger prawn tropomyosin for the detailed cloning and sequencing experiments to deduce its monoclonal antibody-binding site and to subsequently extrapolate this information to other tropomyosins from crustaceans, as well as molluscs. The recombinant form of tropomyosin from black tiger prawn was subsequently generated and its antibody binding was evaluated. The application of this recombinant protein as an allergen standard will enable future improved quantification approaches for the detection of the major shellfish allergen, using specific antibodies.

Next, we attempted to predict the mAb binding site on tropomyosin. Three representative crustacean and mollusc species were selected, since the tropomyosin sequence identity was high within these two sub-groups. Published amino acid sequences from GenBank were compared, in order to understand the underlying molecular characteristics of tropomyosin across the different species. There were 22 specific amino acid substitutions along the primary structure of tropomyosin. Three major amino acid substitutions were revealed near the N-terminal of the protein by amino acid sequence comparison. Moreover, an identified tropomyosin fragment (residue 16–266) was able to elicit IgE binding but exhibited no mAb reactivity. This indicated the possibility of the presence of a specific mAb epitope at the N- or C-terminal of the protein. Based on the antibody binding data, and using amino acid sequence analysis, the most likely mAb-binding epitope was identified to lie between amino acid residues 9–19.

In summary, we have shown that heating has a profound effect on the detection of the major shellfish allergen tropomyosin, which could have considerable implications for the detection and quantification of tropomyosin in processed food. Further studies are needed to characterise the multiple tropomyosin variants formed during heating or various “cooking” processes. We have successfully demonstrated the analytical application of a specific anti-tropomyosin antibody for the differentiation of tropomyosin from crustaceans and molluscs at a molecular level. While this mAb was able to detect tropomyosin from all crustacean species tested, it detected few mollusc tropomyosins. A specific antibody target was identified in the N-terminal region of shellfish tropomyosin to enable the differentiation between crustacean and mollusc allergens. More specific assays may be developed by applying this approach for better food labelling for consumer protection.

Acknowledgements

This study received financial support from the Asthma Foundation Victoria. AL is holder of Australian Research Council–Future Fellowship. Patient sera were kindly provided by Robyn E. O’Hehir and Jennifer Rolland, The Alfred Hospital, Prahran, Melbourne, VIC, Australia. The authors would like to thank Bob Helleur for access to

the Mass Spectrometry facility, Memorial University, Newfoundland, Canada.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2013.06.105>.

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			Crustaceans											Molluscs							
Crustaceans		Specimen no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
	Black tiger prawn	1	100																		
	King prawn	2	95	100																	
	Vannamei prawn	3	100	95	100																
	Banana prawn	4	100	95	100	100															
	Green tiger prawn	5	•	•	•	•	•														
	Blue swimmer crab	6	97	94	97	97	•	100													
	Sand crab	7	•	•	•	•	•	•	•												
	Snow crab	8	89	88	89	89	•	88	•	100											
	Slipper lobster	9	97	95	97	97	•	99	•	88	100										
	Rock lobster	10	95	100	95	95	•	94	•	88	95	100									
Yabby	11	98	95	98	98	•	98	•	89	98	95	100									
Molluscs	Green mussel	12	55	57	55	55	•	55	•	54	55	57	55	100							
	Blue mussel	13	57	58	57	57	•	56	•	56	56	58	57	94	100						
	Scallop	14	•	•	•	•	•	•	•	•	•	•	•	•	•	•					
	Oyster	15	61	62	61	61	•	61	•	59	61	62	61	78	78	•	100				
	Sea snail	16	60	61	60	60	•	60	•	58	60	61	60	68	72	•	73	100			
	Octopus	17	63	64	63	63	•	63	•	63	63	64	63	69	70	•	76	76	100		
	Squid	18	62	63	62	62	•	62	•	62	63	63	63	70	71	•	75	76	91	100	

Supplementary figure 1

Supplementary table 1: Start and end position numbers, calculated molecular weights and amino acid sequences of peptides identified for each IgE reactive tropomyosin using mass spectrometry

MW of protein from SDS-PAGE	Start-end	MW, calc.	Peptide sequence
38 kDa Tropomyosin molecule	67-74	914.07	ANIQLVEK
	153-160	950.02	FLAEEADR
	141-149	1061.22	MDALENQLK
	190-198	1129.28	IVELEEEELR
	67-76	1157.33	ANIQLVEKDK
	169-178	1146.32	LAMVEADLER
	92-101	1257.41	IQLLEEDLER
	168-178	1274.50	KLAMVEADLER
	113-125	1376.40	LAEASQAADESER
	91-101	1413.59	RIQLLEEDLER
	77-90	1414.54	ALSNAEGEVAALNR
	252-260	1587.70	EVDRLEDELVNEK
	169-182	1631.82	LAMVEADLERAEER
	92-105	1758.90	IQLLEEDLERSEER
	16-30	1748.84	DNAMDRA DTLEQQNK
26 kDa Tropomyosin fragment	67-74	914.07	ANIQLVEK
	153-160	950.02	FLAEEADR
	141-149	1061.22	MDALENQLK
	190-198	1129.28	IVELEEEELR
	169-178	1146.32	LAMVEADLER
	67-76	1157.33	ANIQLVEKDK
	169-178	1146.32	LAMVEADLER
	168-178	1274.50	KLAMVEADLER
	256-264	1345.47	LEDELVNEKEK
	113-125	1376.40	LAEASQAADESER
	77-90	1414.54	ALSNAEGEVAALNR
	252-260	1587.70	EVDRLEDELVNEK
	169-182	1631.82	LAMVEADLERAEER
	75-90	1657.8	DKALSNAEGEVAALNR
	113-126	1663.78	LAEASQAADESERMR
	16-30	1748.84	DNAMDRA DTLEQQNK
	92-105	1758.90	IQLLEEDLERSEER
	134-149	1878.04	SLSDEERMDALENQLK