

Malaysian cockle (*Anadara granosa*) allergy: Identification of IgE-binding proteins and effects of different cooking methods

Zailatul, H.M.Y.^{1,2*}, Rosmilah, M.², Faizal, B.¹, Noormalin, A.¹ and Shahnaz, M.¹

¹Allergy and Immunology Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

²Department of Biology, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjong Malim, Perak, Malaysia

*Corresponding author email: zailatul@imr.gov.my

Received 30 May 2014; received in revised form 18 July 2014; accepted 8 September 2014

Abstract. The purpose of this study was to evaluate the effect of different cooking methods on the allergenicity of cockle and to identify proteins most frequently bound by IgE antibodies using a proteomics approach. Raw, boiled, fried and roasted extracts of the cockle were prepared. The protein profiles of the extracts were obtained by separation using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and 2-dimensional gel electrophoresis (2-DE). IgE-immunoblotting was then performed with the use of individual sera from patients with cockle allergy and the major IgE-binding proteins were analyzed by mass-spectrometry. SDS-PAGE of raw extract showed 13 protein bands. Smaller numbers of protein bands were detected in the boiled, fried and roasted extracts. The 2-DE gel profile of the raw extract further separated the protein bands to ~50 protein spots with molecular masses between 13 to 180 kDa and isoelectric point (pI) values ranging from 3 to 10. Immunoblotting of raw extract exhibited 11 IgE-binding proteins with two proteins of 36 and 40 kDa as the major IgE-binding proteins, while the boiled extract revealed 3 IgE-binding proteins. Fried and roasted extracts only showed a single IgE-binding protein at 36 kDa. 2-DE immunoblotting of raw extract demonstrated 5 to 20 IgE reactive spots. Mass spectrometry analysis led to identification of 2 important allergens, tropomyosin (36 kDa) and arginine kinase (40 kDa). Heated extracts showed a reduction in the number of IgE-reactive bands compared with raw extract, which suggest that thermal treatment can be used as a tool in attempting to reduce cockle allergenicity. The degree of allergenicity of cockle was demonstrated in the order raw > boiled > fried ≈ roasted. Two important allergens reacting with more than 50% of patients' sera identified using mass spectrometric approaches were tropomyosin and arginine kinase. Thus, allergens found in this study would help in component-based diagnosis, management of cockle allergic patients and to the standardisation of allergenic test products as tools in molecular allergology.

INTRODUCTION

The marine bivalve (*Anadara granosa*) cockle, locally known as 'kerang' is classified in the phylum Mollusca, class Bivalvia, order Arcoidea and family Arcidae. It is one of the popular gourmet shellfish in Malaysia and its cultivation is a good revenue earner. In 2012, total production of cockles in Malaysia was approximately 43,000 tons and it was estimated that the retail value of cockle

production amounted to RM 104,811 million (DOF, 2013).

However, hypersensitivity to shellfish including cockle appears to be a significant cause of allergy among local patients with asthma and allergic rhinitis (Shahnaz *et al.*, 2001). Shellfish protein is a potent allergen and can provoke symptoms by skin contact, inhalation or ingestion (Woo & Bahna, 2011). Sensitized individuals develop allergic reactions affecting one or more target organs:

the skin (urticaria, angio-oedema), respiratory tract (rhinitis, asthma), gastrointestinal tract (pain, emesis, diarrhoea) and cardiovascular system (anaphylactic shock) (Lopata *et al.*, 2010; Lopata & Jeebhay, 2013).

Currently, allergy diagnosis and specific immunotherapy are performed using allergenic extracts containing a variety of allergenic and non-allergenic components that make them too complex to be standardized (Ciardiello *et al.*, 2013). The lack of standardization of allergenic extracts for allergy tests, mainly the result of incomplete knowledge of their main allergens, results in low diagnostic accuracy (Zuidmeer-Jongejan *et al.*, 2012; Ciardiello *et al.*, 2013). Thus proper identification and characterization of proteins causing IgE-mediated allergies would be expected to lead to significant improvement in diagnosis and management of patients (Woo & Bahna, 2011; Zuidmeer-Jongejan *et al.*, 2012; Ciardiello *et al.*, 2013). To date, there are only few reports on the identification of cockle allergens. Tropomyosin is the only allergen identified in different cockle species. Emoto *et al.* (2009) isolated 37 kDa allergens from bloody cockle *Anadara broughtonii* and Japanese cockle *Fulvia mutica* and identified them as tropomyosins based on the determined partial amino acid sequences.

Tropomyosin is a 34 to 38 kDa heat-stable protein that belongs to a highly conserved family of actin filament binding proteins, which plays a functional role in contractile activities in muscle cells (Thammathongchat *et al.*, 2010; Suzuki *et al.*, 2011; Lopata & Kamath, 2012). Tropomyosin is the most common and well documented allergen for crustaceans and molluscs (Emoto *et al.*, 2009; Abdel Rahman *et al.*, 2010; Rosmilah *et al.*, 2012a; Rosmilah *et al.*, 2012b; Yadzir *et al.*, 2012a; Yadzir *et al.*, 2012b; Abramovitch *et al.*, 2013). IgE cross-reactivity of tropomyosin can be seen clinically and experimentally amongst crustaceans, amongst molluscs, between crustaceans and molluscs and between crustaceans and terrestrial arthropods, such as cockroaches and mites (Leung *et al.*, 1996; Reese *et al.*,

1999; Ayuso *et al.*, 2002; Lopata & Kamath, 2012; Abramovitch *et al.*, 2013).

Since allergens are mainly proteins, their structure may be changed by various types of processing methods resulting in alteration of allergenicity (Chatterjee *et al.*, 2006; Nowak-Wegrzyn & Fiocchi, 2009; Abramovitch *et al.*, 2013). Alteration in protein structure can lead to epitope destruction, modification, masking or unmasking thereby decreasing, increasing or having no effect on allergenicity (Sathe *et al.*, 2005; Nowak-Wegrzyn & Fiocchi, 2009; Liu *et al.*, 2010). Cockles are usually subjected to some form of heat treatment, including boiling, frying or roasting prior to consumption. Since there is a paucity of information available about heat-sensitive or heat-resistant cockle allergens, the aim of our present study is to evaluate the effect of different cooking methods on the allergenicity of cockle and to identify proteins most frequently bound by IgE antibodies using a proteomics approach.

MATERIALS AND METHODS

Preparation of cockle extracts

For the preparation of the raw cockle (*Anadara granosa*) extract, the shell was split open and the inner muscle tissue used for extraction. About 20 g of the muscle mass was homogenized in 200 ml of 0.1 M phosphate buffered saline (PBS), pH 7.2 for 10 min using a Waring blender. This homogenate was then agitated overnight at 4°C, followed by centrifugation at 4,500 and 14,000 rpm, for 30 and 15 min, respectively. The clear supernatant was then recovered and sterilized by passing through a 0.22 µm syringe filter, frozen and lyophilized. The lyophilized extracts were stored at -20°C until use. The boiled cockle extract was prepared by boiling muscle tissue with 0.1 M of PBS (pH 7.2) for 10 min at 100°C before being homogenized using a Waring blender. The fried cockle extract was carried out by frying muscle tissue with vegetable oil for 10 min and subsequently placed on filter paper to remove the oil. The fried cockle muscle was then homogenized in 0.1 M of PBS (pH 7.2)

and extracted as above. The roasted cockle extract was prepared by roasting at 180°C for 10 min followed by homogenization according to the same protocol as above. Protein concentrations of the extracts were determined using the total protein kit (Sigma, USA), according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as the protein standard.

Human sera

Sera from 23 cockle-allergic patients, 12 males and 11 females whose ages ranged from 18 to 64 years (mean age, 36.4 years) were used in this study. The sera were obtained from patients referred to the Allergy Clinic, Kuala Lumpur Hospital. Allergic response was confirmed on the basis of the patient's clinical history and characterized by a positive skin prick test (SPT) to the raw cockle extract. Serum from non-allergic individuals was used as a negative control. All sera were stored at -80°C until use. This study was approved by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia and informed consent was obtained from the patients or their relatives.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and 2-dimensional gel electrophoresis (2-DE)

SDS-PAGE was carried out under denaturing conditions with the stacking and separating gels containing 5% and 12% of acrylamide (BioRad, USA), respectively. Cockle extracts were dissolved in Laemmli sample buffer (BioRad, USA) in the presence of 5% 2-mercaptoethanol and heated at 97°C for 4 min before loading onto the gel. Each lane was loaded with 10 µl, containing 10 µg of protein or protein standards (BioRad, USA) before the proteins were separated for 45 min at 120 mA and 200 V using Mini-Protean 3 system (BioRad, USA). The separated proteins were then visualized by staining with Coomassie brilliant blue R-250 (BioRad, USA). Molecular weights of the protein bands were determined by comparing with the molecular weight markers using an Imaging Densitometer GS800 and Quantity One Software (BioRad, USA).

For 2-DE gel electrophoresis, the lyophilized raw cockle extract was suspended in rehydration buffer containing 8 M urea, 50 mM DTT, 4% CHAPS, 0.2% carrier ampholyte pH 3-10 and 0.0002% bromophenol blue. 50 µg of protein sample was then applied to immobilized non-linear pH 3-10 gradient strip of 7 cm length (BioRad, USA) for rehydration overnight (12-14 h). For the first-dimension isoelectric focusing (IEF), the rehydrated strips were placed into a Protean IEF cell system (BioRad, USA) in which the proteins were separated based on their pI according to the following voltage/ time gradient: 100 V for 1 min, 250 V for 30 min, 4,000 V for 2 h and 4,000 V for 10,000 V-h. After first-dimension separation, the strips were equilibrated in equilibration buffer containing 125 mM Tris-HCL, pH 6.8, 6 M urea, 2% SDS, 30% glycerol and 0.01% bromophenol blue supplemented with 65 mM dithio-threitol for 10 min at room temperature followed by 135 mM iodoacetamide in equilibration buffer for another 10 min at room temperature and directly applied onto 12% of separating gels of SDS-PAGE with 5% of stacking gels and sealed in place using ReadyPrep overlay agarose (BioRad, USA) for second-dimension, as described above.

Immunoblot analysis

Proteins used in immunoblotting were first separated by SDS-PAGE or 2-DE and then electroblotted onto 0.45 µm nitrocellulose membranes (BioRad, USA) in transfer buffer containing 25 mM Tris, 192 mM glycine and 20% methanol. Blotting was performed for 70 min at 100 V using a Mini Trans-Blot system (BioRad, USA). After confirming the transfer of the proteins by staining with Ponceau S solution, the blots were washed (10 min/wash) three times in TTBS (TBS buffer containing 0.05% Tween-20), before being blocked in TBS containing 5% non-fat milk for 1 h at room temperature. The blots were then incubated with the individual patients' sera (primary antibody) in blocking buffer (1:100 dilution) for overnight at 4°C. After three washes with TTBS, the blots were then reacted with biotinylated goat-antihuman IgE (Kirkegaard and Perry Laboratories,

UK) as second antibody for 30 min at room temperature, with a working dilution of 1:1000. The blots were washed a further three times in TTBS, followed by incubation with streptavidin-conjugated alkaline phosphatase (BioRad, USA) for 30 min at room temperature. After a final three washes, the IgE reactivity was revealed by colorimetric detection using alkaline phosphatase conjugate substrate kit (BioRad, USA) as described by the manufacturer. Serum from a non-allergic individual was used as a negative control, while a blot without a serum sample was used as a blank.

Mass spectrometry analysis

The Coomassie-stained protein spots corresponding to those recognized by the above sera were manually excised and transferred to microcentrifuge tubes. These protein spots were analyzed using mass spectrometry analysis by First Base Laboratories Sdn Bhd, Malaysia. Protein samples were trypsin digested and peptides extracted according to standard techniques. Peptides were analyzed by matrix-assisted laser desorption-ionization time of flight

(MALDI-TOF) mass spectrometer using a 4800 Proteomics Analyzer. Spectra were analyzed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with Ludwig NR Database and taxonomy set to other metazoa.

RESULTS

Comparison of protein fractions in raw, boiled, fried and roasted cockles by means of SDS-PAGE

Coomassie brilliant blue staining of the SDS-PAGE gel loaded with raw extract of cockle demonstrated ~13 protein bands with molecular masses ranging from about 13 to 180 kDa (Figure 1). On the other hand, smaller numbers of protein bands were detected in the boiled extract as several protein bands between 20 to 30 kDa and 40 to 90 kDa were denatured, whereas most protein bands in fried and roasted extracts were completely denatured. In the case of the heated cockle extracts, the 36 kDa protein was clearly observed even after boiling, frying or roasting.

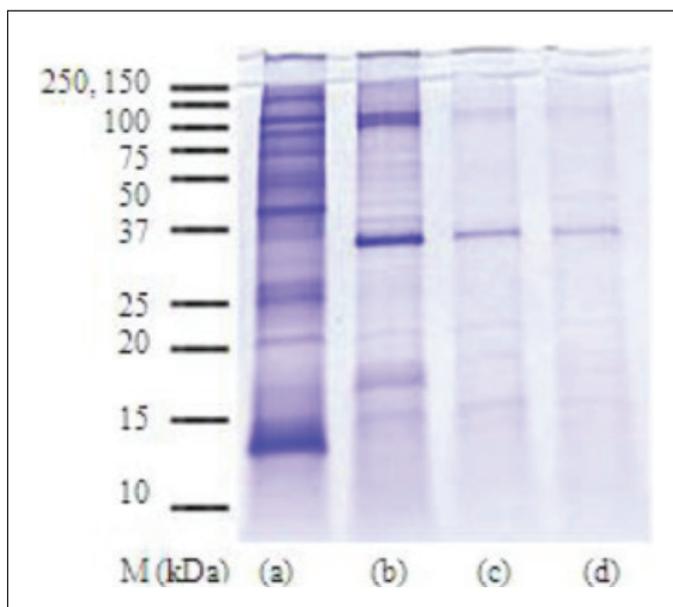


Figure 1. SDS-PAGE profiles of raw (a), boiled (b), fried (c) and roasted (d) extracts of *Anadara granosa* (Malaysian cockle). Lane M, molecular mass markers.

Comparison of IgE binding to raw, boiled, fried and roasted cockles

When serum samples were tested on immunoblots for IgE binding to the raw extract, the patient serum samples exhibited IgE binding to 11 protein bands (Figure 2a). The percentage of IgE binding to more than 50% was found for two protein bands with the estimated molecular weights of these proteins are 36 and 40 kDa. Further, 25, 50, 65, 75 and 90 kDa proteins were identified at frequencies in the range of 30–43%. Less frequently detected IgE-reactive protein bands were 16, 30, 100 and 150 kDa which were seen in only 9-17% of the patients. However, as shown by the immunoblots, not all proteins present in the raw extract were recognized by these patients' sera. Remarkably, two protein bands of ~13 and ~20 kDa, which are clearly present on SDS-PAGE (Figure 1), did not bind any IgE.

The allergenicity of the heated extracts which include boiled, fried and roasted extracts were further studied using 10 allergic patients' sera, as shown in Figure 2b, c and d. These patients' sera were selected based on having IgE-binding to raw extract in the immunoblotting above. It was shown that all these patients' sera retained an IgE-binding protein at 36 kDa which confirmed this protein as a heat-stable in cockle and more staining to this band was observed in most sera tested. Smear IgE-reactive proteins of ~100-150 kDa were also recognized with some of the sera in the boiled extract. No IgE-binding was observed with the negative control serum.

2-DE profiles and immunoblots

Figure 3 shows the 2-dimensional gel map of proteins from the raw cockle extract. Typically 50 protein spots were detected by Coomassie blue staining. These proteins were located between isoelectric point (pI) 4 to 8 and from 13 to 180 kDa. Immunoblotting of 2-DE gel was performed using sera from 10 patients identified to have IgE-binding in the 1-DE immunoblotting. These 2-DE immunoblots exhibited remarkable heterogeneity (only four 2-DE immunoblots are shown). The immunoblots with sera No. 16 (Fig. 4b) and No. 18 (Fig. 4c) showed less

than ten different IgE-binding protein spots while the blots with sera No. 15 (Fig. 4a) and No. 22 (Fig. 4d) revealed up to 20 different protein spots. The most frequently recognized IgE-binding spots were numbers 1, 2a, 2b and 2c with 100% of reactions.

Allergen identification

By comparing with established databases, the protein showing the highest correlation with spot 1 (36 kDa) was found to be tropomyosin. Spots 2a, b, and c (40 kDa) actually represented one protein and showed homology to arginine kinase. Table 1 summarizes the results of mass spectrometry analysis of the spots.

DISCUSSION

In Malaysia, cockle is one of the important allergenic shellfish. However, there is almost no data on local cockle allergens. This study has, for the first time, identified major local cockle allergens using a proteomics approach and evaluates the effect of different cooking methods on their allergenicity.

Our study demonstrated that protein bands between 20 to 30 kDa and 40 to 90 kDa were no longer seen in SDS-PAGE of the boiled cockle extract compared to SDS-PAGE of the raw extract. We found that both frying and roasting altered the proteins in a similar way. Most of the protein bands seemed to be clearly abolished compared with those of the raw and boiled extracts. The disappearance of proteins in boiled, fried and roasted extracts might be due to loss of almost all secondary and tertiary protein structures, causing the protein to exhibit a wide range of characteristics from loss of solubility, fragmentation to random-coil aggregation as consequence of high temperature (Davis and Williams, 1998; Samson *et al.*, 2004). In contrast to this, a protein of about 36 kDa is very stable to heating as evidenced in this study; it can be detected in SDS-PAGE even after boiling, frying or roasting.

Not all proteins present in the raw cockle extract were allergenic. Remarkably, two protein bands of ~13 and ~20 kDa, which are clearly present on SDS-PAGE (Figure 1), did

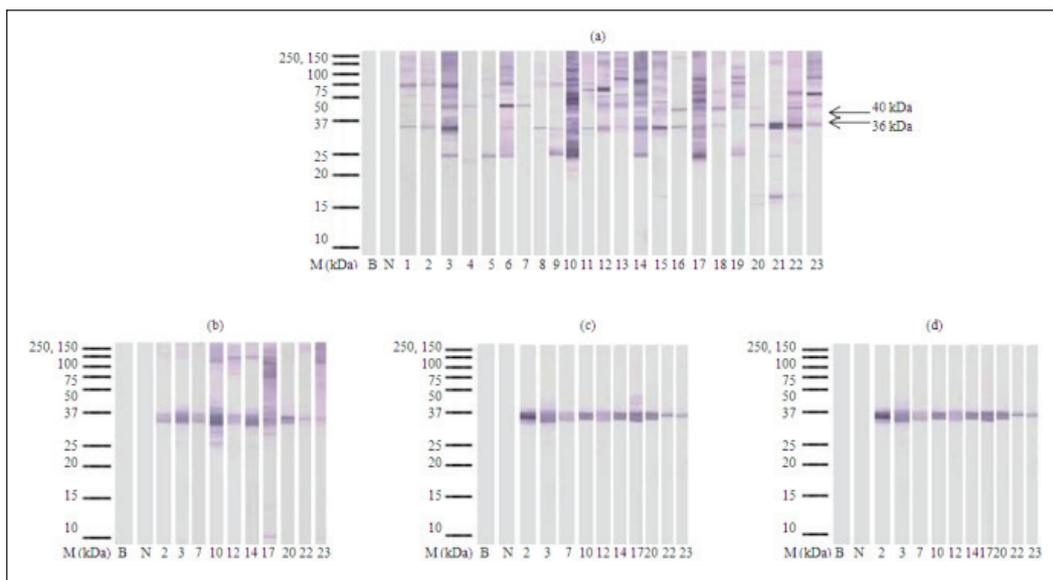


Figure 2. Immunoblotting of raw (a), boiled (b), fried (c) and roasted (d) extracts of *Anadara granosa* (Malaysian cockle). Lane M, molecular mass markers; lanes 1-23, immunoblots showing binding of IgE from different serum samples; lane N, immunoblot using serum from a non-allergic individual; and lane B, blank.

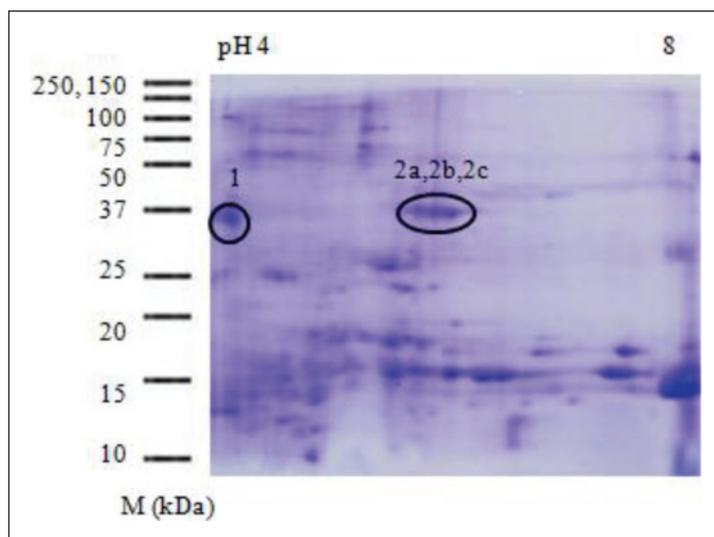


Figure 3. 2-DE profile of raw Malaysian cockle (*Anadara granosa*) protein extract. Lane M, molecular mass markers.

not bind any IgE. This is an aspect to be considered in the process of allergy diagnosis, as well as of the validation of real allergens (Mari, 2008). The allergen profile of an allergenic source must include only validated allergens. The IgE-binding proteins

for which a clinical relevance has not been demonstrated cannot be considered as components of the allergen profile of an allergenic source (Ciardiello *et al.*, 2013). Therefore, the identification of allergens is of the utmost importance because it is

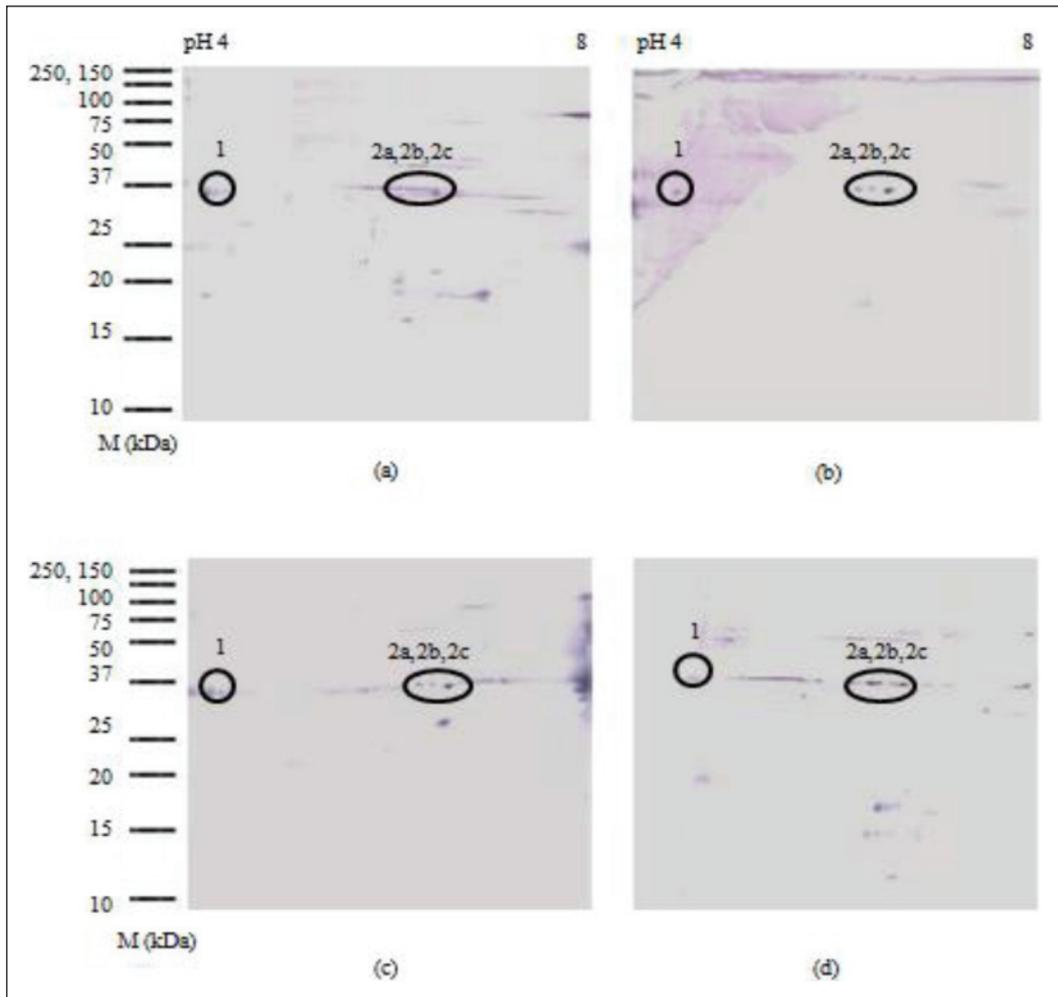


Figure 4. 2-DE immunoblotting of raw Malaysian cockle (*Anadara granosa*) protein extract. Lane M, molecular mass markers. The circle shows the spots analyzed by mass spectrometry.

Table 1. Identities of major protein spots of *A.granosa* identified by mass spectrometry analysis

Spot	MW (kDa) and pI of matched proteins: Observed/predicted	Protein Identification	Organism	Accession No.	Residue numbers of matched regions	Coverage of protein sequence
1	36/32.5 kDa, 4.6/4.57	Tropomyosin	<i>Tegillarca granosa</i>	G8XWU2	16-28, 77-90, 106,125, 134-149, 168-182, 190-226	40%
2a	40/39.3 kDa, 6.1/6.29	Arginine Kinase	<i>Anadara broughtonii</i>	Q760P7	73-85, 274-282, 302-320	11%
2b	40/39.3 kDa, 6.2/6.29	Arginine Kinase	<i>Anadara broughtonii</i>	Q760P7	73-85, 99-116, 302-320	14%
2c	40/39.3 kDa, 6.3/6.29	Arginine Kinase	<i>Anadara broughtonii</i>	Q760P7	73-85, 99-116	8%

essential for the understanding of the specific IgE mediated immune response. Moreover, their identification would aid in reliable diagnostic tests and management of patients (Woo & Bahna, 2011; Zuidmeer-Jongejan *et al.*, 2012; Ciardiello *et al.*, 2013).

‘Major allergens’ mean proteins that elicit IgE binding in the majority (more than 50%) of patients; while those, which elicit allergic reactions only in a minor fraction (less than 50%) of patients allergic to the specific source, are regarded as ‘minor allergens’ (Bangping *et al.*, 2011). According to this definition, the 36 kDa protein could potentially be the major allergen of cockle while other proteins which had specific IgE binding capacity at less than 50% could be considered as minor allergens. Aside from 36 kDa, an IgE-binding protein with a molecular mass of 40 kDa was also noted as a major allergen of cockle.

Allergenic proteins normally contain numerous and structurally identical or different IgE-binding sites. After food has been processed, it might induce changes in the structure and quantity of those sites, which alters IgE binding (Sathe *et al.*, 2005; Chatterjee *et al.*, 2006; Yu *et al.*, 2011; Abramovitch *et al.*, 2013). Our result revealed modification of the antigenic profile of cockle after heat treatment. The most prominent major protein band in heated extracts corresponded to a protein of about 36 kDa and demonstrated markedly enhanced IgE binding intensity. High temperatures may enhance allergenicity of this 36 kDa protein as a result of the alteration of IgE epitopes by unmasking the epitopes with more surfaces accessible to bind the IgE antibody more effectively over that of the 36 kDa protein of raw extract (Sathe *et al.*, 2005; Nowak-Wegrzyn & Fiocchi, 2009; Liu *et al.*, 2010). In boiled extract, smeared IgE-reactive proteins of ~100-150 kDa were also recognized by some of the patients’ sera. Similar results have been previously reported for the other bloody cockle *Anadara broughtonii* by Emoto *et al.* (2009).

Immunoblots of the heated extracts demonstrated that there was an absence of the 40 kDa IgE-reactive protein. In contrast, this 40 kDa major protein and several

additional minor proteins were detected only in the raw extract. Unlike the 36 kDa protein, the disappearance of the 40 kDa protein in the heated extracts suggested that it was heat-labile, denaturing to an insoluble protein upon heating. The disappearance of this 40 kDa might be also due to heating causing fragmentation to small peptides not observed on the gel. Heat treatment may alter cockle extracts by masking the allergenic epitopes thereby reducing allergen recognition and therefore potentially reducing allergenicity (Sathe *et al.*, 2005; Nowak-Wegrzyn & Fiocchi, 2009; Liu *et al.*, 2010).

The 1-DE and 2-DE immunoblots obtained with different sera showed a remarkable heterogeneity in recognized cockle allergens. Such variation in IgE reactivity may be due to different exposures to the allergenic proteins and/or different genetic background of the patients (Sander *et al.*, 2001; Kukreja *et al.*, 2008). It was also reported that the difference in IgE-binding pattern may reflect different symptom patterns of allergy (Gill *et al.*, 2009).

Mass spectrometry analysis of the peptide fragments isolated from the digested spot of 36 kDa was similar to tropomyosin. Consistent with this finding, Emoto *et al.* (2009) also identified tropomyosins as major allergens from bloody cockle *Anadara broughtonii* and the Japanese cockle *Fulvia mutica*. Tropomyosin with a molecular weight about 34 to 38 kDa, is a group of highly conserved actin-binding proteins present in muscle and non-muscle cells and plays a central role in muscle contraction (Thammathongchat *et al.*, 2010; Suzuki *et al.*, 2011; Lopata & Kamath, 2012). Our findings confirmed and strengthened earlier reports on the heat stability of tropomyosin. After heat treatment, only tropomyosin exhibited the IgE-binding capacity. Tropomyosin is heat resistant and retains its IgE-binding ability even after prolonged heating (Leung *et al.*, 1998; Motoyama *et al.*, 2007; Kamath *et al.*, 2013). The ability of tropomyosin to withstand heat-treatment and most known type of food processing techniques can be attributed to its exceptionally stable alpha helical coiled-coil secondary structure (Reese *et al.*, 1999; Kamath *et al.*, 2013).

In addition to tropomyosin, the 40 kDa protein showed a significant homology with an enzyme, arginine kinase. In this study, arginine kinase is the second major allergen of cockle, beside tropomyosin. Arginine kinase, which is involved in cell metabolism of invertebrates, is a major invertebrate phosphagen kinase that plays an important role in regenerating ATP during bursts of cellular activity (Abdel Rahman *et al.*, 2011; Shen *et al.*, 2011; Rosmilah *et al.*, 2012b; Chen *et al.*, 2013). Prior to this study, arginine kinase has already been established to be one of the allergens in crustacean and some invertebrates such as shrimps or prawns (Garcia-Orozco *et al.*, 2007; Ortea *et al.*, 2009; Yadzir *et al.*, 2012a), crayfish (Chen *et al.*, 2013), crab (Abdel Rahman *et al.*, 2011; Shen *et al.*, 2011; Rosmilah *et al.*, 2012a; Rosmilah *et al.*, 2012b), moth (Binder *et al.*, 2001), house dust mite (Hales *et al.*, 2007), cockroach (Sookrung *et al.*, 2006; Tungtrongchitr *et al.*, 2009) and spider (Bobolea *et al.*, 2011). However, arginine kinase is obviously less IgE reactive and also less thermostable than tropomyosin, suggesting that arginine kinase contributes less to the adverse reactions induced after ingestion of cockle, especially cooked cockle, than tropomyosin.

In conclusion, thermal treatment can be used as a tool to reduce cockle allergenicity by reducing the number of IgE-reactive bands. We also showed that proteomics provides a powerful tool for the identification of allergenic proteins. Previously, allergens other than tropomyosin has not been identified in cockle. Our study is the first to report arginine kinase as the second major allergen of cockle. Thus, allergens found in this study would help in component-based diagnosis, management of cockle allergic patients and to the standardisation of allergenic test products as tools in molecular allergology.

Acknowledgements. The authors would like to thank the Director General of the Ministry of Health of Malaysia (MOH) for permission to publish this paper.

REFERENCES

- Abdel Rahman, A.M., Kamath, S.D., Lopata, A.L., Robinson, J.J. & Helleur, R.J. (2011). Biomolecular characterization of allergenic proteins in snow crab (*Chionoecetes opilio*) and de novo sequencing of the second allergen arginine kinase using tandem mass spectrometry. *Journal of Proteomics* **74**(2): 231-241.
- Abdel Rahman, A.M., Lopata, A.L., O'Hehir, R.E., Robinson, J.J., Banoub, J.H. & Helleur, R.J. (2010). Characterization and de novo sequencing of snow crab tropomyosin enzymatic peptides by both electrospray ionization and matrix-assisted laser desorption ionization QToF tandem mass spectrometry. *Journal of Mass Spectrometry* **45**(4): 372-381.
- Abramovitch, J.B., Kamath, S., Varese, N., Zubrinich, C., Lopata, A.L., O'Hehir R.E. & Rolland, J.M. (2013). IgE reactivity of blue swimmer crab (*Portunus pelagicus*) Tropomyosin, Por p 1, and other allergens; cross-reactivity with black tiger prawn and effects of heating. *PLoS One* **8**(6): e67487.
- Ayuso, R., Reese, G., Leong-Kee, S., Plante, M. & Lehrer, S.B. (2002). Molecular basis of arthropod cross-reactivity: IgE-binding cross-reactive epitopes of shrimp, house dust mite and cockroach tropomyosins. *International Archives of Allergy and Immunology* **129**(1): 38-48.
- Bangping, W., Zhenxing, L., Lina, Z., Yixuan, L. & Hong, L. (2011). Identification and characterization of a new IgE-binding protein in mackerel (*Scomber japonicus*) by MALDI-TOF-MS. *Journal of Ocean University of China* **10**(1): 93-98.
- Binder, M., Mahler, V., Hayek, B., Sperr, W.R., Scholler, M., Prozell, S., Wiedermann, G., Valent, P., Valenta, R. & Duchene, M. (2001). Molecular and immunological characterization of arginine kinase from the Indianmeal moth, *Plodia interpunctella*, a novel cross-reactive invertebrate pan-allergen. *Journal of Immunology* **167**: 5470-5477.

- Bobolea, I., Barranco, P., Pastor-Vargas, C., Iraola, V. & Quirce, S. (2011). Arginine kinase from a cellar spider (*Holocnemus pluchei*): a new asthma-causing allergen. *International Archives of Allergy and Immunology* **155**(2): 180-186.
- Chatterjee, U., Mondal, G., Chakraborti, P., Patra, H.K. & Chatterjee, B.P. (2006). Changes in the allergenicity during different preparations of pomfret, hilsa, bhetki and mackerel fish as illustrated by enzyme-linked immunosorbent assay and immunoblotting. *International Archives of Allergy and Immunology* **141**: 1-10.
- Chen, H., Mao, H., Cao, M., Cai, Q., Su, W., Zhang, Y. & Liu, G. (2013). Purification, physicochemical and immunological characterization of arginine kinase, an allergen of crayfish (*Procambarus clarkii*). *Food and Chemical Toxicology* **62**: 475-484.
- Ciardello, M.A., Tamburrini, M., Liso, M., Crescenzo, R., Rifaiani, C. & Mari, A. (2013). Food allergen profiling: A big challenge. *Food Research International* **54**: 1033-1041.
- Davis, P.J. & Williams, S.C. (1998). Protein modification by thermal processing. *Allergy* **53**(46): 102-105.
- Department of Fisheries (DOF) Malaysia, 2014. Available from: URL: <http://www.dof.gov.my> [2 March 2014]
- Emoto, A., Ishizaki, S. & Shiomi, K. (2009). Tropomyosins in gastropods and bivalves: Identification as major allergens and amino acid sequence features. *Food Chemistry* **114**(2): 634-641.
- Garcia-Orozco, K.D., Aispuro-Hernandez, E., Yepiz-Plascencia, G., Calderon-de-la-Barca & Sotelo-Mundo, R.R. (2007). Molecular characterization of arginine kinase, an allergen from shrimp *Litopenaeus vannamei*. *International Archives of Allergy and Immunology* **144**: 23-28.
- Gill, B.V., Rice, T.R., Cartier, A., Gautrin, D., Neis, B., Horth-Susin, L., Jong, M., Swanson, M. & Lehrer, S.B. (2009). Identification of crab proteins that elicit IgE reactivity in snow crab-processing workers. *Journal of Allergy and Clinical Immunology* **124**: 1055-1061.
- Hales, B.J., Laing, I.A., Pearce, L.J., Hazell, L.A., Mills, K.L., Chua, K.Y., Thornton, R.B., Richmond, P., Musk, A.W., James, A.L., Lesouef, P.N. & Thomas, W.R. (2007). Distinctive immunoglobulin E anti-house dust allergen-binding specificities in a tropical Australian Aboriginal community. *Clinical Experimental of Allergy* **37**(9): 1357-1363.
- Kamath, S.D., Abdel Rahman, A.M., Komoda, T. & Lopata, A.L. (2013). Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies. *Food Chemistry* **141**: 4031-4039.
- Kukreja, N., Singh, B.P., Arora, N., Gaur, S.N. & Sridhara, S. (2008). Identification of *Epicoccum purpurascens* allergen by two-dimensional immunoblotting and mass spectrometry. *Immunobiology* **213**: 67-73.
- Leung, P.S.C., Chen, Y.C., Gershwin, M.E., Wong, S.H., Kwan, S.H. & Chu, K.H. (1998). Identification and molecular characterization of *Charybdis feriatus* tropomyosin, the major crab allergen. *Journal of Allergy and Clinical Immunology* **102**: 847-852.
- Leung, P.S., Chow, W.K., Duffey, S., Kwan, H.S., Gershwin, M.E. & Chu, K.H. (1996). IgE reactivity against a cross-reactive allergen in Crustacea and Mollusca: Evidence for tropomyosin as the common allergen. *Journal of Allergy and Clinical Immunology* **98**(5 Pt 1): 954-961.
- Liu, G.M., Cheng, H., Nesbit, J.B., Su, W.J., Cao, M.J. & Maleki, S.J. (2010). Effects of boiling on the IgE-binding properties of tropomyosin of shrimp (*Litopenaeus vannamei*). *Journal of Food Science* **75**(1): T1-5.

- Lopata, A.L. & Kamath, S. (2012). Shellfish allergy diagnosis- gaps and needs. *Current Allergy and Clinical Immunology* **25**(2): 60-66.
- Lopata, A.L. & Jeebhay, M.F. (2013). Airborne seafood allergens as a cause of occupational allergy and asthma. *Current Allergy and Asthma Reports* **13**(3): 288-297.
- Lopata, A.L., O'Hehir, R.E. & Lehrer, S.B. (2010). Shellfish allergy. *Clinical and Experimental Allergy* **40**(6): 850-858.
- Mari, A. (2008). When does a protein become an allergen? Searching for a dynamic definition based on most advanced technology tools. *Clinical and Experimental Allergy* **38**(7): 1089-1094.
- Motoyama, K., Suma, Y., Ishizaki, S., Nagashima, Y. & Shiomi, K. (2007). Molecular cloning of tropomyosin identified as allergens in six species of crustaceans. *Journal of Agricultural and Food Chemistry* **55**: 985-991.
- Nowak-Wegrzyn, A. & Fiocchi, A. (2009). Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. *Current Opinion in Allergy and Clinical Immunology* **9**: 234-237.
- Ortea, I., Cañas, B. & Gallardo, J.M. (2009). Mass spectrometry characterization of species-specific peptides from arginine kinase for the identification of commercially relevant shrimp species. *Journal of Proteome Research* **8**: 5356-62.
- Reese, G., Ayuso, R. & Lehrer, S.B. (1999). Tropomyosin: an invertebrate pan-allergen. *International Archives of Allergy and Immunology* **119**(4): 247-258.
- Rosmilah, M., Shahnaz, M., Zailatul, H.M.Y., Noormalin, A. & Normilah, I. (2012a). Identification of tropomyosin and arginine kinase as major allergens of *Portunus pelagicus* (blue swimming crab). *Tropical Biomedicine* **29**(3): 467-478.
- Rosmilah, M., Shahnaz, M., Zailatul, H.M.Y. & Noormalin, A. (2012b). Identification of the major allergens of *Charybdis feriatus* (red crab) and its cross-reactivity with *Portunus pelagicus* (blue crab). *Asian Pacific Journal of Allergy and Immunology* **30**: 285-293.
- Samson, K.T.R., Chen, F.H., Miura, K., Odajima, Y., Iikura, Y., Rivas, M.N., Minoguchi, K. & Adachi, M. (2004). IgE binding to raw and boiled shrimp proteins in atopic and non-atopic patients with adverse reactions to shrimp. *International Archives of Allergy and Immunology* **133**: 225-232.
- Sander, I., Flagge, A., Merget, R., Halder, T.M., Meyer, H.E. & Baur, X. (2001). Identification of wheat flour allergens by means of 2-dimensional immunoblotting. *Journal of Allergy and Clinical Immunology* **107**(5): 907-913.
- Sathe, S.K., Teuber, S.S. & Roux, K.H. (2005). Effects of food processing on the stability of food allergens. *Biotechnology Advances* **23**: 423-429.
- Shahnaz, M., Gendeh, B.S. & Nasuruddin, A. (2001). Skin test reactivity to inhalant and food allergens in patients with allergic rhinitis. *International Medical Research Journal* **5**(2): 69-73.
- Shen, Y., Cao, M.J., Cai, Q.F., Su, W.J., Yu, H.L., Ruan, W.W. & Liu, G.M. (2011). Purification, cloning, expression and immunological analysis of *Scylla serrata* arginine kinase, the crab allergen. *Journal of Science and Food Agriculture* **91**(7): 1326-1335.
- Sookrung, N., Chaicumpa, W., Tungrongchitr, A., Vichyanond, P., Bunnag, C., Ramasoota, P., Tongtawe, P., Sakolvaree, Y. & Tapchairsri, P. (2006). *Periplaneta americana* arginine kinase as a major cockroach allergen among Thai patients with major cockroach allergies. *Environmental Health Perspectives* **114**: 875-80.

- Suzuki, M., Kobayashi, Y., Hiraki, Y., Nakata, H. & Shiomi, K. (2011). Paramyosin of the disc abalone *Haliotis discus discus*: Identification as a new allergen and cross-reactivity with tropomyosin. *Food Chemistry* **124**: 921-926.
- Thammathongchat, S., Hagiwara, T. & Sakiyama, T. (2010). Adsorption of tropomyosin from pink shrimp (*Pandalus eosus*) on stainless steel surface. *Food Control* **21**(9): 1250-1253.
- Tungtrongchitr, A., Sookrung, N., Indrawattana, N., Sae-Lim, J., Punduang, S., Phonrat, B., Wanachiwanawin, D. & Chaicumpa, W. (2009). Seasonal levels of the major American cockroach allergen *Per a 9* (arginine kinase) in Bangkok and their relevance for disease severity. *Asian Pacific Journal of Allergy and Immunology* **27**: 1-7.
- Woo, C.K. & Bahna, S.L. (2011). Not all shellfish "allergy" is allergy!. *Clinical and Translational Allergy* **1**: 3.
- Yadzir, Z.H.M., Misnan, R., Abdullah, N., Bakhtiar, F., Arip, M. & Murad, S. (2012a). Identification of the major allergen of *Macrobrachium rosenbergii* (giant freshwater prawn). *Asian Pacific Journal of Tropical Biomedicine* **2**(1): 50-54.
- Yadzir, Z.H.M., Misnan, R. & Murad, S. (2012b). Identification of tropomyosin as major allergen of white squid (*Loligo edulis*) by two-dimensional immunoblotting and mass spectrometry. *Southeast Asian Journal of Tropical Medicine and Public Health* **43**(1): 185-191.
- Yu, H.L., Cao, M.J., Cai, Q.F., Weng, W.Y., Su, W.J. & Liu, G.M. (2011). Effects of different processing methods on digestibility of *Scylla paramamosain* allergen (tropomyosin). *Food and Chemical Toxicology* **49**: 791-798.
- Zuidmeer-Jongejan, L., Fernandez-Rivas, M., Poulsen, L., Neubauer, A., Asturias, J., Blom, L., Boye, J., Bindslev-Jensen, C., Clausen, M., Ferrara, R., Garosi, P., Huber, H., Jensen, B.M., Koppelman, S., Kowalski, M.L., Lewandowska-Polak, A., Linhart, B., Maillere, B., Mari, A., Martinez, A., Mills, C.E., Nicoletti, C., Opstelten, D.J., Papadopoulos, N.G., Portoles, A., Rigby, N., Scala, E., Schnoor, H.J., Sigurdardottir, S.T., Stavroulakis, G., Stolz, F., Swoboda, I., Valenta, R., van den Hout, R., Versteeg, S.A., Witten, M., van Ree, R. (2012). FAST: Towards safe and effective subcutaneous immunotherapy of persistent life-threatening food allergies. *Clinical and Translational Allergy* **2**(1): 5.