



# Identification of novel allergen in edible insect, *Gryllus bimaculatus* and its cross-reactivity with *Macrobrachium* spp. allergens



Chutima Srinroch<sup>a,b</sup>, Chantragan Srisomsap<sup>c</sup>, Daranee Chokchaichamnankit<sup>c</sup>, Phaibul Punyarit<sup>d,e</sup>, Pharima Phiriyangkul<sup>a,b,\*</sup>

<sup>a</sup> Division of Biochemistry, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng-Saen Campus, Nakhon-Pathom 73140, Thailand

<sup>b</sup> Bioproducts Science, Department of Science, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng-Saen Campus, Nakhon-Pathom 73140, Thailand

<sup>c</sup> Laboratory of Biochemistry, Chulabhorn Research Institute, Bangkok 10210, Thailand

<sup>d</sup> Army Institute of Pathology, Medical Center and Government Building, Thanon Phaya Thai, Bangkok 10400

<sup>e</sup> Department of Pathology, Phramongkutklao College of Medicine, Bangkok 10400, Thailand

## ARTICLE INFO

### Article history:

Received 11 September 2014

Received in revised form 23 February 2015

Accepted 19 March 2015

Available online 28 March 2015

### Keywords:

*Macrobrachium rosenbergii*

Hepatopancreas

*Macrobrachium lanchesteri*

*Gryllus bimaculatus*

Edible insect

Allergen

IgE-immunoblotting

SDS–PAGE

LC–MS/MS

## ABSTRACT

Edible insects have recently been promoted as a source of protein and have a high nutrition value. Identification of allergens and cross-reactivity between *Macrobrachium* spp. and the field cricket (*Gryllus bimaculatus*) is necessary for food safety control and to assist in the diagnosis and therapy of allergy symptoms. Denaturing polyacrylamide gel electrophoresis (SDS–PAGE) was used to separate proteins. Allergens were determined and identified by IgE-immunoblotting with pooled sera from prawn-allergic patients ( $n = 16$ ) and LC–MS/MS. Arginine kinase (AK) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined as the important allergens in muscle of *Macrobrachium rosenbergii* whereas, hemocyanin (HC) was identified as an allergen in *Macrobrachium* spp. The allergens in *Macrobrachium lanchesteri* were identified as AK and HC. In addition, hexamerin1B (HEX1B) was identified as a novel and specific allergen in *G. bimaculatus*. The important allergen in *G. bimaculatus* and *Macrobrachium* spp. is AK and was found to cross-react between both species.

© 2015 Elsevier Ltd. All rights reserved.

## 1. Introductions

Food hypersensitivity type I is an IgE-mediated food allergy, that accounts for the majority of food allergic reactions, and is characterized by the presence of antigen-specific serum IgE antibodies. Food allergy symptoms vary depending on the age of the subjects, the allergen involved and the amount of food eaten, among other symptoms. The symptoms of food allergy may include urticaria, angioedema, laryngoedema, diarrhea, nausea, vomiting, bronchospasm or hypotension (Nauta et al., 2008). Edible crustaceans mostly belong to the class Malacostraca order Decapoda, and include shrimps, prawn, crawfish, lobster and crab, which are widely consumed as delicious foods around the world. However, they are simultaneously recognized as one of the most common causes of IgE-mediated food allergy from eight allergenic foods (Food and Agriculture Organization of the United Nations,

2002; Suma et al., 2007). The dominant freshwater prawn family in Southeast Asia is Palaemonidae, of which *Macrobrachium* spp. is the principal genus (Shuhaimi-Othman, Yakub, Ramle, & Abas, 2011). Giant freshwater prawns (*Macrobrachium rosenbergii*) are found extensively in Southeast Asia and are grown in aquaculture in tropical and subtropical areas of the world, such as Thailand, India, Sri Lanka, Malaysia, the Philippines and Vietnam (Davassi, 2011). Culturing of *M. rosenbergii* has developed in every continent, particularly in Asia and the Americas. Global production has increased to over 200,000 tonnes per year (Food and Agriculture Organization of the United Nations, 2002). Many recipes around the world involve cooking prawns with the head intact to preserve the natural juices and enhance the delicate flavor from the hepatopancreas which is located in this section. The hepatopancreas is a popular ingredient for cooking and is exported in canned food products under the name shrimp fat in oil paste. The whole body of the prawn, including muscle and the hepatopancreas, has been widely used for cooking in Asia in recipes including fried rice with shrimp fat in oil paste, batter-fried prawns and grilled prawns. *M. rosenbergii* has been increasingly recognized in the world-market

\* Corresponding author at: Division of Biochemistry, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng-Saen Campus, Nakhon-Pathom 73140, Thailand. Tel.: +66 827265078.

E-mail address: [faasprm@ku.ac.th](mailto:faasprm@ku.ac.th) (P. Phiriyangkul).

due to its texture and palatability. Lanchester's freshwater prawn (*Macrobrachium lanchesteri*) is a small freshwater prawn commonly found in the vicinity of agricultural areas and widely consumed as food throughout Southeast Asia (Tongbai & Damrongphol, 2011).

Moreover, insects have a high nutritional value and are a healthy food source with high levels of protein, vitamins, minerals and fat content that vary over the wide range of edible insect species (Belluco et al., 2013). Many species of insect have served as traditional foods among indigenous people, especially in warmer climates, and have played an important role in the history of human nutrition in Africa, Asia and Latin America. Insects generally comprise 80% of the edible invertebrates in northeastern Thailand (Food and Agriculture Organization of the United Nations, 2010). *Gryllus bimaculatus*, the field cricket, is commonly consumed as a food around Asia. As of 2012, there were about 20,000 cricket farmers in Thailand, and this is of interest from an economic standpoint because this species has an energy content of approximately 120 kcal per 100 g of fresh weight of the whole insect (Food and Agriculture Organization of the United Nations, 2013). There have been two reports of allergic symptoms, such as anaphylactic shock and an itchy sensation in the mouth and face after consuming edible insects, silkworm pupa (*Bombyx mori*) (Ji, Zhan, Chen, & Liu, 2008) and mopane worm (*Gonimbrasia belina*) (Okezie, Kgomotso, & Letswiti, 2010). Arginine kinase (AK), with a molecular mass of 40 kDa, has been reported as the allergen in shellfish—black tiger prawn (*Penaeus monodon*) (Yu, Lin, Chiang, & Chow, 2003), Pacific white shrimp (*Litopenaeus vannamei*) (García-Orozco, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, & Sotelo-Mundo, 2007), *M. rosenbergii* (Yadzir et al., 2012) and in indoor insect species—moth (*Plodia interpunctella*) (Binder et al., 2001), American cockroach (*Periplaneta americana*) (Sookrung et al., 2006) and German cockroach (*Blattella germanica*) (Chuang, Su, Chiang, Lee, & Chow, 2010). Liu et al. (2009) identified AK in silkworm (*B. mori*) as a food allergen. The major allergen in crustaceans is 35 to 38 kDa tropomyosin (TM) present in Indian white prawn (*Penaeus indicus*) (Shanti, Martin, Nagpal, Metcalfe, & Rao, 1993), spiny lobster (*Panulirus stimpsoni*) (Leung et al., 1998), brown shrimp (*Penaeus aztecus*) (Daul, Slattey, Reese, & Lehrer, 1994), Pacific white shrimp (Liu et al., 2011) and black tiger prawn (Abdel Rahman, Kamath, Lopata, & Helleur, 2010).

The aim of this study was to identify allergens and to investigate the cross-reactivity of allergens in *G. bimaculatus*, *M. lanchesteri* and organ-specific allergens in muscle and the hepatopancreas via proteomic procedures using *M. rosenbergii*. Proteins were identified by liquid chromatography–two-dimensional mass spectrometry (LC–MS/MS).

## 2. Materials and methods

### 2.1. Prawn allergic patients

The sera used in this study were allergen-specific to prawns and were obtained from 16 prawn-allergic patients at Phramongkutklao Hospital and Phayathai Hospital (Bangkok, Thailand). Most of the patients presented with urticarial dermatitis and/or angioedema with one case of developed asthma. The 16 patients consisted of 12 males and 4 females, with an age range from 4 to 41 years, and they had been subjected to a quantitative serum IgE level and/or skin test to food allergens. The use of sera were approved by the Institutional Review Board at Phramongkutklao Hospital (R101h/2009). The sera from allergic patients was confirmed with levels of IgE greater than 0.35 kU/L using EUROLINE inhalation Southeast Asian test strips (EUROIMMUN AG; Lübeck, Germany) and was used as positive sera. The quantitative IgE levels of all 16

patients ranged from 0.47 to 46.00 kU/L. Control sera were obtained from 30 healthy subjects without reported adverse reactions to prawns. After confirmation of the IgE levels for all 16 patients, the sera from allergic patients were pooled and frozen at  $-80^{\circ}\text{C}$  until use.

### 2.2. Sample preparation

Adult *M. rosenbergii*, each weighing approximately 30 to 50 g and *M. lanchesteri*, with a pooled weight of approximately 40 g, were purchased from a local market in Nakhon-Pathom, Thailand. Muscle and the hepatopancreas of *M. rosenbergii* were obtained after cold shock and removal of the heads and shells. Whole bodies of live *M. lanchesteri* and *G. bimaculatus* were pooled and immediately frozen in liquid nitrogen. All samples were frozen at  $-80^{\circ}\text{C}$  until use.

### 2.3. Sample extracts

Samples were homogenized with liquid nitrogen in a pestle and mortar until reduced to a powder. 300  $\mu\text{g}$  of each sample powder was added to 600  $\mu\text{l}$  of phosphate buffer saline (PBS; 9 mM  $\text{Na}_2\text{HPO}_4$ , 1.13 mM  $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$  and 0.152 M NaCl, pH 7.4) with a protease inhibitor cocktail (Sigma–Aldrich; MO, USA) and incubated for 30 min at  $4^{\circ}\text{C}$ . The homogenates were centrifuged twice at  $16,215\times g$  for 30 min and the supernatant was collected. Protein concentrations were determined using UV–VIS spectrophotometry (UV 1700; Shimadzu; Kyoto, Japan) with the Bio-Rad protein assay (Bio-Rad; CA, USA). Bovine serum albumin was used as a standard.

### 2.4. SDS–PAGE and immunoblotting with IgE

A protein sample with a concentration of 30  $\mu\text{g}$  was loaded onto a 12.5% separating gel with a 4% stacking gel using a Mini-PROTEAN Tetra system (Bio-Rad; CA, USA). The protein standard for SDS–PAGE was a PageRuler™ prestained protein ladder (Thermo Fisher Scientific; MA, USA). All proteins were separated in triplicate by SDS–PAGE. After electrophoresis, the separated proteins on gels were either stained with Coomassie Brilliant Blue R-250 (CBB) or transferred onto Immobilon-P transfer membranes (Millipore Corporation; MA, USA) with a pore size of 0.45  $\mu\text{m}$ . A constant voltage gradient of 100 V was applied for 1.5 h at  $4^{\circ}\text{C}$ . The blotted membranes were then either stained directly with CBB or blocked with Block Ace (Dainippon Sumitomo Pharma; Osaka, Japan) and incubated with a 1:25 dilution of pooled serum from the 16 prawn-allergic patients in 10% skimmed milk for 16 h at  $4^{\circ}\text{C}$ . Horseradish peroxidase (HRP) conjugated goat anti-human IgE with peroxidase (Immunology consultants Laboratory Inc, OR, USA) was used as a secondary antibody for detection of IgE binding with a dilution of 1:5000 in 3% skimmed milk. Incubated membranes were exposed to Amersham ECL Plus™ western blotting detection reagents (GE Healthcare Bio-Sciences AB; Uppsala, Sweden). The membrane was exposed using Amersham® Hyperfilm® ECL™ (GE Healthcare Bio-Sciences AB; Uppsala, Sweden) in a dark room and immunoreactive bands were visualized. This process used enhanced chemiluminescence (ECL) as a substrate and HRP as an enzyme. The reaction presented at 428 nm under low-intensity light. The approximate molecular weight of the allergens identified on X-ray film was estimated using standard molecular weight markers.

### 2.5. Protein image analysis

Images of protein-stained gel and immunoreactive bands from films were scanned using an image scanner (ImageScannerIII; GE Healthcare Bio-Sciences AB; Uppsala, Sweden) and an image

scanner program (Labscan 5.0.0.0: GE Healthcare Bio-Sciences AB; Uppsala, Sweden). The analyzed images of protein gels and films were processed using ImageQuant TL Plus 7.0 software (GE Healthcare Bio-Sciences AB; Uppsala, Sweden). The volume was determined as the sum of the intensities of every pixel in the band which could be thought of as a three-dimensional band, where intensity was the third dimension. The raw volume of the band represented the uncalibrated quantity of material in the band, after the background intensity had been removed. The band volumes were detected and calculated from immunoreactive bands and the resultant data were shown in an interleaved bar graph with mean  $\pm$  SE using GraphPad Prism 5 (GraphPad Software, Inc; CA, USA) to compare the allergenicity of IgE binding proteins between samples.

## 2.6. Tryptic in-gel digestion

Bands of protein of interest were excised from SDS–PAGE, washed twice with 50  $\mu$ l of 0.1 M  $\text{NH}_4\text{HCO}_3$  in 50% (v/v) acetonitrile (ACN) for 20 min at 30 °C to remove the CBB. After washing, the solvent was removed and the gel pieces were dried in a speed vacuum (CentriVap Console: Labconco; MO, USA), followed by swelling the gel pieces in 50  $\mu$ l buffer solution (0.1 M  $\text{NH}_4\text{HCO}_3$ , 10 mM DTT, and 1 mM EDTA) and incubating at 60 °C for 45 min using a thermomixer (R Mixer: Eppendorf; Hamburg, Germany). The excess liquid was removed and quickly replaced by the same volume of freshly prepared 100 mM iodoacetamide (IAA) in 0.1 M  $\text{NH}_4\text{HCO}_3$  solution and incubated at room temperature in the dark for 30 min. The solvent was removed and washed with 50% (v/v) ACN in water, 3 times for 10 min, and completely dried in a speed vacuum. The reaction mixture solvent; 50  $\mu$ l aliquot of digestion buffer (0.05 M TrisHCl, 10% ACN, 1 mM  $\text{CaCl}_2$ , pH 8.5) and 1  $\mu$ l aliquot of trypsin (Promega Corporation; Madison WI, USA) (1 mg trypsin in 10  $\mu$ l 1% acetic acid) were added to the gel piece and incubated at 37 °C overnight. The digestion buffer was removed and saved. The gel pieces were then extracted by adding 60  $\mu$ l of 2% freshly prepared trifluoroacetic acid and incubating for 30 min at 60 °C. The extract and the saved digestion buffer were pooled and dried in a speed vacuum and stored at –20 °C until analyzed using LC–MS/MS.

## 2.7. Protein identification by LC–MS/MS

Tandem mass spectrometry (LC–MS/MS) was carried out using a Waters capillary LC system coupled to a Q-TOF mass spectrometer (Micromass; Manchester, UK) equipped with a Zspray ion source working in the nanoelectrospray mode according to [Srisomsap et al. \(2010\)](#). The MS/MS data were extracted and output as searchable .PKL files for independent searches using the Mascot search engine (Matrix Science Ltd.; London, United Kingdom), as this is associated with more protein databases and provides the number of hits for peptides. A mascot MS/MS ions search (<http://www.matrixscience.com>) against, the Mascot search tool available on the Matrix Science site screening NCBI nr was used. [Table 1](#) shows the parameters used for the database search. The proteins were identified at a probability level of  $P < 0.05$  and Mascot scores  $> 35$  were considered as promising hits.

## 3. Results

The electrophoretic separation of prawn protein extracts from *Macrobrachium* spp. and *G. bimaculatus* was determined by SDS–PAGE and staining with CBB, which showed multiple bands ranging from ~10 to ~170 kDa ([Fig. 1](#), lane 1A–4A).

**Table 1**  
Parameters used for database search.

| Parameter                        | Parameter selected                                                                    |
|----------------------------------|---------------------------------------------------------------------------------------|
| Type of search                   | MS/MS ions search                                                                     |
| Databases                        | NCBI nr                                                                               |
| Enzyme                           | Trypsin                                                                               |
| Missed cleavages allowance       | 1                                                                                     |
| Taxonomy                         | All entries                                                                           |
| Fixed modifications              | –                                                                                     |
| Variable modifications           | Carbamidomethyl (C)<br>Oxidation (HW)<br>Oxidation (M)<br>Phospho (ST)<br>Phospho (Y) |
| Peptide mass tolerance MS/MS ion | $\pm 1.2$ Da                                                                          |
| MS/MS tolerance                  | $\pm 0.2$ Da                                                                          |
| Peptide charge state             | 1 <sup>+</sup> , 2 <sup>+</sup> and 3 <sup>+</sup>                                    |
| Mass values                      | Monoisotopic                                                                          |
| Quantitation                     | None                                                                                  |
| Data format                      | Micromass (.PKL)                                                                      |
| Report top                       | Auto hits                                                                             |

The immunoblotting results revealed allergens in the muscle and hepatopancreas of *M. rosenbergii*, as well as in the whole body of *M. lanchesteri* and *G. bimaculatus* from lane 1B to 4B ([Fig. 1](#)), respectively. The results of immunoblotting with pooled sera from the 16 prawn-allergic patients showed that the protein bands had molecular masses ranging from ~32 to ~77 kDa. The identification of allergens by LC–MS/MS analysis is shown in [Table 2](#). These protein bands could be identified as allergens in *Macrobrachium* spp. and *G. bimaculatus*. Allergens in muscle of *M. rosenbergii* ([Fig. 1](#), lane 1B) had a molecular mass of ~32 kDa being glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and of ~40 kDa being arginine kinase (AK), whereas the hepatopancreas proteins had a molecular mass of ~65 kDa ([Fig. 1](#), lane 2B) being hemocyanin (HC). Allergens in *M. lanchesteri* ([Fig. 1](#), lane 3B) were detected as HC (~67 to ~77 kDa) and AK (~40 kDa). Furthermore, 2 allergens were detected in *G. bimaculatus* (Gb) ([Fig. 1](#), lane 4B); ~40 kDa as hexamerin1B (HEX1B) and ~60 kDa as AK. No positive band was detected when using non-allergic pooled sera as shown in lanes 1C to 4C ([Fig. 1](#)).

The results from immunoblotting indicated that pooled allergic-patient sera has different immunological reactions to different species and organs. The band intensity of the IgE-binding protein is shown in [Fig. 2](#) indicating there are 4 allergen proteins—AK, HC, GAPDH and HEX1B—that from band intensities on the graph are allergens of *M. rosenbergii*, *M. lanchesteri* and *G. bimaculatus*. In *M. rosenbergii*, AK was a major allergen and GAPDH was a minor allergen in the muscle, whereas HC was a specific allergen in this organ. In *M. lanchesteri*, HC and AK were identified as allergens. AK was a major and HEX1B was a minor allergen in *G. bimaculatus*.

## 4. Discussion

Prawn is an important food source around the world and a major allergenic food ([Lopata, O'Hehir, & Lehrer, 2010](#)). Insects are considered as future sources of food and feed for humans that can be used as an emergency food source to prevent starvation, and are highly nutritious and naturally abundant. Thus they can be part of a planned diet throughout the year, as well as seasonally, which means insects can be used as a commercial food in local markets and around the world ([Wang, Bai, Li, & Zhang, 2004; Yhoun-Aree, Puwastein, & Attig, 1997](#)). Many of these studies have focused on social, nutritional, and environmental aspects, but few have addressed the safety concerns. In the current study, the IgE reactivity of sera from subjects sensitive to prawns was tested with allergenic proteins in the muscle and the hepatopancreas of *M.*

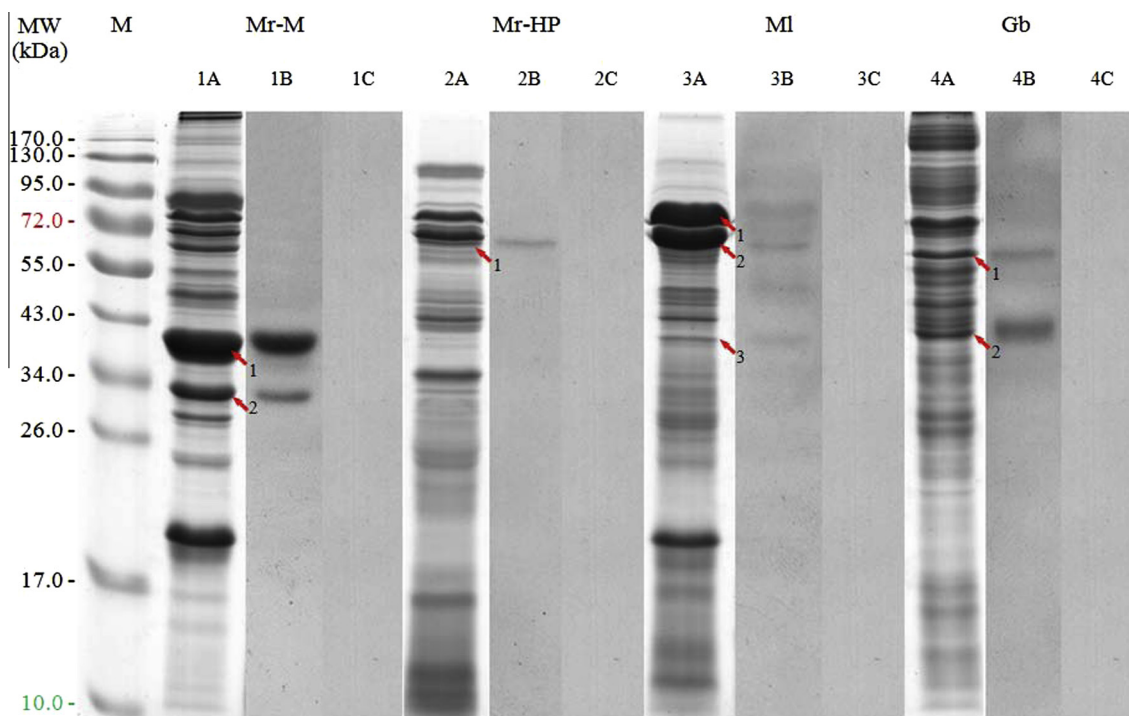


**Fig. 1.** SDS-PAGE pattern and immunoreactive bands from *M. rosenbergii* for muscle protein extracts (Mr-M) and for hepatopancreas protein extracts (Mr-HP), from *M. lanchesteri* for protein extracts (MI) and from *G. bimaculatus* for protein extracts (Gb); lane M, molecular weight; lanes 1A–4A, gel stained of total sample protein by CBB staining; lanes 1B–4B, IgE immunolabeling with pooled sera from 16 patients; lane 1C–4C, labeling with pooled control sera from 30 healthy subjects. The arrow labels represent the IgE-binding proteins identified by LC–MS/MS.

**Table 2**

Identified allergens from fresh muscle and hepatopancreas of *M. rosenbergii*, *M. lanchesteri* and *G. bimaculatus* by LC–MS/MS.<sup>a</sup>

| Band    | Accession number | Protein name/species                                                 | Theoretical |      | LC-MS/MS analysis |                 |           |
|---------|------------------|----------------------------------------------------------------------|-------------|------|-------------------|-----------------|-----------|
|         |                  |                                                                      | MW (kDa)    | pI   | Score             | Peptide matches | %Coverage |
| Mr-M-1  | gi:307715368     | Arginine kinase 1/ <i>Macrobrachium rosenbergii</i>                  | 39.489      | 6.17 | 175               | 7               | 20        |
| Mr-M-2  | gi:31338868      | Glyceraldehyde-3-phosphate dehydrogenase/ <i>Procambarus clarkii</i> | 35.686      | 6.54 | 105               | 4               | 19        |
| Mr-HP-1 | gi:330858322     | Hemocyanin/ <i>Macrobrachium nipponense</i>                          | 76.602      | 5.42 | 210               | 6               | 5         |
| MI-1    | gi:330858322     | Hemocyanin/ <i>Macrobrachium nipponense</i>                          | 76.602      | 5.42 | 41                | 2               | 2         |
| MI-2    | gi:330858322     | Hemocyanin/ <i>Macrobrachium nipponense</i>                          | 76.602      | 5.42 | 205               | 5               | 7         |
| MI-3    | gi:307715368     | Arginine kinase 1/ <i>Macrobrachium rosenbergii</i>                  | 39.489      | 6.17 | 59                | 3               | 9         |
| Gb-1    | gi:283046829     | Hexamerin 1B precursor/ <i>Tribolium castaneum</i>                   | 93.661      | 6.67 | 66                | 2               | 1         |
| Gb-2    | gi:53830710      | Putative arginine kinase/ <i>Oncometopia nigricans</i>               | 39.958      | 5.80 | 361               | 10              | 20        |

<sup>a</sup> Identified protein data are according to assigned band number (Fig. 1).

*rosenbergii*, and in the whole body from *M. lanchesteri* and *G. bimaculatus*. Allergenic proteins were identified by LC–MS/MS as ~40 kDa arginine kinase (AK), ~67 to ~77 kDa hemocyanin (HC), ~32 kDa glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ~70 kDa hexamerin1B (HEX1B). GAPDH and AK were identified as allergens in the muscle of *M. rosenbergii*, whereas only one allergen (HC) was detected in the hepatopancreas. Testing the whole body of *M. lanchesteri* found HC and AK as allergens. In addition, *G. bimaculatus* had two bands of IgE reactivity with the patient sera as allergens (HEX1B and AK). The band volume of IgE binding proteins (Fig. 2) suggested that AK is the major allergen in the muscle of *M. rosenbergii*, and is found in *Macrobrachium* spp. and *G. bimaculatus*. GAPDH is a minor allergen in *M. rosenbergii*, with HC being the important specific organ allergen in the hepatopancreas. In this study, HEX1B was identified as a novel allergen for *G. bimaculatus*. Allergen information from Arthropoda has been searched for, using the Allergen Nomenclature database (<http://www.allergen.org/index.php>), approved by the World Health Organization and International Union of Immunological Societies

(WHO/IUIS) Allergen Nomenclature Sub-committee. Allergens from Arthropoda reported as food are: tropomyosin (TM), myosin light chain, AK, troponin C, troponin I and sarcoplasmic calcium-binding protein. Allergenic proteins characterized in crustaceans and insects are shown in Table 3. Note that our results did not determine tropomyosin as an allergen in *M. rosenbergii*, *M. lanchesteri* and *G. bimaculatus*.

Recently, GAPDH has been reported as a major allergen in banana shrimp (*Fenneropenaeus merguensis*) (Khanaruksombat, Srisomsap, Chokchaichamnankit, Punyari, & Phiriyangkul, 2014) whereas in this study, GAPDH was a minor allergen in the muscle of *M. rosenbergii*. Moreover, GAPDH has been reported as an allergen in indoor mould (*Aspergillus versicolor*) (Benndorf et al., 2008), dimorphic fungus (*Candida albicans*) (Martínez, Gil, López-Ribot, & Chaffin, 1998), common wheat (*Triticum aestivum*) (Sander et al., 2011) and suggested as an important allergen in occupational sensitization to fish (*Sardinops sagax*) (Van der Ventel et al., 2011). GAPDH is a soluble protein that harbors multiple intracellular functions and is considered as a classical glycolytic protein, used

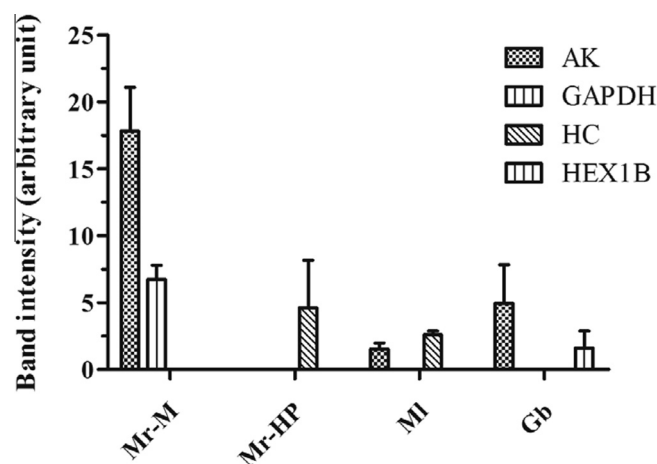


**Fig. 2.** Band intensity of SDS-PAGE/immunoblot binding of IgE from pooled human sera to 4 allergens; arginine kinase (AK), hemocyanin (HC), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hexamerin1B (HEX1B). Graph compares volume ratio of each allergen to protein from muscle (Mr-M) and hepatopancreas (Mr-HP) of *M. rosenbergii*, whole body of *M. lanchesteri* (MI) and whole body of *G. bimaculatus* (Gb).

as a model protein for the analysis of protein structure and enzyme mechanisms (Tarze et al., 2007).

AK has been identified and characterized as an allergen in many species of shrimp, including: black tiger shrimp (*P. monodon*) (Yu et al., 2003), Pacific white shrimp (*L. vannamei*) (García-Orozco, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, & Sotelo-Mundo, 2007), banana shrimp (Khanaruksombat et al., 2014) and *M. rosenbergii* (Yadzir et al., 2012). AK has been identified as an allergen in several kinds of indoor insects including: German cockroach (*B. germanica*) (Chuang et al., 2010), Indian meal moth (*P. interpunctella*) (Binder et al., 2001) and silkworm (*B. mori*) larvae (Liu et al., 2009). AK is a phosphotransferase that plays a critical role in energy metabolism in invertebrates and plays an

important role in the coupling of energy production for muscle mobilization, utilization and the immune response in prawns (Yao, Ji, Kong, Wang, & Xiang, 2009). This corresponds well with our experimental results that AK was detected in the band intensity of an allergen in both *Macrobrachium* spp. and *G. bimaculatus*, especially with high values in muscle protein, whereas it was not detected in the hepatopancreas of *M. rosenbergii*. Our results agreed with Binder et al. (2001) that AK is a novel, cross-reactive, invertebrate pan-allergen.

In this study, HC was shown to be an allergen in the hepatopancreas of *M. rosenbergii* and in the whole body of *M. lanchesteri*, but was not present in the muscle of *M. rosenbergii*. HC was not detected in the muscle of *M. rosenbergii* as an allergen, perhaps because the presence of HC is very rare in this organ as reported by Wang et al. (2012). HC was identified as an allergen in banana shrimp (Khanaruksombat et al., 2014) as well being reported as a novel, non-cross reactive allergen in *M. rosenbergii* (Piboonpocanun, Jirapongsananuruk, Tipayanon, Boonchoo, & Goodman, 2011). HC consists of giant oxygen transport protein complexes with molecular masses of about 75 kDa and is found in the hemolymph of the invertebrates phyla or in arthropod species, being composed of six subunits, or multiples thereof depending on the species (Jaenicke, Pairet, Hartmann, & Decker, 2012; Olianias et al., 2006).

Hexamerin1B precursor (HEX1B) was found as a novel allergen in *G. bimaculatus*. Hexamerins consist of six subunits with a molecular mass of 80 kDa and are found in all insects (Pick, Hagner-Holler, & Burmester, 2008). Hexamerins are mainly considered as storage proteins that support nutrition during non-feeding, resting periods, such as molting, and are abundant in the hemolymph (Telfer & Kunkel, 1991). From an evolutionary viewpoint, hexamerins are derived from HC, with which they share many characteristics in terms of structure. In contrast to the ancestral molecule, they have lost the capacity of Cu-binding histidine residues because hexamerins do not bind with oxygen transport and copper-binding histidine residues have been replaced by other amino acids (Martins, Anhezini, Dallacqua, Simoes, & Bitondi, 2011). In this experiment, HEX1B was suggested as a novel and

**Table 3**  
Allergenic proteins characterized in crustaceans and insects.

| Allergenic protein                       | Functions                 | Molecular weight (kDa) | Species                              | Common name                   | References                       |
|------------------------------------------|---------------------------|------------------------|--------------------------------------|-------------------------------|----------------------------------|
| Arginine kinase                          | Muscle regulator          | ~40                    | (1) <i>Penaeus monodon</i>           | Black tiger shrimp            | (1) Yu et al. (2003)             |
|                                          |                           | ~40                    | (2) <i>Litopenaeus vannamei</i>      | Pacific white shrimp          | (2) García-Orozco et al. (2007)  |
|                                          |                           | ~40                    | (3) <i>Fenneropenaeus merguensis</i> | Banana shrimp                 | (3) Khanaruksombat et al. (2014) |
|                                          |                           | ~40                    | (4) <i>Blattella germanica</i>       | German cockroach              | (4) Chuang et al. (2010)         |
|                                          |                           | ~40                    | (5) <i>Plodia interpunctella</i>     | Indian meal moth              | (5) Binder et al. (2001)         |
|                                          |                           | ~40                    | (6) <i>Bombyx mori</i>               | Silkworm                      | (6) Liu et al. (2009)            |
|                                          |                           | ~40                    | (7) <i>Macrobrachium rosenbergii</i> | Giant freshwater prawn        | (7) Yadzir et al. (2012)         |
|                                          |                           | ~40                    | (8) <i>Macrobrachium lanchesteri</i> | Lanchester's freshwater prawn | In this study                    |
|                                          |                           | ~40                    | (9) <i>Gryllus bimaculatus</i>       | Field cricket                 | In this study                    |
| Glyceraldehyde 3-phosphate dehydrogenase | Metabolism                | ~32                    | (1) <i>Fenneropenaeus merguensis</i> | Banana shrimp                 | (1) Khanaruksombat et al. (2014) |
|                                          |                           | ~32                    | (2) <i>Macrobrachium rosenbergii</i> | Giant freshwater prawn        | In this study                    |
| Hemocyanin                               | Oxygen-transport proteins | ~75                    | (1) <i>Macrobrachium rosenbergii</i> | Giant freshwater prawn        | (1) Piboonpocanun et al. (2011)  |
|                                          |                           | ~75                    | (2) <i>Fenneropenaeus merguensis</i> | Banana shrimp                 | (2) Khanaruksombat et al. (2014) |
|                                          |                           | ~65                    | (3) <i>Macrobrachium rosenbergii</i> | Giant freshwater prawn        | In this study                    |
|                                          |                           | 67–77                  | (4) <i>Macrobrachium lanchesteri</i> | Lanchester's freshwater prawn | In this study                    |
| Hexamerin1B                              | Storage proteins          | ~60                    | (1) <i>Gryllus bimaculatus</i>       | Field cricket                 | In this study                    |

specific allergen in *G. bimaculatus* because hexamerins have only been found in the hemolymph of insect species. From the similarity found between HC and hexamerins, sequence comparisons have revealed that both arthropod HC and insect hexamerins belong to the same superfamily (Burmester, 2002). Note that HC, an allergen protein from *Macrobrachium* spp., is related to the hexamerins based on phylogenetic analysis.

The effect of microbial and chemical contaminants, toxicological and allergens hazards are specifically related to the consumption of insects (Belluco et al., 2013). The Food and Agriculture Organization of the United Nations (FAO) and the International Agency for Atomic Energy (IAEA) has reported that the Hazard Analysis and Critical Control Point (HACCP) system is a preventive system and can increase confidence in food safety (Food and Agriculture Organization of the United Nations/International Agency for Atomic Energy, 2011). Many useful preservation methods are recommended for insect consumption, such as the application of heating/cooling step, acidifying the insects with vinegar and freeze-dried (Food and Agriculture Organization of the United Nations, 2013). Insects have various micro-organisms in their gut flora, but many people in tropical countries widely consume whole bodies of insects without degutting. On a household level, raw insects should be prepared hygienically and sufficient heat treatment applied to ensure a microbiologically safe food product. Klunder, Wolkers-Rooijackers, Korpela, and Nout (2012) demonstrated that the effect of drying/acidifying without a refrigerator can eliminate enterobacteria and bacterial spores from mealworm and crickets. Chemical hazards, due to bioaccumulation and heavy metal content in insect bodies, have been investigated. The risk of food allergy from insects requires more study to distinguish between toxic and allergic symptoms. According to Gaylor, Harvey, and Hale (2012), soil-dwelling insects, such as crickets, can also become contaminated from solid waste in disposal sites and accumulate the chemical hazardous in insects. In china, it has been reported that the anaphylactic reactions, after consumption of fried silkworm pupa, may be cross-reactive among related taxonomy (Ji et al., 2008). Cross-reactivity might also help in identifying specific allergens between two species that can induce an allergic reaction, by antibodies (Food and Agriculture Organization of the United Nations, 2013). It has been reported that cross-reactivity, between seafood and insect allergen, suggests that people with seafood allergenicity could also suffer allergic reactions after consumption of edible insects; TM shows a high degree sequence similarity among crustaceans, and has an immunological relationship between crustaceans and insects (Leung et al., 1996). AK is an important allergen from silkworm and this enzyme may cross-react with cockroach (Chuang et al., 2010; Liu et al., 2009).

In summary, HEX1B is a novel allergen in *G. bimaculatus*. GAPDH was found as a novel allergen in the muscle of *M. rosenbergii*. AK is the important allergen found in *Macrobrachium* spp. and *G. bimaculatus*, which could suggest it as a cross-reactive, invertebrate pan-allergen. HC was found to be a hemolymph allergen in *Macrobrachium* spp. and has structural similarity to HEX1B, an allergen in *G. bimaculatus*. This supports the suggestion that *Macrobrachium* spp. and *G. bimaculatus* have cross-reactivity to each other. Thus, this experiment assumed that prawn-allergic patients might also be allergic to edible insects and should include GAPDH, HEX1B and AK for prawn and insect allergy diagnostic and therapeutic strategies. These results can warn entomophagists about a possible allergic reaction for people allergic to raw prawns and insects. However, the application of HACCP, such as advances in equipment design, processing procedures or technological developments system, can ensure the safety of food and toxicology. So, optimal preservation methods are a critical factor in the commercialization of edible insects on a global scale. Moreover, studying the epitope of allergens can aid in gaining a greater

understanding of cross-reactivity and how it can cause allergic symptoms. A complete allergen database could be an important instrument for developing new and reliable diagnostic and therapeutic strategies. There is a need for more analysis of allergens from edible insects in order to develop industrial processes for preparation of food using insects around the world.

## Acknowledgments

This work was supported by a Graduate Scholarship for International Publication from the Kasetsart University Research and Development Institute (KURDI). The authors thank Prof. Dr. M.R. Jisnusun Svasti and the Laboratory of Biochemistry, Chulabhorn Research Institute for their kind advice and the use of their facilities for this project. Moreover, the authors thank Lt. Gen. Clinical Professor Phanuvich Pumhirun for his recommendations and would also like to thank Phramongkutklao Hospital and Phyathai Hospital, Thailand for allowing access to sera from patients, who were allergic to prawn.

## References

- Abdel Rahman, A. M., Kamath, S., Lopata, A. L., & Helleur, R. J. (2010). Analysis of the allergenic proteins in black tiger prawn (*Penaeus monodon*) and characterization of the major allergen tropomyosin using mass spectrometry. *Rapid Communications in Mass Spectrometry*, 24, 2462–2470.
- Belluco, S., Losasso, C., Maggioletti, M., Alonzi, C. C., Paoletti, M. G., & Ricci, A. (2013). Edible insects in a food safety and nutritional perspective: A critical review. *Comprehensive Reviews in Food Science and Food Safety*, 12, 296–313.
- Benndorf, D., Müller, A., Bock, K., Manuwald, O., Herbarth, O., & von Bergen, M. (2008). Identification of spore allergens from the indoor mould *Aspergillus versicolor*. *Allergy*, 63, 454–460.
- Binder, M., Mahler, V., Hayek, B., Sperr, W. R., Schöller, M., Prozell, S., et al. (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.
- Burmester, T. (2002). Origin and evolution of arthropod hemocyanins and related proteins. *Journal of Comparative Physiology – Biochemical, Systemic, and Environmental Physiology*, 172, 95–107.
- Chuang, J. G., Su, S. N., Chiang, B. L., Lee, H. J., & Chow, L. P. (2010). Proteome mining for novel IgE-binding proteins from the German cockroach (*Blattella germanica*) and allergen profiling of patients. *Proteomics*, 10, 3854–3867.
- Daul, C. B., Slattey, M., Reese, G., & Lehrer, S. B. (1994). Identification of the major brown shrimp (*Penaeus aztecus*) allergen as the muscle protein tropomyosin. *International Archives of Allergy and Immunology*, 105, 49–55.
- Davassi, L. A. (2011). Survival and growth of the freshwater prawn *Macrobrachium rosenbergii* in relation to different nutrients composition. *Journal of Fisheries and Aquatic Science*, 6, 649–654.
- Food and Agriculture Organization of the United Nations/the International Agency for Atomic Energy. (2001). Manual on the application of the HACCP system in Mycotoxin prevention and control. FAO Food and Nutrition paper, No. 73, Rome, Italy.
- Food and Agriculture Organization of the United Nations. (2002). A manual for the culture of the giant river prawn (*Macrobrachium rosenbergii*). FAO fisheries technical paper, No. 428, Rome, Italy.
- Food and Agriculture Organization of the United Nations. (2010). Forest insects as food: Humans bite back. Proceedings of a workshop on Asia-Pacific resources and their potential for development. 19–21 February 2008, Chiang Mai, Thailand.
- Food and Agriculture Organization of the United Nations. (2013). Edible insects: Future prospects for food and feed security. FAO Forestry paper 171, Rome, Italy.
- García-Orozco, K. D., Aispuro-Hernández, E., Yepiz-Plascencia, G., Calderón-de-la-Barca, A. M., & Sotelo-Mundo, R. R. (2007). Molecular characterization of arginine kinase, an allergen from the shrimp *Litopenaeus vannamei*. *International Archives of Allergy and Immunology*, 144, 23–28.
- Gaylor, M. O., Harvey, E., & Hale, R. C. (2012). House crickets can accumulate polybrominated diphenyl ethers (PBDEs) directly from polyurethane foam common in consumer products. *Chemosphere*, 86, 500–505.
- Jaenicke, E., Pairet, B., Hartmann, H., & Decker, H. (2012). Crystallization and preliminary analysis of crystals of the 24-meric hemocyanin of the emperor scorpion (*Pandinus imperator*). *PLoS ONE*, 7, e32548.
- Ji, K. M., Zhan, Z. K., Chen, J. J., & Liu, Z. G. (2008). Anaphylactic shock caused by silkworm pupa consumption in China. *Allergy*, 63, 1407–1408.
- Khanaruksombat, S., Srisomsap, C., Chokchaichamnankit, D., Punyarit, P., & Phiriyaikul, P. (2014). Identification of novel allergen from muscle and various organs in banana shrimp (*Fenneropenaeus merguensis*). *Annals of Allergy, Asthma & Immunology*, 113, 301–306.

- Klunder, H. C., Wolkers-Rooijackers, J., Korpela, J. M., & Nout, M. J. R. (2012). Microbiological aspects of processing and storage of edible insects. *Food Control*, 26, 628–631.
- Leung, P. S., Chen, Y. C., Mykles, D. L., Chow, W. K., Li, C. P., & Chu, K. H. (1998). Molecular identification of the lobster muscle protein tropomyosin as a seafood allergen. *Molecular Marine Biology and Biotechnology*, 7, 12–20.
- 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, 954–961.
- Liu, G. M., Huang, Y. Y., Cai, Q. F., Weng, W. Y., Su, W. J., & Cao, M. J. (2011). Comparative study of in vitro digestibility of major allergen, tropomyosin and other proteins between Grass prawn (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*). *Journal of the Science of Food and Agriculture*, 91, 163–170.
- Liu, Z., Xia, L., Wu, Y., Xia, Q., Chen, J., & Roux, K. H. (2009). Identification and characterization of an arginine kinase as a major allergen from silkworm (*Bombyx mori*) larvae. *International Archives of Allergy and Immunology*, 150, 8–14.
- Lopata, A. L., O'Hehir, R. E., & Lehrer, S. B. (2010). Shellfish allergy. *Clinical and Experimental Allergy*, 40, 850–858.
- Martínez, J. P., Gil, M. L., López-Ribot, J. L., & Chaffin, W. L. (1998). Serologic response to cell wall mannoproteins and proteins of *Candida albicans*. *Clinical Microbiology Reviews*, 11, 121–141.
- Martins, J. R., Anhezini, L., Dallacqua, R. P., Simoes, Z. L. P., & Bitondi, M. M. G. (2011). A honey bee hexamerin, HEX 70a, is likely to play an intranuclear role in developing and mature ovarioles and testicles. *PLoS ONE*, 6, e29006.
- Nauta, A. J., Engels, F., Knippels, L. M., Garssen, J., Nijkamp, F. P., & Redegeld, F. A. (2008). Mechanisms of allergy and asthma. *European Journal of Pharmacology*, 585, 354–360.
- Okezie, O. A., Kgomotso, K. K., & Letswiti, M. M. (2010). Mopane worm allergy in a 36-year-old woman: A case report. *Journal of Medical Case Reports*, 4, 42.
- Olianas, A., Sanna, M. T., Messina, I., Castagnola, M., Masia, D., Manconi, B., et al. (2006). The hemocyanin of the shrimplike crab *Calappa granulata*: Structural-functional characterization. *Journal of Biochemistry*, 139, 957–966.
- Piboonpocanun, S., Jirapongsananurak, O., Tipayanon, T., Boonchoo, S., & Goodman, R. E. (2011). Identification of hemocyanin as a novel non cross-reactive allergen from the giant freshwater shrimp *Macrobrachium rosenbergii*. *Molecular Nutrition & Food Research*, 55, 1492–1498.
- Pick, C., Hagner-Holler, S., & Burmester, T. (2008). Molecular characterization of hemocyanin and hexamerin from the firebrat *Thermobia domestica* (Zygentoma). *Insect Biochemistry and Molecular Biology*, 38, 977–983.
- Sander, I., Rozynek, P., Rihs, H. P., van Kampen, V., Chew, F. T., Lee, W. S., et al. (2011). Multiple wheat flour allergens and cross-reactive carbohydrate determinants bind IgE in baker's asthma. *Allergy*, 66, 1208–1215.
- Shanti, K. N., Martin, B. M., Nagpal, S., Metcalfe, D. D., & Rao, P. V. (1993). Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *Journal of Immunology*, 151, 5354–5363.
- Shuhaimi-Othman, M., Yakub, N., Ramle, N. A., & Abas, A. (2011). Sensitivity of the freshwater prawn, *Macrobrachium lanchesteri* (Crustacea: Decapoda), to heavy metals. *Toxicology and Industrial Health*, 27, 523–530.
- Sookrung, N., Chaicumpa, W., Tungtrongchitr, A., Vichayanond, P., Bunnag, C., Ramasoota, P., et al. (2006). *Periplaneta americana* arginine kinase as a major cockroach allergen among Thai patients with major cockroach allergies. *Environmental Health Perspectives*, 114, 875–880.
- Srisomsap, C., Sawangareetrakul, P., Subhasitanont, P., Chokchaichamnankit, D., Chiablaem, K., Bhudhisawasdi, V., et al. (2010). Proteomic studies of cholangiocarcinoma and hepatocellular carcinoma cell secretomes. *Journal of Biomedicine and Biotechnology*, 437143.
- Suma, Y., Ishizaki, S., Nagashima, Y., Lu, Y., Ushio, H., & Shiomi, K. (2007). Comparative analysis of barnacle tropomyosin: Divergence from decapod tropomyosins and role as a potential allergen. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 147, 230–236.
- Tarze, A., Deniaud, A., Le Bras, M., Maillier, E., Molle, D., Larochette, N., et al. (2007). GAPDH, a novel regulator of the pro-apoptotic mitochondrial membrane permeabilization. *Oncogene*, 26, 2606–2620.
- Telfer, W. H., & Kunkel, J. G. (1991). The function and evolution of insect storage hexamers. *Annual Review of Entomology*, 36, 205–228.
- Tongbai, W., & Damrongphol, P. (2011). Bioactivation of chlorpyrifos in the riceland prawn, *Macrobrachium lanchesteri*. *Journal of Biological Sciences*, 11, 275–281.
- Van der Ventel, M. L., Nieuwenhuizen, N. E., Kirstein, F., Hikuam, C., Jeebhay, M. F., Swoboda, I., et al. (2011). Differential responses to natural and recombinant allergens in a murine model of fish allergy. *Molecular Immunology*, 48, 637–646.
- Wang, D., Bai, Y., Li, J., & Zhang, C. (2004). Nutritional value of the field cricket (*Gryllus testaceus walker*). *Insect Science*, 11, 275–283.
- Wang, W., Xia, X., Liu, F., Chen, X., Yang, H., & Ning, Q. (2012). Cloning and characterization of the hemocyanin gene of prawn *Macrobrachium nipponense*. *Turkish Journal of Biochemistry*, 37, 348–355.
- Yadzir, Z. H., Misnan, R., Abdullah, N., Bakhtiar, F., Arip, M., & Murad, S. (2012). Identification of the major allergen of *Macrobrachium rosenbergii* (giant freshwater prawn). *Asian Pacific Journal of Tropical Biomedicine*, 2, 50–54.
- Yao, C. L., Ji, P. F., Kong, P., Wang, Z. Y., & Xiang, J. H. (2009). Arginine kinase from *Litopenaeus vannamei*: Cloning, expression and catalytic properties. *Fish & Shellfish Immunology*, 26, 553–558.
- Yhoang-Aree, J., Puwastein, P., & Attig, G. A. (1997). Edible insects in Thailand: An unconventional protein source. *Ecology of Food and Nutrition*, 36, 133–149.
- Yu, C. J., Lin, Y. F., Chiang, B. L., & Chow, L. P. (2003). Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. *Journal of Immunology*, 170, 445–453.