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**TITLE:** Proteomics Alteration of Banana Shrimp (*Fenneropenaeusmerguiensis*) Allergen after Various Food Processing

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THESIS

# PROTEOMICS ALTERATION OF BANANA SHRIMP (*FENNEROPENAEUS MERGUIENSIS*) ALLERGEN AFTER VARIOUS FOOD PROCESSING

SUPARADA KHANARUKSOMBAT

A Thesis Submitted in Partial Fulfillment of
The Requirements for the Degree of
Master of Science (Bioproducts Science)
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Suparada Khanaruksombat 2014: Proteomics Alteration of Banana Shrimp (*Fenneropenaeus merguiensis*) Allergen after Various Food Processing. Master of Science (Bioproducts Science), Major Field: Bioproducts Science, Division of Science. Thesis Advisor: Mrs. Pharima Phiriyangkul, Ph.D. 201 pages.

Shellfish refers to crustacean and mollusk such as shrimp, crab and squid. It is a major cause of adverse food reaction type I. Banana shrimp (*Fenneropenaeus merguiensis*) is a native species considered to be economically important in Thailand. Proteomic methods were used to investigate the allergenic proteins from banana shrimp by denaturing, gel electrophoresis (1/2D-PAGE) and immunoblotting with sera from patients who allergic to shrimp. Liquid chromatography-two-dimensional tandem mass spectrometry (LC-MS/MS) used for identifying IgE-binding protein and then analyzed by Mascot MS/MS software. The electrophoresis pattern of raw muscle and each treated muscle were clearly different. IgE-binding proteins in *Fenneropenaeus merguiensis* were identified and compare how the food processing treatments may alter the allergenicity of this shrimp. Vitellogenin (VG) was suggested as a major and novel allergen in banana shrimp ovarian. Ovarian peritrophin 1 precursor (SOPs), β-actin and 14-3-3 zeta were suggested as minor and novel allergens in the ovary at different stages of ovarian development. Myosin heavy chain (MHC), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hemocyanin (HC) and enolase were demonstrated as novel allergens in raw muscle of banana shrimp. Food processing treatment can alter the allergenicity of shrimp allergens, including tropomyosin (TM), arginine kinase (AK), sarcoplasmic calcium-binding protein (SCP) and GAPDH. These novel allergens should improve diagnostic allergic methods for shrimp allergic patient, because presently the only measurement of IgE to tropomyosin in shrimp allergy diagnosis can be missed. The allergenicity alteration from food processing may be the new ways to produce hypoallergenic food in the future.

| _________________________ | _________________________ | ___ / ___ / ___ |
|---|---|---|
| Student's signature | Thesis Advisor's signature | |

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Suparada Khanaruksombat
July 2014

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| °C | = | Degree Celsius |
| µg | = | Microgram |
| µL | = | Microliter |
| 1D | = | One-dimension |
| 2D | = | Two-dimension |
| ACN | = | Acetonitrile |
| AK | = | Arginine kinase |
| APS | = | Ammonium persulphate |
| CBB | = | Coomassie brilliant blue R-250 |
| CHAPs | = | 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate |
| cm | = | Centimeter |
| DBPCFC | = | Double-blind placebo controlled food challenge |
| Dot blot | = | Dot immunoblotting |
| DTT | = | Dithiothreitol |
| EAST | = | Enzyme allergosorbent test |
| ECL | = | Enhanced chemiluminescence |
| EDTA | = | Ethylenediaminetetraacetic acid |
| ELISA | = | Enzyme-linked immunosorbent assay |
| FAO | = | Agriculture Organization of the United Nations |
| FBPA | = | Fructose-bisphosphate aldolase |
| g | = | Gram |
| GAPDH | = | Glyceraldehyde 3-phosphate dehydrogenase |
| GHz | = | Gigahertz |
| GI | = | gastrointestinal systems |
| GRPL | = | Glucan pattern-recognition lipoprotein |
| GSI | = | Gonadosomatic index |
| h | = | hour |
| HC | = | Hemocyanin |

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# LIST OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| HRP | = | Horseradish peroxidase |
| IAA | = | Iodoacetamide |
| IEF | = | Isoelectric focusing |
| IgE | = | Immunoglobulin E |
| IgG | = | Immunoglobulin G |
| IgY | = | Immunoglobulin Y |
| IPG | = | Immobilized pH gradient |
| kDa | = | Kilo Dalton |
| kg | = | Kilogram |
| kHz | = | Kilohertz |
| kPa | = | Kilopascal |
| kU | = | Kilo Units |
| L | = | Liter |
| LC-MS/MS | = | Liquid chromatography-two-dimensional tandem mass spectrometry |
| m | = | meter |
| M | = | Molar |
| mA | = | Milliampere |
| mg | = | Milligram |
| MHC | = | Myosin heavy chain |
| MHz | = | Megahertz |
| min | = | Minute |
| mL | = | Milliliter |
| MLC | = | Myosin light chain |
| mm | = | Millimeter |
| mM | = | Millimolar |
| $mm^2$ | = | Square Millimeters |
| MW | = | Molecular-weight |

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## LIST OF ABBREVIATIONS (Continued)

| | | |
|---|---|---|
| ng | = | Nanogram |
| OAS | = | Oral allergy syndrome |
| PAGE = | = | Polyacrylamide gel electrophoresis |
| *pI* | = | Isoelectric point |
| PK | = | Pyruvate kinase |
| PMF | = | Peptide mass fingerprinting |
| RAST | = | Radio allergosorban Test |
| RIE | = | Rocket immunoelectrophoresis |
| SCP | = | Sarcoplasmic calcium-binding protein |
| SDS | = | Sodium dodecyl sulphate |
| sec | = | Second |
| SOPs | = | shrimp ovarian peritrophins |
| SPT | = | Skin prick test |
| TEMED | = | N,N,N’,N’-Tetramethylethylenediamaine |
| TFA | = | Trifluoroacetic acid |
| TM | = | Tropomyosin |
| V | = | Voltage |
| VG | = | Vitellogenin |
| W | = | Watt |
| WHO | = | World Health Organization |
| WSSV | = | white spot syndrome virus |

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# PROTEOMICS ALTERATION OF BANANA SHRIMP (*FENNEROPENAEUS MERGUIENSIS*) ALLERGEN AFTER VARIOUS FOOD PROCESSING

## INTRODUCTION

Food allergies are growing public health and safety concern in the United States and around the world. It is now becoming a major concern for governments, doctor, families, and the food service industry. As the number of food allergies increase, it need to take steps to more education (Schaefer, 2011).

Shellfish allergy is a long-lasting disorder, usually persisting throughout life (Steensma, 2003). Patients who are allergic to some foods have several symptoms, such as urticaria, asthma, diarrhea and even life-threatening anaphylaxis (Emoto *et al*., 2009). A large variety of crustaceans play an important role as a food source for humans and the consumption of shrimp has tripled since 1970 (Sicherer *et al*., 2004). In coastal countries, shellfish including crustaceans and mollusks are the most common causes of food allergy (Ayuso *et al*., 2009). Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) suggested that crustaceans are one of the eight important allergenic foods (Food and Agriculture Organization of the United Nations [FAO]/World Health Organization [WHO], 2001).

The banana shrimp, *Fenneropenaeus merguiensis*, is distributed in the Indo-Pacific region from the Persian Gulf to Thailand, Hong Kong, the Philippines, Indonesia, New Guinea, New Caledonia and northern Australia (Grey *et al*., 1983). *F. merguiensis* is an important species for commercial fisheries, with the total global banana shrimp capture reported to FAO for 2011 being 102,091 tonne and has tended to increase annually (FAO, 2014a). *F. merguiensis* is an important contributor to Thailand's shrimp production, numbers are inferior to white shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*). In 2011, banana shrimp

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production for whole marine fishery of Thailand is more than 7,000 tonne valued at 1,476.1 million Baht (Fishery Information Technology Center, Department of Fisheries, Ministry of Agriculture and Cooperatives of Thailand, 2014). However, *F. merguiensis* is an important species for commercial fisheries, but information about *F. merguiensis* is limited.

Moreover, these shrimp is valued by consumers for their good taste and high dietary quality (Yamagata and Low, 1995). In general, food processing is necessary prior to human consumption to increase the taste and for food safety. Shrimp is often consumed after a certain degree of heating. To date, boiling (blanching) has been the most common way to cook shellfish and is often used to study the effect of allergenicity. In the past few years, new sustainable and environmentally friendly techniques for the preservation of foods, such as ultrasound assisted (ultrasonication), water immersion sterilization (autoclave/retort) and microwave, have rapidly developed for improving the quality of food products while also reducing the chemical/physical hazard associated with live bacteria. Furthermore, this both methods for food processing is important in food and pharmaceutical industries especially, food canning technology and/or ready to eat food product in the market (Chemat *et al*., 2011; Teixeira, 2007). Cooking processing around the world almost always uses the whole shrimp, especially in East and Southeast Asia, where a shrimp dish comprised of the whole body is very popular because shrimp can make food taste better and has high nutritional value. The food processing such as boiling can alter the allergenicity of shellfish allergen but there is still the allergenic property to cause the allergic symptom (Liu *et al*., 2010).

Tropomyosin (TM) has been reported as a major allergen in shellfish (Reese *et al*., 1999) and minor allergen is arginine kinase (AK) (Lopata and Lehrer, 2009). Both of allergens were also known as pan-allergen in invertebrate. This gives rise to the so called "panallergen" concept, with the Greek prefix "pan" meaning "all". The panallergen concept encompasses families of related proteins, which are involved in general vital processes and thus, widely distributed throughout nature (Hauser *et al*., 2010). However, the effect of ultrasonic and autoclaving on the allergenicity of *F.*

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*merguiensis* allergen has not yet been demonstrated. Consequently, some patients might be allergic to protein allergens from other organs apart from muscle. An understanding of the allergenic properties of shrimp organs is critical especially for individuals who are allergic to shrimp. The shell, ovaries and hepatopancreas are organs that are most likely to be consumed. Shrimp shell is contacted to muscle. Ovarian and extra-ovarian tissues such as the hepatopancreas have been suggested to be the tastiest part of whole shrimp. Thus, these various organs may have allergenic properties. Thus, the study of allergenicity of *F.merguiensis* allergen from food processing and various organs is necessary to understand more and can be use this knowledge to future diagnostic and therapeutic strategies for allergic treatment.

At present, the famous way to study allergenicity of food allergen is proteomic processing. Thus, methods in this study were based on proteomic techniques. For the separation of protein, Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), isoelectric focusing electrophoresis (IEF) and two-dimensional gel electrophoresis (2D-PAGE) were preform. Then, immunoblotting with atopic and non-atopic serum can be used to detect IgE-binding proteins and liquid chromatography-two-dimensional tandem mass spectrometry (LC-MS/MS) methodologies follow by MASCOT MS/MS Ions Search then can be applied for protein identification.

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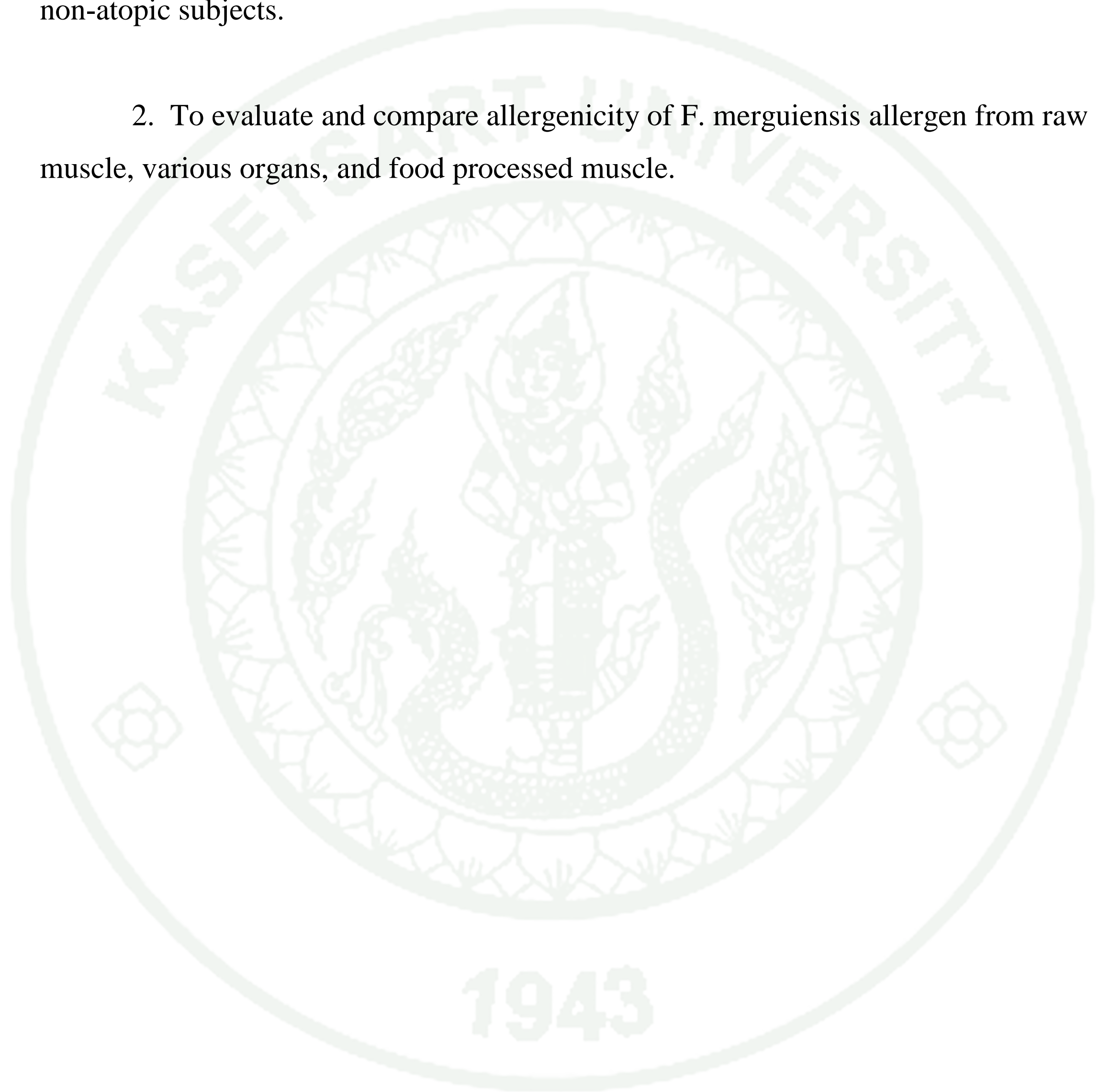
# OBJECTIVES

1. To identify IgE-binding proteins in the raw muscle, various organs, and food processed muscle of *Fenneropenaeus merguiensis* with sera from atopic and non-atopic subjects.

2. To evaluate and compare allergenicity of F. merguiensis allergen from raw muscle, various organs, and food processed muscle.



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# LITERATURE REVIEW

***Fenneropenaeus merguiensis* (De Man, 1888) (banana shrimp)**

1. General background

1.1 Geographic distribution and its habitat

Banana shrimp, *Fenneropenaeus merguiensis* is distributed across the Indo-West Pacific, from the Persian Gulf to Thailand, Hong Kong, the Philippines, Indonesia , New Guinea, New Caledonia and Northern Australia (Figure 2A) (Grey *et al*., 1983). This species has been report that distinguished from other alien penaeid shrimp in the Mediterranean in its distinctive color pattern (Özcan *et al*., 2006). In Thailand, banana shrimp was distributed in both of the Gulf of Thailand as Chon Buri, Rayong, Chanthaburi, Trat, Chachoengsao, Samut Sakhon, Samut Songkhram, Phetchaburi, Prachup Khiri Khan, Chumphon, Surat Thani, Nakhon Si Thammarat and Narathiwat; and the Andaman Sea as Trang, Ranong, Phuket, Phangnga, Krabi and Satun (Figure 2B) (Naiyanetr, 2007). *F. merguiensis* is commonly captured on muddy and sandy bottoms, mostly at depths of 10-45 m.

**Figure 1** *Fenneropenaeus merguiensis* (banana shrimp)

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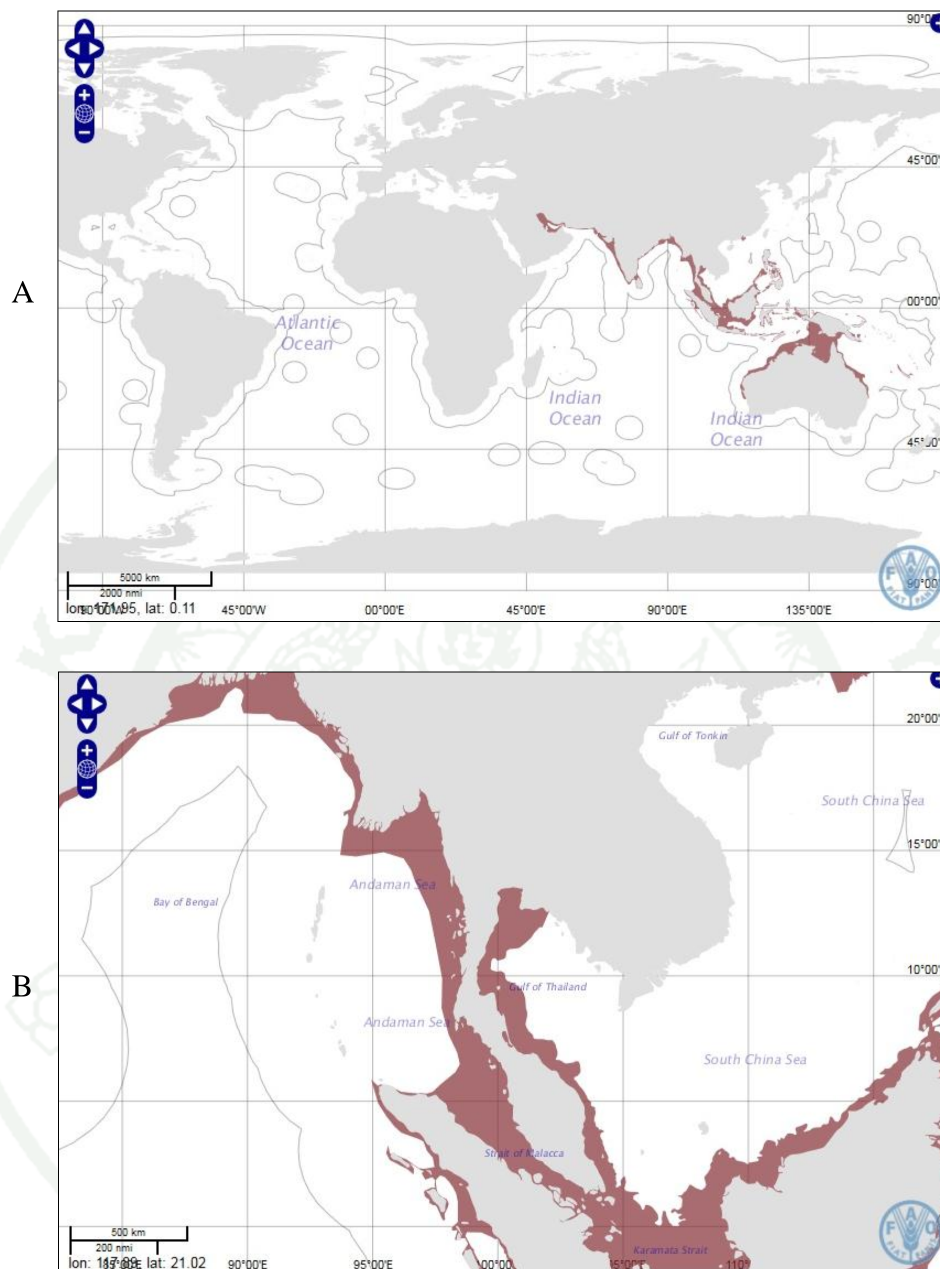


**Figure 2** Geographical distribution for *Fenneropenaeus merguiensis*. A: distribution across the world, B; distribution in Thailand

**Source**: FAO (2014b)

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1.2 Banana shrimp production and economic importance

The world catch of shrimp in 2000 was recorded at over three million tonne. From 1970 to 2000 Penaeidae on average comprised 42.2% of the world catch of shrimps. Some of the largest shrimp fisheries of the world are in Indonesia, Thailand, India and the Gulf of Mexico (Khorshidian, 2002). The total banana shrimp production around the world reported to FAO for 2011 was 116,173 tonne with aquaculture production of 14,082 tonne and natural captured 102,091 tonne (FAO, 2014a). In Thailand, banana shrimp production for whole marine fishery reported to Ministry of Agriculture and Cooperatives of Thailand was 74,000 tonne valued at 1,476.1 million baht (Fishery Information Technology Center, Department of Fisheries, Ministry of Agriculture and Cooperatives of Thailand, 2014).

Raised on extensive farms throughout Southeast Asia, *F. merguiensis* is a also a "white" shrimp that has attracted attention because it tolerates low water quality better than *Penaeus monodon*, it can be grown at high densities, and it is readily available in the wild. But banana shrimp has been ignored by modern shrimp farmers because it does not grow as fast as the major culture species, *P. monodon*, and their limited information on biology and culture, low survival in intensive ponds (not confirmed by research), die quickly at harvest, and no species-specific commercial feeds. The farm operation intensity and the species cultured changed from an extensive banana shrimp (*F. merguiensis*) farm to semi-intensive or intensive and tiger shrimp (*P. monodon*) to extensive aiming the export to fit well with the Japanese taste and requirements (Coastal Resources Institute [CORIN], 2000; Rosenberry, 2014). However, *F. merguiensis* almost catch up from the natural, this makes none of the material culture contamination and they have given a good sense of taste and naturally high levels of many nutrients which contribute to a healthy lifestyle (Australian Seafood Cooperative Research Centre, 2014)

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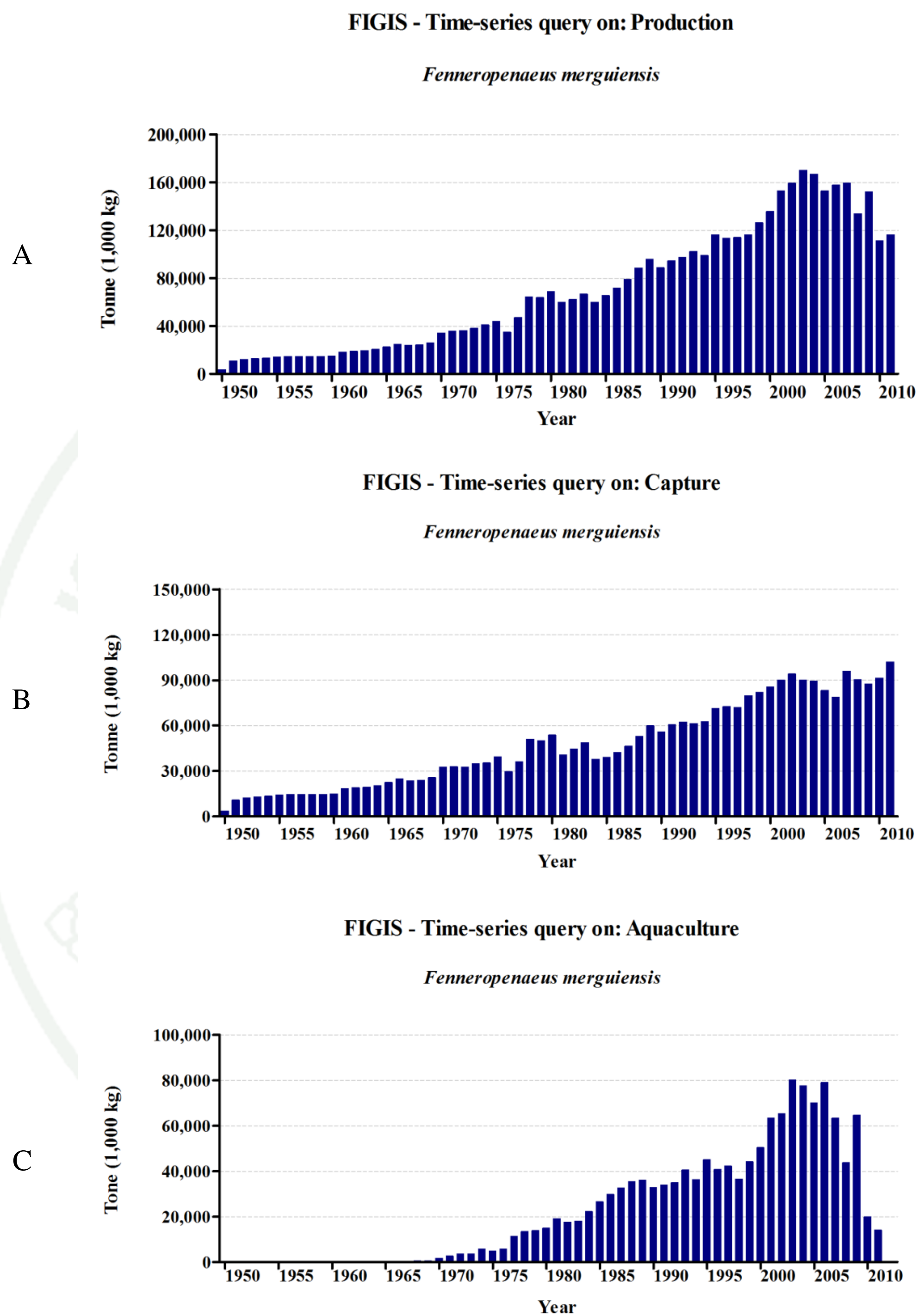


**Figure 3** Fisheries Global Information System (FIGIS) of FAO reported on global *F. merguiensis* production (A), global wild capture production (B) and global aquaculture production (C).

**Source:** FAO (2014a)

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**Biology of *Fenneropenaeus merguiensis***

1. Banana shrimp taxonomy

*F. merguiensis* is member of the genus Penaeus. The genus has been reorganized following a proposition of Pérez Farfante and Kensley based on morphological differences, in particular the genital characteristics of these animals. Following the revision, many species formerly in the genus Penaeus have been reassigned to new genera in the family Penaeidae: *Farfantepenaeus*, *Fenneropenaeus*, *Litopenaeus* and *Marsupenaeus* (Pérez Farfante and Kensley, 1997).

PHYLUM Arthropoda
SUBPHYLUM Crustacea
CLASS Malacostraca
SUBCLASS Eumalacostraca
SUPERORDER Eucarida
ORDER Decapoda
SUBORDER Dendrobranchiata
SUPERFAMILY Penaeoidea
FAMILY Penaeidae
GENUS *Fenneropenaeus*
SPICIES *merguiensis*

Commercial names / Local Names:

Pakistan: Jaira, Jairo (also used for other species of *Fenneropenaeus*)
Malaysia: Udang kaki merah
Indonesia: Udang putih
Thailand: Kung chaebauy
Hong Kong: Pak ha, White prawn
Australia: Banana prawn, White prawn

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There are 4 species belonging to subgenus *Fenneropenaeus* in Indo-West Pacific: *Fenneropenaeus merguiensis*, *Fenneropenaeus chinensis*, *Fenneropenaeus indicus* and *Fenneropenaeus penicillatus* (FAO, 1980). The common names of these *Fenneropenaeus* are showed in Table 1.

**Table 1** The old and new scientific name and common names of the closely related species.

| Old scientific name | New scientific name | Common name(s) |
|---|---|---|
| ***Penaeus merguiensis*** | *Fenneropenaeus merguiensis* | banana shrimp, banana prawn |
| ***Penaeus chinensis*** | *Fenneropenaeus chinensis* | fleshy prawn, Chinese white shrimp, oriental shrimp |
| ***Penaeus indicus*** | *Fenneropenaeus indicus* | Indian prawn |
| ***Penaeus penicillatus*** | *Fenneropenaeus penicillatus* | redtail prawn |

**Source:** FAO (1980)

2. Penaeid shrimp morphology

The body of banana shrimp (*F. merguiensis*) is often yellow or translucent with no banding on body or antennae and speckled with reddish brown dots (hence the common name) (Grey *et al*., 1983). The exterior of penaeid shrimp is distinguished by a cephalothorax with characteristic hard rostrum, and by a segmented abdomen (Figure 4). Most organ, such as gills, digestive system and heart, are located in the cephalothorax, while the muscles concentrate in the abdomen. Appendages of the cephalothorax vary in appearance and function (Primavera, 1990). In the head region, antennules and antennae perform sensory functions. In the thorax region, the maxillipeds are the first three pairs of appendages, modified for food handing, and the remaining five pairs are the walking legs (pereiopods). Five pairs of swimming leg (pleopods) are found on the abdomen (Bell and Lightner, 1988; Baily-Brock and Moss, 1992).

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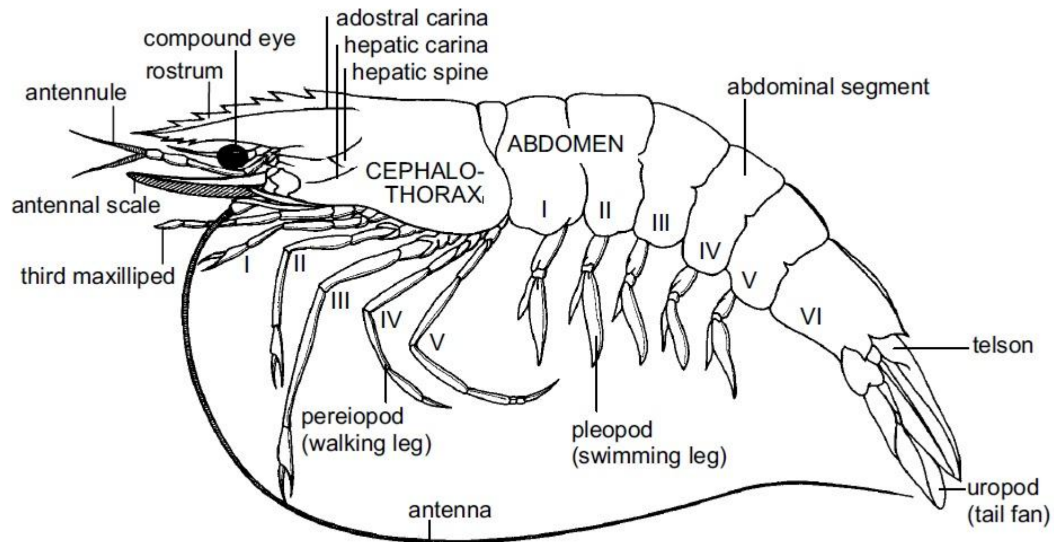


**Figure 4** The lateral view of the external morphology of penaeid shrimp.

**Source:** Primavera (1990)

Penaeids and other arthropods have an open circulatory system. So, the blood and the blood cells are called haemolymph and haemocytes, respectively. Crustaceans have a muscular heart that is dorsally located in the cephalothorax. The valve haemolymph vessels leave the heart throughout the body, where exchange of substances takes place (Bauchau, 1981). A large part of the cephalothorax in penaeid shrimp is occupied by the hepatopancreas. This digestive gland consists of diverticula of the intestine. Spaces between these hepatopancreatic tubules are haemolymph sinuses. The main functions of the hepatopancreas are the absorption of nutrients, storage of lipids and production of digestive enzymes (Johnson, 1980). The internal morphology of penaeid shrimp is showed in Figure 5. The ovary lies dorsal to the gut and extends from the cephalothorax along the entire length of the tail (Figure 6) (Hall *et al*., 1999).

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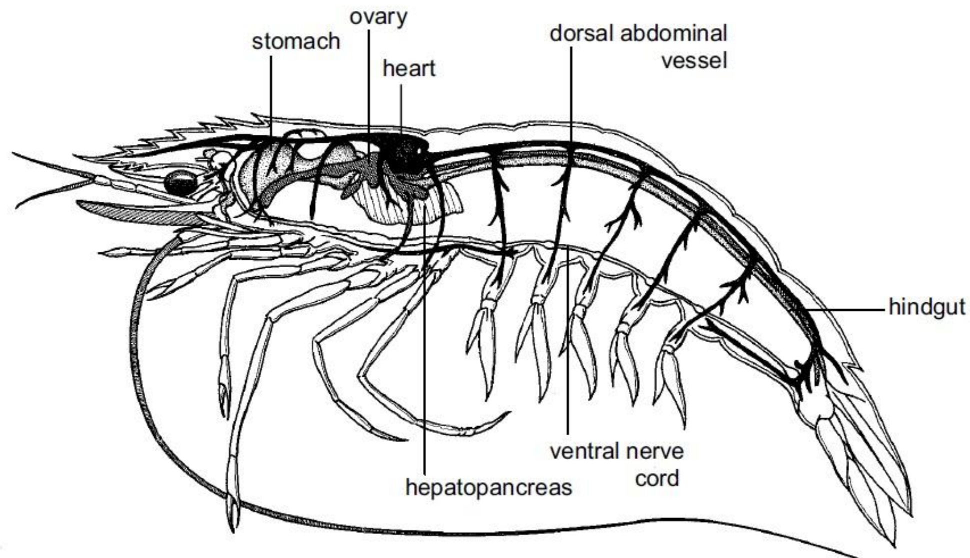


**Figure 5** The lateral view of the internal morphology of penaeid shrimp.

**Source:** Primavera (1990)

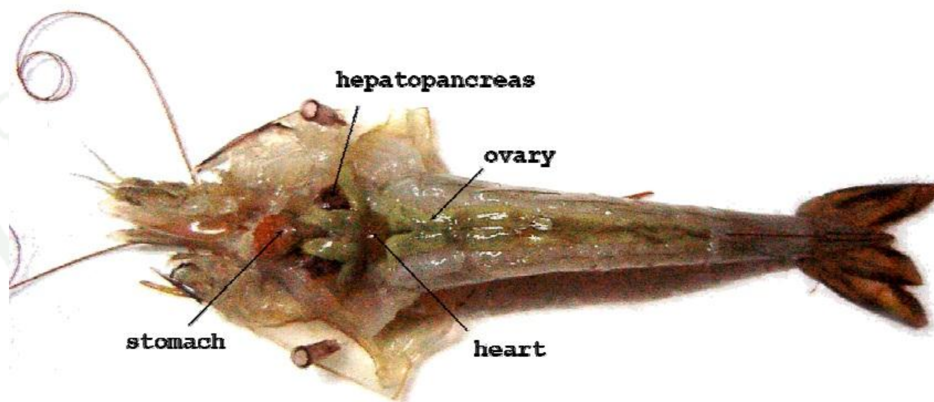


**Figure 6** The top view of the internal anatomy of a female banana shrimp (*Fenneropenaeus merguiensis*).

**Source:** Phiriyangkul (2006)

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3. Life cycle of penaeid shrimp

In the wild, juvenile mature shrimps typically make seasonal migrations into coastal and off shore waters where they can later spawn planktonic eggs (Figure 7). After these hatch, there are several stages of planktonic larvae lasting for about three weeks before settlement on the bottom as postlarvae which then migrate towards inshore nursery areas (such as seagrass and mangroves). To encourage the shrimps to mate and spawn in captivity, hatchery operators try to recreate the environmental conditions that are present when the adults move into deeper waters — very low light levels, oceanic salinities and stable temperatures. Females produce between 50,000-1,000,000 eggs per spawning (Rosenberry, 2014). These environmental manipulations approximate conditions in the species' natural spawning grounds, and assist in reducing stress. In captivity, well-balanced nutrition and artificial stimulation of the endocrine system through techniques such as eyestalk ablation (destruction of a gland at the base of an eyestalk, causing gonad development) can initiate final maturation and spawning. The phases of the moon are also taken into consideration when planning husbandry practices due to their effect on the breeding cycle (for example, moulting and subsequent mating peak in adults around the full moon).

Banana shrimps are known to spawn in comparatively shallow waters (10–30 m deep), and even within river systems. Baby shrimps or postlarvae hatch out of semi-floating eggs and live as part of the sea plankton before settling in the nursery areas of estuaries. Juvenile banana shrimps predominate in mud-mangrove habitats, these species are known to move into rivers and travel well upstream into areas of occasional low salinity (The State of Queensland, Department of Primary Industries and Fisheries, 2006). Adults may be 5-8 inches in length, are usually found in the ocean, mature and breed, which completes the life cycle. The maximum life span is approximately 12-18 months. To enable growth, shrimp (and all other crustaceans) periodically loosen their extracellular cuticle from the underlying epidermal layer called moulting (Phiriyangkul, 2006).

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From Figure 7, egg hatch within 16 h after fertilization. The larval stages comprise nauplius (6 stages in 2 day), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days) and megalopa (6-35 days). The mega and early juvenile are called postlarvae. Transition from juvenile to subadult takes 135-255 days and subsequently completion of sexual maturity occurs within 10 months. This information is modified from Motoh 1981. Pictures are not in proportion to actual size and scale.

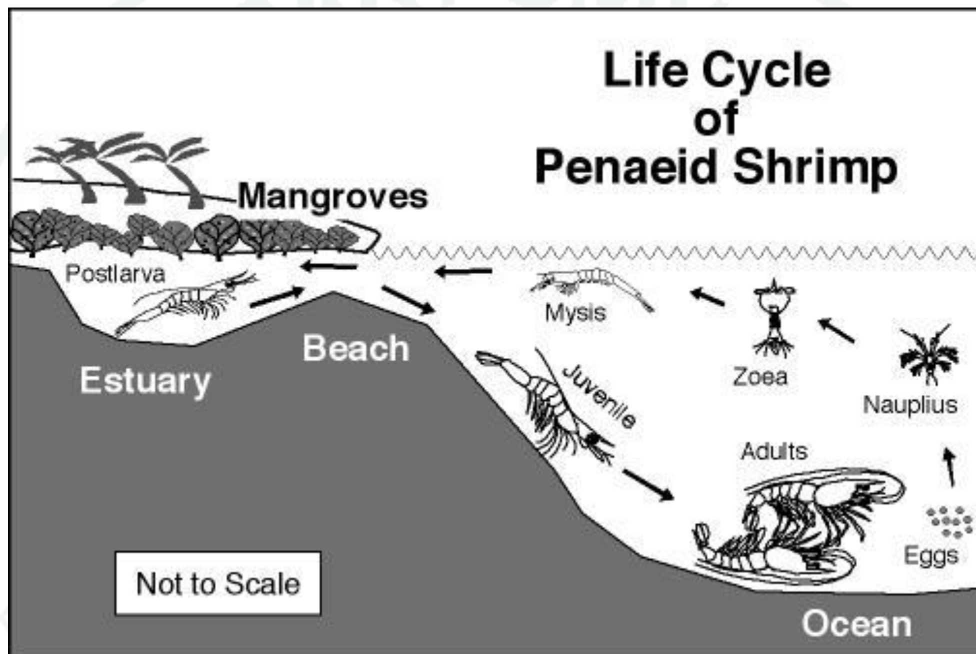


**Figure 7** Life cycle of shrimp showing distribution with depth.

**Source:** Stewart (2014)

The shrimp species which are commercially fished are bisexual, that is, the genders are separate, with the mature female being larger than the male. Males and females can thus be readily identified on the presence or absence of a petasma or a thelycum. The maturation process has been arbitrarily categorized into five successive stages of a development, based mainly on ovum size and gonad expansion plus the color for the purpose of describing the degree of sexual maturity (Motoh, 1981). The determination of ovarian development by means of a bright underwater torch beam being passed along her side, only reveals the shadow of the ovary in the tail region

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and is scored from 1 to 5 as shown in Figure 8A. And the intense pigmentation of the shell in this region prevents the visualization of any ovarian outline as show in Figure 8B (Hall *et al*., 1999).

Stage 1 Immature, undeveloped ovary or previtellogenic stage; the ovary either does not cast any shadow or a thin opaque line is seen along the length of the tail. The ovary is translucent and unpigmented.

Stage 2 Developing ovary or early exogenous vitellogenic stage; the ovary can be visualized with a light beam as a large centrally located opaque rope-like structure.

Stage 3 Early mature, nearly ripe ovary or late exogenous vitellogenic stage; the ovary is visible through the exoskeleton and has light green color.

Stage 4 Mature of fully ripe ovary; the shadow cast by the ovary is large, resulting in a very distinct dark thick region extending the length of the abdomen, with an enlarged bulbous region directly behind the carapace, called the saddle. The wide saddle of ovarian tissue directly behind the carapace is indicative of an immediate pre-spawning female. A female scored as a stage 4 during the day is most likely to spawn that night. The light green color of ovary is changed into dark green color.

Stage 5 Spawning; the shrimp has already spawned. The ovary is translucent and unpigmented, similar to stage 1 (Phiriyangkul, 2006).

Gonadosomatic Index (GSI) is common tools used as quantitative methods to verify the gonadal development and represent the percentage of these organs to the total weight of the animal (Magalhães *et al*., 2012).

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A

B

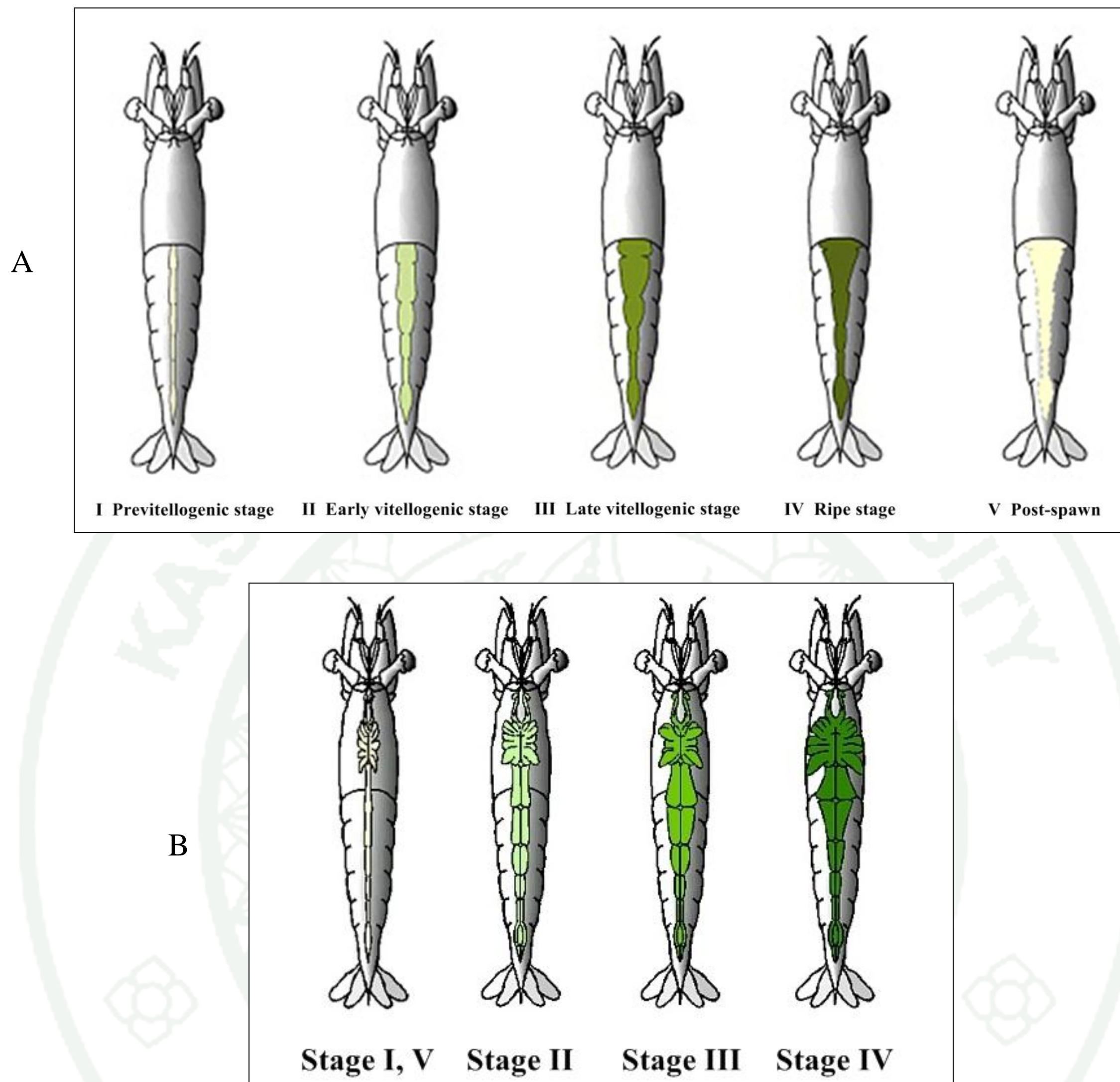


**Figure 8** The view observed by hatchery operators when female broodstock are graded for ovarian development by torchlight (A) and the complete ovary extends from head to tail, the majority of the ovarian mass is within the cephalothorax region which cannot be observed by torchlight (B).

**Source:** Hall *et al*. (1999)

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**Food allergy**

Adverse reactions to food can be divided into toxic and non-toxic reaction. Toxic reactions depend on the food itself and will occur in any exposed individual provided that the dose is high enough. Non-toxic reactions depend on the individual susceptibility to the food, and can be classified into immune-mediated or non-immune-mediated. Immune-mediated reaction can be further divided into IgE and non-IgE-mediated reaction (Fernández-Rivas *et al*., 2007). FAO/WHO (2001) suggested that there are eight most common food allergies comprises milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat and soybean.

Allergy is defined as a hypersensitivity reaction to intrinsically harmless antigens, most of which are environmental, and the process is initiated by specific immunologic mechanisms. The term food allergy has been recommended when an adverse reaction to food is mediated by immunologic mechanisms. Food allergens are defined as the antigenic molecules giving rise to the immunologic response. Proteins are the food constituents responsible for eliciting immune-mediated adverse responses to food. Hence, the eliciting dietary proteins are known as allergens (Pulido, 2010).

1. The allergic response and the role of IgE

Allergic reactions (or hypersensitivity reactions) are based on four different immunological mechanisms (Type I, II, III, IV) as first classified by Coombs and Gell (1975). These same mechanisms apply for food allergies and for allergic reactions to pollens, mold spores, animal danders, insect venoms, and drugs. The Type I mechanism also is called “immediate hypersensitivity,” and involves the formation of Immunoglobulin E (IgE). IgE-mediated reactions are the most important type of food allergy (Taylor *et al*., 2001).

IgE has a molecular weight of 190 kDa. IgE is found in extraordinarity low concentrations in the serum of unparasitized individuals, varying from 20-500 ng/mL. This is approximately 1/40,000 of the concentration of Immunoglobulin G (IgG). IgE

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is a heat-labile immunoglobulin of conventional four-chain structure. An IgE molecule contains two κ or λ light chains and two ε heavy chain. The Fc region of IgE binds strongly to high-affinity receptors on must cells and basophils and, together with antigen, mediates the release of inflammatory agents from these cells (Tizard, 1995). Molecule structure model of an IgE molecule is shown in Figure 9.

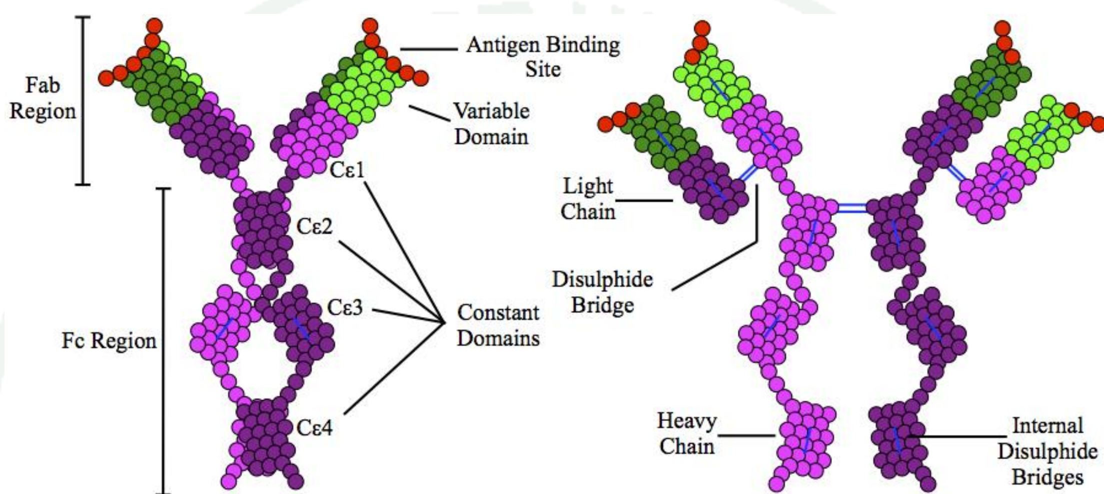


**Figure 9** The structure of the Immunoglobulin E (IgE) antibody.

**Source:** Sabban (2011)

Usually food allergens are proteins or glycoproteins with a molecular mass ranging from 10 to 70 kDa. Only a small percentage of proteins in foods are allergenic. These allergens belong to a variety of different protein families. Generally even traces of food allergens are capable of inducing allergic symptoms. In some cases the amount of allergen in certain foods which elicited an allergic reaction could be determined (Besler *et al*., 2001).

The development of allergy is a multistep process, and the mechanisms leading to sensitization, production of IgE antibodies, and allergic diseases are complex and not fully understood. In IgE-mediated food allergies, the allergen-specific antibodies are produced in response to stimulus of the antibody-forming B cells by a food allergen, usually a naturally occurring protein present in the food. The

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IgE antibodies bind to the surfaces of mast cells in the tissues or basophils in the blood. When the same food allergen is encountered on a subsequent occasion, the allergen-IgE complex is bound to high affinity IgE receptors (FcεR I) present on mast cells and basophils, and at least two IgE receptors are cross-linked by this event. This precipitates a cascade of biochemical events which causes cell membrane disruption and the release of a variety of mediators contained within granules existing in the mast cells and basophils. The granules in mast cells and basophils contain most of the important mediators of the allergic reaction to induced triggering marked allergic inflammatory responses. While more than 60 substances have been identified as chemical mediators emanating from mast cell and basophils, histamine is responsible for most of the immediate effects of allergic reactions (Bredehorst and David, 2001; Taylor *et al*., 2001).

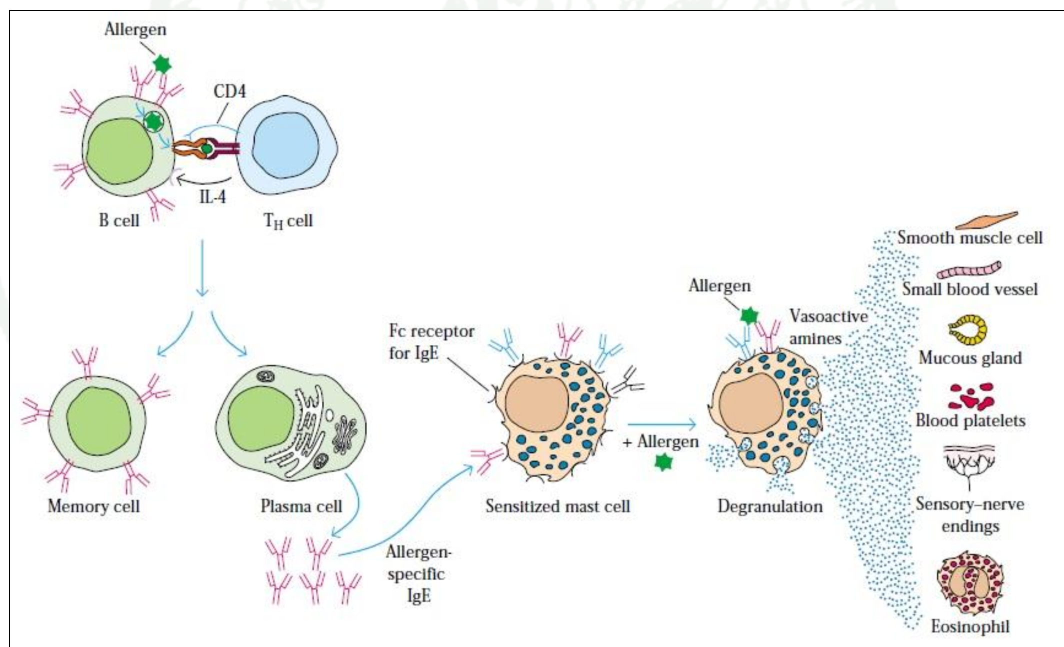


**Figure 10** General mechanism underlying a type I hypersensitive reaction.

**Source:** Goldsby *et al*. (2002)

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2. Symptoms caused by the allergic reaction

Food allergy is an immune system–mediated adverse reaction to food proteins that can affect multiple organs. When an allergen is eaten, two type of allergic response are seen. Activation of mucosal mast cell associated with the gastrointestinal tract lead to transpithelial fluid loss and smooth muscle contraction include abdominal pain, nausea, vomiting, and diarrhea (Janeway *et al*., 2001; Allan-Bock and Sampson, 2003; Helm and Burks, 2008). The allergens may be absorbed and cause mast-cell degranulation elsewhere in the body. Thus local reactions the skin can cause the development of edematous lesions commonly called hives, or urticaria. Respiratory symptoms such as asthma may also develop as a result of food allergies (Tizard, 1995).

Urticaria is the one of the most recognizable symptoms of immediate hypersensitivity. Acute urticarial triggered by food proteins typically appears within minutes of the ingestion of a food. Their brief duration, typically less than 24 hours, is an important characteristic of food-induced hives. Atopic dermatitis is more often food related, especially in young children (Bindslev-Jensen and Oesterballe, 2003; Allan-Bock and Sampson, 2003). Respiratory manifestations of immediate hypersensitivity reactions include rhinitis, sneezing, cough, laryngospasm, wheezing, tightness of the chest, and breathlessness. Ocular symptoms are often included with respiratory symptoms. In severe reactions, swelling of oral structures may occur that threaten the integrity of the airway (Allan-Bock and Sampson, 2003). The symptoms normally present together and sign from other organ systems, such as the respiratory or gastrointestinal (GI) systems. Moreover, the life-threating symptom is the cardiovascular manifestations of immediate hypersensitivity which primarily hypotension and the attendant changes in the circulation, leading rapidly to circulatory failure and death if not promptly and aggressively treated. It is important to note that severe food allergic reactions may present with the patient in shock, with no other symptoms or sign.

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Oral allergy syndrome (OAS) id the clinical term used to refer to food allergy symptoms involving the mouth and the pharynx. The mane of the symptom focuses on the need for direct contact of the oral mucosa with the offending food to trigger local symptoms, usually in the form of oral itching, lip swelling, and labial angioedema, but occasionally also glottal edema. OAS symptoms arise immediately (within 1-5 minutes) after the culprit foods come in contact with the oral mucosa. Symptoms recur regularly after each new exposure to the culprit foods (Pastorello and Ortolani, 2003).

Clinical anaphylaxis or anaphylactic shock is a symptoms associated with the classic features of IgE-mediated hypersensitivity. The symptoms results from the generation and release of a variety of potent biologically active mediators and their concerted effects by cutaneous, respiratory, cardiovascular, and GI signs and symptoms occurring singly or combination (Bruks and Sampson, 2003). This anaphylaxis can occur in some allergic patients but can cause the dead in several minutes.

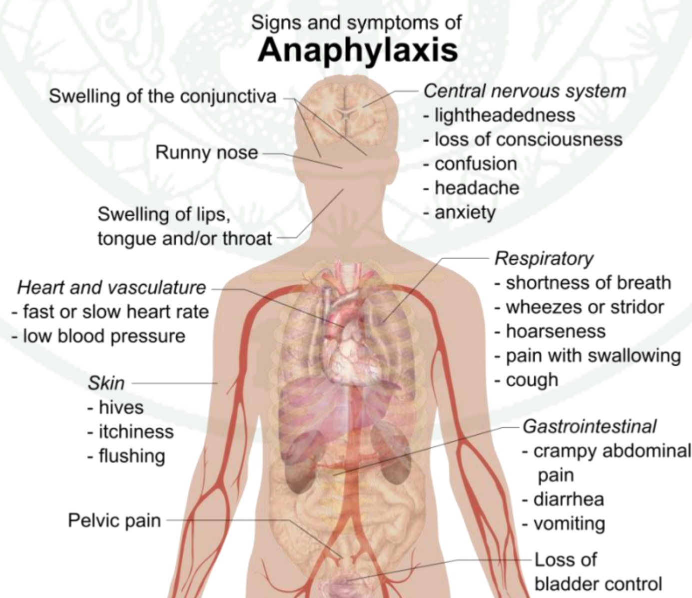


**Figure 11** Signs and symptoms of Anaphylaxis.

**Source:** Dr.K.T.S (2014)

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The primary therapy for food allergy is to avoid the causal food or foods. Education about avoidance includes careful attention to label reading, care in obtaining foods from restaurants/food establishments, and avoidance of cross-contact of foods with an allergen during meal preparation, such as avoiding shared cutting boards, slicers, and mixers (Sicherer and Sampson, 2010). Steensma (2003) has report about the importance of carefully counseling all patients who are allergic to food that they must avoid exposure to offending proteins via all potential routes of contact, including kissing and touching. Furthermore, presumed passionate kissing (between romantic partners on the lips or mouth) resulted in more significant manifestations compared with a casual kiss on the cheek, which usually caused local symptoms (contact urticaria) (Maloney *et al*., 2006).

3. Technique used to diagnose allergy

In food allergy, an accurate diagnosis is extremely important in particular to prevent patients from unnecessary and even potentially heath threatening diets. The very first step in the diagnostic work-up of food allergy starts with a detailed case history and a physical examination of the patient. Asking for clinical features, severity of reactions, type of food, time interval between ingestion of the incriminated food and the appearance of symptoms, or the procession state of the food eliciting symptoms (raw, cooked, frozen, etc.) provides important information to triage patients with adverse reactions to foods into ‘likely’ and ‘unlikely’ allergies (Fernández-Rivas *et al*., 2007).

3.1 *In-vivo* Test

1) Skin Prick Test (SPT)

Skin Prick testing is a cheap, rapid and accurate way of identifying causative allergens in an atopic individual (Potter, 1994). Skin testing to food antigen is usually performed by the skin prick test, in which a drop of 1:10 or 1:20 glycerinated food extract, a positive histamine control, and a negative saline control

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are placed on the skin (Metcalfe, 2003). A SPT reaction is considered to be positive in case of a mean wheal diameter of 3 mm or a wheal area of 7 $mm^2$ , respectively (Fernández-Rivas *et al*., 2007).

2) The atopy patch test

Procedure involving epicutaneous patch tests with allergens known to elicit IgE-mediated reactions and the evaluation of eczematous skin lesions after 24–72 hours in patients with aeroallergen- and food-triggered atopic dermatitis are called the atopy patch test. It has been standardized with regard to the use of vehicle and dose response relationships. Although there is increasing evidence that small subsets of patients with atopic dermatitis show atopy patch test positivity and serum-specific IgE negativity to the same allergen, as far as food allergy is concerned, the atopy patch test still requires standardization (Demoly *et al*., 2009).

3) Double Blind Placebo Controlled Oral Food Challenge Test (DBPCFC)

A double-blind placebo controlled food challenge (DBPCFC), the golden standard for the diagnosis of food allergy, is important to prevent adverse effects associated with an unnecessary elimination diet (Dambacher *et al*., 2013). The food challenge is administered in a fasting state, starting with a dose is then doubled every 15-30 minutes until the maximum dose is reached. It is important that the first dose is below the individual threshold level. However, patients may not be dismissed after an appropriate observation time of at least 2 hours after the intake of the last dose. A randomized challenge with an equal number of placebo and food allergens is necessary to control for a variety of confounding factors (Fernández-Rivas *et al*., 2007; Demoly *et al*., 2009).

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4) Skin Prick Test (SPT)

Since allergenicity of the food might be affected by processing the food for the blinded meal, a negative DBPCFC should wherever possible be followed by an open challenge. Open challenge are less time and personnel consuming and easier to perform. Thus, it is advisable to choose an open challenge as the first approach when a negative outcome of the challenge is expected (Fernández-Rivas *et al*., 2007).

3.2 *In-vitro* Tests

The direct demonstration of IgE binding to a particular allergen using immunochemical methods offers some advantages over skin tests because of the ease of automation, standardization, and efficiency. All such assays use immobilized allergens and sensitive methods for detecting IgE that has bound, and are known collectively as allergosorbent tests (AST) (Stewart et al., 2012).

1) Radio Allergosorbant Test (RAST) and Enzyme Allergosorbent Test (EAST)

To identify allergens in food products the RAST/EAST has been modified by pre-incubation of the human serum with protein extracts from the respective foods. RAST /EAST inhibition is the ideal tool for characterization of IgE binding properties of food allergens in various forms (crude protein extracts, purified allergen preparations, allergenic activities of different varieties or species and various food products) (Besler, 2001).

In enzyme-linked and radioallergosorbent assays (EAST/RAST) used for allergen characterization purposes, increasing concentrations of allergen are coupled to a solid phase and then incubated with a constant volume of serum from an allergic donor. For diagnostic purposes, a constant amount of allergen is coupled to the solid phase and varying dilutions of serum are then added. The amount of IgE

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bound is detected using a labeled anti-IgE. In each case, a positive dose response curve is obtained (Figure 12B). In EAST/ RAST-inhibition, allergic serum is incubated with fluid phase allergen prior to incubation with solid phase allergen (Figure 12A). The more potent the fluid phase allergen, the less free IgE is available to bind to the solid phase allergen. This gives rise to a negative dose response curve, which can then be converted to a percent inhibition dose responsive curve (Figure 12B) (Stewart *et al*., 2012).

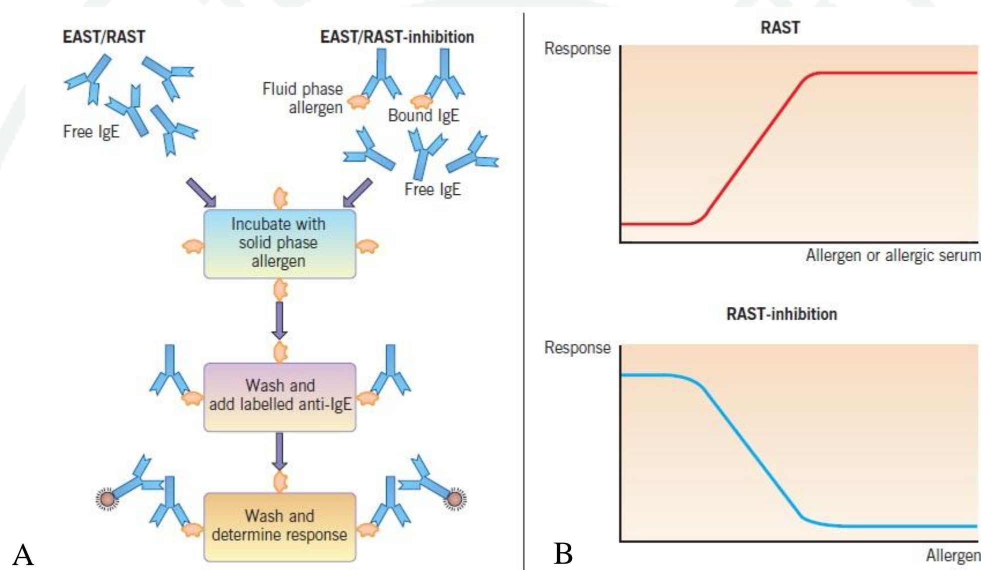


**Figure 12** Outline of the steps involved in allergosorbent and allergosorbent-inhibition assays.

**Source:** Stewart *et al*. (2012)

A major disadvantage of RAST or EAST inhibition is the difficulty of standardization due to its reliance on human sera, amounts of which are often limited. Although RAST /EAST inhibition specifically detects food allergens, variable specificity of human IgE antibodies hinders use in a wider range of analytical laboratories (Besler, 2001).

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For the example about application of the RAST inhibition method is the detection of alpha-Lactalbumin in baby food and food quality lactose. They use human IgE as antisera. For the result, the standard curve gave a range of detection from 100 ng/mL to 10 µg/mL, resulting in a limit of detection of 1 mg/kg in the samples (Frémont *et al.* 1996).

2) SDS-PAGE and / or IEF-PAGE immunoblotting

The electrophoretic separation of food protein extracts by isoelectric focusing (IEF) and SDS-PAGE (or both in two-dimensional electrophoresis) allows the identification of individual allergens after immunoblotting. SDS-PAGE immunoblotting has been involved in the identification and characterization of almost every known food allergen. Two-dimensional PAGE is often used to identify isoallergens with the same molecular mass but differing in isoelectric points due to minor differences in amino acid sequence (Besler, 2001). For more information about SDS-PAGE, 2D-PAGE and immunoblotting will be described in the topic of proteomic method.

3) Double immunodiffusion (Ouchterlony)

Immunodiffusion in gels are classified as single diffusion and double diffusion. In ouchterlony double diffusion, both antigen and antibody are allowed to diffuse into the gel. This assay is frequently used for comparing different antigen preparation. In the Ouchterlony assay, solutions of an antigen and an antibody are placed in nearby wells cut out of a thin layer of agarose, and allowed to stand for a few hours or a day or two. During that time they diffuse toward each other, and where they meet they will form a visible line of precipitation. The pattern in which adjacent lines cross one another yields considerable information about the antigenic relationships between different antigens (Irvine College of Medicine, University of California, 2014).

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Malmheden-Yman *et al.* (1994) reported the detection of gluten in pasta and buckwheat products by double immunodiffusion. Major limitations are the qualitative use only, poor sensitivity, and the time-consuming though very easy procedure (24-48 hours).

4) Dot immunoblotting

Dot immunoblotting (also known as dot blot) immunoassays, used extensively in immunochemical research, have great potential significance for diagnostic testing in the clinical laboratory. The potential for diagnostic applications includes: assay of antigens from pathologic serum samples, body fluids, and tissue; assay of patient serum samples for antibody against known antigen; and separation and assay of patient immunoglobulin. Dot immunoblotting, in a similar fashion, permits assays of multiple specimens simultaneously on single strips of blotting media using sample sizes as small as 0.1 μL (Renner, 1988).

In the application of dot blot is detection of peanut protein in food. Peanut protein extract was spotted on polyester cloth pre-coated with anti-peanut antibodies (chicken IgY). Bound peanut were detected by sequential reactions with peroxidase-conjugated anti-peanut antibodies and chromogenic substrate. So, positive results with samples containing less than 1 ppm peanut protein were detected (Blais and Philippe, 2000).

5) Rocket immunoelectrophoresis (RIE)

Rocket immunoelectrophoresis (also referred to as electroimmunoassay) is a simple, quick, and reproducible method for determining the concentration of a specific protein in a protein mixture (Walker, 1984). This analytical method involves an antibody containing gel. The sample proteins migrate according to their electrophoretic mobility until antigen-antibody complexes precipitate in the gel building ‘rockets’ at a constant antigen/antibody ratio. The height of the rocket-shaped precipitation zone is proportional to the applied amount of sample protein.

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Precipitates can be either Coomassie stained or (more sensitively) immunostained (Besler, 2001).

Holzhauser *et al.* (1998) has reported about the detection of peanut allergen in processed food with RIE techniques. The result show that this technique had detecting sensibility about 20 ng/mL peanut protein in chocolate extract, equivalent to 0.0002% peanut in chocolate, could still be detected. And described a reliable, specific, and sensitive RIE application for the determination of peanut proteins in chocolate and other food products.

6) ELISA

The ability of an ELISA method to detect food allergen proteins in a test sample is affected by the efficiency with which these proteins are extracted from the sample, as well as the efficiency with which the antibody or antibodies used in the ELISA detect these proteins in the sample extract (Abbott *et al*., 2010). Although there are a huge number of variations based on the original ELISA principle, but the most useful and routinely performed: (i) the indirect sandwich ELISA-providing high sensitivity (Figure 13B) and specificity and (ii) the basic direct ELISA-useful when only one antibody to the sample antigen is available (Figure 13A) (Jordan, 2002).

During the indirect sandwich ELISA, an antibody specific for the substance to be measured is first coated onto a high-capacity protein binding microtiter plate. Any vacant binding sites on the plate are then blocked with the use of an irrelevant protein such as Bovine serum albumin (BSA). The samples, standards, and controls are then incubated on the plate, binding to the capture antibody. The bound sample can be detected using a secondary antibody recognizing a different epitope on the sample molecule, thus creating the “sandwich”. In some cases, however, only one specific antibody may be available, and in such a small quantity that directly conjugating it to biotin would be impractical. In this situation a direct ELISA should be used. During the direct ELISA the sample itself is coated directly onto the microtiter plate and is then detected using the specific antibody (Jordan,

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2002). And, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate.

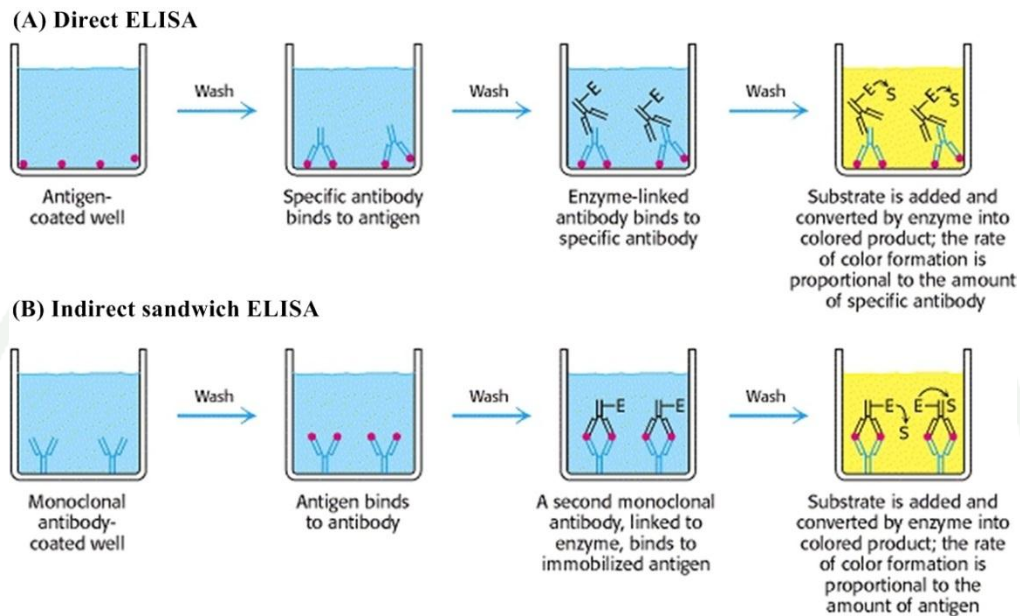


**Figure 13** Outline of ELISA setups.

**Source:** Chakravarthy (2014)

**Crustacean shellfish allergy and its allergen**

Crustacean shellfish is common food on many menus and important part of the healthy diet. A large variety of crustaceans play an important role as a food source for humans and the consumption of shrimp has tripled since 1970 (Sicherer *et al*., 2004). Crustacean shellfish such as shrimp, crab, and lobster, and mollusks such as squid, scallop, clams, and snails are one of the eight important allergenic foods suggested by FAO/WHO (2001). Thus, increased production and consumption of crustaceans can increase the number of shellfish-allergic patients and result in the majority of emergency department visits, not only in adults but also in children (Clark *et al*., 2004). According to the study of Ross *et al*. (2008) and Liu *et al.* (2008), Shrimp is

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the most frequently implicated food from the hospital emergency department. Life-threatening and fatal anaphylaxis cases have been reported occasionally.

In a random digit-dial telephone survey conducted in the United States of almost 15,000 individuals, crustacean shellfish allergy was identified as the most common food allergy, with an estimated prevalence of 1.9% of the population, or approximately 5.4 million American (Sicherer et al., 2004). Crustacean shellfish allergy is common not only in western but also in Asian countries where allergic reactions to seafood and particularly shellfish are significant among children and adults. Chiang *et al*. (2007) has reported that 39% of Asian children and 33% of Asian adults has the shellfish allergy by the skin tests of 227 symptomatic allergic disease patients.

Crustacean shellfish belong to the Anthropoda phylum. Crustacean shellfish include shrimp, prawns, crab, lobster, crayfish, and barnacles. The majority of individuals with allergies to crustacean shellfish appear to be allergic to all crustacean species, including shrimp, crab, and lobster. However, some individuals appear to be reactive only to specific species (Lee and Taylor, 2011). From Jirapongsananuruk *et al.* (2008) study, food challenges identified specific allergy to seawater shrimp (*P. monodon*) and freshwater shrimp (*Macrobrachium rosenbergii*) found that a food challenge is not feasible because the relative between two species shrimp allergen.

Crustacean shellfish appear to contain multiple allergenic proteins. However, the 34-39 kDa muscle protein, tropomyosin (TM) is viewed as a particularly important major allergen and a pan-allergen responsible for most of the widespread cross-reactivity observed between various crustacean shellfish species (Lee and Taylor, 2011). TM comprises a class of highly conserved proteins with multiple isoforms found in both muscle and non-muscle cells of all species of vertebrates and invertebrates. It is an abundant and heat-stable protein that constitutes up to 20% of total protein in the edible part of the animal. It is physically associated with actin and myosin in muscle fibers and other motile filaments (Shanti *et al*., 1993; Reese *et al*., 1999).

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In addition to tropomyosin, other allergens have been identified and characterized in crustaceans such as the 40 kDa arginine kinase (AK), which might be a new class of invertebrate pan-allergens (Lopata and Lehrer, 2009). Yu *et al.* (2003) was identified a novel allergen from *P. monodon* by two-dimensional immunoblotting using sera from subjects with shrimp allergy, followed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of the peptide digest. AK is a phosphotransferase that plays a critical role in energy metabolism in invertebrates and play an important role in the coupling of energy production for muscle mobilization and utilization and the immune response in shrimps. AK was present in very large quantities in shrimp muscle tissue (France *et al*., 1997; Yao *et al*., 2009).

Ayuso *et al*. (2008) demonstrated IgE binding to a 20 kDa allergen in 55% of patients reacting to the white pacific shrimp (*Litopenaeus vannamei*). The recombinant protein was subsequently produced and identified as a myosin light chain (MLC) with high amino acid similarity to Bla g 8 from cockroaches. Myosin light chain, a well-known cytoskeleton gene, regulates multiple processes that are involved in material transport, muscle shrink and cell division (Han *et al*., 2010)

Moreover, the 20 kDa, Sarcoplasmic calcium-binding protein (SCP) was suggested as a new allergen in shrimp. Shiomi *et al*. (2008) purified SCP protein extract by salting-out, anion-exchange HPLC and reverse-phase HPLC from *P. monodon*. SCP valuated for IgE reactivity by ELISA and confirmed the allergenicity by immunoblotting surveyed with 5 species of crustaceans. Ayuso *et al*. (2009) has studied by immunoblotting demonstrated IgE binding by 31 of 52 (59.6%) of the sera to a 20-kd shrimp protein. The protein was identified as a SCP. Amplified cDNA encoding SCP was isolated and sequenced. SCP is also a muscle protein. It has been speculated that invertebrate SCP may serve a similar function as vertebrate parvalbumins-that is, promoting rapid muscle relaxation by facilitating calcium translocation from myofibrils to the sarcoplasmic reticulum-and may protect against high calcium concentration inside the cell (Takagi and Konishi, 1984; Gao et al., 2006).

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A recent study by Piboonpocanun *et al*. (2011) demonstrated IgE binding to ~60–80 kDa proteins, hemocyanin (HC) using immunoblotting and inhibition ELISA from 12 of 13 *Macrobrachium rosenbergii* allergic subjects. HC is identified as heat-stable, non-cross-reactive, high-molecular-weight (MW) allergens in *M. rosenbergii*. Since circulatory organs are not always removed during food preparation, high concentrations of HC may be present along with shrimp meat, which contains the known cross-reactive tropomyosin protein.

In the summary, the allergenic proteins characterized in Crustacean shellfish were show in Table 2.

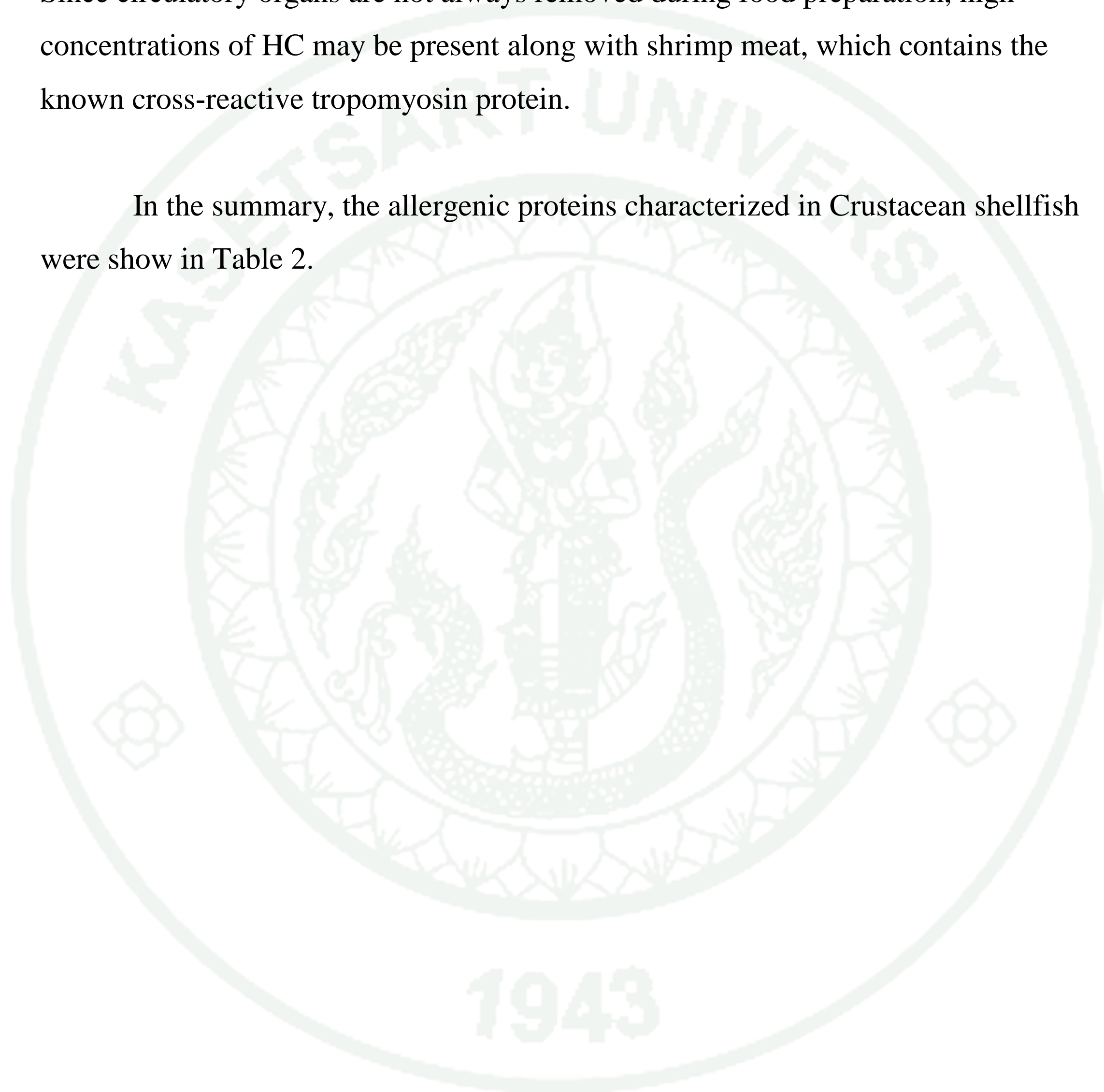

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**Table 2** Allergenic proteins characterized in Crustacean shellfish.

| Allergen name | Allergen Nomenclature | MW (kDa) | Species | Common Name | References |
|---|---|---|---|---|---|
| Tropomyosin | 1) Pan s 1<br>2) Hom a 1<br>3) Met e 1<br>4) Pen i 1<br>5) Pen f 1<br>6) Pen a 1 | 34 - 39 | 1) *Panulirus stimpsom*<br>2) *Homarus americanus*<br>3) *Metapenaeus ensis*<br>4) *Panaeus indicus*<br>5) *Parapenaeus fissurus*<br>6) *Panaeus aztecus* | Chinese spiny lobster<br>American lobster<br>Greasyback shrimp<br>Indian prawn<br>Neptune rose shrimp<br>Brown shrimp | 1) Leung *et al*. (1998)<br>2) Leung *et al*. (1998)<br>3) Leung *et al*. (1994)<br>4) Shanti *et al*. (1993)<br>5) Lin *et al*. (1993)<br>6) Daul *et al*. (1994) |
| Arginine kinase | 1) Pen m 2<br>2) Lit v 2 | ~40 | 1) *Panaeus monodon*<br>2) *Lipopenaeus vannamei* | Giant tiger prawn<br>Whiteleg shrimp | 1) Yu *et al*. (2003)<br>2) Garcia-Orozco *et al*. (2007) |
| Myosin light chain | Lit v 3 | 20 | *Litopenaeus vannamei* | Whiteleg shrimp | Ayuso *et al*. (2008) |
| sarcoplasmic calcium-binding protein | 1) -<br>2) Lit v 4.0101 | ~20 | 1) *Panaeus monodon*<br>2) *Litopenaeus vannamei* | Giant tiger prawn<br>Whiteleg shrimp | 1) Shiomi *et al*. (2008)<br>2) Ayuso *et al*. (2009) |
| Hemocyanin | - | ~60-80 | *Macrobrachium rosenbergii* | Giant fresh water prawn | Piboonpocanun *et al*. (2011) |



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During the manufacture of food their allergenicity may be altered by various processing parameters. The allergenic activity may be unchanged, decreased or even increased by food processing (Besler *et al*., 2001). TM has been detected in the cooking water, but in general there have been few studies on the impact of cooking on shellfish and crustacean allergenicity (Mills *et al*., 2012). Thus, it is necessary to study the impact of food processing. This can provide the novel diagnostic and therapeutic strategy in the future.

**Food processing treatment**

The aims of food processing are to ensure microbiological and chemical safety of foods, adequate nutrient content and bioavailability, and acceptability to the consumer and caregiver with regard to sensory properties and ease of preparation (WHO, 1998). Numerous foods are processed in different ways at home, in institutional settings, and in industry depending on the processing method and the food (Sathe and Sharma, 2009). In the present, raw shrimp may be cooked under a variety of condition with heat using such as boiling (blanching), Microwave oven process, ultrasonicated or water immersion sterilization (autoclave/retort).

1. Boiled (blanching) treatment

In general, it is conventional to cook shrimp in water or in brine solution at the temperature of from about 70-100 °C for a specified time (Gillies, 1975). The heat received by a food during boiling or blanching inevitably causes some changes to sensory and nutritional qualities. Minerals, water-soluble vitamins and other water-solution components are lost during boiling or blanching. Cupisti *et al*. (2006) suggested that boiled foods can reduce dietary phosphate while preserving protein intake, namely reducing the effective phosphate intake per gram of dietary protein. Moreover, Boiling and blanching brightens the color of some foods and thus altering the wavelength of reflected light. The time and temperature of boiling also influence the change in food pigment (Fellows, 1988).

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2. Autoclaved treatment

The autoclave/retort exist from the first techniques of the preserving food, it evolved as well on the products as on the packaging or canning food products. An autoclave is a device used to sterilize equipment and supplies by subjecting them to high pressure saturated steam at 121 °C for around 15-20 minutes depending on the size of the load and the contents (Black, 1993). The purpose of heat sterilization is to extend the shelf life of foods while minimizing the changes in nutritive value and eating quality.

The time-temperature combinations used in canning have a substantial effect on most naturally occurring pigments in food. Maillard browning and caramelisation also contribute to the color of sterilized meats but this is an acceptable change in cooked meats. In canned meats, changes in texture are caused by coagulation and a loss of the water-holding capacity of protein, which produces shrinkage and stiffening of muscle tissue. Autoclaving process causes the hydrolysis of carbohydrates and lipids, but these nutrients remain available and the nutritive value of the food is not affected. Protein are coagulated and loss of 10-20% amino acids (Fellows, 1988).

3. Ultrasonicated treatment

Nowadays, power ultrasound is considered to be an emerging and promising technology for industrial food processing (Dolatowski *et al*., 2007). Ultrasound is defined as mechanical waves with a frequency above the threshold of human hearing (16 to 20 kHz). It can be divided into two frequency ranges; high frequency ultrasound (low-power ultrasound) uses frequencies in the 5-10 MHz range with low intensity levels. It is mainly used in diagnostic analysis of food materials. Low-frequency ultrasound (high-power ultrasound) uses frequencies in the 20-100 KHz range with much high intensity levels. The applications of high-power ultrasound in the food industry include extraction (release of plant material),

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inactivation of microorganisms, enzyme inhibition, homogenization, emulsification, filtration, crystallization, and deforming (Fahmi *et al*., 2011).

Today, ultrasound technology is routinely used by the beef industry for reduced the beef tenderness (Dolatowski *et al*., 2007). Moreover, ultrasound treatment was used to extend the shelf life of roasted peanuts by removing oils on peanut kernel surface (Baysal and Demirdoven, 2012). Ultrasound processing can be used as an alternative method to thermal treatments in the food preservation without destroying nutrients of foods (Ercan and Soysal, 2013). Tiwari, et al. (2009) found that vitamin C retention of orange juice after ultrasonic treatment is higher when it is compared to thermal processing. Also, Dubrović *et al*. (2011) demonstrated that total anthocyanin content in strawberry juice was greater in more than 85 % of the selected ultrasound treatments compared to pasteurized juices.

4. Microwaved treatment

Started as a by-product of the radar technology developed during World War II, microwave heating is now used in about 92% of homes in the USA and Thailand was the second largest seller inferior to China, holding 13% of the sales market, producing 395 million dollars (Thompson, 2014). Microwave heating takes place due to the polarization effect of electromagnetic radiation at frequencies between 300 MHz and 300 GHz. Electromagnetic waves are waves of electrical and magnetic energy moving together through space including gamma rays, x-rays, ultraviolet radiation, visible light, infrared radiation, microwaves and the less energetic radio waves. Microwaves can pass through materials like glass, paper, plastic and ceramic, and be absorbed by foods and water; but they are reflected by metals (Food and Environmental Hygiene Department [FEHD], The Government of the Hong Kong Special Administrative Region, 2005).

Microwave heating has also found applications in the food industry, including tempering of frozen foods for further processing, pre-cooking of bacon for institutional use, and final drying of pasta products (Tang *et al*., 2002). Proteins would

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be denatured with the modification in molecular structure upon heating. The degradation rates depend on the heating time and temperature. It has been shown that the nutritive value of proteins in foods treated by conventional and microwave heating are comparable (FEHD, The Government of the Hong Kong Special Administrative Region, 2005).

**Food processing effects to food allergen**

Food processing, which includes the many step a food goes through between the producer and the ultimate consumer can modify proteins which can change the functionality, nutritional quality, or safety of the food (Finley, 1988). In addition to alteration of allergenic potencies the problem of allergen contamination is present in processed foods. There are no reliable data on the frequency of contamination of processed foods with undeclared allergens (Besler *et al*., 2001). According to shrimp allergen reported previously, several studies proved the heat stability of the allergenic potential of shrimps after food processing.

IgE - binding epitopes are either conformational or linear in nature. Processing can have a disruptive effect on the native protein structure of an allergen, which can result in disruption of the IgE binding epitopes and consequently modify the ability of an allergen to elicit a reaction. On the other hand, food processing could also unveil or create new epitopes, sometimes termed neo-epitopes. This may occur through protein unfolding, which unravels inner portions of the protein structure, not generally available for antibody binding or the covalent modifi cation of a protein by sugars or other food components (Wal, 2003; Maleki and Sathe, 2006). Thus, cooking process might be alter the allergenicity of banana shrimp, may destroy existing epitopes on a protein or may generate neo-allergenic formations (Maleki *et al*., 2000; Taylor *et al*., 2008).

However, the study of how heat processing can alter the allergenicity of food is a way to develop the hypoallergenic food.  Therefor, Clinical investigations of the allergenic potential of proteins must take into consideration both the potential

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decrease as well as the possible increase of antibody-binding capacity, which can result from protein unfolding during processing. Several chemical and technological approaches have been considered to produce non- or hypoallergenic foods by removal, destruction, proteolytic modifi cation, or masking of epitopes (Boye *et al*., 2010).

Liu *et al.* (2010) demonstrated the major allergen, TM, of shrimp (*Litopenaeus vannamei*) allergenicity by iELISA and dot-blot analysis. The inhibition ELISA (iELISA) results showed that extracts from raw shrimp bound higher IgE than extracts from boiled shrimp; dot-blot assay demonstrates higher IgE binding to purified TM from boiled shrimp than raw shrimp. For the diagnosis of seafood allergic individuals, *in vivo* SPT results showed that shrimp boiled extracts induced statistically significant larger wheals than raw extracts. More patients with positive results were also detected with shrimp boiled extracts; this result was confirmed by *in vitro* direct ELISA (Carnés *et al*., 2007). Moreover, water-soluble shrimp allergens released during boiling were characterized and compared to allergen extracts from boiled shrimp muscle by RAST and RAST inhibition. Results demonstrated that shrimp water is an excellent source of shrimp allergens (Lehrer *et al*., 1990).

*Penaeus vannamei* muscle was treated by high-intensity ultrasound were analyzed by SDS-PAGE, Western blots and competitive inhibition ELISA (Ci-ELISA) to determine the shrimp allergenicity. The results shown a significant decrease in the level of the major shrimp allergen, Pen a 1, when the samples were treated by high-intensity ultrasound compared with raw muscle extract (Zhenxing *et al*., 2006).

Nowadays, the studies in food processing on the shrimp allergenicity is limited but there are more reported about other food allergenicity after food processing treatments. Alvarez-Alvarez *et al*. (2005) has studies the effect of food processing on allergenicity of lupine seeds. IgE-immunoblotting and CAP Inhibition assays were used to analyze the IgE-binding ability of food processed lupine. The result showed

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that autoclave processing can ostensibly reduce the lupine allergenicity whereas boiled and microwave has slightly reduced the lupine allergenicity.

Hazelnut allergen after autoclave processing was analyzed by IgE-binding immunoblotting and *in vivo* via Prick-Prick test. A severe reduction *in vitro* in allergenicity to hazelnut after autoclave processing was observed in the allergic clinic-patients studied (López *et al*., 2012). Stanic-Vucinic *et al*. (2012) had studied the allergenicity of β-lactoglobulin upon exposure to high-intensity ultrasound. Sonication had a minor effect on IgE-binding properties of β-lactoglobulin from the result of IgE binding ability and skin prick testing in 41 cow's milk allergy patients.

Food processing may help inactivate certain conformational epitopes on some, but not all allergens and is unlikely to eliminate linear epitopes. When food allergens are present in trace quantities (e.g. as "hidden allergens" or as "contaminants"), avoidance of the offending agent requires the foreknowledge of their presence. Therefore, there is a critical need to develop robust, reliable, sensitive, and accurate allergen detection methods (Sathe *et al*., 2005).

**Proteomic approaches for identifying allergens**

The term “proteomics” was introduced to refer to the study of the proteome. In a more recent definition, the proteome is the set of all the proteins of a cell, organism or biological medium at a given moment. This includes all the proteins modified by alternative splicing of primary transcripts, posttranslational processing or a combination of both (González-Buitrago *et al*., 2007). In the field of allergy, the former approach is mainly used to discover new allergens (Figure 14).

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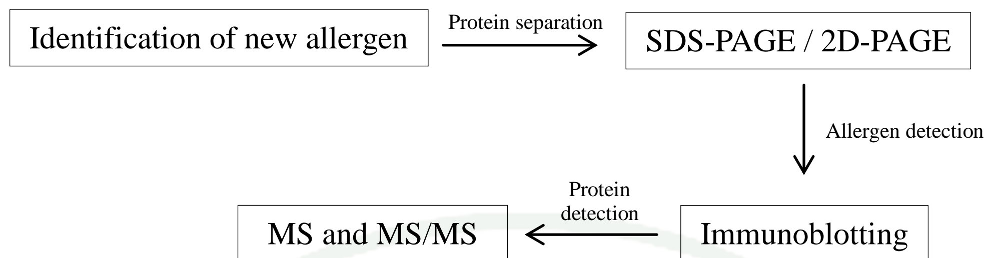


**Figure 14** Proteomic technological approaches used in allergy.

1. Protein separation

Separation of peptides and proteins represents a key element in proteomics analysis, with the choice of separation method depending strictly on experimental needs (Monaci and Visconti, 2009). To date, the standard method for protein separation is polyacrylamide gel electrophoresis. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) is a major application in the protein separation by molecular size. Moreover, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is more suitable for separation protein by molecular size and isoelectric point (*pI*). The proteins are first subjected to isoelectric focusing (IEF), which separates them in the first dimension according to their isoelectric point (*pI*), and subsequently, in the second dimension, to SDS-PAGE, which separates them on the basis of their relative masses (González-Buitrago *et al*., 2007).

In selecting condition for the separation of a particular protein mixture, a buffer pH that gives the greatest difference in the charge carried by each individual protein results in the greatest difference in velocities and in the final distances moved. In practice, buffer of pH 8.6 are most frequently used. The zones or band of protein that develop during electrophoresis can be precipitated in the pore of the supporting medium by trichloroacetic acid and stained using a suitable dye (Coomassie blue, Silver stain, etc.) (Holme and Peck, 1998). Proteins resolved by electrophoresis are electrotransferred onto a nitrocellulose membrane or suitable transfer membrane for allergen detection process.

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2. Allergen detection

Antibodies may also be used to determine the presence or identity of soluble antigens by a process known generally as immunoblotting. Once on the membrane antibodies may be used to probe for the presence of particular allergen either directly or indirectly (Figure 15). Non-specific binding site may be 'blocked' using other non-specific protein such as bovine serum albumin or casein before washing to remove unbound antibody. The labeled molecule may be visualized by the colorimetric detection, chemiluminescent, redioactive detection, or fluorescent detection (Holme and Peck, 1998).

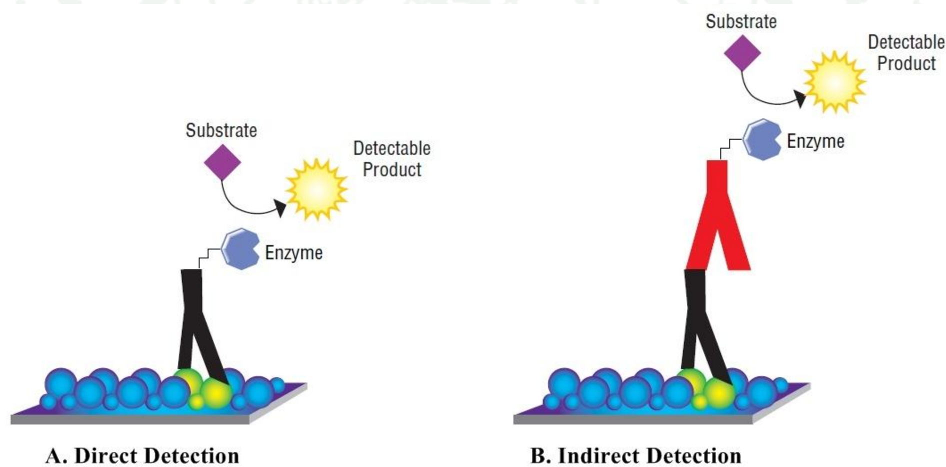


**Figure 15** In the direct detection method, labeled primary antibody binds to antigen on the membrane and reacts with substrate, creating a detectable signal (A). In the indirect detection method, unlabeled primary antibody binds to the antigen. Then, a labeled secondary antibody binds to the primary antibody and reacts with the substrate (B).

**Source:** Pierce Biotechnology (2004)

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3. Protein identification

In proteomic analyses the identification of proteins is accomplished by means of mass spectrometry (MS). To identify a protein by MS using trypsin digestion of the proteins, two main approaches can be used. The first is peptide mass fingerprinting and the second is peptide sequencing. Peptide mass fingerprinting (PMF) is usually performed with MALDI-TOF MS, whereas peptide sequencing uses tandem MS (MS/MS) (González-Buitrago *et al*., 2007).

While mono-stage MS usually performs PMF, MS/MS is most used for peptide sequencing. MS/MS also provides detailed structural features of peptides that can be inferred from analysis of the resulting fragments. MS/MS is commonly used in product-ion mode to determine the amino-acid sequence of a specific peptide. After ionization, the mixture of charged peptides enters the first mass spectrometer, where the peptides are separated according to their mass/charge ratios. This technique is available on all instruments equipped with MS/MS capabilities (González-Buitrago *et al*., 2007; Monaci and Visconti, 2009).

For final protein identification, manual interpretation of the complex fragment spectra is complicated and very time consuming. Therefore, computer programs were developed which could accomplish ‘‘online’’ data evaluation. The most commonly used are Sequest, Mascot and MS-tag. These results are usually very discriminating and highly significant in identifying a protein present in a data base. By these approaches, the peptides identified are compiled into a protein ‘‘hit list’’. Here, not only proteins with a fully determined amino acid sequence stored in the data base can be identified (Bayard and Lottspeich, 2001; Monaci and Visconti, 2009).

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# MATERIALS AND METHODS

## Materials

### Banana shrimp, *Fenneropenaeus merguiensis*

Live banana shrimp, each weighing approximately 30 to 40 g, were purchased in Chonburi province, Thailand. After cold shock and washing, shrimp were peeled and organs—muscle, shell, hepatopancreas and ovaries with 3 different levels of gonadosomatic index (GSI)—were collected. Shrimp were defined by different stages of their developing ovaries as follows: stage 1 (GSI = 0.995 ± 0.104) as previtellogenic; stage 2 (GSI = 2.998 ± 0.175) as early vitellogenic and stage 3-4 (GSI = 8.273 ± 0.092) as vitellogenic (Phiriyangkul et al., 2007). The gonadosomatic index measures the cyclic changes in gonad weight in relation to total tissue weight and is represented by the formula: GSI = [gonad weight/total tissue weight] x 100 (Barbar and Blake, 2006).

### Human serum

The sera used in this study were allergen-specific to shrimp and were obtained from 21 shrimp-allergic patients at Phramongkutklao Hospital and Phyathai Hospital (Bangkok, Thailand). Control sera were obtained from 30 healthy subjects without any shrimp allergy and no reported adverse reactions to any food stuff at Phramongkutklao Hospital. The uses of sera were approved by the Institutional Review Board at Phramongkutklao Hospital (R101h/2009).

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**Chemicals and Reagent**

1. Analytical or/and molecular biology grade

Chemicals used were analytical or/and molecular biology grade and purchased from the following companies.

**Table 3** Analytical grade chemicals used.

| **Chemical** | **Company** |
|---|---|
| 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPs ) | USB Corporation |
| Acetic acid | RCI Labscan Limited |
| Acetonitrile (ACN) | Carloerba |
| Acrylamide, 99% | Bio-Rad |
| Agarose | CAMBREX |
| Amersham ECL Plus™ western blotting detection reagents | GE Healthcare |
| Ammonium bicarbonate ($NH_4HCO_3$) | Merck |
| Ammonium persulphate (APS) | SIGMA |
| Ampholines pH 3-10 | Serva |
| β-Mercaptoethanol | Acros Organics |
| Bisacrylamide (N,N'-Bis-methyleneacrylamide) | Bio-Rad |
| BlockACE | Dainippon Pharmaceutical |
| Bradford reagent | Bio-Rad |
| Bromophenol blue | SIGMA |
| Calcium chloride ($CaCl_2$) | Merck |

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**Table 3** (Continued)

| Chemical | Company |
| --- | --- |
| Coomassie brilliant blue R-250 | Serva |
| Dithiothreitol (DTT) | USB Corporation |
| Ethanol | Merck |
| Ethylenediaminetetraacetic acid (EDTA) | Fluka Chemical |
| Formic acid | Merck |
| Glycerol | USB Corporation |
| Glycine | USB Corporation |
| Hydrochloric acid | Labachemie |
| Iodoacetamide (IAA) | SIGMA |
| IPG buffer pH 3-10 | GE Healthcare |
| Medical X-ray film developer and replenisher | Kodak GBX |
| Medical X-ray film fixer and replenisher | Kodak GBX |
| Methanol | Fisher Chemical |
| Protease inhibitor cocktail #P8340 | SIGMA |
| Skim milk | - |
| Sodium chloride (NaCl) | RFCL limited |
| Sodium dihydrogen phosphate dehydrate ($NaH_2PO_4$•$2H_2O$) | Merck |
| Sodium dodecyl sulphate (SDS) | SIGMA |
| Sodiumhydrogenphosphate ($Na_2HPO_4$) | Merck |
| TEMED (N,N,N’,N’-Tetramethylethylenediamaine) | GE Healthcare |
| Thiourea | Amersham Bioscience |
| Trifluoroacetic Acid (TFA) | APPILED Bio Systems |

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**Table 3** (Continued)

| Chemical | Company |
| --- | --- |
| Tris-base | USB Corporation |
| Tween 20 | EMD chemical (OmmiPur) |
| Trypsin, sequencing grade modified | Promega |
| Trypsin resuspension buffer | Promega |
| Urea | Bio-Rad |

2. Antibody

Horseradish peroxidase (HRP) conjugated goat anti-human IgE was purchased form Immunology consultants Laboratory Inc. (Newberg, OR, USA).

3. Protein standard marker

PageRuler Prestained Protein Ladder, product number #26616 was purchased from Thermo Fisher Scientific.

4. Scientific test kit

GelCode® Glycoprotein Staining Kit was purchased Thermo Fisher Scientific.

**Equipment and instruments**

1. Immunoblot equipment

Immobilon-P transfer membrane was purchased from Millipore Corporation. Amersham® Hyperfilm® ECL™ was purchased from GE Healthcare Bio-Sciences AB.

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2. Instruments

Instruments used from the following model and companies.

**Table 4** Instruments used.

| **Instrument** | **Model** | **Company** |
|---|---|---|
| Analytical Balance | XS205 DualRange | METTLER TOLEDO |
| Autoclave | SS325 | Tomy Digital Biology |
| Battery powered pipette controller | Pipet-X | METTLER TOLEDO |
| SpeedVAC | CentriVap Console | LABCONCO |
| Electrophoresis and Blotting Systems | mini-PROTEAN tetra cells | Bio-Rad |
| | SE 250 Mini | Hoefer |
| Heat box | Multi-Blok Heater | Lab-Line |
| High speed refrigerated centrifuge | CR20B2 | Kitachi |
| High speed refrigerated microliter centrifuge | 1-15K | SIGMA |
| Image scanner | ImageScanner III | Amersham Biosciences |
| Ion trap mass spectrometers | amaZon speed | Bruker Daltonics |
| Laboratory fume hood | IIM | I-LAB |
| Magnetic stirrer | MR Hei-Mix L | Heidolph |
| Manual Single channel Micropipettes | RAININ Classic | METTLER TOLEDO |
| | Pipet-Lite | METTLER TOLEDO |
| Microlitre centrifuge | MIKRO 120 | Hettich Instruments |
| Microwave oven | EMM2007X | Electrolux |
| Multipurpose shaker | Orbit 1000 | Labnet International |

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**Table 4** (Continued)

| Instrument | Model | Company |
|---|---|---|
| Nanoflow LC coupled | - | Bruker Daltonics |
| pH meter | Seven Easy | METTLER TOLEDO |
| Power Supply | PowerPac™ Basic | Bio-Rad |
| | PowerPac™ HC | Bio-Rad |
| Protein IEF system | Ettan™ IPGphor™ 3 | GE Healthcare |
| Rocking shaker | MS-NRK | Cleaver Scientific |
| Spectrophotometer | UV-1700 | Shimadzu |
| Thermomixer | R Mixers 022670000 | Eppendorf |
| Ultrasonic sonicator | Transonic digitals | Elma |
| | D-78224 | Singen/Htw |
| Upright freezers (-86 ◦C) | 900 Series | Thermo Scientific |
| Vortex mixer | Vortex-Genie 2 | Scientific Industries |

3. Computer software

Image scanner program software is Labscan 5.0.0.0 from GE Healthcare Bio-Sciences AB. ImageQuant TL Plus 7.0 Software and ImageMaster 2D Platinum 7.0 Software were using for analyzed protein spot and band from GE Healthcare Bio-Sciences AB. The statically software is SPSS software version 15.0 and GraphPad Prism 5 form GraphPad Software. For the protein identification software is Bruker Compass 1.4 software from Bruker Daltonics and Mascot MS/MS ions search from Matrix Science Ltd. (http://www.matrixscience.com).

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## Methods

The experimental method was done as described by the flow chart as demonstrated in Figure 16.

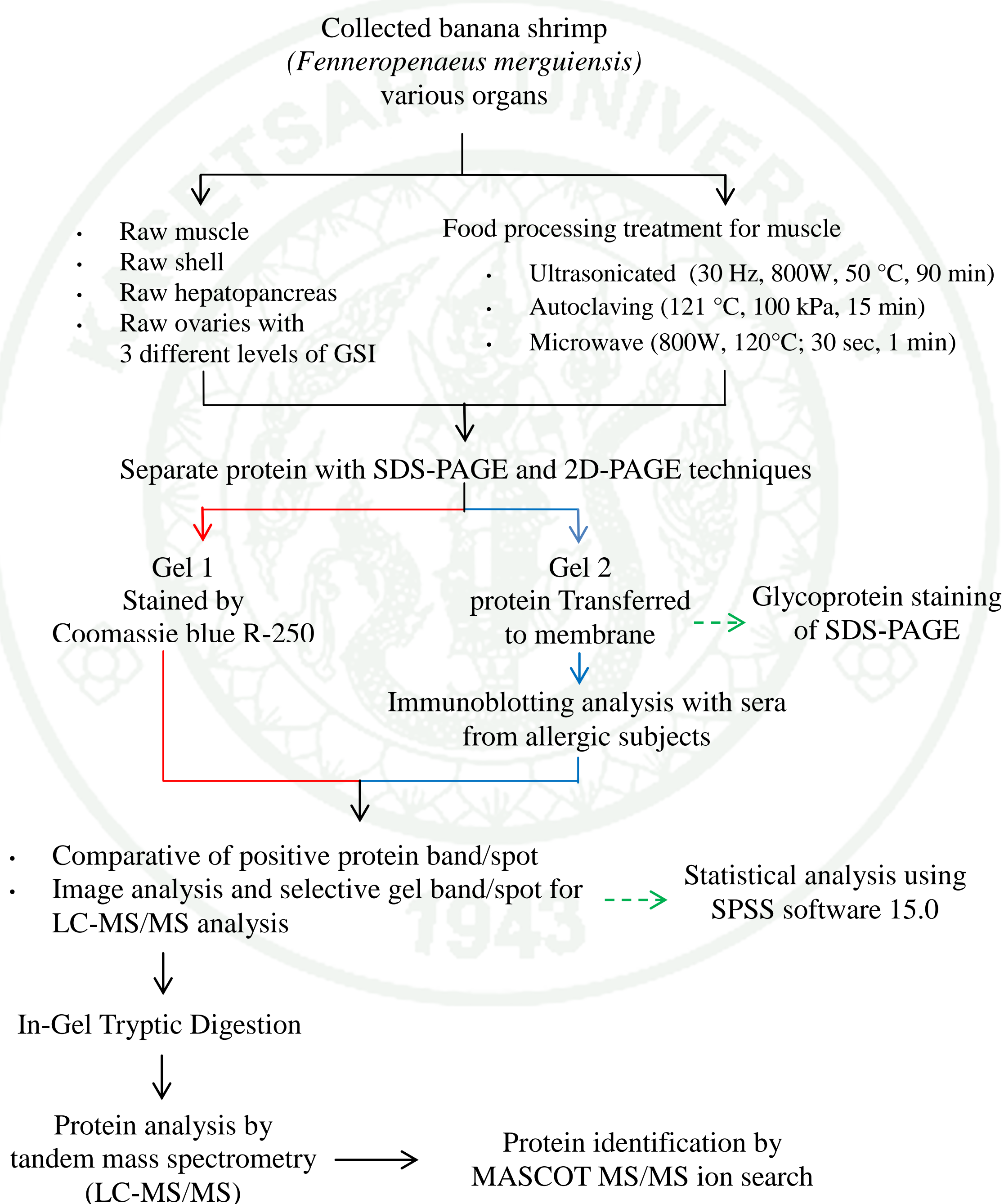


**Figure 16** Flow chart indicates the identification of allergen analysis details.

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**Sample preparation**

1. Allergic subjects' sera

The level of the antibody IgE in the sera from allergic patients was verified to be greater than 0.35 kU/L using modern EUROLINE inhalation Southeast Asian test strips (EUROIMMUN AG; Schleswig Holstein, Lübeck, Germany). After incubation of the test strip with patient serum and chromogen substrate, each test strip was scanned with a EUROBlotScanner and the results appeared on a EUROLineScan result sheet. This processing was done at Phyathai Hospital (Bangkok, Thailand). The serum data sheet is reported as Appendix Table C1. After verification of the IgE antibody, the sera from 12 shrimp-allergic patients were aliquot for used as individual immunoblot analysis and the rest of allergic subjects' sera were pooled for 2D-immunoblot analysis. Every healthy subject's sera were pooled for 1D/2D-immunoblot analysis as well. The sera were frozen at -80°C until use.

2. Food processing treatment for banana shrimp muscle

Raw muscle was prepared by food processing follow by:

- Autoclave process at 121°C, 100 kPa for 15 min.
- Ultrasound-assisted processing in sonicator (30Hz, 800 W) at $50^{0}$C for 90 min (Zhenxing *et al*., 2006).
- Microwave process at electrical energy 800 W, $102^{0}$C for 30 sec and 1 min, respectively.

Raw shrimp muscle and various organs were removed without any further treatment and raw shrimp was used as the control. The shrimp muscles from all food processing treatments were frozen at -80°C until use.

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**Banana shrimp protein extraction**

Separate samples of frozen shrimp muscles and various organs from each treatment were ground to a powder in a mortar and pestle using liquid nitrogen.

1. Phosphate buffer saline (PBS) extract

The sample powder of each treatment was solubilized in phosphate buffer saline (PBS) for SDS-PAGE analysis. A sample of 200 µg of shrimp powder protein was added to 400 µL of PBS buffer with a protease inhibitor cocktail and incubated for 1 h at 4°C. The mixture was centrifuged at 16,215×g for 30 min at 4°C and the supernatant was collected.

2. Lysis buffer extract

The sample powder of each treatment was solubilized in lysis buffer for 2D-PAGE. A sample of 200 µg of shrimp powder protein was added to 400 µL of lysis buffer and incubated for 1 h at 4°C. The mixture was centrifuged at 16,215×g for 30 min at 4°C and the supernatant was collected.

**Protein concentration measurement**

Protein concentration was determined with the Bio-Rad protein assay that modified from Bradford (1976). Protein extract was diluted with distilled water at 1:5000 ratios. Then, 200 µL of dilution protein was mixed with 800 µL of Bradford reagent in test tube. Distilled water was used as blank sample. Incubated sample test tube at room temperature for 2 min. Protein concentration was determined by means of UV-VIS spectrophotometry. Protein concentration was calculated from a standard curve is known concentrations of bovine serum albumin (BSA).

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**Polyacrylamide gel electrophoresis (PAGE)**

1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extract from raw muscle, shell, hepatopancreas, ovaries with 3 different and every food processing treatment shrimp muscle were performed.

Polyacrylamide gel using slab gel 8×10 cm with 1 mm thick follow by 1.5 cm stacking gel and 6.5 gel separating gel.

The crude extract of shrimp muscle was separated in triplicate by SDS-PAGE according to the method of Laemmli (1970). Samples proteins were loaded onto 12.5% separating gel and 4% stacking gel. Gel composition was following by:

| **Composition** | **Stacking gel 4% (2.5 ml)** | **Separating gel 12.5% (6.5 ml)** |
|---|---|---|
| 30% acrylamide-0.8% bisacrylamide | 0.33 ml | 2.638 ml |
| 0.5 M Tris-Hcl, pH 6.8 | 0.63 ml | - |
| 1.5 M Tris-Hcl, pH 8.8 | - | 1.625 ml |
| 10% SDS | 25 µl | 65 µl |
| 10% Ammonium persulphate | 12.5 µl | 32.5 µl |
| TEMED | 2.5 µl | 3.25 µl |
| Distilled water | 1.5 ml | 2.136ml |

1.1 Protein sample and protein standard maker

Protein crude extract from each treatment Samples with a concentration of 30 µg were mixed with sample buffer as 1:4 ratios. Then, the sample was incubated in 95°C water for 5 min. Protein standard for SDS-PAGE was a PageRuler Prestained Protein Ladder, product no. #26616 from Thermo Fisher Scientific was used.

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1.2 Electrophoresis

Samples with a concentration of 30 µg were loaded onto 12.5% separating gel and 4% stacking gel of SDS- polyacrylamide tris/glycine gel at 15 mL/lane and electrically separated by the Mini-PROTEAN Tetra system. The protein gel was separate in 1X running buffer using constant electric current at 15 mA for 2 h.

2. Two dimensional polyacrylamide gel electrophoresis (SDS-PAGE)

2.1 Protein sample and protein standard maker

150 µg of protein extract from each treatment was suspended in 75 µL rehydration buffer, this method modified from Beyer *et al.* (2002). Then, applied protein solution to an ImmobilineTM DryStrip pH 3-10 NL (7 cm) gradient strip for re-swelling the gel strips overnight.

2.2 Isoelectric focusing (IEF)

Gel strips from rehydration process were laid on the strip holder along with anodic and cathode electrode and then put the moist paper wick on strip. Lid adapters will put to attach the paper wick by copper line through direct both terminals. Isoelectric focusing was performed in Ettan™ IPGphor™ 3 protein IEF system at 20°C and following by:

| **Phase** | **Voltage (V)** | **Vhr** |
|---|---|---|
| 1 step | 300 | 200 |
| 2 Gradient | 1000 | 300 |
| 3 Gradient | 5000 | 4500 |
| 4 step | 500 | 2000 |

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2.3 IPG strip equilibration

After isoelectric focusing, strips were equilibrated in equilibration buffer I for 15 min, and then in another similar buffer, equilibration buffer II for 15 min.

2.4 SDS - polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extract from raw muscle and every food processing treatment shrimp muscle were performed.

Polyacrylamide gel using slab gel 10×10.5 cm with 1.5 mm thick follow by 2.5 cm stacking gel and 8 gel separating gel.

Protein in the strip was separated in triplicate by SDS-PAGE according to the method of Laemmli (1970). Samples proteins were loaded onto 14% separating gel and 4% stacking gel. Gel composition was following by:

| **Composition** | **Stacking gel 4% (2.5 mL)** | **Separating gel 14% (11 mL)** |
|---|---|---|
| 30% acrylamide-0.8% bisacrylamide | 0.33 mL | 5 mL |
| 0.5 M Tris-Hcl, pH 6.8 | 0.63 mL | - |
| 1.5 M Tris-Hcl, pH 8.8 | - | 2.75 mL |
| 10% SDS | 25 µL | 110 µL |
| 10% Ammonium persulphate | 12.5 µL | 55 µL |
| TEMED | 2.5 µL | 5.5 µL |
| Distilled water | 1.5 mL | 3.08 mL |

2.5 Electrophoresis

Load protein strip and Protein standard above stacking gel. Then, laid 0.5% agarose with 0.1% CBB on strip and electrically separated by Electrophoresis and

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Blotting Systems from Hoefer. The protein gel was separate in 1X running buffer using constant electric current at 12 mA for 3 h.

**Coomassie blue protein staining**

Polyacrylamide gels from electrophoresis were stained with 0.1% CBB solution for 12 h. Gel were washing with de-staining solution I for 1 h and following by de-staining solution II until appear blue band of protein.

**Glycoprotein staining of SDS-PAGE**

The protein membrane was stained using a GelCode® glycoprotein staining kit (Thermo Fisher Scientific; Waltham, MA, USA). The membrane was washed by 3% acetic acid and transferred to 10 mL of oxidizing solution. Membrane was washed by 3% acetic acid before being to GelCode® glycoprotein staining reagent. After that, the membrane was transferred to reducing solution then washed extensively with 3% acetic acid and then with ultrapure water. Glycoprotein staining membranes were produced in triplicate.

**Western blot (Immunoblot) analysis with antibody IgE**

For immunodetection of IgE-binding proteins, the separated proteins from the SDS-PAGE and 2D-PAGE gels were electroblotted onto an Immobilon-P transfer membrane with a constant voltage of 100 V for 1.5 h at 4°C, according to the methods of Towbin *et al.* (1979).

After that, transfer membranes were blocked with Block Ace for 1 h and washed the membrane with TBS/T 10 min for 3 times. Then, incubated the membrane for 16 h at 4°C with a 1/25 dilution of the individual serum with TBS/T - 10% skim milk from 12 allergic subjects or/and pooled sera from shrimp-allergic patients. After that washed the membrane with TBS/T 10 min for 3 times.

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The bound IgE antibody was detected using HRP conjugated goat anti-human IgE with dilution of 1/5000 TBST - 3% skim milk for 1 h. After washing the membranes with TBS/T buffer (Tris-Buffered Saline/Tween 20), they were incubated with Amersham ECL Plus™ western blotting detection reagents to done chemiluminescence immunoassay. Finally, the immunoreactive bands were visualized using exposure with Amersham® Hyperfilm® ECL™ in a dark room after fix the film with developer and fixer solution, respectively.

**Protein image analysis and statistical analysis**

Stained gels and immunoreactive bands/spots from the films were scanned using by image scanner and image scanner program. Image of protein gels and films were analyzed by ImageQuant TL Plus 7.0 Software for 1D-gel. ImageMaster 2D Platinum 7.0 Software was used for analysis of protein spot for 2D-gel. The intensity values of each 2D-immunoreactive spot from the films were detected. Significant differences in intensity values of each allergen spots were determined by statistical analysis using SPSS software version 15. Data are presented as interleaved bars graph with mean±SEM by GraphPad Prism 5 to compare the allergenicity of IgE binding protein between each cooked treatment of banana shrimp muscle. One-way ANOVA were used followed by Bonferroni's multiple comparison tests. *P*-values less than 0.05 were considered statistically significant.

**Trypsin in-gel digestion**

Interested protein bands/spots were excised and transferred to 0.5 mL microcentrifuge tubes. CBB were removed by using 0.1 M $NH_4HCO_3$/ 50% CAN (Solution A) until the gel pieces were colorless. After drying by speed vacuum, reduction and alkylation were performed by swelling the gel pieces in 50 µL buffer solution (Solution B; 0.1 M $NH_4HCO_3$, 10 mM DTT and 1 mM EDTA) and incubating at 60ºC for 45 min by thermomixer. The liquid was removed and then the gel slices were covered in 100 mM IAA/ 0.1 M $NH_4HCO_3$ solution (Solution C) and incubated at room temperature in a dark room for 30 min. The residual of IAA solution was

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removed and the gel pieces were washed with 0.5 M Tris-HCl, pH 8.5/ 50% CAN (Solution D) and dried in a speed vacuum. Aliquots of trypsin (1 µg trypsin: 10 µL 1% acetic acid) were prepared and stored at -20ºC. 50 µL of digestion buffer (Solution $E_1$; 0.05 M tris HCl, 10% ACN, 1 mM $CaCl_2$, pH 8.5) and 0.1 µg/µL of trypsin (Solution $E_{2)}$ were added.

After incubating at 37ºC overnight, the digestion buffer was removed and transferred to a new 0.5 mL microcentrifuge tube. the gel pieces were extracted by adding 60 µL of 2% freshly prepared of TFA (Solution F) and incubating for 30 min at 60ºC. Then, add 40 µL of extracting buffer (Solution G; 0.05 M Tris-HCl pH 8.5, 1 mM $CaCl_2$) incubated at 30ºC for 10 min by thermomixer and then sonicated for 5 min. Extracting buffer was removed and transferred to a new 0.5 mL microcentrifuge tube. the gel pieces were extracted more by 50% ACN incubated at 30ºC for 10 min by thermomixer and then sonicated for 5 min. Then, add 2.5% formic acid/ 50% ACN (Solution H) to the gel pieces incubated at 30ºC for 10 min by thermomixer and then sonicated for 5 min. The solutions were pooled in new microcentrifuge tube and dried by speed vacuum and stored at -20ºC until analyze.

**Protein Identification by LC-MS/MS**

Nanoflow liquid chromatography coupled with the amaZon speed ion trap mass spectrometry from Bruker Daltonics was utilized to identify protein spots. A 75 µm id × 100 mm $C_{18}$ EASY-nLC™ Column from Thermo Fisher Scientific was used to concentrate and desalt all trypsinized peptides. After that, 0.1% formic acid in water (solution A) and 0.1% formic acid in ACN (solution B) were added respectively to elude peptide out. Sample (6 µL) was injected into nano-LC system for separation at a flow rate of 0.0005 mL/min for 30 min using these gradient: 0 min 95%A, 20 min 60%A, 20.5 min 5%A, 29 min 5%A, and 29.5 min 95%A followed by MS/MS equipped with the CaptiveSpray™ source using 1.0 s automatic scan rate with 0.1 sec interscan delay. Parent mass peaks with range from 50 to 3000 *m/z* were selected for MS/MS analysis which collision energy was fixed at 1300V. AutoMS$^n$ was applied

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with SMART isolation and fragmentation (at 60% amplitude), and scan ranges that were automatically scaled to the individual precursor mass in each MS/MS spectrum.

MS/MS data were processed and converted to .mgf files by Bruker Compass 1.4 software and Mascot MS/MS ions search (http://www.matrixscience.com) was used. The search parameters were set as follows:

| **Parameter** | **Parameter selected** |
|---|---|
| Type of search | Mascot MS/MS Ion Search |
| Database | NCBInr |
| Enzyme | Trypsin |
| Missed cleavage allowance | 1 |
| Taxonomy | All entries |
| Fixed modifications | - |
| Variable modifications | Carbamidomethyl (C), Oxidation (HW), Oxidation (M), Phospho (ST) and Phospho (Y) |
| Peptide mass tolerance | 1.2 Da |
| MS/MS ion mass tolerance | 0.2 Da |
| Peptide charges limited | $1^+$, $2^+$ and $3^+$ |
| Mass values | Monoisotopic |
| Data format | Mascot generic (.mgf) |

The proteins were identified at a probability level $P < 0.05$ and Mascot scores >35 were considered as promising hits.

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# RESULTS AND DISCUSSION

## Results

### Total protein extraction and SDS-PAGE

The electrophoretic pattern of shrimp extracts from various organs showed multiple protein bands ranging from 17 to 170 kDa with different patterns (Figure 17a). The protein pattern of hepatopancreas was not clear with smears on the protein bands and low molecular masses of proteins when compared with the other shrimp protein samples. The protein pattern of the ovaries showed the three different stages with similar patterns but with some slight differences in the intensity of some proteins. The intensity of the proteins was dependent on the different vitellogenic stages.

The SDS-PAGE profiles of the sample extracts with each food processing condition showed multiple protein bands ranging from ~20 to ~170 kDa. The electrophoretic patterns are shown in lane R (Figure 19) for raw muscle, lane A (Figure 20) for autoclave treated muscle, lane S (Figure 21) for ultrasonic treated muscle, lane MS for 30 sec microwave treated muscle (Figure 22) and lane MM for 1 min microwave treated muscle (Figure 23).

### 1D-immunoblotting and allergen identification

To identify the IgE-binding proteins from various organs of banana shrimp extract, the allergens were analyzed using western blotting. Pooled sera from 21 shrimp-allergic patients were tested on immunoblotting for IgE binding to shrimp extract. IgE binding to the protein bands ranged with molecular masses of ~20 - ~170 kDa (Figure 17b). These protein bands could be identified as banana shrimp allergens. Lane R was a protein from raw muscle extract that contained six immunoreactive bands. Lane SH was a protein from *F. merguiensis* shell that contained four

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immunoreactive bands. Lane HP was a protein from *F. merguiensis* hepatopancreas that did not have an immunoreactive band, which may have been due to smearing and the low molecular mass pattern of the protein. Lanes OV1-OV3 were proteins from *F. merguiensis* ovarian at different vitellogenic stages that contained six immunoreactive bands. No immunoreactive band was detected using serum from non-allergic individuals (data not shown).

The identification of allergens was show in Table 5. Six proteins could be identified as muscle allergens with the highest volume ratio being for arginine kinase (AK), followed by a myosin heavy chain (MHC), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hemocyanin (HC), sarcoplasmic calcium-binding protein (SCP) and enolase, respectively. In the shell, three proteins were identified based on the volume ratio as AK, SCP and GAPDH. Three allergens were identified as vitellogenin (VG), AK and ovarian peritrophin 1 precursor in the stage 1 ovaries. Allergens from ovaries at vitellogenic stage 2 were VG and 14-3-3 protein and from the vitellogenic stage 3, VG, HC, β-actin and 14-3-3 protein were identified.

**Glycoprotein staining of SDS-PAGE**

Staining can identify glycoprotein. Glycoproteins appear on the membrane as magenta bands as shown in Figure 18. In this study, there were three glycoproteins with allergenic properties from muscle that were identified as enolase, AK and GAPDH. In the ovaries with different vitellogenic stages, three allergens were identified as glycoproteins: SOPs in an ovary at the vitellogenic stage 1 and 14-3-3 protein in ovaries at the vitellogenic stages 2 and 3. Moreover, VG was identified as a glycoprotein at every vitellogenic stage.

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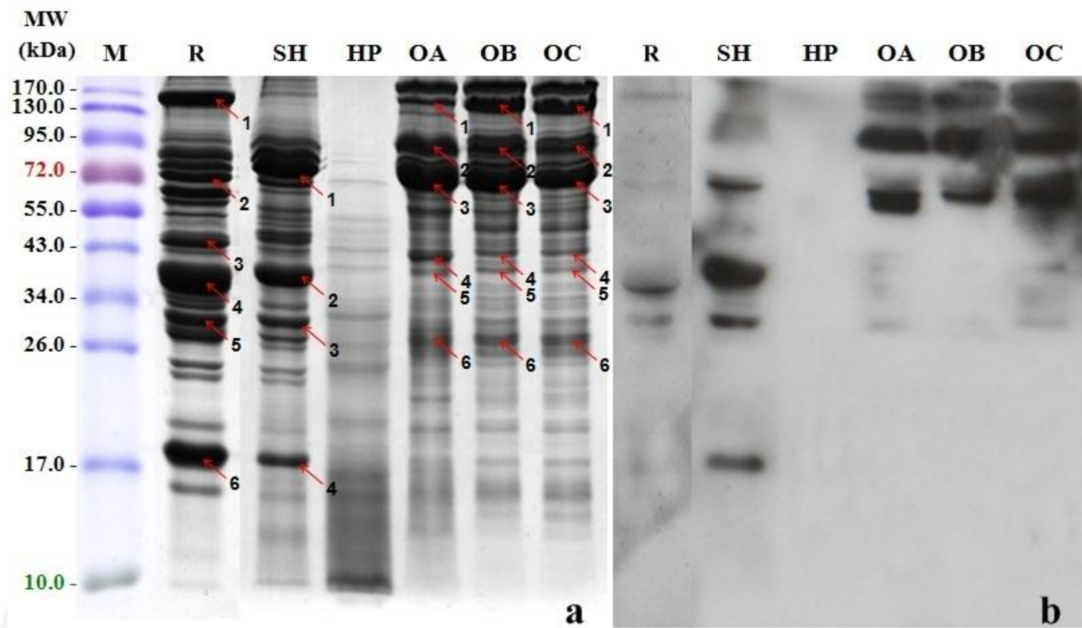


**Figure 17** SDS-PAGE protein patterns with CBB staining (a) and film-based images of IgE immunoreactive bands (b) of the various raw banana shrimp organ extract; lane M, molecular weight of protein ladder. Arrows label represent the IgE-binding protein that was identified by LC-MS/MS.

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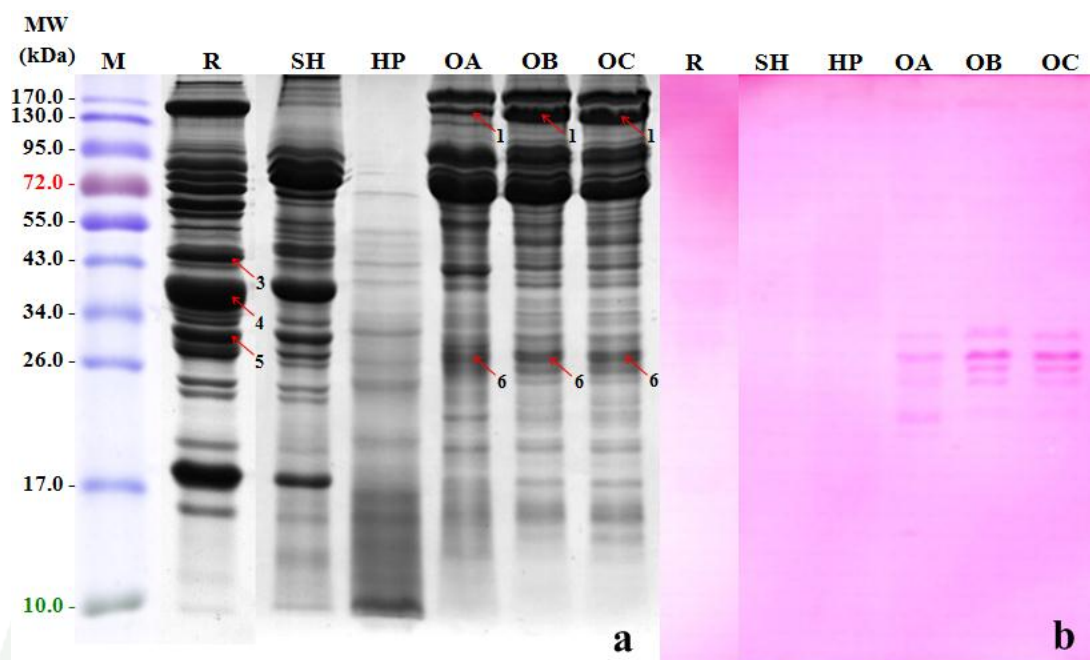


**Figure 18** SDS-PAGE protein patterns with CBB staining (a) and magenta bands (b) represented glycoproteins; M, molecular weight of protein ladder. The arrow labels indicate glycoprotein bands with allergenic properties were identifying using LC-MS/MS.

**Table 5** Identified allergens from various organs of *Fenneropenaeus merguiensis* using LC-MS/MS[1]

| Band[2] | Protein Name Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| R_1 | **Myosin Heavy Chain type b** *Marsupenaeuse japonicus*/ gi\|343183155 | 21.887 | 6.00 | 172 | 15 | 10 |
| R_2 | **Hemocyanin** *Litopenaeus vannamei*/ gi\|854403 | 74.984 | 5.27 | 70 | 11 | 11 |
| R_3 | **Enolase** *Penaeus monodon*/ gi\|3885968 | 47.235 | 6.18 | 249 | 6 | 17 |

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**Table 5** (Continued)

| Band[2] | Protein Name Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| R_4 | **Arginine kinase** *Penaeus monodon*/ gi\|308154236 | 40.115 | 6.05 | 273 | 41 | 44 |
| R_5 | **Glyceraldehyde 3-phosphate dehydrogenase** *Marsupenaeus japonicus*/ gi\|62701385 | 17.017 | 6.56 | 192 | 13 | 39 |
| R_6 | **Sarcoplasmic calcium-binding protein** *Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 59 | 5 | 18 |
| R_5 | **Glyceraldehyde 3-phosphate dehydrogenase** *Marsupenaeus japonicus*/ gi\|62701385 | 17.017 | 6.56 | 192 | 13 | 39 |
| SH_1 | **Sarcoplasmic calcium-binding protein** *Litopenaeus vannamei*/ gi\|223403273 | 22.064 | 4.73 | 156 | 6 | 20 |
| SH_2 | **Arginine kinase** *Homarus gammarus*/ gi\|585342 | 39.958 | 6.05 | 168 | 3 | 8 |
| SH_3 | **Glyceraldehyde-3-phosphate dehydrogenase** *Nephropidae*/ gi\|229256 | 35.694 | 6.24 | 82 | 17 | 18 |
| SH_4 | **Sarcoplasmic calcium-binding protein** *Litopenaeus vannamei*/ gi\|223403273 | 22.064 | 4.73 | 64 | 16 | 47 |
| HP | **Unidentified** | | | | | |
| OA_1 | **Vitellogenin** *Fenneropenaeus merguiensis*/ gi\|74860193 | 282.850 | 6.36 | 740 | 18 | 5 |

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**Table 5** (Continued)

| Band[2] | Protein Name Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| OA_2 | **Vitellogenin** *Fenneropenaeus merguiensis*/ gi\|74860193 | 282.850 | 6.36 | 378 | 9 | 3 |
| OA_3 | **Vitellogenin** *Fenneropenaeus chinensis*/ gi\|86129739 | 283.466 | 6.13 | 314 | 8 | 2 |
| OA_4 | **Vitellogenin** *Fenneropenaeus merguiensis*/ gi\|74860193 | 282.850 | 6.36 | 317 | 8 | 3 |
| OA_5 | **Arginine kinase** *Homarus gammarus*/ gi\|585342 | 39.958 | 6.05 | 113 | 3 | 8 |
| OA_6 | **Ovarian peritrophin 1 precursor** *Penaeus monodon*/ gi\|21218359 | 30.870 | 4.80 | 90 | 2 | 6 |
| OB_1 | **Vitellogenin** *Fenneropenaeus merguiensis*/ gi\|256861071 | 283.130 | 6.47 | 1038 | 26 | 9 |
| OB_2 | **Vitellogenin** *Fenneropenaeus merguiensis*/ gi\|74860193 | 282.850 | 6.36 | 390 | 10 | 3 |
| OB_3 | **Vitellogenin** *Fenneropenaeus merguiensis*/ gi\|74860193 | 282.850 | 6.36 | 622 | 16 | 6 |
| OB_4 | **β-actin** *Litopenaeus vannamei*/ gi\|10304437 | 41.841 | 5.30 | 90 | 5 | 9 |
| OB_5 | **Arginine kinase** *Penaeus monodon*/ gi\|27463265 | 40.087 | 6.05 | 179 | 6 | 17 |
| OB_6 | **14-3-3 zeta** *Fenneropenaeus merguiensis*/ gi\|298570899 | 27.915 | 4.68 | 113 | 3 | 10 |

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**Table 5** (Continued)

| Band[2] | Protein Name Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| OC_1 | **Vitellogenin** *Fenneropenaeus merguiensis*/ gi\|74860193 | 282.850 | 6.36 | 764 | 15 | 5 |
| OC_2 | **Vitellogenin** *Fenneropenaeus merguiensis*/ gi\|74860193 | 282.850 | 6.36 | 206 | 4 | 1 |
| OC_4 | **β-actin** *Litopenaeus vannamei*/ gi\|10304437 | 41.841 | 5.30 | 130 | 3 | 8 |
| OC_5 | **Arginine kinase** *Litopenaeus vannamei*/ gi\|115492980 | 40.134 | 6.19 | 107 | 3 | 8 |
| OC_6 | **14-3-3-like protein** *Penaeus monodon*/ gi\|66774602 | 27.834 | 4.61 | 151 | 3 | 10 |

[1] The protein identified data were according to assigned band number (Figure 17-18).

[2] Band from SDS-PAGE; e.g. R_1 means the band number 1 in SDS-PAGE of raw shrimp muscle.

To identify IgE-binding proteins for food processed muscle for 1D-immunoblotting. Western blotting and immunoblotting with individual serum samples from 12 shrimp-allergic patients were tested. The identification of allergens was show in Table 6. In raw muscle, six IgE binding protein bands have molecular mass ranging from ~20 – ~170 kDa (Figure 19, lane 1-12). These protein bands could be identified as banana shrimp allergens in raw muscle. The predominant bands from raw muscle protein extract were ~30 and ~40 kDa which recognized by all 12 patients (Table 7). There are 6 allergens have identified in raw muscle from 1D-immunobotting as MHC, HC, enolase, AK, GAPDH and SCP.

There is only an IgE-binding protein band identified in this treated muscle and demonstrated as predominant allergen band with molecular mass of ~40 kDa

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tropomyosin (TM) from autoclave condition. In ultrasound treated muscle, four IgE binding protein band have molecular masses ranging ~20 - ~75 kDa and predominant bands were ~34 and ~75 kDa which are HC, β-actin, TM and SCP.

Moreover, seven IgE binding protein band for 30 sec microwave treated identified as TM and SCP; and six IgE binding protein band for 1 min microwave treated were identified as actin, TM and SCP. The predominate allergen bands were ~40 kDa from both microwave condition.

The remaining IgE-binding components with various molecular masses were detected at lower intensity. No positive band was detected using serum from non-allergic individuals shown in lane NC in every food processing conditions.

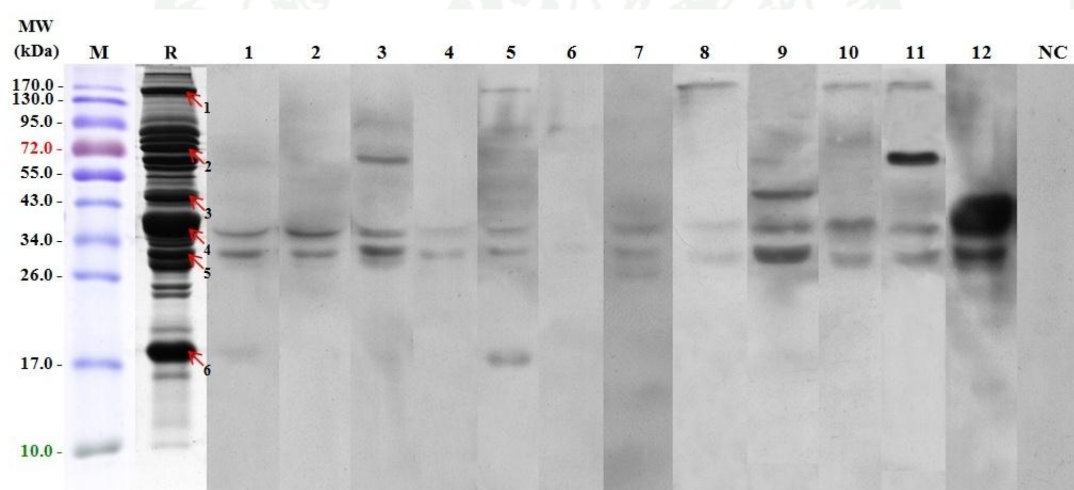


**Figure 19** SDS-PAGE pattern (lane R) and film-based images of IgE immunoreactive bands from 12 individual patient rerum (lane 1-12) of raw muscle. Lane M: molecular weight protein ladder; lane NC, band detected using serum from non-allergic individuals; arrows indicated the bands were identified protein by LC-MS/MS.

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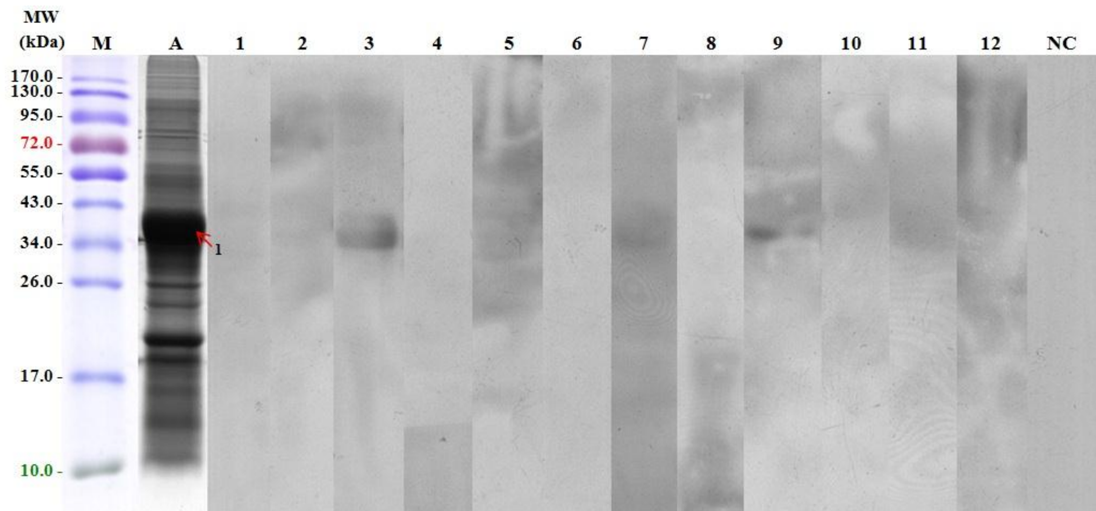


**Figure 20** SDS-PAGE pattern (lane A) and film-based images of IgE immunoreactive bands from 12 individual patient rerum (lane 1-12) of autoclave treated muscle. Lane M: molecular weight protein ladder; lane NC, band detected using serum from non-allergic individuals; arrows indicated the bands were identified protein by LC-MS/MS.

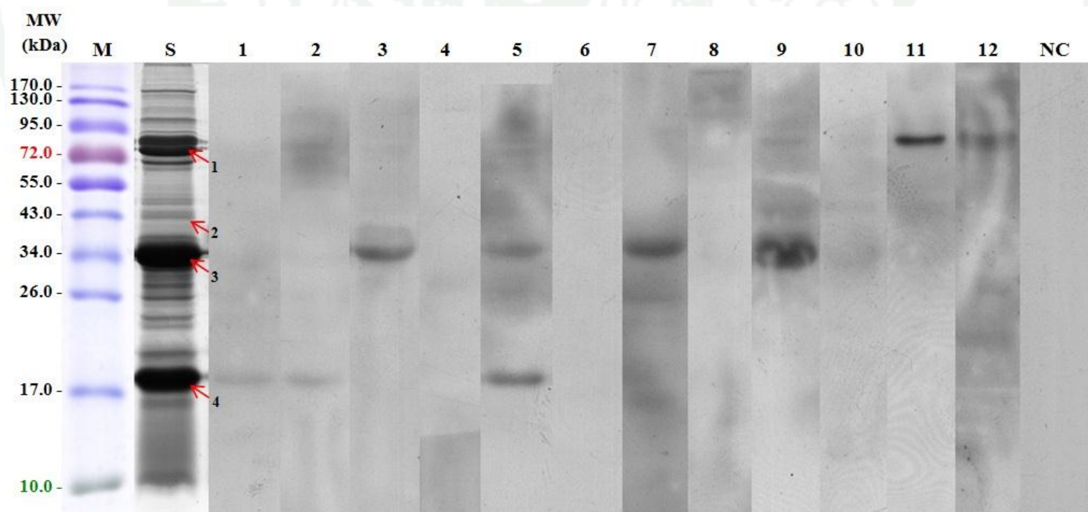


**Figure 21** SDS-PAGE pattern (lane S) and film-based images of IgE immunoreactive bands from 12 individual patient rerum (lane 1-12) of ultrasound treated muscle. Lane M: molecular weight protein ladder; lane NC, band detected using serum from non-allergic individuals; arrows indicated the bands were identified protein by LC-MS/MS.

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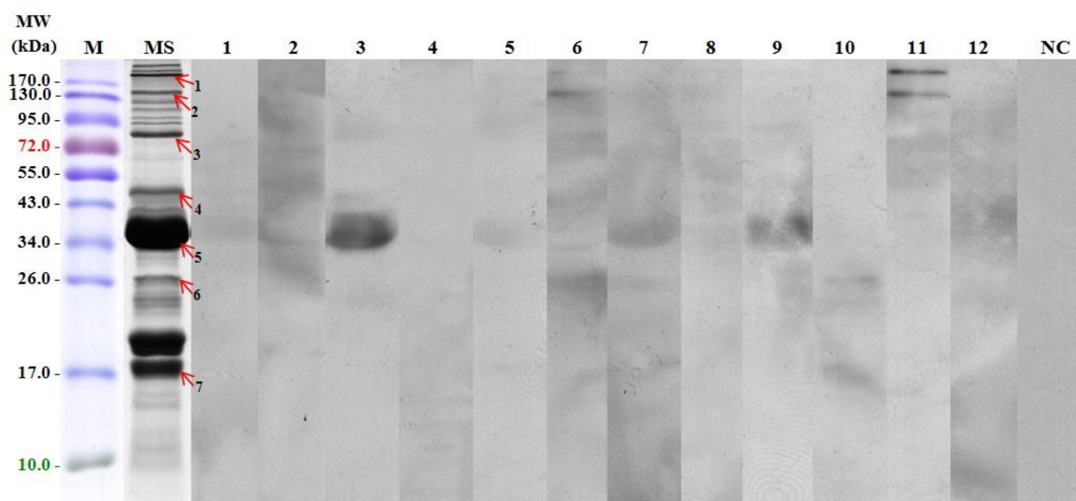


**Figure 22** SDS-PAGE pattern (lane MS) and film-based images of IgE immunoreactive bands from 12 individual patient rerum (lane 1-12) of 30 sec microwave treated muscle. Lane M: molecular weight protein ladder; lane NC, band detected using serum from non-allergic individuals; arrows indicated the bands were identified protein by LC-MS/MS.

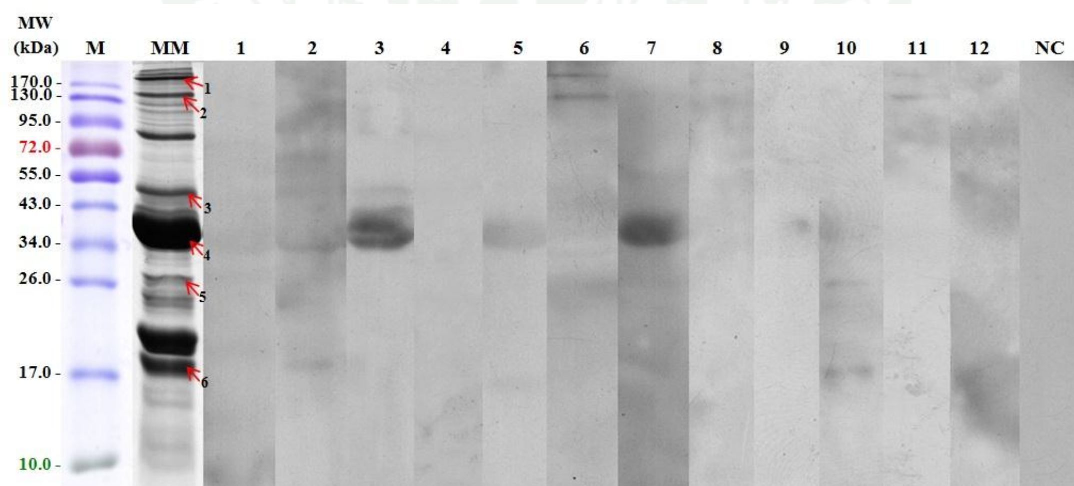


**Figure 23** SDS-PAGE pattern (lane MM) and film-based images of IgE immunoreactive bands from 12 individual patient rerum (lane 1-12) of 1 min microwave treated muscle. Lane M: molecular weight protein ladder; lane NC, band detected using serum from non-allergic individuals; arrows indicated the bands were identified protein by LC-MS/MS.

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**Table 6** 1D-immunoblotting identified protein bands from *Fenneropenaeus merguiensis* with various food processing muscle extract by LC-MS/MS[1]

| Band[2] | Protein Name Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| F_1 | **Myosin Heavy Chain type b** *Marsupenaeuse japonicas*/ gi\|343183155 | 218.870 | 6.00 | 172 | 15 | 10 |
| F_2 | **Hemocyanin** *Litopenaeus vannamei*/ gi\|854403 | 74.984 | 5.27 | 70 | 11 | 11 |
| F_3 | **Enolase** *Penaeus monodon*/ gi\|3885968 | 47.235 | 6.18 | 249 | 6 | 17 |
| F_4 | **Arginine kinase** *Penaeus monodon*/ gi\|308154236 | 40.115 | 6.05 | 273 | 41 | 44 |
| F_5 | **Glyceraldehyde 3-phosphate dehydrogenase** *Marsupenaeus japonicas*/ gi\|62701385 | 17.017 | 6.56 | 192 | 13 | 39 |
| F_6 | **Sarcoplasmic calcium-binding protein** *Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 59 | 5 | 18 |
| A_1 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 321 | 8 | 25 |
| S_1 | **Hemocyanin** *Litopenaeus vannamei*/ gi\|854403 | 74.934 | 5.27 | 104 | 4 | 4 |
| S_2 | **β-actin** *Penaeus monodon*/ gi\|118136261 | 41.973 | 5.00 | 109 | 3 | 7 |
| S_3 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 122 | 5 | 14 |

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**Table 6** (Continued)

| Band[2] | Protein Name Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| S_4 | **Sarcoplasmic calcium-binding protein** *Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 72 | 6 | 25 |
| MS_1 | **Unidentified** | | | | | |
| MS_2 | **Unidentified** | | | | | |
| MS_3 | **Unidentified** | | | | | |
| MS_4 | **Unidentified** | | | | | |
| MS_5 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 626 | 15 | 42 |
| MS_6 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 285 | 5 | 15 |
| MS_7 | **Sarcoplasmic calcium-binding protein** *Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 457 | 9 | 41 |
| MM_1 | **Myosin heavy chain type 2** *Litopenaeus vannamei*/ gi\|410509312 | 219.056 | 5.84 | 309 | 9 | 4 |
| MM_2 | **Unidentified** | | | | | |
| MM_3 | **Actin 1** *Penaeus monodon*/ gi\|3907620 | 41.716 | 5.23 | 230 | 5 | 13 |
| MM_4 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 511 | 11 | 32 |
| MM_5 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 345 | 7 | 21 |

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**Table 6** (Continued)

| Band[2] | Protein Name Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| MM_6 | **Sarcoplasmic calcium-binding protein** *Litopenaeus vannamei*/ gi\|223403273 | 22.064 | 4.73 | 105 | 3 | 16 |

[1] The protein identified data were according to assigned band number (Figure 19-23).

[2] Band from SDS-PAGE; e.g. R_1 means the band number 1 in SDS-PAGE of raw shrimp muscle.

**Table 7** Evaluation of positivity as obtained by cut-off based densitometric analysis and prevalence of IgE-binding at/or above the cut-off level from 1D-immunoboltting of each food process treatment

| Band[1] | Protein[2] | Shrimp allergic subjects | | | | | | | | | | | | Prevalence |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| R_1 | MHC | - | - | - | - | + | - | - | + | - | + | + | - | 33.33% |
| R_2 | HC | - | - | + | - | - | - | - | - | - | - | + | - | 16.67% |
| R_3 | Enolase | | - | - | - | - | - | - | - | + | - | - | - | 8.33% |
| R_4 | AK | + | + | + | + | + | + | + | + | + | + | + | + | 100.00% |
| R_5 | GAPDH | + | + | + | + | + | + | + | + | + | + | + | + | 100.00% |
| R_6 | SCP | - | - | - | - | + | - | - | - | - | - | - | - | 8.33% |
| A_1 | TM | - | - | + | - | - | - | + | - | + | - | - | - | 25.00% |
| S_1 | HC | - | + | + | - | + | - | - | - | + | - | + | + | 50.00% |
| S_2 | β-actin | - | - | - | - | - | - | - | - | + | + | + | - | 25.00% |
| S_3 | TM | - | - | + | - | + | - | + | - | + | + | + | - | 50.00% |
| S_4 | SCP | + | + | - | - | + | - | - | - | - | - | - | - | 25.00% |
| MS_1 | Unidentified | - | - | - | - | - | + | - | - | - | - | + | - | 16.67% |
| MS_2 | Unidentified | - | - | - | - | - | + | - | - | - | - | + | - | 16.67% |
| MS_3 | Unidentified | - | + | - | - | - | + | - | - | + | - | - | - | 25.00% |

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**Table 7** (Continued)

| Band[1] | Protein[2] | Shrimp allergic subjects | | | | | | | | | | | | Prevalence |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| MS_4 | Unidentified | - | - | + | - | - | - | - | - | - | - | - | - | 8.33% |
| MS_5 | TM | - | - | + | - | + | + | + | - | + | - | - | - | 41.67% |
| MS_6 | TM | - | - | - | - | - | + | + | - | - | + | - | - | 25.00% |
| MS_7 | SCP | - | - | - | - | - | - | + | - | - | + | - | - | 16.67% |
| MM_1 | MHC | - | - | - | - | - | + | - | - | - | - | + | - | 16.67% |
| MM_2 | Unidentified | - | - | - | - | - | + | - | - | - | - | + | - | 16.67% |
| MM_3 | Actin 1 | - | - | + | - | - | - | - | - | - | - | - | - | 8.33% |
| MM_4 | TM | - | + | + | - | + | - | + | + | - | - | - | - | 41.67% |
| MM_5 | TM | - | - | - | - | - | - | - | - | - | + | - | - | 8.33% |
| MM_6 | SCP | - | + | - | - | - | - | - | - | - | + | - | - | 16.67% |

[1] The protein identified data were according to assigned band number (Figure 19-23).

[2] Band from SDS-PAGE; e.g. R_1 means the band number 1 in SDS-PAGE of raw shrimp muscle.

**Total protein extraction and 2D-PAGE**

Protein extracts were separated by means of 2D-PAGE and stained with CBB. The electrophoresis profiles of each food processing conditions are clearly differentiated and have shown in Figure 24a – 27a; lane M: molecular weight protein ladder; arrows indicated the spots were identified protein by LC-MS/MS.

**2D-immunoblotting and allergen identification**

Identification the IgE-binding proteins in shrimp extract from 2D-PAGE were analyzed by immunoblot with pooled sera from 21 patients who allergic to shrimp (Figure 24b – 27b). Banana shrimp's allergens have molecular masses ranging from ~17- ~40 kDa and isoelectric point ~3 - ~7. Proteomic maps and immunoblot revealed

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that every food processing treated muscle have impact on allergenicity in banana shrimp. The identification of allergens was show in Table 8.

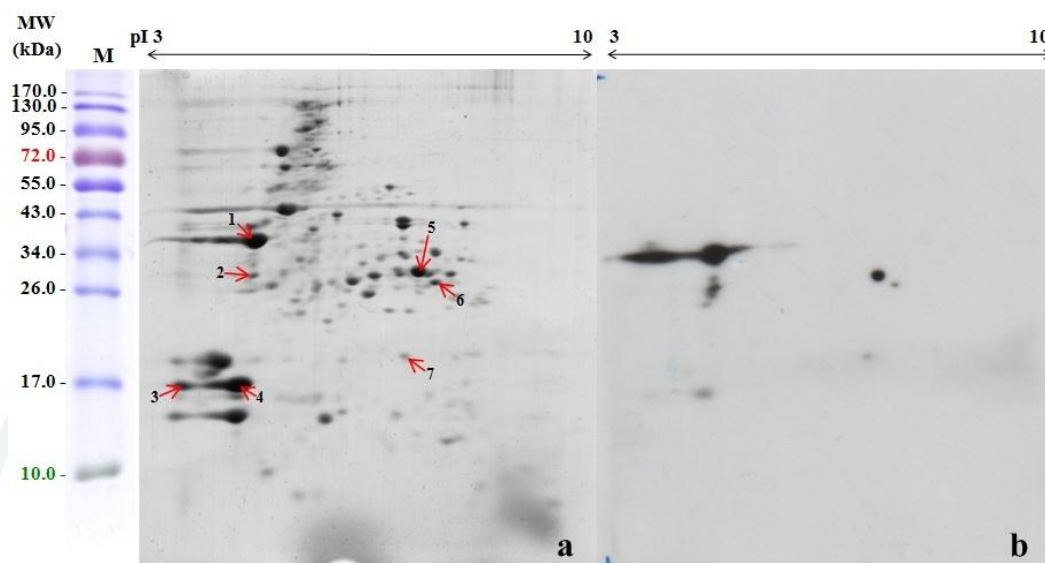


**Figure 24** 2D-PAGE profiles of raw muscle (a) and their immunoblot analysis (b) with pooled sera of 21 shrimp-allergic patients.

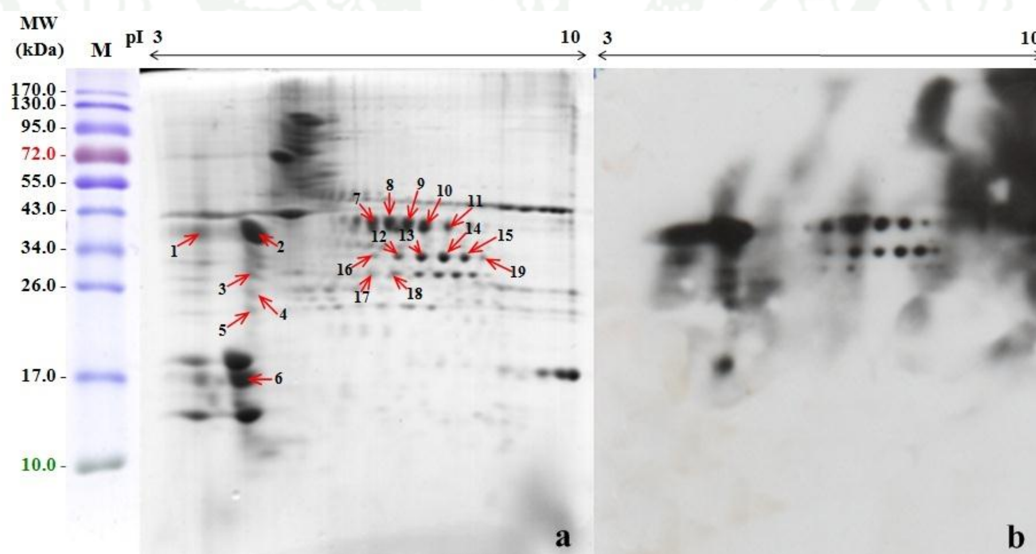


**Figure 25** 2D-PAGE profiles of autoclave treated muscle (a) and their immunoblot analysis (b) with pooled sera of 21 shrimp-allergic patients.

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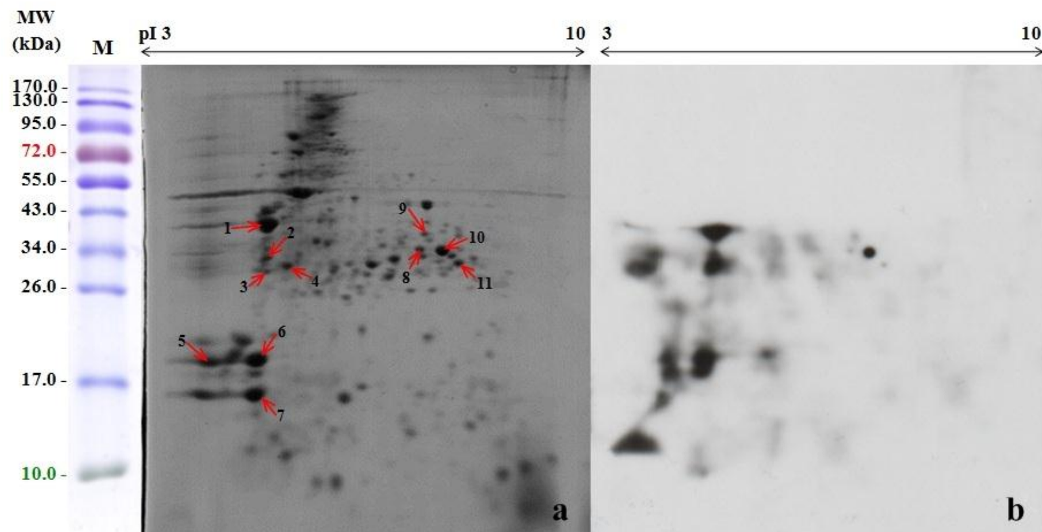


**Figure 26** 2D-PAGE profiles of ultrasound treated muscle (a) and their immunoblot analysis (b) with pooled sera of 21 shrimp-allergic patients.

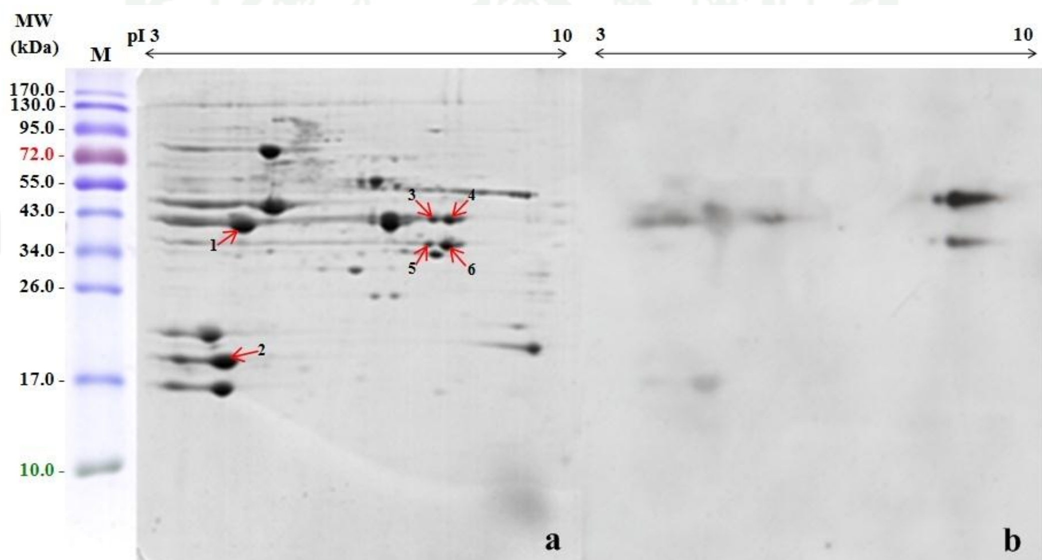


**Figure 27** 2D-PAGE profiles of 1 min microwave treated muscle (a) and their immunoblot analysis (b) with pooled sera of 21 shrimp-allergic patients.

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**Table 8** 2D-immunoblotting identified protein bands from *Fenneropenaeus merguiensis* with various food processing muscle extract by LC-MS/MS[1]

| Spot[2] | Protein Name Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| R_1 | **Tropomyosin** *Procambarus clarkia*/ gi\|225348412 | 32.794 | 4.73 | 459 | 16 | 33 |
| R_2 | **Tropomyosin** *Metapenaeus ensis*/ gi\|6094504 | 31.686 | 4.66 | 187 | 5 | 13 |
| R_3 | **Sarcoplasmic calcium-binding protein** *Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 106 | 6 | 30 |
| R_4 | **Sarcoplasmic calcium-binding protein** *Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 142 | 4 | 20 |
| R_5 | **Glyceraldehyde 3-phosphate dehydrogenase** *Marsupenaeus japonicas*/ gi\|62701385 | 17.017 | 6.56 | 89 | 9 | 13 |
| R_6 | **Glyceraldehyde 3-phosphate dehydrogenase** *Marsupenaeus japonicas*/ gi\|62701385 | 17.017 | 6.56 | 174 | 3 | 24 |
| R_7 | **Glyceraldehyde 3-phosphate dehydrogenase** lobster/ gi\|229256 | 35.694 | 6.24 | 74 | 3 | 4 |
| A_1 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 321 | 8 | 25 |
| A_2 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 757 | 15 | 42 |

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**Table 8** (Continued)

| Spot[2] | Protein Name Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| A_3 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 605 | 13 | 40 |
| A_4 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 609 | 12 | 36 |
| A_5 | **Tropomyosin** *Fenneropenaeus merguiensis*/ gi\|335347355 | 31.685 | 4.66 | 661 | 16 | 42 |
| A_6 | **Sarcoplasmic calcium-binding protein** *Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 420 | 9 | 41 |
| A_7 | **Arginine kinase** *Penaeus monodon*/ gi\|308154236 | 40.115 | 6.05 | 560 | 16 | 37 |
| A_8 | **Allergen Pen m 2, arginine kinase** *Penaeus monodon*/ gi\|27463265 | 40.087 | 6.05 | 510 | 14 | 33 |
| A_14 | **Glyceraldehyde-3-phosphate dehydrogenase** *Homarus americanus*/ gi\|6016080 | 35.693 | 6.53 | 285 | 8 | 23 |
| A_15 | **Glyceraldehyde-3-phosphate dehydrogenase** *Homarus americanus*/ gi\|6016080 | 35.693 | 6.53 | 276 | 7 | 17 |
| A_16 | **Myosin heavy chain type 1** *Penaeus monodon*/ gi\|410509306 | 219.450 | 5.77 | 195 | 4 | 1 |

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**Table 8** (Continued)

| Spot[2] | Protein Name<br>Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| A_17 | **Myosin heavy chain type 2**<br>*Penaeus monodon*/ gi\|410509308 | 219.033 | 5.85 | 134 | 3 | 1 |
| A_18 | **Glyceraldehyde 3-phosphate dehydrogenase**<br>lobster/ gi\|229256 | 35.694 | 6.24 | 135 | 3 | 9 |
| A_19 | **Glyceraldehyde 3-phosphate dehydrogenase**<br>lobster/ gi\|229256 | 35.694 | 6.24 | 150 | 3 | 10 |
| S_4 | **Enolase**<br>*Penaeus monodon*/ gi\|3885968 | 47.235 | 6.18 | 248 | 5 | 11 |
| S_5 | **Sarcoplasmic calcium-binding protein**<br>*Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 366 | 8 | 34 |
| S_6 | **Sarcoplasmic calcium-binding protein**<br>*Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 383 | 9 | 34 |
| S_7 | **Sarcoplasmic calcium-binding protein**<br>*Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 340 | 7 | 31 |
| S_8 | **Glyceraldehyde 3-phosphate dehydrogenase**<br>lobster/ gi\|229256 | 35.694 | 6.24 | 104 | 3 | 8 |
| S_9 | **Arginine kinase**<br>*Fenneropenaeus chinensis*/ gi\|56182374 | 40.104 | 5.92 | 237 | 7 | 17 |
| S_10 | **Glyceraldehyde 3-phosphate dehydrogenase**<br>lobster/ gi\|229256 | 35.694 | 6.24 | 141 | 5 | 14 |

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**Table 8** (Continued)

| Spot[2] | Protein Name Species/Accession no. | Theoretical value | | LC-MS/MS analysis | | |
|---|---|---|---|---|---|---|
| | | MW (kDa) | *pI* | Score | Peptide matches | %Coverage |
| S_11 | **Glyceraldehyde-3-phosphate dehydrogenase** *Homarus americanus*/ gi\|6016080 | 35.693 | 6.53 | 63 | 2 | 6 |
| MM_2 | **Tropomyosin** *Portunus trituberculatus*/ gi\|151505281 | 32.767 | 4.73 | 546 | 13 | 28 |
| MM_3 | **Sarcoplasmic calcium-binding protein** *Penaeus monodon*/ gi\|380503696 | 22.106 | 4.76 | 377 | 9 | 34 |
| MM_4 | **Arginine kinase** *Homarus gammarus*/ gi\|585342 | 39.958 | 6.05 | 239 | 6 | 16 |
| MM_5 | **Glyceraldehyde 3-phosphate dehydrogenase** lobster/ gi\|229256 | 35.694 | 6.24 | 143 | 5 | 11 |
| MM_6 | **Glyceraldehyde-3-phosphate dehydrogenase** *Panulirus versicolor*/ gi\|6016083 | 35.701 | 6.94 | 138 | 4 | 12 |

[1] The protein identified data were according to assigned band number (Figure 24-27).

[2] Spot from 2D-PAGE; e.g. R_1 means the band number 1 in 2D-PAGE of raw shrimp muscle.

**Food processing on allergenicity**

Food processing have affect to the allergenicity of banana shrimp allergen. The results from immunoblotting refer that each allergic patient has difference immunological reactions with differ allergens as shown in intensity values of IgE binding from 2D-immunoblotting (Figure 28) to compare the allergenicity of IgE binding protein between raw and cooked treatment of banana shrimp muscle.

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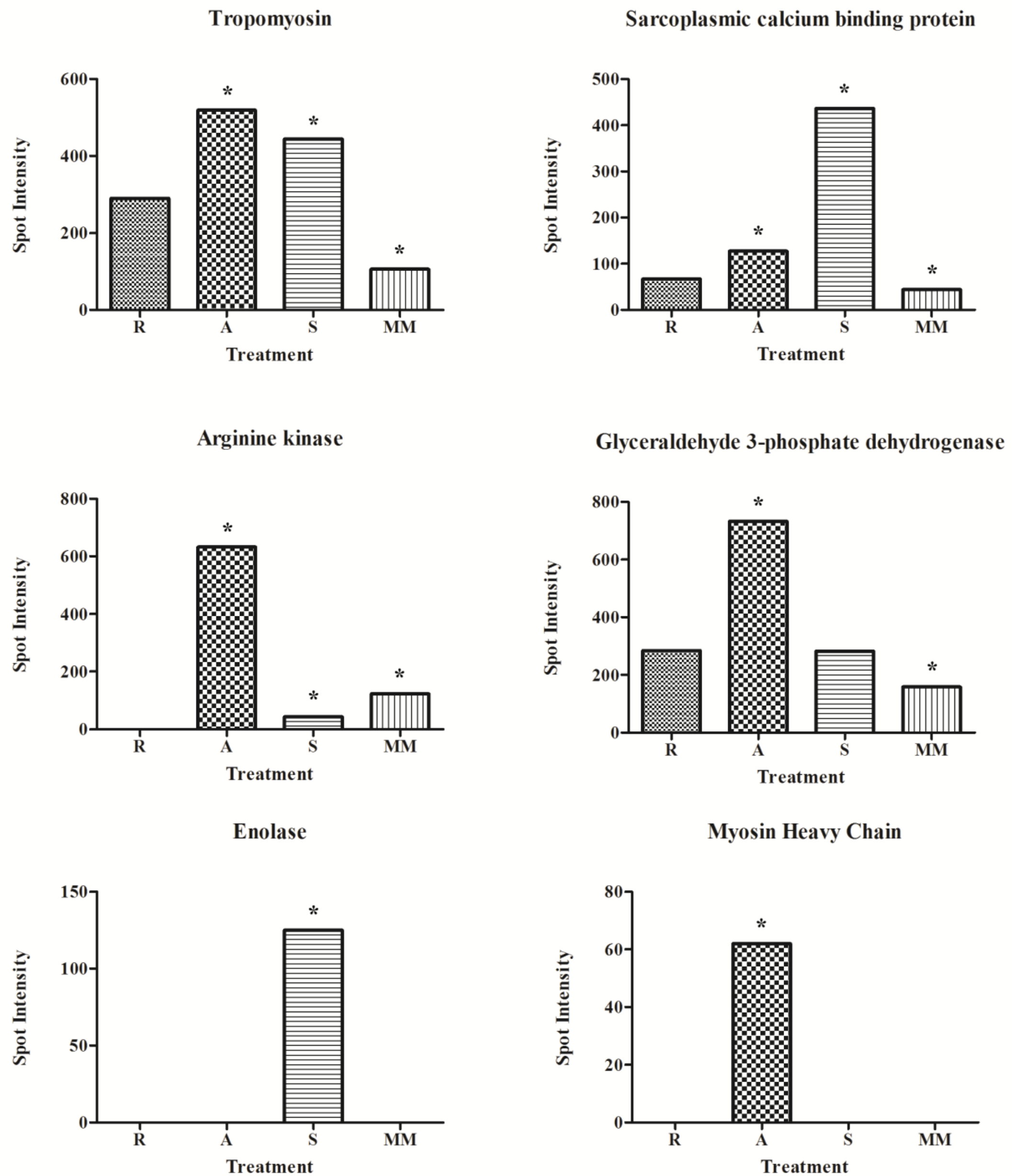


**Figure 28** The spot intensity of IgE binding protein to pooled human sera by 2D-immunoblotting from raw muscle (R), autoclave treated muscle (A), ultrasound treated muscle (S), and 1 min microwave treated muscle (MM). The spot intensity of every allergens was presented as mean±SEM. *$P < 0.05$ vs. raw muscle condition.

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**Table 9** Allergenic proteins characterized in *Fenneropenaeus merguiensis* from this study

| Protein | Organs | | | | | Food processing treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1D-immunobotting** | | | | | **1D-immunobotting** | | | | | **2D-immunobotting** | | | |
| | **SH** | **HP** | **OA** | **OB** | **OC** | **F** | **A** | **S** | **MS** | **MM** | **F** | **A** | **S** | **MM** |
| Tropomyosin | Unidentified | | | | | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Sarcoplasmic calcium-binding protein | ✓ | | | | | ✓ | | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Arginine kinase | ✓ | | ✓ | ✓ | ✓ | ✓ | | | | | | ✓ | ✓ | ✓ |
| glyceraldehyde 3-phosphate dehydrogenase | ✓ | | | | | ✓ | | | | | ✓ | ✓ | ✓ | ✓ |
| Hemocyanin | | | | | | ✓ | | ✓ | | | | | | |
| Enolase | | | | | | ✓ | | | | | | | ✓ | |
| β-actin | | | | ✓ | ✓ | | | ✓ | | ✓ | | | | |
| Myosin heavy chain | | | | | | ✓ | | | | ✓ | | ✓ | | |
| Vitellogenin | | | ✓ | ✓ | ✓ | | | | | | | | | |
| Shrimp ovarian peritrophin | | | ✓ | | | | | | | | | | | |
| 14-3-3 protein | | | | ✓ | ✓ | | | | | | | | | |



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**Discussion**

Allergens were identified in four organs: muscle, shell, hepatopancreas and ovaries with three different levels of the gonadosomatic index (GSI). Allergenic proteins were recognized using the IgE of pooled sera from patients who suffered from shrimp allergy. The same three proteins were identified in the muscle and shell of banana shrimp. Subsequent LC-MS/MS analysis of a 22 kDa protein identified the sarcoplasmic calcium-binding protein, ~40 kDa arginine kinase (AK) and ~30 kDa glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In addition, muscle also contained three other proteins that may be allergens in banana shrimp—namely, a myosin heavy chain (MHC), hemocyanin (HC) and enolase (known as phosphopyruvate hydrates).

In this study, no allergen was identified in the hepatopancreas of banana shrimp. This may have been due to the low molecular mass result in the SDS-PAGE/immunoblotting analysis. SDS-PAGE used glycine-SDS-PAGE for separation of proteins in the range from 1 to 100 kDa. For very small proteins, it is necessary to increase the acrylamide concentrations or use tricine as the trailing ion to allow for the resolution of small proteins at lower acrylamide concentrations (Schagger and Jagow, 1987). Before extraction, shrimp muscle and the other organs were stored at -80°C for several weeks and the hepatopancreas is an organ where proteases are present and there are enzymatic activities occurring under various conditions that cannot be completely inhibited by protease inhibitor (Auttarat *et al*., 2006). The enzymatic properties may cause the degradation of shrimp allergens (Oh *et al*., 2000). Sen *et al*. (2002) suggested that peanut allergen disulfide bonds reduction and their accessibility to IgE-binding epitopes can reduce overall allergenicity. Thus, the structure/epitope degradation of the allergen may reduce the allergenicity.

In the ovaries of banana shrimp at different vitellogenic stages, arginine kinase was identified as in other organs. However, vitellogenin (VG) had a high intensity in all the vitellogenic stages which suggests it is an important allergen in ovaries. The

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other identical proteins were ovarian peritrophin 1 precursor, β-actin and 14-3-3 protein.

VG is a female-specific protein that has been found in the hemolymph of most crustacean species studied. This protein is very important during ovarian development in crustaceans but its function is still not clear and study on it is incomplete. However, it has been suggested that VG is synthesized in extraovarian tissue, secreted to the hemolymph, taken up by developing ovaries and intracellularly processed to generate vitellin, the major yolk protein in the eggs (Avarre *et al*., 2003; Auttarat *et al*., 2006; Rao *et al*., 2007) . The Vg gene is expressed only by mature *F. merguiensis* females and both the ovary and hepatopancreas are possible sites for VG synthesis in this species (Phiriyangkul and Utarabhand, 2006). The major site for vitellogenesis in banana shrimp is the ovary which was investigated by real-time PCR to determine the VG mRNA level (Phiriyangkul *et al*., 2007) and the protein level at all stages of ovarian development was found to correlate well with the GSI values (Auttarat *et al*., 2006). This protein is a complex lipo-glyco-ceroteno-protein (Fatima *et al*., 2013). VG has been suggested as an allergen in many species. VG has also been reported as an allergen in salmonic roe (Kondo *et al*., 2005; Shimizu *et al*., 2009) and Beluga caviar (Perez-Gordo *et al*., 2008). In addition, there are the reports that VG is an allergen in insects such as *Blattella germanica* (German cockroach) (Chuang *et al*., 2010) and in the venom of *Apis mellifera* (western honey bee) and *Vespula vulgaris* (common wasp) (Blank *et al*., 2013).

In the current study, ovarian peritrophin 1 precursor was found as a part of shrimp ovarian peritrophins (SOPs). These proteins were found in ovary stage 1 because this stage is prepared for spawning and the proteins are extruded from the egg cortical crypts to form a protective layer around eggs immediately after spawning (Du *et al*., 2006). β-actin is a member of the actin family which exists in invertebrate and vertebrate cells in several different isoforms (Sun *et al*., 2007). The 14-3-3 protein group of dimeric acidic proteins is involved in many biological processes acting as molecular chaperones and is found in many species of plants, animal and fungi (Inoue

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*et al*., 2005). Kaeodee *et al*. (2011) have suggested that 14-3-3b is likely to be involved in hyper-osmotic regulation in *Penaeus monodon*. The study of the true functions of beta-actin and 14-3-3 protein is still incomplete but there are many suggestions that these proteins are involved in shrimp infection including yellow head virus, white spot syndrome virus (WSSV) and Taura syndrome (Chongsatja *et al*., 2007; Wongpanyaa *et al*., 2007; Pongsomboon *et al*., 2008). SOPs, beta-actin and 14-3-3 protein have not been reported as allergens in shrimp but the current study suggests these three proteins are novel minor allergens in *F. merguiensis*.

The most important post-translational modification in newly synthesized proteins is called glycosylation. Without exception, glycosylation occurs in the integral membrane proteins of higher organisms (Khan *et al*, 2003). From the glycoprotein staining results, six allergens (enolase, AK, VG, GAPDH, SOPs and 14-3-3 protein) have features of glycoprotein that suggest that these six allergens are allergenic glycoproteins. Many studies about the structural analysis of glycan moieties of allergenic glycoproteins showed isolated glycoprotein binding to the IgE of allergic patients when compared with the non-IgE binding properties of pure glycan. The occurrence of allergens with glycan moieties suggests that there are additional IgE epitopes, but no there is no direct evidence for their involvement in allergenicity (Fötisch and Vieths, 2001). Thus, the study of allergenic glycoproteins is important because they take part in inducing an IgE response.

There are 2 proteins as HC and β-actin can identified as allergens in *F.merguiensis* by 1D-immunoblotting but 2D-immunoblotting cannot identified these four proteins. However, it is interesting that 2D-immunoblotting can identify AK and GAPDH as allerges in food processed shrimp muscle whereas 1D-immunoblotting cannot identify these allergens in food processed shrimp muscle. So, it is possible that the isolated protein with SDS-PAGE is not elaborate enough. So, 2D-PAGE is the most frequently for protein separation in proteomic studies because it can provide the spot with single protein. This technique can prove identification of protein (Becker

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and Reese, 2001; González-Buitrago *et al*., 2007). However, this study will include the result of 1D and 2D immunoblotting to determinations.

The purpose of SDS-PAGE separate proteins according to protein molecular mass, but 2D-PAGE separate proteins with two properties in two dimensions, isoelectric point (*pI*) and molecular mass. It is not able to use SDS-PAGE to separate two proteins at the same molecular mass from each other. So, 2D-PAGE has been the most frequently for protein separation in proteomics studies because it can provide the spot with single protein (González-Buitrago *et al*., 2007). PBS buffer and cell lysis buffer are the most physiological buffers used in proteomics study because there are isotonic and non-toxic to cells. Moreover, they have the ability to maintain protein osmolality (Morris *et al.*, 2001). From SDS-PAGE, PBS buffer was chosen because there is some component as sodium chloride (NaCl) can increase the solubility of proteins in solution. Inyang and Iduh (1996) reported about influence of pH and salt concentration on protein solubility of sesame protein, suggested that optimize of salt concentration can increasing pprotein solubility. In addition to the advantages of PBS extraction buffer that mentioned above, there are some disadvantages due to sodium chloride (NaCl) in buffer. NaCl is able to disturb the process of isoelectric focusing which effect the separation by isoelectric point (*pI*). Some component as thiourea and urea can raise the solvent capacity of the cell-lysis buffer as well (Carpentier *et al*., 2005). Moreover, the different detergents and reducing agents in each extraction buffer can cause the different extracting properties. Reducing agent such as dithioerythritol (DTT) or β-mercaptoethanol can cleaves the disulfide bonds in the proteins but β-mercaptoethanol is not recommended for 2D-PAGE because it disturbance the alkaline end of the pH gradient in IEF technique (Righetti *et al*., 1982).

1D-immunoblotting experiments were probed with IgE antibody from the 12 individual serum but 2D-immunoblotting experiments were probed with pooled sera from 21 shrimp allergic patients. So, it may provide the different results between treatments because sera of patients were different in many ways as gender, age, race

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and allergic IgE reactive from each subject has different active recognition sites (Li *et al*., 2007) and there are different amount of IgE antibody (Appendix Table C1).

In 1D-immunoblotting, every food processing treatment can increase allergenicity of tropomyosin (TM) while immunoblotting of 2D-PAGE, autoclave muscle and ultrasonicate muscle have increased of TM allergenicity and microwave muscle has decreased of TM allergenicity. TM has identified as major allergen in every treatment (Figure 28).

The effect of heating may appear in two different ways by unfolding of molecules and heat-induced aggregation. A consequence of unfolding and aggregation during cooking is destroys the conformational epitopes, IgE-reactivity and ability to trigger a reaction in sensitized individuals is generally reduced by food processing (Nakai and Li-Chan, 1988; Mills *et al*., 2007). On the other hand, food processing could also unveil or create new epitopes, sometimes termed neo-epitopes. This may occur through protein unfolding, which unravels inner portions of the protein structure, not generally available for antibody binding or the covalent modifi cation of a protein by sugars or other food components (Wal, 2003; Maleki and Sathe, 2006). Thus, cooking process might be alter the allergenicity of banana shrimp, may destroy existing epitopes on a protein or may generate neo-allergenic formations (Maleki et al., 2000; Taylor et al., 2008).

For the TM, this protein comprises a class of highly conserved proteins with multiple isoforms found in both muscle and non-muscle cells of all species of vertebrates and invertebrates (Leung and Chu, 1998) It is an abundant and heat-stable protein that constitutes up to 20% of total protein in the edible part of the animal and physically associated with actin and myosin in muscle fibers and other motile filaments (Leung *et al*., 1998; Reese *et al*., 1999). Historically, linear and conformational epitopes are defined as two types of epitopes. The IgE recognition specificity depends on the amino acid sequence may be a simple stretch along the primary structure (linear epitope) or a three dimensional motif of the protein structure

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(conformational epitope) (Sathe *et al*., 2005; Chen and Gao, 2012). Thus, linear epitopes are typically expected to be less susceptible to heat processing induced destruction than conformational epitopes on the same allergen. In this study, we can assume that tropomyosin has the linear epitopes because it is not destroyed by high-heat and pressure.

Recently, researchers have demonstrated that autoclave treatment can decrease the binding ability of porcine serum albumin (PSA) to IgG from sera of pork allergic patient (Kim *et al*., 2011). In 2002, Venkatachalam *et al.* (2002) suggested in the same way that autoclave processing can decreases allergenicity of almond determined by competitive inhibition ELISA. Moreover, López *et al*. (2012) has report about effect of autoclave on allergenicity of hazelnut protein. They suggested that autoclave can reduce the allergenicity of hazelnut protein but not completely, epitopes study predicted to carry linear epitopes, as they are located in the external faces of the protein hexamer and thus exposed to the solvent.

High-intensity ultrasound waves interact with the food system. A part of the wave energy is absorbed and converted into mechanical and thermal energy to change the properties of the food system (Zhenxing *et al*., 2006). From the previous study about ultrasonic on the allergenicity of shrimp by ELISA with pooled serum of allergic patients found that ultrasonicated treatment can reduced the allergenicity of shrimp allergen (Li *et al*., 2006). Moreover, the allergenicity of major peanut allergen after ultrasonic treated was demonstrated. The results shown peanut allergen after ultrasonicated has lower IgE binding ability than non-treated peanut allergen (Li *et al*., 2013). In this study, the high intensity ultrasound (30Hz, 800 W) at $50^{0}$C for 1.5 h is the combination between heat and ultrasound can alter the allergenicity of IgE binding protein in increasing and decreasing ways. While the study of Zhenxing *et al*. (2006) with same condition of ultrasound treatment shown the reducing of allergenicity of whiteleg shrimp (*P. vannamei*) allergen. The different results might due to shrimp species specific and/or sera of patients were different in many ways as gender, age, race and allergic IgE reactive.

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Moreover, microwave is the common ways to household cooking. The study about microwave treatment on allergenicity of IgE-binding protein is limited. Alvarez-Alvarez *et al*. (2005) has studies the effect of food processing on allergenicity of lupine seeds. The result showed that microwave has slightly reduced the lupine allergenicity whereas autoclave processing can ostensibly reduce the lupine allergenicity (Liu *et al*., 2010; Carnés *et al*., 2007).

There is the allergenicity of AK in every cooked treatment from 2D-immunoblotting whereas AK has been found as an non-allergenicity protein in raw muscle (Appendix Figure E1; Appendix Table E14). In 1D-immunoblotting, AK can suggested as major allergen in raw *F. merguiensis* protein because there one hundred percent prevalent of IgE binding ability with shrimp allergic patient sera (Table 7). AK was present in very large quantities in shrimp muscle tissue and as phosphotransferase that plays a critical role in the coupling of energy production for muscle mobilization and utilization (France *et al*., 1997; Yao *et al*., 2009). Moreover, sarcoplasmic calcium-binding protein (SCP) can identify as shrimp allergen from both 1D and 2D-immunoblotting in almost every condition of food processing and there have the different allergenicity with statistically significant between autoclave, ultrasonic and microwave treatment compared with raw muscle. It is acidic allergen with molecular mass ~15 - ~22 kDa. It has been speculated that invertebrate SCP may serve a similar function as vertebrate parvalbumins, promoting rapid muscle relaxation by facilitating calcium translocation from myofibrils to the sarcoplasmic reticulum and may protect against high calcium concentration inside the cell (Takagi and Konishi., 1984; Gao *et al*., 2006). Thus; TM, AK and SCP are all involved in muscle regulator and structure that can suggested as major allergen in *F. merguiensis*.

GAPDH has also recognized 2D-immunoblotting data from subjects who allergic to shrimp in every food process treatment in this work. Autoclave treatment can increase allergenicity of GAPDH while microwave can decrease it when compare with raw muscle with statistical significance. GAPDH was considered a classical glycolytic protein examined for its pivotal role in energy production. It was also used

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as a model protein for analysis of protein structure and enzyme mechanisms (Sirover, 1999). GAPDH is found as minor allergen in bread wheat (*Triticum aestivum*) (Sander *et al*., 2011) and also been report as allergen in indoor mould (*Aspergillus versicolor*) (Benndorf *et al*., 2008). GAPDH has been identified as an important allergen in occupational sensitization to pilchard (*Sardinops sagax*) (Ventela *et al.,* 2011). Until now, there is no report that GAPDH is an allergen in shrimp, results suggest that GAPDH is the novel allergen and major in *F.merguiensis*.

From 1D-immunoblotting, HC was found in raw and ultrasound muscle. This protein has functioned as giant oligomeric oxygen carrier protein present in the hemolymph of arthropods and mollusks (Paoli *et al*., 2007). β-actin was found in ultrasound and 1 min microwave muscle by 1D-immunoblotting. This might be occur from muscle protein denaturalize under critical condition from food processing. β-actin is subunit of actin proteins that involved in muscle organization, cellular cytoskeleton, cell motility and cell division (Cesar and Yang, 2007; Sun *et al*., 2007).

There were found another two proteins, furthermore, that have recognized immunoblotting data from subjects who allergic to shrimp. MHC was found in ultrasonic and microwave muscle by 1D-immunoblotting and found in autoclave muscle by 2D-immunoblotting. MHC is a large subunit of the myosin molecule functional as the motor protein of muscle thick filaments (Wells *et al*., 1996; Koyama *et al*., 2012). There are two isoforms show in this study: MHC type 1 (MHC type a) and MHC type 2 (MHC type b). MHC is the important allergen from brown garden snail (*Helix aspersa*), famous French cuisine and popularity as the chief ingredient in skin creams and gels in the USA (Martins *et al*., 2005).

This study also found that enolase has allergenicity in raw muscle by 1D-immunoblotting but 2D-immunoblotting found enolase in ultrasound. Enolase and GAPDH are in the group of glycolytic enzymes. There is evidence for the existence of glycolytic and pentose phosphate pathways in various crustacea. In general the metabolism of crustacea does not differ in broad outline from that of higher animal

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phyla and the principal pathways are more or less similar in crustacea as in vertebrates (Claybrook, 1983). Mathew *et al*. (2007) demonstrated that WSSV infected in *P. monodon* will alter the glycolytic enzymes activity. However, carbohydrate metabolism has only been partially studied in shrimp. And some glycolytic enzyme such as enolase also identified as stress protein causing by hypoxia (Leu *et al*., 2007). Enolase is the new allergen found in fish; cod, tuna and salmon (Liu *et al*., 2011; Tomm *et al*., 2013). As the GAPDH, there is no previous reported the IgE binding ability of MHC, enolase and β-actin in Crustacean.

Crustaceans are responsible for food-induced allergic reactions in both children and adults. Increasing shellfish consumption can result in increased levels of hypersensitivity symptoms. Avoidance of the offending foods is the only therapy recommended for food allergic subjects. Steensma (2003) underscores the importance of carefully counseling all patients who are allergic to food that they must avoid exposure to offending proteins via all potential routes of contact. The current study suggests that muscle and other organs also contain IgE-binding proteins. MHC, HC, enolase, AK, GAPDH, SCP, VG, 14-3-3 protein and β-actin, were identified as allergens in banana shrimp. MHC, enolase, GAPDH, ovarian peritrophin 1 precursor, 14-3-3 protein, beta-actin and VG are novel allergens in shrimp.

In autoclave treatment muscle, 1D-immunoblotting can increase the allergenicity of TM and 2D-immunoblotting can increase the allergenicity of TM, SCP, AK, GAPDH and MHC with statistic significant. Ultrasounic treatment was make a neo-allergen, β-actin in 1D-immunoblotting and increase the allergenicity of TM, SCP, AK and enolase with statistic significant. Moreover, microwave treatment can alter IgE binding protein by increase TM in 1D-immunoblotting and AK in 2D-immonoblotting but decrease the allergeinicity of TM, SCP and GAPDH with statistic significant.

Food processing may permit masking of allergenic epitopes by altering allergen recognition and potentially altering allergenicity of the food. Food processing

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is the first point of interest and the most expedient way to study the alteration of allergenicity in food and can generate the hypoallergenic food in the future. The present study can appear critical to include these allergens in future diagnostic and therapeutic strategies. This may be one of important step in food safety protocol to developed food product for food allergic patients in the future.

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# CONCLUSIONS AND RECOMMENDATIONS

## Conclusions

From the experimental results and discussion of this study, can be concluded this work as follows:

1. Food processing alters the protein forms. The electrophoresis profiles of *F.merguiensis* raw muscle and each food processing conditions showed clear differentiation between treatments.

2. VG, SOPs, 14-3-3 protein and β-actin were demonstrated as novel allergen in *Fenneropenaeus merguiensis* ovarian and suggested VG as major allergen in ovarian. GAPDH, HC and enolase were demonstrated as novel allergen in *F.merguiensis* raw muscle. In addition; TM, AK, SCP and GAPDH were suggested as important allergen in *F.merguiensis* muscle in every food processing treatment.

3. Food processing can alter the allergenicity of shrimp allergen in many ways and also generate new IgE-binding properties for non-allergenic protein as called the neo-allergen; β-actin in autoclave and microwave muscle.

## Recommendations

1. The purification or recombinant protein from allergen cDNA performs with *in vivo* and another *in vitro* to diagnose allergenicity is necessary to confirm the IgE-binding ability.

2. The allergenicity can be altered by food processing, may be caused by changes in patterns of protein epitopes. A study of epitope patterns for each type of allergen is necessary in further studies to even more understand in allergy.

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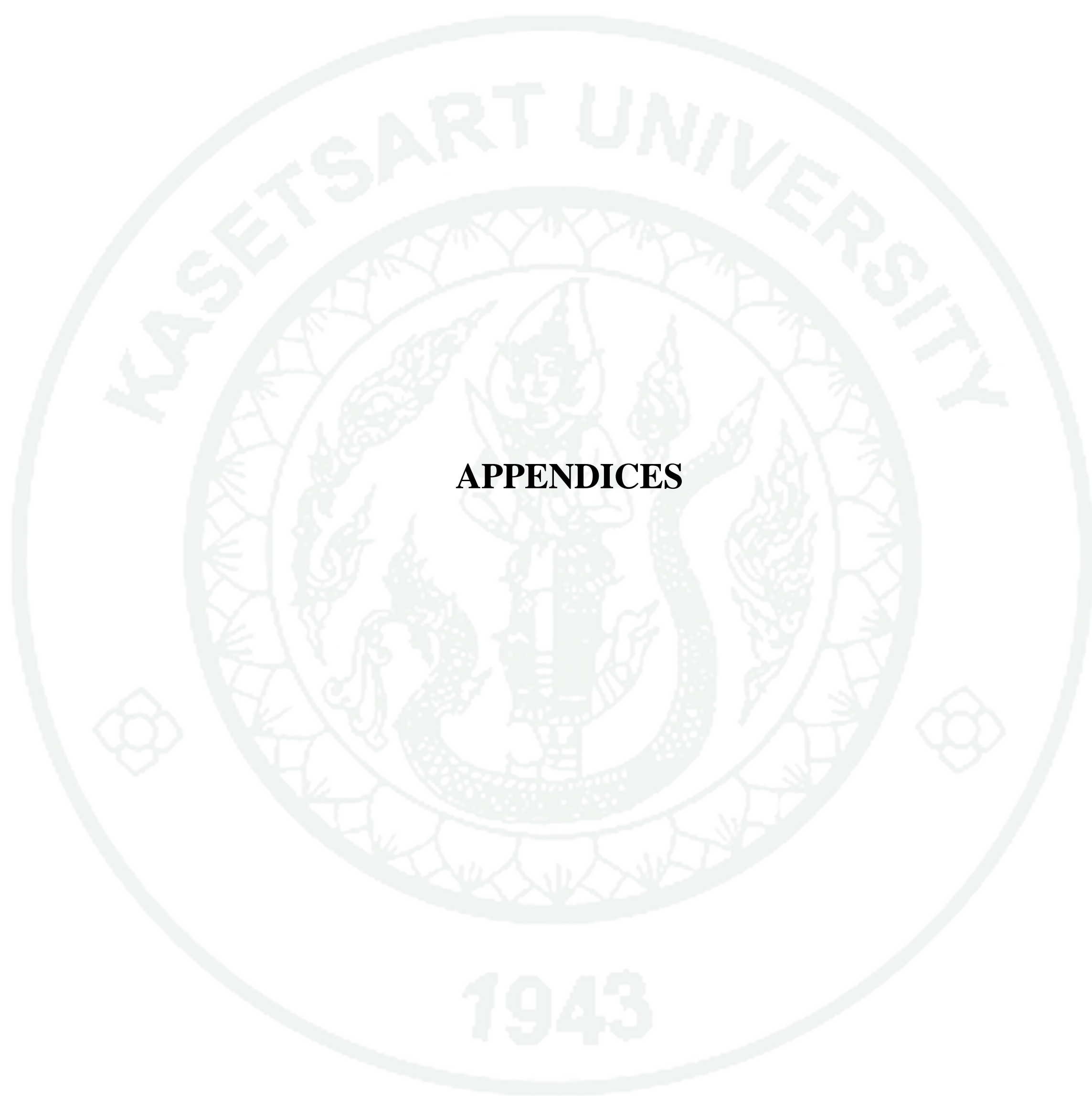

# APPENDICES

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**Appendix A**

Buffers and solutions preparation

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# Buffers and solutions preparation

## 1. Protein extraction

1.1 Phosphate buffered saline (PBS)

1) Dissolve the following in 80 ml distilled water.

| | | |
|---|---|---|
| $Na_2HPO_4$ | 0.128 | g |
| $NaH_2PO_4 \bullet 2H_2O$ | 0.0156 | g |
| NaCl | 0.890 | g |

2) Adjust pH to 7.4 with HCl.
3) Adjust volume to 1L with additional distilled water.

1.2 Lysis buffered

1) Dissolve the following together.

| | | |
|---|---|---|
| Urea | 4.2 | g |
| Thiourea | 1.525 | g |
| CHAPs | 0.4 | g |
| DTT | 0.2 | g |
| Ampholines pH 3-10 | 500 | µl |
| Protease inhibitor cocktail | 100 | µl |
| 10% SDS | 400 | µl |

2) Adjust volume to 10 ml with additional distilled water.

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**2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

2.1 5X running (electrode) buffer, pH 8.3*

1) Dissolve the following in 800 ml distilled water.

| | |
|---|---|
| Tris-base | 15 g |
| Glycine | 72 g |
| SDS | 5 g |

2) Adjust volume to 1L with additional distilled water.

* Store at 4°C, warm to 37°C before use if precipitation occurs.

2.2 1X running (electrode) buffer, pH 8.3

Dilute 200 ml 5X running buffer with 800 ml distilled water.

2.3 10% (w/v) SDS, stock solution

Dissolve 10 g SDS in 80 ml distilled water and adjust volume to 100 ml with additional distilled water.

2.4 30% Acrylamide/Bis*

1) Dissolve the following in 80 ml distilled water.

| | |
|---|---|
| Acrylamide | 30.0 g |
| N,N'-Bis-methyleneacrylamide | 72.0 g |

2) Adjust volume to 100 ml with additional distilled water.

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* Store the solution in dark bottles at room temperature for less than 3 months.

2.5 1.5 M Tris-HCl, pH 8.8

1) Dissolve 18.15 g Tris-base in 80 ml distilled water.
2) Adjust pH to 8.8 with HCl.
3) Adjust volume to 100 ml with additional distilled water.

2.6 0.5 M Tris-HCl, pH 6.8

1) Dissolve 6 g Tris-base in 80 ml distilled water.
2) Adjust pH to 6.8 with HCl.
3) Adjust volume to 100 ml with additional distilled water.

2.7 10% Ammonium persulfate (APS)

Dissolve 20 mg APS in 200 µl distilled water.

2.8 0.05% (w/v) bromophenol blue

Dissolve 0.25 mg bromophenol blue in 500 µl distilled water.

2.9 0.05% (w/v) coomassie blue R-250

Dissolve 0.25 mg coomassie blue R-250 in 500 µl distilled water.

2.10 5X sample buffer (SDS reducing buffer) with 30% glycerol

Mixed the following together.

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| | |
|---|---|
| Distilled water | 2.2 ml |
| 0.5 M Tris-HCl, pH 6.8 | 1.0 ml |
| Glycerol | 2.4 ml |
| 10% SDS | 1.6 ml |
| β-Mercaptoethanol | 0.4 ml |
| 0.05% (w/v) bromophenol blue | 0.4 ml |

2.11 Rehydration buffer

Mixed the following together.

| | | |
|---|---|---|
| Distilled water | 320 | μl |
| Urea | 0.24 | g |
| CHAPs | 10 | mg |
| DTT | 1.4 | mg |
| IPG buffer, pH 3-10 | 2.5 | μl |
| 0.05% (w/v) bromophenol blue | 2 μl | |

2.12 Equilibration buffer

Mixed the following together.

| **Composition** | **Equilibration I** | **Equilibration II** |
|---|---|---|
| 0.5 M Tris-HCl, pH 6.8 | 0.3 ml | 0.3 ml |
| Urea | 1.08 g | 1.08 g |
| Glycerol | 0.9 ml | 0.9 ml |
| SDS | 30 mg | 30 mg |
| Distilled water | 1.005 ml | 1.005 ml |
| DTT | 30 mg | - |
| IAA | - | 75 mg |

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2.9 0.5% agarose

Dissolve 5 mg agarose in 1000 µl distilled water.

**3. Coomassie blue staining**

3.1 0.1% coomassie blue R-250*

1) Dissolve 0.5 g of coomassie blue R-250 in:

| | |
|---|---|
| Methanol | 200 ml |
| Distilled water | 250 ml |
| Acetic acid | 50 ml |

* Filter with filter paper no. 4 to remove particulates and store at room temperature in a sealable container.

3.2 Destaining solution

Mixed the following together.

| **Composition** | **Destaining Solution I** | **Destaining Solution II** |
|---|---|---|
| Methanol | 400 ml | 100 ml |
| Ethanol | 100 ml | 50 ml |
| Distilled water | 500 ml | 850 |

**4. Westernblot (immunoblot) analysis**

4.1 5X Tris-glycine (stock for blotting buffer)

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1) Dissolve the following in 800 ml distilled water.

| | | |
|---|---|---|
| Tris-base | 15 | g |
| Glycine | 72 | g |

2) Adjust volume to 1L with additional distilled water.

4.2 1X blotting buffer*

Mixed the following together.

| | |
|---|---|
| 5X Tris-glycine | 200 ml |
| Methanol | 200 ml |
| Distilled water | 600 ml |

* Store at 4°C until used.

4.3 10X Tris-buffered saline (TBS), pH 7.6

1) Dissolve the following in 800 ml distilled water.

| | | |
|---|---|---|
| Tris-base | 24.2 | g |
| NaCl | 80 | g |

2) Adjust pH to 7.6 with HCl.
3) Adjust volume to 1L with additional distilled water.
4) Sterilize by autoclaving and store at 4°C

4.4 Tris-Buffered Saline/Tween 20 (TBS/T)*

Mixed the following together.

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| | |
|---|---|
| 10X TBS | 100 ml |
| Tween 20 | 1 ml** |
| Distilled water | 899 ml |

* Store at 4°C, warm to room temperature before use

** Final concentration = 0.1% Tween

4.5 10% Skim milk

Dissolve 1 g skim milk in 10 ml distilled water.

4.6 3% Skim milk

Dissolve 0.6 g skim milk in 20 ml distilled water.

**5. Glycoprotein staining**

5.1 3% Acetic acid

Dilute 3 ml acetic acid with 97 ml distilled water.

**6. Trypsin in-gel digestion (stock solution)**

6.1 10 mM $CaCl_2$

Dissolve 73.51 mg $CaCl_2$ in 50 ml MilliQ water.

6.2 100 mM EDTA

Dissolve 1.8612 g EDTA in 50 ml MilliQ water.

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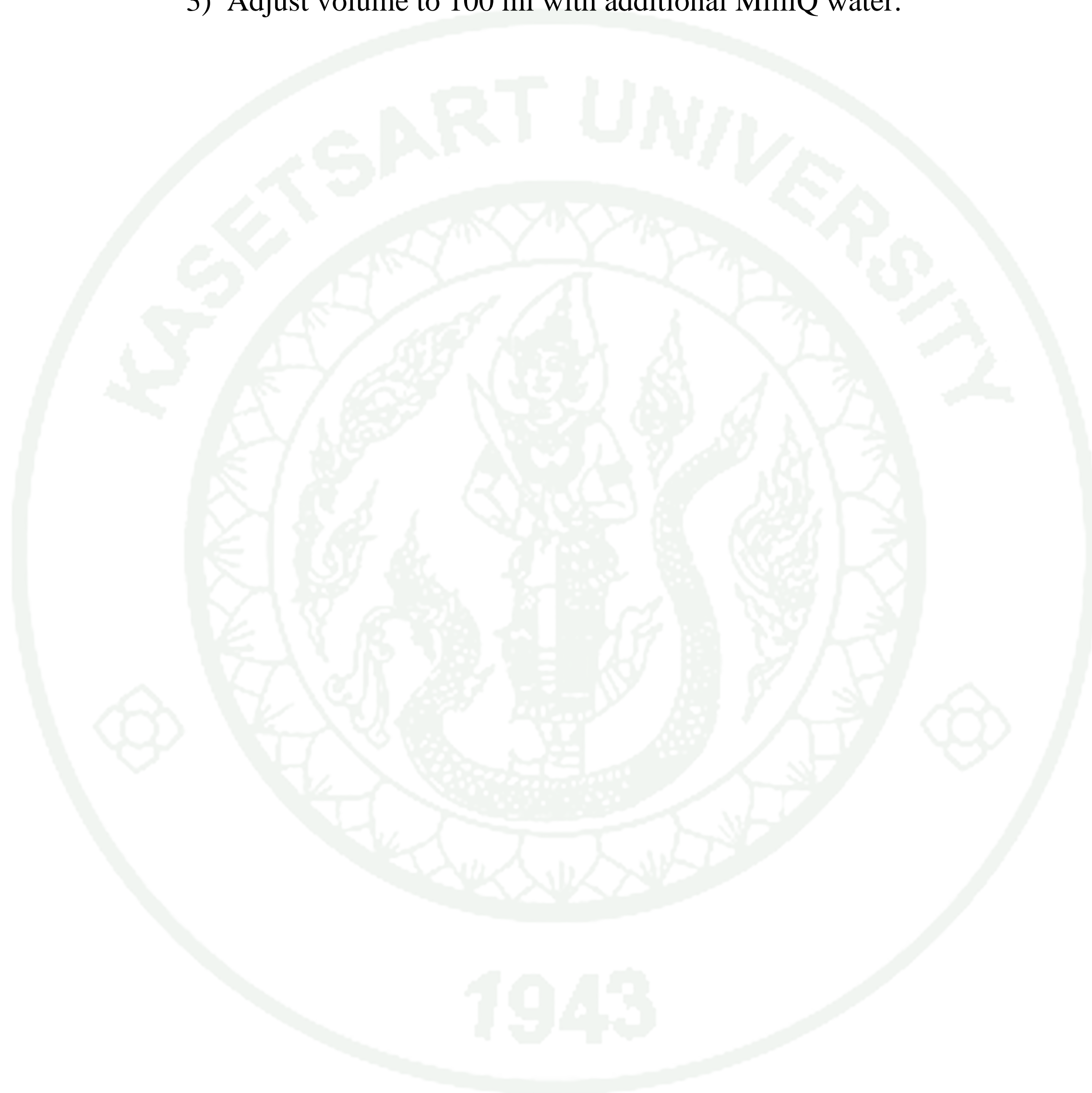
6.3 0.1 M Tris-HCl, pH 8.5

1) Dissolve 1.2114 g Tris-base in 80 ml MilliQ water.
2) Adjust pH to 8.5 with HCl.
3) Adjust volume to 100 ml with additional MilliQ water.



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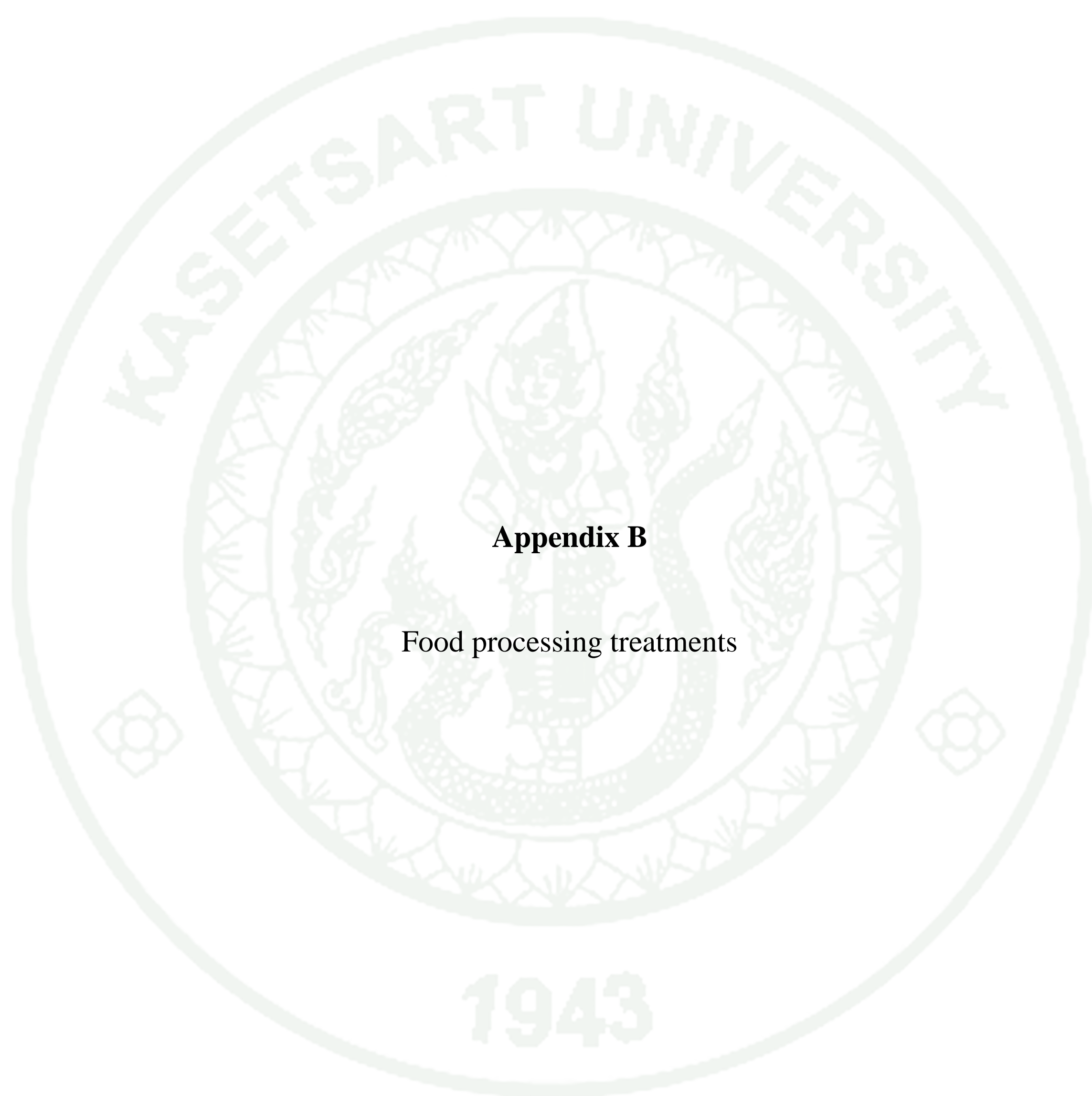

**Appendix B**

Food processing treatments

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# Food processing treatment for muscle

## 1. Autoclave treatment

Autoclave treatment for shrimp muscle with 121 °C, 100 kPa for 15 min (SS325: Tomy Digital Biology; Nerima-ku, Tokyo, Japan).

**Appendix Figure B1** Autoclave treatment.

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**2. Ultrasonic treatment**

Ultrasonic treated for shrimp muscle with 30 Hz, 800W, 50 °C for 90 min by ultrasonic sonicator (D-78224: Singen/Htw; Singen, Baden-Württemberg, Germany).

**Appendix Figure B2** Ultrasonicated treatment.

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**3. Microwave treatment**

Microwave treated for shrimp muscle with 800W, 120°C for 30 sec and 1 min by Microwave oven (EMM2007X: Electrolux; Stadshagen, Stockholm, Sweden).

**Appendix Figure B3** Microwave treatment.

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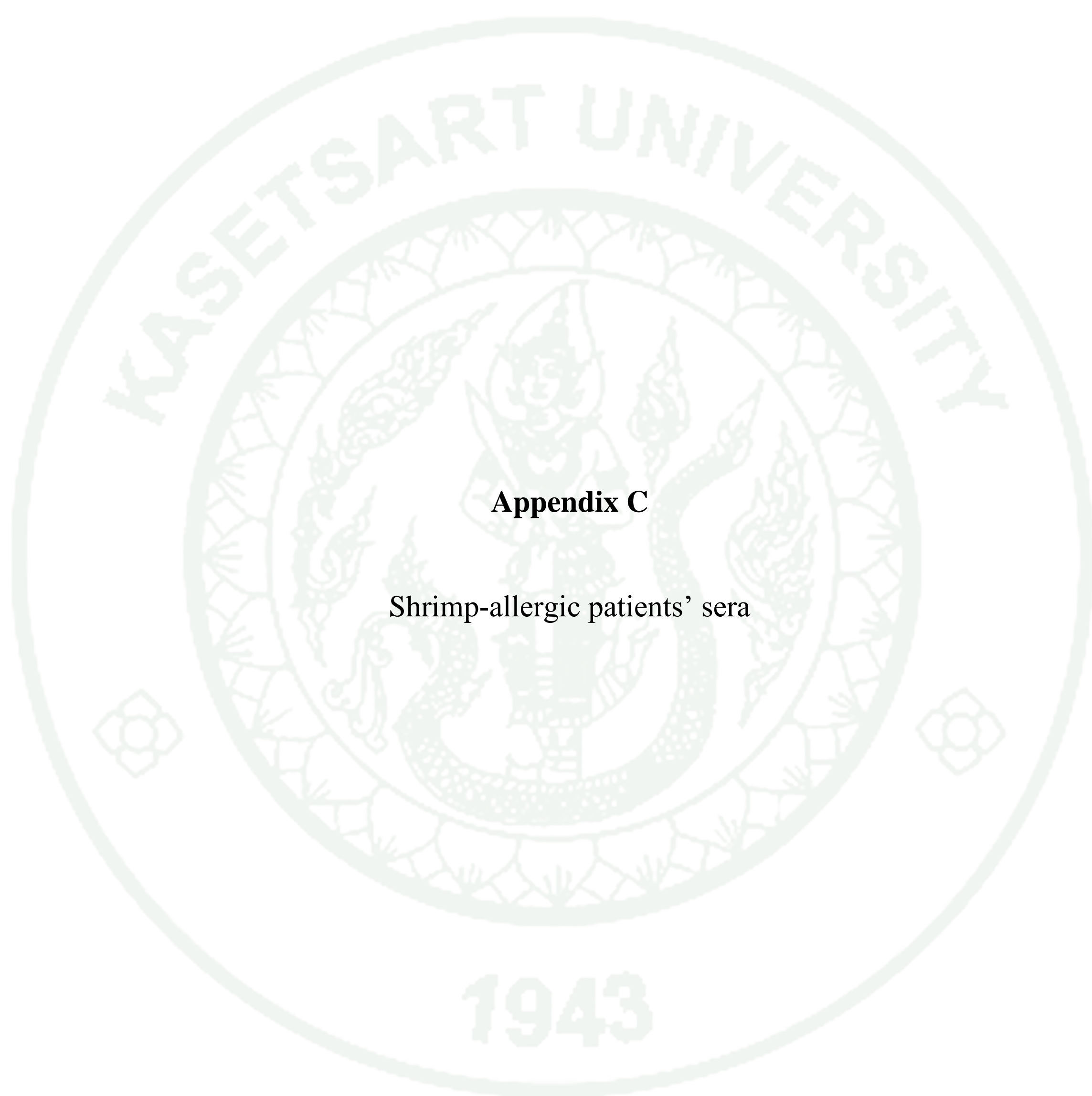


## Appendix C

Shrimp-allergic patients' sera

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**Appendix Table C1** Clinical characteristics of the 21 shrimp-allergic patients' sera

| Code | Gender | Source | EUROLINE test | | Using |
|---|---|---|---|---|---|
| | | | IgE verified | Class* | |
| SA001 | Male | Phramongkutklao hospital | NT | - | 1D/2D-immunoblot Pooled |
| SA002 | Male | Phramongkutklao hospital | NT | - | 1D/2D-immunoblot Pooled |
| SA003 | Female | Phramongkutklao hospital | NT | - | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA004 | Male | Phramongkutklao hospital | NT | - | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA005 | Male | Phyathai Hospital | 5.50 kU/L | 3 | 1D/2D-immunoblot Pooled |
| SA006 | Male | Phyathai Hospital | 32.0 kU/L | 4 | 1D/2D-immunoblot Pooled |
| SA007 | Male | Phyathai Hospital | 2.10 kU/L | 2 | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA008 | Female | Phyathai Hospital | 19.00 kU/L | 4 | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA009 | Male | Phyathai Hospital | 90.00 kU/L | 5 | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA010 | Female | Phyathai Hospital | 9.50 kU/L | 3 | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA011 | Male | Phyathai Hospital | 13.50 kU/L | 3 | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA012 | Male | Phyathai Hospital | 9.50 kU/L | 3 | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA013 | Male | Phyathai Hospital | 15.50 kU/L | 3 | 1D/2D-immunoblot Pooled |
| SA014 | Female | Phyathai Hospital | 1.70 kU/L | 2 | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA015 | Female | Phyathai Hospital | 20.00 kU/L | 4 | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |



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**Appendix Table C1** (Continued)

| Code | Gender | Source | EUROLINE test | | Using |
|---|---|---|---|---|---|
| | | | IgE verified | Class* | |
| SA016 | Female | Phyathai Hospital | 19.00 kU/L | 4 | 1D/2D-immunoblot Pooled |
| SA017 | Male | Phyathai Hospital | 2.10 kU/L | 2 | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA018 | Male | Phramongkutklao hospital | NT | - | 1D-immunoblot Individual; 1D/2D-immunoblot Pooled |
| SA019 | Female | Phyathai Hospital | 13.50 kU/L | 3 | 1D/2D-immunoblot Pooled |
| SA020 | Male | Phyathai Hospital | 5.50 kU/L | 3 | 1D/2D-immunoblot Pooled |
| SA021 | Female | Phyathai Hospital | 0.70 kU/L | 2 | 1D/2D-immunoblot Pooled |

* Class 1, often no clinical symptoms; Class 2, existing sensitization; Class 3, clinical symptoms are often present; Class 4, almost always with existing symptoms; Class 5, always with existing symptoms; NT, not tested.



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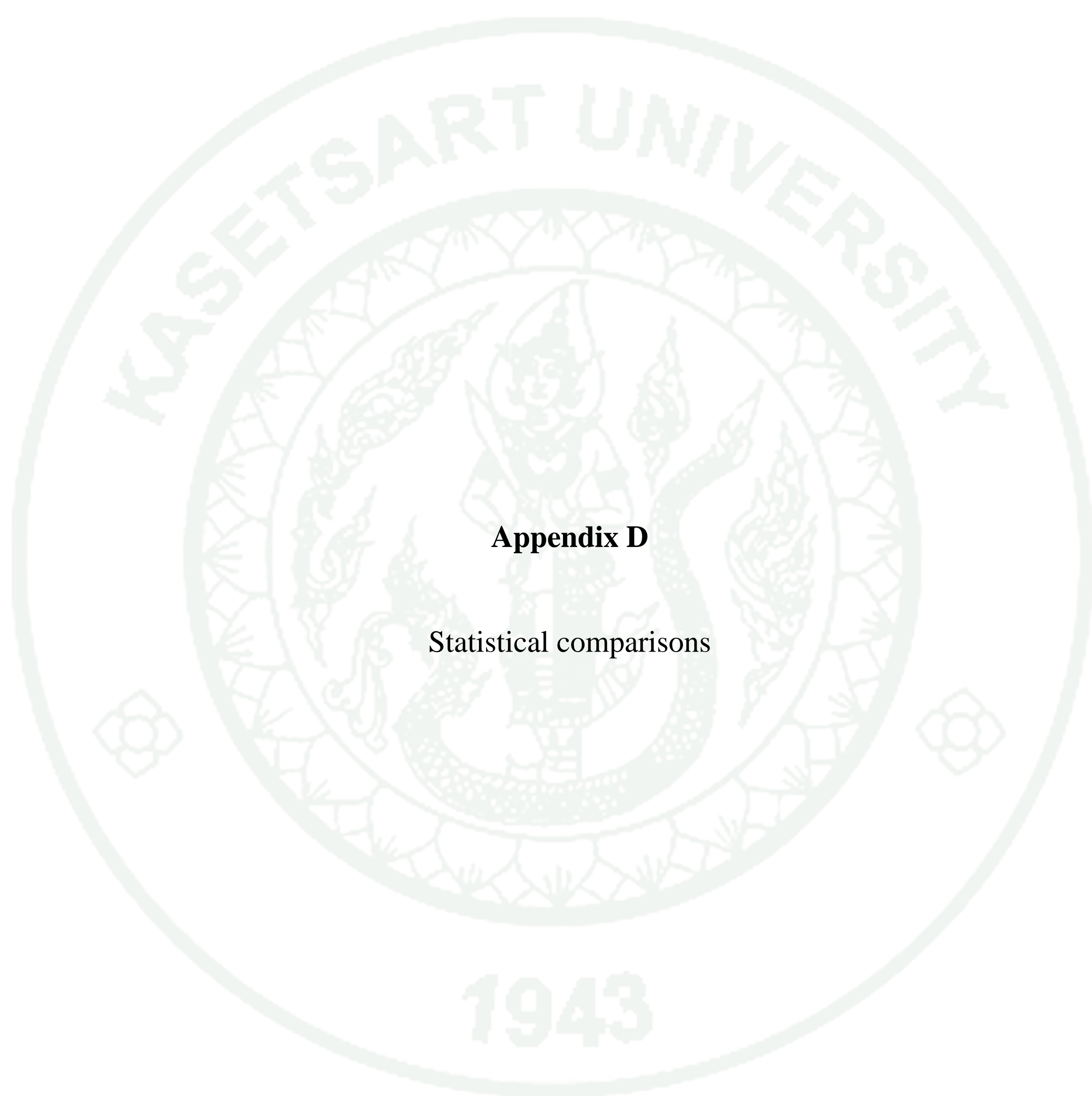


# Appendix D

Statistical comparisons

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Significant differences in intensity values of each allergen were determined by statistical analysis using SPSS software version 15.0. One-way ANOVA were used followed by Bonferroni's multiple comparison tests. *P*-values less than 0.05 were considered statistically significant.

Symbols used in tables

SS = the sum of squares for each of the estimates of variance.
df = the degrees of freedom for each estimate of variance.
MS = the estimates of variance (the mean squares).
F = F ratio. ($MS_{[between\text{-}groups]}$/ $MS_{[within\text{-}groups]}$).
Sig. = the significance of the F ratio. This is the *P*-value.

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**Appendix Table D1** Analysis of variance in intensity values between food processing treatments of tropomyosin

| Source of variation | SS | df | MS | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 200225.50 | 3 | 66741.83 | 33370.92 | .000 |
| Within Groups | 8.00 | 4 | 2.00 | | |
| Total | 200233.50 | 7 | | | |

**Appendix Table D2** Bonferroni's multiple comparison tests of intensity's mean values shows which treatment differed from each other of tropomyosin

| **Treatment** | **x̄** | **R** | **A** | **S** | **MM** |
|---|---|---|---|---|---|
| | | **290.00** | **519.00** | **444.00** | **106.00** |
| **R** | **290.00** | | 229.00* | 154.00* | 184.00* |
| **A** | **519.80** | | | 75.00* | 413.00* |
| **S** | **444.00** | | | | 338.00* |
| **MM** | **106.00** | | | | |

* Significant statistical difference (P < 0.05)

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**Appendix Table D3** Analysis of variance between food processing treatments of sarcoplasmic calcium-binding protein

| Source of variation | SS | df | MS | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 197627.37 | 3 | 65875.79 | 40538.95 | .000 |
| Within Groups | 6.50 | 4 | 1.63 | | |
| Total | 197633.87 | 7 | | | |

**Appendix Table D4** Bonferroni's multiple comparison tests of intensity's mean values shows which treatment differed from each other of sarcoplasmic calcium-binding protein

| Treatment | x̄ | R | A | S | MM |
|---|---|---|---|---|---|
| | | **67.00** | **127.00** | **435.50** | **44.00** |
| **R** | **67.00** | | 60.00* | 368.50* | 23.00* |
| **A** | **127.00** | | | 308.50* | 83.00* |
| **S** | **435.50** | | | | 391.50* |
| **MM** | **44.00** | | | | |

* Significant statistical difference ($P < 0.05$)

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**Appendix Table D5** Analysis of variance between food processing treatments of arginine kinase

| Source of variation | SS | df | MS | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 513777.50 | 3 | 171259.17 | 114172.78 | .000 |
| Within Groups | 6.00 | 4 | 1.50 | | |
| Total | 513783.50 | 7 | | | |

**Appendix Table D6** Bonferroni's multiple comparison tests of intensity's mean values shows which treatment differed from each other of arginine kinase

| Treatment | x̄ | R | A | S | MM |
|---|---|---|---|---|---|
| | | **.00** | **632.00** | **44.00** | **123.00** |
| **R** | **.00** | | 632.00* | 44.00* | 123.00* |
| **A** | **632.00** | | | 588.00* | 509.00* |
| **S** | **44.00** | | | | 79.00* |
| **MM** | **123.00** | | | | |

* Significant statistical difference (P < 0.05)

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**Appendix Table D7** Analysis of variance between food processing treatments of glyceraldehyde 3-phosphate dehydrogenase

| Source of variation | SS | df | MS | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 381641.50 | 3 | 127213.83 | 63606.92 | .000 |
| Within Groups | 8.00 | 4 | 2.00 | | |
| Total | 381649.50 | 7 | | | |

**Appendix Table D8** Bonferroni's multiple comparison tests of intensity's mean values shows which treatment differed from each other of glyceraldehyde 3-phosphate dehydrogenase

| **Treatment** | **x̄** | **R** | **A** | **S** | **MM** |
|---|---|---|---|---|---|
| | | **284.00** | **732.00** | **283.00** | **158.00** |
| **R** | **284.00** | | 448.00* | 1.00 | 126.00* |
| **A** | **732.00** | | | 449.00* | 574.00* |
| **S** | **283.00** | | | | 125.00* |
| **MM** | **158.00** | | | | |

* Significant statistical difference ($P < 0.05$)

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**Appendix Table D9** Analysis of variance between food processing treatments of enolase

| Source of variation | SS | df | MS | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 23437.50 | 3 | 7812.50 | 15625.00 | .000 |
| Within Groups | 2.00 | 4 | .50 | | |
| Total | 23439.50 | 7 | | | |

**Appendix Table D10** Bonferroni's multiple comparison tests of intensity's mean values shows which treatment differed from each other of enolase

| Treatment | x̄ | R | A | S | MM |
|---|---|---|---|---|---|
| | | **.00** | **.00** | **125.00** | **.00** |
| **R** | **.00** | | .00 | 125.00* | .00 |
| **A** | **.00** | | | 125.00* | .00 |
| **S** | **125.00** | | | | 125.00* |
| **MM** | **.00** | | | | |

* Significant statistical difference ($P < 0.05$)

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**Appendix Table D11** Analysis of variance between food processing treatments of myosin heavy chain

| Source of variation | SS | df | MS | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 5766.00 | 3 | 1922.00 | 3844.00 | .000 |
| Within Groups | 2.00 | 4 | .50 | | |
| Total | 5768.00 | 7 | | | |

**Appendix Table D12** Bonferroni's multiple comparison tests of intensity's mean values shows which treatment differed from each other of myosin heavy chain

| **Treatment** | **x̄** | **R** | **A** | **S** | **MM** |
|---|---|---|---|---|---|
| | | **.00** | **62.00** | **.00** | **.00** |
| **R** | **.00** | | 62.00* | .00 | .00 |
| **A** | **62.00** | | | 62.00* | 62.00* |
| **S** | **.00** | | | | .00 |
| **MM** | **.00** | | | | |

* Significant statistical difference ($P < 0.05$)

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**Appendix E**

Allergen identification lists table from immunoblotting

using LC-MS/MS

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Liquid chromatography-two-dimensional tandem mass spectrometry (LC-MS/MS) methodologies follow by MASCOT MS/MS Ions Search were used for identified the allergens from F.merguiensis. The allergen identified data were according to assigned band/spot number in Figure 17-27.

Symbols used in tables

| | |
|---|---|
| 1D: | Band from 1D-immunoblotting |
| 2D: | Spot from 2D-immunoblotting |
| R | Raw muscle sample |
| SH: | Raw shell sample |
| OA: | Raw ovarian with state 1 |
| OB: | Raw ovarian with state 2 |
| OC: | Raw ovarian with state 3-4 |
| A: | Autoclave treated muscle |
| S: | Ultrasonic treated muscle |
| MS: | 30 sec microwave treated muscle |
| MM: | 1 min microwave treated muscle |

For example, 1D-R_1 mean the band number 1 from SDS-PAGE of raw shrimp muscle. 2D-A_4 mean the spot number 4 from 2D-PAGE of shrimp muscle with autoclave treated.

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**Appendix Table E1** Identified allergens from raw muscle of *Fenneropenaeus merguiensis* by SDS-PAGE using LC-MS/MS

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-R_1 | gi\|343183155 | Myosin heavy chain type b [*Marsupenaeus japonicas*] | 218.870/6.00 | 51 | 6 | 4 | R.SKYESEGVAR.A<br>R.NLEHDLDGLR.E<br>K.VLSYFANVGATSK.K<br>R.QIEEAEEIAALNLAK.Y<br>K.AVYEENLEHLDSVR.R<br>K.KLESDIGELEIALDHANK. |
| | gi\|343183153 | Myosin heavy chain type a [*Marsupenaeus japonicas*] | 219.368/5.79 | 37 | 5 | 3 | R.SKYESEGVAR.A<br>R.DLMDQIGEGGR.A + Oxidation (M)<br>K.IEDEQALVYR.D<br>R.LDEAGGATAAQIEINK.K<br>R.QIEEAEEIAALNLAK.F |
| 1D-R_2 | gi\|854403 | Hemocyanin [*Litopenaeus vannamei*] | 74.934/5.27 | 211 | 5 | 6 | R.IFAWPHK.D<br>K.YMDNIFK.E + Oxidation (M)<br>R.WNAIELDK.F<br>K.FEDVDDVAR.I<br>R.IRDMVIVESR.I + Oxidation (M) |
| 1D-R_3 | gi\|3885968 | Phosphopyruvate hydratase (Enolase) [*Penaeus monodon*] | 47.235/6.18 | 249 | 6 | 17 | K.ACNCLLLK.V + 2 Carbamidomethyl (C)<br>R.IEEELGGNAK.F<br>K.GENIYDLDFK.T<br>K.AGAAELGIPLYR.H<br>R.AAVPSGASTGVHEALEMR.D + Oxidation (M)<br>K.VNQIGSVTESIDAHLLAK.K |



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**Appendix Table E1** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-R_4 | gi\|227955305 | Arginine kinase [*Fenneropenaeus merguiensis*] | 40.124/6.19 | 218 | 5 | 17 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.GTYYPLTGMSK.E + Oxidation (M)<br>R.IISMQMGGDLGQVFR.R + 2 Oxidation (M)<br>R.GEHTEAEGGIYDISNK.R |
| | gi\|227955305 | Arginine kinase [*Fenneropenaeus merguiensis*] | 40.124/6.19 | 186 | 5 | 12 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.LTSAVNEIEK.R<br>R.MGLTEFQAVK.E + Oxidation (M)<br>R.LTSAVNEIEKR.I<br>R.GEHTEAEGGIYDISNK.R |
| | gi\|115492980 | Arginine kinase [*Litopenaeus vannamei*] | 40.134/6.19 | 72 | 9 | 17 | R.EKLEEVAGK.Y<br>K.KLEAATDCK.S + Carbamidomethyl (C)<br>R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.LTSAVNEIEK.R<br>R.MGLTEFQAVK.E + Oxidation (M) (+2)<br>R.IISMQMGGDLGQVFR.R + 2 Oxidation (M) (+1) |



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**Appendix Table E1** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-R_5 | gi\|62701385 | Glyceraldehyde 3-phosphate dehydrogenase [*Marsupenaeus japonicas*] | 17.017/6.56 | 95 | 5 | 24 | K.ASAHFQGGAK.K<br>K.AEDGCLVVDGHK.I + Carbamidomethyl (C)<br>K.VVSNASCTTNCLAPVAK.V + 2 Carbamidomethyl (C) |
| | gi\|134312 | Sarcoplasmic calcium-binding protein [*Penaeus sp.*] | 21.966/4.63 | 51 | 9 | 35 | R.SAFAEVK.E<br>R.NTLIEGR.G<br>K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.VFIANQFK.A<br>K.VFIANQFK.A<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.M<br>R.GEFSAADYANNQK.I |
| 1D-R_6 | gi\|380503696 | Sarcoplasmic calcium-binding protein [*Penaeus monodon*] | 22.106/4.76 | 59 | 5 | 18 | K.AGGLTLER.Y<br>K.YGEFPGAFK.V (+1)<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q |



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**Appendix Table E2** Identified allergens from raw shell of *Fenneropenaeus merguiensis* by SDS-PAGE using LC-MS/MS

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-SH_1 | gi\|223403273 | Sarcoplasmic calcium-binding protein [*Litopenaeus vannamei*] | 22.064/4.73 | 156 | 6 | 20 | K.AGGLTLER.Y<br>K.EIDDAYNK.L<br>K.DGEVTVDEFK.Q<br>R.GEFSADAYANNQK.I (+2) |
| 1D-SH_2 | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 168 | 3 | 8 | K.KLEAATDCK.S + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.VSSTLSGLEGELK.G |
| | gi\|332145607 | Arginine kinase [*Xyleborinus saxeseni*] | 26.800/5.83 | 117 | 3 | 9 | K.QKLEEVAGK.Y<br>K.SKLEEVAGK.Y + Phospho (ST)<br>K.VSSTLSGLEGELK.G |
| | gi\|400271640 | Arginine kinase [*Amphicranus sp*. BHJ-2012] | 30.350/6.03 | 61 | 1 | 4 | K.VSSTLSALDGELK.G |
| 1D-SH_3 | gi\|229256 | Dehydrogenase,glycerol dehydephosphate [Lobster] | 35.694/6.24 | 82 | 17 | 18 | R.VIDLLK.H (+1)<br>K.AGIQLSK.T<br>U R.SSIFDAK.A<br>U K.ASAHFKGGAK.K<br>U R.VPTPDVSVVDLTVR.L (+9)<br>K.AGAEYIVESTGVFTTIEK.A (+1) |



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**Appendix Table E2** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-SH_3 | gi\|262316945 | Glyceraldehyde-3-phosphate dehydrogenase [*Manihot michaelis*] | 11.340/7.82 | 64 | 12 | 13 | R.VPTVIVSVVDLTVR.L (+10)<br>R.VPTVNVSVVDLTVR.L |
| | gi\|62701385 | Glyceraldehyde 3-phosphate dehydrogenase [*Marsupenaeus japonicus*] | 17.017/6.56 | 62 | 5 | 35 | K.ASAHFQGGAK.K<br>K.AEDGCLVVDGHK.I + Carbamidomethyl (C)<br>K.VVSNASCTTNCLAPVAK.V + 2 Carbamidomethyl (C)<br>K.AGAEYIVESTGVFTTIEK.A (+1) |
| 1D-SH_4 | gi\|223403273 | Sarcoplasmic calcium-binding protein [*Litopenaeus vannamei*] | 22.064/4.73 | 64 | 16 | 47 | R.SAFAEVK.E<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V<br>K.VFIANQFK.A (+2)<br>K.EIDDAYNK.L<br>K.LTTEDDRK.A<br>K.NDFECLAVR.N + Carbamidomethyl (C) (+3)<br>K.DGEVTVDEFK.Q<br>R.GEFSADAYANNQK.I (+1)<br>R.YMYDIDNNGFLDK.N + Oxidation (M) |



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**Appendix Table E3** Identified allergens from raw ovary with previtellogenic stage (stage 1) of *Fenneropepaeus merguiensis* by SDS-PAGE using LC-MS/MS

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OA_1 | gi\|74860193 | Vitellogenin [*Fenneropenaeus merguiensis*] | 282.850/6.36 | 740 | 18 | 5 | K.AETYLVK.A<br>R.AEAFLTSR.M<br>R.LAGTAVAGLK.M<br>K.DLNLNTAR.T<br>K.VISTVEYK.N<br>R.VEADAPILK.T<br>R.AVAYAATFGK.Y (+1)<br>K.ACDAAQAYR.T + Carbamidomethyl (C)<br>K.YASVIAYEK.L<br>K.VVLAAHGSHR.L<br>R.LMPSGAAASNPR.D (+1)<br>K.MNVEAAGASTPR.R<br>K.VEAGIEQGAEGR.Y<br>K.MNVEAAGASTPR.R + Oxidation (M)<br>R.ANALPLGEPAIAK.L<br>R.SIDSSVIADFFGK.L |



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**Appendix Table E3** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OA_1 | gi\|45774386 | Vitellogenin [*Litopenaeus vannamei*] | 283.283/6.55 | 303 | 8 | 2 | K.AETYLVK.A<br>K DLNLNTAR T<br>K.VLSTVEYK.N<br>R.LMPSGAAASNPR.D (+1)<br>K.MNVEAAGASTPR.R<br>K.MNVEAAGASTPR.R + Oxidation (M)<br>R.ANALPLGEPAIAK.L |
| 1D-OA_2 | gi\|74860193 | Vitellogenin [*Fenneropenaeus merguiensis*] | 282.850/6.36 | 430 | 9 | 3 | R.LAGTAVAGLK.M<br>K.VISTVEYK.N<br>R.VEADAPILK.T<br>K.LILTTPSQK.T<br>K.YASVIAYEK.L<br>K.MNVEAAGASTPR.R<br>K.VEAGIEQGAEGR.Y<br>R.ANALPLGEPAIAK.L<br>R.SIDSSVIADFFGK.L |



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**Appendix Table E3** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OA_3 | gi\|86129739 | Vitellogenin [*Fenneropenaeus chinensis*] | 283.466/6.13 | 314 | 8 | 2 | R.DVGIVR.E<br>R.AFAAEAEK.V<br>K.NVATAEVGR.S<br>K.APLPIEESK.S<br>K.AVAYAATFGK.Y<br>K.ITGTLETQR.I<br>R.LMPSGAAASNPR.D<br>R.LMPSGAAASNPR.D + Oxidation (M) |
| | gi\|327533501 | Hemocyanin, partial [*Penaeus monodon*] | 44.371/5.97 | 160 | 4 | 9 | K.YSHHLDR.K<br>R.DLLIVESR.I<br>R.HWFSLFNPR.Q<br>R.DAIAHGYIVDR.A |
| 1D-OA_4 | gi\|74860193 | Vitellogenin [*Fenneropenaeus merguiensis*] | 282.850/6.36 | 317 | 8 | 3 | K.MELLNIK.A + Oxidation (M)<br>R.LAGTAVAGLK.M<br>K.DLNLNTAR.T<br>R.GYLVTAAIK.N<br>R.RAEMTLSYTK.E<br>K.MNVEAAGASTPR.R<br>R.SIDSSVIADFFGK.L<br>R.ANALPLGEPAIAKLYVEK.A |



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**Appendix Table E3** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OA_5 | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 113 | 3 | 8 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.VSSTLSGLEGELK.G |
| | gi\|1708615 | Arginine kinase [*Marsupenaeus japonicus*] | 39.965/6.36 | 85 | 2 | 5 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.LTSAVNEIEK.R |
| 1D-OA_6 | gi\|298570899 | 14-3-3 zeta [*Fenneropenaeus merguiensis*] | 27.915/4.68 | 120 | 2 | 7 | R.VISSIEQK.T<br>YLAEVATGDAR.N |
| | gi\|21218359 | Ovarian peritrophin 1 precursor [*Penaeus monodon*] | 30.870/4.80 | 90 | 2 | 6 | R.YSCPESYR.W + Carbamidomethyl (C)<br>R.SVTADNHPYSK.L |



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**Appendix Table E4** Identified allergens from raw ovary with early vitellogenic stage (stage 2) of *Fenneropepaeus merguiensis* by SDS-PAGE using LC-MS/MS

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OB_1 | gi\|256861071 | Vitellogenin [*Fenneropenaeus merguiensis*] | 283.130/6.47 | 1038 | 26 | 9 | K.YNVISK.M<br>K.AETYLVK.A<br>R.AFAAEAEK.L<br>R.AEAFLTSR.M<br>R.LAGTAVAGLK.M<br>K.NVATAEVGR.S<br>K.DLNLNTAR.T<br>R.GYLVTAAIK.N<br>K.ITGTLETQR.I<br>K.YASVIAYEK.L<br>K.VVLAAHGSHR.L<br>K.GFADVHIAEK.K<br>R.LMPSGAAASNPR.D + Oxidation (M)<br>R.RAEMTLSYTK.E<br>K.MNVEAAGASTPR.R<br>R.AGETHGVAVAAVK.L<br>K.VEAGIEQGAEGR.Y<br>K.MNVEAAGASTPR.R + Oxidation (M)<br>R.TVLEASGPIMAR.F<br>R.TVLEASGPIMAR.F + Oxidation (M)<br>R.LIQCEALLGIR.S + Carbamidomethyl (C)<br>R.YITDVLPPYPR.T<br>R.SIDSSVIADFFGK.L<br>R.YTLESAITYGQR.T<br>R.FTRPSPTSYTMQMR.T+2Oxidation (M)<br>R.TATVVGGSEILTFSGLVVR.A |



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**Appendix Table E4** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OB_2 | gi\|74860193 | Vitellogenin [*Fenneropenaeus merguiensis*] | 282.850/6.36 | 622 | 16 | 6 | K.AETYLVK.A<br>K.NVATAEVGR.S<br>R.GYLVTAAIK.N<br>R.VEADAPILK.T<br>K.GFADVHIAEK.K<br>R.SHTHAELFAR.F<br>R.RAEMTLSYTK.E<br>K.VEAGIEQGAEGR.Y<br>K.MNVEAAGASTPR.R + Oxidation (M)<br>R.TVLEASGPIMAR.F<br>R.TVLEASGPIMAR.F + Oxidation (M)<br>R.SIDSSVIADFFGK.L<br>R.YTLESAITYGQR.T<br>K.FLAEIPGYIQPVK.V<br>K.MGASLEVLQAAGNEK.V<br>K.LVLTAANLVEHMEGENGFK.L |
| | gi\|82754312 | Vitellogenin [*Penaeus monodon*] | 283.809/6.19 | 130 | 4 | 1 | K.AETYLVK.A<br>R.GYLVTAAIK.N<br>R.SHVHADLFAR.F + Oxidation (HW)<br>K.LVLTAANLVEHMEGENGFK.L |



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**Appendix Table E4** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OB_3 | gi\|74860193 | Vitellogenin [*Fenneropenaeus merguiensis*] | 282.850/6.36 | 372 | 11 | 4 | R.AFAAEAEK.L<br>R.AEAFLTSR.M<br>K.APLPIEESK.S<br>R.AVAYAATFGK.Y<br>K.VVLAAHGSHR.L<br>K.EEGSHIVSAK.L<br>K.YEIETEGEK.V<br>R.AGETHGVAVAAVK.L<br>R.LIQCEALLGIR.S + Carbamidomethyl (C)<br>K.TAAHNAAATFTVK.M<br>R.YITDVLPPYPR.T |
| | gi\|256861071 | Vitellogenin [*Fenneropenaeus merguiensis*] | 283.130/6.47 | 341 | 9 | 3 | R.AFAAEAEK.L<br>R.AEAFLTSR.M<br>K.APLPIEESK.S<br>R.AVAYAATFGK.Y<br>K.ITGTLETQR.I<br>R.LMPSGAAASNPR.D+Oxidation(M)<br>R.AGETHGVAVAAVK.L<br>K.TAAHNAAATFTVK.M<br>R.YITDVLPPYPR.T |
| | gi\|16612121 | Hemocyanin [*Penaeus monodon*] | 51.120/5.10 | 196 | 5 | 9 | K.YMDNIFK.E<br>R.DLLIVESR.I<br>K.GQEVLATVR.I<br>R.WNAIELDK.F<br>R.DAIAHGYIVDR.A |



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**Appendix Table E4** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OB_4 | gi\|10304437 | Beta-actin [*Litopenaeus vannamei*] | 41.841/5.30 | 90 | 5 | 9 | R.AVFPSIVGR.A<br>K.AGFAGDDAPR.A<br>R.HQGVMVGMGQK.D + Oxidation (M)<br>K.DSYVGDEAQSK.R<br>R.HQGVMVGMGQK.D + 2 Oxidation (M) |
| | gi\|319893874 | Alpha actin-2 [*Rachycentron canadum*] | 42.026/5.16 | 81 | 5 | 9 | K.ILTER.G<br>K.AGFAGDDAPR.A<br>R.HQGVMDGMGQK.D<br>K.DSYVGDEAQSK.R<br>R.HQGVMDGMGQK.D + Oxidation (M) |
| 1D-OB_5 | gi\|27463265 | Arginine kinase [*Penaeus monodon*] | 40.087/6.05 | 179 | 6 | 17 | K.YVISTR.V<br>R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.LTSAVNEIEK.R<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.GTYYPLTGMSK.E + Oxidation (M)<br>R.GEHTEAEGGIYDISNK.R |



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**Appendix Table E4** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OB_5 | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 170 | 4 | 13 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.VSSTLSGLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |
| | gi\|171473123 | Arginine kinase [*Uca pugilator*] | 22.905/7.21 | 141 | 4 | 24 | R.FLQAANACR.F + Carbamidomethyl (C)<br>K.GTYYPLTGMSK.E + Oxidation (M)<br>K.VSSTLSGLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |
| 1D-OB_6 | gi\|298570899 | 14-3-3 zeta [*Fenneropenaeus merguiensis*] | 27.915/4.68 | 113 | 3 | 10 | R.VISSIEQK.T<br>R.NLLSVAYK.N<br>K.SYQEAFDIAK.A |
| | gi\|6979112 | Peritrophin-like protein 2 [*Penaeus semisulcatus*] | 30.127/5.16 | 61 | 2 | 4 | R.FFSCK.D + Carbamidomethyl (C)<br>R.VDPYDPQR.F |



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**Appendix Table E5** Identified allergens from raw ovary with vitellogenic stage (stage 3) of *Fenneropenaeus merguiensis* by SDS-PAGE using LC-MS/MS

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OC_1 | gi\|74860193 | Vitellogenin [*Fenneropenaeus merguiensis*] | 282.850/6.36 | 764 | 15 | 5 | R.DVGIVR.E<br>K.AETYLVK.A<br>R.AFAAEAEK.L<br>R.AEAFLTSR.M<br>R.LAGTAVAGLK.M<br>K.NVATAEVGR.S<br>R.GYLVTAAIK.N<br>K.YASVIAYEK.L<br>K.VVLAAHGSHR.L<br>K.EEGSHIVSAK.L<br>R.LMPSGAAASNPR.D<br>K.VEAGIEQGAEGR.Y<br>K.MNVEAAGASTPR.R + Oxidation (M)<br>R.ANALPLGEPAIAK.L<br>R.SIDSSVIADFFGK.L |



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Appendix Table E5 (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OC_1 | gi\|256861071 | Vitellogenin [*Fenneropenaeus merguiensis*] | 283.130/6.47 | 762 | 15 | 5 | K.AETYLVK.A<br>R.AFAAEAEK.L<br>R.AEAFLTSR.M<br>R.LAGTAVAGLK.M<br>K.NVATAEVGR.S<br>R.GYLVTAAIK.N<br>K.ITGTLETQR.I<br>K.YASVIAYEK.L<br>K.VVLAAHGSHR.L<br>K.EEGSHIVSAK.L<br>R.LMPSGAAASNPR.D<br>K.VEAGIEQGAEGR.Y<br>K.MNVEAAGASTPR.R + Oxidation (M)<br>R.ANALPLGEPAIAK.L<br>R.SIDSSVIADFFGK.L |
| 1D-OC_2 | gi\|74860193 | Vitellogenin [*Fenneropenaeus merguiensis*] | 282.850/6.36 | 126 | 3 | 1 | K.VISTVEYK.N<br>R.VEADAPILK.T<br>K.MNVEAAGASTPR.R |



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**Appendix Table E5** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-OC_3 | gi\|74860193 | Vitellogenin [*Fenneropenaeus merguiensis*] | 282.850/6.36 | 213 | 5 | 1 | R.DVGIVR.E<br>R.ISENAIR.A<br>R.AFAAEAEK.L<br>R.AVAYAATFGK.Y<br>R.LMPSGAAASNPR.D |
| | gi\|23138909 | Apolipocrustacein [*Penaeus semisulcatus*] | 283.211/6.23 | 115 | 2 | 0 | K.ITGTLETQR.I<br>K.ACDAAQAYR.T + Carbamidomethyl (C) |
| | gi\|16612121 | Hemocyanin [*Penaeus monodon*] | 51.120/5.10 | 67 | 1 | 2 | K.GQEVLATVR.I |
| 1D-OC_4 | gi\|10304437 | Beta-actin [*Litopenaeus vannamei*] | 41.841/5.30 | 130 | 3 | 8 | K.AGFAGDDAPR.A<br>R.GYTFTTTAER.E<br>K.EITALAPSTMK.I + Oxidation (M) |
| 1D-OC_5 | gi\|115492980 | Arginine kinase [*Litopenaeus vannamei*] | 40.134/6.19 | 107 | 3 | 8 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.LTSAVNEIEK.R<br>K.GTYYPLTGMSK.E + Oxidation (M) |
| 1D-OC_6 | gi\|298570899 | 14-3-3 zeta [*Fenneropenaeus merguiensis*] | 27.915/4.68 | 86 | 2 | 6 | R.VISSIEQK.T<br>R.EKVETELR.E |
| | gi\|119655552 | Peritrophin 3 precursor [*Penaeus monodon*] | 34.604/5.22 | 97 | 3 | 7 | R.FFSCK.A + Carbamidomethyl (C)<br>K.TLVQCVK.G + Carbamidomethyl (C)<br>R.SVTADNHPYSK.L |



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**Appendix Table E6** Identified allergens from autoclaved treatment muscle of *Fenneropepaeus merguiensis* by SDS-PAGE using LC-MS/MS

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-A_1 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 155 | 4 | 14 | K.ANIQLVEK.D<br>K.IVELEEELR.V<br>R.IQLLEEDLER.S<br>R.ALSNAEGEVAALNR.R |
| | gi\|6094504 | Tropomyosin [*Metapenaeus ensis*] | 31.686/4.66 | 91 | 3 | 12 | K.IVELEEELR.V<br>K.LAMVEADLER.A + Oxidation (M)<br>K.ALSNAEGEVAALNR.R |



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**Appendix Table E7** Identified allergens from ultrasonic treatment muscle of *Fenneropepaeus merguiensis* by SDS-PAGE using LC-MS/MS

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-S_1 | gi\|854403 | Hemocyanin [*Litopenaeus vannamei*] | 74.934/5.27 | 104 | 4 | 4 | R.FLLPK.G<br>R.WNAIELDK.F<br>K.FDLPPGVLEHFETATR.D<br>K.FDLPPGVLEHFETATR.D |
| | gi\|148841021 | Hemocyanin subunit Y [*Marsupenaeus japonicus*] | 75.092/5.38 | 79 | 4 | 3 | R.FLLPK.G<br>K.STFTGTK.K<br>R.WNAIELDK.F<br>K.FKSTFTGTK.K |
| | gi\|410509308 | Myosin heavy chain type 2 [*Penaeus monodon*] | 219.033/5.85 | 48 | 1 | 0 | R.GQLELSQVR.Q |
| | gi\|148841019 | Hemocyanin subunit L [*Marsupenaeus japonicus*] | 77.184/5.29 | 48 | 2 | 2 | R.FLLPK.G<br>K.IYGDIRDDALK.A |
| 1D-S_2 | gi\|118136261 | Beta-actin [*Penaeus monodon*] | 41.973/5.00 | 109 | 3 | 7 | R.GYSFTTTAER.E<br>K.ELTALAPSTIK.I |
| 1D-S_3 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 383 | 8 | 24 | K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>K.IVELEEELR.V<br>K.LAMVEADLER.A + Oxidation (M)<br>K.SEEEVHNLQK.R<br>R.KLAMVEADLER.A + Oxidation (M)<br>R.ALSNAEGEVAALNR.R |



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Appendix Table E7 (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-S_3 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 122 | 5 | 14 | K.ANIQLVEK.D<br>K.IVELEEELR.V<br>K.LAMVEADLER.A + Oxidation (M)<br>R.KLAMVEADLER.A + Oxidation (M)<br>R.ALSNAEGEVAALNR.R |
| | gi\|257071009 | Troponin I [*Fenneropenaeus chinensis*] | 18.511/9.25 | 103 | 3 | 11 | K.AAEFNFR.N<br>K.AAEFNFR.N<br>K.NLDGANEDALR.A |
| 1D-S_4 | gi\|380503696 | Sarcoplasmic calcium-binding protein [*Penaeus monodon*] | 22.106/4.76 | 72 | 6 | 25 | K.VFIANQFK.A<br>K.YGEFPGAFK.V<br>K.NDFECLAVR.N<br>+ Carbamidomethyl (C)<br>K.DGEVTVDEFK<br>R.NLWNEIAELADFNK.D<br>R.NLWNEIAELADFNKDGEVTVD EFK |



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**Appendix Table E8** Identified allergens from 30 sec microwave treatment muscle of *Fenneropepaeus merguiensis* by SDS-PAGE using LC-MS/MS

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-MS_1 | gi\|156402899 | Predicted protein, partial [*Nematostella vectensis*] | 18.221/7.89 | 52 | 1 | 5 | R.GPFVSAIGK.S |
| 1D-MS_2 | gi\|156402899 | Predicted protein [*Nematostella vectensis*] | 18.221/7.89 | 59 | 1 | 5 | R.GPFVSAIGK.S |
| 1D-MS_3 | gi\|156385004 | Predicted protein [*Nematostella vectensis*] | 48.578/5.37 | 46 | 1 | 1 | K.LKAIIDR.S |
| 1D-MS_4 | gi\|546679612 | Hypothetical protein D910_07407 [*Dendroctonus ponderosae*] | 81.637/5.45 | 93 | 2 | 2 | K.KIALSIR.I<br>K.GLDPEALTGK.Y |
| 1D-MS_5 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 626 | 15 | 42 | R.FLAEEADR.K<br>R.LEDELVNEK.E<br>K.IVELEEELR.V<br>K.IVELEEELR.V<br>K.LAMVEADLER.A + Oxidation (M)<br>K.SEEEVHNLQK.R<br>R.IQLLEEDLER.S<br>K.LAEASQAADESER.M<br>K.ALSNAEGEVAALNR.R<br>K.ALSNAEGEVAALNR.R<br>K.ALSNAEGEVAALNR.R<br>K.ALSNAEGEVAALNR.R<br>K.EVDRLEDELVNEK.E<br>K.SITDELDQTFSELSGY.-<br>R.MQQLENDLDQVQESLLK.A |



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**Appendix Table E8** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-MS_6 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 285 | 5 | 15 | R.KYDEVAR.K<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>K.IVELEEELR.V<br>K.LAMVEADLER.A + Oxidation (M) |
| 1D-MS_7 | gi\|380503696 | Sarcoplasmic calcium-binding protein [*Penaeus monodon*] | 22.106/4.76 | 457 | 9 | 41 | R.NTLIEGR.G<br>K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V<br>K.VFIANQFK.A<br>K.YGEFPGAFK.V<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q<br>R.GEFSADAYANNQK.I |
| | gi\|223403273 | Sarcoplasmic calcium-binding protein [*Litopenaeus vannamei*] | 22.064/4.73 | 435 | 9 | 40 | R.NTLIEGR.G<br>K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V<br>K.VFIANQFK.A<br>K.EIDDAYNK.L<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q<br>R.GEFSADAYANNQK.I |



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**Appendix Table E8** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-MS_7 | gi\|134312 | Sarcoplasmic calcium-binding protein [*Penaeus sp.*] | 21.966/4.63 | 402 | 8 | 36 | R.NTLIEGR.G<br>K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V<br>K.VFIANQFK.A<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.M<br>R.GEFSAADYANNQK.I |
| | gi\|225711822 | Sarcoplasmic calcium-binding protein, beta chain [*Lepeophtheirus salmonis*] | 22.136/4.77 | 112 | 2 | 8 | K.KGGITLER.Y<br>K.NDFECLAVR.N + Carbamidomethyl (C) |



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**Appendix Table E9** Identified allergens from 1 min microwave treatment muscle of *Fenneropepaeus merguiensis* by SDS-PAGE using LC-MS/MS

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-MM_1 | gi\|410509312 | Myosin heavy chain type 2 [*Litopenaeus vannamei*] | 219.056/5.84 | 309 | 9 | 4 | R.IEDEINK.L<br>R.YPIYTNR.T<br>K.AAEMTFQK.S + Oxidation (M)<br>R.MQYPDFK.H + Oxidation (M)<br>R.VVSQAPAER.G<br>R.AQGLANAAER.R<br>K.DLEQTIQR.K<br>R.GQLELSQVR.Q<br>R.IQEKEEEFDATR.K |
| | gi\|410509306 | Myosin heavy chain type 1 [*Penaeus monodon*] | 219.450/5.77 | 214 | 7 | 3 | K.EISAITAK.I<br>R.GEEELEK.L<br>R.YPIYTNR.A<br>R.TALIVMQR.N + Oxidation (M)<br>R.VISQSPAER.G<br>R.TQLELSQVR.Q<br>R.AAAELDDLHASAER.A |
| 1D-MM_2 | gi\|156402899 | Predicted protein [*Nematostella vectensis*] | 18.221/7.89 | 59 | 1 | 5 | R.GPFVSAIGK.S |



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**Appendix Table E9** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-MM_3 | gi\|3907620 | Actin 1 [*Penaeus monodon*] | 41.716/5.23 | 230 | 5 | 13 | R.AVFPSIVGR.A<br>K.AGFAGDDAPR.A<br>R.GYSFTTTAER.E<br>K.EITALAPSTIK.I<br>K.DAYVGDEAQSK.R |
| | gi\|62126068 | Actin 1 [*Fenneropenaeus chinensis*] | 41.801/5.30 | 218 | 5 | 11 | R.DLTAYLMK.I + Oxidation (M)<br>K.AGFAGDDAPR.A<br>R.GYSFTTTAER.E<br>K.EITALAPSTIK.I<br>K.DAYVGDEAQSK.R |
| 1D-MM_4 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 511 | 11 | 32 | R.MDALENQLK.E<br>K.IVELEEELR.V<br>K.IVELEEELR.V<br>K.LAMVEADLER.A<br>K.LAMVEADLER.A + Oxidation (M)<br>K.SEEEVHNLQK.R<br>R.IQLLEEDLER.S<br>R.IQLLEEDLER.S<br>K.LAEASQAADESER.M<br>K.ALSNAEGEVAALNR.R<br>K.SITDELDQTFSELSGY.- |



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**Appendix Table E9** (Continued)

| Band No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 1D-MM_5 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 345 | 7 | 21 | R.KYDEVAR.K<br>K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>K.IVELEEELR.V<br>K.LAMVEADLER.A + Oxidation (M)<br>K.SEEEVHNLQK.R |
| 1D-MM_6 | gi\|223403273 | Sarcoplasmic calcium-binding protein [*Litopenaeus vannamei*] | 22.064/4.73 | 105 | 3 | 16 | K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q<br>R.GEFSADAYANNQK.I |



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**Appendix Table E10** Identified allergens from raw muscle of *Fenneropepaeus merguiensis* by 2D-PAGE using LC-MS/MS

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-R_1 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 515 | 16 | 37 | K.SLEVSEEK.A<br>R.FLAEEADR.K<br>R.MDALENQLK.E + Oxidation (M)<br>R.LEDELVNEK.E<br>R.EEAYKEQIK.T<br>K.LAMVEADLER.A + Oxidation (M)<br>K.SEEEVHNLQK.R<br>R.IQLLEEDLER.S (+3)<br>K.LAEASQAADESER.M<br>K.ALSNAEGEVAALNR.R (+2)<br>K.EVDRLEDELVNEK.E |
| 2D-R_2 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 187 | 5 | 13 | K.YDEVAR.K<br>K.SLEVSEEK.A<br>R.IQLLEEDLER.S<br>K.ALSNAEGEVAALNR.R (+1) |
| 2D-R_3 | gi\|380503696 | Sarcoplasmic calcium-binding protein [Penaeus monodon] | 22.106/4.76 | 106 | 6 | 30 | K.AIDVNGDGK.V<br>K.VFIANQFK.A<br>K.YGEFPGAFK.V<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q<br>R.YMYDIDNNGFLDK.N + Oxidation (M) |



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**Appendix Table E10** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-R_3 | gi\|229256 | Dehydrogenase, glyceraldehydephosphate [Lobster] | 35.694/6.24 | 80 | 3 | 14 | R.VPTPDVSVVDLTVR.L<br>K.AGAEYIVESTGVFTTIEK.A<br>K.ITVFNEMKPENIPWSKAGAEYIVESTGVFTTIEK.A + Oxidation |
| 2D-R_4 | gi\|380503696 | Sarcoplasmic calcium-binding protein [*Penaeus monodon*] | 22.106/4.76 | 142 | 4 | 20 | K.LTTEDDRK.A<br>K.YGEFPGAFK.V<br>K.DGEVTVDEFK.Q<br>R.GEFSADAYANNQK.I |
| 2D-R_5 | gi\|116582732 | Cytosolic glyceraldehyde-3-phosphate dehydrogenase, partial [*Mesostigma viride*] | 35.281/6.09 | 194 | 10 | 9 | R.VPTPDVSVVDLTVR.L (+8)<br>K.VVSNASCTTNCLGPLAK.V + 2 Carbamidomethyl (C) |
| | gi\|62701385 | Glyceraldehyde 3-phosphate dehydrogenase [*Marsupenaeus japonicus*] | 17.017/6.56 | 147 | 6 | 24 | K.ASAHFQGGAK.K<br>K.AEDGCLVVDGHK.I + Carbamidomethyl (C) (+2)<br>K.VVSNASCTTNCLAPVAK.V + 2 Carbamidomethyl (C) (+1) |
| | gi\|262316945 | Glyceraldehyde-3-phosphate dehydrogenase [*Manihot michaelis*] | 11.340/7.82 | 89 | 9 | 13 | R.VPTVIVSVVDLTVR.L (+8) |



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**Appendix Table E10** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-R_5 | gi\|126697350 | Glyceraldehyde 3-phosphate dehydrogenase [*Haliotis discus discus*] | 31.457/6.12 | 61 | 6 | 15 | R.SSIFDAK.A (+1)<br>K.ASAHFKGGAK.K<br>R.GAAQNIIPSSTGAAK.A (+1)<br>R.VPVPDVSVVDLTVR.L |
| | gi\|155966165 | Glyceraldehyde-3-phosphate dehydrogenase [*Lepeophtheirus salmonis*] | 35.711/6.32 | 60 | 3 | 4 | R.SSIFDAK.A (+1)<br>K.YDSTHGMCK.K |
| 2D-R_6 | gi\|62701385 | Glyceraldehyde 3-phosphate dehydrogenase [*Marsupenaeus japonicas*] | 17.017/6.56 | 174 | 3 | 24 | K.ASAHFQGGAK.K<br>K.AEDGCLVVDGHK.I + Carbamidomethyl (C)<br>K.VVSNASCTTNCLAPVAK.V + 2 Carbamidomethyl (C) |
| | gi\|6016083 | Glyceraldehyde-3-phosphate dehydrogenase [*Panulirus versicolor*] | 35.701/6.94 | 152 | 4 | 14 | R.VIDLIK.H<br>K.ASAHFKGGAK.K<br>R.GAAQNIIPSSTGAAK.A<br>K.VVSNASCTTNCLAPVAK.V + 2 Carbamidomethyl (C) |
| | gi\|300518909 | Glyceraldehyde-3-phosphate dehydrogenase [*Cancer borealis*] | 35.701/6.60 | 139 | 5 | 15 | R.VIDLLK.H<br>R.GAAQNIIPSSTGAAK.A<br>K.VVSWYDNEFGYSNR.V<br>K.VVSWYDNEFGYSNR.V<br>K.VVSNASCTTNCLAPVAK.V + 2 Carbamidomethyl (C) |



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**Appendix Table E10** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-R_7 | 671058A | Dehydrogenase, glyceraldehydephosphate [lobster] | 35.694/6.24 | 3 | 4 | 74 | K.LTGMAFR.V + Oxidation (M)<br>K.LTGMAFR.V + Oxidation (M)<br>K.ECSYDDIK.A + Carbamidomethyl (C) |
|  | AAV83993 | Arginine kinase [*Fenneropenaeus chinensis*] | 40.104/5.92 | 2 | 5 | 69 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>K.LIDDHFLFK.E |



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**Appendix Table E11** Identified allergens from autoclaved treatment muscle of *Fenneropepaeus merguiensis* by 2D-PAGE using LC-MS/MS

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_1 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 321 | 8 | 25 | R.KYDEVAR.K<br>K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>R.ADTLEQQNK.E<br>R.LEDELVNEK.E<br>K.SEEEVHNLQK.R<br>R.IQLLEEDLER.S |
| | gi\|27463265 | Allergen Pen m 2 (Arginine kinase) [*Penaeus monodon*] | 40.087/6.05 | 297 | 7 | 20 | K.AVFDQLK.E<br>R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.LTSAVNEIEK.R<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.GTYYPLTGMSK.E + Oxidation (M)<br>R.GEHTEAEGGIYDISNK.R |
| | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 265 | 5 | 16 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.VSSTLSGLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_2 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 757 | 15 | 42 | R.KYDEVAR.K<br>K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>R.ADTLEQQNK.E<br>R.MDALENQLK.E<br>R.LEDELVNEK.E<br>K.LAMVEADLER.A<br>K.LAMVEADLER.A + Oxidation (M)<br>K.SEEEVHNLQK.R<br>K.SEEEVHNLQK.R<br>R.IQLLEEDLER.S<br>R.KLAMVEADLER.A + Oxidation (M)<br>K.LAEASQAADESER.M<br>R.ALSNAEGEVAALNR.R |
| 2D-A_2 | gi\|40548517 | Tropomyosin [*Squilla aculeata*] | 32.804/4.62 | 548 | 10 | 29 | K.SLEVSEEK.A<br>R.ADTLEQQNK.E<br>R.MDALEDQLK.E + Oxidation (M)<br>R.LEDELVNEK.E<br>K.LAMVEADLER.A<br>K.LAMVEADLER.A + Oxidation (M)<br>R.IQLLEEDLER.S<br>R.KLAMVEADLER.A + Oxidation (M)<br>K.LAEASQAADESER.M<br>K.ALSNAEGEVAALNR.R |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_3 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 609 | 12 | 36 | R.KYDEVAR.K<br>K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>R.MDALENQLK.E + Oxidation (M)<br>R.LEDELVNEK.E<br>K.IVELEEELR.V<br>K.LAMVEADLER.A + Oxidation (M)<br>K.SEEEVHNLQK.R<br>K.SEEEVHNLQK.R<br>R.IQLLEEDLER.S<br>K.LAEASQAADESER.M |
| | gi\|298570899 | 14-3-3 zeta [*Fenneropenaeus merguiensis*] | 27.915/4.68 | 356 | 9 | 31 | K.VFYLK.M<br>K.LAEQAER.Y<br>R.VISSIEQK.T<br>R.NLLSVAYK.N<br>R.AGVVDDSQK.S<br>R.YDDMAAAMK.Q + 2 Oxidation (M)<br>R.YLAEVATGDAR.A<br>K.SYQEAFDIAK.A<br>K.DSTLIMQLLR.D + Oxidation (M) |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_3 | gi\|14423954 | Tropomyosin [*Dermatophagoides pteronyssinus*] | 32.881/4.78 | 318 | 6 | 18 | R.KYDEVAR.K<br>K.SLEVSEEK.A<br>R.MEGLENQLK.E + Oxidation (M)<br>K.IVELEEELR.V<br>K.LAMVEADLER.A + Oxidation (M)<br>R.IQLIEEDLER.S |
| | gi\|380003174 | 14-3-3 protein [*Scylla paramamosain*] | 28.070/4.78 | 298 | 8 | 26 | K.VFYLK.M<br>K.LAEQAER.Y<br>R.VISSIEQK.T<br>R.NLLSVAYK.N<br>R.AVVVDDSQK.S<br>R.YDDMAAAMK.Q + 2 Oxidation (M)<br>K.SYQEAFDIAK.S<br>K.DSTLIMQLLR.D + Oxidation (M) |
| 2D-A_4 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 605 | 13 | 40 | R.KYDEVAR.K<br>K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>R.ADTLEQQNK.E<br>R.MDALENQLK.E + Oxidation (M)<br>R.LEDELVNEK.E<br>K.IVELEEELR.V<br>K.SEEEVHNLQK.R (+1)<br>R.IQLLEEDLER.S<br>R.KLAMVEADLER.A + Oxidation (M)<br>K.LAEASQAADESER.M |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_4 | gi\|14423954 | Tropomyosin [*Dermatophagoides pteronyssinus*] | 32.881/4.78 | 255 | 5 | 15 | R.KYDEVAR.K<br>K.SLEVSEEK.A<br>K.IVELEEELR.V<br>R.IQLIEEDLER.S<br>R.KLAMVEADLER.A + Oxidation (M) |
| | gi\|257071009 | Troponin I [*Fenneropenaeus chinensis*] | 18.511/9.25 | 100 | 2 | 11 | K.AAEFNFR.N<br>K.NLDGANEDALR.A |
| | gi\|380503696 | Sarcoplasmic calcium-binding protein [*Penaeus monodon*] | 22.106/4.76 | 73 | 2 | 8 | K.SLEVSEEK.A<br>K.LQLIEEDLER.S |
| 2D-A_5 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/ 4.66 | 661 | 16 | 42 | R.SLSDEER.M<br>R.KYDEVAR.K<br>K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>R.ADTLEQQNK.E<br>R.MDALENQLK.E<br>R.MDALENQLK.E + Oxidation (M)<br>R.LEDELVNEK.E<br>K.IVELEEELR.V<br>K.LAMVEADLER.A<br>K.LAMVEADLER.A + Oxidation (M)<br>K.SEEEVHNLQK.R (+1)<br>R.IQLLEEDLER.S<br>K.LAEASQAADESER.M |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_5 | gi\|14423954 | Tropomyosin [*Dermatophagoides pteronyssinus*] | 32.881/4.78 | 323 | 7 | 18 | R.KYDEVAR.K<br>K.SLEVSEEK.A<br>R.MEGLENQLK.E + Oxidation (M)<br>K.IVELEEELR.V<br>K.LAMVEADLER.A<br>K.LAMVEADLER.A + Oxidation (M)<br>R.IQLIEEDLER.S |
| 2D-A_5 | gi\|40548521 | Tropomyosin [*Neoscona nautica*] | 32.898/4.66 | 207 | 4 | 11 | R.KYDEVAR.K<br>K.SLEVSEEK.N<br>K.LMAEEADR.K + Oxidation (M)<br>K.IVELEEELR.V |
| 2D-A_6 | gi\|380503696 | Sarcoplasmic calcium-binding protein [*Penaeus monodon*] | 22.106/4.76 | 402 | 9 | 41 | R.NTLIEGR.G<br>K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V<br>K.VFIANQFK.A<br>K.YGEFPGAFK.V<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q<br>R.GEFSADAYANNQK.I |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_6 | gi\|223403273 | Sarcoplasmic calcium-binding protein [*Litopenaeus vannamei*] | 22.064/4.73 | 376 | 9 | 40 | R.NTLIEGR.G<br>K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V<br>K.VFIANQFK.A<br>K.EIDDAYNK.L<br>K.NDFECLAVR.N + Carbamidomethyl<br>K.DGEVTVDEFK.Q<br>R.GEFSADAYANNQK.I |
| 2D-A_7 | gi\|308154236 | Arginine kinase [*Penaeus monodon*] | 40.115/ 6.05 | 560 | 16 | 37 | K.LEAGFK.K<br>K.YVISTR.V<br>K.LEEVAGK.Y<br>K.AVFDQLK.E<br>K.LEAATDCK.S + Carbamidomethyl<br>R.IPFSHHDR.L<br>K.KLEAATDCK.S + Carbamidomethyl<br>R.FLQAANACR.Y + Carbamidomethyl<br>R.LTSAVNEIEK.R<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.GTYYPLTGMSK.E + Oxidation (M)<br>K.EMQDGILELIK.M + Oxidation (M)<br>K.VSSTLSSLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_7 | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 374 | 9 | 21 | K.LEAATDCK.S + Carbamidomethyl (C)<br>K.KLEAATDCK.S + Carbamidomethyl (C)<br>R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.EMQDGILELIK.I + Oxidation (M)<br>K.VSSTLSGLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |
| 2D-A_8 | gi\|27463265 | Allergen Pen m 2 (Arginine kinase) [*Penaeus monodon*] | 40.087/6.05 | 510 | 14 | 33 | K.LEEVAGK.Y<br>K.AVFDQLK.E<br>K.LEAATDCK.S + Carbamidomethyl (C)<br>R.IPFSHHDR.L<br>R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.LTSAVNEIEK.R<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.GTYYPLTGMSK.E + Oxidation (M)<br>R.LTSAVNEIEKR.I<br>K.EMQDGILELIK.M + Oxidation (M)<br>K.VSSTLSSLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_8 | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 323 | 8 | 21 | K.LEAATDCK.S + Carbamidomethyl (C)<br>R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.EMQDGILELIK.I + Oxidation (M)<br>K.VSSTLSGLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |
| 2D-A_9 | gi\|308154236 | Arginine kinase [*Penaeus monodon*] | 40.115/6.05 | 527 | 16 | 31 | K.YVISTR.V<br>K.LEAATDCK.S + Carbamidomethyl (C)<br>R.IPFSHHDR.L<br>K.KLEAATDCK.S + Carbamidomethyl (C)<br>R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.LTSAVNEIEK.R<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.GTYYPLTGMSK.E<br>K.GTYYPLTGMSK.E + Oxidation (M)<br>R.LTSAVNEIEKR.I<br>K.EMQDGILELIK.M<br>K.EMQDGILELIK.M + Oxidation (M)<br>K.TFLVWVNEEDHLR.I<br>R.GEHTEAEGGIYDISNK.R |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_9 | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 432 | 10 | 21 | K.LEAATDCK.S + Carbamidomethyl (C)<br>K.KLEAATDCK.S + Carbamidomethyl (C)<br>R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.EMQDGILELIK.I<br>K.EMQDGILELIK.I + Oxidation (M)<br>K.VSSTLSGLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_10 | gi\|308154236 | Arginine kinase [*Penaeus monodon*] | 40.115/6.05 | 590 | 15 | 36 | K.LEAGFK.K<br>K.YVISTR.V<br>K.LEEVAGK.Y<br>K.LEAATDCK.S + Carbamidomethyl (C)<br>R.IPFSHHDR.L<br>K.KLEAATDCK.S + Carbamidomethyl (C)<br>R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.GTYYPLTGMSK.E + Oxidation (M)<br>K.EMQDGILELIK.M + Oxidation (M)<br>K.VSSTLSSLEGELK.G<br>R.GEHTEAEGGIYDISNK.R<br>R.LGFLTFCPTNLGTTVR.A + Carbamidomethyl (C) |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_11 | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 211 | 6 | 16 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.EMQDGILELIK.I<br>K.VSSTLSGLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |
| | gi\|405973252 | Fructose-bisphosphate aldolase [*Crassostrea gigas*] | 38.965/7.01 | 155 | 3 | 5 | K.ATVVALSR.G<br>R.IANAIVAPGK.G<br>R.RIANAIVAPGK.G |
| 2D-A_12 | gi\|229256 | Dehydrogenase, glyceraldehydephosphate [Lobster] | 35.694/6.24 | 239 | 6 | 15 | K.LTGMAFR.V + Oxidation (M)<br>K.LTGMAFR.V + Oxidation (M)<br>K.IGIDGFGR.I<br>K.ECSYDDIK.A + Carbamidomethyl (C)<br>R.GAAQNIIPSSTGAAK.A<br>R.VPTPDVSVVDLTVR.L |
| | gi\|317039786 | Glyceraldehyde 3-phosphate dehydrogenase [*Coenobita violascens*] | 18.471/8.65 | 196 | 5 | 24 | K.AAAEGPLK.G<br>K.LTGMAFR.V + Oxidation (M) (+1)<br>R.GAAQNIIPSSTGAAK.A<br>R.VPTPDVSVVDLTVR.L |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_13 | gi\|229256 | Dehydrogenase, glyceraldehydephosphate [Lobster] | 35.694/6.24 | 309 | 9 | 23 | R.SSIFDAK.A<br>K.LTGMAFR.V + Oxidation (M) (+1)<br>K.ASAHFKGGAK.K<br>K.ECSYDDIK.A + Carbamidomethyl (C)<br>R.GAAQNIIPSSTGAAK.A<br>R.VPTPDVSVVDLTVR.L (+1)<br>K.AGAEYIVESTGVFTTIEK.A |
| | gi\|317039786 | Glyceraldehyde 3-phosphate dehydrogenase [*Coenobita violascens*] | 18.471/8.65 | 211 | 6 | 24 | K.AAAEGPLK.G<br>K.LTGMAFR.V + Oxidation (M) (+1)<br>R.GAAQNIIPSSTGAAK.A<br>R.VPTPDVSVVDLTVR.L (+1) |
| 2D-A_14 | gi\|6016080 | Glyceraldehyde-3-phosphate dehydrogenase [*Homarus americanus*] | 35.693/6.53 | 285 | 8 | 23 | R.SSIFDAK.A<br>K.LTGMAFR.V + Oxidation (M)<br>K.IGINGFGR.I<br>K.ECSYDDIK.A + Carbamidomethyl (C)<br>R.GAAQNIIPSSTGAAK.A<br>R.VPTPDVSVVDLTVR.L<br>R.VPTPDVSVVDLTVR.L<br>K.AGAEYIVESTGVFTTIEK.A |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_14 | gi\|317039786 | Glyceraldehyde 3-phosphate dehydrogenase [*Coenobita violascens*] | 18.471/8.65 | 169 | 5 | 24 | K.AAAEGPLK.G<br>K.LTGMAFR.V + Oxidation (M)<br>R.GAAQNIIPSSTGAAK.A<br>R.VPTPDVSVVDLTVR.L (+1) |
| 2D-A_15 | gi\|6016080 | Glyceraldehyde-3-phosphate dehydrogenase [*Homarus americanus*] | 35.693/6.53 | 276 | 7 | 17 | R.SSIFDAK.A<br>K.LTGMAFR.V + Oxidation (M)<br>K.IGINGFGR.I<br>K.ECSYDDIK.A + Carbamidomethyl (C)<br>R.GAAQNIIPSSTGAAK.A<br>R.VPTPDVSVVDLTVR.L (+1) |
| | gi\|317039786 | Glyceraldehyde 3-phosphate dehydrogenase [*Coenobita violascens*] | 18.471/8.65 | 205 | 5 | 24 | K.AAAEGPLK.G<br>K.LTGMAFR.V + Oxidation (M)<br>R.GAAQNIIPSSTGAAK.A<br>R.VPTPDVSVVDLTVR.L (+1) |
| 2D-A_15 | gi\|217795253 | Glyceraldehyde-3-phosphate dehydrogenase [*Portunus trituberculatus*] | 35.643/6.60 | 188 | 5 | 13 | K.AAAEGPLK.G<br>R.SSIFDAK.A<br>K.LTGMAFR.V + Oxidation (M)<br>K.IGINGFGR.I<br>R.GAAQNIIPSSTGAAK.A |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_16 | gi\|410509306 | Myosin heavy chain type 1 [*Penaeus monodon*] | 219.450/5.77 | 195 | 4 | 1 | R.MQDLVDK.L + Oxidation (M)<br>R.TLLEQSDR.G<br>R.LEESESAAMK.A + Oxidation (M)<br>R.HAETELNDAR.E |
| | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 182 | 3 | 8 | R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.VSSTLSGLEGELK.G |
| | gi\|6016080 | Glyceraldehyde-3-phosphate dehydrogenase [*Homarus americanus*] | 35.693/6.53 | 142 | 4 | 11 | K.LTGMAFR.V + Oxidation (M)<br>K.IGINGFGR.I<br>K.ECSYDDIK.A + Carbamidomethyl (C)<br>R.GAAQNIIPSSTGAAK.A |
| | gi\|114159495 | Arginine kinase [*Platytrigona hobbyi*] | 20.011/4.78 | 127 | 2 | 12 | K.IIDDHFLFK.E<br>K.VSSTLSGLEGELK.G |
| | gi\|410509308 | Myosin heavy chain type 2 [*Penaeus monodon*] | 219.033/5.85 | 114 | 3 | 1 | R.MQDLVDK.L + Oxidation (M)<br>R.TLLEQSDR.G<br>R.LDEFESSAHK.T |



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**Appendix Table E11** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-A_17 | gi\|410509308 | Myosin heavy chain type 2 [*Penaeus monodon*] | 219.033/5.85 | 134 | 3 | 1 | R.MQDLVDK.L + Oxidation (M)<br>R.TLLEQSDR.G<br>K.AQQELETVQR.S |
| | gi\|3885968 | Phosphopyruvate hydratase [*Penaeus monodon*] | 47.235/6.18 | 120 | 2 | 5 | K.AGAAELGIPLYR.H<br>K.DALTLIQESIEK.A |
| 2D-A_18 | gi\|229256 | Dehydrogenase, glyceraldehydephosphate [Lobster] | 35.694/6.24 | 135 | 3 | 9 | K.LTGMAFR.V + Oxidation (M)<br>K.ECSYDDIK.A + Carbamidomethyl (C)<br>R.GAAQNIIPSSTGAAK.A |
| 2D-A_19 | gi\|229256 | Dehydrogenase, glyceraldehydephosphate [Lobster] | 35.694/6.24 | 150 | 3 | 10 | K.LTGMAFR.V + Oxidation (M)<br>R.GAAQNIIPSSTGAAK.A<br>R.VPTPDVSVVDLTVR.L |



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**Appendix Table E12** Identified allergens from ultrasonic treatment muscle of *Fenneropepaeus merguiensis* by 2D-PAGE using LC-MS/MS

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-S_1 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 557 | 12 | 37 | R.KYDEVAR.K<br>K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>R.ADTLEQQNK.E<br>R.MDALENQLK.E<br>R.MDALENQLK.E + Oxidation (M)<br>R.LEDELVNEK.E<br>K.LAMVEADLER.A<br>K.SEEEVHNLQK.R<br>R.IQLLEEDLER.S<br>R.ALSNAEGEVAALNR.R |
| 2D-S_2 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 393 | 8 | 25 | R.KYDEVAR.K<br>K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>R.ADTLEQQNK.E<br>R.MDALENQLK.E<br>K.LAMVEADLER.A<br>R.IQLLEEDLER.S |



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**Appendix Table E12** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-S_2 | gi\|225718350 | Tropomyosin [*Caligus clemensi*] | 32.840/4.79 | 243 | 5 | 14 | R.KYDEVAR.K<br>K.SLEVSEEK.A<br>R.FLAEEADR.K<br>K.IIELEEELR.V<br>R.IQLLEEDLER.S |
| | gi\|14423954 | Tropomyosin [*Dermatophagoides pteronyssinus*] | 32.881/4.78 | 242 | 5 | 15 | R.KYDEVAR.K<br>K.SLEVSEEK.A<br>R.MEGLENQLK.E<br>K.LAMVEADLER.A<br>R.IQLIEEDLER.S |
| 2D-S_3 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 142 | 3 | 8 | K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K |
| | gi\|310743896 | Glycogen phosphorylase [*Marsupenaeus japonicus*] | 97.956/6.82 | 107 | 3 | 2 | R.TIAQYGR.E<br>K.AIMNIASSGK.F<br>K.VVYLENYR.V |
| 2D-S_4 | gi\|3885968 | Phosphopyruvate hydratase (Enolase) [*Penaeus monodon*] | 47.235/6.18 | 248 | 5 | 11 | K.ITGDQLR.D<br>K.ACNCLLLK.V + 2 Carbamidomethyl (C)<br>K.GENIYDLDFK.T<br>K.AGAAELGIPLYR.H<br>K.DALTLIQESIEK.A |
| | gi\|410509312 | Myosin heavy chain type 2 [*Litopenaeus vannamei*] | 219.056/5.84 | 131 | 3 | 1 | R.AEEIEAAR.L<br>K.EEEFDATR.K<br>R.AQGLANAAER.R |



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**Appendix Table E12** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-S_5 | gi\|380503696 | Sarcoplasmic calcium-binding protein [*Penaeus monodon*] | 22.106/4.76 | 366 | 8 | 34 | R.NTLIEGR.G<br>K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V<br>K.VFIANQFK.A<br>K.YGEFPGAFK.V<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q |
| 2D-S_6 | gi\|380503696 | Sarcoplasmic calcium-binding protein [*Penaeus monodon*] | 22.106/4.76 | 383 | 9 | 34 | R.NTLIEGR.G<br>K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V<br>K.AIDVNGDGK.V<br>K.VFIANQFK.A<br>K.YGEFPGAFK.V<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q |



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**Appendix Table E12** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-S_7 | gi\|380503696 | Sarcoplasmic calcium-binding protein [*Penaeus monodon*] | 22.106/4.76 | 340 | 7 | 31 | K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V<br>K.VFIANQFK.A<br>K.YGEFPGAFK.V<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q |
| 2D-S_8 | gi\|229256 | Dehydrogenase, glyceraldehydephosphate [Lobster] | 35.694/6.24 | 104 | 3 | 8 | K.LTGMAFR.V + Oxidation (M)<br>K.ECSYDDIK.A + Carbamidomethyl (C)<br>R.VPTPDVSVVDLTVR.L |
| 2D-S_9 | gi\|56182374 | Arginine kinase [*Fenneropenaeus chinensis*] | 40.104/5.92 | 237 | 7 | 17 | K.AVFDQLK.E<br>R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>R.LTSAVNEIEKR.I<br>R.GEHTEAEGGIYDISNK.R |



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**Appendix Table E12** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-S_9 | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 236 | 6 | 16 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.VSSTLSGLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |
| | gi\|405973252 | Fructose-bisphosphate aldolase [*Crassostrea gigas*] | 38.965/7.01 | 101 | 2 | 4 | K.ATVVALSR.G<br>R.IANAIVAPGK.G |
| 2D-S_10 | gi\|229256 | Dehydrogenase, glyceraldehydephosphate [Lobster] | 35.694/6.24 | 141 | 5 | 14 | K.LTGMAFR.V + Oxidation (M)<br>K.LTGMAFR.V + Oxidation (M)<br>K.ECSYDDIK.A + Carbamidomethyl (C)<br>R.VPTPDVSVVDLTVR.L<br>K.AGAEYIVESTGVFTTIEK.A |
| 2D-S_10 | gi\|62701385 | Glyceraldehyde 3-phosphate dehydrogenase [*Marsupenaeus japonicus*] | 17.017/6.56 | 69 | 2 | 18 | K.AEDGCLVVDGHK.I + Carbamidomethyl (C)<br>K.AGAEYIVESTGVFTTIEK.A |
| 2D-S_11 | gi\|6016080 | Glyceraldehyde-3-phosphate dehydrogenase [*Homarus americanus*] | 35.693/6.53 | 63 | 2 | 6 | K.ECSYDDIK.A + Carbamidomethyl (C)<br>R.VPTPDVSVVDLTVR.L |



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**Appendix Table E13** Identified allergens from 1 min microwave treatment muscle of *Fenneropenaeus merguiensis* by 2D-PAGE using LC-MS/MS

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-MM_1 | gi\|335347355 | Tropomyosin [*Fenneropenaeus merguiensis*] | 31.685/4.66 | 732 | 18 | 40 | R.KYDEVAR.K<br>K.ANIQLVEK.D<br>K.SLEVSEEK.A<br>R.FLAEEADR.K (+1)<br>R.ADTLEQQNK.E<br>R.MDALENQLK.E<br>R.MDALENQLK.E + Oxidation (M)<br>R.LEDELVNEK.E<br>K.IVELEEELR.V<br>1 K.LAMVEADLER.A<br>1 K.LAMVEADLER.A + Oxidation (M)<br>K.SEEEVHNLQK.R (+1)<br>R.IQLLEEDLER.S<br>R.KLAMVEADLER.A + Oxidation (M)<br>R.ALSNAEGEVAALNR.R (+1) |



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**Appendix Table E13** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-MM_2 | gi\|151505281 | Tropomyosin [*Portunus trituberculatus*] | 32.767/4.73 | 546 | 13 | 28 | R.KYDEVAR.K<br>K.SLEVSEEK.A<br>R.FLAEEADR.K (+1)<br>R.ANTLEQQNK.E<br>R.MDALENQLK.E<br>R.MDALENQLK.E + Oxidation (M)<br>R.LEDELVNEK.E<br>K.IVELEEELR.V<br>K.LAMVEADLER.A<br>K.LAMVEADLER.A + Oxidation (M)<br>R.IQLLEEDLER.S<br>R.KLAMVEADLER.A + Oxidation (M) |
| 2D-MM_3 | gi\|380503696 | Sarcoplasmic calcium-binding protein [*Penaeus monodon*] | 22.106/4.76 | 377 | 9 | 34 | R.NTLIEGR.G<br>K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V (+1)<br>K.VFIANQFK.A<br>K.YGEFPGAFK.V<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q |



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**Appendix Table E13** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-MM_3 | gi\|223403273 | Sarcoplasmic calcium-binding protein [*Litopenaeus vannamei*] | 22.064/4.73 | 352 | 9 | 34 | R.NTLIEGR.G<br>K.AGGLTLER.Y<br>K.VGLDEYR.L<br>K.AIDVNGDGK.V (+1)<br>K.VFIANQFK.A<br>K.EIDDAYNK.L<br>K.NDFECLAVR.N + Carbamidomethyl (C)<br>K.DGEVTVDEFK.Q |
| | gi\|225711822 | Sarcoplasmic calcium-binding protein, beta chain [*Lepeophtheirus salmonis*] | 22.136/4.77 | 108 | 2 | 8 | K.KGGITLER.Y<br>K.NDFECLAVR.N + Carbamidomethyl (C) |
| 2D-MM_4 | gi\|585342 | Arginine kinase [*Homarus gammarus*] | 39.958/6.05 | 239 | 6 | 16 | R.FLQAANACR.Y + Carbamidomethyl (C)<br>R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.VSSTLSGLEGELK.G<br>R.GEHTEAEGGIYDISNK.R |
| | gi\|405973252 | Fructose-bisphosphate aldolase [*Crassostrea gigas*] | 38.965/7.01 | 183 | 3 | 5 | K.ATVVALSR.G<br>R.IANAIVAPGK.G<br>R.RIANAIVAPGK.G |



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**Appendix Table E13** (Continued)

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-MM_5 | gi\|229256 | Dehydrogenase,glyceraldehydephosphate [lobster] | 35.694/6.24 | 143 | 5 | 11 | K.LTGMAFR.V + Oxidation (M)<br>K.LTGMAFR.V + Oxidation (M)<br>K.IGIDGFGR.I<br>K.ECSYDDIK.A + Carbamidomethyl (C)<br>R.VPTPDVSVVDLTVR.L |
| 2D-MM_6 | gi\|6016083 | Glyceraldehyde-3-phosphate dehydrogenase [*Panulirus versicolor*] | 35.701/6.94 | 138 | 4 | 12 | K.LTGMAFR.V + Oxidation (M)<br>K.IGINGFGR.I<br>K.ECSYDDIK.A + Carbamidomethyl (C)<br>K.VVSNASCTTNCLAPVAK.V + 2 Carbamidomethyl (C) |



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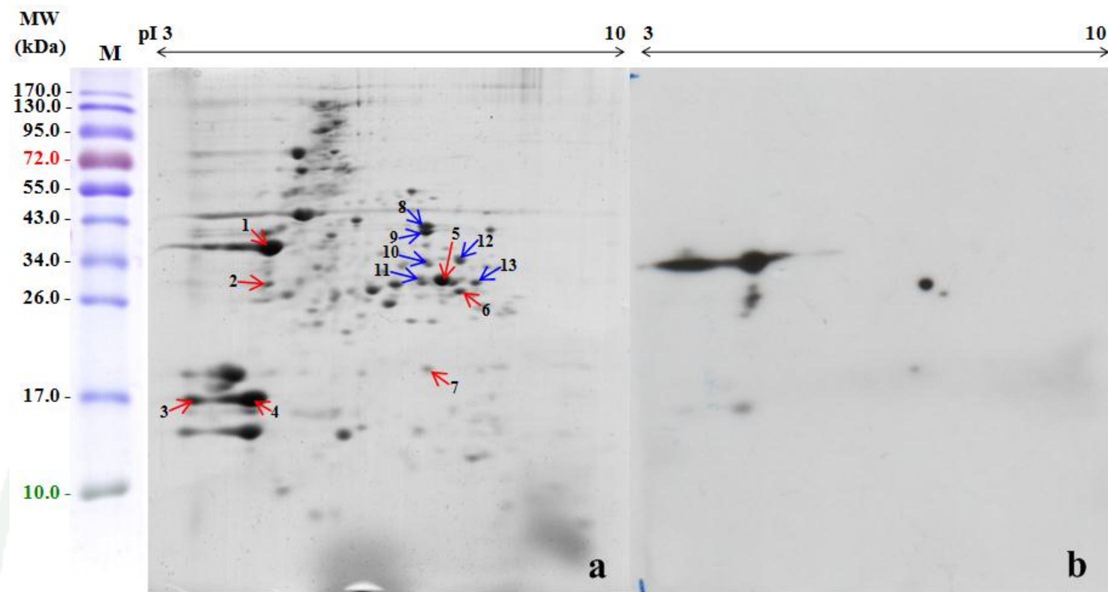
Non-IgE binding protein identified for prove that arginine kinase is the neo-allergen in food processing muscle.




**Appendix Figure E1** 2D-PAGE profiles of raw muscle (a) and their immunoblot analysis (b) with pooled sera of 21 shrimp-allergic patients. Red arrow; IgE binding protein as identied in Appendix Table E10, blue arrow; non-IgE binding protein identifiedin Appendix Table E14.

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**Appendix Table E14** Identified allergens from raw muscle of *Fenneropepaeus merguiensis* by 2D-PAGE using LC-MS/MS (In addition for arginine kinase proved).

| Spot No. | Accession numbers | Description | MW/pI | Score | Peptide matches | %Coverage | Peptide sequences |
|---|---|---|---|---|---|---|---|
| 2D-R_8 | gi\|585342 | Arginine kinase [Homarus gammarus] | 39.958/6.05 | 151 | 4 | 8 | R.MGLTEFQAVK.E<br>R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.VSSTLSGLEGELK.G |
| 2D-R_9 | gi\|585342 | Arginine kinase [Homarus gammarus] | 39.958/6.05 | 120 | 3 | 9 | R.MGLTEFQAVK.E<br>K.EMQDGILELIK.I + Oxidation (M)<br>K.VSSTLSGLEGELK.G |
| 2D-R_10 | gi\|585342 | Arginine kinase [Homarus gammarus] | 39.958/6.05 | 185 | 3 | 8 | R.MGLTEFQAVK.E + Oxidation (M)<br>K.LIDDHFLFK.E<br>K.VSSTLSGLEGELK.G |
| 2D-R_11 | gi\|585342 | Arginine kinase [Homarus gammarus] | 39.958/6.05 | 51 | 1 | 3 | K.VSSTLSGLEGELK.G |
| 2D-R_12 | gi\|585342 | Arginine kinase [Homarus gammarus] | 39.958/6.05 | 136 | 2 | 6 | R.MGLTEFQAVK.E + Oxidation (M)<br>K.VSSTLSGLEGELK.G |
| 2D-R_13 | gi\|585342 | Arginine kinase [Homarus gammarus] | 39.958/6.05 | 66 | 1 | 3 | K.VSSTLSGLEGELK.G |



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