Ann Allergy Asthma Immunol xxx (2014) 1–6

Contents lists available at ScienceDirect

Identification of a novel allergen from muscle and various organs in banana shrimp (*Fenneropenaeus merguiensis*)

Suparada Khanaruksombat, BSc [*,†,‡]; Chantragan Srisomsap, PhD [§]; Daranee Chokchaichamnankit, MSc [§]; Phaibul Punyarit, PhD [¶]; and Pharima Phiriyangkul, PhD [*,†,‡]

[*] *Division of Biochemistry, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand*
[†] *Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University, Kasetsart University, Chatuchak, Bangkok, Thailand*
[‡] *Bioproducts Science, Department of Science, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand*
[§] *Laboratory of Biochemistry, Chulabhorn Research Institute, Bangkok, Thailand*
[¶] *Department of Pathology, Phramongkutklao College of Medicine, Bangkok, Thailand*

ARTICLE INFO

Article history:
Received for publication December 28, 2013.
Received in revised form April 8, 2014.
Accepted for publication June 5, 2014.

ABSTRACT

Background: The increasing consumption of shellfish can cause an increase in allergic symptoms. Shrimp allergy can be species specific, but specific allergies in different organs have not been studied. Identification of allergens in muscle and others organs of banana shrimp is necessary for improved diagnostics of allergies for shrimp and food safety control.
Objective: To identify the IgE-binding proteins in various organs of *Fenneropenaeus merguiensis* by immunoblotting and tandem mass spectrometry.
Methods: Proteomic methods were used to investigate the allergenic proteins from banana shrimp. Proteins from muscle and various organs were separated by denaturing polyacrylamide gel electrophoresis. Allergens were analyzed by immunoblotting with pooled sera from shrimp allergic patients (n = 21) and tandem mass spectrometry.
Results: The important allergens in banana shrimp are arginine kinase, sarcoplasmic calcium-binding protein, myosin heavy chain, hemocyanin, enolase, and glyceraldehyde-3-phosphate dehydrogenase, which can be demonstrated by immunoblotting in muscle and shell. Moreover, vitellogenin, ovarian peritrophin 1 precursor, β-actin, and 14-3-3 protein were suggested as allergens in the ovary at different stages of ovarian development.
Conclusion: Ten allergens were identified as allergens in various organs, and they are suggested as novel allergens in banana shrimp. The major allergen in muscle and shell from this shrimp is arginine kinase, whereas the major allergen in the ovary is vitellogenin.
© 2014 American College of Allergy, Asthma & Immunology. Published by Elsevier Inc. All rights reserved.

Introduction

The Food and Agricultural Organization and World Health Organization suggest that crustaceans are 1 of 8 important allergenic foods[1] and that the number of allergic cases due to crustaceans tends to increase every year, perhaps because the consumption of crustaceans (shrimp, crab, and lobster) and mollusks (squid, scallop, clams, and snail) has been increasing since 1970.[2] Thus, shellfish allergy is a public concern in industrialized and/or coastal countries[3] because it can last a lifetime and, in cases of sensitization, may cause life-threatening anaphylaxis.[4] Shellfish is the most frequently implicated food in allergies in not only adults but also children,[5,6] which has resulted in food allergy symptoms being the most common reason for visits by allergy patients in the pediatric emergency department.[7] The major allergen in shellfish has been identified as the 34- to 39-kDa muscle protein tropomyosin.[8–12] Minor allergens have been identified, such as arginine kinase (AK; a 40-kDa protein)[13,14] and the sarcoplasmic calcium-binding protein (SCP; a 20-kDa protein).[15,16] Proteins that cause allergies are most studied in shrimp muscle, so it is extremely interesting to study these proteins in other organs of shrimp.

The banana shrimp, *Fenneropenaeus merguiensis*, is distributed in the Indo-Pacific region.[17] Cooking processing around the world almost always uses the whole shrimp, especially in East and Southeast Asia, where a shrimp dish composed of the whole body is popular for a better taste and high dietary value.[18] The muscle,

Reprints: Pharima Phiriyangkul, PhD, Division of Biochemistry, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand; E-mail: faasprm@ku.ac.th.
Disclosures: Authors have nothing to disclose.
Funding: This study was supported by Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University (023-2554), and grant MRG5280238 from the Thailand Research Fund and Office of the Higher Education Commission.

http://dx.doi.org/10.1016/j.anai.2014.06.002
1081-1206/© 2014 American College of Allergy, Asthma & Immunology. Published by Elsevier Inc. All rights reserved.

ovaries, and hepatopancreas are organs that are most likely to be consumed. 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 aim of this study is to determine the allergens in the various organs of banana shrimp (shell, hepatopancreas, and ovaries from different states of ovarian development) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting with pooled sera from 21 patients allergic to shrimp and by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Methods

Shrimps and Human Serum

Live banana shrimp, each weighing approximately 30 to 40 g, were purchased in Chonburi, 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 the different stages of their developing ovaries as follows: stage 1 (mean [SD] GSI, 0.995 [0.104]) as previtellogenic, stage 2 (mean [SD] GSI, 2.998 [0.175]) as early vitellogenic, and stage 3 (mean [SD] GSI, 8.273 [0.092]) as vitellogenic.[19] The GSI measures the cyclic changes in gonad weight in relation to total tissue weight and is represented by the following formula: [Gonad Weight/Total Tissue Weight] × 100.[20] 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). Of the 21 patients, 13 were male and 8 were female, ranging in age from 4 to 41 years. Most patients presented with urticarial dermatitis and/or angioedema, with 1 patient having asthma. The patients had their quantitative serum IgE levels measured and/or underwent skin tests to food allergen. The uses of sera were approved by the institutional review board at Phramongkutklao Hospital. The antibody IgE in the sera from allergic patients was verified at the levels greater than 0.35 kU/L using modern EUROLINE inhalation Southeast Asian test strips (EUROIMMUN AG; Schleswig Holstein, Lübeck, Germany). Control sera were obtained from healthy individuals without reported adverse reactions to shrimp and any food stuff. The quantitative IgE levels of all 21 patients ranged from 2.1 to 90.0 kU/L. After verification of the IgE antibody, the sera from shrimp allergic patients were pooled. Shrimp organs and human sera were frozen at −80°C until use.

Shrimp Extract Preparation

Separate samples of frozen shrimp organs were ground to a powdered liquid nitrogen. The samples were solubilized by adding 200 μg of shrimp powder to 400 μL of phosphate-buffered saline extraction buffer (9 mM Na_2HPO_4, 1.13 mM NaH_2PO_4 H_2O, and 0.152 M NaCl, pH 7.4) with a protease inhibitor cocktail (P8340: Sigma-Aldrich, St Louis City, Missouri) and incubated for 1 hour at 4°C. The mixture was centrifuged at 16,215*g* for 30 minutes at 4°C, and the supernatant was collected. Protein concentration was determined using UV-VIS spectrophotometry (UV-1700; Shimadzu, Nakagyo-Ku, Kyoto, Japan) with the Bio-Rad protein assay (Bio-Rad, Hercules, California). The protein concentration was calculated from a standard curve of known concentrations of bovine serum albumin.

Total Protein Determination and IgE-Binding Immunodetection

Each shrimp organ extract was separated by SDS-PAGE, using the method of Laemmli et al.[21] Samples with a concentration of 30 μg were loaded onto a 12.5% separating gel and 4% stacking gel of SDS–polyacrylamide Tris/glycine gel and electrically separated by the Mini-PROTEAN Tetra system (Bio-Rad). The protein standard for SDS-PAGE was a PageRuler prestained protein ladder (Thermo Fisher Scientific, Waltham, Massachusetts). The gels were stained with Coomassie Brilliant Blue R-250.[22] For immunodetection of IgE-binding proteins, the separated proteins from the SDS-PAGE gels were electroblotted onto an Immobilon-P transfer membrane (Millipore Corporation, Billerica, Massachusetts) with a constant voltage according to the methods of Towbin et al.[23] After that, transfer membranes were blocked with Block Ace (Dainippon Sumitomo Pharma, Fukushima-Ku, Osaka, Japan) and incubated with pooled sera from shrimp allergic patients. The bound IgE antibody was detected using horseradish peroxidase–conjugated goat anti-human IgE (Immunology Consultants Laboratory Inc, Newberg, Oregon). Finally, the immunoreactive bands were visualized using Amersham ECL Plus Western blotting detection reagents (GE Healthcare Bio-SciencesAB, Little Chalfont, Buckinghamshire, England) exposure with Amersham Hyperfilm ECL (GE Healthcare Bio-Sciences AB) in a dark room.

Glycoprotein Staining of SDS-PAGE

The protein membrane was stained using a GelCode glycoprotein staining kit (Thermo Fisher Scientific). The membrane was washed with 3% acetic acid and transferred to 10 mL of oxidizing solution. Membrane was washed with 3% acetic acid before being added 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.

Protein Image Analysis

The stained gels and immunoreactive bands from the films were scanned using an image scanner (ImageScannerIII; GE Healthcare Bio-Sciences AB). Images of the protein gels and films were analyzed using ImageQuant TL Plus 7.0 software (GE Healthcare Bio-Sciences AB). The volume values for immunoreactive bands from the films were detected and calculated by volume ratio. The data were presented as interleaved bar graphs with a mean value of the volume ratio from each protein using GraphPad Prism 5 software (GraphPad Software Inc, La Jolla, California) to compare the allergenicity of the IgE-binding protein between each organ.

Protein Identification by LC-MS/MS

The protein bands of interest were excised, destained, and digested with trypsin for LC-MS/MS analysis performed using a Waters capillary liquid chromatography system coupled to a quadruple-time-of-flight (Q-TOF) mass spectrometer (Micromass; Wythenshaw, Manchester, United Kingdom) equipped with a Zspray ion source working in the nanoelectrospray mode according to Srisomsap et al.[24] The Mascot search engine (Matrix Science Ltd, London, United Kingdom) is associated with more protein databases than others and also provides the number of hits for peptides. For some proteins that were difficult to find, the Mascot search tool available on the Matrix Science site screening NCBInr was used. Mascot MS/MS ions search (http://www.matrixscience.com) was used, and the search parameters were set as follows: peptide mass tolerance, 1.2 Da; MS/MS ion mass tolerance, 0.2 Da; enzyme, trypsin; allowance, up to one missed cleavage; and peptide charges, limited to 1^+, 2^+, and 3^+. The proteins were identified at $P \leq .05$, and Mascot scores greater than 35 were considered promising hits.

Results

Total Protein Extraction From SDS-PAGE Separation

The electrophoretic pattern of shrimp extracts from various organs revealed multiple protein bands ranging from 17 to 170 kDa

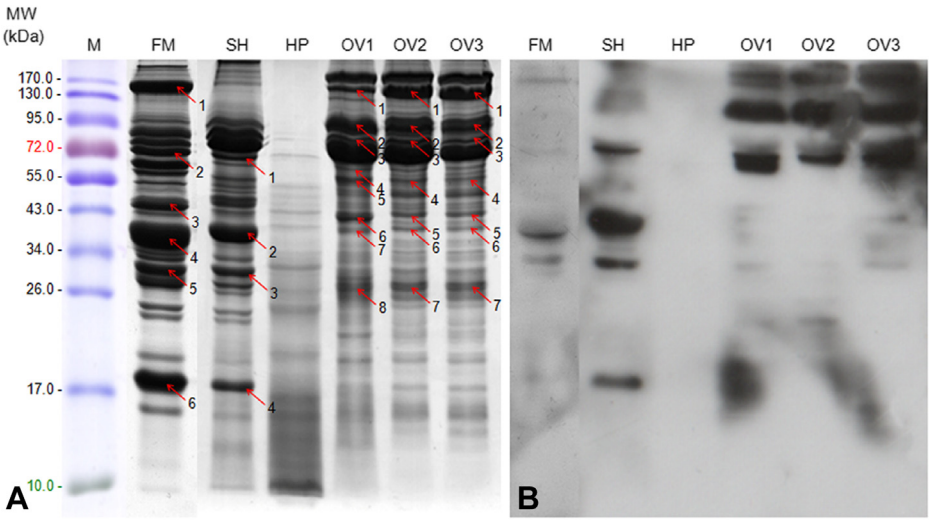


Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern with Coomassie Brilliant Blue R-250 staining (A) and immunoreactive bands (B) of various banana shrimp organ extracts. MW indicates molecular weight of the protein ladder. The arrows represent allergen identified by liquid chromatography tandem mass spectrometry.

with different patterns (Fig 1A). The protein pattern of hepatopancreas was not clear with smears on the protein bands and had low molecular masses of proteins of less than 17 kDa of molecular mass when compared with the other shrimp protein samples. The protein pattern of the ovaries revealed the 3 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. Fingerprint and identification of the allergen protein are given in Table 1.

Immunodetection of IgE-Binding Protein

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 in molecular mass from 17 to 170 kDa (Fig 1B). These protein bands could be identified as banana shrimp allergens. Lane FM was a protein from *F merguiensis* muscle extract that contained 6 immunoreactive bands. Lane SH was a protein from *F merguiensis* shell extract that contained 4 immunoreactive bands. Lane HP was a protein from *F merguiensis* hepatopancreas extract 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 to OV3 were proteins from *F merguiensis* ovarian extracts at different vitellogenic stages that contained 6 immunoreactive bands. No immunoreactive band was detected using serum from nonallergic individuals (data not shown).

The various organs revealed allergenic reactions to *F merguiensis*. The results from immunoblotting indicated that each allergic patient had different immunologic reactions to different allergens and different intensities of the IgE-binding protein shown in Figure 2. Six proteins could be identified as muscle allergens, with the highest volume ratio for AK, followed by a myosin heavy chain (MHC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hemocyanin (HC), SCP, and enolase, respectively. In the shell, the 3 proteins AK, SCP, and GAPDH were identified based on the volume ratio. Three allergens were identified as vitellogenin (VG), AK, and ovarian peritrophin 1 precursor (SOPs) in the stage 1 ovaries. Allergens from ovaries were VG and 14-3-3 protein at vitellogenic stage 2 and VG, HC, β-actin, and 14-3-3 protein at vitellogenic stage 3.

Glycoprotein Staining of SDS-PAGE

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

Discussion

Allergens were identified in 4 organs: muscle, shell, hepatopancreas, and ovaries with 3 different levels of the GSI. Allergenic proteins were recognized using the IgE of pooled sera from patients with shrimp allergy. The same 3 proteins were identified in the muscle and shell of banana shrimp. Subsequent LC-MS/MS analysis of a 22-kDa protein identified SCP, AK (approximately 40 kDa), and GAPDH (approximately 30 kDa). Both SCP and AK have been suggested as minor allergens in whiteleg shrimp (*Litopenaeus vannamei*)[14,16] and giant tiger prawn (*Panaeus monodon*).[13] GAPDH has not been reported as an allergen in shellfish, but it was confirmed as the most abundant allergen in indoor mold (*Aspergillus versicolor*)[25] and common wheat (*Triticum aestivum*).[26] Moreover, GAPDH was suggested as an important allergen in occupational sensitization to fish.[27] In addition, muscle also contained 3 other proteins that may be allergens in banana shrimp, namely, MHC, HC, and phosphopyruvate hydrates or enolase. The volume ratio of the IgE-binding proteins suggests that AK is the most important allergen in muscle and shell, whereas VG is the major allergen in ovaries (Fig 2).

AK, SCP, and MHC are muscle proteins and play important roles in muscle mobilization and use.[28,29] Moreover, the muscle protein found in shell may be due to the connective tissue between shell and muscle. In the current research, HC was identified as an allergen in banana shrimp and giant freshwater prawn (*Macrobrachium rosenbergii*).[30] This protein is important in the hemolymph system of crustaceans and mollusks as an oxygen-carrying protein.[31] GAPDH was considered a classic glycolytic protein and has been examined for its pivotal role in energy production; it was also used as a model protein in the analysis of protein structure and enzyme mechanisms.[32] Enolase is homodimeric enzymes that

Table 1
Identified allergens from various organs of *Fenneropenaeus merguiensis* Using LC-MS/MS

Band	Protein name/species/accession No.	Theoretical value		LC-MS/MS analysis		
		Molecular weight, kDa	*pI*	Score	No. of peptidematches	Coverage, %
FM-1	Myosin heavy chain type b/*Marsupenaeuse japonicus*/BAK61430	21.887	6.00	172	15	10
FM-2	Hemocyanin/*Litopenaeus vannamei*/CAA57880	74.984	5.27	70	11	11
FM-3	Enolase (phosphopyruvate hydrates)/*Penaeus monodon*/AAC78141	47.235	6.18	249	6	17
FM-4	Arginine kinase/*P monodon*/C7E3T4	40.115	6.05	273	41	44
FM-5	Glyceraldehyde-3-phosphate dehydrogenase/*M japonicus*/BAD95643	17.017	6.56	192	13	39
FM-6	Sarcoplasmic calcium-binding protein/*P monodon*/BAL72725	22.106	4.76	59	5	18
SH-1	Sarcoplasmic calcium-binding protein/*L vannamei*/ACM89179	22.064	4.73	156	6	20
SH-2	Arginine kinase/*Homarus gammarus*/P14208	39.958	6.05	168	3	8
SH-3	Glyceraldehyde-3-phosphate dehydrogenase/Nephropidae/671058A	35.694	6.24	82	17	18
SH-4	Sarcoplasmic calcium-binding protein/*L vannamei*/ACM89179	22.064	4.73	64	16	47
OV1-1	Vitellogenin/*F merguiensis*/Q6RG02	282.850	6.36	740	18	5
OV1-2	Vitellogenin/*F merguiensis*/Q6RG02	282.850	6.36	378	9	3
OV1-3	Vitellogenin/*F merguiensis*/ACV32381	283.130	6.47	468	11	4
OV1-4	Vitellogenin/*F merguiensis*/Q6RG02	282.850	6.36	317	8	3
OV1-5	Arginine kinase/*Homarus gammarus*/P14208	39.958	6.05	113	3	8
OV1-6	Ovarian peritrophin 1 precursor/*P monodon*/AAM44049	30.870	4.80	90	2	6
OV2-1	Vitellogenin/*F merguiensis*/ACV32381	283.130	6.47	1038	26	9
OV2-2	Vitellogenin/*F merguiensis*/Q6RG02	282.850	6.36	390	10	3
OV2-3	Vitellogenin/*F merguiensis*/Q6RG02	282.850	6.36	622	16	6
OV2-4	β-Actin/*L vannamei*/AAG16253	41.841	5.30	90	5	9
OV2-5	Arginine kinase/*P monodon*/AAO15713	40.087	6.05	179	6	17
OV2-6	14-3-3Z/*F merguiensis*/ADI87601	27.915	4.68	113	3	10
OV3-1	Vitellogenin/*F merguiensis*/Q6RG02	282.850	6.36	764	15	5
OV3-2	Vitellogenin/*F merguiensis*/Q6RG02	282.850	6.36	206	4	1
OV3-3	Vitellogenin/*F merguiensis*/Q6RG02	282.850	6.36	126	3	1
OV3-4	β-Actin/*L vannamei*/AAG16253	41.841	5.30	130	3	8
OV3-5	Arginine kinase/*L vannamei*/ABI98020	40.134	6.19	107	3	8
OV3-6	14-3-3–like protein/*P monodon*/AAY56092	27.834	4.61	151	3	10

Abbreviation: LC-MS/MS, liquid chromatography tandem mass spectrometry; pI, Isoelectric point.

catalyze the reversible dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate as part of the glycolytic and gluconeogenesis pathways. There are no reports that enolase is an allergen in shellfish, but it has been suggested as an allergen in many fish species, such as Wuchang bream (*Megalobrama amblycephala*), Nile perch (*Lates niloticus*), and Atlantic cod (*Gadus morhua*).[33,34] Furthermore, it has been found in insects, such as German cockroach (*Blattella germanica*).[35]

In this study, no allergen was identified in the hepatopancreas of banana shrimp. This may have been due to the low molecular mass in the SDS-PAGE/immunoblotting analysis. SDS-PAGE used glycine and 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.[36] 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 that occur under various conditions that cannot be completely inhibited by protease inhibitor.[37] The enzymatic properties may cause the degradation of shrimp allergens.[38] Sen et al[39] suggested that peanut allergen disulfide bond reduction and their accessibility to IgE-binding epitopes can reduce overall allergenicity. Thus, the structure/epitope degradation of the allergen may reduce the allergenicity.

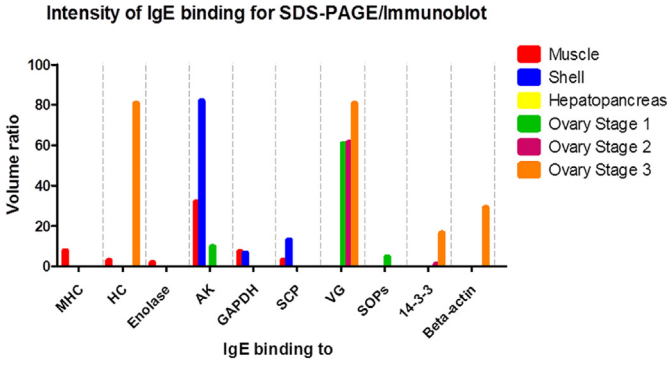


Figure 2. Volume ratio of sodium dodecyl sulfate–polyacrylamide gel electrophoresis/immunoblot binding of IgE from pooled human sera to banana shrimp allergens from various organs. Nine allergens were identified for banana shrimp.

In the ovaries of banana shrimp at different vitellogenic stages, AK was identified as in other organs. However, VG had a high intensity in all the vitellogenic stages, which suggests it is an important allergen in ovaries. The other identical proteins were SOPs, 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 research is incomplete. In addition, VG has been suggested to be 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.[37,40,41] 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.[42] The major site for vitellogenesis in banana shrimp is the ovary, which was investigated by real-time polymerase chain reaction to determine the VG messenger RNA level,[20] and the protein level at all stages of ovarian development was found to correlate well with the GSI levels.[37] This protein is a complex lipo-glycocarotenoprotein.[43] VG has been suggested as an allergen in many species. VG has also been reported as an allergen in salmonic roe[44,45] and Beluga caviar.[46] In addition, there are reports that VG is an allergen in insects such as German cockroach[35] and in the venom of western honey bee (*Apis mellifera*) and common wasp (*Vespula vulgaris*).[47]

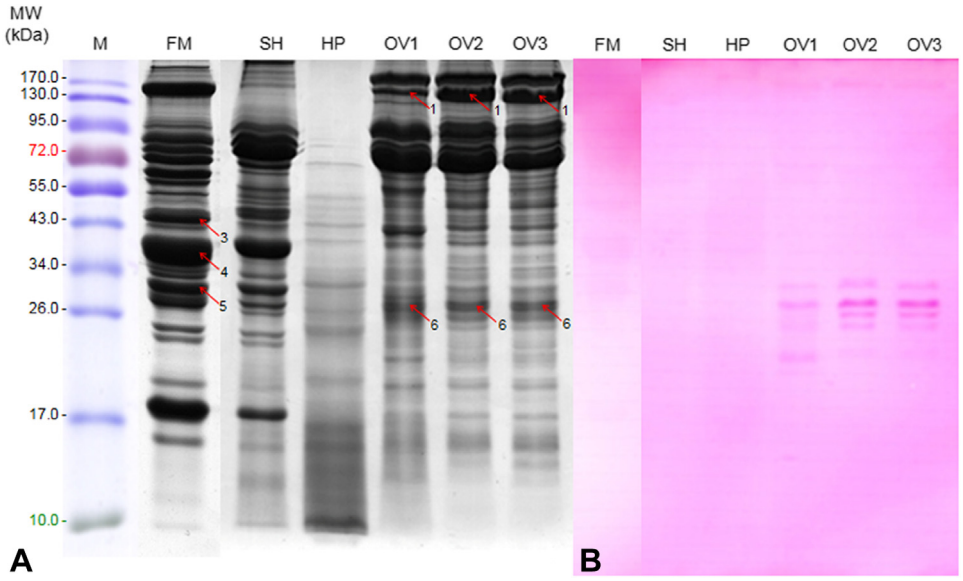


Figure 3. Electrophoretic pattern with arrows (A) and magenta bands (B) indicating glycoproteins. MW indicates molecular weight of the protein ladder. The arrows indicate glycoprotein bands with allergenic properties identified by liquid chromatography tandem mass spectrometry.

In the current study, SOPs were found as a part of shrimp ovarian peritrophins. These proteins were found in the stage 1 ovary 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.[48] β-Actin is a member of the actin family that exists in invertebrate and vertebrate cells in several different isoforms.[49] 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.[50] Kaeodee et al[51] suggested that 14-3-3b is likely to be involved in hyperosmotic regulation in *P monodon*. The study of the true functions of β-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, and Taura syndrome.[52–54] SOPs, β-actin, and 14-3-3 protein have not been reported as allergens in shrimp, but the current study suggests these 3 proteins are novel minor allergens in *F merguiensis*.

The most important posttranslational modification in newly synthesized proteins is called glycosylation. Without exception, glycosylation occurs in the integral membrane proteins of higher organisms.[55] From the glycoprotein staining results, 6 allergens (enolase, AK, VG, GAPDH, SOPs, and 14-3-3 protein) have features of glycoprotein that suggest that these 6 allergens are allergenic glycoproteins. Many studies about the structural analysis of glycan moieties of allergenic glycoproteins reported 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 there is no direct evidence of their involvement in allergenicity.[56] Thus, the study of allergenic glycoproteins is important because they take part in inducing an IgE response.

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, SOPs, 14-3-3 protein, β-actin, and VG are novel allergens in shrimp. Thus, shrimp allergic patients can react to the whole body of shrimp. Tropomyosin can cause an allergenic reaction in approximately 71.4% of patients with shrimp allergy.[57] These novel allergens may be improved diagnostic allergic methods for shrimp allergic patients because the only measurement of IgE to tropomyosin in shrimp allergy diagnosis can be missed. Avoidance of the offending foods is the only therapy recommended for food allergic individuals. Steensma[58] 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. Moreover, greater understanding of the resistance of food allergens to food processing with heat is needed. Further work should study the effect of food processing on the allergenicity of shrimp. An increased understanding of food allergens can be applied to manage food quality and food safety procedures. Food processing 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.

Acknowledgments

We thank Dr M. R. Jisnuson Svasti and the Laboratory of Biochemistry, Chulabhorn Research Institute, for their kind advice and the use of their facilities for this project. We also thank Lt Gen Phanuvich Pumhirun for his recommendations. Moreover, we thank Phramongkutklao Hospital and Phyathai Hospital, Thailand, for providing the sera of patients allergic to shrimp.

References

[1] Food and Agricultural OrganizationWorld Health Organization. Evaluation of allergenicity of genetically modified foods. In: *Report of a Joint FAO/WHO Expert Consultation of Allergenicity of Foods Derived from Biotechnology*. Geneva, Switzerland: World Health Organization; 2001:22–25.

[2] Sicherer SH, Munoz-Furlong A, Sampson HA. Prevalence of seafood allergy in the US determined by a random telephone survey. *J Allergy Clin Immunol*. 2004;114:159–165.

[3] Emoto A, Ishizaki S, Shiomi K. Tropomyosins in gastropods and bivalves: identification as major allergens and amino acid sequence features. *Food Chem*. 2009;114:634–641.

[4] Ayuso R, Grishina G, Bardina L, et al. Myosin light chain is a novel shrimp allergen, Lit v 3. *J Allergy Clin Immunol*. 2008;122:795–802.

[5] Clark S, Bock SA, Gaeta TJ, Brenner BE, Cydulka RK, Camargo CA. Multicenter study of emergency department visits for food allergies. *J Allergy Clin Immunol*. 2004;113:347–352.

[6] Ross MP, Ferguson M, Street D, Klontz K, Schroeder T, Luccioli S. Analysis of food-allergic and anaphylactic events in the National Electronic Injury Surveillance System. *J Allergy Clin Immunol*. 2008;121:166–171.

[7] Liu TH, Lin YR, Yang CC, Chou CC, Chang YJ, Wu HP. First attack of acute urticaria in pediatric emergency department. *Pediatr Neonatal*. 2008;49:58–64.

ARTICLE IN PRESS

[8] Shanti KN, Martin BM, Nagpal S, Metcalfe DD, Rao PV. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J Immunol*. 1993;151:5354–5363.

[9] Leung PSC, Chu KH, Chow WK, et al. Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen. *J Allergy Clin Immunol*. 1994;94:882–890.

[10] Leung PSC, Chen YC, Mykles DL, Chow WK, Li CP, Chu KH. Molecular identification of the lobster muscle protein tropomyosin as a seafood allergen. *Mol Mar Biol Biotechnol*. 1998;7:12–20.

[11] Lin RY, Shen HD, Han SH. Identification and characterization of a 30 kDa major allergen from *Parapenaeus fissurus*. *J Allergy Clin Immun*. 1993;92:837–845.

[12] Daul CB, Slattery M, Reese G, Lehrer SB. Identification of the major brown shrimp (*Penaeus aztecus*) allergen as the muscle protein tropomyosin. *Int Arch Allergy Appl Immunol*. 1994;105:49–55.

[13] Yu CJ, Lin YF, Chiang BL, Chow LP. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 21. *J Immunol*. 2003;170:445–453.

[14] Garcia-Orozco KD, Aispuro-Hernandez E, Yepiz-Plascencia G, Calderón-de-la-Barca AM, Sotelo-Mundo RR. Molecular characterization of arginine kinase, an allergen from the shrimp *Litopenaeus vannamei*. *Int Arch Allergy Immunol*. 2007;144:23–28.

[15] Shiomi K, Sato Y, Hamamoto S, Mita H, Shimakura K. Sarcoplasmic calcium-binding protein: Identification as a new allergen of the black tiger shrimp Penaeus monodon. *Int Arch Allergy Immunol*. 2008;146:91–98.

[16] Ayuso R, Grishina G, Ibanez MD, et al. Sarcoplasmic calcium-binding protein is an EF-hand–type protein identified as a new shrimp allergen. *J Allergy Clin Immunol*. 2009;124:114–120.

[17] Grey DL, Dall W, Baker A. *A Guide to the Australian Penaeid Prawns*. Darwin, Australia: Northern Territory Government Printing Office; 1983.

[18] Yamagata M, Low LK. Banana shrimp, *Penaeus merguiensis*, quality changes during iced and frozen storage. *J Food Sci*. 2006;60:721–726.

[19] Phiriyangkul P, Puengyam P, Jakobsen IB, Utarabhand P. Dynamics of vitellogenin mRNA expression duringvitellogenesis in the banana shrimp *Penaeus (Fenneropenaeus) merguiensis*using real-time PCR. *Mol Reprod Dev*. 2007;74:1198–1207.

[20] Barbar BJ, Blake NJ. Reproductive physiology. In: Shumway SE, Parsons GJ, eds. *Scallops: Biology, Ecology and Aquaculture*. Developments in Aquaculture and Fisheries Science 35. New York, NY: Elsevier Sciences; 2006:357–416.

[21] Laemmli UK. Cleavage of structural protein during assembly of the head of bacteriophage T4. *Nature*. 1970;227:680–695.

[22] Smith I, Cromie R, Stainsby K. Seeing gel wells well. *Anal Biochem*. 1988;169:370–371.

[23] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. 1979;76:4350–4354.

[24] Srisomsap C, Sawangareetrakul P, Subhasitanont D, et al. Proteomic studies of cholangiocarcinoma and hepatocellular carcinoma cell secretomes. *J Biomed Biotechnol*. 2010;2010:437143.

[25] Benndorf D, Müller A, Bock K, Manuwald O, Herbarth O, Bergen MV. Identification of spore allergens from the indoor mould *Aspergillus versicolor*. *Allergy*. 2008;63:454–460.

[26] Sander I, Rozynek P, Rihs HP, et al. Multiple wheat flour allergens and cross-reactive carbohydrate determinants bind IgE in baker's asthma. *Allergy*. 2011;66:1208–1215.

[27] Ventela ML, Nieuwenhuizen NE, Kirstein F, et al. Differential responses to natural and recombinant allergens in a murine model of fish allergy. *Mol Immunol*. 2011;48:637–646.

[28] Yao CL, Ji PF, Kong P, Wang ZY, Xiang JH. Arginine kinase from *Litopenaeus vannamei*: cloning, expression and catalytic properties. *Fish Shellfish Immun*. 2009;26:553–558.

[29] Gao Y, Gillen CM, Wheatly MG. Molecular characterization of the sarcoplasmic calcium-binding protein (SCP) from crayfish *Procambarus clarkii*. *Comp Biochem Physiol B Biochem Mol Biol*. 2006;144:478–487.

[30] Piboonpocanun S, Jirapongsananuruk O, Tipayanon T, Boonchoo S, Goodman RE. Identification of hemocyanin as a novel noncross-reactive allergen from the giant freshwater shrimp *Macrobrachium rosenbergii*. *Mol Nutr Food Res*. 2011;55:1492–1498.

[31] Paoli M, Giomi F, Hellmann N, et al. The molecular heterogeneity of hemocyanin: Structural and functional properties of the 4x6-meric protein of *Upogebia pusilla* (Crustacea). *Gene*. 2007;398:177–182.

[32] Sirover MA. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim Biophys Acta*. 1999;1432:159–184.

[33] Liu R, Krishnan HB, Xue W, Liu C. Characterization of allergens isolated from the freshwater fish blunt snout bream (*Megalobrama amblycephala*). *J Agric Food Chem*. 2011;59:458–463.

[34] Tomm JM, van Do T, Jende C, et al. Identification of new potential allergens from Nile perch (*Latesniloticus*) and cod (*Gadus morhua*). *J Investig Allergol Clin Immunol*. 2013;23:159–167.

[35] Chuang JG, SU SN, Chiang BL, Lee HJ, Chow LP. Proteome mining for novel IgE-binding proteins from the German cockroach (*Blattella germanica*) and allergen profiling of patients. *Proteomics*. 2010;10:3854–3867.

[36] Schagger H, Jagow GV. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem*. 1987;166:368–379.

[37] Auttarat J, Phiriyangkul P, Utarabhand. Characterization of vitellin from the ovaries of the banana shrimp *Litopenaeus merguiensis*. *Comp Biochem Physiol B: Biochem Mol Biol*. 2006;43:27–36.

[38] Oh ES, Kim DS, Kim JH, Kim HR. Enzymatic properties of a protease from the hepatopancreas of shrimp, Penaeus orientalis. *J Food Biochem*. 2000;24:251–264.

[39] Sen M, Kopper R, Pons L, Abraham EC, Burks AW, Bannon GA. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J Immunol*. 2002;169:882–887.

[40] Avarre JC, Michelis R, Tietz A, Lubzens E. Relationship between vitellogenin and vitellin in a marine Shrimp (*Penaeus semisulcatus*) and molecular characterization of vitellogenin complementary DNAs. *Biol Reprod*. 2003;69:355–364.

[41] Rao MS, Rajitha B, Pavitra E, Anjaneyulu N. Identification and changes of copper profile in different during vitellogenesis of white shrimp *Litopenaeus vannamei*. *J Biol Sci*. 2007;6:989–992.

[42] Phiriyangkul P, Utarabhand P. Molecular characterization of a cDNA encoding vitellogenin in the banana shrimp, *Penaeus (Litopenaeus) merguiensis* and sites of vitellogenin mRNA expression. *Mol Reprod Dev*. 2006;73:410–423.

[43] Fatima H, Ayub Z, Ali SA, Siddiqui G. Biochemical composition of the hemolymph, hepatopancreas, ovary, and muscle during ovarian maturation in the penaeid shrimps *Fenneropenaeus merguiensis* and *F. penicillatus* (Crustacea: Decapoda). *Turk J Zool*. 2013;37:334–347.

[44] Kondo Y, Kakami M, Koyama H, et al. IgE cross-reactivity between fish roe (salmon, herring and pollock) and chicken egg in patients anaphylactic to salmon roe. *Allergol Int*. 2005;54:317–323.

[45] Shimizu Y, Nakamura A, Kishimura H, Hara A, Watanabe K, Saeki H. Major allergen and its IgE cross-reactivity among salmonid fish roe allergy. *J Agric Food Chem*. 2009;57:2314–2319.

[46] Gordo MP, Garcia S, Cases B, Pastor C, Vivanco F, Herranz JC. Identification of vitellogenin as an allergen in Beluga caviar allergy. *Allergy*. 2008;63:479–480.

[47] Blank S, Seismann H, McIntyre M, et al. Vitellogenins are new high molecular weight components and allergens (Api m 12 and Ves v 6) of *Apis mellifera* and *Vespula vulgaris* venom. *PLoS One*. 2013;8:e62009.

[48] Du XJ, Wang JX, Liu N, Zhao XF, Li FH, Xiang JH. Identification and molecular characterization of a peritrophin-like protein from fleshy prawn (*Fenneropenaeus chinensis*). *Mol Immunol*. 2006;43:1633–1644.

[49] Sun PS, Soderlund M, Vanzon NC Jr, Ye D, Lu Y. Isolation and characterization of two actins of the Pacific white shrimp, *Litopenaeus vannamei*. *Mar Biol*. 2007;151:2145–2151.

[50] Inoue M, Nakamura Y, Yasuda K, et al. The 14-3-3 protein of *Trypanosoma brucei* function in motility, cytokinesis, and cell cycle. *J Biol Chem*. 2005;280:14085–14096.

[51] Kaeodee M, Pongsomboon S, Tassanakajon S. Expression analysis and response of *Penaeus monodon* 14-3-3 genes to salinity stress. *Comp Biochem Physiol B: Biochem Mol Biol*. 2011;159:244–251.

[52] Wongpanyaa R, Aokib T, Hironob I, Yasuikeb M, Tassanakajona A. Analysis of gene expression in haemocytes of shrimp *Penaeus monodon* challenged with white spot syndrome virus by cDNA microarray. *ScienceAsia*. 2007;33:165–174.

[53] Chongsatja P, Bourchookarn A, Lo CF, Thongboonkerd V, Krittanai C. Proteomic analysis of differentially expressed proteins in *Penaeus vannamei* hemocytes upon Taura syndrome virus infection. *Proteomics*. 2007;7:3592–3601.

[54] Pongsomboon S, Tang S, Boonda S, et al. Differentially expressed genes in *Penaeus monodon* hemocytes following infection with yellow head virus. *BMB Rep*. 2008;41:670–677.

[55] Khan RH, Rasheedi S, Hoq SK. Effect of pH, temperature and alcohols on the stability of glycosylated and deglycosylated stem bromelain. *J Biosci*. 2003;28:709–714.

[56] Fötisch K, Vieths S. N- and O-linked oligosaccharides of allergenic glycoproteins. *Glycoconjugate J*. 2001;18:373–390.

[57] Yang AC, Arruda LK, Santos AB, et al. Measurement of IgE antibodies to shrimp tropomyosin is superior to skin prick testing with commercial extract and measurement of IgE to shrimp for predicting clinically relevant allergic reactions after shrimp ingestion. *J Allergy Clin Immunol*. 2010;125:872–878.

[58] Steensma DP. The kiss of death: a severe allergic reaction to a shellfish induced by good-night kiss. *Mayo Clin Proc*. 2003;78:221–222.