

Identification of pyruvate kinase as a novel allergen in whiteleg shrimp (*Litopenaeus vannamei*) by specific-IgE present in patients with shrimp allergy

Chih-Hung Lee^a, Chia-Ching Wu^b, Yu-Chang Tyan^{c,d,e}, Wei-Tai Yu^f, Edward S. Huang^g, Hsu-Sheng Yu^{b,*}

^a Department of Dermatology, Kaohsiung Chang Gung Memorial Hospital and College of Medicine, Chang Gung University, Kaohsiung 833, Taiwan

^b Department of Food Science, National Pingtung University of Science and Technology, Pingtung 912, Taiwan

^c Department of Medical Imaging and Radiological Sciences, and Center for Infectious Disease and Cancer Research, and Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan

^d Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan

^e Institute of Medical Science and Technology, National Sun Yat-sen University, Kaohsiung 804, Taiwan

^f Department of Dermatology, Kaohsiung Medical University Hospital and Graduate Institute of Clinical Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan

^g Department of Gastroenterology, Palo Alto Medical Foundation, Mountain View, CA 94040, USA

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ABSTRACT

Food allergy is one of the most important health issues worldwide. In Taiwan, current literature suggests shrimps and crabs are the most common causes of food allergy, and are frequently associated with acute allergic reactions such as urticaria, atopic dermatitis, and asthma. However, knowledge regarding the shrimp allergens remains limited. Thus, there is an urgent need to establish comprehensive information for elucidating underlying triggers for food allergy. In this study, whiteleg shrimp (*Litopenaeus vannamei*) was used to evaluate the IgE-binding properties of various shrimp proteins to 7 allergic patients' sera by western blot. A 63 kDa protein was found in raw and cooked shrimp bound to specific-IgEs in 7 and 4 patients' sera, respectively. This protein was further identified as pyruvate kinase based on the proteomic mass spectrometry. This study identifies an important shrimp allergen unique to Taiwan and further testing and prevention measures might be implemented in the allergen analysis.

1. Introduction

Food allergy is an important issue in the field of food safety. The World Health Organization (WHO) has recognized food allergy as the fourth most critical public health issue (Kirsch et al., 2009). It is estimated up to 10% of the population worldwide suffers from food allergy (Leung et al., 2014). Studies have demonstrated crustacean, tree nut, milk, egg, peanut, soy bean, wheat, and fish account for more than 90% of all food allergies (Monaci & Visconti, 2009; Poms, Klein, & Anklaam, 2004).

Seafood such as crustacean, shellfish and mollusc have both high commercial and nutritional value. However, with its increasing

popularity, there has also been higher incidence of food allergy, which is associated with congestive heart failure, peripheral vascular disease, chronic pulmonary disease, rheumatologic disease and renal disease in the Taiwanese population (Yu, Tu, Hong, & Lee, 2016). Current literature has shown that shrimp and crab are the most common causes of food allergy in Taiwan, resulting in 50% of all emergency department visits for allergy (Chan, Chen, Huang, & Wu, 2014). As immune reactions are quite variable, the same allergen exposure may trigger different reactions based on ethnicity. However, most studies use Europeans and Americans as study subjects. Thus, it is important to investigate various kinds of crustacean allergens in East Asian populations to provide consumers with more

* Corresponding author.

E-mail address: hsyu@mail.npust.edu.tw (H.-S. Yu).

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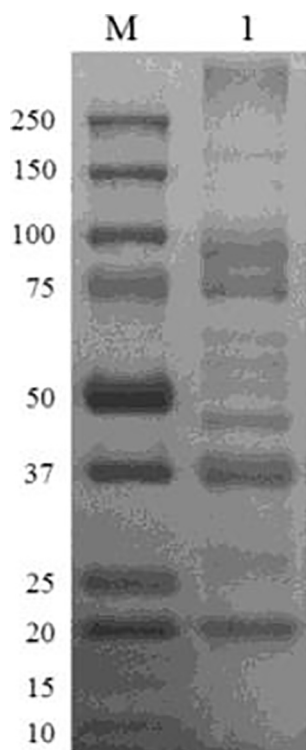


Fig. 1. SDS-PAGE of shrimp extract. M: protein marker, 1: Shrimp extract of *Litopenaeus vannamei*.

information for allergy prevention.

In spite of the high prevalence of shrimp and crab allergy, there is little knowledge of the allergens involved in the allergic response. Recognizing crustacean allergens can be critical for the diagnosis and treatment of crustacean allergies. To date, various shrimp and crab allergens were identified in numerous studies. Candidate protein allergens were detected using Western blot and subsequently identified using mass spectrometry (Giuffrida, Villalta, Mistrello, Amato, & Asero, 2014; Piboonpocanun, Jirapongsananuruk, Tipayanon, Boonchoo, & Goodman, 2011). Currently, the major crustacean allergen is tropomyosin (Hajeb & Selamat, 2012; Yu et al., 2011; Zhang, Lu, Ushio, & Shiomi, 2014), which accounts for 80% of shrimp and crab allergies (Jeoung et al., 1997). This allergen is a heat-stable protein with an α -helical coiled-coil structure (Huang et al., 2010), and has a molecular weight ranging from 34 to 38 kDa. The amino acid sequence of tropomyosin is highly conserved among certain species. Thus, crustacean tropomyosin has cross-reactivity with tropomyosin in cockroach and dustmite (Kamath et al., 2013). In addition, minor allergens including arginin kinase (AK), sarcoplasmic calcium-binding protein (SCP), myosin light chain (MLC), myosin heavy chain (MHC), troponin C (TnC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triose phosphate isomerase (TIM), enolase (EA), hemocyanin (HC) and smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA), have been reported in numerous studies during the last decade (Ayuso et al., 2008; Bauermeister et al., 2011; Khanaruksombat, Srisomsap, Chokchaichamnankit, Punyarit, & Phiriyangkul, 2014; Rahman, Kamath, Gagne, Lopata, & Helleur, 2013; Rahman, Kamath, Lopata, Robinson, & Helleur, 2011; Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008; Yu, Lin, Chiang, & Chow, 2003).

Although numerous shrimp allergens have been reported, little is known about the patterns of antigen sensitization for Taiwanese patients. In this study, the aim was to demonstrate the binding activities of specific-IgEs in allergic patients' sera with proteins in whiteleg shrimp (*Litopenaeus vannamei*), the most commonly consumed shrimp in Taiwan. Also, the aim was to further identify new candidate allergic proteins in this model shrimp.

2. Materials and methods

2.1. Study strategy

In this study, the allergic response was measured between shrimp allergen and specific-IgEs in 7 patients' sera. In this study, whiteleg shrimp was profiled on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and screened against allergic patients' sera, and allergic proteins from this profile were identified using mass spectrometry.

2.2. Sample extraction

Samples of whiteleg shrimp were obtained from a local market at Pingtung, Taiwan. The protein extraction method by Sahabudin et al. was used with minor modifications (Sahabudin et al., 2011). Briefly, 5 g of sample was mixed with 25 ml of cold PBS buffer (pH 7.4, 0.137 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4), and homogenized for 1 min. Then the sample was centrifuged at 7000g for 30 min at 4 °C. Next, the filtrate was centrifuged at 15,000g for 15 min at 4 °C, and the supernatant was taken out as protein extracts. Protein extracts were stored at −20 °C. Cooked shrimps were steamed in laboratory for 5 min followed by the same protein extraction.

2.3. Patients' sera

Sera of 7 patients diagnosed with shrimp and crab allergy (age 22–48, 4 males and 3 females) were collected in Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan, and were approved by the Institutional Review Board (104-1569B). Written informed consent was obtained from each patient. Serum from one healthy subject without a history of shrimp and crab allergy was used as a negative control. All sera were stored at −80 °C until use.

2.4. SDS-PAGE and western blot analysis

The diluted sample protein extracts were heated to 100 °C for 5 min, and 10 μl were loaded into each well. SDS-PAGE was performed by a Mini-PROTEAN® electrophoresis system (Bio-Rad, USA) with 60 V for 20 min, then 80 V for 120 min at room temperature. Precision Plus Protein™ Dual Color Standards (Bio-Rad, USA) were used as protein markers. The separated proteins were stained with Bio-Safe™ Coomassie G-250 Stain (Bio-Rad, USA).

Separated protein extracts were transferred to polyvinylidene fluoride (PVDF) membrane and blocked with 5% skimmed milk powder in PBS-T buffer for 1 h. The membrane was subsequently incubated with diluted patients' sera overnight. After washing three times with PBS-T buffer, mouse monoclonal B3102E8 anti-human IgE Fc (HRP) (Abcam, USA) at 1/40,000 dilution was used as a secondary antibody, and incubated for 1 h. After final washing, Clarity™ Western ECL Substrate (Bio-Rad, USA) was used as a chemiluminescent substrate. Finally, the PVDF membrane was

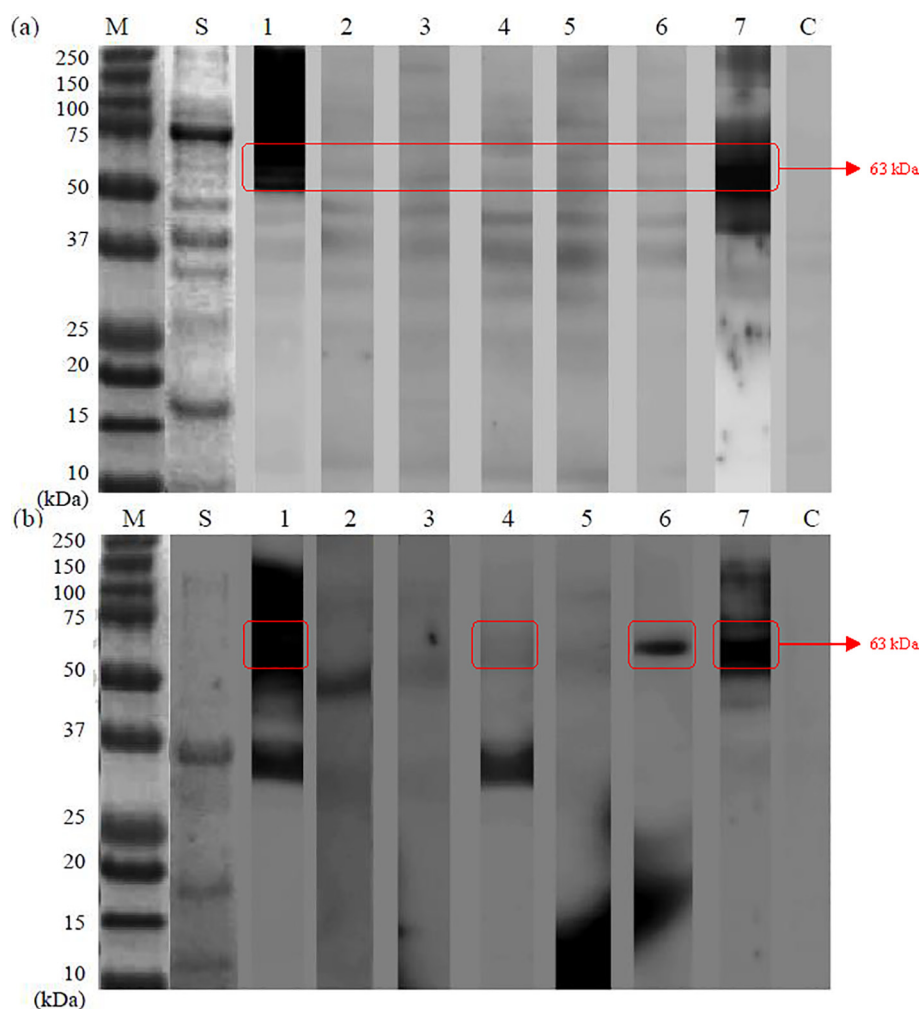


Fig. 2. Binding of proteins in *Litopenaeus vannamei* with specific-IgEs in patients' sera. M: protein marker, S: SDS-PAGE of shrimp extract, 1–7: patients' sera, C: serum of control subject.

photographed by a chemiluminescence imaging system (G:BOX Chemi XX8, Syngene, UK).

2.5. Identification of novel allergen in whiteleg shrimp

The integrated protein lane on SDS-PAGE was excised manually, and transferred into siliconized 0.5 ml Eppendorf tubes. After trypsin digestion and peptide extraction, target peptides were separated by mass spectrometry. Briefly, the protein tryptic digests were fractionated using a flow rate of 300 nl/min with a nano-UPLC system (nanoACQUITY UPLC, Waters, USA) coupled to an ion trap mass spectrometer (LTQ Orbitrap Discovery Hybrid FTMS, Thermo, USA) equipped with an electrospray ionization source.

For protein identification, Mascot software (ver 2.2.1, Matrix Science, UK) was used to search the NCBI metazoa protein sequence database. For proteolytic cleavages, only tryptic cleavage was allowed, and the number of maximal internal (missed) cleavage sites was set to 2. Variable modifications of carbamidomethyl (C), deamidated (NQ), Gln- > pyro-Glu (N-term Q), Glu- > pyro-Glu (N-term E) and Oxidation (M) were allowed. Proteins were annotated by similar searches using UniProtKB/Swiss-Prot databases (SIB Swiss Institute of Bioinformatics, Switzerland).

3. Results

3.1. Protein profile of shrimp extract

Fig. 1 shows the protein profile of shrimp extract (raw whiteleg shrimp) by SDS-PAGE. Various allergens were observed, including the 35 kDa tropomyosin, 40 kDa arginine kinase, 20 kDa myosin light chain, and other important shrimp allergens which were published in previous studies (Ayuso et al., 2008; Bauermeister et al., 2011; Khanaruksombat et al., 2014; Rahman et al., 2011, 2013; Shiomi et al., 2008; Yu et al., 2003).

3.2. The reaction of shrimp proteins and specific-IgE in patients' sera

In Fig. 2a, line 2, proteins with 18, 24, 27, 35, 38, 47, 63, 75, 100 and 200 kDa are observed in extracted raw whiteleg shrimp. By western blot, specific proteins were found binding to sera of 7 allergic patients. A specific-IgE in patient no. 1 was found bound to 50–200 kDa proteins. Fig. 2b also shows different binding patterns in patients' sera with proteins of cooked whiteleg shrimp. For example, patient no. 1 showed high binding activity to proteins with 35 kDa and 47–100 kDa. Our control serum did not show any binding.

Table 1

The reactivity of possible allergens with specific-IgE in patients' sera.

Shrimp allergens ^a	Molecular weight of protein (kDa)		Number of patients' sera							Reactivity ^b (%)
			1	2	3	4	5	6	7	
MHC	200	F	+	–	+	–	+	–	+	57
		C	–	–	–	–	–	–	–	0
SERCA	100	F	+	+	+	+	+	+	–	86
		C	+	+	+	–	+	–	–	57
HC	75	F	+	+	+	+	+	+	+	100
		C	+	+	+	–	+	–	+	71
Allergen identified in this study	63	F	+	+	+	+	+	+	+	100
		C	+	–	–	+	–	+	+	57
EA	47	F	+	+	+	+	+	+	+	100
		C	+	+	+	–	–	–	+	57
GAPDH, AK	38	F	+	+	+	+	+	+	–	86
		C	–	–	–	–	–	–	–	0
TM	35	F	+	+	+	+	+	+	+	100
		C	+	+	+	+	+	–	+	86
TIM	27	F	+	+	+	+	+	–	–	71
		C	–	–	–	–	–	–	–	0
TnC	24	F	–	+	+	+	+	–	–	57
		C	–	–	–	–	–	–	–	0
MLC, SCP	18	F	–	–	+	–	–	–	–	14
		C	–	–	–	–	–	–	–	0

+: Positive reaction between specific-IgE and the protein.

–: No reaction between specific-IgE and the protein.

F: fresh shrimp extract, C: cooked shrimp extract.

^a MLC: myosin light chain, SCP: sarcoplasmic calcium-binding protein, TnC: troponin C, TIM: triose phosphate isomerase, TM: tropomyosin, AK: arginine kinase, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, EA: enolase, HC: hemocyanin, SERCA: smooth endoplasmic reticulum Ca²⁺ ATPase, MHC: myosin heavy chain.

^b Reactivity = numbers of sera that bound to the protein/total numbers of sera.

3.3. The binding activities of shrimp proteins

Table 1 lists the reactivity of each shrimp protein and patients' sera (Reactivity = number of sera that bound to this protein/total number of sera). Our results showed each protein in both raw and cooked whiteleg shrimp bound to specific IgEs in individual patients. These proteins were possible allergens. The strength of reactivity of proteins in raw shrimp in descending order were 35, 47 and 75 kDa (100%) > 38 and 100 kDa (86%) > 27 kDa (71%) > 24 and 200 kDa (57%) > 18 kDa (14%), while in cooked shrimp they were 34 and 75 kDa (71%) > 47 and 100 kDa (57%) > 18, 24, 27, 38 and 200 kDa (0%).

3.4. Identification of trypsin digested peptides in Mascot database

The fragmentation spectra obtained by the RP-nano-UPLC-ESI-MS/MS analysis in gradient detection mode were compared with a non-redundant protein database using Mascot software. In this study, a summary of the protein identifications achieved is shown in Table 2. The Mascot search of the data yielded pyruvate kinase as the top hit with the score of 1442, and the sequence coverage of ~63%, corresponding to 29 peptide matches (Table 3).

3.5. Mass spectra of the identified peptides

Fig. 3 shows typical mass spectra of the identified peptides. The mass spectra identified the amino acid sequences of tryptic peptides, which were double- and triple-charged peptides with m/z of 734.44 and 495.60. The amino acid sequences of the tryptic peptides were VLVV-PSTGPTASIVK and IGHSTYPVAIALDTK. Peaks matched as b and y ions

of the peptide sequence were labeled.

4. Discussion

This is the first study to identify pyruvate kinase, a 63 kDa protein, as a novel shrimp allergen. Using the Expert Protein Analysis System, the proteomics server of the Swiss Institute of Bioinformatics (SIB), to identify known functions of proteins, pyruvate kinase was found, an enzyme that catalyzes the conversion and the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP). Thus, this protein is involved in a subpathway that synthesizes pyruvate from D-glyceraldehyde 3-phosphate. Previous studies also suggested pyruvate kinase was an important enzyme during the life stage of the crustacean (Lemos, Salomon, Gomes, Phan, & Buchholz, 2003; Salomon, Mayzaud, & Buchholz, 2000). Our results showed specific-IgE binding to this novel allergen in raw and cooked shrimp extract by 7 (100%) and 4 (57%) of 7 allergic patients' sera, respectively. Additionally, specific-IgE was found binding to tropomyosin in raw and cooked shrimp extract by 7 (100%) and 6 (86%) of 7 allergic patients' sera, respectively. Although tropomyosin appears to be the most sensitized protein in our study, pyruvate kinase also showed high binding to most of the patients. Therefore, it is important to explore properties and characteristics of pyruvate kinase in future studies and confirm the levels of this protein in various crustacean species.

Proteins were initially annotated by similarity searches using NCBI PubMed. In this study, most Mascot results were manually confirmed by visual assessment of the MS/MS spectra for their overall quality. In addition, the criterion for manual validation was required using a readily observable series of at least 4 y ions. Besides pyruvate kinase,

Table 2

Proteins identified and peptides matched sorted by residue number. The protein was identified in the band of the SDS-PAGE gel.

gi 126571553				Pyruvate kinase
Start-End	Observed	Mr(expt)	Mr(calc)	Peptide
4–33	1070.8561	3209.5464	3209.5485	K.VAPMQLGAADTHTQVDHMAALDIDSKPFSK.R + Oxidation (M)
4–33	1070.8616	3209.5629	3209.5485	K.VAPMQLGAADTHTQVDHMAALDIDSKPFSK.R + Oxidation (M)
4–33	803.6468	3210.5581	3210.5325	K.VAPMQLGAADTHTQVDHMAALDIDSKPFSK.R + Deamidated (NQ); Oxidation (M)
4–33	1076.1904	3225.5495	3225.5435	K.VAPMQLGAADTHTQVDHMAALDIDSKPFSK.R + 2 Oxidation (M)
4–33	1076.8602	3227.5587	3227.5115	K.VAPMQLGAADTHTQVDHMAALDIDSKPFSK.R + 2 Deamidated (NQ); 2 Oxidation (M)
35–47	686.8859	1371.7572	1371.7544	R.LSGIICITGPVSR.S + Carbamidomethyl (C)
55–64	570.2528	1138.4911	1138.4933	K.MMEAGMNIAR.M + Oxidation (M)
55–64	570.2542	1138.4939	1138.4933	K.MMEAGMNIAR.M + Oxidation (M)
55–64	578.2521	1154.4897	1154.4882	K.MMEAGMNIAR.M + 2 Oxidation (M)
55–64	578.7546	1155.4947	1155.4722	K.MMEAGMNIAR.M + Deamidated (NQ); 2 Oxidation (M)
93–106	742.9094	1483.8043	1483.8035	K.IGHSYPVAIALDTK.G
112–126	757.4102	1512.8058	1512.8035	R.TGLLEGGPSAEIELK.E
133–142	595.7831	1189.5517	1189.5503	K.LTTDASYYEK.C
165–177	702.4108	1402.8071	1402.8072	R.IFVDDGLISLIAK.D
178–197	1034.9475	2067.8805	2067.8725	K.DVGSDSIDCEVENGMLGSK.K + Carbamidomethyl (C)
178–197	1035.4382	2068.8619	2068.8565	K.DVGSDSIDCEVENGMLGSK.K + Carbamidomethyl (C); Deamidated (NQ)
178–197	1042.9416	2083.8687	2083.8674	K.DVGSDSIDCEVENGMLGSK.K + Carbamidomethyl (C); Oxidation (M)
178–197	1043.4349	2084.8553	2084.8514	K.DVGSDSIDCEVENGMLGSK.K + Carbamidomethyl (C); Deamidated (NQ); Oxidation (M)
198–215	607.0159	1818.0258	1818.0251	K.KGVNLPGVVPDLPAVSEK.D
198–217	697.3939	2089.1598	2089.1532	K.KGVNLPGVVPDLPAVSEKDR.G
198–217	697.7280	2090.1621	2090.1372	K.KGVNLPGVVPDLPAVSEKDR.G + Deamidated (NQ)
199–215	845.9736	1689.9327	1689.9301	K.GVNLPGVVPDLPAVSEK.D
199–217	654.6930	1961.0572	1961.0582	K.GVNLPGVVPDLPAVSEKDR.G
199–217	655.0275	1962.0606	1962.0422	K.GVNLPGVVPDLPAVSEKDR.G + Deamidated (NQ)
226–237	677.8632	1353.7119	1353.7115	K.MGVDIVFASFIR.D
270–285	591.9612	1772.8617	1772.8614	K.NIDDIIEEGDGIMAR.G
270–285	895.4363	1788.8581	1788.8564	K.NIDDIIEEGDGIMAR.G + Oxidation (M)
286–296	571.3089	1140.6032	1140.6026	R.GDLGIEIPAEK.V
311–327	953.9957	1905.9769	1905.9726	K.VGKPVICATQMLSESMVK.K + Carbamidomethyl (C); Oxidation (M)
311–327	641.6629	1921.9669	1921.9675	K.VGKPVICATQMLSESMVK.K + Carbamidomethyl (C); 2 Oxidation (M)
311–327	962.4919	1922.9693	1922.9515	K.VGKPVICATQMLSESMVK.K + Carbamidomethyl (C); Deamidated (NQ); 2 Oxidation (M)
334–358	841.3978	2521.1715	2521.1676	R.AEVSDVGNAILDGADCVMLSGETAK.G + Carbamidomethyl (C)
359–367	510.7680	1019.5215	1019.5222	K.GGYPLVQVR.T + Carbamidomethyl (C)
368–374	388.7080	775.4015	775.4010	R.TMANIAR.E
368–374	396.7052	791.3958	791.3959	R.TMANIAR.E + Oxidation (M)
375–383	527.7698	1053.5250	1053.5243	R.EAEAAIWHK.Q
384–413	1085.5539	3253.6397	3253.6143	K.QLFTELSQQVHLPTDSTHTTAIAAVEASF.K + Deamidated (NQ); Gln- > pyro-Glu (N-term Q)
384–413	1085.8892	3254.6457	3254.5983	K.QLFTELSQQVHLPTDSTHTTAIAAVEASF.K + 2 Deamidated (NQ); Gln- > pyro-Glu (N-term Q)
439–446	458.2577	914.5009	914.5008	R.CPIVAVTR.Y + Carbamidomethyl (C)
447–452	367.2034	732.3922	732.3919	R.YPQVAR.Q
459–473	854.4633	1706.9120	1706.9104	R.GIPIHYTVPQNAER.I
459–473	854.9652	1707.9159	1707.8944	R.GIPIHYTVPQNAER.I + Deamidated (NQ)
474–484	681.8091	1361.6037	1361.6034	R.IEDWMNDVNAR.V
474–484	682.3106	1362.6067	1362.5874	R.IEDWMNDVNAR.V + Deamidated (NQ)
474–484	682.8126	1363.6107	1363.5714	R.IEDWMNDVNAR.V + 2 Deamidated (NQ)
474–484	689.8048	1377.5951	1377.5983	R.IEDWMNDVNAR.V + Oxidation (M)
474–484	690.3006	1378.5866	1378.5823	R.IEDWMNDVNAR.V + Deamidated (NQ); Oxidation (M)
485–493	521.7640	1041.5135	1041.5131	R.VDYAVQYGK.E
494–512	706.0349	2115.0829	2115.0823	K.ECGFIKPGDPVVVVGTGWQK.G + Carbamidomethyl (C)
513–521	485.7241	969.4337	969.4338	K.GAGFTNTMR.V + Oxidation (M)
522–536	734.4448	1466.8750	1466.8709	R.VLVVPTGTASIVK.L

Variable modifications of amino acids were underlined.

the Mascot search of the data yielded pyruvate kinase 2 as the second potential hit with the scores of 1042, and the sequence coverage of ~63%, corresponding to 26 peptide matches (data not shown). Although these two proteins were similar, there are still some differences in the amino sequences. A previous study showed that pyruvate kinase 2 is an isozyme of pyruvate kinase (Ma et al., 2015); hence, pyruvate kinase was selected as the most plausible target protein.

The present study evaluates the sera of 7 allergic patients by examining their reactivity with shrimp allergens. The sensitization patterns for Taiwanese patients to various kinds of allergens were clearly shown in our study. Studies reported non-specific lipid transfer proteins

were important food allergens for South Europeans, but not for Northern Europeans (Scheurer et al., 2001; Schocker et al., 2004). This suggested that designation as minor and major allergens is not necessarily uniform across ethnicities. In other words, sensitized allergic responses were highly dependent on ethnicities. Thus, pyruvate kinase was not found in allergic patients recruited in previous studies, but was found to sensitize Taiwanese allergic patients. The results of our research will improve our understanding of crustacean allergy research in Taiwan and perhaps in East Asia.

Various shrimp and crab allergens were reported in previous studies. According to the previous publications, Rahman et al. identified

Table 3

Protein view and matched peptides shown in the identified proteins. Matched peptides were shown in **bold letter**.

Match to: gi|126571553; Score: 1442
 pyruvate kinase [Litopenaeus vannamei] (Pacific white shrimp)
 Nominal mass (M_r): 63737; Calculated pI value: 7.58
 Sequence Coverage: 63%

1 MSKVAPMQLG AADTHTQVDH MAALDIDSKP FSKRLSGIIC TIGPVSRVSE
 51 MLEKMMMEAGM NIARMNFSHG THEYHSETMM NVRKAAQKYS DKIGHSYPPVA
 101 IALDTKGPEI RTGLLEGGPS AEIELKEGAT IKLTDDASY EKCSDEVLYL
 151 DYVNITKVVK PGNRIFVDDG LISLIAKDVG SDSIDCEVEN GGMLGSKKGV
 201 NLPGVVPDLP AVSEKDRGDL LLGVKMGVDI VFASFIRDA GVREIRDVLG
 251 EKGKNIKIS KIENHQGCKN IDDIIEGDG IMIARGDLGI EIPAEEKVFA
 301 QKQMIACNK VGGPVICATQ MLESMVKKPR PTRAEVSDVG NAILDGADCV
 351 MSLGETAKGG YPLVCVRTMA NIAREAAAI WHKQLFELS QQVHLPTDST
 401 HTTAIAAVEA SFKAMATAII VITPTGRSAH LVSKYRPRCP IVAVTRYPOV
 451 ARQCHLYRGI IPIHYTVPQN AERIEDWMND VNARVDYAVQ YGKEGCFIKP
 501 GGPVVVVVTGW QKGAGFTNTM RVLVVPSTGP TASIVKLGAH SSVKSGPVKS
 551 GPQASGEHQR TGKAQPDIPW PSFLAHMGPI HLCAAIVANK I

SCP, tropomyosin and AK as important allergens in *Pandalus borealis* (Rahman et al., 2013). Ayuso et al. identified MLC as a minor allergen in *Litopenaeus vannamei* (Ayuso et al., 2008), while Bauermeister et al. identified SCP, TIM and TnC as allergens in *Crangon crangon*

(Bauermeister et al., 2011). Other investigators have identified GADPH, MHC, EA and HC as allergens in *Fenneropenaeus merguensis*, and SERCA as an allergen in *Chionoecetes opilio* (Khanaruksombat et al., 2014; Leung et al., 1998; Rahman, Kamath, Lopata, & Helleur, 2010). Similarly these crustacean allergens were found in our experiments. Based on this, it was speculated that the proteins with 18, 24, 27, 35, 38, 47, 75, 100 and 200 kDa in whiteleg shrimp correspond to MLC/SCP, TnC, TIM, tropomyosin, AK, EA, HC, SERCA and MHC, respectively. This will need to be validated with other studies to confirm these possible allergens in Taiwanese patients.

A limitation of this study was the small sample size. Nevertheless, it was possible to observe the different sensitization patterns in each patient. The difference of binding reactivity between raw and cooked shrimp extract was further elucidated. The specific-IgEs had higher binding reactivity to proteins in raw shrimp extract compared to those in cooked shrimp extract, which is consistent with previous study (Chen et al., 2013; Rahman et al., 2010). Tropomyosin was also found to have the best heat stability among these allergens, similar to previous studies. Furthermore, pyruvate kinase was found in raw and cooked shrimp bound to specific-IgEs in 100% and 57% patients' sera, respectively, showing the moderate heat stability of this protein. Therefore, pyruvate kinase, which was recognized by more than 50% of the allergic patients in our study, may play an important role as a shrimp

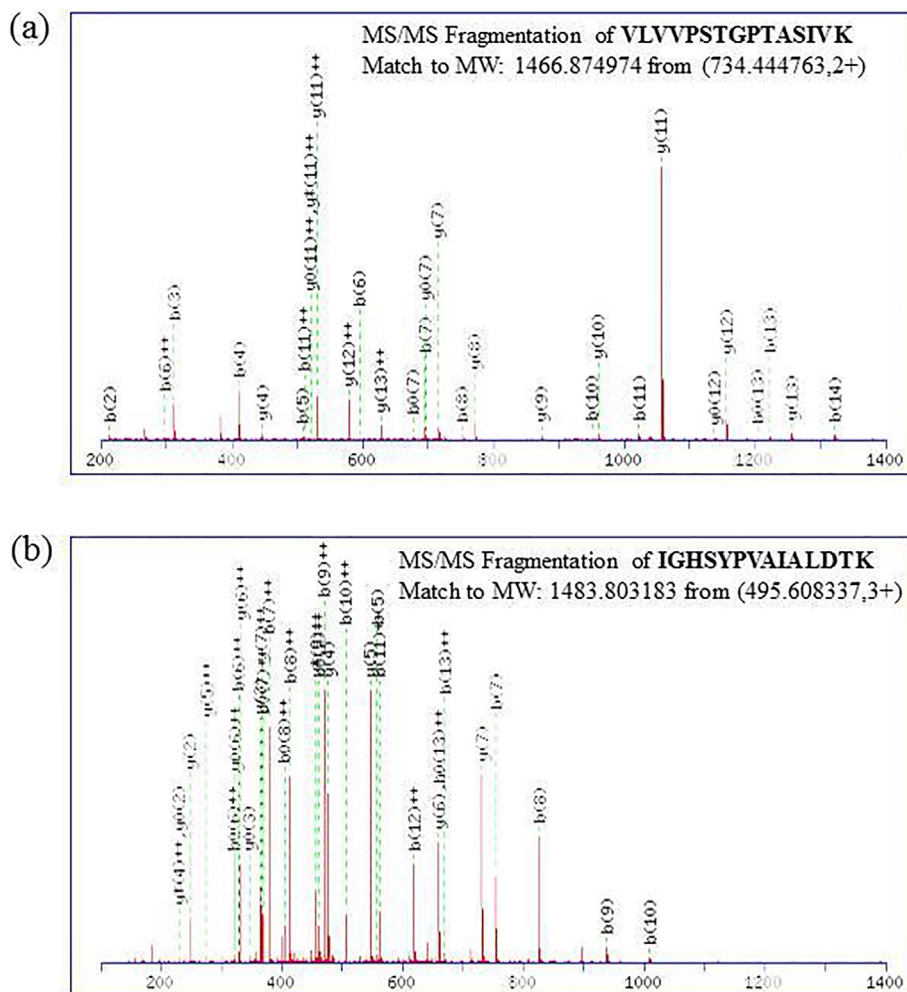


Fig. 3. Two examples of significant match (product ion scan data against NCBI sequence database) derived from (A) doubly, and (B) thrice charged precursor ions, respectively. Peaks matched as b and y ions of the peptide sequence are labeled.

allergen. These results will need to be replicated with larger samples sizes to clarify the role of pyruvate kinase in crustacean allergy.

5. Conclusions

In summary, this is the first study to identify pyruvate kinase as a novel shrimp allergen by proteomic mass spectrometry. Also, this is the first study to demonstrate various allergens from raw/cooked shrimps sensitized to Taiwanese patients with crustacean allergy. These findings may contribute to the future research of food allergy and could be useful in allergy diagnosis.

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Conflicts of interest

The authors declare no conflict of interest.

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