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Biomolecular characterization of allergenic proteins in snow crab (*Chionoecetes opilio*) and *de novo* sequencing of the second allergen arginine kinase using tandem mass spectrometry

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ABSTRACT

Snow crab (*Chionoecetes opilio*) proteins have been recognized as an important source of both food and occupational allergens. While snow crab causes a significant occupational allergy, only one novel allergen has recently been fully characterized. The muscle proteins from snow crab legs were profiled by SDS-PAGE. Several of these proteins were characterized using tandem mass spectrometry. Five proteins were identified; sarcoplasmic Ca-binding (20 kDa), arginine kinase (40), troponin (23 kDa) and α -actine (42 kDa) and smooth endoplasmic reticulum Ca^{2+} ATPase (113 kDa).

Immunoblotting using serum of sixteen allergic patients resulted in strong reactivity with the 40-kDa protein in seven patients (43%). This protein was purified by chromatography and subsequently *de novo* sequenced using matrix assisted laser desorption ionization and electrospray tandem mass spectrometry. We identified a second important allergen, arginine kinase, in snow crab, designated Chi o 3. Based on identity and homology analysis, using bioinformatics tools, a signature peptide was identified as a chemical surrogate for arginine kinase. The suitability of this signature peptide was tested for analytically representing the arginine kinase, by performing a multi-reaction monitoring tandem mass spectrometry approach on actual air filter samples collected from a simulated crab processing plant.

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1. Introduction

The snow crab (SC) fishery in Atlantic Canada represents the world's largest SC fishery; it accounts for almost 90% of world landings (by weight) in 2004, with over half of these landings coming from Newfoundland and Labrador. In 2007, the NL fishery alone landed 50,000 t, valued at \$177 million [1]. Unfortunately, SC meat can be one of the most important causes of severe acute hypersensitivity reactions, including

fatal anaphylaxis and severe asthma among the fishermen and processing plant workers [2]. Fish and shellfish are a leading cause of IgE-mediated food hypersensitivity [2–4]. IgE-mediated reactions that cause nausea, vomiting, abdominal pain, and diarrhea may be triggered within minutes of ingestion [5].

The molecular structure of tropomyosin, the major allergen in crustaceans [5–7], was recently characterized in snow crab and black tiger prawn using mass spectrometry [8,9]. Besides tropomyosin, other allergenic proteins from crustaceans have

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been reported. In 2003, Yu et al. [10] identified a novel shrimp allergen designated as Pen m 2 from (*Penaeus monodon*) by two-dimensional immunoblotting using sera from shrimp allergic patients. The allergen was identified using cDNA cloning. The open reading frame encoded 356 amino acids with a theoretical molecular weight of ~40 kDa. The amino acid sequence of this protein showed 60% similarity to arginine kinase of the crustacean, Kuruma prawn (*Penaeus japonicus*) [10].

Arginine kinase (AK) was recently reported as an allergen in different crustacean and invertebrate species. AK was identified in white shrimp (*Litopenaeus vannamei*) [11], gulf shrimp (*Penaeus aztecus*) [12], Chinese shrimp (*Fenneropenaeus chinensis*) [13], and other shrimp species using a proteomics approach [14]. Moreover, AK has been identified in other invertebrates such as the house dust mite (*Dermatophagoides farinae*) [15], Indian-meal moth (*Plodia interpunctella*) [16], silkworm larvae (*Bombyx mori*) [17], and American cockroach (*Periplaneta americana*) [18–20].

Monitoring airborne allergens in SC harvesting and processing workplaces is essential to reducing the worker's risk of developing allergenic airway diseases [21]. Normally, allergens are characterized and measured through immunological reactivity by enzyme-linked immunosorbent assay (ELISA) [22–24], radioallergosorbent test (RAST) [25], and immunoblotting [22–26]. These techniques evaluate the total protein concentration which includes non-allergenic proteins in addition to non-SC allergens [27].

Quantifying the major SC allergens as a way of correlating their amounts with the severity of the allergen exposure and in determining the threshold values requires a highly sensitive, specific, and reproducible technique. Isotopic dilution mass spectrometry has played a crucial role in protein quantification in the last two decades, provoking the use of this technique for the present study [27].

Snow crab tropomyosin was previously characterized and *de novo* sequenced [8]. Arginine kinase is also a protein of interest since it has been identified in other crustaceans but not snow crab [11–14].

In this study snow crab crude extract was profiled on SDS-PAGE and screened against patients' sera. Different allergenic proteins from this profile were characterized using tandem mass spectrometry. Previously, we identified and characterized the major allergen tropomyosin [8,9]. Arginine kinase, which is one of the most immunoactive protein was isolated and purified from the crude extract. The allergenicity of purified AK was examined by immunoblotting with allergenic patients' sera. The tryptic peptides were generated along with different types of derivatization reactions used in amino acid sequencing. The most abundant peptides were characterized using both ESI and MALDI ion sources, for protein identification and *de novo* sequencing using peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF), respectively. The mass spectra data were uploaded to the Mascot database search engine. The AK homology between snow crab and orange mud crab, which is the closest species, was studied. The active site motifs were evaluated using selected bioinformatics algorithms to confirm a signature pattern for AK. A unique and abundant tryptic peptide was selected and evaluated as a chemical surrogate for AK. Finally, this signature peptide and its deuterated isotopic homolog using

d_3 -L-alanine-were chemically synthesized and used in a preliminary study to develop a sensitive and specific quantification method for AK using multi-reaction monitoring (MRM) LC-MS/MS. The levels of the snow crab AK, for the first time, were monitored in actual air samples collected from a simulated processing plant. A tryptic-digested snow crab crude extract sample was used as a positive control.

2. Experimental

2.1. Chemicals and materials

All chemicals were used without further purification. Ammonium sulfate, acetonitrile, hydrochloric acid, and methanol were supplied by ACP (Montreal, Canada). Trypsin sequencing grade enzymes were purchased from Promega (WI, USA). Tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), formic acid (FA), ammonium bicarbonate, o-methylisourea hemisulfate, ammonium hydroxide, horseradish peroxidase (HRP), Chemiluminescent substrate, Sodium dodecylsulphate (SDS), ammonium formate and α -cyano-4-hydroxycinnamic acid (HCCA) matrix were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Bradford assay kit and PVDF immunoblot membrane were from BioRad (Hercules, CA, USA). The dialysis bags were purchased from Fischer Scientific (Roncho Dominguez, CA, USA). For desalting, the ZipTip C₁₈ filters were purchased from Millipore Corporation (Bedford, MA, USA). The skimmed milk powder was purchased from the local supermarket. Tris Buffered Saline (TBS) and Phosphate Buffered Saline (PBS) tablets were purchased from Amresco, USA. The photo-sensitive films were purchased from Amersham Bioscience, Germany. The developer and fixer were purchased from Kodak, USA. RapiGest SF surfactant from Waters Corporation (Milford, MA, USA), TopTip filters with Poly-Hydroxyethyl (HILIC) resin from PolyLC Inc. (Columbia, MD, USA), and 33-mm polytetrafluoroethylene (PTFE) filters for air sampling was bought from SKC Inc. (Eighty Four, PA, USA). The signature peptide, LVSAVNEIEK (purity > 98.33%; molar mass = 1101.27 Da) and its deuterated isotopic homolog using d_3 -L-alanine (purity > 96.80%; molar mass 1104.27 Da) were purchased from GeneMed Synthesis (San Francisco, CA, USA).

2.2. Arginine kinase purification and SDS profiling

Arginine kinase was purified from crab leg muscle extract by ammonium sulfate precipitation following a protocol developed by Garcia-Orozco et al. [11].

The total protein concentration of the crude extract and the AK isolate was determined using the Bradford assay. Since this crude extract does not contain many different proteins we utilized a 12% SDS-PAGE gel electrophoresis for protein profiling of the crude extract, the ammonium sulfate precipitated fractions, and the purified AK. A protein solution (10 μ g) was added to each of the wells, and electrophoresis was run at 170 V for 45 min, or until the tracker dye was seen at the base of the gel. One gel was treated with Coomassie Brilliant Blue R-250, using standard protocol. For the second gel, proteins were transferred to a PVDF membrane at 15 V for 20 min. After the transfer was completed, the membrane was placed in a

blocking solution (5% skimmed milk in Tris-buffered saline (TBS)) for immunoblotting.

2.3. Immunoblotting

To demonstrate the allergenicity of the isolated crab proteins, different bands were analyzed for IgE antibody binding from allergic patients with marked allergic symptoms to shellfish. Patients for this study were selected based on clinical reactivity to shellfish. Ethics approval for this study was acquired by Monash University as part of an ongoing survey. For immunoblotting, protein extracts were electrophoretically separated (see SDS gels above), proteins transferred and incubated with human serum (diluted 1:10 in 1% skimmed milk) overnight at 4 °C using a slot-blot apparatus (Idea Scientific, USA). Subsequently, blots were washed three times with PBS-T and the membrane incubated for 1 h in 5 mL of HRP labeled rabbit polyclonal antihuman IgE antibody (DAKO, USA) (diluted 1:2000) in PBS-T containing 1% skimmed milk. After washing the membrane with PBS-T three times the blots were incubated with the chemiluminescent substrate and analyzed for IgE reactivity using ECL technique [8,22].

2.4. In-gel digestion and guanidation

Six immunoreactive proteins, as identified by SDS-gel electrophoresis and subsequent immunoblotting with allergic patient serum, were excised from SDS-PAGE gels. An in-gel guanidation procedure was performed on these selected proteins for subsequent MALDI-QqToF-MS using the protocol developed by Sergeant et al. [28]. This procedure increases the sensitivity of the lysine-containing peptides by changing the lysine residue to the homoarginine. The gel pieces were destained by washing three times with 200 mM of $(\text{NH}_4)_2\text{CO}_3$ in solution of 50% acetonitrile in dH_2O , at 30 °C for 30 min. The destained piece was dried under a stream of N_2 , and then covered by a solution of 50 mM $(\text{NH}_4)_2\text{CO}_3$ pH 7.8, containing 5 ng/ μL trypsin in ice for 30 min for rehydration. After rehydration, the excess solution was sucked out. The gel was covered by a solution of 50 mM of $(\text{NH}_4)_2\text{CO}_3$ and incubated at 37 °C overnight to enhance protein digestion. The water soluble peptides were extracted twice with the incubation solution and other remaining peptides extracted twice with 0.15%TFA in 50% ACN after a 2 min vortex mixing. The samples were lyophilized, and reconstituted, prior to analysis with 10 μL of 0.1% TFA and desalted with C_{18} ZipTip.

2.5. MALDI-QqToF-MS

2.5.1. Matrix/sample preparation

The two-layer sample/matrix preparation for plate spotting was employed [20]. The first layer solution consisted 20 mg of HCCA in 1 mL (1/9) methanol/acetone. The second layer solution consisted 40% ACN of H_2O saturated by HCCA. 0.5 μL of the first layer matrix solution was applied to a MALDI target. 1 μL of the second layer matrix solution was mixed with 1 μL of sample. 1 μL of the sample/matrix mixture was deposited onto the first layer and allowed to dry, followed by an on-target wash step. By adding 1 μL of water on top of the dry spot and blowing the water off using

a pulse of air (after 10 s), a subsequent amount of salt was removed.

2.6. MALDI-QqToF-MS and CID-MS/MS

MALDI-MS and low energy collision (CID) analyses were carried out on a QSTAR XL hybrid quadrupole–quadrupole/time-of-flight tandem mass spectrometer (QqToF-MS/MS) (Applied Biosystems/MDS Sciex, Foster City, USA) equipped with an o-MALDI ion source (Applied Biosystems, Foster City, CA).

2.7. LC-ESI-QqToF-MS

Peptide separation was conducted using a DIONEX Ulti-Mate3000 Nano LC System (Germering, Germany). A 250 fmol sample of protein digest dissolved in 0.1% TFA was loaded onto a pre-column (300 μm ID $\times$ 5 mm, C_{18} PepMap 100, 5 μm (LC Packing, Sunnyvale, CA) for desalting and concentrating. Peptides were then eluted from the pre-column and separated on a nanoflow analytical column (75 μm ID $\times$ 15 cm, C_{18} PepMap 100, 3 μm , 100 Å, (LC Packing, Sunnyvale, CA) at 180 nL/min using the following gradient. The aqueous mobile phases consisted of (A) 0.1% formic acid/0.01% trifluoroacetic acid/2% ACN and (B) 0.08% formic acid/0.008% trifluoroacetic acid/98% ACN. A gradient of 0% B for 10 min, 0–60% B in 55 min, 60–90% in 3 min, and 90% B in 5 min was applied. Including a regeneration step one run was 106 min long.

The ESI-MS of the LC-eluting peptides were measured with the same hybrid QqToF-MS/MS system equipped with a nanoelectrospray source (Protana XYZ manipulator). The nanoelectrospray was generated from a PicoTip needle (10 μm i.d., New Objectives, Woburn, USA) at a voltage of 2400 V.

This protein was further analyzed by CID-MS/MS and the resulting peptides spectra were searched by using the National Center for Biotechnology Information non-redundant database (NCBIInr) with the Matrix Science (Mascot) search engine (precursor and product ion mass tolerance set at 0.2 Da. Methionine oxidation was allowed as a variable modification and guanidinylation (K) as a fixed modification since the guanidation derivatisation has been performed. Peptides were considered identified if the Mascot score was over 95% confidence limit.

2.8. Air sample collection, protein extraction and tryptic digestion

Airborne snow crab proteins were generated by processing crab in a simulated crab processing station and air samples were collected as described in a previous study that measured airborne crab tropomyosin [27]. The procedures for air sample collection, protein extraction from filters, tryptic digestion, and detergent removal prior to LC-MS/MS analysis were performed as described elsewhere [27].

2.9. Multi reaction monitoring LC-MS/MS quantification method

Signature peptide analysis was performed following the MRM LC-MS/MS protocol developed by Abdel Rahman et al. [27] with the exception of the mass spectrometric conditions: the

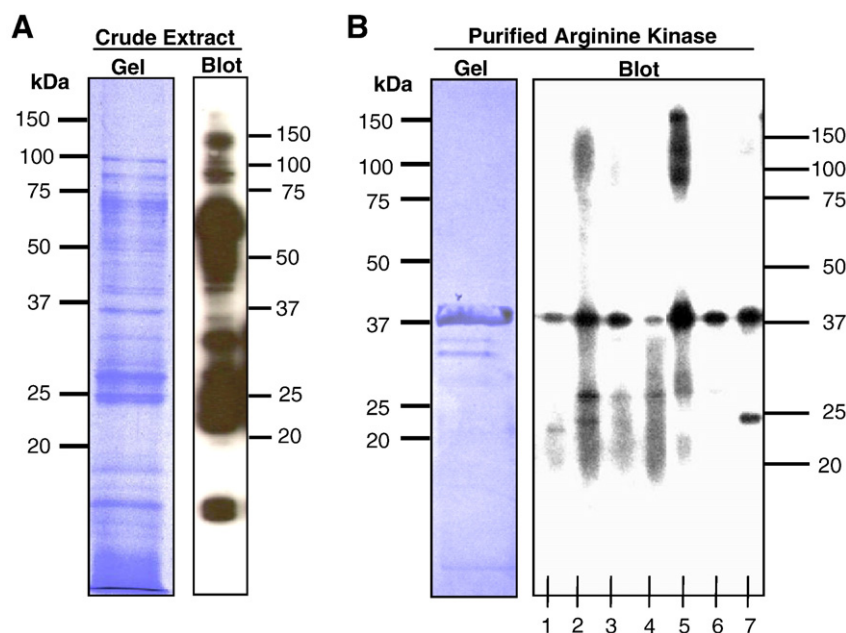


Fig. 1 – (A) Crude extract of snow crab muscle protein analyzed by SDS-PAGE Coomassie staining and characterized by immunoblotting using a pool of patients' IgE antibody. (B) Purified arginine kinase analyzed by SDS-PAGE Coomassie staining, and by immunoblot IgE antibody binding using serum from seven patients.

eluted peptides' droplets were desolvated at the ESI with gas flow rate of 400 L/h and a temperature of 250 °C. The ions were accelerated through the capillary and orifice cone at 3.02 kV

and 40 V, respectively. The precursor ions were fragmented at low energy CID using argon gas and the collision energy of 13 eV. The $[M+2H]^{2+}$ precursor ions of the unlabeled and

Table 1 – List of the allergenic muscle proteins from snow crab and the *de novo* sequences of their relevant peptides.

Protein name	Molecular mass (kDa)	Mascot score	Selected peptides
Sarcoplasmic Ca-binding	20	44	¹⁰⁸ VATVSLPR ¹¹⁵
Troponin	23	118	³⁸ KGFMTPER ⁴⁵ ¹⁶² AAEFNFR ¹⁶⁸
Tropomyosin	33	494	Ref. [8]
Arginine kinase	40	885	Table 2
α-Actine	42	891	²¹ AGFAGDDAPR ³⁰ ³¹ AVFPSIVGRPR ⁴¹ ⁵³ DAYVGDEAQSQR ⁶⁴ ⁶⁴ RGILTLK ⁷⁰ ⁹⁸ IAPEESPVLLTEAPLNPK ¹¹⁵ ¹⁵⁰ TTGIVLDTGDGVTHTVPIYEGYCLPHAILR ¹⁷⁹ ¹⁸⁰ LDLAGRDLTAYLT ¹⁹³ ¹⁹⁹ GYSFTTTAEREIVR ²¹² ²⁴¹ SYELPDGQVITIGNER ²⁵⁶ ²⁸⁷ CDIDIRK ²⁹³ ²⁹³ KDLFANNVLSGGTTMYPGIADR ³¹⁴ ³¹⁸ EITALAPPTIK ³²⁸ ³²⁹ IKIILAPPER ³³⁷ ³³¹ LIAPPERK ³³⁸ ³⁶² EEYDESGPGIVHR ³⁷⁴ ³⁶ YGNELPAEEGK ⁴⁷ ¹¹¹ NAESAIEALKEYEPEMGK ¹²⁸ ¹⁴⁴ EIVPGDLVEISVGDKIPADLR ¹⁶⁴ ¹⁷⁵ IDQSILTGESVSVIK ¹⁸⁹ ²⁰⁶ NILFSGTINVAAGK ¹⁸ ²³⁷ TQMAETEEIKTPLQK ²⁵² ⁴³⁷ VGEATETALIVLGEK ⁴⁵¹ ⁴⁸² EFTLEFSR ⁴⁸⁹ ⁶²¹ VIVITGDNK ⁶²⁹ ⁷¹³ KAEIGIAMSGTAVAK ⁷²⁸

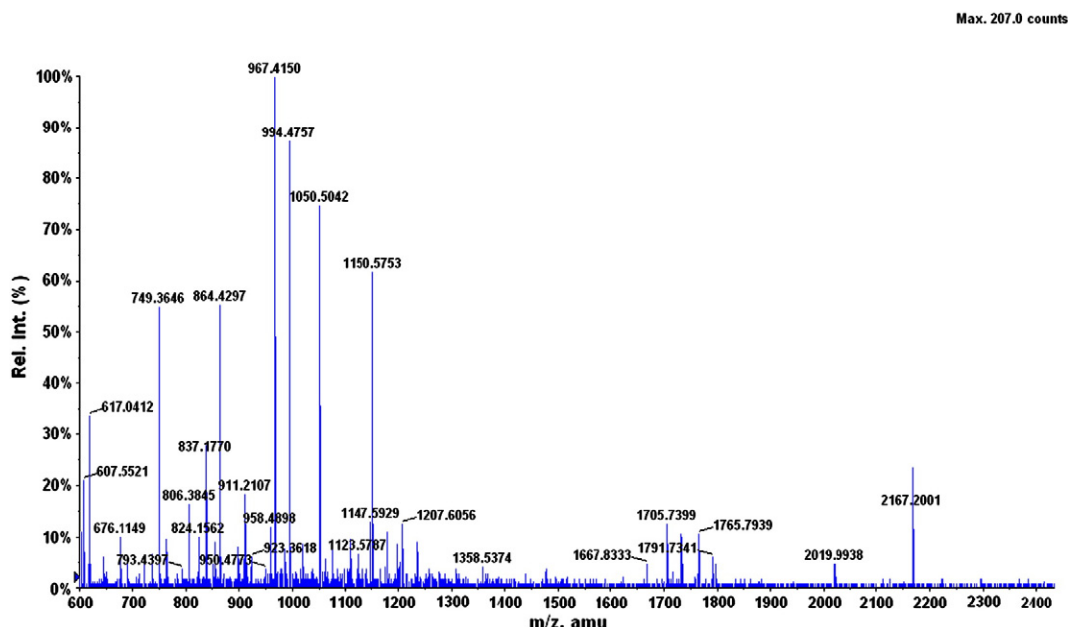


Fig. 2 – An ESI precursor spectrum from snow crab AK in-solution tryptic digestion. Spectrum was uploaded to PMF Mascot mode for protein characterization.

labeled forms of the signature peptide were 551.52 and 552.64 m/z , respectively. Data processing was performed with Mass Lynx 4.1 software.

3. Result and discussion

The crude extract of the SC proteins (from leg sections) was profiled by SDS-PAGE as shown in Fig. 1A. Proteins from numerous bands were excised and treated with tryptic digestion for further characterization by *bottom up* tandem mass spectrometry approach. Several protein bands within the range 20–113 kDa were analyzed and their relevant peptides *de novo* sequenced using PFF by tandem mass spectrometry. The precursor and product spectra of the peptides were uploaded and searched in Mascot search engine against the NCBI nr database. The proteins' identity and their relevant information are summarized in Table 1.

Remarkably, the number of peptides that has been used in protein identification varied with the natural abundance of each protein in the crude extract. On the other hand, the lack of specific genomic information in the databases for snow crab made the matching of the MS results very difficult since most of the identified proteins were obtained by comparison with the closest species available in the databases.

All the SC proteins identified in Table 1 are also immunoreactive as these proteins demonstrate serum IgE antibody reactivity by immunoblotting in our study. This current study identifies, for the first time, the proteins which were previously identified as being involved in allergic reactions to snow crab, ranging from 20 to 113 kDa [7]. One of these allergens was recently sequenced by our group as the 33 kDa tropomyosin novel snow crab allergen [8]. Subsequently, arginine kinase (AK) (40-kDa) was isolated in the present study from frozen non-processed SC meat and purified using

ammonium sulphate precipitation. The purification steps were monitored by SDS-PAGE (results are not shown) and the final purified AK shown by its SDS band in Fig. 1B. Further, the allergenicity of the AK extract was evaluated by sixteen different patients' sera with allergic symptoms. Seven of these patients' IgE have strong reactivity with AK (43%). The IgE binding profile for these seven patients is demonstrated by Fig. 1B. Serum of a non-allergic patient did not show any IgE reactivity (data not shown). Interestingly, the 43% of the patients' sera reacted with snow crab AK, confirming that AK is one of the important crab allergens along with tropomyosin. The AK band was excised and its identity confirmed by PMF mass spectrometry. The precursor ion spectra (Fig. 2) of the generated peptides were uploaded to the Mascot database search engine. The probability based Mowse score was 80 for arginine kinase, where the data of any protein scored >75 is

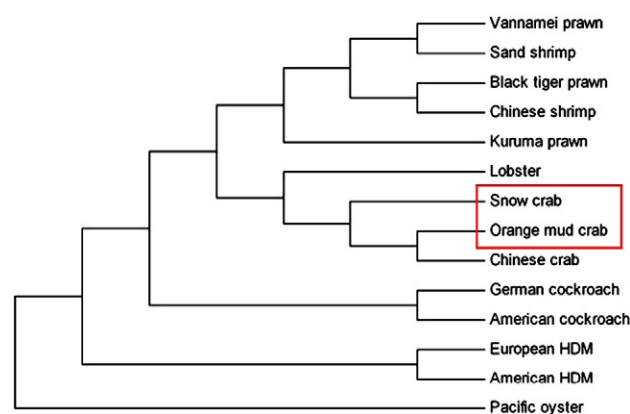


Fig. 3 – Phylogenetic tree based on the amino acid sequences of arginine kinase from snow crab (this study) in comparison with other invertebrate and vertebrates.

highly significant ($P < 0.05$). This search was matched with AK from orange mud crab (*Scylla olivacea*), marbled crab (*Pachygrapsus marmoratus*), and green crab (*Carcinus maenas*). A comparison was performed among the different crustaceans using multisequence alignment freeware. A phylogenetic tree

based on AK's amino acid sequence was constructed by the neighbour joining method. A cladogram was described based on the maximum probability of distances. Orange mud crab was found to have the closest similarity in evolution to snow crab as shown in Fig. 3.

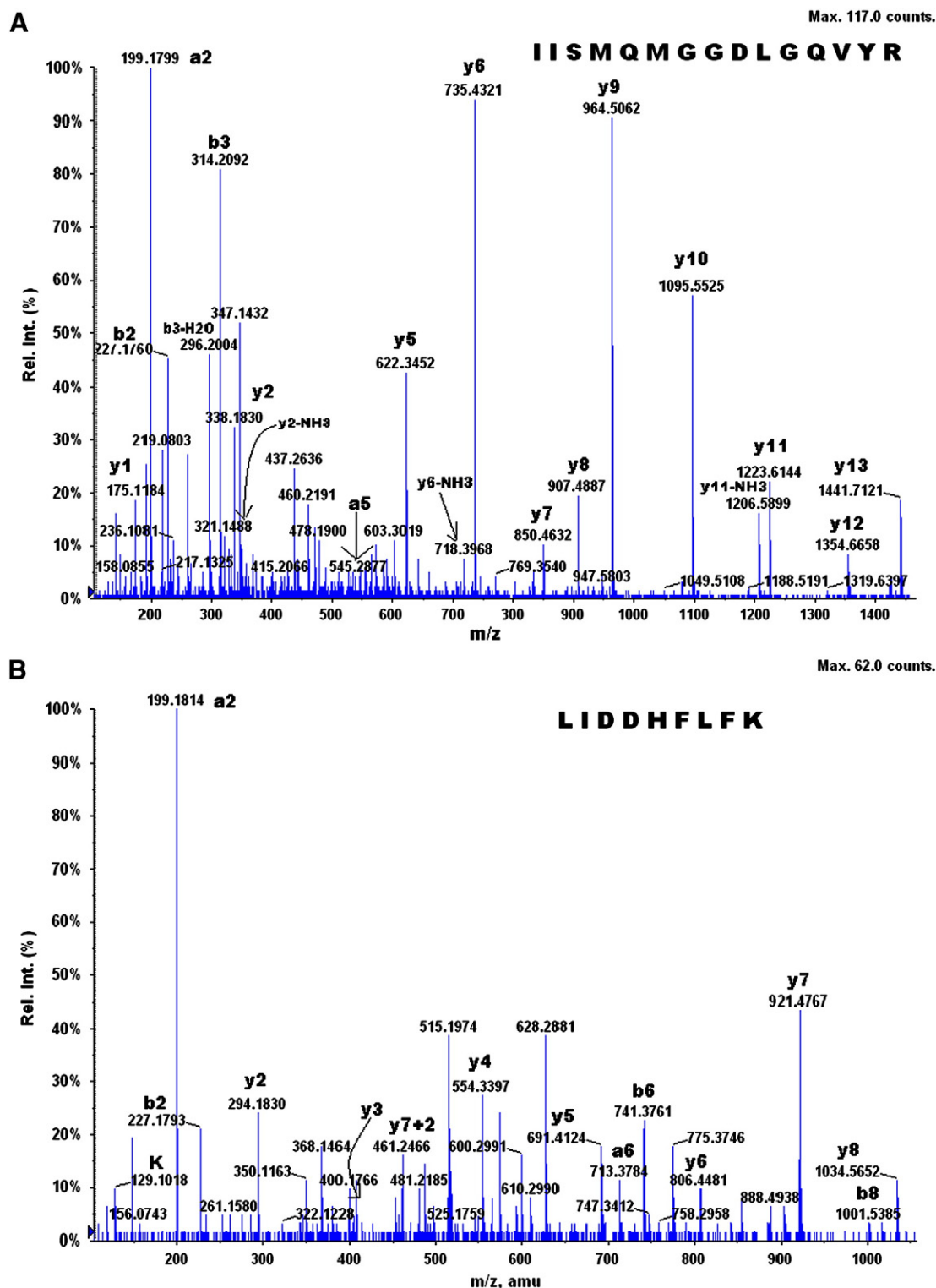


Fig. 4 – Representative ESI product ion spectra of arginine kinase tryptic peptides. The selected precursor ions are (A) $[M + 2H]^+ = m/z$ 834.4 and (B) $[M + 2H]^+ = m/z$ 574.3. The amino acid sequence is shown for each spectrum.

Several of the snow crab AK tryptic peptides produced by both in-solution and in-gel digestions were *de novo* sequenced using bottom up tandem MS approach. MALDI and ESI ion sources were used in order to obtain a maximum number of product ions. The peptide's amino acid sequence was interpreted manually and later confirmed using bioinformatics tools. The MALDI ion source is known to ionize preferentially the arginine-containing peptides. To increase the sensitivity of the lysine-containing peptides, guanidation of lysine was performed whereby lysine residues were converted to homo-arginine. This amino acid has the same chemical behaviour and proton affinity as arginine in a MALDI source (results are not shown). For minimizing ion suppression effect and increasing the sensitivity of mass spectrometry, the tryptic peptides were also separated based on their hydrophobicity using nano-HPLC prior to ionization by an ESI source.

The peptide ions from both the ESI (Fig. 2) and MALDI precursor ion spectra, (i.e. $[M+H]^+ m/z$ 923.3618, 850.4348,

1020.5053, 864.4297, etc.) were exposed to low energy collision induced dissociation (CID) for peptide *de novo* sequencing using a quadrupole time-of-flight (QqToF) tandem mass spectrometer. Data interpretation was performed manually and the peptide sequence confirmed by uploading them to the Mascot MS/MS mode. Two representative product ion spectra are shown in Fig. 4. The Mascot software was tuned to search against non-redundant proteins NCBI nr database. Since the orange mud crab is the closest species to the SC, most of the SC AK's tryptic peptides matched with the orange mud crab's AK thus meeting the Mascot criteria for identity and extensive homology with an average ~1185 as orange mud crab AK (*S. olivacea*).

The amino acid sequence of snow crab AK (this study) that matched totally or partially with orange mud crab AK is summarized in Table 2. The protein sequence data reported in this paper will appear in the UniProt knowledgebase under the accession number P86699 for AK (*Chionoecetes opilio*). Using

Table 2 – De novo sequencing of the product ion spectra obtained from ESI-QqToF and MALDI-QqToF experiments of the major tryptic peptides from snow crab AK.

Start-end	Mass			Charge (z)	Peptide <i>de novo</i> sequence	Ion source
	Observed	Expected	Calculated			
10–16	425.746	849.4791	849.4596	+2	LEEGFKK	ESI
181–189	574.3178	1146.6210	1146.6073	+2	LIDDHFLFK	
181–193	535.6201	1603.8384	1603.7995	+3	LIDDHFLFKEGDR	
194–202	525.7632	1049.5119	1049.5076	+2	FLQAANACR	
230–244	834.4453	1666.8761	1666.8171	+2	IISMQMGGDLGQVYR	
230–245	608.6669	1822.9788	1822.9182	+3	IISMQMGGDLGQVYRR	
246–255	551.3182	1100.6219	1100.6077	+2	LVS AVNEIEK	
246–256	629.3746	1256.7346	1256.7088	+2	LVS AVNEIEKR	
256–264	575.8096	1149.6047	1149.5792	+2	RVPSHHDR	
257–264	497.7533	993.4921	993.4781	+2	VPFSHHDR	
265–280	898.9819	1795.9492	1795.9291	+2	LGFLTFPCPTNLGTTVR	
281–289	497.7584	993.5022	993.4781	+2	ASVHIKLPK	
295–303	501.7672	1001.5198	1001.5393	+2	EKLEEVAGK	
297–309	746.4394	1490.8642	1490.8093	+2	LEE VAGKYS LQVR	
310–328	673.9952	2018.9639	2018.9294	+3	GTRGEHTEAEGGVYDISNK	
313–328	569.2693	1704.7859	1704.7591	+3	GEHTEAEGGVYDISNK	
313–329	621.3206	1860.9400	1860.8602	+3	GEHTEAEGGVYDISNKR	
330–340	640.3662	1278.7179	1278.6754	+2	RMGLTEFQAVK	
331–340	562.3232	1122.6318	1122.5743	+2	MGLTEFQAVK	
341–351	644.8727	1287.7308	1287.6744	+2	EMQDGILELIK	
341 – 354	829.9928	1657.9711	1657.8960	+2	EMQDGILELIKIEK	
1–9	923.3618	922.3545	922.4430	+1	M [*] ADAATISK	MALDI
10–16	850.4348	849.4275	849.4596	+1	LEEGFKK	
16–24	1020.5053	1019.4980	1019.5070	+1	KLQGATDCK	
34–40	864.4297	863.4224	863.4389	+1	DVFDQLK	
95–102	967.4150	966.4077	966.4883	+1	QTDKHPNK	
103–118	1765.7939	1764.7866	1764.7955	+1	DFGDVNQFVNVDPDGK	
119–124	722.4092	721.4019	721.4123	+1	FVISTR	
119–126	977.4974	976.4901	976.5818	+1	FVISTRVR	
181–189	1147.5929	1146.5856	1146.6073	+1	LIDDHFLFK	
194–202	1050.5042	1049.4969	1049.5076	+1	FLQAANACR	
203–216	1706.7247	1705.7174	1705.7961	+1	YWPSGRGIYHNDNK	
230–244	1667.7269	1666.7196	1666.8171	+1	IISMQMGGDLGQVYR	
246–256	1257.6976	1256.6903	1256.7088	+1	LVS AVNEIEKR	
256–264	1150.5753	1149.5680	1149.5792	+1	RVPSHHDR	
257–264	994.4757	993.4684	993.4781	+1	VPFSHHDR	
265–280	1796.9241	1795.9168	1795.9291	+1	LGFLTFPCPTNLGTTVR	
304–309	765.4236	764.4163	764.4181	+1	YSLQVR	
331–340	1123.5787	1122.5714	1122.5743	+1	MGLTEFQAVK	
352–357	793.4397	792.4324	792.3687	+1	IEKEMQ	

SCAK:	1	MADAATISKLEEGFKKLQGATDCKSLKKYLTKDVFQKAKKTSLGATLLDVIQSGVEN	60
OMCAK:	1	MADAATI+KLEEGFKKL+ ATDCKSLKKYLTK VFDQK KKTSLGATLLDVIQSGVEN	60
SCAK:	61	LDSGVGVYAPDAEAYTLFAPLFDPIIEDYHKGFKQTDKHPNKDFGDNQFVNVDPDGKFV	120
OMCAK:	61	LDSGVGVYAPDAEAYTLFAPLFDPIIEDYHKGFKQTDKHPNKDFGDNQFVNVDPDGKFV	120
SCAK:	121	ISTRVRCGRSMEGYPFNPCLTEAQYKEMEAKVSSTLKGLEGLKGSFYPLTGMAKDQQK	180
OMCAK:	121	ISTRVRCGRSMEGYPFNPCLTEAQYKEME+KVSSTL LEGELKG++YPLTGM KDQQK	180
SCAK:	181	LIDHFLFKEGDRFLQAANACRYWPSGRGIFHNDKKTFLVWCNEEDHLRIISMOMGGDLG	240
OMCAK:	181	LIDHFLFKEGDRFLQAANACRYWP+GRGI+HND KTFVWCNEEDHLRIISMOMGGDLG	240
SCAK:	241	QVYRRLVSAVNEIEKRVFFSHHDLRGFLTFPCPTNLGTTVRASVHIKLPKLAANREKLEEV	300
OMCAK:	241	QVYRRLVSAVNEIEKRVFFSHHDLRGFLTFPCPTNLGTTVRASVHIKLPKLAANREKLEEV	300
SCAK:	301	AGKYSLQVRGTRGEHTEAEGGVYDISNKRMRGLTEFQAVKEMQDGILELIKIEKEMQ	357
OMCAK:	301	AGKYSLQVRGTRGEHTEAEGGVYDISNKRMRGLTEFQAVKEMQDGILELIKIEKEMQ	357

Fig. 5 – Amino acid alignment between snow crab AK (SCAK) and organic mud crab AK (OMCAK) using the SIB BLAST Network Service. The match identity is 96% and the positive value is 98%.

BLAST 2.0 (http://www.ch.embnet.org/cgi-bin/blast20_parser.pl) the alignment scores for the two AK sequenced proteins was 701 bits (1810) with 96% identity (Fig. 5).

Interestingly, the N-terminal peptide ($^1\text{MADAATISK}^9$) identified in MALDI experiments, indicates that the methionine residue is not acetylated, a structural feature which is not

common in eukaryotic proteins [8,9]. The kinase active site of the snow crab AK was evaluated using the Prosit bioinformatics tool. Sequence hits were with Guanido-kinase model (PS00112) as ATP guanido phosphotransferases, matching the AK sequence at site ($^{271}\text{CPTNLGT}^{277}$). The ATP guanido phosphotransferases active site model has a consensus

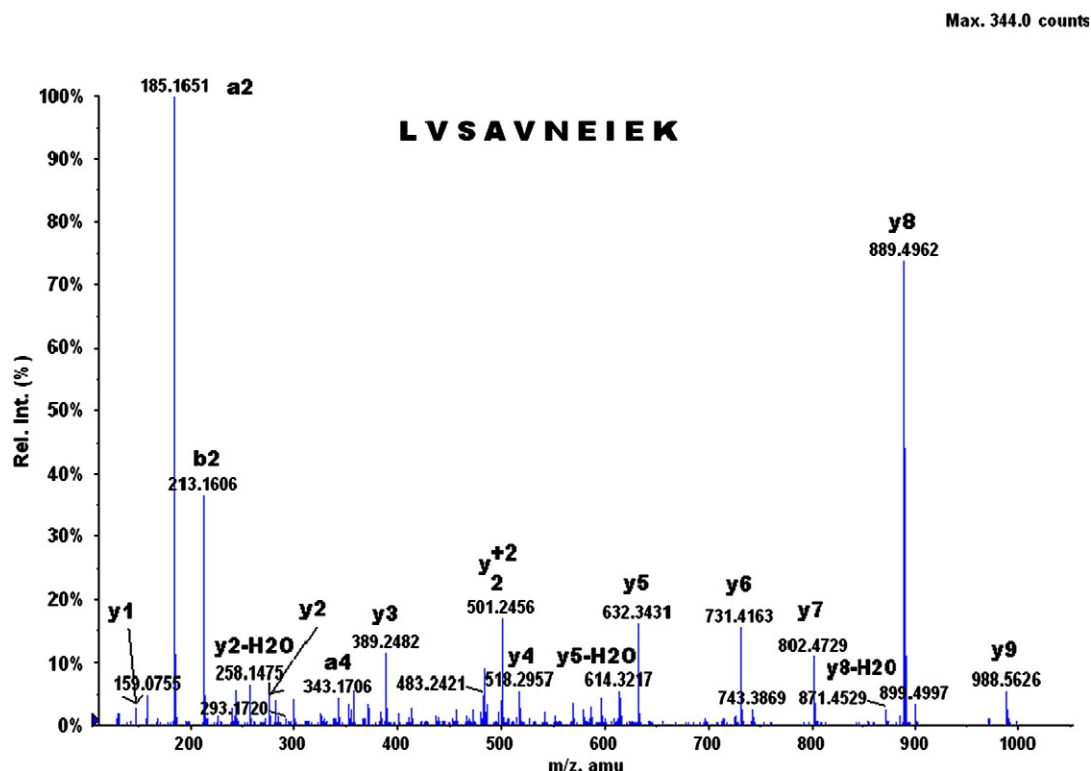


Fig. 6 – ESI product spectrum for the snow crab AK signature peptide to be selected for quantification purposes of snow crab AK.

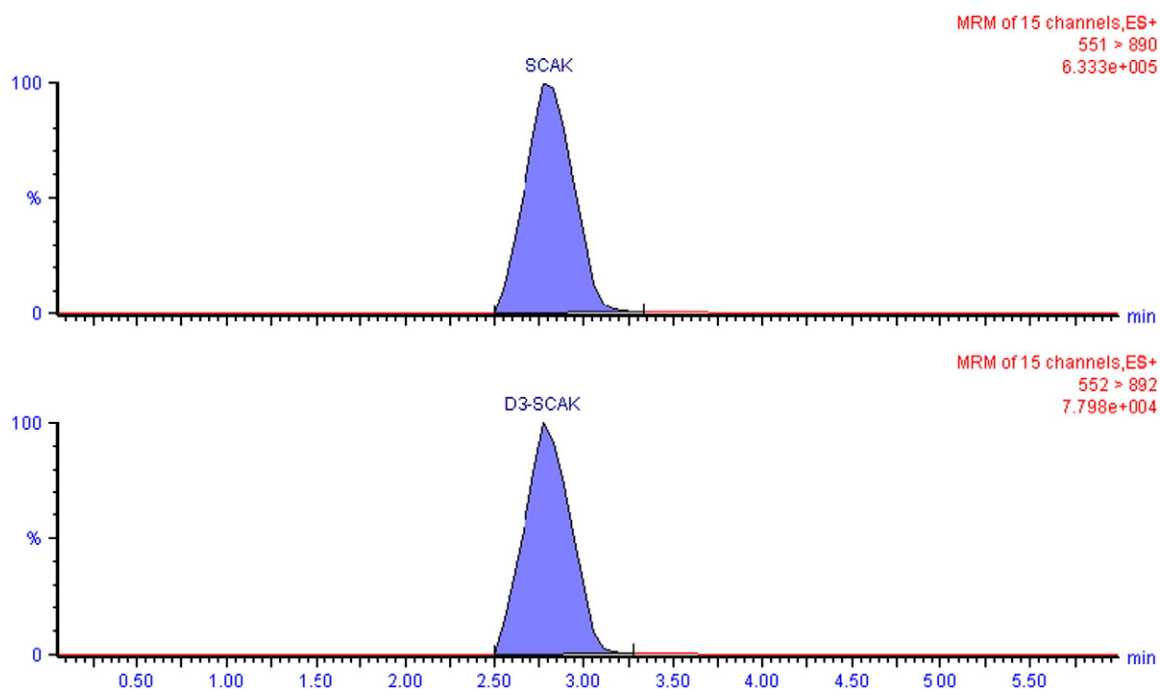


Fig. 7 – An MRM LC-MS/MS chromatogram for the signature peptide (SCAK) and its isotopic analog (d₃-SCAK) present in the digested sample of SC crude extract.

pattern of (C-P-X (0, 1)-[ST]-N-[ILV]-G-T), and represents a family of structurally and functionally related enzymes that reversibly catalyze the transfer of phosphate between ATP and various phosphogens (e.g. creatine, glycoamine, arginine kinase, etc). The cysteine residue in the site is implicated in the catalytic activity of these enzymes, and the amino acid region near this active site residue is highly conserved and could be used as signature pattern [29].

Developing an analytical method for quantifying snow crab AK by isotopic dilution mass spectrometry in different matrices requires assigning a signature peptide as a stoichiometric surrogate for the target protein. Ideally the signature peptide should be unique only for the target protein, shorter than 15 amino acids, and free of chemically reactive residues such as methionine and cysteine [8,27,30]. Following this criteria several candidate peptides as listed in Table 2, were examined and evaluated. The signature peptide of AK was determined to be ²⁴⁶LVSANVEIEK²⁵⁵. The ESI product spectrum for the candidate signature peptide is given in Fig. 6. The BLAST test shows that this peptide is not only specific for AK from snow crab, but also can be utilized as a universal signature peptide for other crustaceans' AK.

The AK signature peptide was chemically synthesized in light and heavy forms as analytical standards, which were subsequently used to develop a quantification method similar to a previous study [27]. The MRM transitions for both the signature peptide and its deuterated form were tuned to be 551>890 and 552>892, respectively. A chromatogram for the digested SC crude extract is shown in Fig. 7, which is routinely used as a positive control for actual air samples analyses. Air samples were collected from different sites of a simulated processing plant, where the proteins were collected, extracted from filter papers, and tryptic digested for quantification purposes.

The levels of the SCAK (as its surrogate peptide) are summarized in Table 3. Noteworthy is the fact that arginine kinase, for the first time, is being molecularly identified as one of the aerosolized allergens in seafood processing environment. The levels of SCAK are shown to vary among sampling locations. For example, high levels of SCAK were collected on the personal breathing zone filter during butchering, while the lowest was from a filter sample 3 m away from butchering. These experiments demonstrate the validity of the use of a signature peptide to surrogate chemically the presence of AK in crude extract and in air sample. This will be useful in

Table 3 – Levels of snow crab AK in different work stations inside a simulated crab processing plant.

Sample name	Conc. as solution (nM)	Amount per filter (pmol)	Volume of air (m ³)	μg/m ³
Butchering (PBZ)	10.48	1.0484	0.045	0.93
Butchering (Area 1)	26.42	2.6419	0.175	0.60
Butchering (Area 2)	12.32	1.2324	0.190	0.26
Cooking (PBZ)	22.67	2.2675	0.150	0.60
Cooking (Area 1)	21.09	2.1095	0.130	0.65
Cooking (Area 2)	21.52	2.1515	0.175	0.49
Blank (Area 2)	<LOQ	n/a	0.200	n/a

(PBZ=personal breathing zone; Area 1=1 m from activity; Area 2=5 m from activity).

developing a very selective and sensitive quantification method for airborne proteins in the future.

4. Conclusion

Arginine kinase has recently been reported as a major seafood allergen in prawns. The present study evaluates, for the first time, the allergenicity of the snow crab AK, by examining its immunoreactivity with the sera of seven allergic patients. The primary structure of the AK was characterized through amino acid sequencing using *bottom up* tandem mass spectrometry. The amino acid sequence was registered in the UniProt knowledge-base. The active site of AK was determined with a consensus pattern (C-P-X (0, 1)-[ST]-N-[ILV]-G-T). The signature peptide of AK was determined and utilized as a universal signature peptide among the crustaceans' AK. An MRM tandem mass spectrometry quantification method was developed using the designated signature peptide to measure, for the first time, the levels of the aerosolized form of SCAK inside crab processing workplaces. Finally, several other allergenic proteins present in the SC crude extract were identified using the same MS strategy as that followed in the snow crab AK.

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REFERENCES

- [1] Mullowney R, Dawe E. Development of performance indices for the Newfoundland and Labrador snow crab (*Chionoecetes opilio*) fishery using data from a vessel monitoring system. *Fish Res* 2009;100:248–54.
- [2] Lopata AL, O'Hehir RE, Lehrer SB. Shellfish allergy. *Clin Exp Allergy* 2010;40:850–8.
- [3] Lopata AL, Lehrer SB. New insights into seafood allergy. *Curr Opin Allergy Clin Immunol* 2009;9:270–7.
- [4] Jeebhay MF, Robins TG, Lehrer SB, Lopata AL. Occupational seafood allergy: a review. *Occup Environ Med* 2001;58:553–62.
- [5] Chu KH, Tang CY, Wu A, Leung PS. Seafood allergy: lessons from clinical symptoms, immunological mechanisms and molecular biology. *Adv Biochem Eng Biotechnol* 2005;97: 205–35.
- [6] Motoyama K, Suma Y, Ishizaki S, Nagashima Y, Shiomi K. Molecular cloning of tropomyosins identified as allergens in six species of crustaceans. *J Agric Food Chem* 2007;55:985–91.
- [7] Gill BV, Rice TR, Cartier A, Gautrin D, Neis B, Horth-Susin L, Jong M, Swanson M, Lehrer SB. Identification of crab proteins that elicit IgE reactivity in snow crab-processing workers. *J Allergy Clin Immunol* 2009;124:1055–61.
- [8] Abdel Rahman AM, Lopata AL, O'Hehir RE, Robinson JJ, Banoub JH, Helleur RJ. Characterization and *de novo* sequencing of snow crab tropomyosin enzymatic peptides by both electrospray ionization and matrix-assisted laser desorption ionization QqToF tandem mass spectrometry. *J Mass Spectrom* 2010;45:372–81.
- [9] Abdel Rahman AM, Kamath SD, Lopata AL, Helleur RJ. Analysis of the allergenic proteins in black tiger prawn (*Penaeus monodon*) and characterization of the major allergen tropomyosin using mass spectrometry. *Rapid Commun Mass Spectrom* 2010;24:2462–70.
- [10] Yu CJ, Lin YF, Chiang BL, Chow LP. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. *J Immunol* 2003;170:445–53.
- [11] García-Orozco KD, Aispuro-Hernández 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;144:23–8.
- [12] France RM, Sellers DS, Grossman SH. Purification, characterization, and hydrodynamic properties of arginine kinase from gulf shrimp (*Penaeus aztecus*). *Arch Biochem Biophys* 1997;345:73–8.
- [13] Yao CL, Wu CG, Xiang JH, Dong B. Molecular cloning and response to laminarin stimulation of arginine kinase in haemolymph in Chinese shrimp, *Fenneropenaeus chinensis*. *Fish Shellfish Immunol* 2005;19:317–29.
- [14] Ortea I, Cañas B, Gallardo JM. Mass spectrometry characterization of species-specific peptides from arginine kinase for the identification of commercially relevant shrimp species. *J Proteome Res* 2009;8:5356–62.
- [15] Bi XZ, Chew FT. Molecular, proteomic and immunological characterization of isoforms of arginine kinase, a cross-reactive invertebrate pan-allergen, from the house dust mite, *Dermatophagoides farinae*. *J Allergy Clin Immunol* 2004: S226.
- [16] Binder M, Mahler V, Hayek B, Sperr WR, Schöller M, Prozell S, Wiedermann G, Valent P, Valenta R, Duchêne M. Molecular and immunological characterization of arginine kinase from the indian meal moth, *Plodia interpunctella*, a novel cross-reactive invertebrate Pan-allergen. *J Immunol* 2001;167: 5470–7.
- [17] Liu Z, Xia L, Wu Y, Xia Q, Chen J, Roux KH. Identification and characterization of an arginine kinase as a major allergen from Silkworm (*Bombyx mori*) larvae. *Int Arch Allergy Immunol* 2009;150:8–14.
- [18] Brown AE, France RM, Grossman SH. Purification and characterization of arginine kinase from the American cockroach (*Periplaneta americana*). *Arch Insect Biochem Physiol* 2004;56:51–60.
- [19] Sookrung N, Chaicumpa W, Tungtrongchitr A, Vichyanond P, Bunnag C, Ramasoota P, Tongtawe P, Sakolvaree Y, Tapchaisri P. *Periplaneta americana* arginine kinase as a major cockroach allergen among Thai patients with major cockroach allergies. *Environ Health Perspect* 2006;114:875–80.
- [20] Tungtrongchitr A, Sookrung N, Indrawattana N, Sae-Lim J, Punduang S, Phonrat B, Wanachiwanawin D, Chaicumpa W. Seasonal levels of the major American cockroach allergen Per a 9 (arginine kinase) in Bangkok and their relevance for disease severity. *Asian Pac J Allergy Immunol* 2009;27:1–7.
- [21] Jeebhay MF, Cartier A. Seafood workers and respiratory disease: an update. *Curr Opin Allergy Clin Immunol* 2010;10: 104–13.
- [22] Lopata AL, Jeebhay MF, Reese G, Fernandes J, Fenimore B, Elliott A, Robins TG, Lehrer SB. Detection of fish antigens aerosolized during fish processing using newly developed immunoassays. *Int Arch Allergy* 2005;138:21–8.

- [23] Malo JL, Chretien P, Mccants M, Lehrer SB. Detection of snow-crab antigens by air sampling of a snow-crab production plant. *Clin Exp Allergy* 1997;27:75–8.
- [24] Kluwe L, Maeda K, Miegel A, Fujita-Becker S, Maeda Y, Talbo G, Houthaeve T, Kellner R. Rabbit skeletal muscle $\alpha\alpha$ -tropomyosin expressed in baculovirus-infected insect cells possesses the authentic N-terminus structure and functions. *J Muscle Res Cell Motil* 1995;16:103–10.
- [25] Reese G, Schick Tanz S, Lauer I, Randow S, Luttkopf D, Vogel L, Lehrer SB, Vieths S. Structural, immunological and functional properties of natural recombinant Pen a 1, the major allergen of brown shrimp, *Penaeus aztecus*. *Clin Exp Allergy* 2006;36: 517–24.
- [26] Rosmilah M, Shahnaz M, Masita A, Noormalin A, Jamaludin M. Identification of major allergens of two species of local snappers: *Lutjanus argentimaculatus* (merah/red snapper) and *Lutjanus johnii* (jenahak/golden snapper). *Trop Biomed* 2005;22:171–7.
- [27] Abdel Rahman AM, Lopata AL, Randell EW, Helleur RJ. Absolute quantification method and validation of airborne snow crab allergen tropomyosin using tandem mass spectrometry. *Anal Chem Acta* 2010;681:49–55. (Accepted, *Analytica Chimica Acta*, DOI: 10.1016/j.aca.2010.09.040).
- [28] Sergeant K, Samyn B, Debyser G, Beeumen JV. ‘De novo’ sequence analysis of N-terminal sulfonated peptides after in gel guanidination. *Proteomics* 2005;5:2369–80.
- [29] Stein D, Harn A, David R. A cloned ATP: guanidino kinase in the trematode *Schistosoma mansoni* has a novel duplicated structure. *J Biol Chem* 1990;265:6582–8.
- [30] Brun V, Masselon C, Garin J, Dupuis A. Isotope dilution strategies for absolute quantitative proteomics. *J Proteomics* 2009;72:740–9.