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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.

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 rest (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), dithiotheritol (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-hydroxycinamic 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 C_{18} 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 μg) 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