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Absolute quantification method and validation of airborne snow crab allergen tropomyosin using tandem mass spectrometry

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ABSTRACT

Measuring the levels of the major airborne allergens of snow crab in the workplace is very important in studying the prevalence of crab asthma in workers. Previously, snow crab tropomyosin (SCTM) was identified as the major aeroallergen in crab plants and a unique signature peptide was identified for this protein. The present study advances our knowledge on aeroallergens by developing a method of quantification of airborne SCTM by using isotope dilution mass spectrometry. Liquid chromatography tandem mass spectrometry was developed for separation and analysis of the signature peptides. The tryptic digestion conditions were optimized to accomplish complete digestion. The validity of the method was studied using international conference on harmonization protocol, Where 2–9% for CV (precision) and 101–110% for accuracy, at three different levels of quality control. Recovery of the spiked protein from PTFE and TopTip filters was measured to be 99% and 96%, respectively. To further demonstrate the applicability and the validity of the method for real samples, 45 kg of whole snow crab were processed in an enclosed (simulated) crab processing line and air samples were collected. The levels of SCTM ranged between 0.36–3.92 $\mu g\,m^{-3}$ and 1.70–2.31 $\mu g\,m^{-3}$ for butchering and cooking stations, respectively.

1. Introduction

Seafood plays an important role in nutrition worldwide, sustained by increasing international trade of a variety of new seafood products [1,2]. During seafood harvesting and processing, certain protein allergens are aerosolized into the working environment of vessels and processing plants. Allergic reactions to seafood are generated by otherwise harmless proteins which are, to some individuals, recognized as allergens. The main aeroallergens causing the shellfish allergy are believed to be tropomyosin [3–5] and possible sarcoplasmic calcium-binding protein [6], myosin light chain [7], and arginine kinase [8–9], which all can become aerosolized by seafood handling and processing. Previous studies have reported the prevalence of occupational seafood processing asthma to range from 7% to 36%, and occupational protein contact dermatitis from 3% to 11% [2]. Many studies have used immunological reactivity techniques to detect TM such as enzyme-linked immunosorbent assay (ELISA) [10], radioallergosorbent test (RAST) [11], and immunoblotting [12]. These techniques measure the levels of allergens, indirectly, by measuring the level of bound immunoglobulin's (IgE) to a standard antigen using radiolabels or colorimetric substrates. As a consequence, the sensitivity, specificity, and selectivity of these indirect techniques are limited in evaluating the levels of proteins recognized by these antibodies in air samples. These techniques therefore recognize various airborne proteins, maybe due to the cross-reactivity to various proteins are not only restricted to allergens and could originate from other sources besides the food processing (e.g. dust mites, microbes, etc.) [5].

Tropomyosin (TM) is a common muscle protein and mediates the interaction between the troponin complex and actin to regulate muscle contraction [10]. Crustacean TM was first identified in shrimp by Hoffman et al. in 1981 [13]. It is a water-soluble and heat-stable protein with molar masses ranging between 34 and 39 kDa [14]. It has also been identified as one of the major airborne allergens present in crab processing plants [15]. Developing a sensitive quantification method specifically for airborne snow crab tropomyosin (SCTM) will be very useful in detecting and control-

Abbreviations: CID, collision induced dissociation; ELISA, enzyme-linked immunosorbent assay; HILIC, hydrophilic interaction chromatography; iCAT, isotope-coded affinity tags; ICH, International Conference on Harmonization; IgE, immunoglobulin E; iTRAQ, isobaric tag for relative and absolute quantitation; LC–MS/MS, liquid chromatography tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; PTFE, polytetrafluoroethylene; RAST, radioallergosorbent test; SCTM, snow crab tropomyosin; SDS, sodium dodecyl sulfate; SILAC, stable isotope labeling by amino acids in cell culture; TSP, tropomyosin signature peptide.

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ling the worker's risk of developing allergic airway disease in the processing and harvesting workplaces [1,4,16].

The difficulty of obtaining highly pure protein standard, such as removal of salts and surfactants, makes direct protein quantification an analytical challenge [17]. The limited resolution of the sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) and poor baseline separation of individual proteins by liquid chromatography are other analytical issues in protein quantification. Traditionally the total protein concentration is measured by spectrophotometry methods such as the Lawry and Bradford techniques. These methods, however, are not protein specific, will measure contaminating proteins and have limited sensitivity. Various approaches have been developed in the last two decades for protein quantification using chromatographic methodologies coupled with tandem mass spectrometry. The combined techniques are sensitive, accurate, and reproducible which awards them an unrivaled technique for protein primary structure characterization and quantification [18]. Stable isotope labeling based approaches such as SILAC, iCAT, and iTRAQ, have been successfully developed for studies of the whole proteome [17–21]. The isotopic labeling dilution concept has been implemented for quantification of proteins in biological samples. This approach takes advantage of the identical chromatographic behavior of an isotope-labeled standard analog to the native peptide, which can be differentiated by their mass-to-charge ratio in mass spectrometry [21]. Absolute quantification by isotopic dilution mass spectrometry first involves determining a signature peptide as a stoichiometric surrogate for the target protein. Ideally the signature peptide should be unique for the target protein, shorter than 15 amino acids, and free of chemically reactive residues such as methionine and cysteine [4,18]. Analytical standards of the signature peptide and its isotopic labeled analogue are then used for developing a quantification method using liquid chromatography tandem mass spectrometry (LC–MS/MS) and multi reaction monitoring (MRM) transitions.

This study describes a method to quantify airborne SCTM, which is recognized as a major snow crab allergen [15,22]. This allergen has recently been characterized and amino acid *de novo* sequenced by mass spectrometry [4]. Abdel Rahman et al. [4] reported the amino acid sequence of a signature tryptic peptide for SCTM which stoichiometrically surrogates this protein. For this study, the signature peptide was chemically synthesized in unlabeled and labeled forms as standard and internal standard, respectively. A quantification method has been developed using LC–MS/MS (triple quadruple mass analyzer). A comprehensive validation study was performed to determine the method's linearity, sensitivity, selectivity, and reproducibility. The linearity and the completeness of the tryptic digestion along with air filter recovery of SCTM were also determined. Finally, real air samples were collected from different processing stages of a simulated snow crab plant and assayed for SCTM using this developed method.

2. Materials and methods

2.1. Chemical and reagent

All chemicals were used without further purification. Standard signature peptide, SQLVENELDHAQEQLSAATHK (purity > 95.3%; molar mass = 2348.53 Da) and its deuterated isotopic homolog using d_3-L-alanine- (purity > 97.1%; molar mass = 2357.53 Da) were purchased from GeneMed Synthesis (San Francisco, CA, USA). Acetonitrile and formic acid were purchased from ACP (Montreal, Canada). Sodium dodecylsulfate (SDS), trypsin, ammonium bicarbonate, and ammonium formate were from Sigma (St. Louis, MO, 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).

Snow crab tropomyosin (SCTM) standard was obtained in a previous study [4] which used a series of extraction and purification techniques to purify SCTM from fresh crab tissue.

2.2. Peptide solutions

Working solutions (10.0 μM) of the light (TSP) and heavy (d_9-TSP) signature peptide of snow crab tropomyosin were prepared in deionised water (dH_2O) from stock solutions to be used as standard and internal standard, respectively. A linear calibration curve from 10.0–1000.0 nM was prepared in 1.00-mL final volumes by serial dilution of the working solution of SP (10.0 μM), while a final concentration 500.0 nM of the d_9-TSP was prepared as internal standard. Three samples with different concentrations, 20.0, 250.0, and 850.0 nM, were used as quality control (QC) samples.

2.3. Air sample collection

Forty five kg of fresh snow crab were processed in a small simulated processing plant designed by the Marine Institute, Memorial University of Newfoundland. Area and personal breathing zones (PBZ) air samplers were used at a flow rate of 3.5 L min^{-1} using 33-mm PTFE filters. Three samplers were used in each processing stage and various background air samples were collected before and after processing. The filters were stored at −80 °C, to avoid proteases activities, until the protein extraction protocol.

2.4. Protein extraction from filters and tryptic digestion

Airborne allergens were collected on 33-mm PTFE filter papers and then extracted using 1-mL of 10% SDS solution [15] containing 500 nM of the d_9-TSP as internal standard [20]. The extracts were freeze dried, then subjected to trypsin digestion. The freeze dried extracts were reconstituted with 100 μL of 50 mM ammonium bicarbonate, 10 μL 0.01% RapiGest SF solution was added followed by boiling for 5 min in a water bath at 100 °C, then cooled. A 100 ng μL^{-1} trypsin solution was made up in 50 mM of ammonium bicarbonate. Protein samples were spiked with the appropriate amount of typsin solution and incubated at 37 °C overnight (18 h). The digestion was quenched by adding an equivalent volume of 1% formic acid to degrade the acid labile surfactant (RapiGest SF). Samples were then vortex mixed and centrifuged for 2 min at 4500 rpm. The supernatant was removed, freeze dried, and stored at −80 °C. These samples were later treated to remove SDS prior to mass spectrometric analysis.

2.5. SDS removal protocol

The tryptic peptide residues were reconstituted with 200 μL of 80:20 (ACN:H_2O), and then introduced to a SDS removal protocol using TopTip filters supplied with HILIC resin. The filters were conditioned twice with 50 μL of releasing solution (50 mM formic acid in 5:95 ACN:H_2O), and twice with 50 μL of binding solution (15 mM ammonium formate, pH 3.0, in 85:15 ACN:H_2O). The peptide sample was loaded onto TopTip filters using a micropipette and the resin bed washed twice with the binding solution to remove any salts and other non-retained materials. The peptides were eluted twice using 75 μL of the releasing solution. The samples were freeze dried and stored at −80 °C. The samples were reconstituted in LC mobile phase before LC–MS/MS analysis.