Characterisation of Oyster Allergens for Improved Diagnosis of Mollusc Allergy

Thesis submitted by

Roni Nugraha

B.Sc., M.Sc.

For the degree of Doctor of Philosophy

in the College of Public Health, Medical and Veterinary Sciences

James Cook University

Townsville, Australia

October 2018

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my advisor and guide **Prof. Andreas Lopata** for bringing me in to the world of allergy. Without his guidance, I would not be able to finish this PhD and this thesis would never have been written. Andreas is not only the source of research inspiration, but his guidance also has profound effects on my personal life. As a lecturer at a university, I learned a lot from Andreas how to be a good researcher and mentor for the students.

Secondly, I would like also to thank my co-supervisors, **Prof. Kyall Zenger** for introducing me to the field of bioinformatics and **Dr. Sandip Kamath** for his assistance and technical expertise during my experiments. Moreover, I would like to thank **Prof. Jennifer Rolland** and **Prof. Robyn O'Hehir** of the Monash University and Alfred Hospital for providing serum samples.

Thirdly, I am very fortunate and proud to be a member of a very amazing research group, Molecular Allergy Research Laboratory: Dr. Fiona Baird, Dr. Elecia Johnston, Dr. Shruti Saptarshi, Dr. Michael Sharp, Dr. Martina Koeberl, Dr. Tiange Liu, Mr. Thimo Ruethers, Mrs. Thu Le, Mr. Kunal Pratap and Mr. Shaymaviswanathan Karnaneedi. Thank you for becoming my second family in Australia.

Lastly, but not the least, I would like to thank my wife for the endless support, the smile and the patience; my kids, Yasmin and Zakiy and also my mother for always calling my name in her prays.

Statement of the Contributions of Others

Nature of Assistance	Contribution	Name and Affiliation
Intellectual support	Project plan and development	• Prof. Andreas Lopata, James Cook University • Prof. Kyall Zenger • Dr. Sandip D Kamath
	Editorial support	• Prof. Andreas Lopata, James Cook University • Dr. Sandip D Kamath • Dr. Aya Taki
	Grant proposal writing	• Prof. Andreas L Lopata, James Cook University
Financial support	Research	• ARC Future Fellowship for Prof. Andreas Lopata • National Health and Medical Research Council for Prof. Andreas Lopata
	Conference travel	• HDR Enhancement Scheme, College of Public Health, Medical and Veterinary • ASI ICI Bursary • JCU Science Research Festival
	Stipend	• James Cook University Postgraduate Scholarship • College Scholarship, College of Public Health, Medical and Veterinary Sciences, James Cook University
	Write-up support	• Doctoral Completion Grant, College of Public Health, Medical and Veterinary Sciences, James Cook University
Data collection	Human blood sample collection	• Prof. Jennifer Rolland, Monash University • Prof. Robyn O'Hehir, Monash University and The Alfred Hospital
Technical support	Recombinant protein assay	• Dr. Sandip Kamath, James Cook University
	Mass spectrometry	• Dr. Martina Koeberl, National Measurement Institute

Publications arising from thesis-Published

1) Thimo Ruethers, Aya C Taki, Elecia B Johnston, **Roni Nugraha**, Thu TK Le, Tanja Kalic, Thomas R McLean, Sandip D Kamath, Andreas L Lopata. 2018. Seafood allergy: A comprehensive review of fish and shellfish allergens. *Molecular immunology. 100:28-57.* **Impact Factor: 3.16**

2) **Roni Nugraha**, Sandip D Kamath, Elecia Johnston, Kyall R Zenger, Jennifer M Rolland, Robyn E O'Hehir, Andreas L Lopata. 2018. Rapid and comprehensive discovery of unreported shellfish allergens using large-scale transcriptomic and proteomic resources. *Journal of Allergy and Clinical Immunology*. 141(4): 1501-1504. e8. **Impact Factor: 13.258**

3) Sandip D Kamath, **Roni Nugraha**, Andreas L Lopata. 2017. *Recent advances in Diagnosis and Management of Shellfi*sh Allergy. Food Allergy: Molecular and Clinical Practice. CRC Press: 122-154. Invited book chapter.

Publications arising from thesis – Submitted

1) **Roni Nugraha**, Sandip D Kamath, Shaymaviswanathan Karnaneedi, Elecia Johnston, Andreas L Lopata. 2018. Conservation analysis of B-cell allergen epitopes to predict clinical cross-reactivity between shellfish and inhalant invertebrate allergens. *Frontiers in Immunology*.

Publications arising from thesis – In Preparation

2) **Roni Nugraha**, Sandip D Kamath, Thimo Ruethers, Shaymaviswanathan Karnaneedi, Elecia Johnston, Andreas L Lopata. 2018. Effects of extraction buffers on the solubility and immunoreactivity of the Pacific Oyster (*Crassostrea gigas*) allergens. *Food Chemistry*.

3) **Roni Nugraha**, Sandip D Kamath, Thimo Ruethers, Shaymaviswanathan Karnaneedi, Elecia Johnston, Andreas L Lopata. Recombinant expression, purification and immunological characterisation of the major allergen of the pacific oyster (*Crassostrea gigas*) – Tropomyosin. *Molecular Nutrition and Food Research*.

Abstract

Discovery and characterisation of allergens in various food and inhalant sources is central to our understanding of the molecular mechanisms of allergic reactions. Allergen characterization is the most important underlying factor for better patient management with improved diagnostics, and the design and development of novel immunotherapeutics. Of the 'Big eight' allergen food groups, shellfish presents a unique challenge in terms of allergen discovery due to the large number and diversity of consumed species, leading to heterogeneity of allergen structure and cross-reactivity among various sources. The group of 'shellfish' comprises of two invertebrate phyla; arthropods and molluscs. Although all shellfish are invertebrate animals, these two groups are very distinct in evolutionary terms and subsequently contain different molecular repertoires of allergenic proteins. Co-sensitisation of patients with crustacean and mollusc allergy is often described, however, the current diagnostic approaches to manage patients is not based on sufficient molecular knowledge of these shellfish allergens. Consequently, mollusc allergy is clinically underreported and allergens are ill-defined. To date, only five mollusc allergens are listed in the WHO/International Union of Immunological Societies (IUIS) Allergen Nomenclature SubCommittee database, all of which are different tropomyosin's (http://www.allergen.org/index.php). Additional mollusc allergens have been reported, but not yet fully characterised. A detailed review of the current status and the diagnosis of mollusc allergy is provided in **Chapter 1**.

Current strategies for allergen identification are time- and resource-consuming, which are highly prone to missing hidden allergens present in low concentrations. Allergenic proteins are traditionally identified based on their serum-specific IgE antibody recognition. Soluble proteins derived from whole protein preparations of a suspected allergen source are screened for IgE antibody binding proteins using sera from individuals with clinically confirmed allergy. Although this approach is the current standard for allergen

identification, there are three major drawbacks. Firstly, this approach often does not detect allergenic proteins present in low abundance. Secondly, cross-reactive allergens are not easily identified due to their possible presence in unrelated allergen sources. Thirdly, the IgE recognition patterns are highly dependent on the demographics of the particular allergic patient cohort under investigation. This PhD thesis presents a comprehensive study on the improvement of allergen discovery from Pacific oyster, the most widely consumed mollusc species and immunological characterisation of the major allergen, tropomyosin, using a cohort of mollusc-sensitised patients in Australia. Furthermore, novel ways to diagnose cross-reactivity between crustacean and mollusc species were developed.

Chapter 2 describes a comprehensive discovery pipeline, for allergenic proteins, that accounts for biological and molecular variability using allergenomics, high-throughput screening of genomic databases and high-resolution mass spectrometry. This methodological approach was successful in identifying 24 previously unreported allergens from over 25,000 proteins from the Pacific oyster. This is the first study to demonstrate the presence of 24 hidden allergens, also found in very different allergen sources from animals, including fish and mites, as well as plant allergens from pollen, latex and fungi. Importantly all of these allergenic proteins identified are reactive with shellfish allergic patients' IgE antibodies.

However, it was demonstrated in chapter 2 that not all allergens present in the genome and transcriptome of oyster are also detected in the extracted proteome. Allergens are often overlooked during the extraction process due to the use of inappropriate buffers which might affect the in vitro diagnostic methods that use whole proteins extracts. In **Chapter 3** various buffer compositions covering a wide range of pH were evaluated to improve the detection of the unreported allergens described in chapter 2. The IgE-reactivity of protein extracts from each buffer was determined against a pool of serum from five shellfish allergic patients. In addition, the protein composition of the

Pacific oyster was analysed using high-resolution mass spectrometry. High concentrations of protein were recovered after extraction using high salt content or high pH buffers, subsequently revealing more IgE-reactive bands on the immunoblotting. Low pH buffers, however, resulted in poor protein recovery and affected negatively patient IgE-reactivity. Mass spectrometry analysis discovered that the novel IgE-reactive proteins, particularly of high molecular weight, emerged due to an increased abundance in the allergen extract. Overall, increasing the ionic strength and pH of the extraction buffers improves the solubility of allergenic proteins.

In **Chapter 4,** a detailed analysis of immunological characteristics of the Pacific oyster extracts and the major allergen, tropomyosin were conducted. Twenty-one oyster-sensitised patients were analysed to determine the prevalence of each allergen in the patient cohort. Eighteen out of 21 patients showed reactivity to tropomyosin although the binding intensity varied between patients. Patients who lacked IgE-binding to tropomyosin were shown to be sensitised to other oyster allergens. These allergenic proteins were preliminary abundant in the raw extract of the Pacific oyster. Further investigation was carried out with the Pacific oyster tropomyosin. The natural wild type and recombinant tropomyosin were successfully purified, and their structural properties observed. Both purified natural and recombinant tropomyosin had very similar structural and immunological properties. Cross-reactivity analysis using ELISA demonstrated patients who demonstrated IgE reactivity to the Pacific oyster tropomyosin were also reactive to other tropomyosin's from Black tiger prawn as well as the House dust mite. The degree of cross-reactivity correlated well with the tropomyosin amino acid sequence identity. Sequence alignment of tropomyosin from those three species revealed five protein regions containing predicted IgE-binding epitopes responsible for the strong cross-reactivity observed.

Finally, in **Chapter 5** to improve the prediction of clinical cross-reactivity between crustaceans and molluscs as well as other invertebrate species,

conservation analysis of IgE-binding epitopes of four shrimp allergens were carried out. The results demonstrated that within a large directory of shrimp IgE-binding epitopes there are a substantial fraction of epitopes that are highly conserved across various invertebrate species. Shrimp TM and AK shared a higher number of conserved epitopes compared to shrimp SCP and MLC; in fact, no conserved epitope could be found for SCP, while MLC only shared two epitopes in one region with cockroach MLC. These results suggest that TM and AK are the major contributing proteins in immunological and clinical cross-reactivity between crustacean and other invertebrate groups. Furthermore, comparative evaluation of the number of conserved epitopes in TM and AK revealed a clear cross-reactive hierarchy where cockroach has the highest number followed by mite, and molluscs are on the bottom of this hierarchy.

In conclusion, the outcomes of this thesis have demonstrated that many unreported allergens have been overlooked due to the limitations of the current allergen discovery methodology. The utilisation of transcriptome data and proteomic techniques in addition to the well-established allergenomic approach improves discovery of unreported allergens. While tropomyosin seems to be a clinically relevant cross-reactive major allergen, the presence of additional allergens that has never been reported in mollusc species, suggests mollusc species contain different molecular repertoires of allergens. Supported by the findings of the in-depth bioinformatics analysis of IgE-binding epitopes, component-resolved diagnostics for mollusc allergy could be developed, enabling precise identification of patients sensitised to a specific mollusc group and distinguish from patients with extensive cross-reactivity to ingested and inhaled allergens from other invertebrate sources.

Table of Content

Acknowledgements ..... **ii**
Statement of the Contributions of Others ..... **iii**
Publications arising from thesis ..... **iv**
Abstract ..... **v**
Table of Content ..... **ix**
List of Figures ..... **xiii**
List of Tables ..... **xix**

CHAPTER 1. MOLLUSC ALLERGY AND ALLERGENS– CURRENT ADVANCES AND FUTURE DIRECTIONS **1**
1.1 Introduction **2**
1.2 Prevalence of Mollusc Allergy **4**
1.3 Routes of Exposure and Clinical Features of Mollusc Allergy **7**
1.4 Mollusc Allergens **10**
1.4.1 Tropomyosin 10
1.4.2 Paramyosin 17
1.4.3 Arginine kinase 18
1.4.4 Less characterised allergens 18
1.5 Effect of Food Processing on Mollusc Allergens **19**
1.6 Cross-Reactivity of Allergens **20**
1.6.1 Within the mollusc group 21
1.6.2 Between mollusc and crustacean 23
1.6.3 Cross-reactivity of mollusc with other invertebrates 26
1.7 Diagnosis and Management of Mollusc Allergy **26**
1.8 Conclusions **29**
1.9 References **31**

CHAPTER 2. IDENTIFICATION OF UNREPORTED ALLERGENS FROM PACIFIC OYSTER (*CRASSOSTREA GIGAS*) USING A COMBINATION OF BIOINFORMATICS, PROTEOMICS AND ALLERGENOMIC APPROACH **43**
2.1 Summary **44**

2.2 Introduction 45
2.3 Aims 46
2.4 Material and Methods 47
2.4.1 In silico identification of potential allergens from Pacific Oyster 47
2.4.2 Gene expression analysis of potential allergens in the Pacific Oyster 48
2.4.3 Preparation of protein extracts 49
2.4.4 Proteomic analysis of the Pacific oyster extracts 49
2.4.5 Patient selection 50
2.4.6 Allergenomic analysis of the Pacific oyster extracts 51
2.4.7 Mass Spectrometry Analysis 53
2.4.8 Protein Identification 54
2.5 Results 55
2.5.1 Potential allergens of the Pacific oyster 55
2.5.2 Expression profiles of potential allergens 56
2.5.3 Analysis of the Pacific oyster proteome 58
2.5.4 Identification of IgE-reactive proteins in the Pacific oyster 60
2.5.5 Identification of unreported allergens of Pacific oyster 64
2.6 Discussion 71
2.7 References 75

CHAPTER 3. EFFECTS OF EXTRACTION BUFFER ON THE SOLUBILITY AND IMMUNOREACTIVITY OF THE PACIFIC OYSTER ALLERGENS 79
3.1 Summary 80
3.2 Introduction 81
3.3 Aims 82
3.4 Materials and Methods 83
3.4.1 Preparation of extraction buffers 83
3.4.2 Preparation of oyster soluble protein extracts 83
3.4.3 Quantification of protein content 83
3.4.4 SDS–PAGE and IgE-reactive analysis of oyster extracts 84
3.4.5 Proteomic profiling of oyster extracts 85
3.4.6 Mass-spectrometry analysis 86
3.4.7 Peptide search and protein identification 86

3.4.8 Statistical analysis and experimental design 86
3.5 Results..... 87
3.5.1 Effects of extraction buffers on soluble protein content..... 87
3.5.2 Protein Profiling by SDS-PAGE..... 88
3.5.3 Effect of extraction buffers on IgE-binding..... 91
3.5.4 Proteomic analysis of the extracts..... 96
3.6 Discussion..... 98
3.7 References 102

CHAPTER 4. RECOMBINANT EXPRESSION, PURIFICATION AND IMMUNOLOGICAL CHARACTERISATION OF THE MAJOR ALLERGEN OF THE PACIFIC OYSTER (*Crassostrea gigas*) – TROPOMYOSIN 105
4.1 Summary 106
4.2 Introduction..... 108
4.3 Aims..... 109
4.4 Materials and Methods 110
4.4.1 Patient selection..... 110
4.4.2 Sample preparation and protein extraction..... 110
4.4.3 Quantification of total protein..... 110
4.4.4 Purification of natural TM from the Pacific oyster..... 110
4.4.5 Cloning, sequencing and expression of recombinant oyster TM.... 111
4.4.6 SDS PAGE and Immunoblotting using patient IgE-antibody..... 113
4.4.7 Amino acid sequencing using mass Spectrometry 113
4.4.8 Analysis of secondary protein structure using CD spectrometry.... 114
4.4.9 Evaluation of IgE-reactivity using ELISA..... 114
4.5 Results..... 115
4.5.1 SDS-PAGE and IgE-antibody reactivity of Pacific oyster extracts.. 115
4.5.2 Sequencing and characterisation of Pacific oyster TM 119
4.5.3 IgE-recognition of oyster-sensitised subjects to natural and recombinant TM..... 124
4.5.4 Immunological IgE-reactivity analysis using ELISA 125
4.5.5 Tropomyosin amino acid sequence comparison and IgE-binding epitope prediction 126
4.6 Discussion..... 128

4.7 References 132

CHAPTER 5. CONSERVATION ANALYSIS OF IGE-BINDING EPITOPES TO PREDICT CLINICAL CROSS-REACTIVITY BETWEEN CRUSTACEAN AND MOLLUSC ALLERGENS 135

5.1 Summary 136

5.2 Introduction 137

5.3 Aims 139

5.4 Materials and Methods 140

5.4.1 Collection of shrimp allergens IgE-binding epitopes 140

5.4.2 Sequence retrieval and phylogenetic analysis 140

5.4.3 Conservation analysis of shrimp allergens in invertebrate species. 140

5.4.4 Conservation analysis of IgE-binding epitopes of shrimp allergens 141

5.4.5 Data analysis 141

5.5 Results 142

5.5.1 Amino acid sequence analysis of shrimp allergens 142

5.5.2 Conservation of IgE binding epitopes of shrimp allergens 144

5.5.3 Invertebrate allergen pan-epitopes 148

5.5.4 Shellfish allergen pan-epitopes 151

5.5.5 Decision tree to identify potential crustacean-mollusc cross-reactivity in shrimp-allergic patients 155

5.6 Discussion 157

5.7 References 163

CHAPTER 6. GENERAL DISCUSSION AND FUTURE DIRECTIONS .. 168

List of Figures

Figure 1-1 Classification of seafood species including fish and shellfish, clarifying the group of mollusc 3

Figure 1-2.Structure modelling of three identified allergens from molluscs species: (A) tropomyosin, (B) paramyosin, and (C) arginine kinase. The model of (A) and (B) were constructed from SWISS-MODEL [78], while the model (C) was predicted using protein modelling software Phyre [79] due to lack of a protein model. 15

Figure 1-3 Protein identity matrix of the major allergen tropomyosin from different species as determined by MUSCLE multiple sequence alignment software [82]. The bootstrap consensus tree inferred from 1000 replicates taken to represent the evolutionary history of the taxa analyzed and constructed using the Neighbor-Joining method in MEGA6 [83]. 16

Figure 1-4 IgE-binding regions of tropomyosin from three species of molluscs: oyster, horned turban and octopus in comparison to crustaceans: brown shrimp and whiteleg shrimp are shaded in yellow. Conserved amino acid regions of the proteins' primary structure are shaded in grey. The regions possibly responsible for cross-reactivity between those species is identified by a solid box..... 25

Figure 1-5 Diagnostic decision tree on how to proceed from the suspicion of mollusc-related allergic symptoms 28

Figure 2-1. Workflow for the identification of potential allergens from the Pacific oyster using bioinformatics analysis. In silico discovery of potential allergens based on protein sequence alignment, amino acid sequence identity ≥ 50% with known allergens and E-values <10-7. 48

Figure 2-2. Workflow for the identification of all proteins present in the extracts of Pacific oyster. The proteins were separated using 1D SDS-PAGE, tryptic-digested and identified using mass spectrometry. 50

Figure 2-4. Workflow for the identification of IgE-reactive proteins from the extracts of Pacific oyster. IgE-reactive proteins were identified by 2D-immunoblotting and subsequent LC-MS/MS..... 53
Figure 2-5.. *In silico* analysis identified 22 proteins as “very likely allergenic” (aa identity ≥70%) and 73 proteins as “likely allergenic” (aa identity ≥ 50%, and ≤70%). 57
Figure 2-6. The expression levels of the “very likely allergenic” proteins were different across development stages and organs of the Pacific Oyster. 58
Figure 2-7. Identification of total proteins from the Pacific oyster extracts. (A). The gels were cut into pieces and digested using trypsin and total proteins were identified. (B). Venn diagram shows shared proteins between raw (N=1,086), heated (N= 130). 59
Figure 2-8. A quantitative measurement of protein abundance, based on the exponential modified protein abundance index (emPAI). 60
Figure 2-9. Gene ontology analysis of total proteins identified in the raw and heated extract. The bar plot shows the top 5 terms of molecular function, cellular component and biological process..... 62
Figure 2-10. Separation of proteins based on isoelectric point using 2D-PAGE. (A) Raw and heated extracts were separated by 2D-PAGE (pH 3 - pH 10, Non-Linear) and stained with Coomassie Brilliant Blue. (B) Separated proteins were transferred to PVDF membrane followed by incubation with serum of pool of five patients with mollusc allergy. Spot numbers indicated on the gel were subjected to mass spectrometry analysis. 63
Figure 2-11. Overview of protein abundance in each IgE-reactive spot. (A) the exponentially modified protein abundance index (emPAI) values (%) of the identified proteins within a 2-DE spot. (B) The bar plot shows the number of proteins contribute to over 50% of the total emPAI in each spot. 65
Figure 2-12. Venn diagram displaying proteins shared by the bioinformatics, the allergenomic and the proteomic approach. Numbers of proteins

shared by the three methods are coloured in red. Twenty-four proteins in the raw extract and four proteins in the heated extract were identified across all three methods. 66
Figure 3-1 SDS-PAGE analysis of the proteins from (A) raw and (B) heated extracts. Samples containing 10 µg of proteins were resolved in 12% SDS-acrylamide gels and run at 170 V for 1 h. M = Marker, 1 = Citrate-3 extract, 2 = Citrate-5 extract, 3 = TBS extract, 4 = TBSN extract, 5 = PBS extract, 6 = PBSN extract, 7 = Carbonate-9 extract and 8 = Carbonate-10 extract. The highlighted bands were cut for mass spectrometry analysis. 90
Figure 3-2 IgE-immunoblotting analysis of the proteins from (A) raw and (B) heated extracts using a pool of serum from five shellfish-allergic patients. M = Marker, 1 = Citrate-3 extract, 2 = Citrate-5 extract, 3 = TBS extract, 4 = TBSN extract, 5 = PBS extract, 6 = PBSN extract, 7 = Carbonate-9 extract and 8 = Carbonate-10 extract. 93
Figure 4-1 IgE reactivity of oyster-sensitised patients to raw and heated Pacific oyster extract. The extracts were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (A and C). Immunoblots were performed with sera from 21 oyster-sensitised patients (1-21, B and D) as well as from 3 atopic individuals (NC). 117
Figure 4-2 Allergrogram analysis of IgE-binding patterns to proteins in the raw (A) and heated (B) extract of the Pacific oyster. IgE-binding intensities were measured using Image Studio software and graded as weak, medium, strong and very strong. Percentrage patient reactivity for each IgE-binding intensity is shown. 118
Figure 4-3 Purification profile of protein extract of heated Pacific oyster using CHT™ Ceramic Hydroxyapatite. The increase in the absorbance is measured at 280 nm (blue line) and 220 nm (red line) and 31 eluted fractions were collected and analysed by SDS-PAGE. The 17th, 18th, and 19th peak contain pure tropomyosin, appearing as a strong band at 39 kDa in the Coomassie-stained SDS-PAGE gel. 120

Figure 4-4 SDS-PAGE purification profile of recombinant Pacific oyster tropomyosin. Recombinant tropomyosin was purified using HisPur Ni-NTA with increasing concentration of imidazole. Purified tropomyosin was observed at 40 kDa due to six-His-tag fused to its N-terminal. Note: CE= crude extract, FT = Flow through, W = Wash, E = Eluent. 121

Figure 4-5 Structural analysis of purified natural and recombinant tropomyosin. A) Circular dichroism (CD) spectroscopy profile of natural (green line) and recombinant tropomyosin (blue line). B). The estimated structure of tropomyosin predicted using K2D3 web servers [1], with the majority of the structure consisting of an alpha-helix. 122

Figure 4-6 Comparison of the mass spectrometry analysis of purified natural and recombinant TM. (A) Representative product spectra of two unique peptides of tropomyosin generated from trypsin digestion. (I) sequence 694.35 m/z (II) sequence 924.5m/z. (B) The amino acid sequence of TM. Peptide sequences identified by mass spectrometry are highlighted in red colour. 123

Figure 4-7 IgE reactivity analysis of natural and recombinant Pacific oyster tropomyosin using immunoblotting with 18 IgE reactive patients showed reactivity to protein at 38 kDa from raw and heated extract. rCra g 1 = purified recombinant Pacific oyster tropomyosin, nCra g 1 = purified natural Pacific oyster tropomyosin. 124

Figure 4-8 Patient serum IgE reactivity analysis by ELISA for three tropomyosins: Cra g 1 (Pacific oyster), Pen m 1 (Black tiger prawn) and Der p 10 (House dust mite) (n = 18). 125

Figure 4-9 Amino acid sequence alignment of tropomyosin sequence from Black tiger prawn (*Penaeus monodon*), house dust mite (*Dermatophagoides pteronyssinus*) and Pacific oyster (*Crassostrea gigas*). Variable amino acids are shaded in yellow. The predicted IgE-binding epitopes responsible for cross-reactivity in those three species are identified by solid boxes 127

Figure 5-1 Molecular evolutionary analysis of shellfish allergens. The phylogenetic tree was drawn using Bayesian and Maximum Likelihood approach for (I) Tropomyosin and (II) Arginine kinase. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. 143

Figure 5-2 Conservation analysis of individual amino acids in tropomyosin (TM) and arginine kinase (AK) determined using the Consurf server. The conservation grades were mapped onto the query sequence and structure using the ConSurf colour-code, with blue-through-red corresponding to a variable (grade 1)-through-conserved (grade 9) positions. 144

Figure 5-3 Heatmap representing number of mismatches in homologous peptide of shrimp TM IgE-binding epitopes in different invertebrate species. The heatmap was generated using Heatmapper [32] and clustered using Manhattan distance metric approach. The colours grading as indicated in the top left represents the number of amino acid mismatches found in the homologous peptides of epitopes. Green indicates no mismatches and red indicates maximum mismatches. 145

Figure 5-4 Percentage of conserved shrimp IgE-binding epitopes between invertebrate species. Ninety-Eight B-cell epitopes from tropomyosin (A) and thirty-nine B-cell epitopes from arginine kinase (B) were examined for their conservation within shellfish and between other allergenic invertebrate species. An epitope was considered conserved if the sequence matched to a homolog or a peptide variant with not more than 2 amino acid substitutions in another species. Significance differences ($p<0.05$) were calculated using One-way ANOVA. 147

Figure 5-5 Venn diagram showing the similarities among the conserved shrimp IgE-binding epitopes across invertebrate species. In total 96 epitopes on TM and 39 on AK were analysed. 149

Figure 5-6 Percentage representation of conserved epitopes in the three mollusc classes: bivalves (square), cephalopod (circle) and gastropod (triangle). Only epitopes which are present in over 50% of each mollusc classes are shown. The epitope allergen sources, their amino acid sequences and positions in the protein are indicated on the left. 153
Figure 5-7 Molecular modelling of the conserved IgE-binding epitopes of (A) tropomyosin (TM) and (B) arginine kinase (AK) that are presented in over 50% of each mollusc class. For tropomyosin, the epitopes were remapped to their consensus tropomyosin sequence and colour-coded based on mollusc classes in which the conserved epitopes are found: yellow (all mollusc classes), green (cephalopod and gastropod), and grey (bivalve and cephalopod). The protein structure of TM and AK were modelled using SWISS-MODEL based on reference proteins 1C1G and 4BG4, respectively. 154
Figure 5-8 Decision tree to diagnose potential molecular cross-reactivity to invertebrate allergens in shrimp allergic patients based on the reactivity to specific IgE epitopes. The region numbers refer to the epitope mapping in Fig 5. 156

List of Tables

Table 1-1 Prevalence of shellfish and mollusc allergy determined in different countries..... 6

Table 1-2. Routes of exposure and clinical features of mollusc allergy..... 8

Table 1-3. Allergenic proteins identified in different mollusc species. 12

Table 2-1 Demographics of patients recruited for this study. Note: NT=Not Tested. 51

Table 2-2. Proteins identified across all three methods with their matched allergens source and routes of sensitization. Proteins identified in both raw and heated extract are shaded. The proteins are sorted based on amino acid sequence identity with their homologous allergens in descending order. 67

Table 3-1 Buffers and their composition used to extract proteins from Pacific oyster 84

Table 3-2. The yield of recovered proteins measured by BCA–protein quantification method. Protein concentration was statistically analysed by one-way ANOVA (Tukey). Values with the same letter in the same column are not significantly different ($p > 0.05$). 88

Table 3-3 Proteins identified using LC-MS from the SDS-PAGE bands corresponding to the IgE-reactive spots. The top three proteins from Mascot search engine result in each spot were presented..... 94

Table 3-4. Summary of the results from mass spectrometry analysis. Total number of proteins was obtained from Peptideshaker and after removal of contaminations. The pI and MW were determined using Compute pI/Mw tool in ExPASy website (https://web.expasy.org/compute_pi/). The protein compositions were matched to the potential allergen identified in Chapter 2 to get the number of potential allergens extracted. 97

Table 4-1 Demographics of patients recruited for this study 112

Table 5-1 Sequences of TM IgE-binding epitopes responsible for cross-reactivity between crustacean, cockroach, mite and mollusc in

prawn allergic patients and their presentation in each invertebrate group. 150
Table 5-2 Sequences of AK IgE-binding epitopes responsible for cross-reactivity between crustacean, cockroach, mite and mollusc in prawn allergic patients and their presentation in each invertebrate group. 151

CHAPTER 1. MOLLUSC ALLERGY AND ALLERGENS– CURRENT ADVANCES AND FUTURE DIRECTIONS

Published in part:
Thimo Ruethers, Aya C Taki, Elecia B Johnston, **Roni Nugraha**, Thu TK Le, Tanja Kalic, Thomas R McLean, Sandip D Kamath, Andreas L Lopata. 2018. Seafood allergy: A comprehensive review of fish and shellfish allergens. *Molecular immunology. 100:28-57.* **Impact Factor: 3.16**

Sandip D Kamath, **Roni Nugraha**, Andreas L Lopata. 2017. Recent advances in Diagnosis and Management of Shellfish Allergy. Food Allergy: Molecular and Clinical Practice. CRC Press: 122-154. Invited book chapter.

1.1 Introduction

Globally, seafood is considered healthy due to its high nutritional value; consumption has therefore increased considerably, from 15.8 kg/year in 2000 to 19.2 kg/year in 2012 [1]. In the fisheries industry, the term ‘seafood’ comprises fish as well as shellfish. The phylum Mollusca is the second richest group in the animal kingdom, after arthropods, and is grouped with crustacean as shellfish (Figure 1-1). Molluscs comprise more than 100,000 species and are divided into seven classes, with the most common consumed species grouped within Gastropoda, Bivalvia, and Cephalopoda [2]. Abalone, snail, limpet, and whelk are the major food sources for humans from the gastropod group, while mussels and clams are bivalves, and squid, cuttlefish, and octopus are primary species of the cephalopods [3].

The increasing production and consumption of seafood have been accompanied by increasing reports of adverse reactions to seafood. While some reactions are due to exposure to marine toxins [4, 5], hypersensitivity reactions to seafood allergens are mediated by the immune system and result in a lifelong inflammatory disease. In the last decade, food allergy has turned into a global burden, affecting more than 500 million people worldwide [6]. Furthermore, direct medical and economical loss caused by food allergy alone increased from a quarter billion in 2007 [7] to $4.3 billion in 2013 [8]. Food allergy impacts quality of life [9] but also increases the demand on food producers to detect and quantify relevant allergens [10, 11]. The current review focuses on mollusc allergy and characterisation of currently known mollusc allergens, as well as the effect of processing on the detection and quantification of allergens in food products.

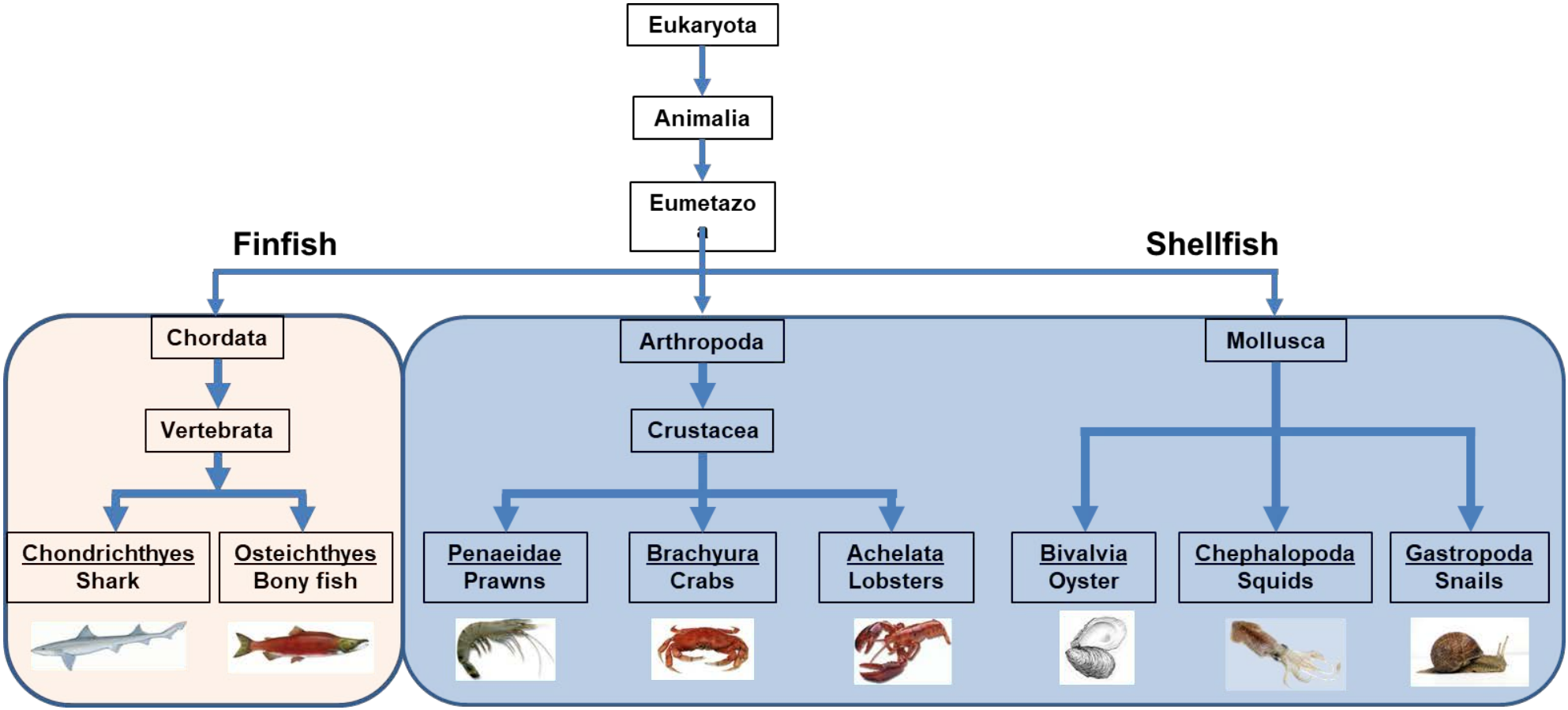


Figure 1-1 Classification of seafood species including fish and shellfish, clarifying the group of mollusc

1.2 Prevalence of Mollusc Allergy

An increasing trend of food allergy prevalence has been reported by Prescott et al. [12] in a survey spanning 89 countries, with the highest incidence observed in children less than five years of age. A similar trend was also reported by the US Centres for Disease Control and Prevention [13], which described an approximate 50% increase in food allergy between 2007 and 2011 among children under 17 years. Studies by Osterballe, Mortz [14] and Rancé, Grandmottet [15] have demonstrated that seafood is a common causative food for allergy symptoms. Although most of the surveys are based on self-reported data with some limitations, there is strong confidence of increasing food allergy prevalence worldwide with shellfish allergy emerging as life-long allergy [16].

The prevalence of allergic reactions to shellfish varied from 0.1% to 24.5% based on self-reporting data [17]; however, this was found to be lower (0.1-5.2%) when more convincing clinical data were included (Table 1-1). The frequency of shellfish allergy seems to be higher in adults as compared to children [18]. The prevalence of allergy specifically to molluscs is not exactly known. The term “shellfish” is used for both crustacean and mollusc, and the patient often fails to identify the offending group due to confusion regarding the different common names used to describe diverse shellfish. The lack of accurate clinical diagnostics to confirm specific food allergy to molluscs, including the double-blind placebo-controlled oral food challenge (DBPCFC), has also resulted in insufficient prevalence data. Previous studies on mollusc allergy were based only on questionnaire-based surveys or retrospective reviews of allergic patient clinical data, which are likely to generate an overestimated prevalence data set [19, 20]. Based on clinical history surveys it seems that the frequency of mollusc allergy ranges between 0.15% and 1.3% [14, 15, 21-23]. The variation in prevalence reports could be explained by two reasons: age of survey participants (children and/or adults) and geographical differences. For example, two reports with low prevalence focused their study on children aged 2.5 to 14 years and 3 to 7 years [15, 23]. Geographically, Asian populations seem to be more allergic to seafood, compared to the

Western population (1.3% vs. 0.4%, respectively). This geographical effect is clearly shown by Shek, Cabrera-Morales [24] where they evaluated the incidence of nuts and shellfish allergy in schoolchildren in Singapore and Philippines and differentiated between expatriate, local, and Western-born children as well as children born in Asia. Local schoolchildren have more allergies to shellfish, compared to nuts, which in contrast has been observed more commonly among expatriate schoolchildren. A similar pattern was also observed when they compared Asia-born and Western-born schoolchildren.

Among allergic individuals, binding of the allergen to IgE antibody on effector cells, including basophils and mast cells, and the subsequent onset of any clinical manifestation is characteristic of an allergic reaction. Furthermore, any food protein has the potential to evoke allergic reactions when they share identical or very similar amino acids on certain protein regions with known allergens. These are known as IgE antibody binding epitopes [25]. Although molluscs are not grouped among the most allergenic foods according to the Codex list [26], most people with crustacean allergy are advised to avoid molluscs, possibly due to potential immunological cross-reactivity. In fact, allergy to crustacean and molluscs is often reported, concurrent [18, 27, 28].

Table 1-1 Prevalence of shellfish and mollusc allergy determined in different countries.

Country	Population	Method of survey	Prevalence	Age	Reference Year conducted
Asia					
Philippines	11,158	Convincing history	Shellfish 5.12%	14-16 y	[24] 2007-2008
Singapore	11,318	Convincing history	Shellfish 3.4%	4-16 y	
Taiwan	30,018	Self-report and expert-screened	Mollusc 1.3%	All ages	[22] 2004
Thailand	452	Parent report, convincing history	Mollusc 0.2%	3-7 y	[23] 2010
Europe, USA, Australia					
Australia	2,848	Skin Prick test	Shellfish 0.9%	11-15 months	[29] 2011
Canada	9,667	Convincing history	Shellfish 1.42%	All	[30] 2008-2009
Denmark	843	Convincing history, SPT	Shellfish 2.4% Mollusc 0.4%	±22 y	[14] 2009
France	2,524	Self-report	Mollusc 0.15%	2.5 – 14 y	[15] 2002
United States	14,948	Self-report	Mollusc 0.4%	All	[21] 2002
	34,480	Parent report, convincing history	Shellfish 1.3%	0-18 y	[31] 2009-2010
	40,104	Parent report, convincing history	Shellfish 1.4%	0-18 y	[32] 2009-2010
	2,714,851	Electronic Health Records	Shellfish 0.9%	All ages	[33] 2000-2013

1.3 Routes of Exposure and Clinical Features of Mollusc Allergy

Gastrointestinal uptake is believed to be one of the major routes of sensitisation to seafood allergens, including molluscs [18]. The clinical manifestations of mollusc allergy through this route are heterogeneous, including vomiting, diarrhoea, and life-threatening anaphylactic shock (Table 1-2). Typically, when food proteins enter the digestive tract, gastric acids and digestive enzymes break the protein down into smaller peptides, which are absorbed and subsequently induce food tolerance. However, some proteins can withstand the digestion process and are often absorbed, whole or in larger fragments, through specialised epithelial cells called microfold (M) cells, intestinal epithelial cells, or dendritic cells [34]. Most of the identified allergenic food proteins are biochemically stable and known to resist gastric digestion and even heating. For example, tropomyosin, the major allergenic protein in crustaceans, and perhaps also molluscs, has been shown to retain its biochemical characteristics and bind the IgE antibody even after 60 min digestion in gastrointestinal fluid [35].

Furthermore, this incomplete digestion of allergenic proteins could increase the risk of allergic sensitisation. This mechanism was demonstrated for codfish in humans [36] and mice [37]. Intake of antacids or food supplements that could elevate gastric pH increased the risk of sensitisation by obstructing protein breakdown. An increase of pH by only one unit significantly reduced the gastric capability to digest the protein and increased the biological activity of allergenic proteins. Moreover, histamine, a mediator of allergy reactions, was released within 10 minutes, demonstrating rapid absorption of pre-gastric codfish proteins. Thus, a person with a clinical history of food allergies should be aware of the biochemical stability of allergens before taking medications that reduce the acidity of the stomach.

Table 1-2. Routes of exposure and clinical features of mollusc allergy.

Route of exposure	Symptoms	Species involved	Reference
Ingestion	Face wheal, erythema and dyspnoea	Freshwater clam (*Corbicula japonica*)	[38]
	Dyspnea, urticaria, nausea, and stomachache	Abalone (*Haliotis* sp)	[39]
	Chronic bronchitis and high blood pressure	King Broderip clam (*Venus antiqua)*	[40]
	Urticaria and anaphylaxis	Scallops (*Pecten* sp)	[41]
	Pruritus and facial angioedema	Razor clam (*Ensis macha*)	[42]
	Anaphylaxis	Limpet (*Patella* sp)	[43]
	Angioedema and urticaria	Octupus (*Octopus vulgaris*)	[44]
	Urticaria and facial angioedema	Limpet (*Patella vulgata*)	[45]
	Edema, widespread wheals with pruritus, sneezes, rhinorrhea, nasal itching, cough, chest tightness, and dyspnea	Razor clam (*Ensis macha*)	[46]
	Anaphylaxis	Snail (*Helix sp*)	[47]
	Oral swelling and pain	Scallop (*Pecten* sp)	[28]
Occupational	Asthma, rhinitis and conjunctivitis	Squid *(Loligo vulgaris*)	[48]
	Asthma	Octopus (*Octopus* sp)	[49]
	Asthma and urticaria	Queen scallop (*Chlamys opercularis*) and king scallop (*Pectin maximus*)	[50]
Skin	Dermatitis	Baby squid (*Loligo vulgaris*)	[51]
	Erythema, oedema, itchin and burning	Squid (*Loligo japonica*)	[52]
	Dermatitis	Pearl oyster (*Pinctada fucata martensii*)	[53]
	Eczema	Squid (*Loligo* sp)	[54]

Occupational sensitization has also been found to trigger allergic reactions against molluscs among seafood-processing workers [55]. Workers exposed to daily doses of particulate allergens in the industrial setting may be at a higher risk of developing respiratory or skin allergies. Occupational asthma particularly is a common adverse respiratory health effect, caused by seafood allergens. The prevalence of occupational asthma caused by mussels could be as high as 23% [56]. The allergenic proteins responsible for asthma have a molecular weight in the ranges of 19 kDa and 43 kDa, suggesting that tropomyosin and arginine kinase may implicate the sensitisation to the mollusc. It is understandable as a considerable amount of tropomyosin within respirable range is produced during shellfish processing. Using a validated immunoassay Kamath, Thomassen [57] quantified aerosolized tropomyosin in a worker- and activity-specific manner. Up to 138.8 ng/m^3 of air-borne tropomyosin were detected in certain workplace, and the highest exposure to tropomyosin was demonstrated during heating and boiling processes.

Contact urticaria and eczematous contact dermatitis are two major allergy manifestations of the skin. Protein contact dermatitis, characterised by itchiness within 10-30 minutes of food handling, was first mentioned by a Danish researcher [58], demonstrating type I (immediate) or type IV (delayed) hypersensitivity experienced by restaurant workers, following contact with certain foods including fish and shellfish. A recent study demonstrated that one-third of a cohort of chefs and culinary trainees in Germany developed an allergy to mussels. Most of these individuals showed symptoms of dermatitis very early in their career as chefs [59], confirmed by a study in Spain on the same profession [60]. It is thought that daily contact with water and seafood products which contain high quantity of protease disrupts the skin barriers and subsequently induces the release of inflammatory substances [61]. As the allergenic proteins from mollusc species are of high molecular weight, disruption of the skin barriers exposes the allergens to the antigen processing cells in the skin. Recurrent contact can prime the immune system to produce

more IgE antibodies and could cause systemic reactions as demonstrated in a murine model [62].

1.4 Mollusc Allergens

Eleven proteins have been implicated in different studies with sensitisation to molluscs, however only three proteins have been fully identified as elicitors of allergic reactions to mollusc species, including tropomyosin, arginine kinase, and paramyosin. Table 1-3 summarises the mollusc allergens known to date, including the mollusc species associated.

1.4.1 Tropomyosin

Tropomyosin is an actin binding protein involved in muscle contraction of vertebrate and invertebrates. This protein is present in muscle as well as non-muscle tissue as an elongated dimeric α-helical coiled-coil structure (

Figure 1-2A). The stability of the coiled-coil structure of tropomyosin is generated from the interaction of repeated sequence patterns of seven-amino acids residues over the entire length of the protein and with hydrophobic amino acid in every first and fourth position. The protein has 284 amino acids among most invertebrates and is relatively highly conserved between all tropomyosin found in the eukaryotic organism [63]. Multiple tropomyosin isoforms are widely present in the muscle, demonstrating a tissue-specific distribution

The first major allergen from a mollusc submitted to the International Union of Immunological Societies (IUIS) was in 1996 isolated from squid *Todarodes pacificus* and designated as Tod p 1. This heat-stable allergen with a molecular weight of 38 kDa was later identified as tropomyosin, after detailed amino acid sequencing and alignment with tropomyosins from other organisms [64]. Subsequently, other tropomyosins were identified in other mollusc groups including oyster [65] and snail [66]. Among different mollusc species, various isoforms have been discovered. Two scallops, *Patinopecten yessoensis* and *Mimachlamys nobilis*, express three different isoforms, while *Meretrix lamarckii*

(hard clam), *Tresus keenae* (horse clam), *Mactra chinensis* (surf clam), *Scapharca broughtonii* (ark shell), *Mytilus galloprovincialis* (mussel), *Atrina pectinata* (surf clam) and *Crassostrea gigas* (Pacific oyster) have two distinct isoforms each. Those isoforms can be classified into two patterns called TMa and TMb. TMa is the common tropomyosin and TMb is found specifically in the opaque portion of the adductor muscle, except in the surf clam (*A. pectinate*) where TMb was found as the common tropomyosin [67, 68]. If this different isoform present with different allergenicity is currently not known.

Invertebrate tropomyosin is very allergenic and presents the second biggest group of animal-derived food allergens (http://www.meduniwien.ac.at/allfam/). Although this protein is a common component of muscle structure among eukaryotes and shares similar physiological function, there is a very sharp division between allergenic invertebrate tropomyosin and non-allergenic vertebrate tropomyosin. The non-allergenic tropomyosin shares greater than 90% similarity with human tropomyosin, while in contrast, the allergenic tropomyosin shares only 54-56% similarity with the human tropomyosin. However, the identity of tropomyosin amino acid sequences among mollusc species is very diverse, ranging from 65-100%, and between mollusc and crustacean, from 56-68% (Figure 1-3).

Table 1-3. Allergenic proteins identified in different mollusc species.

Species		Protein	IUIS Allergen	Molecular Mass	Ref
Gastropod	Abalone (*Haliotis midae*)	Tropomyosin	-	38 kDa	[69]
		NI	Hal m 1	49 kDa	
	Abalone (*Haliotis laevigata x Haliotis rubra*)	Tropomyosin	Hal l 1	33.4 kDa	
	Gastropod (*Turbo cornutus*)	Tropomyosin	Tur c 1	35 kDa	[66]
	Abalone (*Haliotis diversicolor*)	Tropomyosin	-	38 kDa	[70]
	Brown garden snail (*Helix aspersa*)	Tropomyosin	-	36 kDa	[71]
	disc abalone (*Haliotis discus discus*)	Paramyosin	-	100 kDa	[72]
	Common whelk (*Buccinum undatum*)	NI	-	82 kDa 71 kDa 40 kDa	[73]
	Disc abalone (*Haliotis discus discus*)	Tropomyosin	-	37 kDa	[74]
	Turban shell (*Turbo cornutus*)				
	Whelk (*Neptunea polycostata*)				
	Middendorf's whelk (*Buccinum middendorffi*)				

Species		Protein	IUIS Allergen	Molecular Mass	Ref
Bivalve	Bloody cockle (*Scapharca broughtonii*)	Tropomyosin	-	37 kDa	[74]
	Japanese oyster (*Crassostrea gigas*)				
	Japanese cockle (*Fulvia mutica*)				
	Surf clam (*Pseudocardium sachalinensis*)				
	Horse clam (*Tresus keenae*)				
	Razor clam (*Solen strictus*)				
	Shortneck clam (*Ruditapes philippinarum*)				
	Oyster (*Crassostrea gigas*)	Tropomyosin	-	35 kDa	[65, 75, 76]
	Mussle (*Perna viridis*)	Tropomyosin	-	38 kDa	[70]
	Scallop (*Chlamys nobilis*)	Tropomyosin	-	38 kDa	
Cephalopod	Squid (*Todarodes pacificus*)	Tropomyosin	Tod p 1	38 kDa	[64]
	Golden cuttlefish (*Sepia esculenta*)	Tropomyosin	-	35-38 kDa	[77]

Species		Protein	IUIS Allergen	Molecular Mass	Ref
	Big fin reef squid (*Sepioteuthis lessoniana*)				
	Spear squid (*Loligo bleekeri*)				
	Swordtip squid (*Loligo edulis*)				
	Japanese flying squid (*T. pacificus*)				
	Neon flying squid (*Ommastrephes bartrami*)				
	Common octopus (*Octopus vulgaris*)				
	Ocellated octopus (*Octopus ocellatus*)				
	Pacific giant octopus (*Paroctopus doflein*)				
	Octopus (*Octopus fangsiao*)	Arginine kinase	-	38 kDa	[78]
		Triosephosphate isomerase	-	28 kDa	[79]

.

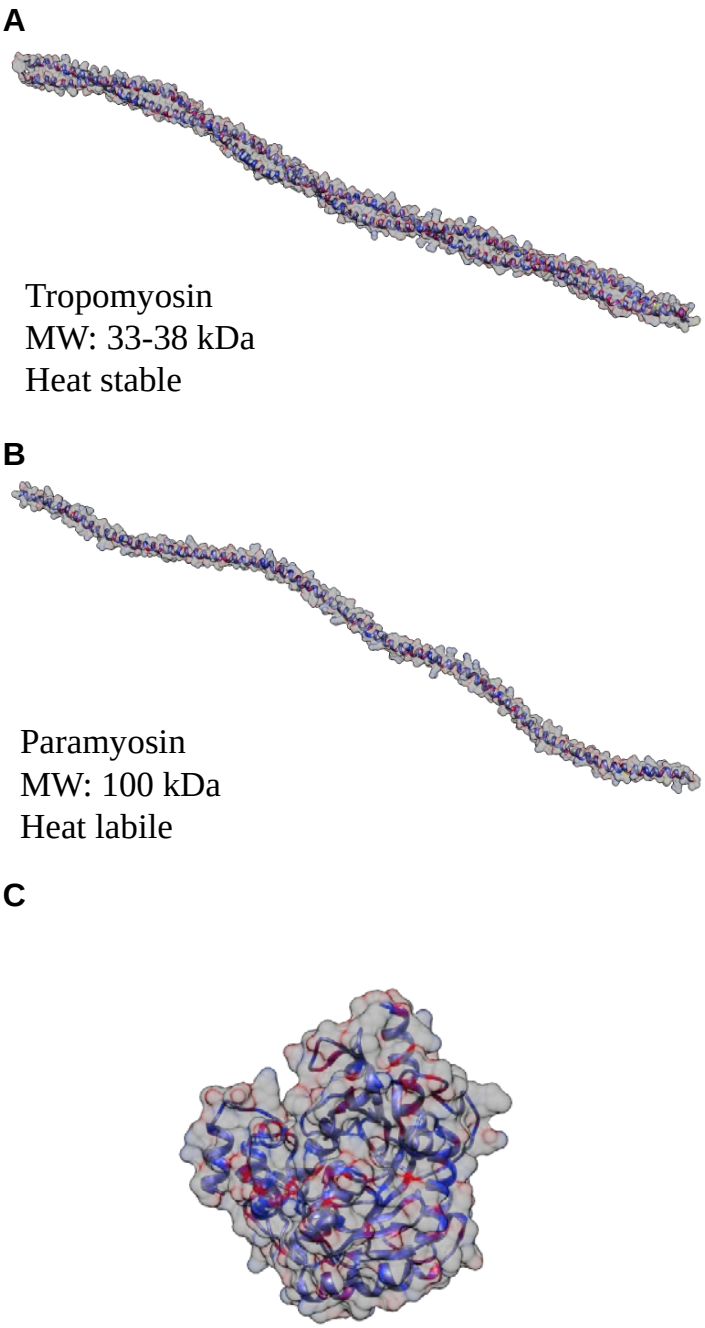


Figure 1-2. Structure modelling of the three identified allergens from molluscs species: (A) tropomyosin, (B) paramyosin, and (C) arginine kinase. The model of (A) and (B) were constructed from SWISS-MODEL [80], while the model (B) was predicted using protein modelling software Phyre [81] due to lack of a protein model.

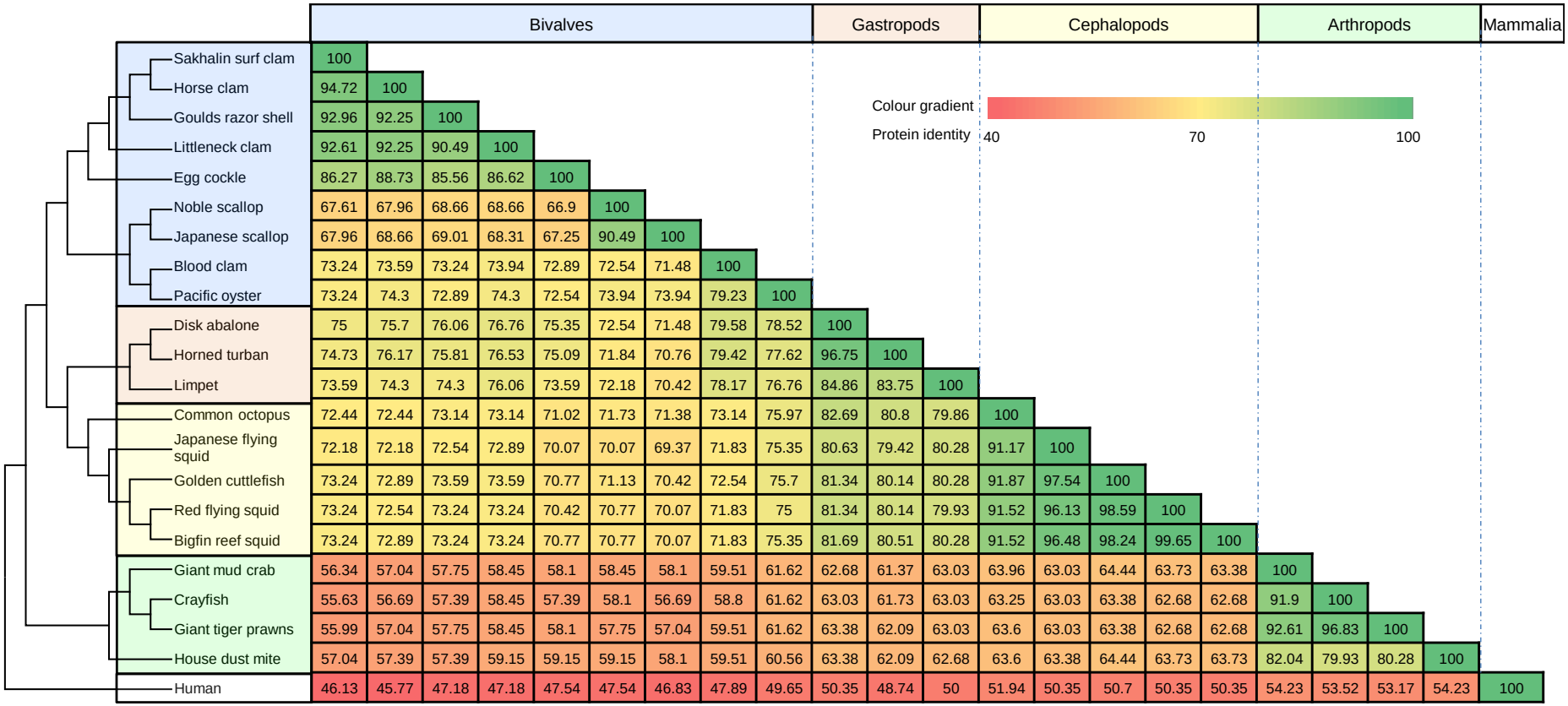


Figure 1-3 Protein identity matrix of the major allergen tropomyosin from different species as determined by MUSCLE multiple sequence alignment software [82]. The bootstrap consensus tree inferred from 1000 replicates taken to represent the evolutionary history of the taxa analyzed and constructed using the Neighbor-Joining method in MEGA6 [83]

1.4.2 Paramyosin

Paramyosin is a major structural component of the invertebrate muscle thick filament and was recently identified as an additional major allergen in abalone (*Haliotis discus discus)* [72, 84]. Western blot studies of the raw abalone extract have shown that 16 out of 18 patients sera tested reacted to a protein with 100 kDa in molecular weight. After purification, the protein was digested with lysylendopeptidase and the produced peptide amino acid sequence matched with corresponding regions of the Mediterranean mussel (*Mytilus galloprovincialis*) paramyosin. The discovery of allergenic paramyosin in mollusc species was not surprising since this protein has been confirmed as a major allergen in other invertebrates such as house dust-mite [85] and anisakis [86]. Furthermore, this protein also forms a significant component of the bivalve myofibril with 38-48% in the white adductor muscle and 15-30% in the red adductor muscle. Meanwhile, the mantle muscle of squid and the foot muscle of turban shell are reported to contain 9-14% of paramyosin in their myofibril [87].

The structure of paramyosin is similar to that of tropomyosin, and is sometimes referred as “water-insoluble tropomyosin” or “tropomyosin-A”. The molecule is composed of two α-helix coiled-coil protein with short non-helical extensions at both the N- and C- termini (Figure 1-2B) [87]. Although paramyosin and tropomyosin have structural similarity, the IgE of allergic patients did not react with paramyosin from abalone heated extract, and paramyosin was therefore also referred to as a heat-labile allergen [72]. This protein also seems to be pH-labile and aggregates during extraction if using extraction buffers containing low salt concentration. This aggregation of paramyosin during extraction indicates that this protein may have been overlooked in a previous study [74]. Interestingly, paramyosin showed cross-reactivity with tropomyosin as demonstrated by inhibition immunoblotting and inhibition ELISA [72]. Amino acid sequence analysis showed a region of paramyosin having up to 64% identity with a tropomyosin antibody binding epitope which could be responsible for the observed cross-reactivity.

1.4.3 Arginine kinase

Arginine kinase is an enzyme that belongs to the phosphagen kinases family, and catalyses the reversible transfer of phosphoryl group ATP to arginine, yielding phosphoarginine and ADP. It is widely distributed throughout the invertebrate groups and found as a monomer, although some dimeric arginine kinases have been observed [88]. The structure of arginine kinase is mainly α-helical and contains an N-terminal region with a specificity loop for specific substrate binding (Figure 1-2).

Arginine kinase is the third mollusc allergen that has been characterised. It was identified from *Octopus fangsiao* in 2012 by Shen et al. [78]. The protein was purified and further identified using mass spectrometry. The immunological activity was characterised using immunoblotting, IgE inhibition immunoblotting assay, and inhibition ELISA. The allergen has a molecular weight of approximately 38 kDa, and the amino acid sequence is 54% similar to that of arginine kinase from crustaceans. Further investigation showed that octopus arginine kinase has a similar structure to that of crab, and cross-reactivity occurred after immunoblotting analysis. However, unlike heat-stable tropomyosin, arginine kinase allergenicity was reduced after thermal and pH treatment. Vertebrate creatine kinase is a homologous protein to arginine kinase and possesses similar functions. Sequence analysis of those proteins concluded that they are derived from a common origin. Similar to tropomyosin, arginine kinase has high potential to be allergenic since the similarity with its human homolog is relatively low, with below 39%.

1.4.4 Less characterised allergens

While only tropomyosin, paramyosin and arginine kinase have been confirmed to be allergenic in molluscs, this does not mean that additional proteins cannot evoke an allergic reaction. Indeed, many unknown proteins have been reported to elicit hypersensitivity. The common whelk *Buccinumun datum* was found to have several IgE binding proteins with molecular weights of 40, 71, and 82 kDa. These proteins were heat-stable but demonstrated rapid

degradation in simulated intestinal fluid and moderate degradation in simulated gastric fluid. Similarly, various proteins from the brown garden snail *Helix aspersa* were found to bind IgE from allergic patient sera [71].

Currently, the identification of allergens employs more sophisticated methods, owing to the growing number of allergen databases and low-cost DNA sequencing. The Codex Alimentarius has established a step-by-step guide for the identification of potentially allergenic proteins, and subsequent characterisation is to be registered with the IUIS (www.allergen.org.). A protein with 35% similarity within 80 amino acids sequences and 6-8 contiguous amino acids identity with known protein is considered as a potential allergen [89]. This approach has been used recently to identify putative allergens in chickpea [90] and Johnson grass pollen [91].

1.5 Effect of Food Processing on Mollusc Allergens

Empirical studies demonstrate that mollusc allergen, raw or heat treated, can evoke immune responses in allergic individuals. Tropomyosin withstands extended heat processing [92, 93] and may retain its allergenicity up to 60 min in simulated gastric fluid [73]. However, little information is available on the effect of processing on allergenicity of mollusc. In a comparative study by Kamath, Abdel Rahman [93] raw and heated protein extracts of seven mollusc species (green mussel, blue mussel, scallop, oyster, sea snail, octopus and squid) were analysed by immunoblotting with a monoclonal anti-tropomyosin antibody. The antibody only binds to the heated proteins, but not to the unheated protein, indicating that some conformational changed occurred during heating, allowing the antibody to bind to this allergen.

Another reason for this differential binding activity could be binding of reducing sugar moieties at a higher temperature to proteins, causing the phenomena called Maillard reaction. Mollusc tropomyosin is known to contain high amounts of lysine (9% of total amino acid) [66, 75], and this amino acid can easily react with reducing sugar moieties. The Maillard reaction enhances the

IgE- binding ability of tropomyosin from scallop by changing the structure of the protein [94]. However, conflicting results were found for squid [95]; when squid tropomyosin reacted with ribose, the antibody binding reactivity of tropomyosin was reduced. These results suggest that the effect of Maillard reaction on allergenicity of molluscs seems to be species-dependent. Furthermore, the amino acid homology of tropomyosin between squid and scallop is only 67%, and 21% of lysine residues are present at different positions. Since amino acid composition plays an important role in tropomyosin IgE-binding epitope, alteration of these amino acids could change the reactivity of IgE against tropomyosin. As for other mollusc allergens, arginine kinase and paramyosin, heat treatment diminished their allergenicity. Arginine kinase and paramyosin develop structural changes after heat treatment, and both allergens became insoluble [72, 78].

The effect of processing on the structure of allergens was prominent in the study of Jin, Deng [96] where they used high hydrostatic pressure to induce structural modification of squid tropomyosin Tod p 1. The tropomyosin α-helix portion was greatly reduced, and up to 53% of α-helix was converted to β-sheet and random coils, making tropomyosin ease digestibility. These structural changes seem to affect the recognition of IgE antibody against the protein, at least in the laboratory setting. Other processing technologies such as Gamma irradiation reduced the amount of tropomyosin in squid, octopuses, and cuttlefish [97].

1.6 Cross-Reactivity of Allergens

Allergenic cross-reactivity is often described in medical reports. Cross-reactivity occurs between allergens derived from different sources that share similar IgE-binding regions on the proteins [98]. Tropomyosin has been shown to be a major pan-allergen among crustacean [99], and conserved regions of the IgE-binding epitope of tropomyosin have been well characterised from prawns [100]. Some of these epitopes seem to be similar in mollusc tropomyosin. Tropomyosin has a linear conformation, meaning the primary

structure similarity, or amino acid sequence is of great importance in determining the degree of cross-reactivity between different shellfish species. This is quite different from other more complex proteins, where conformational IgE binding epitopes are more important [101].

There are only three publications from one research group specifically reporting the IgE-binding epitope of mollusc tropomyosin [66, 75, 102]. The IgE epitope regions on mollusc tropomyosin were determined by using protease digestion followed by a competitive ELISA inhibition assay. Protease digestion methods, however, have limitations in terms of uncontrolled cutting site, the size of peptides and quantity of peptides generated [103]. Indeed, the results of these methods are not very convincing in regards that the single epitope determined in the mollusc 'horned turban' could not answer the occurrence of possible cross-reactivity with other shellfish species. Furthermore, to elicit cross-linking of IgE antibodies on mast cell and/or basophil-bound IgE, the allergenic proteins needs to have multiple IgE-binding sites to initiate degranulation and release of mediators [104]. Further studies need to explore the multiple IgE-binding epitopes on molluscan allergens utilising more sophisticated methodologies, including the use of overlapping peptides. Indeed, those techniques have been successfully applied for crustacean allergens, including arginine kinase, myosin light chain and sarcoplasmic calcium binding protein [105-108].

1.6.1 Within the mollusc group

Immunological cross-reactions within mollusc species are often observed in allergic individuals. Using serum of shellfish allergic patients, Motoyama, Ishizaki [77] determined IgE cross-reactivity between 10 species of cephalopod and established cross-reaction in all species tested. Similar results were also obtained for four species of gastropods (disc abalone, turban shell, whelk, and Middendorf's buccinum) and seven species of bivalves (bloody cockle, Japanese oyster, Japanese cockle, surf clam, horse clam, razor clam, and short neck clam) using serum from 10 shellfish allergic patients [74]. In a

clinical study, 17 patients demonstrated positive RAST test to abalone, however ten of them had positive reactions to snail by RAST [69]. Moreover, the anti-abalone tropomyosin monoclonal antibody did not react with other mollusc species, including squid, oyster and octopus, yet reacted with crustaceans and chicken, confirming the epitope difference within the mollusc group. Carrillo, Castillo [109] reported seven patients with food allergy to squid and shrimp; however, the patients did not show allergic symptoms to octopus or other molluscs.

Several survey-based prevalence studies demonstrated cross-reactivity or co-sensitisation in allergic individuals. Among the individuals surveyed for mollusc allergy, 34 individuals reacted to just one species, 13 individuals to two species, 5 individuals to three species, and 15 individuals to all four mollusc species, including scallops, clams, oysters and mussels [21]. A retrospective analysis of three allergy clinics in the Texas Medical Center reported allergy to mollusc in 7 individuals, and 16.7% were allergic to more than one species [110]. Wu and Williams [111] calculated the probability of having a positive skin test to 5 mollusc species analysed, with 70 patients including 28 patients who reported severe anaphylaxis history. Cross-sensitization was demonstrated within the same group of mollusc, as limpet and abalone are gastropods and oyster, clam and scallop are bivalves. This cross-reactivity could apparently be related to the similarity of tropomyosin proteins within the group, however detailed molecular analysis of these proteins had not been conducted.

This inconsistently reported cross-reactivity raises a question as to whether tropomyosin is the only major allergen in molluscs. Even though tropomyosin is a major mollusc allergen and structurally similar among different species, the overall amino acid sequence of mollusc tropomyosins can differ up to 35%. The identity varies between each mollusc group, with higher identities are noticed between species within the same groups. Cephalopods, including octopus, squid and cuttlefish, share relative high amino acid homology with 91%-100% in their tropomyosin sequence, while gastropods, including snail,

abalone, and whelk, share only 77%-97% and bivalve share 67%-100% similarity. The molecular phylogenetic tree analysis gives a more obvious picture of how tropomyosin is more conserved among species within a related phylogenetic group (Figure 1-3). Nevertheless, patients identified to be allergic to one type of mollusc are often told to avoid other mollusc species.

1.6.2 Between mollusc and crustacean

Allergic cross-reactivity between mollusc and crustacean is not very well defined. Although it is believed that there is a high degree of cross-reactivity between mollusc and crustacean due to a similar major allergen, only few studies address this issue. In a retrospective review of seafood allergic patient data from three allergy clinics in the Texas Medical Centre, Khan, Orson [110] reported only 10.1% of 103 patients demonstrated hypersensitivity towards both, molluscs and crustaceans. This co-sensitisation is lower compared to hypersensitivity within the mollusc and crustacean groups, with 16.7% and 37.5%, respectively. Similar data were also reported by Sicherer, Muñoz-Furlong [21], based on a telephone-based survey conducted in the United States, where only 14.1% of shellfish allergic patients reported allergy to both molluscs and crustaceans. Conversely, the serological analysis demonstrates high cross-sensitisation between mollusc and crustacean. An early study showed significant RAST and skin prick test reactivity to oyster in shrimp-allergic subjects [112]. In another study, Wu and Williams [111] found 27 out of 84 patients who underwent skin testing sensitive to both molluscs and crustaceans. Nonetheless, most of the studies are based on molecular and immunological findings and not necessarily on clinical reactivity. Furthermore, cross-reactivity occurs when the patients have high titre IgE against tropomyosin as demonstrated by a study of Vidal, Bartolomé [113]. Limited cross-reactivity between crustacean and mollusc is partly due to variation of the amino acid sequence in the IgE-binding regions (Figure 1-4). Shrimp tropomyosin has six IgE-binding regions which can be used as biomarkers for clinical shrimp allergy reaction [108, 114]. Alignment of the amino acid

sequences in these regions with mollusc tropomyosins showed only one identical sequence in the position of 248-260.

Figure 1-4 IgE-binding regions of tropomyosin from three species of molluscs: oyster, horned turban and octopus in comparison to crustaceans: brown shrimp and whiteleg shrimp. The IgE-binding regions in mollusc species are shaded in yellow and in crustacean are shaded in grey. The regions possibly responsible for cross-reactivity between those species are identified by solid boxes.

1.6.3 Cross-reactivity of mollusc with other invertebrates

Cross-reactivity of mollusc and other invertebrates was firstly reported by Koshte et al. [115] in 1998, during the study of cross-reactions of inhalant insect allergen-IgE antibodies to cross-reacting carbohydrate determinants (CCD). They showed that sera from confirmed caddis fly allergic patients did not react with CCD-rich material but reacted with a protein in extracts of mussel, oyster, shrimp, crab as well as the honeybee and yellow jacket venom, similar to 13 kDa caddis fly allergen. Positive clinical cross-reactivity between molluscs and other invertebrates have been demonstrated in some populations. HDM-snail cross-reactivity was observed in patients receiving house dust mite (HDM) immunotherapy, although the identity of the problem is still unknown. A different study reported 76% of the patients were sensitised to snail ingestion at the start of HDM immunotherapy [116]. Skin prick testing of 169 children in France showed 31% of the children allergic to HDM were sensitised to snails [117]. The invertebrate pan-allergen tropomyosin seems only to be involved in a minority of the cases. In contrast, the HDM allergens responsible for the HDM-snail cross-reactivity may involve Der p 4, Der p 5, Der p 7, and hemocyanin [118].

1.7 Diagnosis and Management of Mollusc Allergy

Patients with mollusc allergy have often difficulty to identify the offending foods due to the high variety of mollusc species available. Furthermore, cross-reactivity with crustacean species makes identification of the specific mollusc allergens more complicated. Moreover, clinical manifestations due to ingestion of toxin and parasite-contaminated mollusc foods are common and can resemble allergic reactions [119] make a diagnosis of mollusc allergy more challenging. Diagnosis of mollusc allergy is based on clinical history and is aided by a sensitisation-based assays such as skin tests and allergen-specific IgE tests. However, for many mollusc species, particularly outside Europe, there are no commercial assays preparations available. For example, for over 250 edible molluscs, ImmunoCap specific IgE preparations are only available for 9 species. Moreover, the possibility of irrelevant *in-vivo* cross-sensitization

to other invertebrate species, e.g., house dust mites or crustaceans could affect the assays, resulting in false-positive results – especially in patients with poor clinical history. Unlike shrimp allergy, a convinced diagnostic tool for mollusc allergy that gives more than 95% probability of a positive oral food challenge is still lacking. Oral food challenges are the gold standard for food allergy diagnosis and are only performed in the case of the doubtful result between clinical history and blood diagnostics due to high risk of severe reactions.

In a recent review, Lopata et al. [120] recommended seven steps to guide the diagnosis of shellfish allergy. Similar guidance in the form of a decision tree is suggested in this thesis for mollusc allergy, which includes non-immunological adverse reactions due to ingestion of a toxin or parasite-contaminated molluscs (Figure 1-5). Strict avoidance of mollusc containing food is currently the only recommended management of mollusc allergy, although the advice proposed should be based on proven clinical reactions. Patients are often advised to avoid all invertebrate species when they have a confirmed allergy to any shellfish. However, this advice may only be given if the patients are sensitised to the pan-allergen tropomyosin, as reported by Vidal et al. [113].

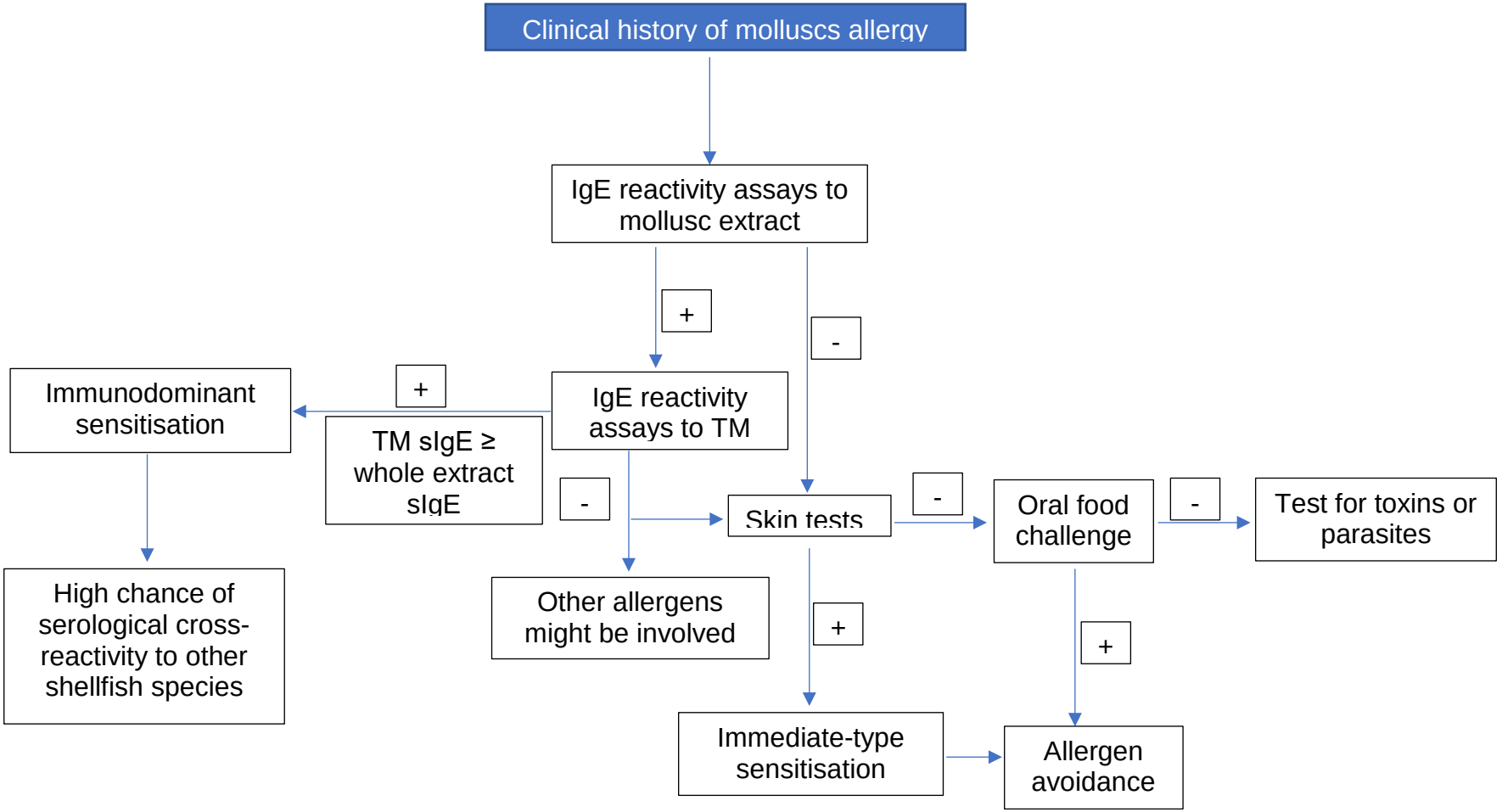


Figure 1-5 Diagnostic decision tree on how to proceed from the suspicion of mollusc-related allergic symptoms

Currently, there is no immunotherapy available for mollusc allergy, however serological cross-reactivity of tropomyosin with crustacean provides the possibility of inducing tolerance to mollusc through desensitisation to shellfish tropomyosin. Two strategies are being developed for clinical management of shellfish allergy. The first strategy is to use hypoallergenic tropomyosin to induce and generate the production of blocking IgG antibodies. Hypoallergenic tropomyosins have recently been produced from *Penaeus aztecus* Pen a 1 [121] and *Metapenaeus ensis* Met e 1 [122]. These hypoallergenic proteins were produced through either direct mutagenesis or deletion of IgE binding epitopes. However, unlike crustacean tropomyosin, where amino acid sequences are highly conserved, mollusc tropomyosin is quite variable, and their IgE-binding epitopes occur in different positions, thus development of a distinct hypoallergenic tropomyosin will be more challenging. Alternatively, peptide-based immunotherapy could be used as a safe and effective therapeutic strategy. Allergen-derived peptides would essentially be small in size, incapable of cross-linking IgE or activating effector cells, but contain the relevant CD4+ T cell epitopes. Allergen-specific T cells have been demonstrated to play an important role in allergic inflammation [123], and the conservation of T-cell epitope drives polysensitization of allergic patients to a broader range of allergen sources [124]. Given that specific tolerance could be established by induction of antigen-specific regulatory T cells or elimination of allergen-specific TH2 cells [125], the conserved T-cell epitopes of tropomyosin could be targeted to induce the desired tolerance.

1.8 Conclusions

With globalisation in the seafood-trading sector, many people have been introduced to and have consumed mollusc species that were previously unknown in the region. Cultural dietary habits and types of food processing can affect the prevalence of mollusc allergy in a specific region. For the latter, however, conflicting results are reported, and it seems that the effect of food processing on the allergenicity of mollusc allergens is species-specific. More

detailed biochemical and immunological studies are needed to fully understand the effect of processing on the allergenicity of mollusc allergens.

The current diagnostic tools for mollusc allergy are based on few species distributed in the Northern Hemisphere and do not represent important species consumed in tropical and southern regions. Compared to crustacean allergy, mollusc allergy is far less studied, resulting in poor diagnosis and management. Current methods for the identification of mollusc allergens limit the discovery of new allergens, especially low-expressed proteins. The combination of advanced proteomic and genomic approaches in conjunction with bioinformatics will allow for the development of more sensitive and specific molecular tools for the quantification of mollusc allergens and development of improved diagnostics for better patient management.

1.9 References

1. FAO, *The State of World Fisheries and Aquaculture: Opportunities and challenges*. 2014, Rome: Food and Agriculture Organization of the United Nations.
2. Telford, Maximilian J. and Graham E. Budd, *Invertebrate Evolution: Bringing Order to the Molluscan Chaos.* Current Biology, 2011. **21**(23): p. R964-R966.
3. Haszprunar, G. and A. Wanninger, *Molluscs.* Current Biology, 2012. **22**(13): p. R510-R514.
4. Prester, L., *Seafood Allergy, Toxicity, and Intolerance: A Review.* J Am Coll Nutr, 2016. **35**(3): p. 271-83.
5. Backer, L. and M. Miller, *Sentinel Animals in a One Health Approach to Harmful Cyanobacterial and Algal Blooms.* Veterinary Sciences, 2016. **3**(2): p. 8.
6. Fiocchi, A., et al., *Food Allergy*, in *WAO White Book on Allergy 2013 Update*, R. Pawankar, et al., Editors. 2013, World Allergy Organization: Wisconsin.
7. Patel, D.A., et al., *Estimating the economic burden of food-induced allergic reactions and anaphylaxis in the United States.* Journal of Allergy and Clinical Immunology, 2011. **128**(1): p. 110-115.
8. Gupta, R., et al., *The economic impact of childhood food allergy in the United States.* JAMA Pediatr, 2013. **167**(11): p. 1026-31.
9. Cummings, A.J., et al., *The psychosocial impact of food allergy and food hypersensitivity in children, adolescents and their families: a review.* Allergy, 2010. **65**(8): p. 933-945.
10. Remington, B.C., et al., *Unintended allergens in precautionary labelled and unlabelled products pose significant risks to UK allergic consumers.* Allergy, 2015. **70**(7): p. 813-819.
11. Koeberl, M., D. Clarke, and A.L. Lopata, *Next generation of food allergen quantification using mass spectrometric systems.* J Proteome Res, 2014. **13**(8): p. 3499-509.

12. Prescott, S., et al., *A global survey of changing patterns of food allergy burden in children.* World Allergy Organization Journal, 2013. **6**(21): p. 1-12.
13. Jackson, K.D., L.D. Howie, and L.J. Akinbami, *Trends in allergic conditions among children: United States, 1997–2011*, in *NCHS data brief, No 121*. 2013, National Center for Health Statistics: Hyattsville, MD.
14. Osterballe, M., et al., *The Prevalence of food hypersensitivity in young adults.* Pediatric Allergy and Immunology, 2009. **20**(7): p. 686-692.
15. Rancé, F., X. Grandmottet, and H. Grandjean, *Prevalence and main characteristics of schoolchildren diagnosed withfood allergies in France.* Clinical & Experimental Allergy, 2005. **35**(2): p. 167-172.
16. Sicherer, S.H. and H.A. Sampson, *Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment.* Journal of Allergy and Clinical Immunology, 2014. **133**(2): p. 291-307.
17. Boye, J.I., *Food allergies in developing and emerging economies: need for comprehensive data on prevalence rates.* Clinical and Translational Allergy, 2012. **2**(1): p. 25.
18. Lopata, A.L., R.E. O'Hehir, and S.B. Lehrer, *Shellfish allergy.* Clinical & Experimental Allergy, 2010. **40**(6): p. 850-858.
19. Taylor, S.L., *Molluscan shellfish allergy.* Adv Food Nutr Res, 2008. **54**(1043-4526 (Print)): p. 139-77.
20. Woods, R.K., et al., *Reported adverse food reactions overestimate true food allergy in the community.* Eur J Clin Nutr, 2002. **56**(1): p. 31-6.
21. Sicherer, S.H., A. Muñoz-Furlong, and H.A. Sampson, *Prevalence of seafood allergy in the United States determined by a random telephone survey.* Journal of Allergy and Clinical Immunology, 2004. **114**(1): p. 159-165.
22. Wu, T.C., et al., *Prevalence of food allergy in Taiwan: a questionnaire-based survey.* Intern Med J, 2012. **42**(12): p. 1310-5.

23. Lao-araya, M. and M. Trakultivakorn, *Prevalence of food allergy among preschool children in northern Thailand.* Pediatrics International, 2012. **54**(2): p. 238-243.
24. Shek, L.P.-C., et al., *A population-based questionnaire survey on the prevalence of peanut, tree nut, and shellfish allergy in 2 Asian populations.* Journal of Allergy and Clinical Immunology, 2010. **126**(2): p. 324-331.e7.
25. Taylor, S.L. and S.L. Hefle, *Will genetically modified foods be allergenic?* Journal of Allergy and Clinical Immunology, 2001. **107**(5): p. 765-771.
26. Hefle, S.L., J.A. Nordlee, and S.L. Taylor, *Allergenic foods.* Critical Reviews in Food Science and Nutrition, 1996. **36**(sup001): p. 69-89.
27. Leung, P.S.C., et al., *IgE reactivity against a cross-reactive allergen in crustacea and mollusca: Evidence for tropomyosin as the common allergen.* Journal of Allergy and Clinical Immunology, 1996. **98**(5, Part 1): p. 954-961.
28. Zhang, Y., H. Matsuo, and E. Morita, *Cross-reactivity among shrimp, crab and scallops in a patient with a seafood allergy.* J Dermatol, 2006. **33**(3): p. 174-7.
29. Osborne, N.J., et al., *Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants.* Journal of Allergy and Clinical Immunology, 2011. **127**(3): p. 668-676.e2.
30. Ben-Shoshan, M., et al., *A population-based study on peanut, tree nut, fish, shellfish, and sesame allergy prevalence in Canada.* Journal of Allergy and Clinical Immunology, 2010. **125**(6): p. 1327-1335.
31. Lau, C.H., et al., *Parent report of childhood shellfish allergy in the United States.* Allergy and Asthma Proceedings, 2012. **33**(6): p. 474-480.
32. Gupta, R.S., et al., *The prevalence, severity, and distribution of childhood food allergy in the United States.* Pediatrics, 2011. **128**(1): p. e9-17.

33. Acker, W.W., et al., *Prevalence of food allergies and intolerances documented in electronic health records.* Journal of Allergy and Clinical Immunology, 2017.
34. Steele, L., L. Mayer, and M. Cecilia Berin, *Mucosal immunology of tolerance and allergy in the gastrointestinal tract.* Immunologic Research, 2012. **54**(1-3): p. 75-82.
35. Guo, Y., Z. Li, and H. Lin, *The effect of simulated gastrointestinal digestion on shrimp Penaeus vannamei allergenicity.* Chinese Journal of Oceanology and Limnology, 2009. **27**(4): p. 703-707.
36. Untersmayr, E., et al., *Incomplete digestion of codfish represents a risk factor for anaphylaxis in patients with allergy.* Journal of Allergy and Clinical Immunology, 2007. **119**(3): p. 711-717.
37. Pali-Schöll, I., et al., *Antacids and dietary supplements with an influence on the gastric pH increase the risk for food sensitization.* Clinical & Experimental Allergy, 2010. **40**(7): p. 1091-1098.
38. Zhang, Y., et al., *Novel allergen from the freshwater clam and the related allergy.* J Dermatol, 2012. **39**(7): p. 672-4.
39. Masuda, K., et al., *Anaphylaxis to abalone that was diagnosed by prick test of abalone extracts and immunoblotting for serum immunoglobulin E.* Int J Dermatol, 2012. **51**(3): p. 359-60.
40. Rodriguez-Del Rio, P., et al., *Selective allergy to Venus antiqua clam.* Allergy, 2009. **64**(5): p. 815.
41. Masuda, K., et al., *Case of anaphylaxis due to fish and shellfish.* The Journal of Dermatology, 2008. **35**(3): p. 181-182.
42. Jiménez, M., et al., *Allergy due to Ensis macha.* Allergy, 2005. **60**(8): p. 1090-1091.
43. Azofra, J. and M. Lombardero, *Limpet anaphylaxis: cross-reactivity between limpet and house-dust mite Dermatophagoides pteronyssinus.* Allergy, 2003. **58**(2): p. 146-9.
44. Damiani, E., et al., *Adverse reaction after ingestion of raw and boiled Octopus vulgaris.* 2010(1398-9995 (Electronic)).

45. Gutierrez-Fernandez, D., et al., *Urticaria-angioedema due to limpet ingestion.* J. Investig. Allergol. Clin. Immunol, 2009. **19**(1): p. 77-79.
46. Martin-Garcia, C., et al., *Selective hypersensitivity to boiled razor shell.* J. Investig. Allergol. Clin. Immunol, 2007. **17**(4): p. 271-273.
47. Peroni, D.G., et al., *Snail anaphylaxis during house dust mite immunotherapy.* Pediatric Allergy and Immunology, 2000. **11**(4): p. 260-261.
48. Wiszniewska, M., et al., *Occupational allergy to squid (Loligo vulgaris).* Occupational Medicine, 2013. **63**(4): p. 298-300.
49. Rosado, A., et al., *Occupational asthma caused by octopus particles.* Allergy, 2009. **64**(7): p. 1101-1102.
50. Barraclough, R.M., et al., *Sensitization to king scallop (Pectin maximus) and queen scallop (Chlamys opercularis) proteins.* Occup Med 2006. **56**(1): p. 63-6.
51. Garcia-Abujeta, J.L., et al., *Occupational protein contact dermatitis in a fishmonger.* Contact Dermatitis, 1997. **36**(3): p. 163.
52. Valsecchi, R., et al., *Contact urticaria from Loligo japonica.* Contact Dermatitis, 1996. **35**(6): p. 367-8.
53. Nakamori, M., I. Matsuo, and M. Ohkido, *Coexistence of contact urticaria and contact dermatitis due to pearl oysters in an atopic dermatitis patient.* Contact Dermatitis, 1996. **34**(6): p. 438.
54. Goday B., J., A. Aguirre, and N. Gil Ibarra, *Allergic contact dermatitis from squid (Loligo opalescens).* Contact Dermatitis, 1991. **24**(4): p. 307.
55. Lopata, A.L. and M.F. Jeebhay, *Airborne Seafood Allergens as a Cause of Occupational Allergy and Asthma.* Current Allergy and Asthma Reports, 2013. **13**(3): p. 288-297.
56. Jeebhay, M.F., et al., *Occupational seafood allergy: a review.* Occupational and Environmental Medicine, 2001. **58**(9): p. 553-562.
57. Kamath, S.D., et al., *Molecular and immunological approaches in quantifying the air-borne food allergen tropomyosin in crab processing facilities.* Int J Hyg Environ Health, 2014. **217**(7): p. 740-50.

58. Hjorth, N. and J. Roed-Petersen, *Occupational protein contact dermatitis in food handlers.* Contact Dermatitis, 1976. **2**(1): p. 28-42.
59. Dickel, H., et al., *Seafood allergy in cooks: a case series and review of the literature.* JDDG: Journal der Deutschen Dermatologischen Gesellschaft, 2014. **12**(10): p. 891-901.
60. Barata, A.R.R. and L. Conde-Salazar, *Protein contact dermatitis - case report.* Anais Brasileiros de Dermatologia, 2013. **88**: p. 611-613.
61. Jeebhay, M.F. and A.L. Lopata, *Chapter 2 - Occupational Allergies in Seafood-Processing Workers*, in *Advances in Food and Nutrition Research*, H. Jeyakumar, Editor. 2012, Academic Press. p. 47-73.
62. Nieuwenhuizen, N., et al., *Differential requirements for interleukin (IL)-4 and IL-13 in protein contact dermatitis induced byAnisakis.* Allergy, 2009. **64**(9): p. 1309-1318.
63. Lees-Miller, J.P. and D.M. Helfman, *The molecular basis for tropomyosin isoform diversity.* BioEssays, 1991. **13**(9): p. 429-437.
64. Miyazawa, H., et al., *Identification of the first major allergen of a squid (Todarodes pacificus).* Journal of Allergy and Clinical Immunology, 1996. **98**(5, Part 1): p. 948-953.
65. Ishikawa, M., et al., *Isolation and Properties of Allergenic Proteins in the Oyster Crassostrea gigas.* Fisheries science, 1997. **63**(4): p. 610-614.
66. Ishikawa, M., et al., *Purification and IgE-Binding Epitopes of a Major Allergen in the Gastropod Turbo cornutus.* Bioscience, Biotechnology, and Biochemistry, 1998. **62**(7): p. 1337-1343.
67. Fujinoki, M., et al., *Heterogeneity and tissue specificity of tropomyosin isoforms from four species of bivalves.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 2006. **143**(4): p. 500-506.
68. Itoh, A. and M. Fujinoki, *Tissue specificity of tropomyosin isoform in the mussel, Mytilus galloprovincialis.* Journal of Electrophoresis, 2008. **52**(3): p. 47-52.

69. Lopata, A.L., C. Zinn, and P.C. Potter, *Characteristics of hypersensitivity reactions and identification of a unique 49 kd IgE-binding protein (Hal-m-1) in abalone (Haliotis midae).* Journal of Allergy and Clinical Immunology, 1997. **100**(5): p. 642-648.
70. Chu, K.H., S.H. Wong, and P.S.C. Leung, *Tropomyosin is the major mollusk allergen: Reverse transcriptase polymerase chain reaction, expression and IgE reactivity.* Marine Biotechnology, 2000. **2**(5): p. 499-509.
71. Asturias, J.A., et al., *Cloning, isolation, and IgE-binding properties of Helix aspersa (brown garden snail) tropomyosin.* Int Arch Allergy Immunol, 2002. **128**(2): p. 90-6.
72. Suzuki, M., et al., *Paramyosin of the disc abalone Haliotis discus discus: Identification as a new allergen and cross-reactivity with tropomyosin.* Food Chemistry, 2011. **124**(3): p. 921-926.
73. Lee, B.J. and H.S. Park, *Common whelk (Buccinum undatum) allergy: identification of IgE-binding components and effects of heating and digestive enzymes.* J Korean Med Sci, 2004. **19**(6): p. 793-9.
74. Emoto, A., S. Ishizaki, and K. Shiomi, *Tropomyosins in gastropods and bivalves: Identification as major allergens and amino acid sequence features.* Food Chemistry, 2009. **114**(2): p. 634-641.
75. Ishikawa, M., et al., *Tropomyosin, the Major Oyster Crassostrea gigas Allergen and its IgE-binding Epitopes.* Journal of Food Science, 1998. **63**(1): p. 44-47.
76. Leung, P.S.C. and K.H. Chu, *cDNA cloning and molecular identification of the major oyster allergen from the Pacific oyster Crassostrea gigas.* Clinical & Experimental Allergy, 2001. **31**(8): p. 1287-1294.
77. Motoyama, K., et al., *Cephalopod tropomyosins: Identification as major allergens and molecular cloning.* Food and Chemical Toxicology, 2006. **44**(12): p. 1997-2002.

78. Shen, H.W., et al., *Purification, cloning, and immunological characterization of arginine kinase, a novel allergen of Octopus fangsiao.* J. Agric. Food Chem, 2012. **60**(1520-5118 (Electronic)).
79. Yang, Y., et al., *Identification of triosephosphate isomerase as a novel allergen in Octopus fangsiao.* Molecular Immunology, 2017. **85**: p. 35-46.
80. Guex, N., M.C. Peitsch, and T. Schwede, *Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: A historical perspective.* Electrophoresis, 2009. **30**(S1): p. S162-S173.
81. Kelley, L.A. and M.J.E. Sternberg, *Protein structure prediction on the Web: a case study using the Phyre server.* Nat. Protocols, 2009. **4**(3): p. 363-371.
82. Edgar, R.C., *MUSCLE: multiple sequence alignment with high accuracy and high throughput.* Nucleic Acids Research, 2004. **32**(5): p. 1792-1797.
83. Tamura, K., et al., *MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0.* Molecular Biology and Evolution, 2013. **30**(12): p. 2725-2729.
84. Suzuki, M., et al., *Paramyosin from the Disc Abalone Haliotis Discus Discus.* Journal of Food Biochemistry, 2014. **38**(4): p. 444-451.
85. Lee, C.S., et al., *Protein sequence analysis of a novel 103-kDa Dermatophagoides pteronyssinus mite allergen and prevalence of serum immunoglobulin E reactivity to rDer p 11 in allergic adult patients.* Clinical & Experimental Allergy, 2004. **34**(3): p. 354-362.
86. Nieuwenhuizen, N.E. and A.L. Lopata, *Anisakis – A food-borne parasite that triggers allergic host defences.* International Journal for Parasitology, 2013. **43**(12-13): p. 1047-1057.
87. Kantha, S.S., S. Watabe, and K. Hashimoto, *Comparative Biochemistry of Paramyosin: A Review.* Journal of Food Biochemistry, 1990. **14**(1): p. 61-88.

88. Uda, K., et al., *Evolution of the arginine kinase gene family.* Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 2006. **1**(2): p. 209-218.
89. Ladics, G.S. and M.K. Selgrade, *Identifying food proteins with allergenic potential: Evolution of approaches to safety assessment and research to provide additional tools.* Regulatory Toxicology and Pharmacology, 2009. **54**(3, Supplement): p. S2-S6.
90. Kulkarni, A., L. Ananthanarayan, and K. Raman, *Identification of putative and potential cross-reactive chickpea (Cicer arietinum) allergens through an in silico approach.* Computational Biology and Chemistry, 2013. **47**(0): p. 149-155.
91. Campbell, B.C., et al., *Total transcriptome, proteome, and allergome of Johnson grass pollen, which is important for allergic rhinitis in subtropical regions.* J. Allergy Clin. Immunol., 2015. **135**(1): p. 133-42.
92. Kamath, S.D., et al., *Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: A comprehensive allergenomic approach.* Molecular Nutrition & Food Research, 2014. **58**(5): p. 1144-1155.
93. Kamath, S.D., et al., *Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies.* Food. Chem., 2013. **141**(4): p. 4031-9.
94. Nakamura, A., et al., *Effect of maillard reaction on allergenicity of scallop tropomyosin.* J Agric Food Chem, 2005. **53**(19): p. 7559-64.
95. Nakamura, A., et al., *Changes in allergenicity and digestibility of squid tropomyosin during the Maillard reaction with ribose.* J Agric Food Chem, 2006. **54**(25): p. 9529-34.
96. Jin, Y., et al., *Allergenic response to squid (Todarodes pacificus) tropomyosin Tod p1 structure modifications induced by high hydrostatic pressure.* Food and Chemical Toxicology, 2015. **76**: p. 86-93.

97. Sinanoglou, V.J., et al., *Microbial population, physicochemical quality, and allergenicity of molluscs and shrimp treated with cobalt-60 gamma radiation.* Journal of Food Protection, 2007. **70**(4): p. 958-966.
98. Bonds, R.S., R. Midoro-Horiuti T Fau - Goldblum, and R. Goldblum, *A structural basis for food allergy: the role of cross-reactivity.* Current Opinion in Allergy and Clinical Immunology 2008. **8**(1528-4050 (Print)): p. 5.
99. Reese, G., R. Ayuso, and S.B. Lehrer, *Tropomyosin: An Invertebrate Pan–Allergen.* International Archives of Allergy and Immunology, 1999. **119**(4): p. 247-258.
100. Matsuo, H., T. Yokooji, and T. Taogoshi, *Common food allergens and their IgE-binding epitopes.* Allergology International, 2015. **64**(4): p. 332-343.
101. Stephen, J.N., et al., *Allergenicity of bony and cartilaginous fish - molecular and immunological properties.* Clinical & Experimental Allergy, 2017. **47**(3): p. 300-312.
102. Ishikawa, M., et al., *Identification of tropomyosin as a major allergen in the octopus Octopus vulgaris and elucidation of its IgE-binding epitopes.* Fisheries Science, 2001. **67**(5): p. 934-942.
103. Bannon, G.A. and T. Ogawa, *Evaluation of available IgE-binding epitope data and its utility in bioinformatics.* Molecular Nutrition & Food Research, 2006. **50**(7): p. 638-644.
104. Lehrer, S.B., R. Ayuso, and G. Reese, *Current Understanding of Food Allergens.* Annals of the New York Academy of Sciences, 2002. **964**(1): p. 69-85.
105. Mao, H.Y., et al., *Structural characterization and IgE epitope analysis of arginine kinase from Scylla paramamosain.* Mol Immunol, 2013. **56**(4): p. 463-70.
106. Zheng, L.-N., et al., *Mapping IgE binding epitopes of major shrimp (Penaeus monodon) allergen with immunoinformatics tools.* Food and Chemical Toxicology, 2011. **49**(11): p. 2954-2960.

107. Ayuso, R., et al., *Greater epitope recognition of shrimp allergens by children than by adults suggests that shrimp sensitization decreases with age.* J Allergy Clin Immunol, 2010. **125**(6): p. 1286-1293 e3.
108. Ayuso, R., et al., *Is epitope recognition of shrimp allergens useful to predict clinical reactivity?* Clinical & Experimental Allergy, 2012. **42**(2): p. 293-304.
109. Carrillo, T., et al., *Squid hypersensitivity: a clinical and immunologic study.* Ann Allergy, 1992. **68**(6): p. 483-7.
110. Khan, F., et al., *Adult seafood allergy in the Texas Medical Center: A 13-year experience.* Allergy & Rhinology, 2011. **2**(2): p. e71-e77.
111. Wu, A.Y. and G.A. Williams, *Clinical Characteristics and Pattern of Skin Test Reactivities in Shellfish Allergy Patients in Hong Kong.* Allergy and Asthma Proceedings, 2004. **25**(4): p. 237-242.
112. Lehrer, S. and M. McCants, *Reactivity of IgE antibodies with crustacea and oyster allergens: Evidence for common antigenic structures.* Journal of Allergy and Clinical Immunology, 1987. **80**(2): p. 133-139.
113. Vidal, C., et al., *Sensitization pattern of crustacean-allergic individuals can indicate allergy to molluscs.* Allergy, 2015. **70**(11): p. 1493-1496.
114. Pascal, M., et al., *Molecular Diagnosis of Shrimp Allergy: Efficiency of Several Allergens to Predict Clinical Reactivity.* The Journal of Allergy and Clinical Immunology: In Practice, 2015. **3**(4): p. 521-529.e10.
115. Koshte, V.L., S.L. Kagen, and R.C. Aalberse, *Cross-reactivity of IgE antibodies to caddis fly with arthropoda and mollusca.* J Allergy Clin Immunol, 1989. **84**(2): p. 174-83.
116. Ree, R., et al., *Possible induction of food allergy during mite immunotherapy.* Allergy, 1996. **51**(2): p. 108-113.
117. Sidenius, K.E., et al., *Allergen cross-reactivity between house-dust mites and other invertebrates.* Allergy, 2001. **56**(8): p. 723-733.
118. Calderón, M.A., et al., *Respiratory allergy caused by house dust mites: What do we really know?* Journal of Allergy and Clinical Immunology, 2015. **136**(1): p. 38-48.

119. Woo, C.K. and S.L. Bahna, *Not all shellfish "allergy" is allergy!* Clinical and Translational Allergy, 2011. **1**(1): p. 3.
120. Lopata, A.L., J. Kleine-Tebbe, and S.D. Kamath, *Allergens and molecular diagnostics of shellfish allergy.* Allergo Journal International, 2016. **25**(7): p. 210-218.
121. Reese, G., et al., *Reduced Allergenic Potency of VR9-1, a Mutant of the Major Shrimp Allergen Pen a 1 (Tropomyosin).* The Journal of Immunology, 2005. **175**(12): p. 8354-8364.
122. Zimmer, J., et al., *Immunization with Hypoallergens of Shrimp Allergen Tropomyosin Inhibits Shrimp Tropomyosin Specific IgE Reactivity.* PLoS ONE, 2014. **9**(11): p. e111649.
123. Kwok, W.W., et al., *Direct ex vivo analysis of allergen-specific CD4+ T cells.* J Allergy Clin Immunol, 2010. **125**(6): p. 1407-1409 e1.
124. Westernberg, L., et al., *T-cell epitope conservation across allergen species is a major determinant of immunogenicity.* J Allergy Clin Immunol, 2016. **138**(2): p. 571-578 e7.
125. Wambre, E., et al., *Differentiation stage determines pathologic and protective allergen-specific CD4+ T-cell outcomes during specific immunotherapy.* J Allergy Clin Immunol, 2012. **129**(2): p. 544-51, 551 e1-7.

CHAPTER 2. IDENTIFICATION OF UNREPORTED ALLERGENS FROM PACIFIC OYSTER (*CRASSOSTREA GIGAS*) USING A COMBINATION OF BIOINFORMATICS, PROTEOMICS AND ALLERGENOMIC APPROACHES

Published in:

Roni Nugraha, Sandip D Kamath, Elecia Johnston, Kyall R Zenger, Jennifer M Rolland, Robyn E O'Hehir, Andreas L Lopata. 2018. Rapid and comprehensive discovery of unreported shellfish allergens using large-scale transcriptomic and proteomic resources. *Journal of Allergy and Clinical Immunology*. 141(4): 1501-1504. e8. Impact Factor: 13.258

2.1 Summary

Shellfish allergy affects over 100 million people worldwide and unexpected clinical cross-reactivities are common; yet the underlying molecular cause is often unknown. Clinical reactions are due to the daily high exposure of individuals to molecular related inhalant allergens including dust-mites, cockroaches as well as stinging insects. Despite recent technological advances, novel allergen discovery is limited by the low abundance of particular allergenic proteins, the large diversity of allergen sources, and the high variability in patient IgE antibody reactivity due to study specific populations. Here we describe a comprehensive discovery pipeline for allergenic proteins from Pacific Oyster that accounts for biological and molecular variability using allergenomics, high-throughput screening of genomic databases and high-resolution mass spectrometry.

Potential allergens were predicted from genome-derived proteome of Pacific oyster by enumerating homology searching based on protein family and sequential sequence alignment. The present of the potential allergens in the Pacific oyster extracts were detected using mass spectrometry and finally, to confirm allergenicity of the potential allergens, IgE immunoblotting using a pool serum of five shellfish allergic patient was applied.

The comparative evaluation of the in silico bioinformatics analysis with the proteomic and allergenomic data generated, confirms that 24 proteins in the raw extract and 4 proteins in the heated extract were identified using all three methods. All proteins share high amino acid identity (>50%) with allergens from different organisms and with different routes of sensitisation. This rapid discovery of allergenic proteins will have significant impact on the current management of patients and the development of new strategies for immunotherapeutics.

2.2 Introduction

Of the ‘Big eight’ allergen food groups, shellfish presents a unique challenge in terms of allergen discovery with the magnitude and diversity of consumed species, leading to heterogeneity of allergen structure and cross-reactivity among various sources. At present, 31 allergens in crustaceans have been officially registered in the WHO/IUIS Allergen Nomenclature Database as compared to 4 allergens in mollusk due to certain pitfalls in current allergy discovery approaches. Co-sensitisation of a patient with crustacean and mollusk allergy is often described, however, the current diagnostic approaches to managing these patients is not based on sufficient molecular knowledge of these shellfish allergens.

Allergen characterization is the most important underlying factor for the design and development of novel hypoallergenic-based immunotherapy to induce tolerance in allergic individuals [1]. Current strategies for allergen identification are time- and resource-consuming, which are highly prone to missing hidden allergens present in low concentrations. Allergenic proteins are traditionally identified based on their IgE antibody recognition. Soluble proteins derived from whole protein preparations of a suspected allergen source are screened for IgE antibody binding proteins using sera from individuals with clinically confirmed allergy. The IgE binding proteins are then isolated and purified using chromatographic methods and identified using bio-analytical techniques including mass spectrometry. Recombinant forms of the target proteins are frequently generated and their IgE binding capacity confirmed using immunological assays. Although this approach is the current standard for allergen identification, there are three major drawbacks. Firstly, this approach often does not detect allergenic proteins present in low abundance. Secondly, cross-reactive allergens are not easily identified due to their possible presence in unrelated allergen sources. Thirdly, the IgE recognition patterns are highly dependent on the demographics of the particular allergic patient cohort under investigation.

Recent advances in the field of genomic, bioinformatics and proteomics have unlocked new opportunities for targeted high-throughput screening and identification of proteins *in silico* based on amino acid homologies, resulting in direct comparative analysis of similar allergens from comprehensive allergen databases [2-4]. Thus, the combinatorial approach using a specific organism's genome data and comparison with a growing number of comprehensive allergen databases, immune-reactivity and high throughput protein sequence assessment can overcome the current limitations of allergen identification.

In this chapter, an innovative strategy was employed for the identification of novel cross-reactive allergenic proteins in the Pacific Oyster, using a combined approach of transcriptomic, proteomic and allergenomic methodologies and high throughput screening of large databases for proteins in addition to specific allergens. The Pacific Oyster is frequently implicated in food-induced anaphylaxis [5], however, the offending allergenic proteins remain undiscovered. Currently, tropomyosin appears to be one allergen implicated in allergic reactions to the heated oyster, although clinical cross-reactivities with house dust mite indicate that other allergens might be involved in allergic reactions [6, 7].

2.3 Aims

1. To develop an allergen identification pipeline by combining bioinformatics, proteomic and allergenomic approach.
2. To discover potential allergenic proteins from the genome of Pacific oyster using bioinformatics approach.
3. To identify the unreported allergens from Pacific oyster using the developed pipeline.

2.4 Material and Methods

2.4.1 In silico identification of potential allergens from Pacific Oyster

Bioinformatics approach was carried out to identify potential allergens of Pacific Oyster (Figure 2-1). For this purpose, two datasets were assembled. The first dataset contained a FASTA file of 25,982 genome-derived proteins of the Pacific Oyster [8] collected from the UniProt database (Proteome ID UP000005408, last modification October 9, 2016). The second dataset contained 2117 allergen sequences compiled from two main allergen databases: the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature (http://www.allergen.org/) [9] and the Food Allergy Research and Resource Program (FARRP) (Version 16, http://www.allergenonline.org/ [10]). Genbank accession IDs of all allergenic proteins were collected from these databases, and the IDs uploaded in the Batch Entrez menu on the NCBI website to obtain the sequence of the protein and remove duplicate proteins. Prior to the sequence alignment, we filtered the oyster proteome by the Pfam domains containing allergens. The latest distribution of protein families from the allergen dataset was defined by running the *hmmscan* program [11] against the Pfam database (version 29.0 [12]). The BLASTP program was employed to align the Pacific Oyster proteins and the repertoire of known allergens using a cut-off E-value of 10^{-7} and sequence identity >50%.

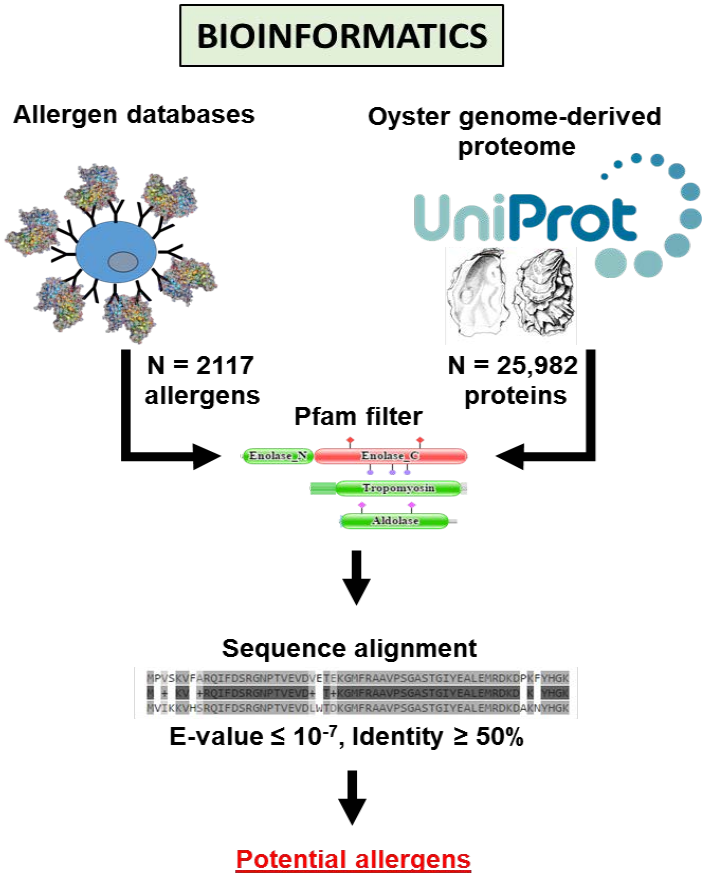


Figure 2-1. Workflow for the identification of potential allergens from the Pacific oyster using bioinformatics analysis. In silico discovery of potential allergens based on protein sequence alignment, amino acid sequence identity ≥ 50% with known allergens and E-values <10-7.

2.4.2 Gene expression analysis of potential allergens in the Pacific Oyster

The expression levels of potential allergen genes were analysed from the available RNA sequencing data from the oyster genome project [8]. The expression profiles were analysed from two developmental stages: spat and juvenile, and from ten adult organs, including the adductor muscle, the digestive gland, the female gonad, the male gonad, the gill, the hemocyte, the labial palp, the outer mantle, the inner mantle, and the remaining tissue. The expression levels were calculated by RPKM (Reads Per Kilobase of transcript per Million mapped reads).

2.4.3 Preparation of protein extracts

Fresh oyster specimens were purchased from the local market and stored at -20 °C prior to use. Protein extracts were prepared according to a method of Kamath et al. [13] with slight modification. The meat of the oyster was finely cut and homogenized in phosphate-buffered saline (PBS, pH 7.2) for 10 min, using an Ultra turrax homogenizer (IKA, Staufen, Germany). After gentle shaking at 4 °C for three h and centrifugation at 20,000 *g* for 20 min, supernatants were clarified through a glass fibre filter, followed by filtration through a 0.45 µm membrane filter (Sartorius AG, Goettingen, Germany) and stored at −80 °C until further use. To produce heated protein extracts, the meat was heated in PBS at 95-100 °C for 20 min instead of heating the raw extract, to mimic the way consumers are exposed to food allergens. The meat was removed after cooling, and the proteins were extracted using the same method as described above. The total protein content of the extracts was quantified using the bicinchoninic acid (BCA) assay (Thermo Scientific™, USA) following the manufacturer's instructions. A pre-diluted set of bovine serum albumin was used as protein standards (Pierce™).

2.4.4 Proteomic analysis of the Pacific oyster extracts

Proteomic analysis of the Pacific Oyster raw and heated extracts was identified after trypsin in-gel digestion as described in Figure 2-2. Briefly, 20 µg of the extracts were loaded onto a 12% polyacrylamide gel and run at 170 V for 1 h. The gels were cut into pieces and washed with 25 mM ammonium bicarbonate (AMBIC). After being dried using a vacuum dryer, the gels were reduced by 20 mM dithiothreitol (DTT) at 65°C for 1 h and alkylated with 50 mM iodoacetamide for 40 min at 37°C in the dark. Gel pieces were washed and dried using a SpeedVac. Dried gel pieces were rehydrated with 20 ng/µl of trypsin for 1 h at room temperature and subsequently incubated overnight at 37°C. The digested proteins were acidified using 0.1% formic acid and the peptides were concentrated on a SpeedVac and subjected to Liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis.

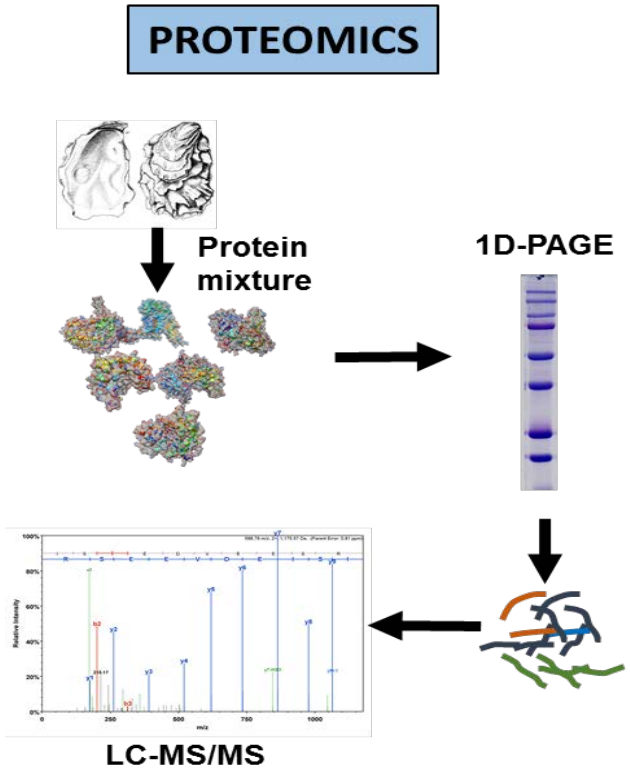


Figure 2-2. Workflow for the identification of all proteins present in the extracts of Pacific oyster. The proteins were separated using 1D SDS-PAGE, tryptic-digested and identified using mass spectrometry.

2.4.5 Patient selection

Five subjects with a convincing clinical history of allergic reactivity to shellfish (Table 2-1) and one non-atopic subject were recruited from The Alfred Hospital Allergy Clinic, Melbourne, Victoria, Australia. Skin prick testing and oral challenge with mollusc extracts were not conducted routinely in these patients, in keeping with the clinicians' preference for safer serum specific allergen IgE testing in adult patients due to comorbidities, together with the clinical history of reactions on exposure. Ethics approval for this study was granted by James Cook University's Ethics Committee (Project number H4313) in collaboration with The Alfred Hospital (Project number 192/07) and Monash University's Ethics Committees (MUHREC CF08/0225).

Table 2-1 Demographics of patients recruited for this study. Note: NT=Not Tested.

Subject	Sex	Age	Total IgE	Specific IgE (ImmunoCAP) kU/L			Other Allergies
				Oyster	Shrimp	HDM	
1	M	50	976	2.04	9.03	13.60	Tuna, Cod
2	F	28	461	0.11	0.36	54.8	NT
3	M	43	194	NT	1.41	0.35	NT
4	F	38	28	3.75	9.82	2.66	NT
5	M	38	183	1.04	6.84	31.70	NT

2.4.6 Allergenomic analysis of the Pacific oyster extracts

Allergenomic analysis of the Pacific oyster extracts was described in the **Error! Reference source not found.**. Proteins were first lyophilized and resuspended in a 8 M urea, 2% CHAPS, 50 mM DTT and 0.2%(w/v) Biolyte 3/10 ampholytes buffer. The extract was subjected to isoelectric focusing using a 3–10 NL pH range 12% ReadyStrip™ IPG Strips (Bio-Rad, USA), as per the manufacturer's instructions. Briefly, 185 µl of rehydration buffer containing 200 µg of raw extract or 100 µg of the heated extract was loaded on to the IPG tray, and a strip was gently placed side down on to the sample and left to incubate overnight at room temperature. Isoelectric focusing was conducted using a PROTEAN IEF cell (Bio-Rad, USA) with a maximum current of 50 µA/strip. After focusing, the IPG strips were equilibrated with equilibration buffer (ES) 1 and ES 2. The ES 1 contained 6 M urea, 2% SDS, 20% glycerol, 0.375 M Tris-HCl pH 8.8 and 2% (w/v) DTT, and the ES 2 contained the same solution as ES 1 except it contained 4% iodoacetamide instead of DTT. The strip was then washed with SDS-PAGE running buffer and laid on top of a 12% polyacrylamide gel. The gels were run at 170 V until bromophenol blue dye reached the bottom of each gel. Gels were either stained with Coomassie Brilliant Blue R-250, or the separated proteins were transferred to

polyvinylidene difluoride (PVDF) membrane. Protein transfer was performed using the Semi-dry TransBlot Apparatus (BioRad, USA). After blocking with 5% (w/v) skim milk powder in phosphate buffered saline with 0.05% Tween (PBS-T), the membrane was incubated with a serum pool from five shellfish-allergic patients at a 1:20 dilution overnight at 4°C with shaking. The membrane was subsequently incubated with 1:10.000 dilution of rabbit anti-human IgE (Dako, France) followed by 1:10.000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibodies (Promega, USA). Specific IgE binding was detected by chemiluminescence and exposed to photographic film (GE Healthcare Biosciences, USA) to visualize the antibody-binding protein spots. Serum from a non-atopic donor was used as a negative control. IgE reactive spots were annotated using the proteome map, and corresponding bands were cut, tryptic digested and analysed using mass spectrometry.

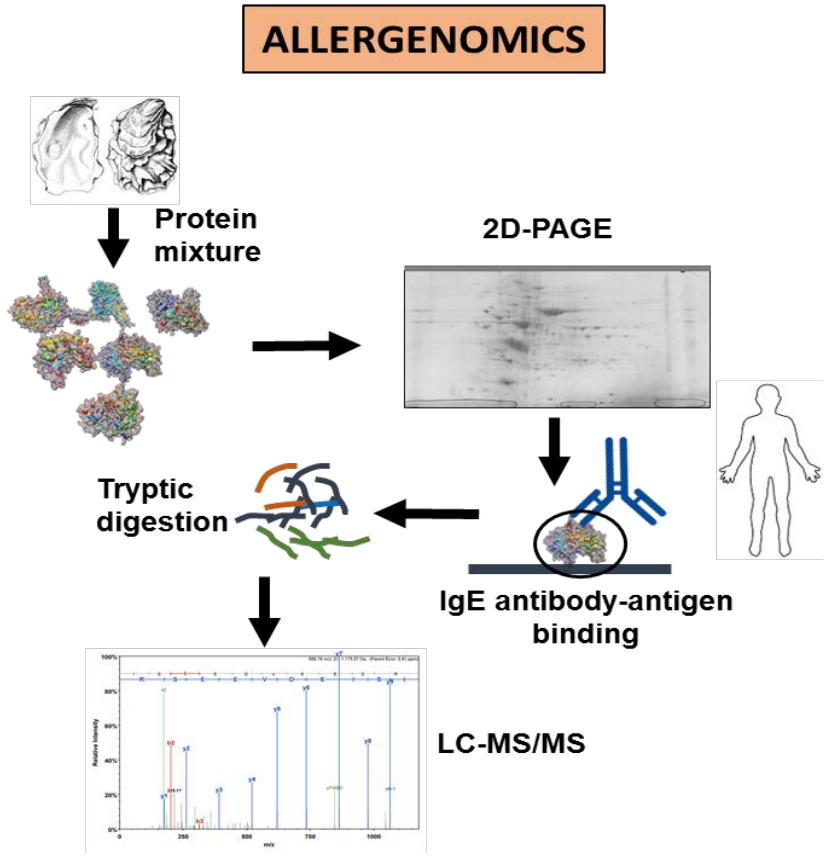


Figure 2-3. Workflow for the identification of IgE-reactive proteins from the extracts of Pacific oyster. IgE-reactive proteins were identified by 2D-immunoblotting and subsequent LC-MS/MS.

2.4.7 Mass Spectrometry Analysis

The LC-MS/MS was carried out on an LTQ Orbitrap Elite (Thermo Scientific) with a nano ESI interface in conjunction with an Ultimate 3000 RSLC nano-HPLC (Dionex Ultimate 3000) at the Bio21 Institute, Melbourne. The LC system was equipped with an Acclaim Pepmap nano-trap column (Dionex-C18, 100 Å, 75 µm x 2 cm) and an Acclaim Pepmap RSLC analytical column (Dionex-C18, 100 Å, 75 µm x 50 cm). The tryptic peptides were injected into the enrichment column at an isocratic flow of 5 µL/min of 3% v/v CH_3CN containing 0.1% v/v formic acid for 5 min before the enrichment column was switched in-line with the analytical column. The eluents were 0.1% v/v formic acid (solvent A) and 100% v/v CH_3CN in 0.l% v/v formic acid (solvent B). The

flow gradient was (i) 0-5 min at 3% B, (ii) 5-25 min, 3-25% B (iii) 25-27 min, 25-40% B (iv) 27-29 min, 40-80% B (v) 29-31 min at 80% B (vii) 31-32 min, 80-3% B and (viii) 32-38 min at 3% B. The LTQ Orbitrap Elite spectrometer was operated in the data-dependent mode with nanoESI spray voltage of 1.8 kV, a capillary temperature of 250°C and S-lens RF value of 55%. All spectra were acquired in positive mode with full scan MS spectra from m/z 300-1650 in the FT mode at 240,000 resolution. Automated gain control was set to a target value of $1.0^{-6,}$ and a lock mass of 445.120025 was used. The top 20 most intense precursors were subjected to rapid collision induced dissociation (rCID) with a normalized collision energy of 30 and activation q of 0.25. A dynamic exclusion of 30 seconds was applied for repeated precursors.

2.4.8 Protein Identification

All MS/MS files were analysed using Mascot v2.4 against the in-house database of the oyster proteome downloaded from the UniProt, supplemented with the common Repository of Adventitious Proteins sequences. Search parameters were as follows: precursor mass tolerance of 200 ppm, fragment mass tolerance of 0.6 Da (CID). Carbamidomethyl (C) was set as a fixed modification and oxidation (M) and deamidated (NQ) were set as variable modifications. Trypsin with a maximum of 3 missed cleavages was used as the cleavage enzyme. Scaffold (version Scaffold_4.7.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm[14] with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm[15]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

2.5 Results

2.5.1 Potential allergens of the Pacific oyster

Promising allergen candidates were selected by enumerating proteins in the first dataset that contain allergen protein domains. These domains were detected by interrogating Hidden Markov Models (HMM) profiles using HMMER3 [11]. The allergen protein domains were derived from the Pfam database version 29.0 [12] and accounted only 273 domains or merely 2% of total 16,295 protein domains specified in the Pfam database. The distribution of allergen family was highly biased toward a few protein families with ten most abundant families represented nearly 50% of all documented allergenic molecules. Among animal allergens, tropomyosin is the most dominant protein family followed by EF-hand protein family. Using HMMER3 2,504 Pacific Oyster proteins were associated to at least one of the allergen protein domains. These proteins were distributed into 186 families

Subsequently, these candidates were submitted to BLASTP program [16] to identify proteins that were homologous to the allergens in the second dataset. A minimum of 50% shared identity [17] and an upper threshold of E = value of 10^{-7} [18] were used as display limits of alignment. Using these two criteria, 95 proteins that have a significant identity with known allergenic proteins were identified. These 95 proteins were categorized two groups based on the percentage of the amino acid identity with known allergens. Twenty-two proteins were categorized as “very likely allergenic” and 73 proteins were “likely allergenic” (Figure 2-4).

Very likely allergenic – Twenty-two proteins were grouped into this cluster due to their high identity (>70%) with known allergens. Eighteen out of 22 very likely allergenic proteins shared high identity with the allergens from related invertebrate species including mollusc, crustacean, mosquito, and mite. Meanwhile, the other four very likely allergenic proteins shared with allergens from fish and plant.

Likely allergenic – The seventy-three proteins in this cluster displayed amino acid identity between 50% and 70%. Unlike the first cluster, proteins in this likely allergenic group shared identity with known allergens from many mollusc-unrelated sources, including fungi, pollens, and plants.

Functional annotations of the potential allergens were conducted using DAVID [19]. DAVID functional annotation clustering measures annotation terms by the degree of their co-association; highly similar annotations are clustered into functional annotation groups. Seventy five oyster potential allergens could be mapped to an internal DAVID ID and grouped into 4 clusters including the Gene Ontology terms “isomerase” (enrichment score 8.72, maximum count 9, p-value 9.4E-13), “heat-shock protein 70” (enrichment score 4.15, maximum count 5, p-value 1.5E-7), “Alpha-tubulin” (enrichment score 2.38, maximum count 3, p-value 2.1E-4) and “Redox-active center” (enrichment score 2.01, maximum count 3, p-value 5.1E-4).

2.5.2 Expression profiles of potential allergens

A comprehensive analysis of the expression of allergen genes across different developmental stages and in various tissues was conducted based on transcriptomic data from the oyster genome project [8]. As illustrated in Figure 2-5, potential allergens were differentially expressed across developmental stages and various tissues of the Pacific Oyster. In general, as well as structural proteins being more highly expressed compared to enzymes, the expression of enzyme-encoding genes was also consistent between spat, juvenile and adult. The structural muscle proteins were generally more highly expressed in the spat, compared to juvenile, and have varying levels of expression in the different tissues of adults. Twenty-four out of 95 Pacific oyster potential allergens were expressed during spat stages and gradually down-regulated during further development. Similarly, fourteen proteins were expressed higher in juvenile than in the spat or adult tissues. Four proteins including two arginine kinases (CGI_10024056 & CGI_10021480), tubulin

alpha-3 chain (CGI_10018930) and heat shock protein 68 (CGI_10002823) and were male-specific proteins.

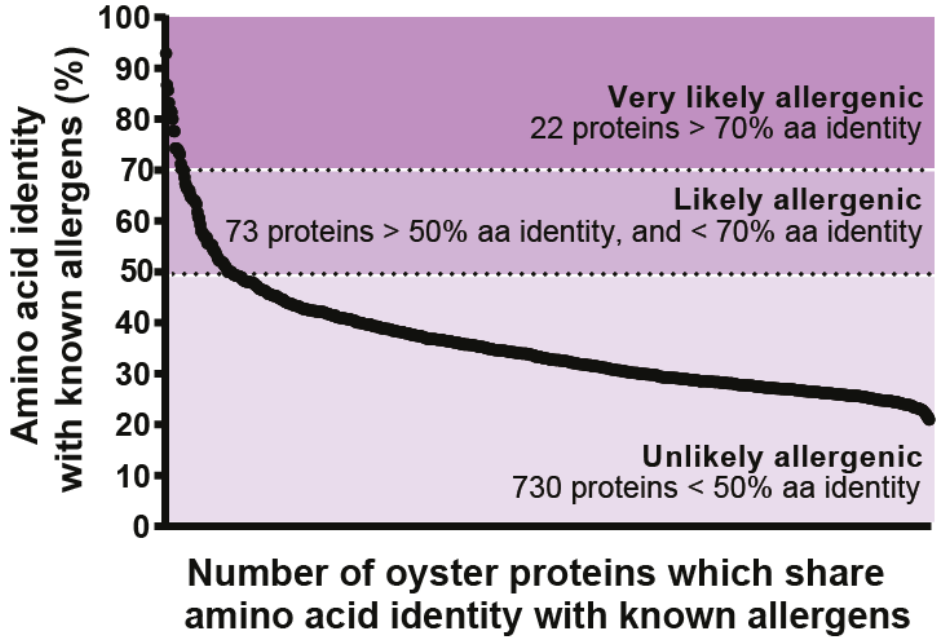


Figure 2-4.. *In silico* analysis identified 22 proteins as “very likely allergenic” (aa identity ≥70%) and 73 proteins as “likely allergenic” (aa identity ≥ 50%, and ≤70%).

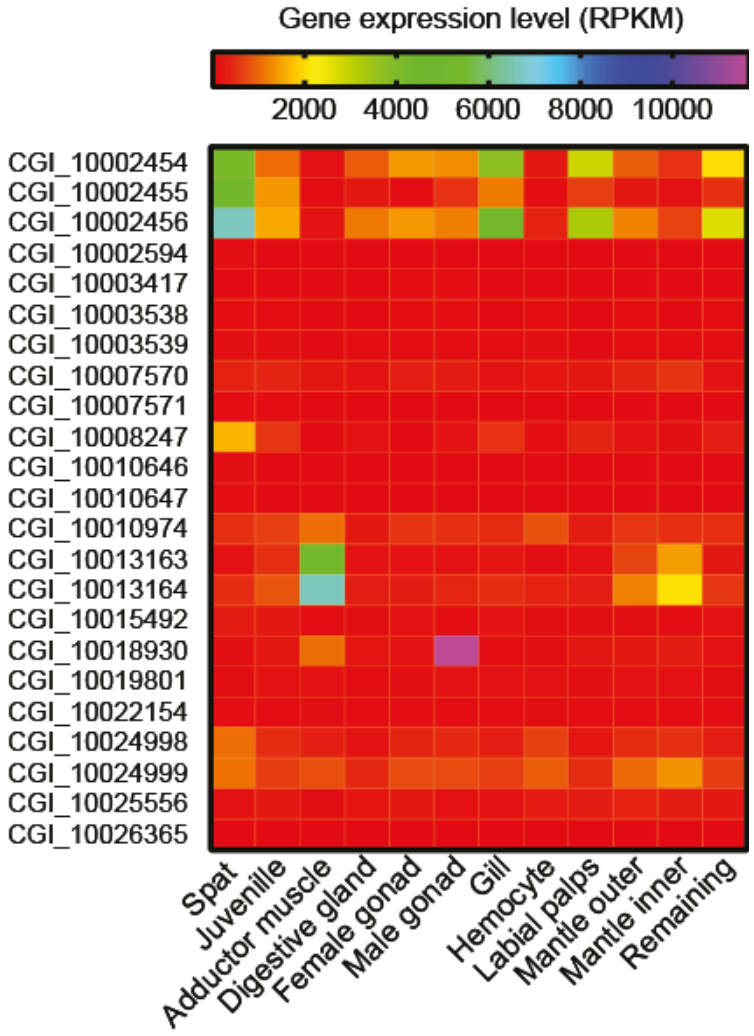


Figure 2-5. The expression levels of the “very likely allergenic” proteins were different across development stages and organs of the Pacific Oyster.

2.5.3 Analysis of the Pacific oyster proteome

Protein compositions of the Pacific oyster extracts were identified using a bottom-up mass spectrometry approach. The proteins were separated on an SDS-PAGE gel, cut into small pieces and tryptic digested (Figure 2-6). The peptide mixtures were then subjected to LC-MS/MS analysis, generating a total of 81831 spectra for the raw extract and 88130 spectra for the heated extract. Mascot searches were conducted against Pacific oyster proteomes, and the identified proteins were validated by Scaffold (version Scaffold_4.7.3)

using the criteria that at least two peptides were present with a 99.0% probability. In total Scaffold identified 1,086 proteins from raw extract and 130 proteins from the heated extract, with 103 proteins being shared between both extracts. A quantitative measurement of protein abundance, based on the exponential modified protein abundance index (emPAI), showed that sarcoplasmic calcium binding protein, arginine kinase, and fatty acid binding protein were the three most abundant proteins in the raw extract, while sarcoplasmic calcium binding protein, tropomyosin and myosin light chain were the most abundant in the heated extract (Figure 2-7). Meanwhile, gene ontology analysis of the identified proteins showed the number of proteins involved in catalytic activity were greatly reduced after heat treatment while binding proteins became dominant (Figure 2-8).

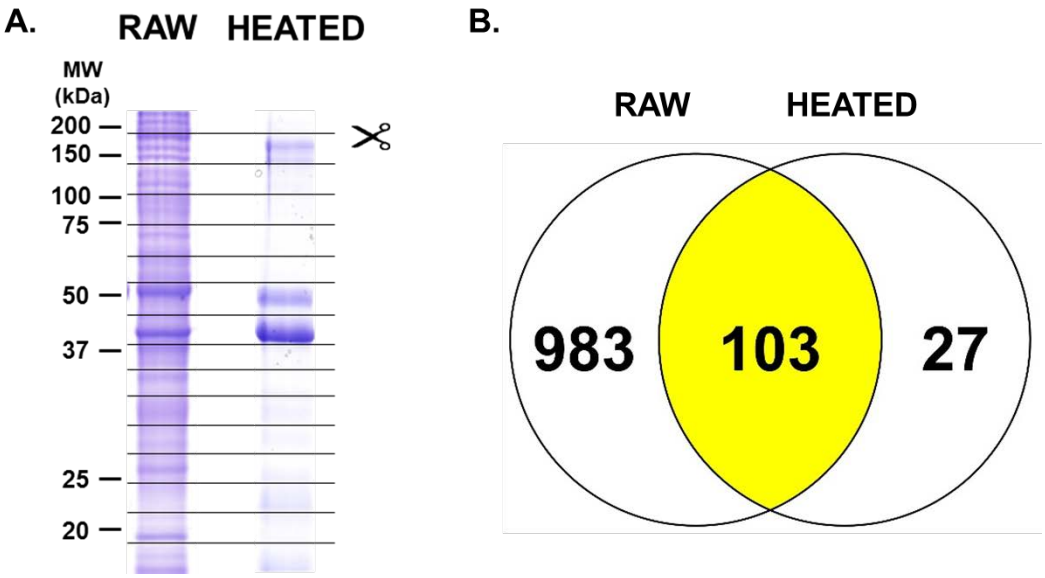


Figure 2-6. Identification of total proteins from the Pacific oyster extracts. **(A)**. The gels were cut into pieces and digested using trypsin and total proteins were identified. **(B)**. Venn diagram shows shared proteins between raw (N=1,086), heated (N= 130).

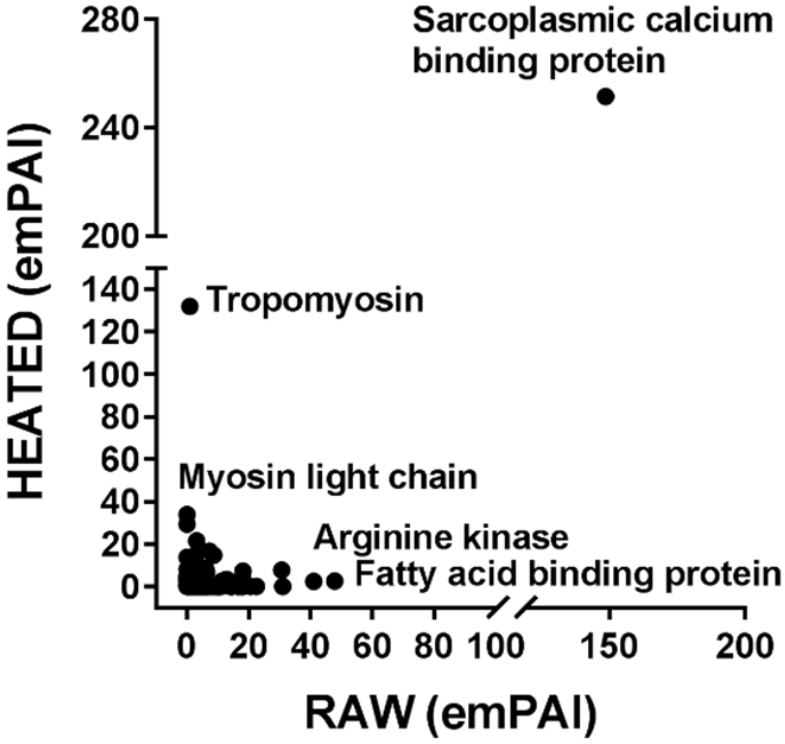


Figure 2-7. A quantitative measurement of protein abundance based on the exponential modified protein abundance index (emPAI).

2.5.4 Identification of IgE-reactive proteins in the Pacific oyster

The next step of the technique involved allergenomics where IgE-binding proteins were identified using a serum pool from five mollusk allergic patients. Protein extracts from raw and heated Pacific Oyster were separated by two-dimensional electrophoresis, and either stained using Coomassie Blue or transferred to PVDF membranes for IgE antibody recognition by immunoblotting (Figure 2-9). Twenty-two IgE-reactive spots were excised from the resolved raw oyster extract and five spots from the heated extract, followed by in-gel tryptic digestion and analyzed using mass spectrometry. A total of 332 and 26 proteins identified in the IgE-reactive spots of the raw and heated extracts, respectively. Due to the complexity of the proteins in the extract, multiple proteins were revealed in single spots, with one spot contained up to

104 proteins. Additionally heterogeneity of the proteins probably caused by gene polymorphisms, alternative splicing, or post-translational modifications [20] were discovered with 62 proteins were found in two spots. However, two proteins including retinal dehydrogenase I and filamin were identified in more than 16 spots. Meanwhile, in the heated extract, only retinal dehydrogenase I was identified in multiple spots. Nonetheless, many of these spots were dominated by one or two proteins (Figure 2-10). For example, spot number 1, 4, 5 and 19 in the raw extract were dominated by a single protein called tropomyosin (80%), 60 kDa heat shock protein (55%), 78kDa glucose regulated protein (49%) and glyceraldehyde-3-phosphate dehydrogenase (74%), respectively, meanwhile spots in the heated extract were dominated by tropomyosin, except spot number 26 where myosin regulatory light chain A was the dominant protein (49%).

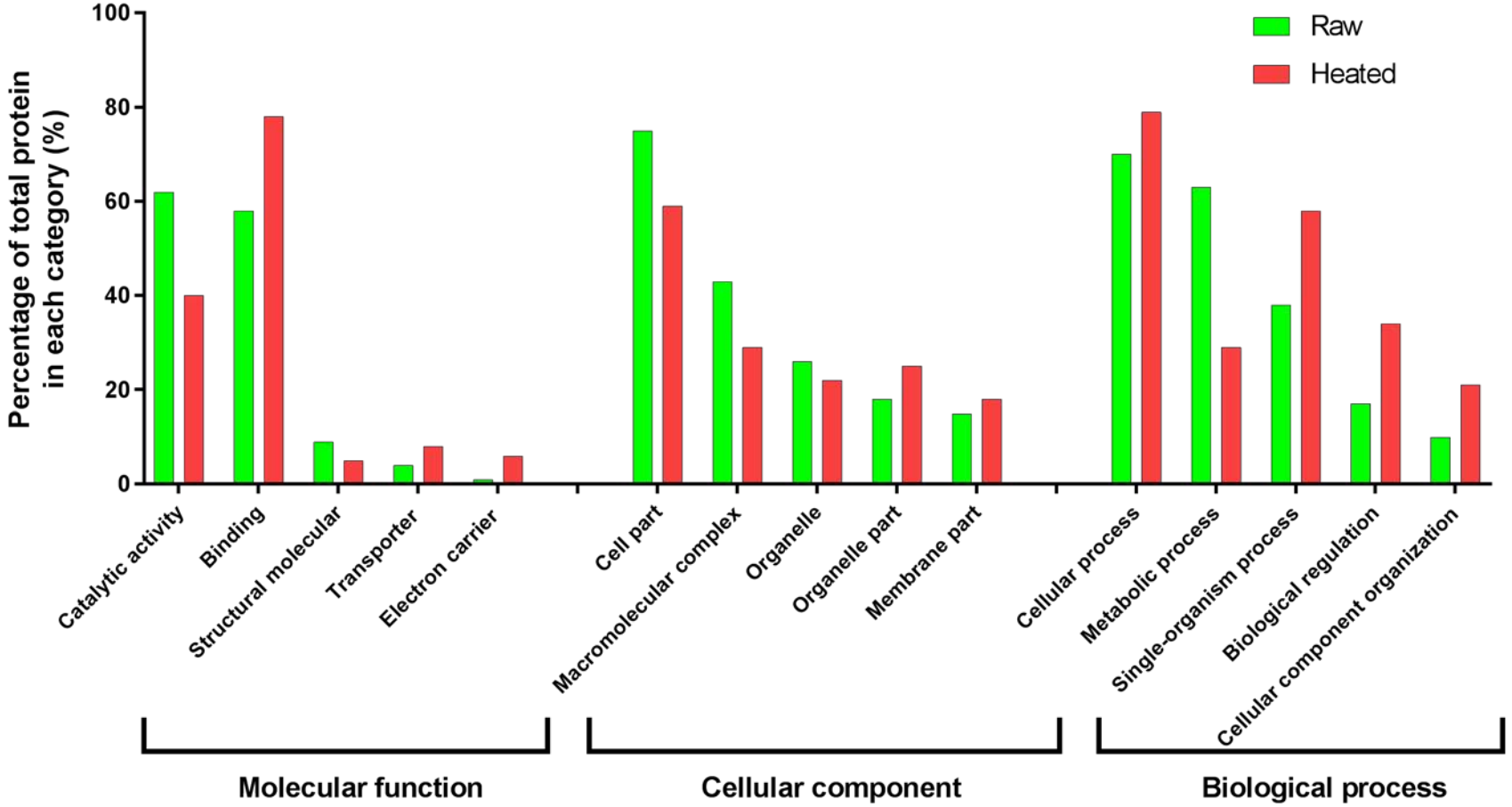


Figure 2-8. Gene ontology analysis of total proteins identified in the raw and heated extracts. The bar plots show the top 5 terms of molecular function, cellular component and biological processes.

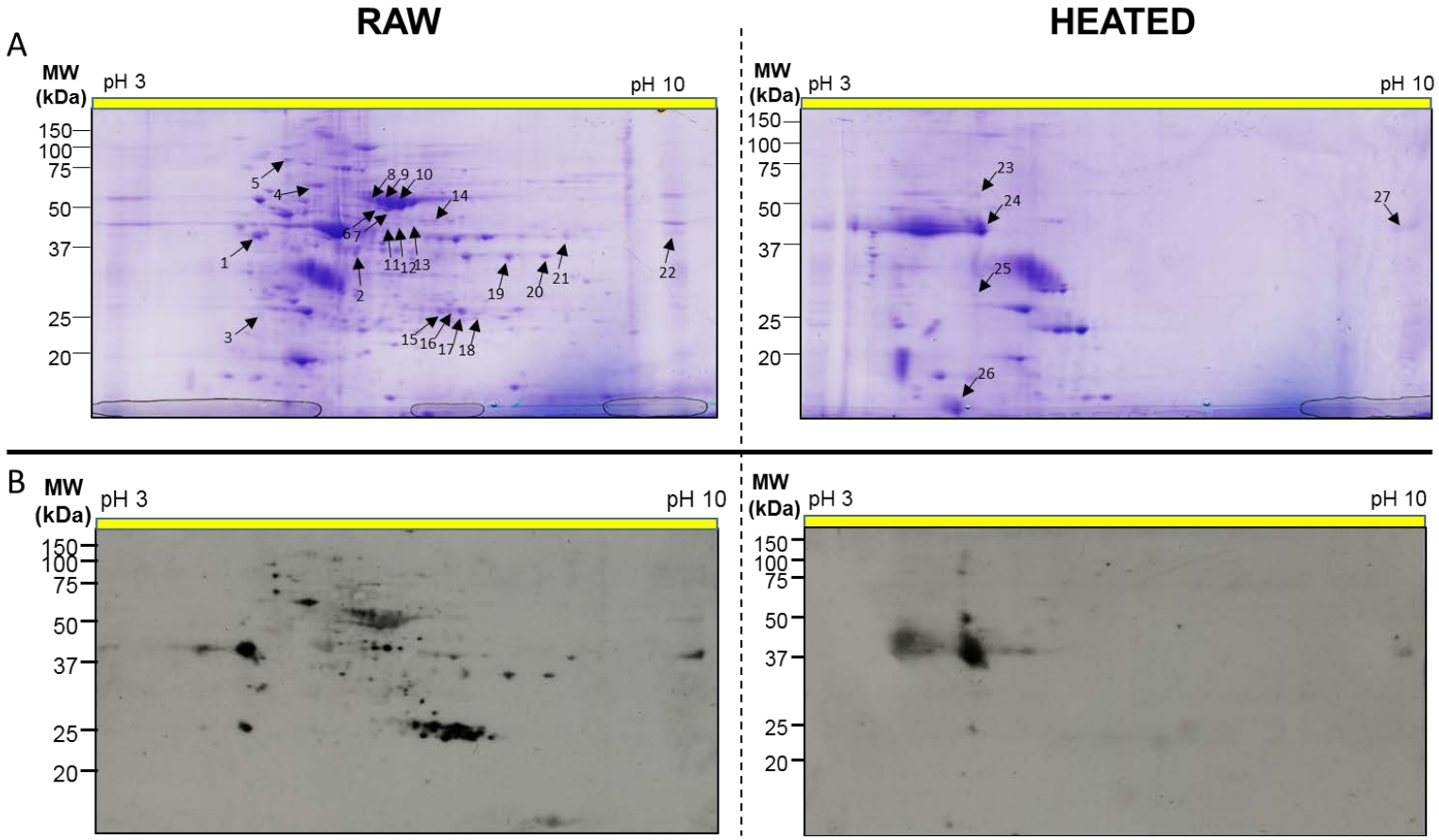


Figure 2-9. Separation of proteins based on isoelectric point using 2D-PAGE. **(A)** Raw and heated extracts were separated by 2D-PAGE (pH 3 - pH 10, Non-Linear) and stained with Coomassie Brilliant Blue. **(B)** Separated proteins were transferred to PVDF membrane followed by incubation with serum of pool of five patients with mollusc allergy. Spot numbers indicated on the gel were subjected to mass spectrometry analysis.

2.5.5 Identification of unreported allergens of Pacific oyster

Unreported allergens of the Pacific oyster was determined by comparative evaluation of the *in silico* bioinformatics analysis with the proteomic and allergenomic data. From 95 potential allergenic proteins identified by the *in silico*, 44 proteins were detected in the proteome analysis of the Pacific oyster extracts. We further analysis these proteins by comparing with the repertoire of proteins identified in the IgE-reactive spots and found 24 proteins were identified in the three methods used, with four of these only identified in the heated extract (Figure 2-11 and Table 2-2). These 24 proteins including tropomyosins, triosephosphate isomerases, enolases, glyceraldehyde-3-phosphate dehydrogenase, 78kDa glucose regulated protein, fructose-bisphosphate aldolases, heat shock protein HSP 90-alpha 1, retinal dehydrogenases 1, aldehyde dehydrogenases, transaldolase, arginine kinase, inorganic pyrophosphatase, stress-70 protein, endoplasmin, protein disulfide-isomerases, peptidyl-prolyl cis-trans isomerase, malate dehydrogenase and paramyosin. The tropomyosins and retinal dehydrogenases 1 were also identified in the heated extract. All proteins share high amino acid identity (>50%, E-value < 10^{-10}) with allergens from different organisms and with different routes of sensitisation.

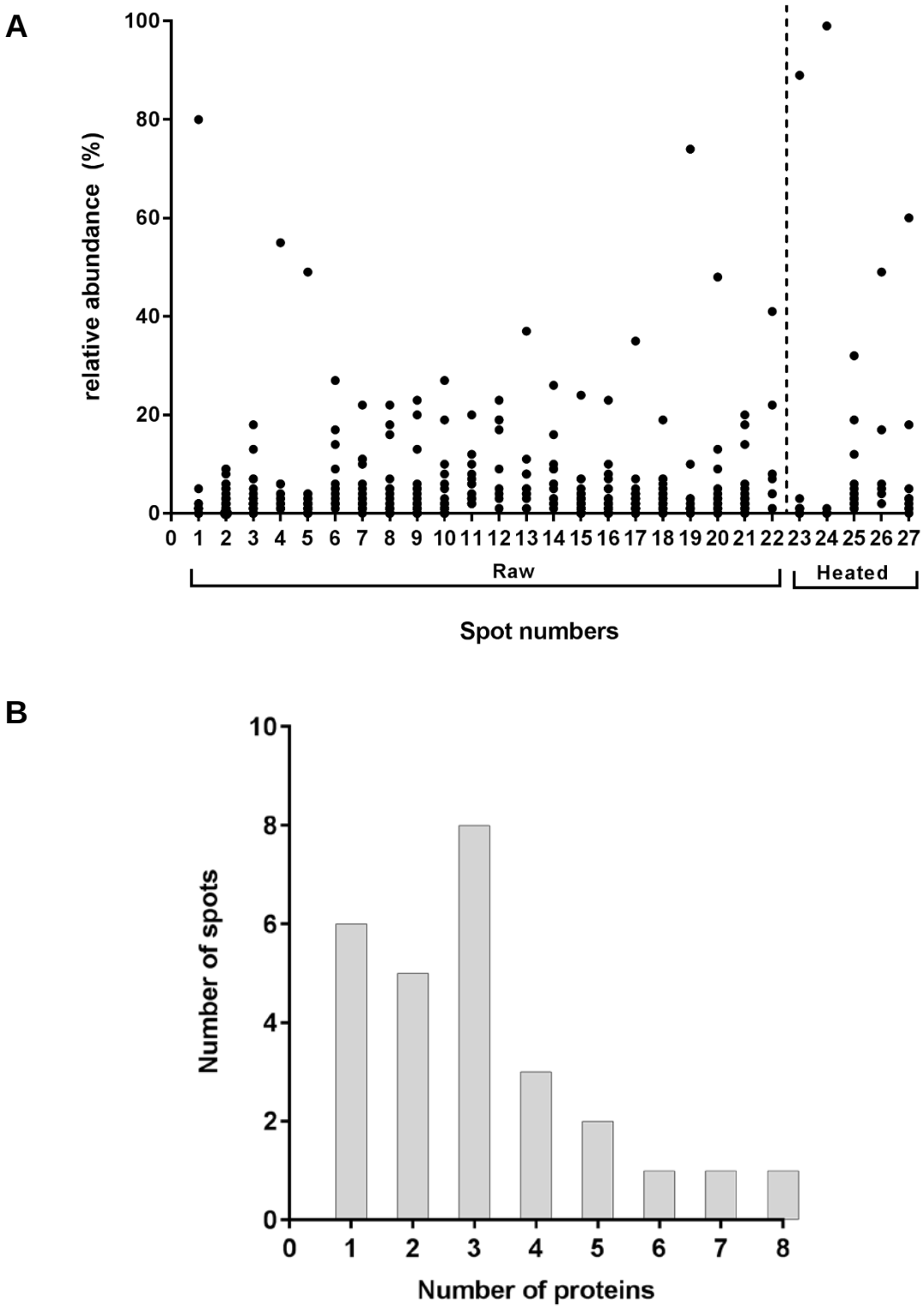


Figure 2-10. Overview of protein abundance in each IgE-reactive spot. **(A)** the exponentially modified protein abundance index (emPAI) values (%) of the identified proteins within a 2-DE spot. **(B)** The bar plot shows the number of proteins contribute to over 50% of the total emPAI in each spot.

Figure 2-11. Venn diagram displaying proteins shared by the bioinformatics, the allergenomic and the proteomic approach. Numbers of proteins shared by the three methods are coloured in red. Twenty-four proteins in the raw extract and four proteins in the heated extract were identified across all three methods.

Table 2-2. Proteins identified across all three methods with their matched allergens source and routes of sensitization. Proteins identified in both raw and heated extract are shaded. The proteins are sorted based on amino acid sequence identity with their homologous allergens in descending order.

No	Entry	Protein name	Homologous allergen in IUIS database*	Amino Acid identity (%)	Overlap (AA)	E-value	Organism	Route of sensitisation	Source
1	**B7XC66**	**Tropomyosin**	Hel as 1	75.7	284	4.00E-133	*Helix aspersa* (Brown garden snail)	Ingestion	Animal
2	**K1PCV6**	**Triosephosphate isomerase**	Cra c 8	74.03	77	2.00E-41	*Crangon crangon* (North sea shrimp)	Ingestion	Animal
3	**K1PJ59**	**Triosephosphate isomerase**	Der f 25	73.37	169	6.00E-92	*Dermatophagoides farinae* (House dust mite)	Inhalation	Animal
4	**K1QX37**	**Enolase**	Thu a 2	72.83	357	0	*Thunnus albacares* (Yellowfin tuna)	Ingestion	Animal
5	**K1Q350**	**Glyceraldehyde-3-phosphate dehydrogenase**	Tri a 34	71.21	330	7.00E-173	*Triticum aestivum* (Wheat)	Ingestion	Plant
6	**Q75W49**	**78kDa glucose regulated protein**	Cor a 10	71.04	618	0	*Corylus avellana* (European hazelnut)	Inhalation	Plant

7	**K1QTC1**	**Paramyosin**‡	-	68.59	799	0	*Haliotis discus discus* (Abalone)	Ingestion	Animal
8	**K1R8R6**	**Fructose-bisphosphate aldolase**	Sal s 3	67.31	364	4.00E-177	*Salmo salar* (Atlantic salmon)	Ingestion	Animal
9	**K1RTQ6**	**Fructose-bisphosphate aldolase**	Sal s 3	66.67	363	1.00E-178	*Salmo salar* (Atlantic salmon)	Ingestion	Animal
10	**K1PNQ5**	**Heat shock protein HSP 90-alpha 1**	Asp f 12	66.26	412	0	*Aspergillus fumigatus*	Inhalation	Fungi
11	**K1QNV6**	**Tropomyosin**	Tod p 1	65.31	271	3.00E-84	*Todarodes pacificus* (Squid)	Ingestion	Animal
12	**K1R266**	**Retinal dehydrogenase 1**	Tyr p 35	61	472	1.00E-170	*Tyrophagus putrescentiae* (Storage mite)	Inhalation	Animal
13	**K1QNT7**	**Aldehyde dehydrogenase, mitochondrial**	Tyr p 35	60	482	1.00E-180	*Tyrophagus putrescentiae* (Storage mite)	Inhalation	Animal

14	**K1QVK0**	**Transaldolase**	Fus p 4	59.2	326	1.00E-125	*Fusarium proliferatum*	Inhalation	Fungi
15	**K1QVG5**	**Retinal dehydrogenase 1**	Tyr p 35	59	474	1.00E-169	*Tyrophagus putrescentiae* (Storage mite)	Inhalation	Fungi
16	**K1PLF9**	**Arginine kinase**	Bomb m 1	59.13	345	3.00E-147	*Bombyx mori* (Slik moth)	Ingestion	Animal
17	**K1Q3F4**	**Inorganic pyrophosphatase**	Der f 32	58.24	261	1.00E-113	*Dermatophagoides farinae* (House dust mite)	Inhalation	Animal
18	**K1Q9Z4**	**Aldehyde dehydrogenase**	Tyr p 35	56	195	8.00E-65	*Tyrophagus putrescentiae* (Storage mite)	Inhalation	Animal
19	**K1P9D0**	**Stress-70 protein, mitochondrial**	Pen c 19	55.22	431	3.00E-163	*Penicillium citrinum*	Inhalation	Fungi
20	**K1QX26**	**Endoplasmin**	Asp f 12	54.04	198	9.00E-61	*Aspergillus fumigatus*	Inhalation	Fungi
21	**K1Q7T5**	**Protein disulfide-isomerase**	Alt a 4	52.27	44	3.00E-11	*Alternaria alternata*	Inhalation	Fungi
22	**K1Q5P7**	**Peptidyl-prolyl cis-trans isomerase**	Cat r 1	52.17	161	5.00E-53	*Catharanthus roseus* (Madagascar periwinkle)	Inhalation	Plant

23	**K1R4Z3**	**Malate dehydrogenase, mitochondrial**	Mala f 4	51.43	280	1.00E-91	*Malassezia furfur*	Inhalation	Fungi
24	**K1Q6X5**	**Protein disulfide-isomerase**	Alt a 4	50	68	1.00E-15	*Alternaria alternata*	Inhalation	Fungi

* Only homologous allergen with the highest identity showed

‡ No paramyosin currently registered in IUIS database. The alignment was determined against allergens from AllergenOnline database

2.6 Discussion

Pacific oyster belongs to bivalve group in mollusc phylum and often referred as shellfish in general term. Shellfish allergy affects over 100 million people worldwide, and unexpected clinical cross-reactivities are common, yet the underlying molecular cause is often unknown. Clinical reactions are due to the daily high exposure of individuals to molecular related inhalant allergens including dust-mites, cockroaches as well as stinging insects. Shellfish allergens are often identified from heat-treated extract to mimic the way of people consume the shellfish. However, unlike other shellfish species, oysters are mainly consumed as raw food and in a whole form. This consumption style implies heat-labile and –stable allergenic proteins could induce allergy reactions without being affected by pre-processing of the oyster. This chapter demonstrates a comprehensive identification of the unreported allergens from Pacific oyster using a combinatorial approach of bioinformatics, proteomics and allergenomics.

Identification of allergens has transformed into more sophisticated methods, thanks to the growth of the allergens database and cheaper next-generation sequencing. Combinations of bioinformatics, proteomic and allergenomic data can identify putative allergens which could be hidden when conventional methods are used. To date, many studies have used bioinformatics to predicted potential allergens from particular species. Recently, 38 and 31 putative allergens were predicted present in *Anisakis simplex* and *A. pegreffi,* respectively [4]. Similar method has been done as well for chickpea [21], peanut [22], citrus [23] and other species. Nevertheless, these study did not further confirm the allergenicity of the putative allergens. Other studies on Johnson grass pollen [3] and ragweed pollen [24], although used patient serum, focused their prediction to identify genes of pollen related allergens.

In silico identification is based on the knowledge that allergenic proteins are distributed only in restricted protein families, 2% of all sequence-based and 5% of all structural protein families [25]. Restricted protein family distribution

of allergens was previously found for food allergens from plants [25] and animals [26] and pollen allergens [27] and is extended in this study to all allergens, irrespective of their source and route of exposure. Allergenic proteins can also be predicted based on sequential sequence similarity (i.e., identical and similar residues) as allergens have the potential to cross-react if two or more allergens share amino acid sequence to the degree that there is shared IgE antibody binding. Based on current studies the best method to identify proteins that are known to be allergens or so similar in the sequence is to use a local alignment method (BLASTP or FASTA) with identity scores of greater than 50% [10]. To increase sensitivity, E-value from BlastP was added as another criterion in selecting the potential allergens. In total, ninety-five proteins were identified as potential allergens after the *in silico* identification against the Pacific oyster proteome, with 22 proteins are categorized as “very likely allergenic” with an amino acid identity ≥70% with known allergens and 73 proteins as “likely allergenic” with amino acid identities between 50% and 70%.

Proteomic data can be used to provide protein-level evidence of gene expression and protein abundance in the sample. According to our screened data, a total of 44 putative allergens is annotated in the raw and heated extract of Pacific oyster, of which 16 proteins are identified in both extracts. As would be expected, structural proteins still remain in the heated extract, while many enzymes could not be detected in that extract.

The comparative evaluation of the *in silico* bioinformatics analysis with the allergenomic and proteomic data generated, confirms that 24 proteins in the raw extract and four proteins in the heated extract were identified using all three methods. All proteins share high amino acid identity (>50%) with allergens from different organisms and with different routes of sensitisation. These allergenic proteins have a high probability to elicit immunological cross-reactivity in oyster allergic patients to other organisms containing the same or highly similar allergenic proteins. For example, triosephosphate isomerase and

78 kDa glucose regulated protein share 74% and 65% amino acid identity with dust-mite allergens, possibly responsible for the clinical mollusk-mite cross-reactivity, previously reported during mite immunotherapy [7]. Oyster enolase shares 61-71% amino acid identity with latex, fish, grass pollen and fungi allergens, however, clinical reactivity due to enolase of oyster and these allergen sources has not yet been reported. The question may arise on how minor proteins could influence IgE binding of a spot in the immunoblotting. We know that many studies assumed dominant proteins based on Mascot score in an IgE-reactive spot were the actual allergens and that assumptions may lead to overlooking the true allergens and to assign non-allergenic protein as an allergen. Many proteins have similar molecular weight and isoelectric point, and some of the proteins are likely allergenic, and the other are non-allergenic. Moreover, a contamination of protein spot with 0.01% of true allergens may have a great impact on the IgE-binding as suggested by many studies [28-31]

The importance of early identification of unreported allergens is corroborated by clinical studies on confirmed cross-allergenicity of specific allergens between shrimp and dust-mite in orthodox Jews. This patient cohort was prohibited from consuming seafood due to strict dietary laws, and therefore the sensitization to the cross-reactive allergen tropomyosin must have occurred through inhalation of HDM [32, 33]. Indeed, in a recent study of serological investigation of patients with shrimp sensitization, 44% of 16 patients negatively challenged to shrimp are sensitized to tropomyosin [34]. Furthermore, using a combination of ten recombinant allergens, they were able to distinguish the severity of clinical reactivity of shrimp allergic patients and to predict cross-reactivity with other allergens sources. Clinical cross-allergenicity can also occur between completely unrelated allergen sources, such as the chicken-fish syndrome, where the offending allergens of this syndrome were only recently characterized [35]. Immunological cross-reactivity due to a high degree of amino acid sequence conservation is also observed in other diseases. Dengue virus-induced antibodies cross-react with Zika virus, both at a patient serum level as well as with monoclonal antibody producing

plasmablast, due to an amino acid sequence identity of >50% between Zika virus envelope (E) protein and dengue virus E protein [36, 37]. Thus, the utilization of cutting-edge technologies and large databases assists in identifying specific proteins, not only for allergens but immunogenic proteins in general.

In summary, this methodological approach utilizing biochemical and computational tools in addition to antibody reactivity was successful in identifying 24 unreported allergens from over 25,000 proteins of the Pacific Oyster. This approach is the first study to demonstrate the presence of 24 unreported allergens, also establish in very different allergen sources from animals, including fish and mites, as well as plant allergens from pollen, latex and fungi. Importantly all of these allergenic proteins identified are reactive to shellfish allergic patients' IgE antibodies.

This comprehensive discovery pipeline is a significant improvement over current approaches for the identification and characterization of allergenic proteins, providing a new tool for researchers developing better diagnostics and novel immunotherapeutics. The preferential discovery of hidden allergenic proteins fills a major gap in the current management of patients at high-risk of concurrent reactivity to diverse allergen sources.

2.7 References

1. Yu, W., D.M.H. Freeland, and K.C. Nadeau, *Food allergy: immune mechanisms, diagnosis and immunotherapy.* Nat Rev Immunol, 2016. **16**(12): p. 751-765.
2. Cui, Y., et al., *Transcriptomic/proteomic identification of allergens in the mite Tyrophagus putrescentiae.* Allergy, 2016. **71**(11): p. 1635-1639.
3. Campbell, B.C., et al., *Total transcriptome, proteome, and allergome of Johnson grass pollen, which is important for allergic rhinitis in subtropical regions.* J Allergy Clin Immunol, 2015. **135**(1): p. 133-42.
4. Baird, F.J., et al., *The Anisakis Transcriptome Provides a Resource for Fundamental and Applied Studies on Allergy-Causing Parasites.* PLoS Negl. Trop. Dis., 2016. **10**(7): p. e0004845.
5. European Food Safety, A., *Opinion of the Scientific Panel on Dietetic products, nutrition and allergies (NDA) related to the evaluation of molluscs for labelling purposes.* EFSA Journal, 2006. **4**(3): p. 25.
6. Bessot, J.C., et al., *Tropomyosin or not tropomyosin, what is the relevant allergen in house dust mite and snail cross allergies?* Eur Ann Allergy Clin Immunol, 2010. **42**(1): p. 3-10.
7. Tsapis, M., et al., *Fatal anaphylaxis after snail ingestion in a child after 3 years of house dust mite immunotherapy.* Revue Francaise D Allergologie, 2013. **53**(4): p. 436-438.
8. Zhang, G., et al., *The oyster genome reveals stress adaptation and complexity of shell formation.* Nature, 2012. **490**(7418): p. 49-54.
9. Radauer, C., et al., *Update of the WHO/IUIS Allergen Nomenclature Database based on analysis of allergen sequences.* Allergy, 2014. **69**(4): p. 413-9.
10. Goodman, R.E., et al., *AllergenOnline: A peer-reviewed, curated allergen database to assess novel food proteins for potential cross-reactivity.* Mol Nutr Food Res, 2016. **60**(5): p. 1183-98.
11. Eddy, S.R., *Accelerated Profile HMM Searches.* PLoS Comput Biol, 2011. **7**(10): p. e1002195.

12. Finn, R.D., et al., *The Pfam protein families database: towards a more sustainable future.* Nucleic Acids Res, 2016. **44**(D1): p. D279-85.
13. Kamath, S.D., et al., *Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies.* Food Chem, 2013. **141**(4): p. 4031-9.
14. Keller, A., et al., *Empirical Statistical Model To Estimate the Accuracy of Peptide Identifications Made by MS/MS and Database Search.* Anal. Chem., 2002. **74**(20): p. 5383-5392.
15. Nesvizhskii, A.I., et al., *A statistical model for identifying proteins by tandem mass spectrometry.* Anal. Chem., 2003. **75**(17): p. 4646-58.
16. Altschul, S.F., et al., *Basic local alignment search tool.* J. Mol. Biol., 1990. **215**(3): p. 403-10.
17. Aalberse, R.C., *Structural biology of allergens.* J. Allergy Clin. Immunol, 2000. **106**(2): p. 228-38.
18. Silvanovich, A., G. Bannon, and S. McClain, *The use of E-scores to determine the quality of protein alignments.* Regul Toxicol Pharmacol, 2009. **54**(3 Suppl): p. S26-31.
19. Huang, D.W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nature Protocols, 2008. **4**(1): p. 44-57.
20. Thiede, B., et al., *High Resolution Quantitative Proteomics of HeLa Cells Protein Species Using Stable Isotope Labeling with Amino Acids in Cell Culture(SILAC), Two-Dimensional Gel Electrophoresis(2DE) and Nano-Liquid Chromatograpohy Coupled to an LTQ-OrbitrapMass Spectrometer.* Molecular & Cellular Proteomics, 2013. **12**(2): p. 529-538.
21. Kulkarni, A., L. Ananthanarayan, and K. Raman, *Identification of putative and potential cross-reactive chickpea (Cicer arietinum) allergens through an in silico approach.* Computational Biology and Chemistry, 2013. **47**: p. 149-155.

22. Chen, X., et al., *Draft genome of the peanut A-genome progenitor (Arachis duranensis) provides insights into geocarpy, oil biosynthesis, and allergens.* Proceedings of the National Academy of Sciences, 2016. **113**(24): p. 6785-6790.
23. Serra, I.A., et al., *The Citrus clementina Putative Allergens: From Proteomic Analysis to Structural Features.* Journal of Agricultural and Food Chemistry, 2013. **61**(37): p. 8949-8958.
24. Ryffel, B., et al., *Identification of Novel Short Ragweed Pollen Allergens Using Combined Transcriptomic and Immunoproteomic Approaches.* Plos One, 2015. **10**(8): p. e0136258.
25. Radauer, C. and H. Breiteneder, *Evolutionary biology of plant food allergens.* Journal of Allergy and Clinical Immunology, 2007. **120**(3): p. 518-525.
26. Jenkins, J.A., H. Breiteneder, and E.N.C. Mills, *Evolutionary distance from human homologs reflects allergenicity of animal food proteins.* Journal of Allergy and Clinical Immunology, 2007. **120**(6): p. 1399-1405.
27. Radauer, C. and H. Breiteneder, *Pollen allergens are restricted to few protein families and show distinct patterns of species distribution.* Journal of Allergy and Clinical Immunology, 2006. **117**(1): p. 141-147.
28. Koppelman, S.J., et al., *Relevance of Ara h1, Ara h2 and Ara h3 in peanut-allergic patients, as determined by immunoglobulin E Western blotting, basophil-histamine release and intracutaneous testing: Ara h2 is the most important peanut allergen.* Clinical <html_ent glyph="@amp;" ascii="&"/> Experimental Allergy, 2004. **34**(4): p. 583-590.
29. Lauer, I., et al., *Hazelnut (Corylus avellana) vicilin Cor a 11: molecular characterization of a glycoprotein and its allergenic activity.* Biochemical Journal, 2004. **383**(2): p. 327-334.
30. Aalberse, R.C., *Is 99.9% purity good enough for allergens?* Allergy, 2011. **66**(9): p. 1131-1132.

31. D'Avino, R., et al., *Kiwifruit Act d 11 is the first member of the ripening-related protein family identified as an allergen.* Allergy, 2011. **66**(7): p. 870-877.
32. Lopata, A.L., J. Kleine-Tebbe, and S.D. Kamath, *Allergens and molecular diagnostics of shellfish allergy: Part 22 of the Series Molecular Allergology.* Allergo. J. Int., 2016. **25**(7): p. 210-218.
33. Fernandes, J., et al., *Immunoglobulin E antibody reactivity to the major shrimp allergen, tropomyosin, in unexposed Orthodox Jews.* Clin. Exp. Allergy., 2003. **33**(7): p. 956-61.
34. Pascal, M., et al., *Molecular Diagnosis of Shrimp Allergy: Efficiency of Several Allergens to Predict Clinical Reactivity.* The Journal of Allergy and Clinical Immunology: In Practice, 2015. **3**(4): p. 521-529.e10.
35. Kuehn, A., et al., *Cross-reactivity to fish and chicken meat - a new clinical syndrome.* Allergy, 2016. **71**(12): p. 1772-1781.
36. Dejnirattisai, W., et al., *Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus.* Nat. Immunol., 2016. **17**(9): p. 1102-8.
37. Priyamvada, L., et al., *Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus.* Proc. Natl. Acad. Sci. U.S.A, 2016. **113**(28): p. 7852-7.

CHAPTER 3. EFFECTS OF EXTRACTION BUFFER ON THE SOLUBILITY AND IMMUNOREACTIVITY OF THE PACIFIC OYSTER ALLERGENS

Published in:

Roni Nugraha, Sandip D Kamath, Thimo Ruethers, Shaymaviswanathan Karnaneedi, Elecia Johnston, Andreas L Lopata. 2018. Effects of extraction buffers on the solubility and immunoreactivity of the Pacific Oyster (Crassostrea gigas) allergens. Food Chemistry. Manuscript in preparation.

3.1 Summary

Over 2,000 allergens have currently been discovered, 907 of which have been assigned a unique nomenclature by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. Despite recent technological advances, novel allergenic protein discovery is limited by their low abundance, often due to particular physical characteristics restricting the recovery of the proteins during extraction process from the allergen source. In particular tissue from molluscs is known to be difficult to extract due to the combination of soft and stringy tissue. In chapter 2 it was ascertained that not all transcriptome derived allergens were actually identified in the proteome of oyster.

In this study, eight different extraction buffers were compared for their ability to recover proteins from Pacific oyster (*Crassostrea gigas*). The IgE-reactivity of each extract was determined against a pool of serum from five shellfish allergic patients. In addition, the protein composition was identified using high resolution mass spectrometry.

Most of the investigated buffers showed good capacity to extract proteins from the Pacific oyster. In general a higher concentration of proteins were recovered using high salt content or high pH buffers and subsequently revealing more IgE-reactive bands on the immunoblotting. In contrast, low pH buffers resulted in a poor protein recovery and negatively affected IgE-reactivity. Mass spectrometry analysis demonstrated that more additional IgE-reactive proteins were detected due to the higher abundance in the extract. In conclusion, increasing the ionic strength and pH of the buffers improves the solubility of allergenic proteins during the extraction process in oyster tissue, and could also apply for other difficult allergen sources..

3.2 Introduction

Food allergy is an over reaction of the human immune system to particular proteins called allergens. Upon exposure to the human immune system, allergenic proteins trigger the production of allergen-specific IgE antibodies which bind to receptors on the surface of mast cells and basophils. Subsequent contact of these allergenic proteins with cell-bound antibodies results in activation of the cells leading to mediator release and clinical symptoms [1]. Currently, over 2,000 allergens have been identified, 907 are analysed in detail and have been assigned a unique nomenclature by the WHO/IUIS Allergen Nomenclature Sub-committee. Mollusc is a major group of shellfish and combine together with crustacean are leading cause of food allergy in adults. However, mollusc allergy is clinically underreported, and their allergens are ill-defined. Despite recent technological advances, novel allergenic protein discovery is limited by their low abundance, often due to particular physical characteristics restricting the recovery of the proteins during the extraction process from the allergen source. In particular, various tissues from molluscs are known to be difficult to extract due to the combination of soft and stringy tissue. Consequently, current in-vitro diagnostic tools of mollusc allergy still rely on the preparations made of raw or cooked extracts [2].

In chapter 2, the bioinformatics analysis of the Pacific oyster genome identified 95 potential allergens. These proteins belong to known protein families of various allergens, and the sequence similarity with their homologous allergens is very high. However, after proteomic analysis of protein extracts from the oyster it was observed that not all identified potential allergens were present in the extract using phosphate buffer. In fact only 44 from the 95 genome derived allergens could be identified in the protein extract. The shortcoming of extractability of commonly used buffers, such as phosphate-buffered saline (PBS) or tris-buffered saline (TBS) have been shown in several studies. Elvin Gomez Cardona, Heathcote [3] et al were unable to extract allergens from mango using TBS without additional treatments. Similarly, paramyosin was overlooked during IgE-binding analysis of abalone *Haliotis discus discus*

proteins extracted using PBS, but was observed after increasing the NaCl concentration in the buffer up to 0.9 M [4]. This highlights the importance of a correct extraction method for a specific allergen and implementation of appropriate buffering system for maximum recovery of allergenic proteins.

Several studies compared different factors that are known to influence extractability of proteins to optimise the extraction of allergens from different food sources such as peanut [5, 6], and shrimp [7]. Most studies, however, focused on the extraction of the major allergens, and thus skipped the presence of other allergens that also contribute to the allergic reaction. Unlike shrimp or other shellfish species, the oyster is often consumed raw. It has been known that some of the shellfish allergens are heat sensititive [8-10], and that the presence of these heat labile allergens may depend on proper extraction procedure. It is therefore, of particular importance to investigate the effect of buffer composition on the protein and allergen content of both raw extracts and heated extracts.

3.3 Aims

The aims of this study were as follows;

1. To compare the extractability of various extraction buffers for improving allergenic protein discovery from the Pacific oyster, and
2. To analyse the difference in IgE-reactivity of extracts from various buffer induced by different allergen composition.

3.4 Materials and Methods

3.4.1 Preparation of extraction buffers

To determine the effects of extraction buffers on the composition of soluble proteins, eight different buffers were prepared for a comparison (Table 3-1). Phosphate-buffered saline (PBS) and Tris-buffered saline (TBS) buffers with low ionic strength, pH 7.4, were included as internal controls since they are the most frequently used buffers for the extraction of proteins. Sodium chloride was used as an additive for the PBS and TBS buffers to prepare high ionic strength buffers. Carbonate buffers with generally high pH are commonly used as coating or coupling buffers in enzyme-linked immunosorbent assay (ELISA) and lateral flow device (LFD) development. Therefore, they were included in this investigation to determine the effect of higher pH. Citrate buffers were chosen as a low-pH buffer of choice to cover a wider pH range for the investigation.

3.4.2 Preparation of oyster soluble protein extracts

Five grams of minced oysters were added to 25 mL of each extraction buffer and homogenised using T 10 basic ULTRA-TURRAX (IKA, Germany) and subsequently stirred overnight at 4 °C. The extracts were centrifuged at 15,000x*g* for 15 min, and the clear supernatant was further filtered through 0.45 µm membrane to attain the final extracts. These extracts were designated as raw extracts. Meanwhile, heated extracts were obtained by heating the aliquot of the raw extracts inside a water bath at 100°C for 15 min. These extracts were then centrifuged and processed as above. All extracts were stored at -20°C until further analysis.

3.4.3 Quantification of protein content

The concentration of protein in each extract was estimated using the bicinchoninic acid assay (BCA) kit (Pierce Biotechnology Inc., Rockford, USA) following the protocol as described in the Material and Method section in Chapter 2.

Table 3-1 Buffers and their composition used to extract proteins from Pacific oyster

Buffer	pH	Chemical composition
Citrate-3	3.0	Citric acid 0.082 M Trisodium citrate 0.018 M
Citrate-5	5.0	Citric acid 0.065 M Trisodium citrate 0.035 M
TBS	7.4	Tris 25 mM Potassium Chloride 3.0 mM Sodium Chloride 140 mM
TBSN	7.4	Tris 25 mM Potassium Chloride 3.0 mM Sodium Chloride 1 M
PBS	7.4	Phosphate 10 mM Potassium Chloride 2.7 mM Sodium Chloride 137 mM
PBSN	7.4	Phosphate 10 mM Potassium Chloride 2.7 mM Sodium Chloride 1 M
Carbonate-9	9.2	Sodium Carbonate 0.01 M Sodium Bicarbonate 0.09 M
Carbonate-10	10.3	Sodium Carbonate 0.07 M Sodium Bicarbonate 0.03 M

3.4.4 SDS–PAGE and IgE-reactive analysis of oyster extracts

The protein components of extracts were profiled using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [11]. A solution of each extract containing 10 µg of protein was mixed with Laemmli buffer and heated at 95°C for 5 minutes. The solution was loaded onto each of the wells of SDS-acrylamide gel and the proteins

were separated at 170 V for 1 h. The resolved protein bands on the gel were stained with Coomassie Brilliant Blue and visualised using the Odyssey ® CLx Imaging System (LI-COR Biosciences).

For IgE binding analysis, after the electrophoresis was run, the proteins were transferred to a nitrocellulose membrane using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Hercules, USA). Subsequently, the membrane was blocked using Casein blocking solution (Sigma, St. Louise, USA) for 1 h at room temperature. The blocked-nitrocellulose membrane was incubated overnight in a serum pooled from five shellfish-allergic patients diluted 1:20 in PBST added with casein. After the washing step, secondary anti-human IgE with (1: 10,000 dilution) was added and incubated for one hour. The membrane was subsequently incubated for 35 minutes with anti-rabbit IgG antibody conjugate with IR (1:10.000 dilution), and IgEantibody binding was visualised using the Odyssey ® CLx Imaging System (LI-COR Biosciences). IgE reactive spots were annotated to the protein profile on SDS-PAGE, and selected corresponding bands were cut, tryptic digested and analysed using mass spectrometry.

3.4.5 Proteomic profiling of oyster extracts

The protein composition of each extract was identified using the shotgun mass spectrometry analysis. Gel-aided sample preparation (GASP) technique was used to prepare the samples following the procedure described by Fischer and Kessler [12]. Fifty microlitres solution containing 100 µg of proteins was denatured for 20 min in the presence of 50 mM of dithiothreitol (DTT) to reduce disulfide bridges. An equal volume of 40% acrylamide-bis solution (37.5:1) (Merck) was added, mixed gently and left at left at room temperature for 20 min. Subsequently, 5 µLof tetramethylethylenediamine (TEMED) and 5 µL of 10% ammonium persulfate (APS) were added and left at room temperature to initiate polymerisation. The gel plug was removed upon the completion of polymerisation and transferred to a minicolumn (Promega) in which the filter membrane had been removed previously by dissolving in acetone. A solution

containing methanol/acetic acid/water (50/40/10) was added to fix the gel pieces. The proteins were then digested following the protocol described in the Material and Method section in Chapter 2.

3.4.6 Mass-spectrometry analysis

The eluted peptides were analysed with an LTQ Orbitrap Elite (Thermo Scientific) with a nano ESI interface in conjunction with an Ultimate 3000 RSLC nano-HPLC (Dionex Ultimate 3000) at the Bio21 Institute, Melbourne, Australia following the procedure described in the Material and Method section in Chapter 2.

3.4.7 Peptide search and protein identification

The Thermo raw files were converted to mgf format using msconvert tools from Proteowizard v3.0.5047. Database searches were conducted within SearchGUI v3.3.3 [13] using its XTandem! Vengenace (2015.12.15.2) search engine against the in-house database of the oyster proteome downloaded from the UniProt (https://www.uniprot.org/proteomes/UP000005408), supplemented with the common Repository of Adventitious Proteins sequences (https://www.thegpm.org/crap/) [14]. The SearchGUI results were further analysed and visualised using PeptideShaker v1.16.26 [15].

3.4.8 Statistical analysis and experimental design

The extraction processes were conducted in triplicates. Differences in protein content of each extract were examined by analysis of variance (ANOVA) using Prism (version 7.03, 2017, GraphPad Software Inc., La Jolla, CA, USA). The Tukey test was used for comparison of the means. The level of significance was set at $p < 0.05$.

3.5 Results

3.5.1 Effects of extraction buffers on soluble protein content

The quantification of protein content on each extract clearly showed that the amount of soluble proteins varies very greatly (p <0.05, Table 3-2). High pH buffers were able to extract a significantly increased amount of proteins than low pH buffers. The carbonate-10 buffer demonstrated the best extraction properties resulting in 10.4 mg/mL of extracted proteins. The carbonate-9 buffer, however, did not differ greatly to the control PBS in its ability to extract proteins (8.0 and 7.7 mg/mL proteins, respectively, p >0.05), while the control TBS resulted in slightly lower protein yield although not significantly different (7.0 mg/mL of protein, p >0.05). Both citrate buffers at low pH showed poor extraction properties, resulting in only 2.3 mg/mL and 3.0 mg/mL of proteins, respectively. Addition of salt up to 1 M to the PBS and TBS buffer significantly increased the ability of the buffers to recover soluble proteins (p <0.05).

Interesting results were observed after extracted proteins underwent heat-treatment in each of the corresponding buffers. The distribution of protein concentrations of the heated extracts was different to the raw extracts. Instead of higher-pH buffers yielding in the higher amounts of protein and vice versa, the amounts of proteins were almost consistent (and low) across all buffers. While most of the proteins heat-treated in TBS, PBS and carbonate buffers were either degraded or aggregated resulting in decreased protein content, the protein concentration for the citrate buffers remained the same, possibly indicating that there was no protein loss. Heat-treatment reduced up to 80% of the protein content in the TBS, PBS and carbonate-9 buffer and up to 60% in the carbonate-10 buffer. A higher ionic strength in buffers did result in an increased amount of recovered protein as seen in both PBSN and TBSN when compared to PBS and TBS, however, it was not significantly different (p >0.05).

Table 3-2. The yield of recovered proteins measured by BCA–protein quantification method. Protein concentration was statistically analysed by one-way ANOVA (Tukey). Values with the same letter in the same column are not significantly different ($p > 0.05$).

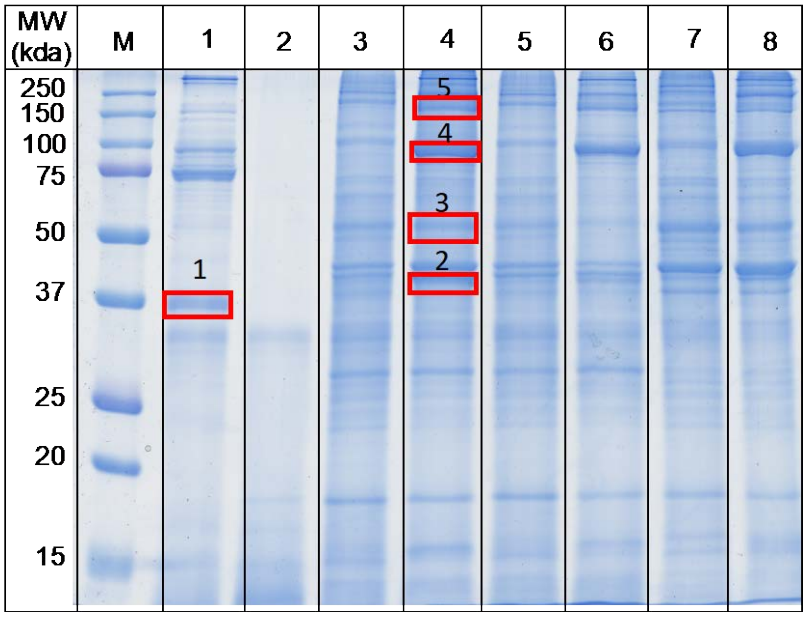
Buffer	Protein concentration (mg/mL)	
	Raw	Heated
Citrate-3	2.26 ± 0.29^{a}	2.35 ± 0.39^{ab}
Citrate-5	3.04 ± 0.14^{a}	2.42 ± 0.09^{a}
TBS	6.99 ± 0.28^{b}	1.61 ± 0.05^{c}
TBSN	9.08 ± 0.29^{c}	1.86 ± 0.05^{cd}
PBS	7.69 ± 0.23^{bd}	1.74 ± 0.04^{ce}
PBSN	9.70 ± 0.97^{be}	2.07 ± 0.08^{bdef}
Carbonate-9	8.04 ± 0.16^{d}	1.88 ± 0.14^{cf}
Carbonate-10	10.43 ± 0.52^{e}	4.29 ± 0.13

3.5.2 Protein Profiling by SDS-PAGE

The protein composition of each buffer was profiled using 12% SDS-acrylamide gels under denaturing condition (Figure 3-1). The raw protein profiles did not vary much between PBS, TBS and carbonate buffers, however, different intensities of the some bands particularly at 40 and 100 kDa were observed (Figure 3-1**A**). Meanwhile, the citrate buffers showed very distinct protein profiles particularly the citrate-5. Extracts from the citrate-3 buffer showed strong protein bands between 70–80 kDa, as well as a prominent 36 kDa band. Although the citrate-5 contained a similar amount of protein with the citrate-3 buffer, the protein profile was different. All bands found in citrate-5 buffer appeared diffused and smeared, particularly proteins above 35 kDa.

The protein profiles in the heated extract were less complex than that of raw extracts (Figure 3-1**B**). While most of the high molecular weight proteins disappeared after heat treatment in most buffers, some lower molecular weight proteins (15 and 18 kDa) emerged with more intense bands. The proteins at 38 kDa, corresponding to the molecular weight range which tropomyosin is often found, also showed more intense bands. With the exception for citrate-3 buffer, the protein profile of heated extract was quite similar with that of raw extracts from the same buffers.

A. Raw Extracts



B. Heated Extracts

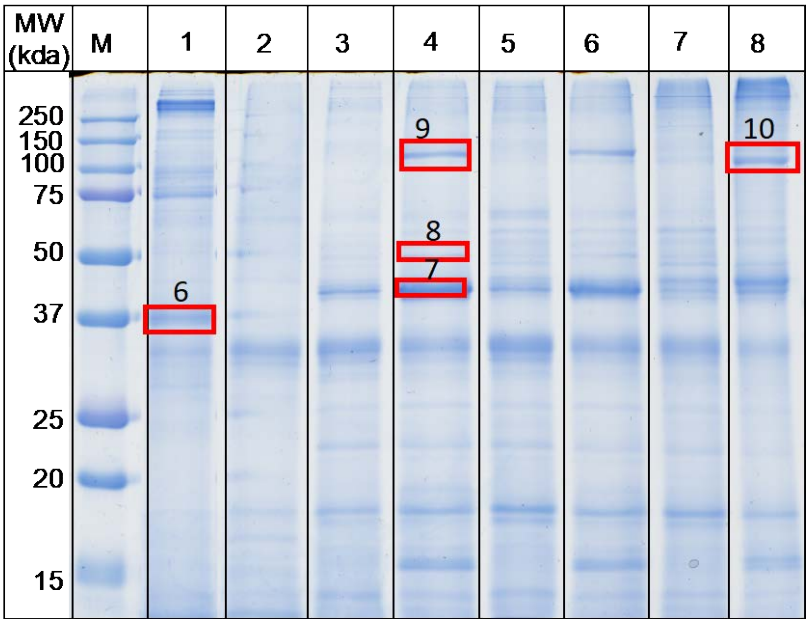


Figure 3-1 SDS-PAGE analysis of the proteins from (**A**) raw and (**B**) heated extracts. Samples containing 10 µg of proteins were resolved in 12% SDS-acrylamide gels and run at 170 V for 1 h. M = Marker, 1 = Citrate-3 extract, 2 = Citrate-5 extract, 3 = TBS extract, 4 = TBSN extract, 5 = PBS extract, 6 = PBSN extract, 7 = Carbonate-9 extract and 8 = Carbonate-10 extract. The highlighted bands were cut for mass spectrometry analysis.

3.5.3 Effect of extraction buffers on IgE-binding

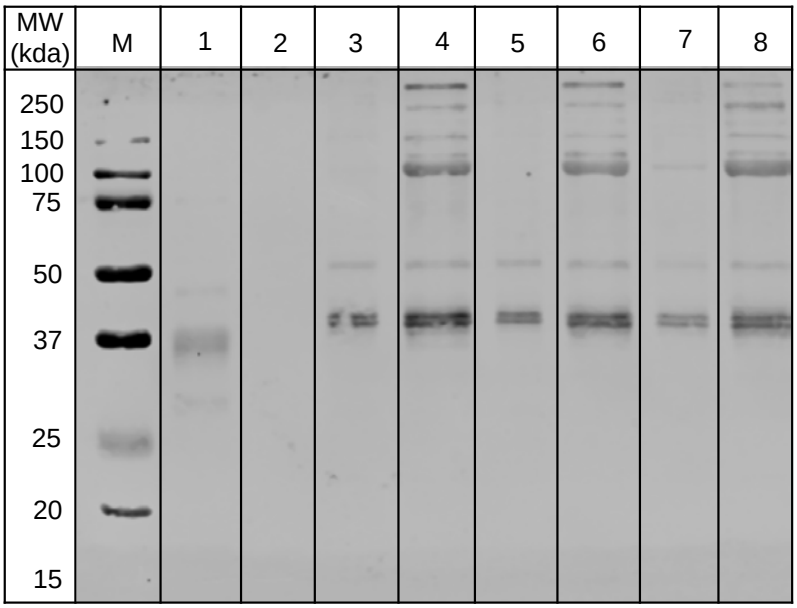
To determine whether the buffers affect the immune-reactivity of the protein extracts, immunoblotting against a pool of serum from five shellfish-allergic patients were conducted. Figure 3-2 shows the different profiles of IgE-reactive bands observed for both raw and heated extracts. The raw extracts, both PBS and TBS extracts showed three prominent bands (at 39, 40 and 50 kDa). Additional strong IgE-reactive bands at high molecular weight regions (100, 120, 150 and 250 kDa) were observed with the PBSN and TBSN extracts as well as carbonate-10 buffer extract. The citrate-3 buffer extract showed weak IgE-bands at 37 kDa and 48 kDa while no IgE-reactivity was detected for the extract of citrate-5 buffer.

Similarly, different patterns of IgE reactivity were observed between the extracts. PBSN, TBSN and carbonate-10 buffer achieved more IgE-reactive bands compared to other buffers. Extracts from those buffers showed five IgE-reactive bands including at 40 kDa, 41 kDa, 50 kDa, 120 kDa and >200 kDa. Meanwhile, the TBS, PBS and carbonate-8 extract lacked the IgE reactive bands at the high molecular weight. Citrate buffers clearly had negative effects on the extractability of allergenic proteins from the Pacific oyster as only one IgE-reactive band was observed in the citrate-3 extract and none in the citrate-5 extract.

To identify the proteins responsible to the IgE reactivity at those spots, selected SDS-PAGE band at each molecular weight were cut and tryptic digested. The top three protein families from Mascot search engine results were listed in Table 3-3. In total, eleven distinct proteins were identified in the raw extracts and six proteins were in the heated extracts. Tropomyosin was identified at the 40 kDa in both the raw and heated extracts, except in the Citrate-3 extract where the protein was identified at 37 kDa. In addition, previously identified Pacific oyster allergens [9] including arginine kinase (40 kDa), retinal dehydrogenase I (50 kDa), aldehyde dehydrogenase (50 kDa) and paramyosin (75 and 100 kDa) were detected in the raw extracts. Interestingly, paramyosin

was also observed in the Carbonate-10 heated extracts. Furthermore, myosin heavy chain, previously identified allergen in other molluscs [8, 16], as well as filamin and troponin C, identified allergens in crustacean [17, 18], were also detected. The other proteins including tubulin alpha-1C chain, alpha-actinin, spectrin-alpha chain, clathrin heavy chain, non-neuronal cytoplasmic intermediate filament protein and adipophilin were identified in the Pacific oyster IgE-reactive spots for the first time.

A. Raw Extracts



B. Heated Extracts

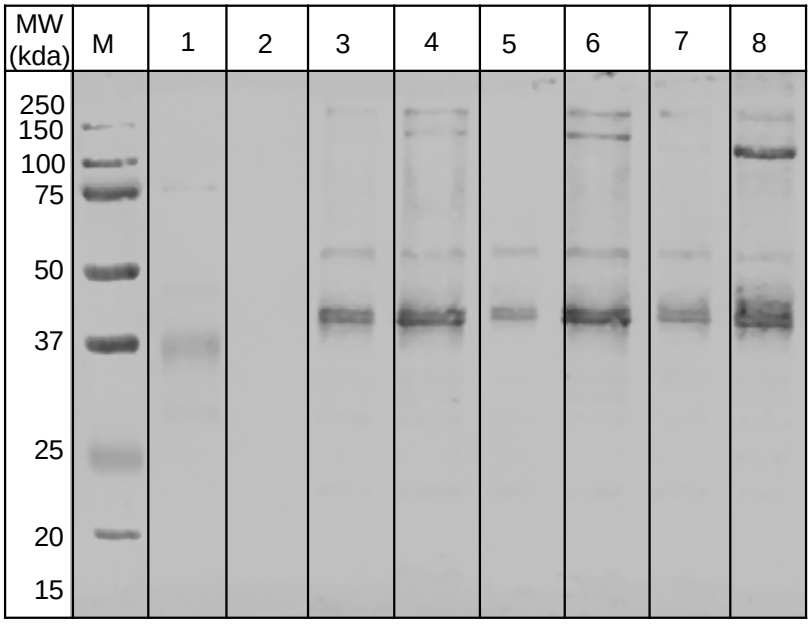


Figure 3-2 IgE-immunoblotting analysis of the proteins from (**A**) raw and (**B**) heated extracts using a pool of serum from five shellfish-allergic patients. M = Marker, 1 = Citrate-3 extract, 2 = Citrate-5 extract, 3 = TBS extract, 4 = TBSN extract, 5 = PBS extract, 6 = PBSN extract, 7 = Carbonate-9 extract and 8 = Carbonate-10 extract.

Table 3-3 Proteins identified using LC-MS from the SDS-PAGE bands corresponding to the IgE-reactive spots. The top three proteins from Mascot search engine result in each spot are presented.

Spot No	Protein	Accession ID	Experimental MW	Theoretical MW	Mascot Score	Coverage (%)	Number of significant peptides	emPAI
Raw								
1	Tropomyosin	B7XC66	37	33	1566	62	19	22.48
	Myosin heavy chain	K1RSS3		230	1463	20	33	1.42
	Filamin	K1PW06		326	553	12	6	0.23
2	Filamin	K1PW06	40	326	2533	32	65	1.17
	Arginine kinase	K1PLF9		40	1749	72	23	34.84
	Tropomyosin	B7XC66		33	1601	55	19	20.33
3	Retinal dehydrogenase I	K1QVG5	50	53	1256	59	43	6.72
	Tubulin alpha-1C chain	K1QII6		51	744	47	14	2.74
	Aldehyde dehydrogenase	K1QNT7		58	648	42	17	3.01
4	Paramyosin	K1QTC1	100	98	6288	74	69	61.54
	Filamin	K1PW06		326	1711	28	50	1.00
	Alpha-actinin	K1RH58		102	1480	56	38	4.44
5	Filamin	K1PW06	150	326	7159	61	151	7.06
	Spectrin alpha chain	K1R401		287	1905	45	77	1.69
	Clathrin heavy chain	K1PNR3		193	1660	44	60	2.22

Heated								
6	Myosin heavy chain	K1RSS3	37	230	1988	27	44	1.56
	Tropomyosin	B7XC66		33	1570	59	21	33.41
	Filamin	K1PW06		326	635	16	23	0.32
7	Tropomyosin	B7XC66	40	33	3535	57	21	60.09
	Troponin T	K1QPC9		21	522	74	10	7.17
	Non-neuronal cytoplasmic intermediate filament protein	K1PBC0		70	410	35	15	1.19
8	Non-neuronal cytoplasmic intermediate filament protein	K1PBC0	50	69.6	1599	49	29	5.32
	Tropomyosin	B7XC66		33.1	822	52	13	5.77
	Adipophilin	K1PJC1		54.4	791	41	16	2.44
9	Myosin heavy chain	K1R1B3	120	80	3998	58	36	11.60
	Filamin	K1PW06		326.2	1796	23	53	0.73
	Paramyosin	K1QTC1		98.1	1523	45	36	2.15
10	Paramyosin	K1QTC1	100	98	7125	63	46	20.17
	Filamin	K1PW06		326	5441	47	107	2.60
	Myosin heavy chain	K1RSS3		230	4644	45	115	1.71

3.5.4 Proteomic analysis of the extracts

To determine the causes of different profile of IgE-reactive proteins in the extracts, proteomics analysis of the whole extract was performed. The protein composition of each extract and their abundance was analysed using PeptideShaker [15]. The summary of protein composition and their physicochemical properties in each extract were presented in Table 3-4. As expected from the protein concentrations measured by BCA assay, citrate buffers contained a fewer number of proteins compared to other buffers. Strikingly, TBS and PBS extract contained more proteins compared to their respective high-salt buffer or carbonate buffers despite the lower concentration measured for the TBS and PBS extracts. Based on the mass spectrometry analysis, proteins extracted by citrate buffers seemed to possess significantly lower average isoelectric points and higher molecular weight. While these proteins in the citrate-3 and citrate-5 were found to be at an average pI of 6.13 and 6.15, respectively, the pI of proteins from other buffers were found to be present between pI 6.5 and 6.6. In the heated extracts, the number of proteins in each extract had reduced, although there were some variabilities in the percentages of reduction. In general, heat treatment had seen a reduction of 30-40% in the numbers of protein, except the proteins in the citrate-5 buffer in which the reduction was found to be only 2%.

Table 3-4. Summary of the results from mass spectrometry analysis. Total number of proteins was obtained from Peptideshaker and after removal of contaminations. The pI and MW were determined using Compute pI/Mw tool in ExPASy website (https://web.expasy.org/compute_pi/). The protein compositions were matched to the potential allergen identified in Chapter 2 to get the number of potential allergens extracted.

Extraction buffer	Raw				Heated			
	Number of proteins	Ave-rage pI	Ave-rage MW (kDa)	Number of potential allergens	Number of proteins	Ave-rage pI	Ave-rage MW	Number of potential allergens
Citrate-3	360	6.13	83.8	19	250	6.24	71.7	15
Citrate-5	425	6.15	83.2	11	414	6.14	75.2	15
TBS	742	6.53	59.3	34	356	6.25	65.2	15
TBSN	594	6.62	58.6	31	352	6.52	60.4	14
PBS	704	6.51	59.2	35	433	6.27	63.0	17
PBSN	549	6.58	60.1	32	381	6.39	65.0	23
Carbonate-9	663	6.56	59.5	33	472	6.25	62.6	28
Carbonate-10	615	6.52	56.7	31	289	6.43	62.2	28

3.6 Discussion

Tris-based (TBS) and phosphate-based (PBS) buffers prepared at neutral pH (7.4) are commonly used for extraction of allergens from various sources. However, it was demonstrated in chapter 2 that not all allergens identified in the genome and transcriptome of oyster are also detected in the extracted proteome. These finding are comparable to previous studies, showing that some allergens could not be recovered using those common buffers [14, 19-21]. Thus, allergens are often overlooked during the discovery of novel and/or undiscovered allergens. In the current study, eight different buffers were evaluated for their capacity to extract a range of allergens from the Pacific oyster (*Crassostrea gigas*). The buffers were prepared to cover a wide pH range of pH 3 to 10. The effect of high concentrations of salt to the tris-based and phosphate-based buffers was also assessed. The protein recovery was compared as well as the soluble protein profile by SDS-PAGE, IgE-reactivity with patient serum was investigated as well as the protein compositions determined using mass spectrometric analysis.

The analysis of the raw extracts demonstrated a significant increase in the protein content of total soluble using high pH buffers for the extraction as compared to the common buffers, TBS and PBS. In contrast, low pH buffers resulted in poor protein extractability, with protein contents 3-fold lower compared to that of TBS or PBS buffer. A similar impact of the pH on the variability of recovered proteins was also observed during extraction of raw samples from peanut [5, 6] and tree nuts [22]. Addition of salt to the TBS and PBS buffer improved the solubility of proteins and therefore, it significantly increased the protein content in the extract. It is known that protein solubility is affected by a complex interplay between the properties of proteins, electrostatic charges and the pH of the buffers. While high pH buffers change the charge of proteins to be more negative, thereby increasing water binding capacity and improving solubility of the proteins [23]. Salts are thought to play a role in improving the extractability of the buffers by associating with the opposite charge in the proteins [24].

Heat treatment of the raw extracts resulted in different effects on each extract. While a significant reduction in the protein concentration of extracts from neutral and high pH buffers was observed, heat treatment did not affect the solubility of proteins in the low pH buffers, particularly the citrate-5 buffer. A significant reduction in the protein content of extracts may be attributed to the denaturation and aggregation of some oyster proteins. Heat treatment unfolds the protein, exposing the hydrophobic residues from its structure and subsequently prompting the formation of insoluble aggregates [25]. Wet-based heat treatments can affect the solubility of proteins greatly as shown by Lasekan and Nayak [7]. While the effects of temperature on the solubility of proteins have been thoroughly studied, the ability of proteins to resist heat treatment at low pH solution is not well understood.

The effect of buffers on the soluble proteins was evident after resolving the proteins in the polyacrylamide gels. Three distinct protein profiles were observed; while the neutral and high pH buffers showed a similar pattern of protein profiles, the low pH buffers exhibited distinct protein profiles. Some proteins were extracted better by the high salt buffers or high pH buffers compared to other buffers as shown by the increase of protein staining intensity in the SDS gels. The change in the abundance seems to affect the IgE reactivity of allergenic proteins, given that additional IgE-reactive bands were revealed in the TBSN, PBSN and carbonate-10 extract. This corresponds to the SDS-PAGE bands with their higher intensity as compared to the PBS or TBS extracts.

The serum IgE analysis by immunoblotting demonstrated the superiority of the high salt or high pH carbonate buffers in solubilising less-abundant but highly immunoreactive proteins as compared to the common buffers. One of the very prominent IgE-reactive bands is paramyosin observed at 100 kDa. Paramyosin is a major structural component of the invertebrate muscle thick filament and was recently identified as an additional major allergen in abalone (*Haliotis discus discus)* [4, 26]. The discovery of allergenic paramyosin in mollusc

species was not surprising since this protein has been confirmed as a major allergen in other invertebrates such as house dust-mite [27] and anisakis [28]. Furthermore, this protein also forms a significant component of the bivalve myofibril with 38-48% in the white adductor muscle and 15-30% in the red adductor muscle [29]. However, paramyosin has a poor solubility in low ionic strength buffers, indicating that a high concentration of salt is required to adequately extract this protein.

Four of eight IgE-reactive bands were heat stable proteins including bands at about 38, 50, 100 and >200 kDa. The 38 kDa IgE-reactive protein was identified as tropomyosin and has been previously identified as major allergen in various mollusc species including squid [30], oyster [31] and abalone [32]. Tropomyosin is a heat stable and water-soluble protein, and due to their abundance in the muscle tissue, the extraction process for this protein is relatively easy. Tropomyosin was also observed at the 50 kDa IgE-reactive spot with a high Mascot score and sequence coverage. The amount of tropomyosin in that spot as indicated by their emPAI value was high as well. This higher molecular weight tropomyosin was also observed in other species including Sydney rock oyster [8] and Black tiger prawn [10]. Strikingly, in contrast to the previous thought that paramyosin is a heat-labile protein, paramyosin was identified in the IgE-reactive spot of heated Carbonate-10 extract at 100 kDa suggesting that paramyosin is structurally heat-stable and could not be identified in the heated extract of other buffers due to poor solubility.

The protein composition was identified using high-resolution mass spectrometry enabling in-depth comparison of each extract. Mass spectrometry analysis showed the number of proteins identified were different in each extract. As expected from the protein content quantification, low pH extracts contained fewer proteins as compared to the neutral or high pH extract. Interestingly, although addition of high salt concentration or high pH increased the total protein content, the numbers of proteins identified in their

extracts were less as compared to the normal TBS or PBS. These findings suggest that an increase in protein content of high salt or high pH buffers was mostly due to the increase in the abundance of some proteins. Further analysis of each extract demonstrated that not only protein composition varied, but the composition of potential allergens was also different in each extract. In total 38 potential allergens could be identified from the extracts. Interestingly, the common buffers, TBS and PBS, extracted more potential allergens than the other buffers. However, the abundance of these potential allergens in those buffers is low and affecting the IgE-reactivity as a result.

In conclusion, buffer compositions affect considerably the protein recovery during the extraction from oyster tissue, resulting in variation of IgE-reactivity. Many allergens are often overlooked during allergen discovery analysis due to low abundance as the common buffers used for protein recovery are unable to sufficiently extract the proteins. This study is the first to investigate in detail the extractability of allergens of animal origin and demonstrated that increasing ionic strength or pH improves the extractability of the buffers, allowing much efficient discovery and identification of IgE binding proteins.

3.7 References

1. Yu, W., D.M.H. Freeland, and K.C. Nadeau, *Food allergy: immune mechanisms, diagnosis and immunotherapy.* Nat Rev Immunol, 2016. **16**(12): p. 751-765.
2. Ruethers, T., et al., *Seafood allergy: A comprehensive review of fish and shellfish allergens.* Molecular Immunology, 2018.
3. Elvin Gomez Cardona, E., et al., *Novel low-abundance allergens from mango via combinatorial peptide libraries treatment: a proteomics study.* Food Chemistry, 2018.
4. Suzuki, M., et al., *Paramyosin of the disc abalone Haliotis discus discus: Identification as a new allergen and cross-reactivity with tropomyosin.* Food Chemistry, 2011. **124**(3): p. 921-926.
5. Poms, R.E., C. Capelletti, and E. Anklam, *Effect of roasting history and buffer composition on peanut protein extraction efficiency.* Molecular Nutrition & Food Research, 2004. **48**(6): p. 459-464.
6. Rudolf, J., et al., *Effects of different extraction buffers on peanut protein detectability and lateral flow device (LFD) performance.* Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 2012. **29**(1): p. 1-11.
7. Lasekan, A.O. and B. Nayak, *Effects of buffer additives and thermal processing methods on the solubility of shrimp (Penaeus monodon) proteins and the immunoreactivity of its major allergen.* Food Chemistry, 2016. **200**: p. 146-153.
8. Rolland, J.M., et al., *Effect of Heat Processing on IgE Reactivity and Cross-Reactivity of Tropomyosin and Other Allergens of Asia-Pacific Mollusc Species: Identification of Novel Sydney Rock Oyster Tropomyosin Sac g 1.* Molecular Nutrition & Food Research, 2018. **62**(14).
9. Nugraha, R., et al., *Rapid and comprehensive discovery of unreported shellfish allergens using large-scale transcriptomic and proteomic resources.* Journal of Allergy and Clinical Immunology, 2018. **141**(4): p. 1501-1504.e8.
10. Kamath, S.D., et al., *Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: A comprehensive allergenomic approach.* Molecular Nutrition & Food Research, 2014. **58**(5): p. 1144-1155.
11. Laemmli, U.K., *Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4.* Nature, 1970. **227**: p. 680.
12. Fischer, R. and B.M. Kessler, *Gel-aided sample preparation (GASP)-A simplified method for gel-assisted proteomic sample generation from protein extracts and intact cells.* Proteomics, 2015. **15**(7): p. 1224-1229.
13. Barsnes, H. and M. Vaudel, *SearchGUI: A Highly Adaptable Common Interface for Proteomics Search and de Novo Engines.* Journal of Proteome Research, 2018. **17**(7): p. 2552-2555.

14. Nugraha, R., et al., *Rapid and comprehensive discovery of unreported shellfish allergens using large-scale transcriptomic and proteomic resources.* J Allergy Clin Immunol, 2018. **141**(4): p. 1501-1504 e8.
15. Vaudel, M., et al., *PeptideShaker enables reanalysis of MS-derived proteomics data sets.* Nature Biotechnology, 2015. **33**: p. 22.
16. Martins, L.M.L., et al., *The Helix aspersa (Brown Garden Snail) Allergen Repertoire.* International Archives of Allergy and Immunology, 2005. **136**(1): p. 7-15.
17. Yang, Y., et al., *Triosephosphate Isomerase and Filamin C Share Common Epitopes as Novel Allergens of Procambarus clarkii.* Journal of Agricultural and Food Chemistry, 2017. **65**(4): p. 950-963.
18. Kalyanasundaram, A. and T.C. Santiago, *Identification and characterization of new allergen troponin C (Pen m 6.0101) from Indian black tiger shrimp Penaeus monodon.* European Food Research and Technology, 2014. **240**(3): p. 509-515.
19. L'Hocine, L. and M. Pitre, *Quantitative and qualitative optimization of allergen extraction from peanut and selected tree nuts. Part 2. Optimization of buffer and ionic strength using a full factorial experimental design.* Food Chemistry, 2016. **194**: p. 820-827.
20. Peiren, N., et al., *The protein composition of honeybee venom reconsidered by a proteomic approach.* Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 2005. **1752**(1): p. 1-5.
21. Van Vaerenbergh, M., et al., *Exploring the hidden honeybee (Apis mellifera) venom proteome by integrating a combinatorial peptide ligand library approach with FTMS.* Journal of Proteomics, 2014. **99**: p. 169-178.
22. L'Hocine, L. and M. Pitre, *Quantitative and qualitative optimization of allergen extraction from peanut and selected tree nuts. Part 1. Screening of optimal extraction conditions using a D-optimal experimental design.* Food Chemistry, 2016. **194**: p. 780-786.
23. Kramer, Ryan M., et al., *Toward a Molecular Understanding of Protein Solubility: Increased Negative Surface Charge Correlates with Increased Solubility.* Biophysical Journal, 2012. **102**(8): p. 1907-1915.
24. Tomé, L.I.N., et al., *Toward an Understanding of the Aqueous Solubility of Amino Acids in the Presence of Salts: A Molecular Dynamics Simulation Study.* The Journal of Physical Chemistry B, 2010. **114**(49): p. 16450-16459.
25. Kamath, S.D., et al., *Impact of heat processing on the detection of the major shellfish allergen tropomyosin in crustaceans and molluscs using specific monoclonal antibodies.* Food. Chem., 2013. **141**(4): p. 4031-9.
26. Suzuki, M., et al., *Paramyosin from the Disc Abalone Haliotis Discus Discus.* Journal of Food Biochemistry, 2014. **38**(4): p. 444-451.
27. Lee, C.S., et al., *Protein sequence analysis of a novel 103-kDa Dermatophagoides pteronyssinus mite allergen and prevalence of serum immunoglobulin E reactivity to rDer p 11 in allergic adult patients.* Clinical & Experimental Allergy, 2004. **34**(3): p. 354-362.

28. Nieuwenhuizen, N.E. and A.L. Lopata, *Anisakis – A food-borne parasite that triggers allergic host defences.* International Journal for Parasitology, 2013. **43**(12-13): p. 1047-1057.
29. Kantha, S.S., S. Watabe, and K. Hashimoto, *Comparative Biochemistry of Paramyosin: A Review.* Journal of Food Biochemistry, 1990. **14**(1): p. 61-88.
30. Miyazawa, H., et al., *Identification of the first major allergen of a squid (Todarodes pacificus).* Journal of Allergy and Clinical Immunology, 1996. **98**(5, Part 1): p. 948-953.
31. Ishikawa, M., et al., *Tropomyosin, the Major Oyster Crassostrea gigas Allergen and its IgE-binding Epitopes.* Journal of Food Science, 1998. **63**(1): p. 44-47.
32. Lopata, A.L., C. Zinn, and P.C. Potter, *Characteristics of hypersensitivity reactions and identification of a unique 49 kd IgE-binding protein (Hal-m-1) in abalone (Haliotis midae).* Journal of Allergy and Clinical Immunology, 1997. **100**(5): p. 642-648.

CHAPTER 4. RECOMBINANT EXPRESSION, PURIFICATION AND IMMUNOLOGICAL CHARACTERISATION OF THE MAJOR ALLERGEN OF THE PACIFIC OYSTER (*Crassostrea gigas*) – TROPOMYOSIN

Published in:

Roni Nugraha, Sandip D Kamath, Thimo Ruethers, Shaymaviswanathan Karnaneedi, Elecia Johnston, Andreas L Lopata. Recombinant expression, purification and immunological characterisation of the major allergen of the pacific oyster (*Crassostrea gigas*) – Tropomyosin. *Molecular Nutrition and Food Research.* Manuscript in preparation.

4.1 Summary

Pacific oyster is an important shellfish species causing food allergy, but knowledge of their allergens and cross-reactivity is limited. In chapter 2 and 3, over 24 allergens where identified using allergen specific antibodies and advanced mass spectrometry. However, only one allergen from the Sydney Rock oyster (*Saccostrea glomerata*) has been recently characterised in detail by our group and registered with the IUSI as allergen and commercial diagnostics utilise only whole protein extracts. These limitations make an optimal diagnosis of oyster allergy difficult, in particular to the Pacific oyster (*Crassostrea gigas*), the most common oyster consumed worldwide. The current study aimed to characterise IgE sensitisation profiles of 21 oyster-sensitised patients to raw and heated extract of the Pacific oyster using immunoblotting and advanced mass spectrometry. Subsequenlty the major allergen tropomosin was identified and characterised on molecular level and expressed as recombinant allergen. In addition the IgE cross-reactivity of these patients to purified tropomyosin from the Pacific oyster, Black tiger prawn and House dust mite was compared using enzyme-linked immunosorbent assay (ELISA).

Tropomyosin was identified as the major allergen in the Pacific oyster in 18 of 21 oyster-sensitised patients and registerd with the IUSI as Cra g 1, the first fully biochemically characterised allergen of the Pacific oyster. In addition immunoblotting demonstrated different IgE-reactivity profiles, with 5 allergens seem to be also of importance, confirming the findings in chapter 2 and 3. Eighteen of the 21 investigated oyster allergic subjects demonstrated IgE reactivity to tropomyosin from Pacific oyster as well as to Black tiger prawn and House dust mite, however, the degree of IgE binding varied between patients, indicating partical cross-reactivity and/or co-sensitisation. Amino acid sequence alignment of tropomyosin from these three species revealed five protein regions that contain predicted IgE-binding epitopes most likely responsible for this cross-reactivity. The major allergen characterised in this study and expressed as pure recombinant allergen will improve the component

resolved diagnosis of oyster allergy and general management of patients with mollusc allergy.

4.2 Introduction

Consumption of shellfish has increased significantly in the last two decades due to the high nutrition content and their health benefits. Two large invertebrate groups named crustacean and mollusc are part of the shellfish group, as detaild in Chatper 1. These two groups have a great diversity of species, giving consumers a variety to choose from. Food and Agricultural Organization (FAO) listed over 60 species of crustaceans and 120 species of molluscs widely consumed. However, the increasing consumption of shellfish has been associated with the increasing incidence of shellfish allergy. Many studies have shown that dietary habits correlate with food allergy prevalence. For example, the Asian population have a higher prevalence of seafood allergy [1], while Australians seem to have a higher prevalence of allergy to nuts [2]. Furthermore, many people are introduced to new shellfish species due to better distribution and globalisation of shellfish-based product.

The Pacific oyster (*Crassostrea gigas*) is one of the most important edible molluscs in the world, collected from the wild and grown in aquaculture. However, the knowledge of their allergens and possible immunological cross-reactivity is limited [3-5]. In the previous two chapters, a serum pool from shellfish allergic patients demonstrated immunological reactivity to one protein band at about 38 kDa, and subsequent analysis using mass spectrometry identified tropomyosin (TM) as the major protein component. Tropomyosin was also demonstrated by our group to be a major allergen for the Sydney rock oyster (*Saccostrea glomerata*) [6]. Tropomyosin, a structural protein involved in muscle contraction, is the major allergen causing allergic reactions in up to 80% of shellfish allergic patients and is found in most shellfish species that have been studied so far [7]. Due to high similarity in TM amino acid structure among shellfish and other invertebrate species, affected individuals often experience allergic reactions to multiple species. However, many clinical studies have demonstrated that the serum IgE antibody repertoire differs in allergic patients [7-9]. Various factors including routes of allergen exposure,

the source of sensitisation e.g. ingestion or inhalantion, could affect the IgE reactivity.

Several studies have demonstrated that the use of purified allergen components can better predict clinical allergy reactivity compared to SPT and/or specific-IgE to whole protein extract [10-12]. Currently, however, no purified allergen components are available for the diagnosis of oyster allergy [13]. In addition, the species used for diagnostics are native to the Northern hemisphere, and several studies demonstrated that allergen components often differ between species of the Northern and Southern hemisphere [14, 15], posing a challenge for reliable diagnostics. Therefore, in this current study TM from the Pacific oyster (*Crassostrea gigas*) was purified and expressed as a recombinant protein, and its structural properties and IgE reactivity characterised. Cross-species IgE-reactivity with one commonly consumed species, the black tiger prawn (*Penaeus monodon*), and major inhalant allergen source, house dust mite, was sought.

4.3 Aims

The aims of this study were as follows:

1. To determine the sensitisation pattern of oyster-sensitised patients to raw and heated extract of Pacific oyster using immunoblotting.
2. To purify and characterise natural and recombinant TM from the Pacific oyster.
3. To investigate IgE-reactivity of oyster-sensitised patients to TM of the Pacific oyster, Black tiger prawn and House dust mite.

4.4 Materials and Methods

4.4.1 Patient selection

Twenty-one subjects with a convincing clinical history of allergic reactivity to shellfish and positive oyster-specific IgE by ImmunoCAP (>0.35 kUA L–1; Phadia Pty Ltd, Uppsala, Sweden; **Table 1**) were recruited from The Alfred Hospital Allergy Clinic, Melbourne, Victoria, Australia. Oral challenge with mollusc extracts were not conducted routinely in these patients, in keeping with the clinicians' preference for safer serum specific allergen IgE testing in adult patients due to comorbidities, together with the clinical history of reactions on exposure. Ethics approval for this study was granted by James Cook University's Ethics Committee (Project number H4313) in collaboration with The Alfred Hospital (Project number 192/07) and Monash University's Ethics Committees (MUHREC CF08/0225).

4.4.2 Sample preparation and protein extraction

Raw and heated Pacific oyster extract were prepared according to the method described in detail in Chapter 2.

4.4.3 Quantification of total protein

Total protein from each extract was estimated using the BCA Assay (Thermo Scientific, Waltham, USA) following the manufacturer's instruction. A pre-diluted set of bovine serum albumin (Pierce, Waltham, USA) was used as protein standards.

4.4.4 Purification of natural TM from the Pacific oyster

Natural TM from Pacific oyster was purified through ion-exchange chromatography using Biologic LP fast protein liquid chromatography system (BioRad, USA). About 20 mg of protein extract was diluted in starting buffer of 5 mM NaPO4, 150 mM NaCl, pH 6.8 and loaded onto a Bio-scale Mini CHT Ceramic Hydroxyapatite Cartridges column (BioRad, Hercules, USA).

Increasing concentration of phosphate was used to elute the proteins. purified proteins were stored at -20°C until further use.

4.4.5 Cloning, sequencing and expression of recombinant oyster TM

Total RNA from *C. gigas* muscle was extracted using TriZol reagent (Life Technologies, Australia) following the manufacturer's instruction. Single-stranded cDNA were generated from RNA using cDNA cDNA Synthesis kit (Bioline, Australia) and used as a template to amplify TM coding region using forward (5' CGC AGA ATT CAT GAC AGC ATC AAG AAG AAG ATG 3') and reverse (5' CGA ACC TGC AGT TAA TAT CCT GCC AGC TCG G 3') primer. The PCR products were cloned into a sequencing vector, pCR 2.1 using the TOPO TA cloning kit (Invitrogen, Carlsbad, USA) and transformed into TOP10 chemical competent *E. coli*. Positive colonies were confirmed by blue-white screening and colony PCR using the gene-specific oligonucleotide primers for the presence of inserts. The plasmid contained the TM open reading frame was sent to Macrogen Inc, South Korea for sequencing.

For the expression of recombinant TM, the coding region of the protein was subcloned into pRSET-A bacterial expression vectors. The cloned vectors were transformed into BL21 (DE3) RIPL *E. coli* competent cells. The cells were grown overnight on LB agar plate agar with 100 µg/ml of ampicillin at 37 °C. A single colony was selected and further grown in 10 mL of LB media overnight. Tropomyosin was expressed by adding 1 mL of overnight LB media to the Novagen® Overnight Express™ Autoinduction Systems (Merck, Kenilworth, USA). After 24 hours, cells were harvested and lysed using a probe sonicator. Expressed TM was purified using HisPur™ Ni-NTA column (Thermo Scientific, Waltham, USA).

Table 4-1 Demographics of patients recruited for this study

Patient ID	Specific IgE ($kU_A\ L^{-1}$)			Symptoms with	Symptoms
	Oyster	Prawn	HDM		
1	0.93	10.10	8.73	Prawn	As, R, U
2	0.92	9.50	14.10	Flounder, prawn, crab	R, A, An
3	2.04	9.03	13.60	Calamari, snapper, tuna	R, An
4	3.75	9.82	2.66	Shellfish, scallops, oyster	U, An, pO
5	4.29	0.20	9.49	Flake, sea perch, rockling	pO, An
6	1.04	6.84	31.70	Mussel, scallops	An
7	0.49	2.57	1.99	Calamari, octopus	An
8	5.99	32.40	6.47	Shellfish	As, R, U, An
9	2.41	8.98	2.36	Shellfish	As, R, U, An
10	1.11	3.63	5.03	Crustaceans/molluscs	R, H, A
11	1.19	4.30	57.5	Prawn, crab meat and marinara mix	As
12	6.68	17.2	13.5	Salmon, crab, lobster, shrimp	An
13	2.59	9.81	16.80	Prawns, calamari, fish	An
14	0.65	3.75	33.80	Oyster	GI
15	5.47	21.60	10.7	Calamari	U, As
16	1.35	5.42	40.20	Mollusc	An, A, U
17	1.08	6.73	6.90	Shellfish	U, A
18	35.8	>100	22.00	Shellfish	U, As
19	0.45	9.74	1.29	Shellfish	As
20	1.08	5.05	1.97	Pipis, squid	pO
21	7.32	2.84	1.95	Oyster	-

As, asthma; R, rhinitis; U, urticaria; A, anaphylaxis; An, angioedema; pO, periorbital edema; H, hypotension; GI, Gastrointestinal.

4.4.6 SDS PAGE and Immunoblotting using patient IgE-antibody

The Pacific oyster extracts and purified TM were resolved using AnykD™ Criterion™ TGX™ Precast Midi Protein Gel (BioRad, Hercules, USA). A solution of protein containing 10 µg of protein or 2 µg of purified TM was added to each well and separated on an electrophoresis apparatus at 170 V for 1 hour. The gel was stained with Coomassie Brilliant Blue using the protocol described in Chapter 2.

To analysis serum IgE binding, 100 µg of extracts or 20µg of purified proteins were resolved. After the electrophoresis, the separated proteins were transferred to a nitrocellulose membrane using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Hercules, USA). Subsequently, the membrane was blocked using Casein blocking solution (Sigma, St. Louis, USA) for 1 h at room temperature. The membrane was washed three times using PBS added with 0.05% Tween (PBST). The blocked-nitrocellulose membrane was incubated overnight in individual serum samples diluted 1:20 in PBST added with casein. After the washing step, anti-human IgE (1: 10,000 dilution) was added and incubated for one hour. The membrane was subsequently incubated for 35 minutes with anti-rabbit IgG antibody conjugate with IR (1:10,000 dilution), and IgE antibody binding was visualised using the Odyssey ® CLx Imaging System (LI-COR Biosciences, Lincoln, USA).

4.4.7 Amino acid sequencing using mass Spectrometry

To confirm the identity of the expressed and purified protein, mass spectrometry was performed using a method described previously [16]. The proteins were digested using a trypsin spin column (Sigma, St. Louis, USA), prepared according to the manufacturer's instructions. See Chapter 2, section xy for details. The eluted peptides were analyzed using an LTQ Orbitrap Elite (Thermo Scientific, Waltham, USA) with a nano ESI interface in conjunction with an Ultimate 3000 RSLC nano-HPLC (Dionex Ultimate 3000) at the Bio21 Institute, Melbourne. The obtained spectra were identified using Mascot search engine (Matrix Science, Boston, USA) against the in-house database

of the oyster proteome downloaded from the UniProt, supplemented with the common Repository of Adventitious Proteins sequences.

4.4.8 Analysis of secondary protein structure using CD spectrometry

To determine the alpha-helical confirmation of natural and recombinant TM, CD spectrometry was performed [17]. Natural and recombinant TM samples were prepared in phosphate buffer, pH 7.2 and adjusted to a final concentration of 3 µM. CD spectroscopy was performed on a J715 Spectropolarimeter (Jasco, USA) with continuous nitrogen flushing at 25◦C. All measurements were performed using a 10 mm quartz cuvette over a wavelength range of 190–260 nm. For wavelength analysis, the TM samples were scanned with a step width of 0.2 nm and bandwidth of 1 nm at 100 nm/min averaging over eight scans. Final data were expressed as mean residual ellipticity (θ) after subtracting the phosphate buffer blank spectrum.

4.4.9 Evaluation of IgE-reactivity using ELISA

100 µL of 10µg/mL TM in carbonate buffer pH 9.6 was added to each well of a 96-well EIA/RIA plate (Costar, St. Louis, MO) and incubated overnight at 4 °C. The plate was washed four times using 0.05% Tween 20/PBS (PBS-T) and subsequently blocked using Casein Blocking Buffer 10x (Sigma-Aldrich) diluted in PBST. After one hour incubation, the plate was washed four times, and wells incubated with 100 µL of serum diluted 1:10 in 0.2× casein/PBST at room temperature for 3 hours with shaking (45 rpm). Rabbit anti-human IgE antibody (1:4000; Dako, Glostrup, Denmark) and goat anti-rabbit IgG-HRP (1:1000; Promega, Madison, WI) were added sequentially to wells and plates incubated at room temperature for 1 hour with gentle shaking. Plates were then washed five times in PBS-T, followed by three washes in PBS. IgE binding was detected using TMB (3,3',5,5'-Tetramethylbenzidine) substrate (Invitrogen, Carlsbad, USA). After 5 minutes, the reaction was terminated using 1 M HCl and the absorbance (O.D.) at 450 nm measured by spectrophotometry (BMG LABTECH, Melbourne, Australia).

4.5 Results

4.5.1 SDS-PAGE and IgE-antibody reactivity of Pacific oyster extracts

The analysis of raw and cooked extracts of Pacific oyster using 1-dimensional SDS-PAGE stained with Coomassie Brilliant Blue showed various proteins with a molecular weight ranging from 10-200 kDa (Figure 1). Heat treatment reduced the number of proteins identified, particularly proteins with high molecular weight (Figure 1C), while heat-stable proteins were oberserved in the cooked extract. In contrast, several protein bands that appeared in cooked extract were absent in the raw extract, demonstrating heat-induced modification of high molecular weight proteins by fragmentation or dimerisation of certain proteins. Tropomyosin, a heat-stable protein, was prominent in the cooked extract at 38-40 kDa. These findings were consistent with the results of the previous study (Chapter 2) analysing the protein profile of raw and cooked extract using 2-dimensional SDS-PAGE and detaeild mass spectrometry to identify the proteins.

IgE-reactivity of proteins in the oyster extracts were determined using immunoblotting against sera from 21 subjects with a positive IgE titre to oyster by Immuno-CAP (Figure 1). The IgE-binding intensity was measured using Image Studio software and the results were summarized in allegrograms in Figure 2. IgE bands were marked as positive when their intensity was above the average of negative controls plus two standard deviations. In general, more IgE-reactive bands in the raw extract (Figure 1B) were observed than in cooked extract (Figure 1D), however, cooked extract demonstrated an increased IgE binding intensity, particularly at 37 kDa. Eighty five percent of the tested patients recognised proteins in the 38-40 kDa region in both extracts. Interestingly, those patients who elicited IgE binding to TM also showed IgE binding to proteins at 48-50 kDa, although with intensity lower than to that of TM. Figure 2 also shows several other bands recognised by the subjects. These IgE binding proteins are most likely paramyosin, myosin heavy

chain and retinal dehydrogenase I, determined already in Chapter 2. However, the subsequent analysis focuses on the major oyster allergen, TM.

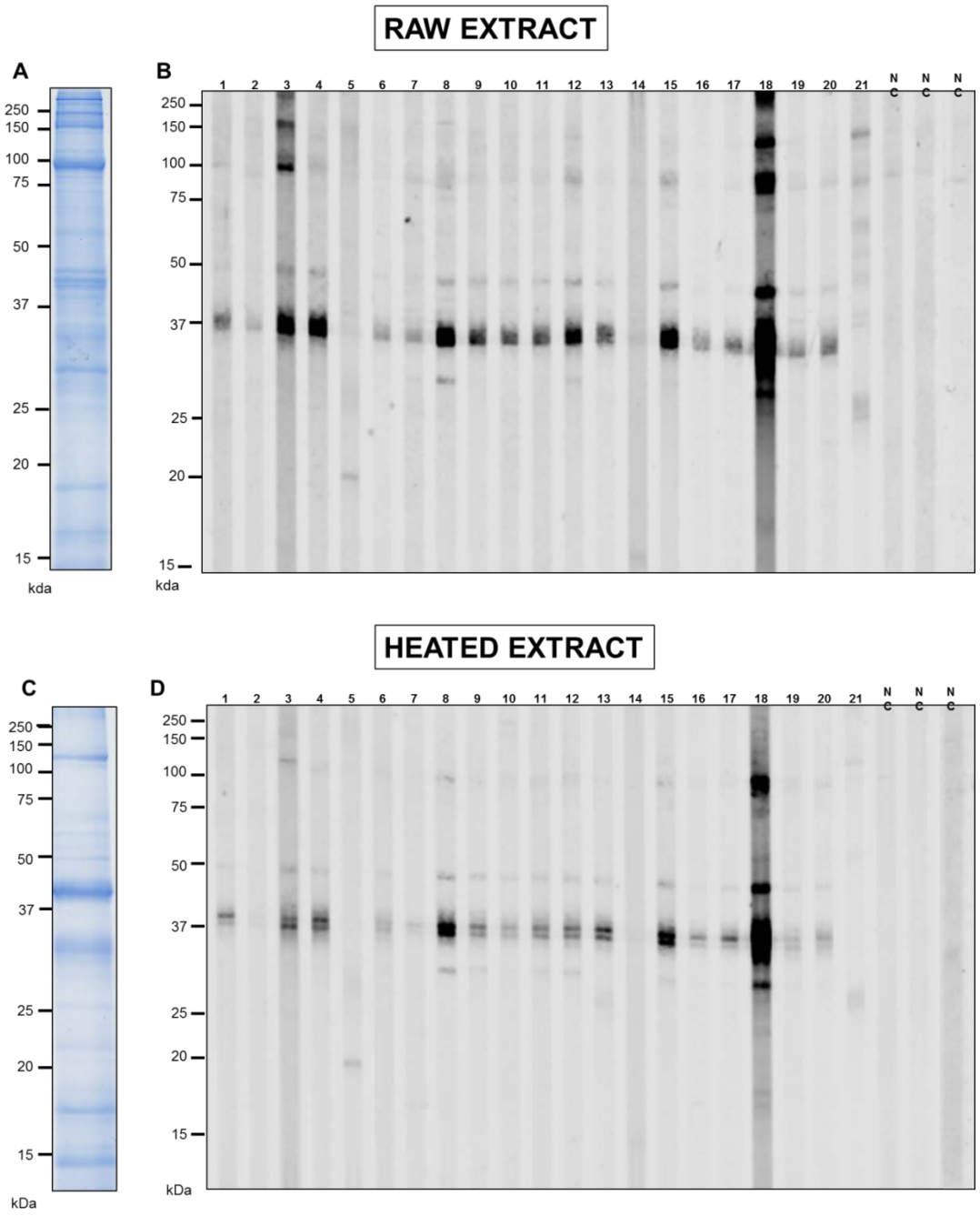


Figure 4-1 IgE reactivity of oyster-sensitised patients to raw and heated Pacific oyster extract. The extracts were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (**A** and **C**). Immunoblots were performed with sera from 21 oyster-sensitised patients (1-21, **B** and **D**) as well as from 3 atopic individuals (NC).

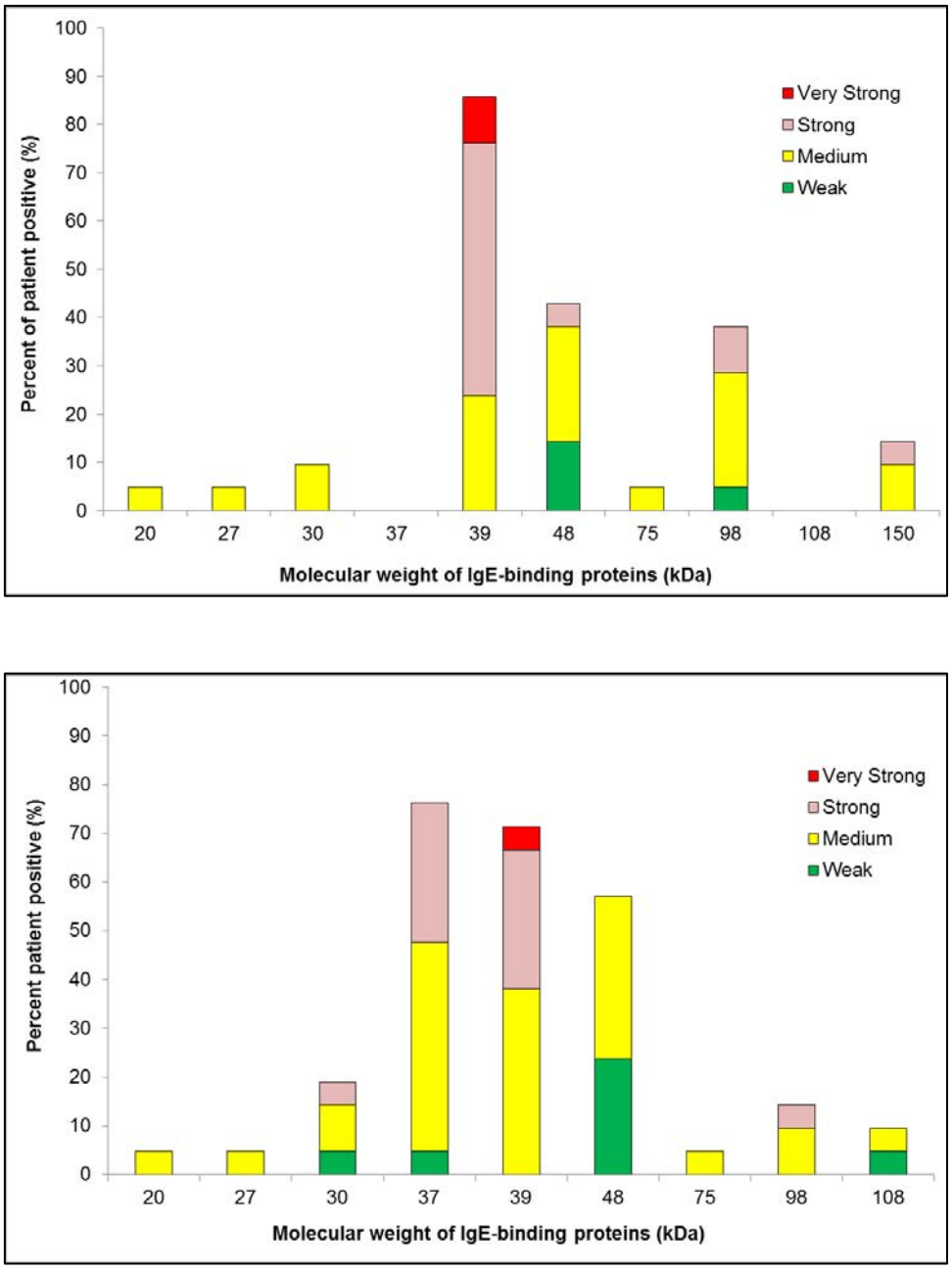


Figure 4-2 Allergogram analysis of IgE-binding patterns to proteins in the raw (**A**) and heated (**B**) extract of the Pacific oyster. IgE-binding intensities were measured using Image Studio software and graded as weak, medium, strong and very strong. Percentage patient reactivity for each IgE-binding intensity is shown.

4.5.2 Sequencing and characterisation of Pacific oyster TM

To confirm the IgE-reactivity of TM, natural TM was purified from oyster extract using CHT™ Ceramic Hydroxyapatite ion-exchange chromatography (Figure 3). Recombinant Pacific oyster TM was also expressed in an *E. coli* expression system and further purified using HisPur Ni-NTA Resin (Figure 4). The cDNA sequence of the TM was published in GenBank under the accession number KY549366.1.

The structural properties of natural and recombinant TM were determined using CD-Spectroscopy (Figure 5) and LC-MS/MS mass spectrometry (Figure 6). Figure 5 confirms the identical secondary structure between natural and recombinant TM, although recombinant TM possessed slightly higher minima of the Mean Residual Ellipticity (MRE) at λ = 209 and 222 nm (Figure 5A). Consistent with previous studies, the structure of TM is dominated by α-helical signal (81.67%) (Figure 5B) and confirmed by the 3D structure modelling of TM (Figure 5C). LC-MS/MS mass spectrometry was used to confirm that the aminoacid sequences of the purified natural and recombinant protein are indeed TM. Figure 6 displays two representatives of unique peptides belong to TM confirming that the purified proteins is TM. Moreover, the sequence coverage of TM is excellent, with the peptides identified by mass spectrometry covering 89% and 76% of natural and recombinant TM sequence, respectively.

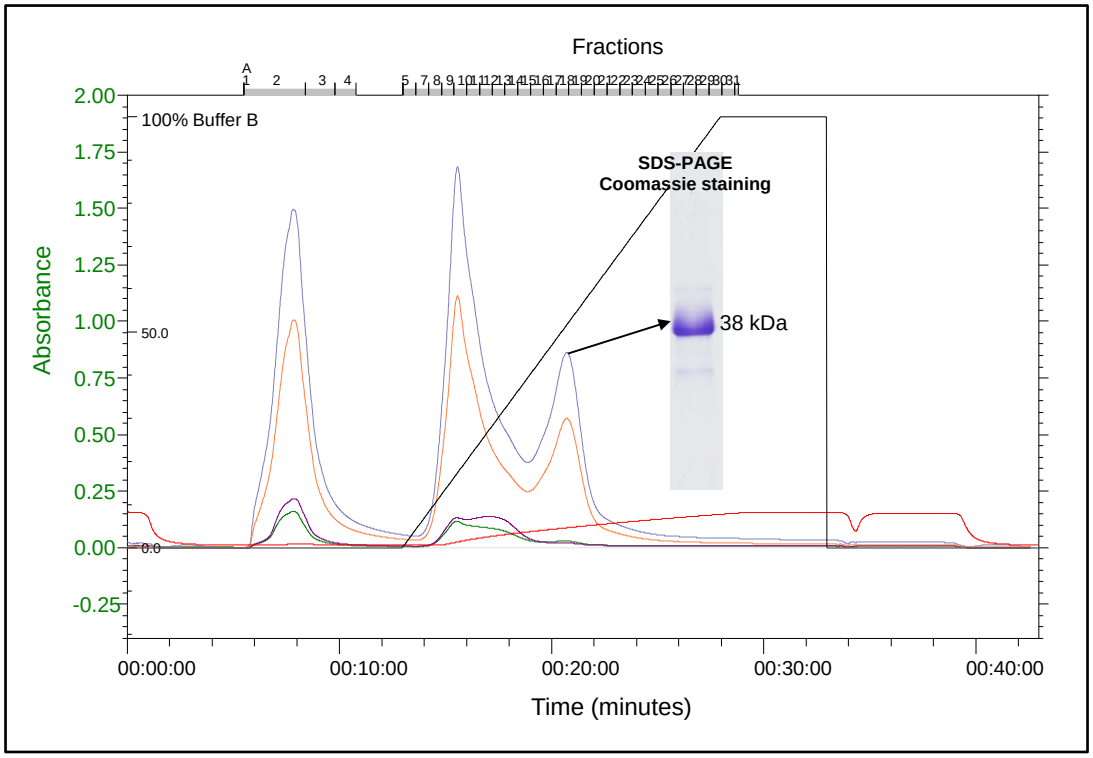


Figure 4-3 Purification profile of protein extract of heated Pacific oyster using CHT™ Ceramic Hydroxyapatite. The increase in the absorbance is measured at 280 nm (blue line) and 220 nm (red line) and 31 eluted fractions were collected and analysed by SDS-PAGE. The 17th, 18th, and 19th peak contain pure tropomyosin, appearing as a strong band at 39 kDa in the Coomassie-stained SDS-PAGE gel.

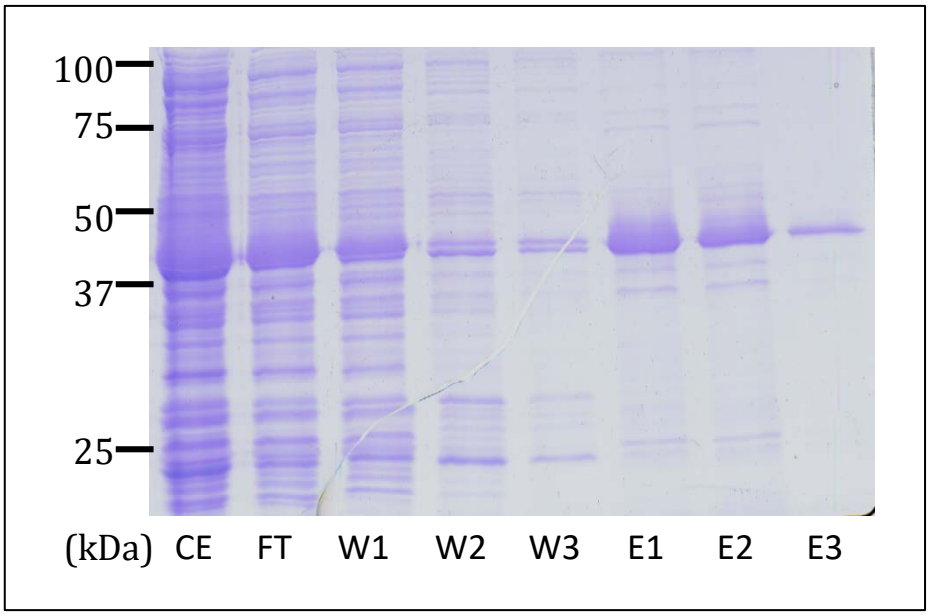


Figure 4-4 SDS-PAGE purification profile of recombinant Pacific oyster tropomyosin. Recombinant tropomyosin was purified using HisPur Ni-NTA with increasing concentration of imidazole. Purified tropomyosin was observed at 40 kDa due to six-His-tag fused to its N-terminal. Note: CE= crude extract, FT = Flow through, W = Wash, E = Eluent.

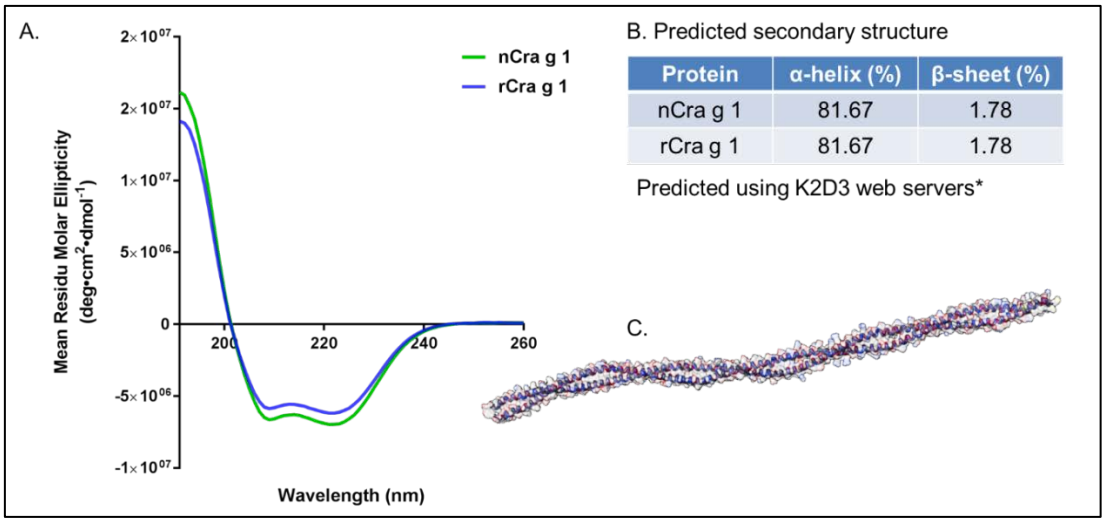
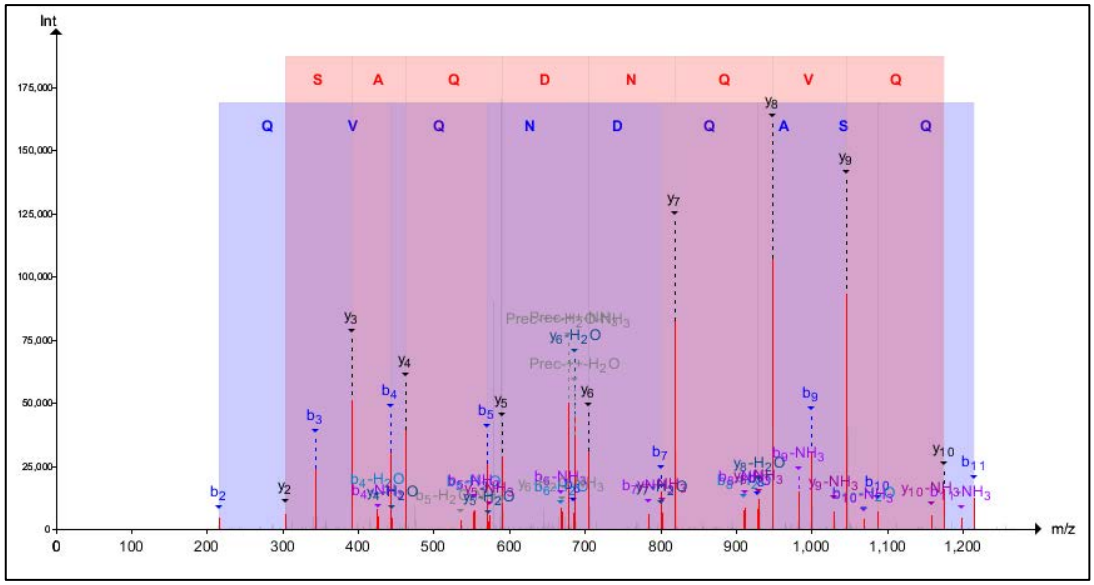


Figure 4-5 Structural analysis of purified natural and recombinant tropomyosin. A) Circular dichroism (CD) spectroscopy profile of natural (green line) and recombinant tropomyosin (blue line). B). The estimated structure of tropomyosin predicted using K2D3 web servers [1], with the majority of the structure consisting of an alpha-helix.

A



B

nCra g 1 Sequence coverage 89%

1 MDSIKK**KMIAMKMEKENAQDRAEQLEQQLRDTEEQKAKIEEDLTSLQKKH**
51 **SNLENEFDTVNEKYQECQTK**LEEAEK**TASEAEQEIQSLNRRIQLLEEDME**
101 **RSEERLQTATEKLEEASKAADESER**NR**KVLENLNNASEERTDVLEKQLTE**
151 **AKLIAEEADKKYDEAARKLAITEVDLERAEARLEAAEAKVYELEEQLSVV**
201 **ANNIKTLQVQNDQASQREDSYEETIRDLTQRLKDAENRATEAER**TVSKLQ
251 K**EVDRLEDELLAEKER**YKAISDELDQTFAELAGY

rCra g 1 sequence coverage 76%

1 MDSIKKKMIAMKMEKENAQDR**AEQLEQQLRDTEEQKAKIEEDLTSLQKKH**
51 **SNLENEFDTVNEKYQECQTKLEEAEKTASEAEQEIQSLNRRIQLLEEDME**
101 **R**SEER**LQTATEKLEEASKAADESER**NR**KVLENLNNASEERTDVLEK**QLTE
151 AK**LIAEEADKKYDEAARKLAITEVDLER**AEARLEAAEAK**VYELEEQLSVV**
201 **ANNIKTLQVQNDQASQREDSYEETIR**DLTQR**LKDAENRATEAER**TVSKLQ
251 K**EVDRLEDELLAEKER**YK**AISDELDQTFAELAGY**

Figure 4-6 Comparison of the mass spectrometry analysis of purified natural and recombinant TM. (**A**) Representative product spectra of unique peptides of tropomyosin generated from trypsin digestion (**B**) The amino acid sequence of TM. Peptide sequences identified by mass spectrometry are highlighted in red colour.

4.5.3 IgE-recognition of oyster-sensitised subjects to natural and recombinant TM

The comparative evaluation of IgE-binding patterns of the patients to natural and recombinant TM confirmed the major reactivity. 76% and 86% of the 21 oyster-sensitised subjects elicited IgE binding to natural and recombinant TM, respectively (Figure 7). The intensity of IgE binding to natural and recombinant TM was comparable, except in subject 6 and 14 where those subjects showed weak IgE binding to recombinant TM, but no binding to natural TM.

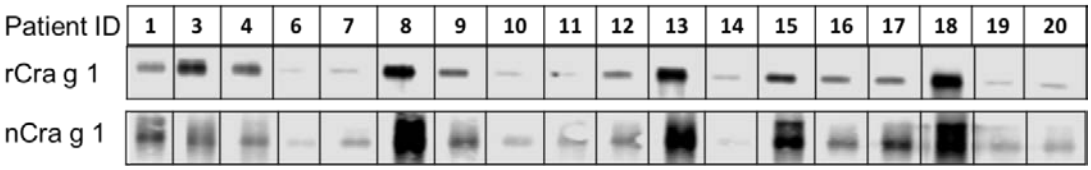


Figure 4-7 IgE reactivity analysis of natural and recombinant Pacific oyster tropomyosin using immunoblotting with 18 IgE reactive patients showed reactivity to protein at 38 kDa from raw and heated extract. rCra g 1 = purified recombinant Pacific oyster tropomyosin, nCra g 1 = purified natural Pacific oyster tropomyosin.

4.5.4 Immunological IgE-reactivity analysis using ELISA

Immunological reactivity between mollusc, crustacean and house dust mite is often reported due to high aminoacid sequence identity of their allergens. Tropomyosin is known as invertebrate pan-allergen, responsible for major IgE reactivity in oyster and cockroach, while much lower immunological reactivity is seen to TM in mites.

In this study, the immunological reactivity to purified Pacific oyster TM (Cra g 1), black tiger prawn TM (Pen m 1) and house dust mite TM (Der p 10) in 18 TM reactive subjects was analysed using ELISA (Figure 4-8). The IgE reacivity to Pen m 1 was markedly higher as compared to Der p 10 and Cra g 1. The median O.D. value for Cra g 1, Pen m 1 and Der p 10 were 1.416, 2.079 and 1.748, respectively. The majority of subjects were significantly more reactive to Pen m 1 than to Cra g 1 or to Der p 10 ($p<0.001$), with one exception for subject 14, where the reactive to Cra g 1 was much higher than to Pen m 1 or to Der p 10 ($p<0.001$).

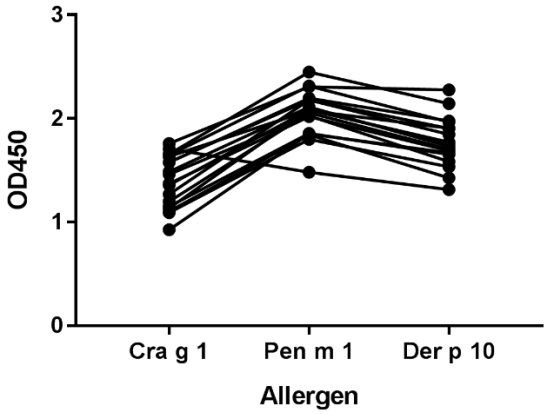


Figure 4-8 Patient serum IgE reactivity analysis by ELISA for three tropomyosins: Cra g 1 (Pacific oyster), Pen m 1 (Black tiger prawn) and Der p 10 (House dust mite) (n = 18).

4.5.5 Tropomyosin amino acid sequence comparison and IgE-binding epitope prediction

To understand the findings of the IgE-reactivity analysis of the Pacific oyster, Black tiger prawn and House dust mite TM using ELISA , a comparison of the amino acid sequence of TM from the three species was generated. The three TM sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log-Expectation) program in Mega 7 software with default parameter sets (Figure 9). Concurrent with the ELISA result of IgE reactivity where the majority of the subjects showed higher reactivity to Pen m 1 and Der p 10, the sequence alignment demonstrated that black tiger prawn is more closely related to house dust mite rather than to Pacific oyster. Seventy-two and 126 amino acid substitutions were observed in Der p 10 and Cra g 1, respectively, compared to Pen m 1. Sequence identity between Pen m 1 and Der p 10 is about 80.28%, while between Pen m 1 and Cra g 1 the identity is much less with about 63.03%. Previously published study [19] showed that cross-reactivity occurs when the IgEs bind to proteins from other species having peptide with maximum two amino acid replacements as compared to the IgE-binding epitopes. Using this knowledge, five regions with two of these regions positioned in the C-terminal of the TM were predicted as the epitopes responsible for the cross-reactivity between Pacific oyster, Black tiger prawn and house dust mite (Figure 4-9).

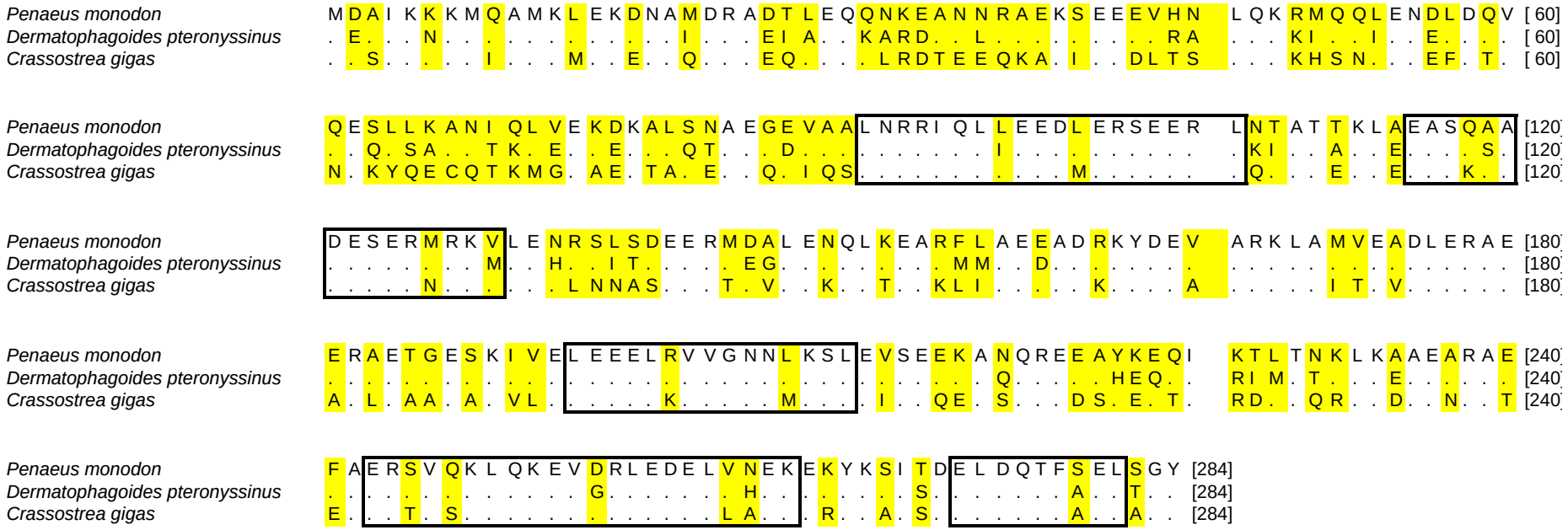


Figure 4-9 Amino acid sequence alignment of tropomyosin sequence from Black tiger prawn (*Penaeus monodon*), house dust mite (*Dermatophagoides pteronyssinus*) and Pacific oyster (*Crassostrea gigas*). Variable amino acids are shaded in yellow. The predicted IgE-binding epitopes responsible for cross-reactivity in those three species are identified by solid boxes

4.6 Discussion

The development of accurate diagnostic tools for mollusc allergy is still facing some difficulties due to the large diversity of edible mollusc species as well as the lack of purified or recombinant clinically relevant allergens. Clinical history is often unreliable as the patients ofte can not recall the offending mollusc species. While food challenge is regarded as the gold standard of food allergy diagnosis, their application has been associated with risk of severe reaction in the case of shellfish challenge due to high allergen potency and adult patient co-morbidities [20]. Several mollusc IgE-reactive proteins have been identified in previous studies. However, they haven't been fully characterised, limiting their application as diagnostic tools for mollusc allergy. In this present study, the major allergen of the Pacific oyster (*Crassostrea gigas*) was characterised, the IgE recognition in a large cohort of oyster allergic patients determining and the IgE reactivity to purified TM of Black tiger prawn (Pen m 1) and House dust mite (Der p 10) compared. Subsequently TM was registed with the IUSI as the first allergen from the Pacific oyster. Immunological studies of natural and recombinant TM from the Pacific oyster have been reported in previous studies [21-23]. However, these studies did not register this major allergen with the IUIS, used only a very limited number of patients, and the reactivity to other TM was determined using IgE derived from pooled serum.

In this study, 21 patients were analysed for their IgE reactivity against the raw and cooked extract of the Pacific oyster. This was the first time screening of the Pacific oyster extracts conducted against a large number of patients. Similarly to the findings of previous chapters, more IgE-binding proteins were observed in the raw compared to the cooked extract. However, the cooked extract demonstrated higher IgE binding intensity than the raw extract for the same IgE reactive proteins. Pacific oyster is the most consumed species of mollusc. While most of the shellfish species are ingested in cooked form such as shrimps and crabs, oysters are mostly consumed raw, thereby potentially presenting both heat-labile and heat-stable allergens to the patient's immune

system. The results presented in this chapter are concurrent with the previous findings in Chapter 2 using a pool of patient sera.

Different IgE-binding patterns of the 21 patientrs were observed, suggesting that different sensitisation profiles are present in oyster-allergic patients. The assessment also demonstrated that not all subjects showed IgE reactivity to TM. Although the majority of the patients recognised TM (>70%), four patients showed no reactivity to TM. Instead these patients IgE recognised other proteins with molecular mass corresponding to those of known mollusc allergens in the previous chapter, Peptidyl-prolyl cis-trans isomerase and paramyosin [24]. Additionally, the fact that over 50% of patients reacted to proteins at molecular weight ~50kDa in the cooked extract is quite intriguing. Mass spectrometry analysis of the band at a similar position in the previous chapter (Chapter 2, spot no 23 at the 2D-PAGE of the cooked extract) revealed the band contained 15 different proteins with TM as the major component. Whether the IgE binding was due to TM isoforms, agregrates or other proteins in the mixture is not clear. However, our recent published study showed that TM from the Sydney rock oyster could be identified at 34, 39, 45 and 72 kDa in the heated extract, possibly due to degradation and aggregation during heat treatment [6]. Therefore, TM-specific IgE testing alone is insufficient for diagnosis of mollusc allergy.

Production of recombinant allergen is essential for the diagnosis of allergy. Traditionally, the diagnosis of allergy is performed using the extracts obtained from different allergen sources. These extracts, however, contain a mixture of non-allergenic and allergenic proteins and is often difficult to standardise the allergen content in these tests. Nowadays, diagnosis of allergy is increasingly conducted at the molecular level collectively referred to as component-resolved diagnosis (CRD) [13, 25]. Purification of the allergens is also an essential criterion for allergen acceptance by the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee. This study reports for the first time the

cloning, full sequence analysis and recombinant expression of the Pacific oyster TM. The recombinant TM shares 97% amino acid sequence similarity with previous published Pacific oyster TM, BAH10152.1. Mass spectrometry and CD spectroscopy analysis of purified natural and recombinant TM demonstrated both TMs have very similar properties. The immunological characteristic of the recombinant TM is also identical to that of natural TM. Based on this study, the Pacific oyster TM has now been accepted and designated Cra g 1.01001 by the Sub-Committee (http://allergen.org/viewallergen.php?aid=902).

Mollusc, together with crustacean, are termed as shellfish in fisheries terminology, although taxonomically those two groups are under different families. Due to this assumption, crustacean allergic patients are often advised to avoid mollusc as well. Although allergen cross-reactivity between crustaceans and mollusc has been documented clinically and experimentally [26], group-specific allergy has also been reported [27], suggesting that the clinical recommendation may not be completely accurate. In this study, IgE reactivity between Pacific oyster TM Cra g 1, Black tiger prawn Pen m 1 and house dust mite Der p 10 was evaluated using ELISA. The results showed that patients which recognised Pacific oyster TM also demonstrated IgE reactivity with Pen m 1 from shrimp and Der p 10 from dust mite. All patients, except patient 14, demonstrated higher IgE reactivity to Pen m 1 and Der p 10 than to Cra g 1, in agreement with the ImmunoCAP results. Tropomyosin is a pan-allergen and has been shown responsible for clinical and immunological cross-reactivity across different invertebrate species [28]. Their highly cross-reactive nature is due to the similarity in amino acid sequence. The IgE reactivity patterns seem to correlate with the degree of amino acid sequence identity. The identity between Pen m 1 and Der p 10 is considerably higher than between Pen m 1 and Cra g 1. Furthermore, the amino acid sequence identity of the predicted IgE-binding epitopes in Pen m 1 showed Cra g 1 has more sequence mismatches compared to that of Der p 10, particularly at the N-terminal and middle part of the TM. Consequently, for patients who showed

high IgE binding to Pen m 1, their immunological reactivity to Cra g 1 is lower than to Der p 10. The number of mismatches on the IgE-binding epitopes have been shown to affect the reactivity to allergenic proteins [29, 30]. However, it is to be noted that cross-reactivity can be symmetrical or asymmetrical. In the symmetric cross-reactivity, two or more allergens can be the sensitising agents, while in asymmetrical only one allergen responsible for the sensitisation and the others are reactive due to similar sequence.

In conclusion, this is the first study on the identification of allergens in the Pacific oyster using a large cohort of oyster-sensitised patients. Tropomyosin was confirmed as the major allergens, Crac g 1, reacting with 18 out of 21 patients analysed. Other allergens were also observed, some corresponding to previously identified allergens from other mollusc species. Patients with reactivity to Pacific oyster TM demonstrated also IgE reactivity to Black tiger prawn and House dust mite TM, although the degree of reactivity varied among patients. In summary, the findings of this chapter provided novel recombinant oyster allergens for the development of reliable component-resolved diagnostic assays for mollusc allergy and enable accurate dietary advice. The following chapter will analyse the IgE binding eptiopes in more detail using advanced bioinformatic tools, to provide clinicians with allergome derived diagnostic decision trees for the prediction and management of patients with multiple allergic sensitisation to shellfish and indoor invertebrate allergens.

4.7 References

1. Wu, T.C., et al., *Prevalence of food allergy in Taiwan: a questionnaire-based survey.* Intern Med J, 2012. **42**(12): p. 1310-5.
2. Prescott, S., et al., *A global survey of changing patterns of food allergy burden in children.* World Allergy Organization Journal, 2013. **6**(21): p. 1-12.
3. Ruethers, T., et al., *Seafood allergy: A comprehensive review of fish and shellfish allergens.* Molecular Immunology, 2018.
4. Nugraha, R., et al., *Rapid and comprehensive discovery of unreported shellfish allergens using large-scale transcriptomic and proteomic resources.* Journal of Allergy and Clinical Immunology, 2018. **141**(4): p. 1501-1504.e8.
5. Taylor, S.L., *Molluscan shellfish allergy.* Adv Food Nutr Res, 2008. **54**(1043-4526 (Print)): p. 139-77.
6. Rolland, J.M., et al., *Effect of Heat Processing on IgE Reactivity and Cross-Reactivity of Tropomyosin and Other Allergens of Asia-Pacific Mollusc Species: Identification of Novel Sydney Rock Oyster Tropomyosin Sac g 1.* Molecular Nutrition & Food Research, 2018.
7. Pascal, M., et al., *Molecular Diagnosis of Shrimp Allergy: Efficiency of Several Allergens to Predict Clinical Reactivity.* The Journal of Allergy and Clinical Immunology: In Practice, 2015. **3**(4): p. 521-529.e10.
8. Kamath, S.D., et al., *IgE reactivity to shrimp allergens in infants and their cross-reactivity to house dust mite.* Pediatr Allergy Immunol, 2017. **28**(7): p. 703-707.
9. Kuehn, A., et al., *Cross-reactivity to fish and chicken meat - a new clinical syndrome.* Allergy, 2016. **71**(12): p. 1772-1781.
10. Gámez, C., et al., *Tropomyosin IgE-positive results are a good predictor of shrimp allergy.* Allergy, 2011. **66**(10): p. 1375-1383.
11. Schoos, A.M.M., et al., *Disagreement between skin prick test and specific IgE in young children.* Allergy, 2015. **70**(1): p. 41-48.

12. Köhler, J., et al., *Component resolution reveals additional major allergens in patients with honeybee venom allergy.* Journal of Allergy and Clinical Immunology, 2014. **133**(5): p. 1383-1389.e6.
13. Ruethers, T., et al., *Seafood allergy: A comprehensive review of fish and shellfish allergens.* Mol Immunol, 2018. **100**: p. 30.
14. Liang, J., et al., *Quantitative analysis of species specificity of two anti-parvalbumin antibodies for detecting southern hemisphere fish species demonstrating strong phylogenetic association.* Food Chemistry, 2017. **237**: p. 588-596.
15. Ruethers, T., et al., *Characterization of Ras k 1 a novel major allergen in Indian mackerel and identification of parvalbumin as the major fish allergen in 33 Asia-Pacific fish species.* Clinical & Experimental Allergy, 2018. **48**(4): p. 452-463.
16. Koeberl, M., et al., *Auto-induction for high yield expression of recombinant novel isoallergen tropomyosin from King prawn (Melicertus latisulcatus) for improved diagnostics and immunotherapeutics.* Journal of Immunological Methods, 2014. **415**(0): p. 6-16.
17. Kamath, S.D., et al., *Molecular and immunological approaches in quantifying the air-borne food allergen tropomyosin in crab processing facilities.* Int J Hyg Environ Health, 2014. **217**(7): p. 740-50.
18. Aalberse, R.C. and R. Crameri, *IgE-binding epitopes: a reappraisal.* Allergy, 2011. **66**(10): p. 1261-74.
19. Ayuso, R., et al., *Molecular Basis of Arthropod Cross-Reactivity: IgE-Binding Cross-Reactive Epitopes of Shrimp, House Dust Mite and Cockroach Tropomyosins.* International Archives of Allergy and Immunology, 2002. **129**(1): p. 38-48.
20. Lopata, A.L., J. Kleine-Tebbe, and S.D. Kamath, *Allergens and molecular diagnostics of shellfish allergy: Part 22 of the Series Molecular Allergology.* Allergo. J. Int., 2016. **25**(7): p. 210-218.

21. Ishikawa, M., et al., *Isolation and Properties of Allergenic Proteins in the Oyster Crassostrea gigas.* Fisheries science, 1997. **63**(4): p. 610-614.
22. Ishikawa, M., et al., *Tropomyosin, the Major Oyster Crassostrea gigas Allergen and its IgE-binding Epitopes.* Journal of Food Science, 1998. **63**(1): p. 44-47.
23. Leung, P.S.C. and K.H. Chu, *cDNA cloning and molecular identification of the major oyster allergen from the Pacific oyster Crassostrea gigas.* Clinical & Experimental Allergy, 2001. **31**(8): p. 1287-1294.
24. Nugraha, R., et al., *Rapid and comprehensive discovery of unreported shellfish allergens using large-scale transcriptomic and proteomic resources.* J Allergy Clin Immunol, 2018. **141**(4): p. 1501-1504 e8.
25. Matricardi, P.M., et al., *EAACI Molecular Allergology User's Guide.* Pediatr Allergy Immunol, 2016. **27 Suppl 23**: p. 1-250.
26. Prester, L., *Seafood Allergy, Toxicity, and Intolerance: A Review.* J Am Coll Nutr, 2016. **35**(3): p. 271-83.
27. Vidal, C., et al., *Sensitization pattern of crustacean-allergic individuals can indicate allergy to molluscs.* Allergy, 2015. **70**(11): p. 1493-1496.
28. Lopata, A.L., R.E. O'Hehir, and S.B. Lehrer, *Shellfish allergy.* Clinical & Experimental Allergy, 2010. **40**(6): p. 850-858.
29. Lapteva, Y.S., V.N. Uversky, and S.E. Permyakov, *Sequence microheterogeneity of parvalbumin, the major fish allergen.* Biochim Biophys Acta, 2013. **1834**(8): p. 1607-14.
30. Huang, R., et al., *Determination of microheterogeneous substitution in shrimp tropomyosin and its effect on IgE-binding capacity.* European Food Research and Technology, 2014. **239**(6): p. 941-949.
31. Aalberse, R.C., *Assessment of allergen cross-reactivity.* Clinical and Molecular Allergy, 2007. **5**(1): p. 2.

CHAPTER 5. CONSERVATION ANALYSIS OF IGE-BINDING EPITOPES TO PREDICT CLINICAL CROSS-REACTIVITY BETWEEN CRUSTACEAN AND MOLLUSC ALLERGENS

Published in

Roni Nugraha, Sandip D Kamath, Shaymaviswanathan Karnaneedi, Elecia Johnston, Andreas L Lopata. 2018. Conservation analysis of B-cell allergen epitopes to predict clinical cross-reactivity between shellfish and inhalant invertebrate allergens. *Frontiers in Immunology***.** Submitted

5.1 Summary

Shellfish allergy affects up to 2% of the world population and persists for life in most patients. The diagnosis of shellfish allergy is however often challenging due to reported clinical cross-reactivity to other invertebrates including mites and cockroaches. Prediction of cross-reactivity can be achieved utilizing an in-depth analysis of a few selected IgE-antibody binding epitopes. In this chapter, available experimentally proven IgE-binding epitopes were combined with informatics-based cross-reactivity prediction modelling to assist in the identification clinical cross-reactivity biomarkers on shellfish allergens. An epitope conservation model using IgE binding epitopes available in the Immune Epitope Database and Analysis Resource (http://www.iedb.org/) was developed. The epitope conservation was applied to a set of four different shrimp allergens, and successfully identified several non-cross-reactive as well as cross-reactive epitopes, which have been experimentally established to cross-react. These findings suggest that this method can be used for advanced component-resolved-diagnosis to identify patients sensitised to a specific shellfish group and distinguish from patients with extensive cross-reactivity to ingested and inhaled allergens from invertebrate sources.

5.2 Introduction

Edible crustacean and mollusc are commonly discussed as ‘shellfish’. However, the group of ‘shellfish’ comprises the two invertebrate phyla arthropods and molluscs. Although all shellfish are invertebrate animals, these two groups are very distinct in evolutionary terms and subsequently contain different molecular repertoires of food allergens. In fact, crustacean are placed closer to insects and arachnids (spiders), and this seems to be the major factor for molecular sensitization and clinical reactivity between crustacean, dust-mite, insects and parasites [1]. Severe acute allergic reactions upon accidental ingestion of different shellfish species, or insect contaminated food, have been observed in several studies [2, 3]. However, lack of specific diagnostic tools is the main problem for correct diagnosis of crustacean and/or mollusc allergy due to extensive immunological cross-reactivity with other invertebrate allergen sources containing similar proteins.

Cross-reactivity occurs when the IgE antibodies recognise identical or very similar protein patches (epitopes) from different proteins as compared to the primary sensitising protein [4]. IgE cross-reactivity to unrelated peanut allergens has recently been demonstrated by Bublin, Kostadinova [5], resulting from amino acid similarities of short peptides. Furthermore, it is known that phylogenetically related species often have similar proteins, and this similarity can implicate IgE cross-reactivity [6]. Allergens which share highly conserved protein sequences, but also structure and function, can be termed pan-allergens if they are responsible for antibody binding cross-reactivity and subsequent clinical cross-reactivity [7].

Cross-reactivity of related proteins could be predicted computationally by comparing the identity of the amino acid sequence to the known allergen. Aalberse [8] reviewed potentially cross-reactive structures of known allergens and noted that proteins with greater than 50% identity throughout the length of the protein compared to an allergen are likely cross-reactive. In Chapter 2, 24 unreported and potentially cross-reactive allergen were identified from Pacific

oyster. However, some recent studies [5, 9, 10] demonstrated that this predictive value is only useful to predict the allergenicity of new proteins but is not accurate to predict cross-reactivity. This is of particular importance for invertebrate tropomyosin which shares over 50% of amino acid identify with human tropomyosin. Moreover, the cross-reactivity is patient-specific as demonstrated in Chapter 4 and seem only to occur when the patient IgE antibodies bind to conserved epitopes. In the context of food allergy, sequential IgE binding epitopes seem to be much more relevant as conformational epitopes are easily degraded due to the digestion in the gastrointestinal tract [11].

Based on these observations a large-scale analysis of sequential IgE epitope conservation is of great importance for predicting clinical cross-reactivity between crustacean and mollusc, as well as mite and cockroach, in allergic patients. While there are eight allergenic proteins known among different shellfish [12], conclusive epitope data are only available for shrimp allergens including TM, AK, SCP and MLC. Positive IgE-binding epitopes of these shrimp allergens were collected from the Immune Epitope Database (IEDB). Subsequently, the conserved epitope sequences responsible for cross-reactivity was determined using the Epitope Conservancy Analysis program [13]. Shrimp allergen epitopes were considered to be conserved if the sequence of the other invertebrate homologous peptide had only up to two amino acid mismatches [14-17]. These epitopes could be used to design better predictive diagnostic tools for shellfish allergic patients.

5.3 Aims

1. To analyse the conservation of the IgE-binding epitopes of four shrimp allergens in crustaceans and mollusc species
2. To determine the IgE-binding epitopes responsible for cross-reactivity between crustacean and mollusc.
3. To develop a decision flow for predicting cross-reactivity between shrimp and molluscs based on the major allergens TM and AK

5.4 Materials and Methods

5.4.1 Collection of shrimp allergens IgE-binding epitopes

The dataset for the subsequent analysis was built from available IgE binding epitopes from four shrimp allergens: tropomyosin (TM), arginine kinase (AK), myosin light chain (MLC) and sarcoplasmic calcium-binding protein (SCBP). The data were assembled from the Immune Epitope Database and Analysis Resource (IEDB) [18]. The collected epitopes were restricted to peptides with positive serum IgE antibody binding from patients with confirmed shrimp allergy.

5.4.2 Sequence retrieval and phylogenetic analysis

Protein sequences were obtained from the UniProt database and aligned using MUSCLE v3.8.31 [19]. Different numbers of protein sequences could be retrieved for TM, AK, MLC and SCBP, with respectively 54, 30, 20 and 10 protein sequences. The selected sequences represent 18 different crustacean and 30 mollusc species within the shellfish group. The subsequent phylogenetic analyses of relatedness of proteins were performed using MrBayes v3.2.6 [20] (50,000 generations) and RAxML v8.2.9 [21] (1000 replicates) via the CIPRES Science Gateway [22].

5.4.3 Conservation analysis of shrimp allergens in invertebrate species

The conservation of amino acid residues for each allergen among the different invertebrate species was estimated using the Rate4Site algorithm in Consurf [23] server by calculating position-specific evolutionary rates under an empirical Bayesian methodology. The rates were normalized and grouped into 9 grades where high conserved residues receive a score of 9, and very variable residues receive a score of 1. The conservation rate of the amino acids was then mapped to the structure model of the allergens using Chimera [24].

5.4.4 Conservation analysis of IgE-binding epitopes of shrimp allergens

The degree of conservation of the epitopes within the sequences of the respective allergens was calculated using conservancy analysis tool [13] on the IEDB website. The degree of conservation of an epitope is calculated as the fraction of the protein sequence that matched the aligned epitope above a chosen identity level. An epitope was considered to be conserved if the homologous peptide had less than two amino acid mismatches.

5.4.5 Data analysis

Data analysis was performed using GraphPad Prism version 7. One-way ANOVA was applied to determine the statistically significant difference of the conserved epitopes between groups of invertebrate species.

5.5 Results

5.5.1 Amino acid sequence analysis of shrimp allergens

Phylogenetic analysis was performed to determine the relationships between shrimp allergen sequences in different invertebrate groups and to infer the evolutionary trends among the wide representation of the allergens. In this study, four shrimp allergens, TM, AK, SCP and MLC were selected based on the availability of the IgE-binding epitopes. A dataset of TM, AK, SCP and MLC protein sequences from crustaceans and molluscs species in UniProt database was assembled to construct a tree using the Maximum Likelihood and Bayesian approach. A consensus tree generated for all protein groups showed similar topologies with good branch support (>70%) for major branches for TM and AK **(**Figure 5-1**)**. These trees, particularly for TM, is in good agreement with previously published TM trees [25, 26], demonstrating the expected distant phylogenetic relationship between crustacean and mollusc. Crustacean clustered closer with other allergy-causing arthropods, including mite and cockroach, while the mollusc forms a distinct cluster.

The degree of evolutionary conservation at individual amino acid sites of TM and AK were determined using Consurf server by applying the Rate4Site algorithm. In ConSurf, the evolutionary rate is estimated based on the evolutionary relatedness between the protein and its homologues and considers the similarity between amino acids as reflected in the substitutions matrix. The conservation grades identified using ConSurf are mapped to the query sequence and/or structure using the ConSurf colour-code, with cyan-through-purple corresponding to a variable (grade 1)-through-conserved (grade 9) positions (Figure 5-2). As the analysis can only be conducted if there are at least five homologous proteins, the conservation analysis could only be conducted for the two major allergens, TM and AK, but not for SCP and MLC. In general, TM had more conserved amino acids than AK. Most of the conserved amino acids in the TM were located at the N- and C-terminal regions. A total of 175 out of 284 TM residues had conservation grades >5,

while 86 residues (30%) had the full grade 9. For AK, 200 out of 356 residues had conservation grades >5, of which 96 residues (27%) had grade 9.

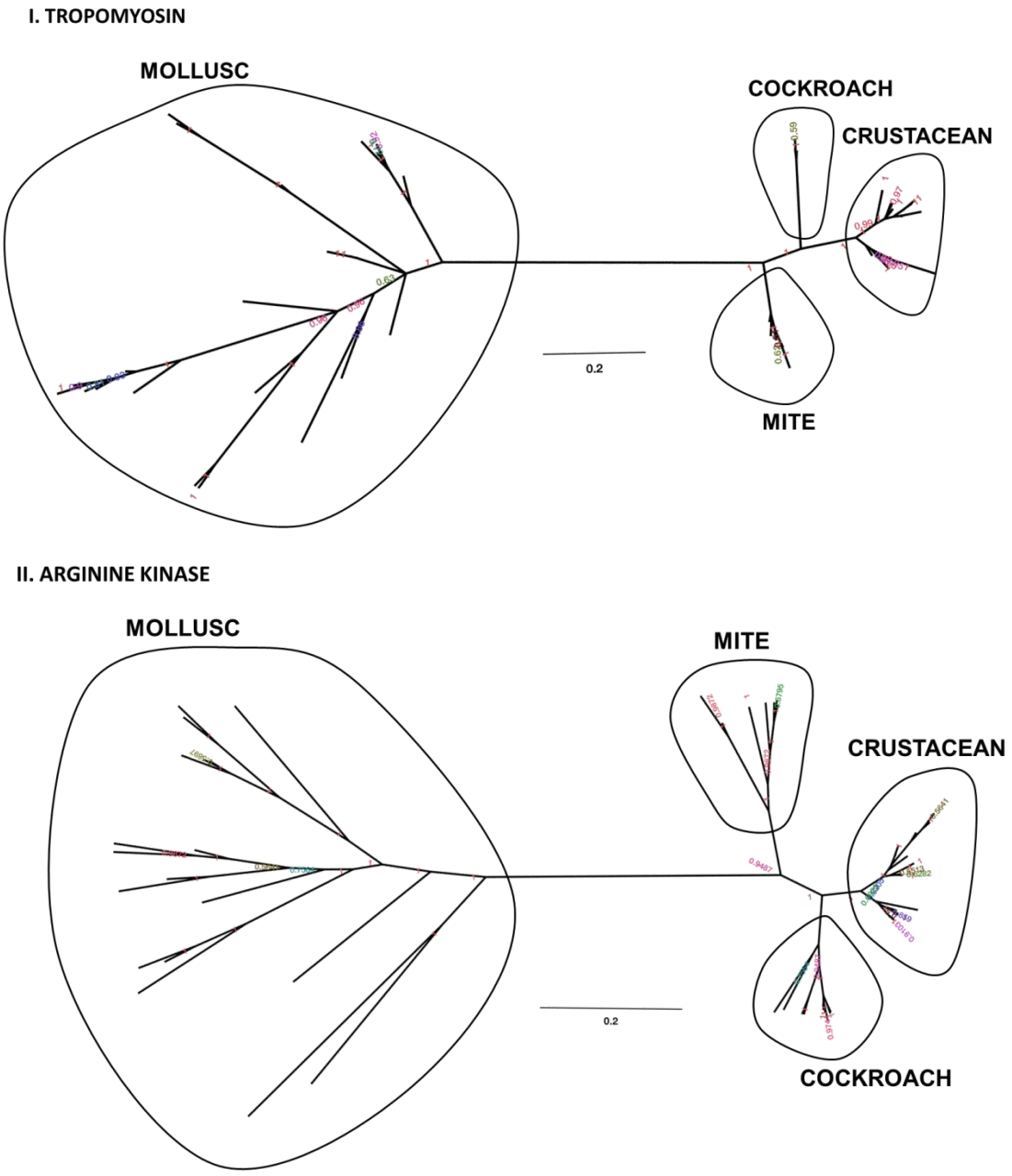


Figure 5-1 Molecular evolutionary analysis of shellfish allergens. The phylogenetic tree was drawn using Bayesian and Maximum Likelihood approach for (**I**) Tropomyosin and (**II**) Arginine kinase. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

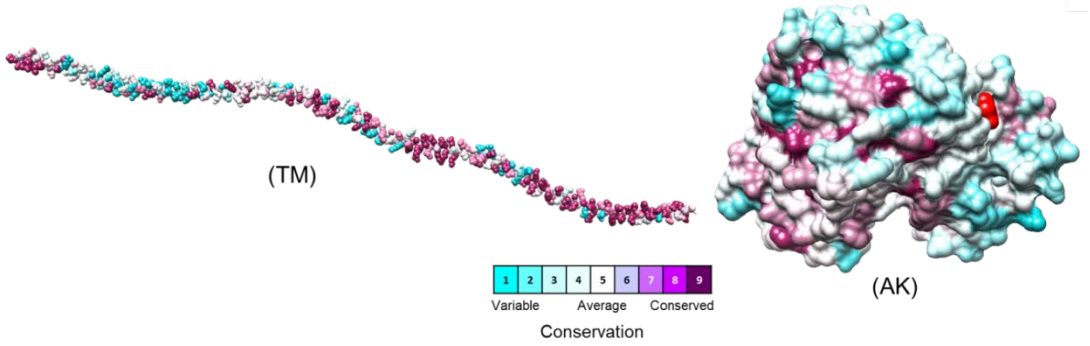


Figure 5-2 Conservation analysis of individual amino acids in tropomyosin (TM) and arginine kinase (AK) determined using the Consurf server. The conservation grades were mapped onto the query sequence and structure using the ConSurf colour-code, with blue-through-red corresponding to a variable (grade 1)-through-conserved (grade 9) positions.

5.5.2 Conservation of IgE binding epitopes of shrimp allergens

To determine the likelihood of cross-reactivity between shrimp and other invertebrates, the conservation analysis of epitopes in those groups was conducted. A database of 176 epitopes was generated, containing 96 epitopes from TM, 39 epitopes from AK, 27 epitopes from MLC and 12 epitopes from SCBP. These epitopes were identified to be recognized by IgE antibodies from over 100 patients with shellfish allergy as determined in previous studies [15, 27-31]. The epitope conservation was determined using epitope conservancy analysis tool within the IEDB webpage. Epitopes were conserved when they share less than two amino acid mismatches within the aligned sequence. The conservation analysis showed a similar trend for TM and AK. In Figure 5-3 the number of conserved epitopes for TM was found to be the highest within the crustacean followed by cockroach, mite and mollusc.

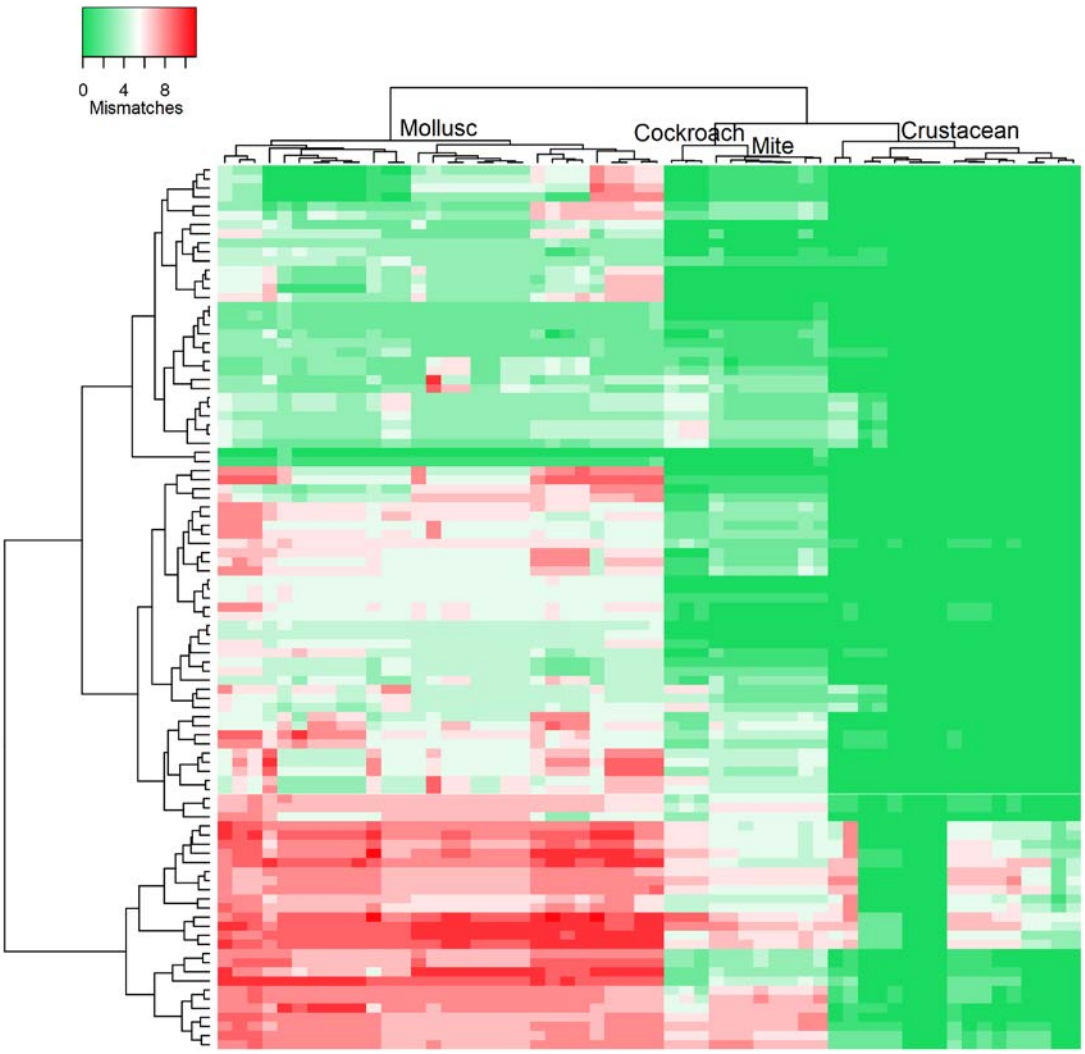


Figure 5-3 Heatmap representing number of mismatches in homologous peptide of shrimp TM IgE-binding epitopes in different invertebrate species. The heatmap was generated using Heatmapper [32] and clustered using Manhattan distance metric approach. The colours grading as indicated in the top left represents the number of amino acid mismatches found in the homologous peptides of epitopes. Green indicates no mismatches and red indicates maximum mismatches.

Approximately, 91% of IgE epitopes could be matched within 16 crustacean species, 56% of the three cockroach species and 48% within the nine mite species. All conserved peptides of crustacean, cockroach and mites epitopes where significantly higher than any of the three mollusc groups. In contrast to the crustacean and insects, within the 30 molluscs less than 20% of TM epitopes were conserved (Figure 5-4A) and even less in AK with 9% of all epitopes (Figure 5-4B). Nevertheless, these conservations were very high with the majority having only one or two amino acid mismatches. Within the molluscs, the cephalopods had the highest number of conserved epitopes, followed by gastropods and bivalves. The analysis of 20 MLC and 10 SCBP proteins resulted in no conserved epitopes to be identified within the mollusc group.

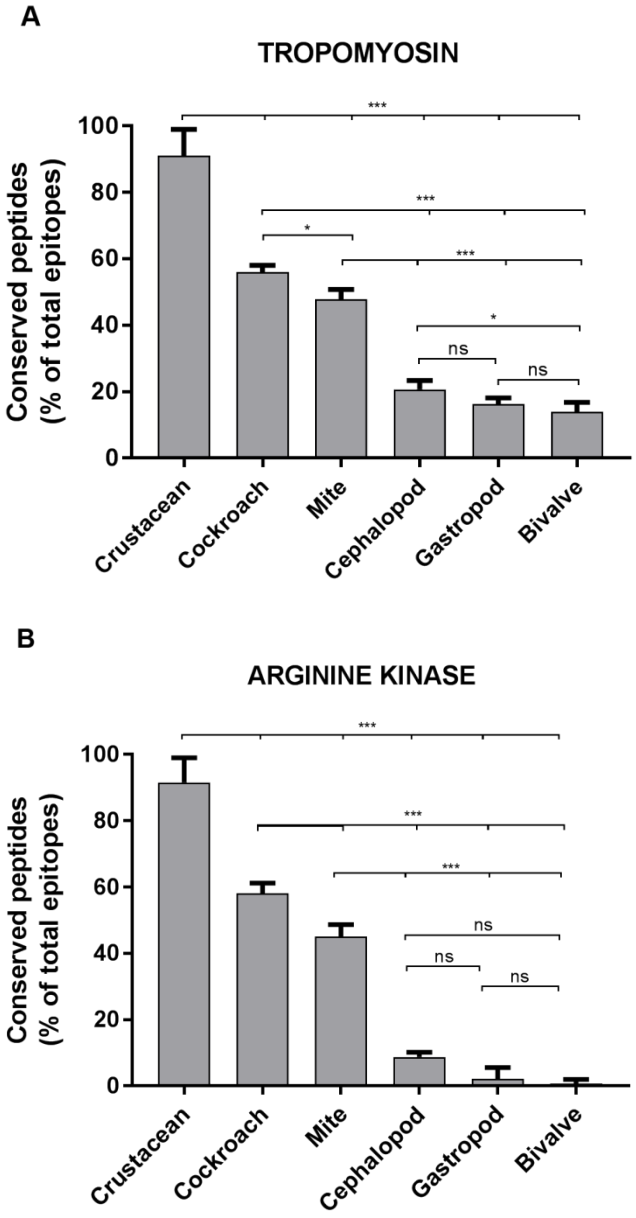
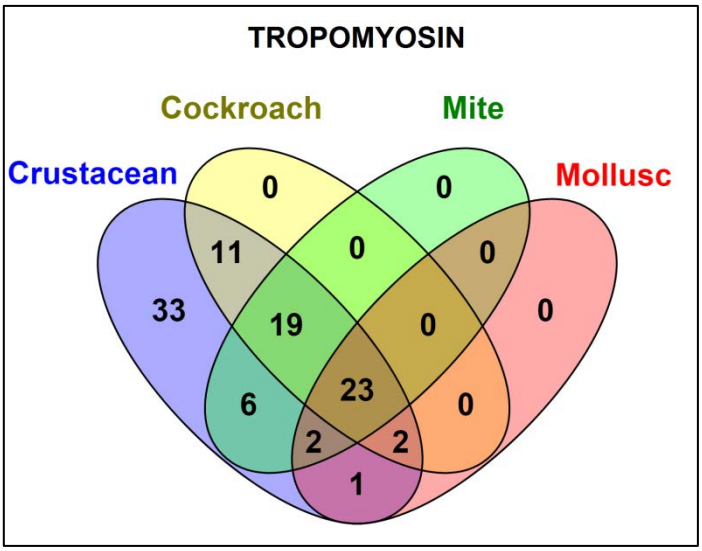


Figure 5-4 Percentage of conserved shrimp IgE-binding epitopes between invertebrate species. Ninety-Eight B-cell epitopes from tropomyosin (A) and thirty-nine B-cell epitopes from arginine kinase (B) were examined for their conservation within shellfish and between other allergenic invertebrate species. An epitope was considered conserved if the sequence matched to a homolog or a peptide variant with not more than 2 amino acid substitutions in another species. Significance differences ($p<0.05$) were calculated using One-way ANOVA.

5.5.3 Invertebrate allergen pan-epitopes

Species-specific conservation analysis of IgE binding epitopes was carried out to identify the allergen epitope sequences that could be termed ‘pan-epitope’. Of the 97 shrimp TM IgE-binding epitopes, 23 invertebrate pan-epitope were identified (Figure 5-5A). These epitopes were conserved and shared by crustacean, cockroach, mite as well as the mollusc. The epitope sequences are summarised in **Error! Reference source not found.**. The species belonging to the Arthropoda - crustacean, cockroach and mite, shared 19 IgE-binding epitopes while crustacean-cockroach and crustacean-mite shared 11 and 6 epitopes respectively. Thirty-three IgE-binding epitopes were specific to crustaceans and may be used to diagnose crustacean-specific IgE sensitisation. **Error! Reference source not found.** shows that most of the invertebrate TM pan-epitopes are located at the N- and C-terminals of the TM sequence. The epitopes at the amino acid position 241 to 264 were of importance for the cross-reactivity across the tested sequences due to high conservation in over 90% of the invertebrate species analysed. In contracts only 5 of the 39 shrimp AK IgE-binding epitopes were conserved across crustacean, cockroach, mite and mollusc (Figure 5-5B and **Error! Reference source not found.**) and unlike TM IgE-binding epitopes, only few AK IgE-binding epitopes were specific to crustacean.



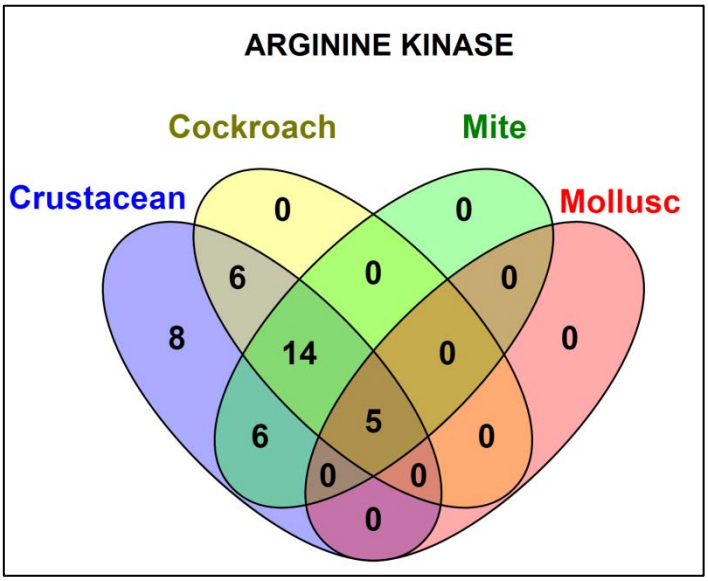


Figure 5-5 Venn diagram showing the similarities among the conserved shrimp IgE-binding epitopes across invertebrate species. In total 96 epitopes on TM and 39 on AK were analysed.

Table 5-1 Sequences of TM IgE-binding epitopes responsible for cross-reactivity between crustacean, cockroach, mite and mollusc in prawn allergic patients and their presentation in each invertebrate group.

Epitope sequence	Position	Presentation (%)			
		Crust	Cockroach	Mite	Mollusc
MDAIKKKMQAMKLEK	1-15	100	100	100	77
IKKKMQAMKLEKDNA	4-18	100	100	100	10
VAALNRRIQLLEEDL	85-99	100	100	100	3
LNRRIQLLEEDLERS	88-102	100	100	100	33
NRRIQLLEEDLERSEER	89-105	100	100	100	33
RIQLLEEDLER	91-101	100	100	100	43
RIQLLEEDLERSEER	91-105	100	100	100	33
EASQAADESERMRK	115-128	100	100	100	50
EASQAADESERMRKV	115-129	100	100	78	50
LENQLKEA	144-151	100	100	100	37
LAEEADRKYDEVARK	154-168	100	100	100	10
EADRKYDEVARKLAM	157-171	100	100	100	10
ESKIVELEEELRVVG	187-201	100	100	100	17
IVELEEELRVVGNNL	190-204	100	100	100	20
LEEELRVVGNNLKSL	193-207	100	100	100	50
KEVDRLEDELVNEKEKYKSI	241-260	100	100	100	60
ERSVQKLQKEVDRLEDE	243-259	100	100	100	90
QKLQKEVDRLEDELV	247-261	100	100	100	93
LQKEVDRLEDELV	249-261	100	100	100	100
QKEVDRLEDELVNEK	250-264	100	100	100	93
KEVDRLEDE	251-259	100	100	100	100
VDRLEDELVNEKEKY	253-267	100	100	100	63

Table 5-2 Sequences of AK IgE-binding epitopes responsible for cross-reactivity between crustacean, cockroach, mite and mollusc in prawn allergic patients and their presentation in each invertebrate group.

Epitope sequence	Position	Present in (%)			
		Crustacean	Cockroach	Mite	Mollusc
SLLKKYLTKEVFDKL	25-39	57	100	33	9
EGGIYDISNKRRMGL	319-333	100	100	67	36
IYDISNKRRMGLTEF	322-336	100	100	67	55
ISNKRRMGLTEFQAV	325-339	100	100	100	45
KRRMGLTEFQAVKEM	328-342	100	50	100	27

5.5.4 Shellfish allergen pan-epitopes

Avoidance of other shellfish species including molluscs is one of the management strategies for shrimp-allergic patients. However, as Figure 5-1 shows, the major allergenic proteins from shrimps and mollusc are distinctly different, supported by previous studies showing that cross-reactivity between shrimp and mollusc is species-specific [33] . Based on this rationale, further detailed analysis of shrimp IgE-binding epitope conservation across three edible mollusc classes, including bivalves, cephalopods and gastropods were carried out. In total, 26 shrimp TM and 5 AK IgE-binding epitope sequences were aligned with less than two amino acid mismatches with at least one mollusc species.

Of the 26 conserved TM epitopes, only 23 epitopes were present in over 50% of each mollusc classes with three epitopes were conserved in all of the mollusc species (Fig 5). Detailed analysis of the conserved epitopes revealed that half of the amino acid residues in shrimp TM were responsible for cross-reactivity to at least one species of mollusc and these amino acids were distributed across the entire protein sequence. Some of the epitopes showed group-specific conservation, such as Epitope 3, 14, 15, 17 and 18 in Fig 5. Unlike TM where the conserved epitopes were aligned with various species of

mollusc, the conserved epitopes of AK were mostly aligned with peptides of the cephalopod. Of the five conserved AK epitopes, only four epitopes were present in over 50% of mollusc species, with two of those were aligned to all species of cephalopods (Figure 5-6). While the complete amino acid sequence of the 4 analysed shrimp TMs are identical, the length and composition of the identified IgE epitopes differs. Nevertheless, the overall trend of high conservation to cephalopod peptides is similar, as well as the low conservation to bivalves.

Combining the conserved epitopes divided the shrimp TM into three possible cross-reactivity scenarios, located on distinct areas on the TM allergen. Three regions with a total of 35 amino acids residues, or 12%, of the total 284 amino acids were conserved across all classes of the mollusc phyla (Figure 5-7, yellow boxes) and responsible for shellfish pan-allergy. Three regions (Figure 5-7, green boxes) were conserved within the cephalopods and gastropods, and one region (Figure 5-7, grey box) was conserved within the bivalves and cephalopods.

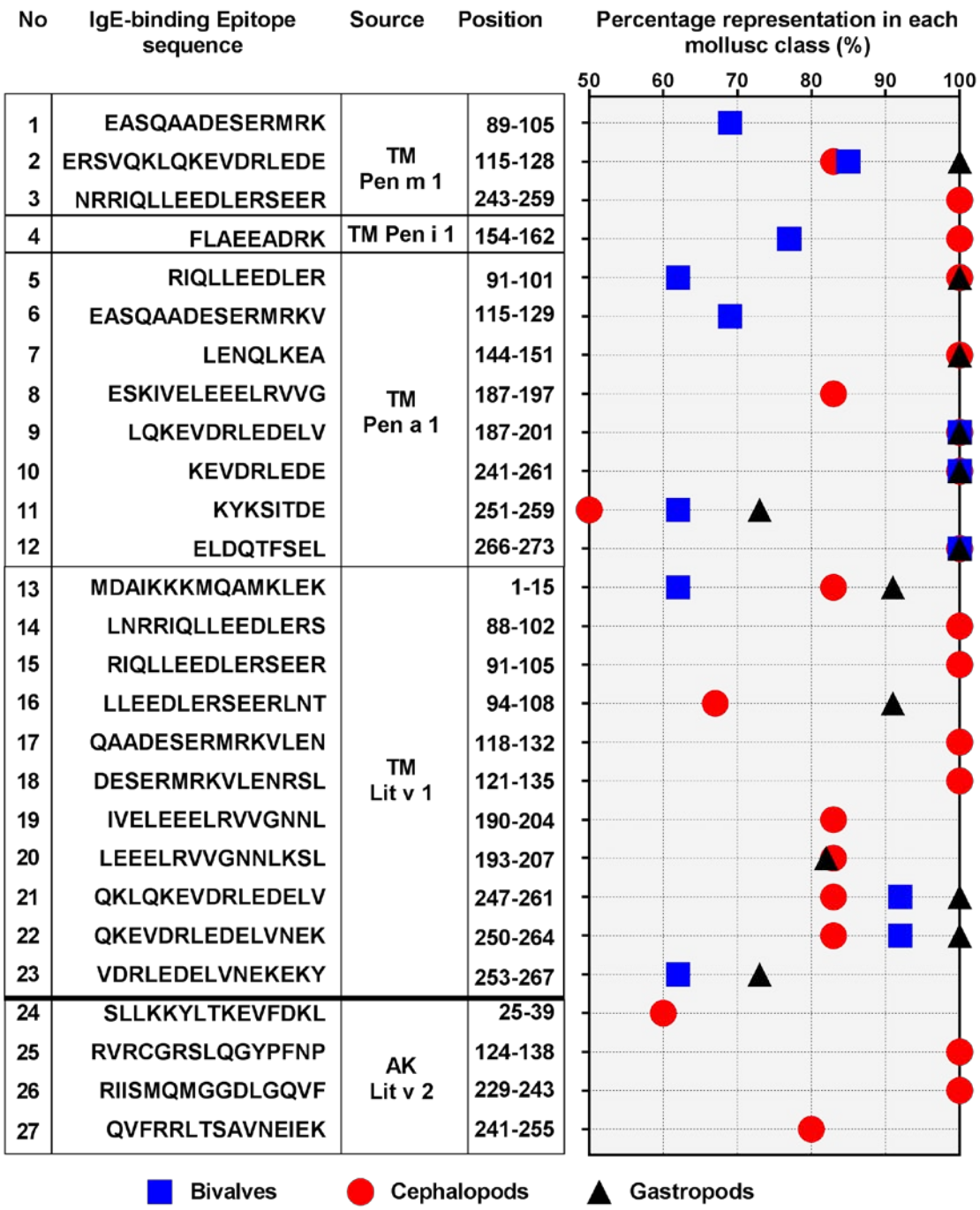
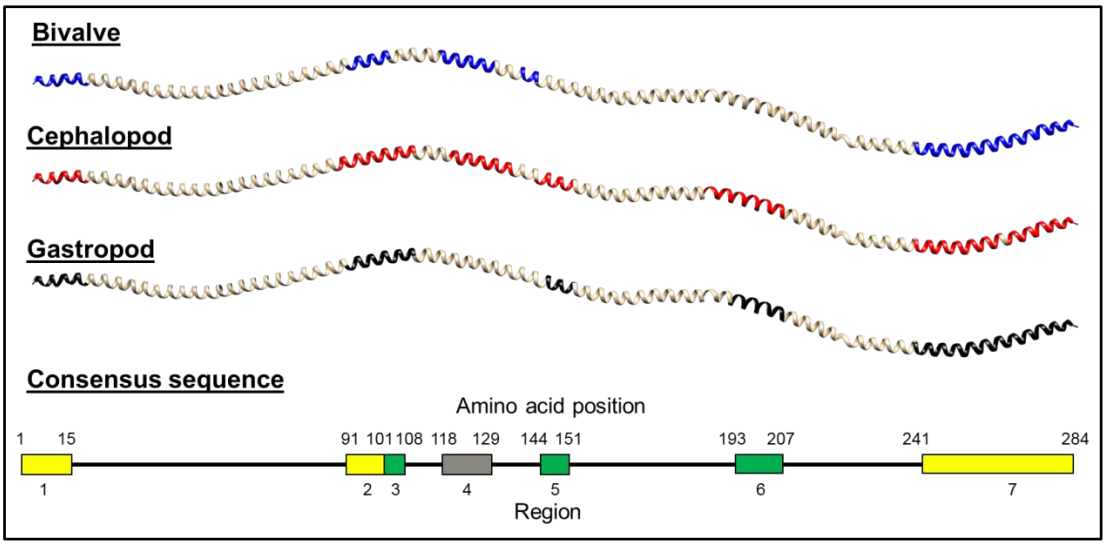


Figure 5-6 Percentage representation of conserved epitopes in the three mollusc classes: bivalves (square), cephalopod (circle) and gastropod (triangle). Only epitopes which are present in over 50% of each mollusc classes are shown. The epitope allergen sources, their amino acid sequences and positions in the protein are indicated on the left.

A



B

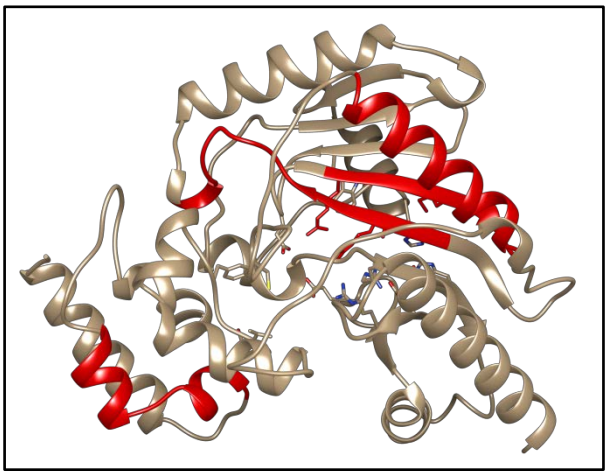


Figure 5-7 Molecular modelling of the conserved IgE-binding epitopes of (A) tropomyosin (TM) and (B) arginine kinase (AK) that are presented in over 50% of each mollusc class. For tropomyosin, the epitopes were remapped to their consensus tropomyosin sequence and colour-coded based on mollusc classes in which the conserved epitopes are found: yellow (all mollusc classes), green (cephalopod and gastropod), and grey (bivalve and cephalopod). The protein structure of TM and AK were modelled using SWISS-MODEL based on reference proteins 1C1G and 4BG4, respectively.

5.5.5 Decision tree to identify potential crustacean-mollusc cross-reactivity in shrimp-allergic patients

A decision tree was developed to identify potential shrimp-mollusc cross-reactivity in shrimp-allergic patients based on the conservation patterns of shrimp TM and AK IgE-binding epitopes in mollusc species (Figure 5-8). In the diagnosis of shrimp allergy, where clinical history does not give a clear conclusion, sensitisation tests against a whole shrimp extract and specific allergens are needed prior to oral food challenge. If TM-specific IgE results are positive, with quantitative IgE-levels to TM being similar than to the whole protein extract, immune-dominant sensitization to shellfish TM is likely, and broad (serological) cross-reactivity to other shellfish species is to be expected. Analysing IgE-binding to shrimp TM epitopes can further improve the diagnosis of cross-reactivity. Four reactivity patterns are suggested including; crustacean mono-reactivity, crustacean-mollusc cross-reactivity, crustacean-cephalopod-gastropod cross-reactivity and crustacean-cephalopod-bivalve cross-reactivity. However, if TM-specific IgE are not present (negative), then cross-reactivity due to sensitisation to AK is still possible, however only Region 1 would be responsible for crustacean-cephalopod cross-reactivity.

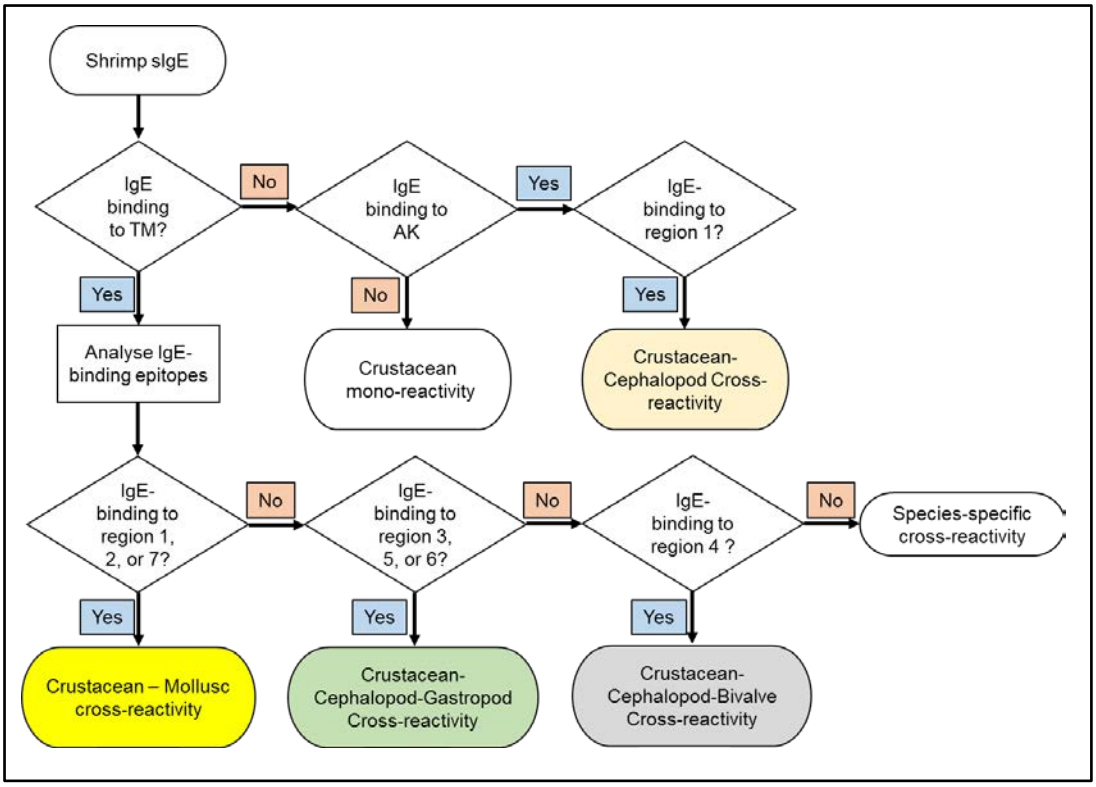


Figure 5-8 Decision tree to diagnose potential molecular cross-reactivity to invertebrate allergens in shrimp allergic patients based on the reactivity to specific IgE epitopes. The region numbers refer to the epitope mapping in Figure 5-7.

5.6 Discussion

The development of safe and reliable diagnostic tools is crucial to diagnose acurately allergic sensitisation in patients and determine the primary allergen sources. Diagnosis of shellfish allergy, in particular, is a major challenge for the management of the allergic patients due to highly cross-reactive nature of some shellfish allergens. This problem is clearly evident in a recent study by Pascal, Grishina [31] where 44% of their negative controls positively reacted with tropomyosin, resulting in an overall in a false-negative rate of 17% in their diagnosis. A preceding study demonstrated that IgE recognition of allergic patients towards identical and/or similar homologous peptides to the allergen epitopes are the basis of the molecular cross-reactivity [17]. Thus, the present study was conducted to determine shrimp IgE-binding epitopes that could be used to predict cross-reactivity toward other invertebrate species in shrimp allergic individuals, through which better predictive diagnostic tools for shellfish allergic patients could be developed.

The present study demonstrates that within a large directory of shrimp IgE-binding epitopes there are a substantial fraction of epitopes that are highly conserved across invertebrate species. These conserved epitopes might play a key role in cross-reactivity between shrimp and other invertebrate species. Shrimp TM and AK shared a higher number of conserved epitopes compared to shrimp SCP and MLC; in fact, no conserved epitope could be found for SCP, while the MLC only shared two epitopes in one region with cockroach MLC. A recent study by Kamath et al, demonstrated the absence of IgE recognition to shrimp SCP and MLC in house dust mite allergic infants [34]. These results suggest that TM and AK are the major contributing proteins in immunological and clinical cross-reactivity between crustacean and other invertebrate groups. Furthermore, comparative evaluation of the number of conserved epitopes in TM and AK revealed a clear cross-reactive hierarchy where cockroach is on the top followed by mite, while molluscs are on the bottom of this hierarchy.

House dust mite and cockroach are the most common sources of indoor allergens worldwide with up to 85% and 60% of asthmatic patients eliciting sensitisation to at least one of the mite or cockroach allergens respectively. In a retrospective study of 95 shrimp-allergic patients in Canada, 90.5% of those patients had a positive test to HDM [35]. Similarly, a study on an Asian population found a majority of patients with shrimp allergy have positive skin tests to HDM and cockroach [36]. Vivid evidence of this cross-reactivity was demonstrated by a study on Orthodox Jews who positively tested against shrimp yet had no prior exposure to seafood due to strict religious laws prohibiting shellfish consumption [37]. TM and AK seem to be the allergens responsible for this cross-reactivity as supported by the current study. Shrimp TM shares about 82% and 81% amino acid identity with cockroach and house dust mite tropomyosin, respectively. Likewise, shrimp AK shares about 82% and 78% amino acid identity with cockroach and house dust mite AK, with 66% of shrimp TM IgE-binding epitopes and 80% of shrimp AK IgE-binding epitopes are also existing within TM and AK of cockroach and house dust mite. These identified IgE-binding epitopes could be used to diagnose shrimp sensitised in patients sensitsed to cockroach or house dust mite, without a previous history of allergic reactions to shrimp. Reciprocally, the non-conserved IgE-binding epitopes can be used to diagnose genuine shrimp sensitisation. These findings are of significant impact on the diagnosis of shrimp allergy as current diagnostic tools using tropomyosin are not specific. Although tropomyosin is a good predictor of allergy reaction to crustacean [38], the rate of false positive reaction is still high due IgE binding of antibodies developed in patients against tropomyosin from other invertebrate sources, in particular house dust mite and cockroach [1]. Furthermore, the identification of specific IgE binding epitopes allows the prediction of allergic reactions to ingested crustacen, in patients allergic to cockroach and house dust mite. As over 30% of the global population is sensitised to dust mite allergens, the developed predictive model in this study could be of major importance.

Crustaceans and molluscs are generally referred to as “shellfish” in the context of seafood consumption and avoidance to both groups are often advised for shellfish allergic patients [39]. Patients with allergy to shellfish may fail to identify the offending seafood species, often as a result of confusion regarding the different common names used to describe diverse seafood. Crustaceans are classified as arthropods together with spiders and insects, while the group of molluscs is a large and diverse group, subdivided into different classes such as bivalves, gastropods, and cephalopods. Precise diagnosis of allergy to crustacean or mollusc species is difficult as no species-specific allergens have been identified so far. Moreover, true sensitisation to shellfish-specific allergens can be hampered due to the highly cross-reactive nature of some allergenic proteins, including TM and AK [40, 41]. While crustacean TMs and AKs show very high amino acid sequence identity (up to 98% and 97% respectively) with demonstrated IgE cross-reactivity [42, 43], the reported sequence identity between crustacean and mollusc TMs and AKs is much lower with only up to 68% and 58% respectively. The gold standard to determine food allergy is a oral food challenge, however due to the risk of severe reactions to shellfish allergens, this test is not frequently performed. This present findings suggest that specific shrimp allergen IgE-binding epitopes could be used as a robust, alternative way to diagnose cross-reactivity between crustacean and mollusc species among shellfish-allergic patients. Among the known 97 shrimp TM IgE-binding epitopes, 71 epitopes are only existing within crustacean TMs, while 26 epitopes are shared with mollusc TMs. Meanwhile, of the 39 shrimp AK IgE-binding epitopes, only five epitopes are shared with cephalopod AK. In contrast no shrimp SCBP and MLC IgE-binding epitopes are present in mollusc SCBP or MLC. These findings indicate that only shellfish-allergic patients sensitised to TM have a risk of reacting to both crustacean and mollusc species. This supports the conclusion of the previous study where crustacean-allergic patients with concurrent mollusc allergy reacted more frequently to tropomyosin than without it (93% vs 35%, respectively, $P = 0.004$), while recognition of the other allergens were not different in both patient cohorts [44].

This chapter findings also demonstrate different patterns of conserved IgE-binding epitopes among the three mollusc classes, suggesting that some crustacean-allergic patients will cross-react to one but tolerate another class of molluscs. The cephalopods have a higher probability to cross-react with crustacean than the other mollusc classes. The cephalopod TM amino acid sequences have a higher identity with crustaceans than those of gastropod and bivalve (68% vs 63 vs 62.3%, respectively), and therefore contained more homologous peptides of shrimp TM and AK IgE-binding epitopes (Figure 5-7). From the study of Vidal, Bartolome [44], of the 14 crustacean-allergic patients with mollusc allergy that were examined by skin prick tests against different mollusc species, 11 patients were positive to cephalopods, and 6 patients were positive to bivalves. While no study identified IgE-cross-reactivity due to AK between crustacean and gastropods or bivalves, cross-reactivity between cephalopods and crustacean has been reported [45]. Nevertheless, immunological cross-reactivity between shrimp and other mollusc classes, the gastropods and bivalves, has been demonstrated in several studies [46-48].

Gastropod and bivalve TMs share only 60% sequence identities with crustacean TMs, however, unlike cephalopod where the identity of TM amino acid sequence is very high among the group, the identity of TM amino acid sequence in the species among those two classes is very variable, particularly among bivalve species. The variability of bivalve TM is very apparent as demonstrated in Fig 5 where out of 23 conserved IgE-binding epitopes, only five epitopes were shared across all species of bivalve providing a molecular basis of selective cross-reactivity [33].

Based on the abovementioned findings on the different pattern of IgE-binding epitope conservations in the three mollusc classes, a decision tree to predict immunological cross-reactivity between shrimp and mollusc classes based on TM and AK was developed. This decision tree could contribute significantly towards patient management particularly on the aspect of food avoidance and

diet. It has been well known that shrimp allergic patients are advised to avoid all shellfish species including mollusc due to the risk of cross-reactivity. The decision tree suggests that this advice should not be generalised as only patients sensitised to TM which account for 70-80% of total shrimp-allergic patients have a risk of cross-reacting with mollusc allergens. Moreover, the cohort of shrimp-allergic patients could be further divided into five groups based on their cross-reactivity patterns to specific IgE binding epitopes.

In conclusion, prediction of immunological cross-reactivity between an allergen and close related proteins based on similarity of the IgE-binding epitopes has been confirmed to be more accurate than the prediction based on similarity of the complete amino acid sequence of the allergenic proteins. Food allergens including shellfish allergens tend to have sequential IgE-binding epitopes due to digestion in the gastrointestinal tract. Thus, epitope sequence comparison is more relevant and conceivable for assessing the potential cross-reactivity of allergenic proteins, then comparing the whole protein sequence. The shrimp allergen IgE-binding epitope conservation results outlined in this study illustrate that a clear hierarchy of cross-reactivity is discovered, with TM being the most cross-reactive allergen among allergenic invertebrate species. This is most likely the main reasons that TM is one, if not the major pan-allergen in inhalant and ingestion animal allergy. The IgE binding epitopes located at the N- and C-terminal regions of TM are highly conserved and could be used as biomarkers to predict allergic cross-reactivity of shrimp-allergic patients. Unexpectedly, more than half of the TM as well as the AK IgE epitopes were found to be conserved in cockroach and mite TM and AK respectively. In contrast, only few shrimp IgE-binding epitopes were conserved across the molluscs. This suggests a low risk of cross-reactivity of shrimp allergic patients to molluscs, while a high risk of cross-reactivity to cockroach or mite is predicted. We developed for the first time a decision tree to predict cross-reactivity between shrimp and molluscs based on the major allergens TM and AK. These fundamental findings could simplify the diagnosis of cross-reactivity

among shellfish-allergic patients, thereby avoiding potential life-threatening food challenges.

5.7 References

1. Aalberse, R.C., *Shrimp Serology: We Need Tests with More and Less Cross-reactivity.* The Journal of Allergy and Clinical Immunology: In Practice, 2015. **3**(4): p. 530-531.
2. Barbi, E., et al., *Fatal allergy as a possible consequence of long-term elimination diet.* Allergy, 2004. **59**(6): p. 668-669.
3. Larramendi, C.H., et al., *Possible consequences of elimination diets in asymptomatic immediate hypersensitivity to fish.* Allergy, 1992. **47**(5): p. 490-494.
4. Aalberse, R.C. and R. Crameri, *IgE-binding epitopes: a reappraisal.* Allergy, 2011. **66**(10): p. 1261-74.
5. Bublin, M., et al., *IgE cross-reactivity between the major peanut allergen Ara h 2 and the nonhomologous allergens Ara h 1 and Ara h 3.* Journal of Allergy and Clinical Immunology, 2013. **132**(1): p. 118-124.e12.
6. Soeria-Atmadja, D., et al., *Multivariate statistical analysis of large-scale IgE antibody measurements reveals allergen extract relationships in sensitized individuals.* Journal of Allergy and Clinical Immunology, 2007. **120**(6): p. 1433-1440.
7. Hauser, M., et al., *Panallergens and their impact on the allergic patient.* Allergy, Asthma & Clinical Immunology, 2010. **6**(1): p. 1.
8. Aalberse, R.C., *Structural biology of allergens.* J. Allergy Clin. Immunol, 2000. **106**(2): p. 228-38.
9. Koeberl, M., et al., *Auto-induction for high yield expression of recombinant novel isoallergen tropomyosin from King prawn (Melicertus latisulcatus) for improved diagnostics and immunotherapeutics.* Journal of Immunological Methods, 2014. **415**(0): p. 6-16.
10. Sharp, M.F., et al., *Differential IgE binding to isoallergens from Asian seabass (Lates calcarifer) in children and adults.* Molecular Immunology, 2014. **62**(1): p. 77-85.

11. Pekar, J., D. Ret, and E. Untersmayr, *Stability of allergens.* Molecular Immunology, 2018.
12. Kamath, S.D., et al., *Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: A comprehensive allergenomic approach.* Mol Nutr Food Res, 2014. **58**(5): p. 1144-55.
13. Bui, H.-H., et al., *Development of an epitope conservancy analysis tool to facilitate the design of epitope-based diagnostics and vaccines.* BMC Bioinformatics, 2007. **8**(1): p. 361.
14. Ayuso, R., et al., *Molecular Basis of Arthropod Cross-Reactivity: IgE-Binding Cross-Reactive Epitopes of Shrimp, House Dust Mite and Cockroach Tropomyosins.* International Archives of Allergy and Immunology, 2002. **129**(1): p. 38-48.
15. Reese, G., et al., *Reduced allergenic potency of VR9-1, a mutant of the major shrimp allergen Pen a 1 (tropomyosin).* J Immunol, 2005. **175**(12): p. 8354-64.
16. Wai, C.Y., et al., *Immunization with Hypoallergens of shrimp allergen tropomyosin inhibits shrimp tropomyosin specific IgE reactivity.* PLoS One, 2014. **9**(11): p. e111649.
17. Huang, R., et al., *Determination of microheterogeneous substitution in shrimp tropomyosin and its effect on IgE-binding capacity.* European Food Research and Technology, 2014. **239**(6): p. 941-949.
18. Vaughan, K., et al., *Strategies to Query and Display Allergy-Derived Epitope Data from the Immune Epitope Database.* International Archives of Allergy and Immunology, 2013. **160**(4): p. 334-345.
19. Edgar, R.C., *MUSCLE: multiple sequence alignment with high accuracy and high throughput.* Nucleic Acids Research, 2004. **32**(5): p. 1792-1797.
20. Huelsenbeck, J.P. and F. Ronquist, *MRBAYES: Bayesian inference of phylogenetic trees.* Bioinformatics, 2001. **17**(8): p. 754-755.
21. Stamatakis, A., *RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies.* Bioinformatics, 2014. **30**(9): p. 1312-1313.

22. Miller, M.A., W. Pfeiffer, and T. Schwartz. *Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees*. in *Gateway Computing Environments Workshop (GCE)*. 2010. New Orleans.
23. Ashkenazy, H., et al., *ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules.* Nucleic Acids Research, 2016. **44**(W1): p. W344-W350.
24. Pettersen, E.F., et al., *UCSF Chimera: A visualization system for exploratory research and analysis.* Journal of Computational Chemistry, 2004. **25**(13): p. 1605-1612.
25. Barua, B., M.C. Pamula, and S.E. Hitchcock-DeGregori, *Evolutionarily conserved surface residues constitute actin binding sites of tropomyosin.* Proceedings of the National Academy of Sciences, 2011. **108**(25): p. 10150-10155.
26. Suma, Y., et al., *Comparative analysis of barnacle tropomyosin: divergence from decapod tropomyosins and role as a potential allergen.* Comp Biochem Physiol B Biochem Mol Biol, 2007. **147**(2): p. 230-6.
27. Ayuso, R., et al., *Greater epitope recognition of shrimp allergens by children than by adults suggests that shrimp sensitization decreases with age.* J Allergy Clin Immunol, 2010. **125**(6): p. 1286-1293 e3.
28. Ayuso, R., et al., *Is epitope recognition of shrimp allergens useful to predict clinical reactivity?* Clin Exp Allergy, 2012. **42**(2): p. 293-304.
29. Zheng, L.N., et al., *Mapping IgE binding epitopes of major shrimp (Penaeus monodon) allergen with immunoinformatics tools.* Food Chem Toxicol, 2011. **49**(11): p. 2954-60.
30. Ayuso, R., S.B. Lehrer, and G. Reese, *Identification of Continuous, Allergenic Regions of the Major Shrimp Allergen Pen a 1 (Tropomyosin).* International Archives of Allergy and Immunology, 2002. **127**(1): p. 27-37.
31. Pascal, M., et al., *Molecular Diagnosis of Shrimp Allergy: Efficiency of Several Allergens to Predict Clinical Reactivity.* The Journal of

Allergy and Clinical Immunology: In Practice, 2015. **3**(4): p. 521-529.e10.

32. Babicki, S., et al., *Heatmapper: web-enabled heat mapping for all.* Nucleic Acids Research, 2016. **44**(W1): p. W147-W153.
33. Wu, A.Y. and G.A. Williams, *Clinical characteristics and pattern of skin test reactivities in shellfish allergy patients in Hong Kong.* Allergy Asthma Proc, 2004. **25**(4): p. 237-42.
34. Kamath, S.D., et al., *IgE reactivity to shrimp allergens in infants and their cross-reactivity to house dust mite.* Pediatr Allergy Immunol, 2017. **28**(7): p. 703-707.
35. Rosenfield, L., et al., *High rate of house dust mite sensitization in a shrimp allergic southern Ontario population.* Allergy, Asthma & Clinical Immunology, 2017. **13**(1).
36. Thalayasingam, M., et al., *Clinical and immunochemical profiles of food challenge proven or anaphylactic shrimp allergy in tropical Singapore.* Clinical & Experimental Allergy, 2015. **45**(3): p. 687-697.
37. Fernandes, J., et al., *Immunoglobulin E antibody reactivity to the major shrimp allergen, tropomyosin, in unexposed Orthodox Jews.* Clin. Exp. Allergy., 2003. **33**(7): p. 956-61.
38. Gámez, C., et al., *Tropomyosin IgE-positive results are a good predictor of shrimp allergy.* Allergy, 2011. **66**(10): p. 1375-1383.
39. Pedrosa, M., et al., *Shellfish Allergy: a Comprehensive Review.* Clinical Reviews in Allergy & Immunology, 2014. **49**(2): p. 203-216.
40. Lopata, A.L., J. Kleine-Tebbe, and S.D. Kamath, *Allergens and molecular diagnostics of shellfish allergy: Part 22 of the Series Molecular Allergology.* Allergo. J. Int., 2016. **25**(7): p. 210-218.
41. Nugraha, R., et al., *Rapid and comprehensive discovery of unreported shellfish allergens using large-scale transcriptomic and proteomic resources.* Journal of Allergy and Clinical Immunology, 2018. **141**(4): p. 1501-1504.e8.
42. Abramovitch, J.B., et al., *IgE Reactivity of Blue Swimmer Crab (Portunus pelagicus) Tropomyosin, Por p 1, and Other Allergens;*

Cross-Reactivity with Black Tiger Prawn and Effects of Heating. PLoS One, 2013. **8**(6): p. e67487.

43. Reese, G., R. Ayuso, and S.B. Lehrer, *Tropomyosin: an invertebrate pan-allergen.* Int Arch Allergy Immunol, 1999. **119**(4): p. 247-58.
44. Vidal, C., et al., *Sensitization pattern of crustacean-allergic individuals can indicate allergy to molluscs.* Allergy, 2015. **70**(11): p. 1493-6.
45. Shen, H.W., et al., *Purification, cloning, and immunological characterization of arginine kinase, a novel allergen of Octopus fangsiao.* J Agric Food Chem, 2012. **60**(9): p. 2190-9.
46. Emoto, A., S. Ishizaki, and K. Shiomi, *Tropomyosins in gastropods and bivalves: Identification as major allergens and amino acid sequence features.* Food Chem, 2009. **114**(2): p. 634-641.
47. Lee, B.J. and H.S. Park, *Common whelk (Buccinum undatum) allergy: identification of IgE-binding components and effects of heating and digestive enzymes.* J Korean Med Sci, 2004. **19**(6): p. 793-9.
48. Asturias, J.A., et al., *Cloning, isolation, and IgE-binding properties of Helix aspersa (brown garden snail) tropomyosin.* Int Arch Allergy Immunol, 2002. **128**(2): p. 90-6.

CHAPTER 6. GENERAL DISCUSSION AND FUTURE DIRECTIONS

Shellfish are a healthy and nutritious food source and considered a sustainable source of proteins. However, the increased consumption of shellfish has been associated with an increase in adverse food reactions. Shellfish allergy is among the top eight food sources responsible for about 90% of food allergic reactions. Shellfish allergy persists into adulthood and currently there is no curative allergen-specific therapy is available. Shellfish is not a taxonomical order; it is a fisheries term for exoskeleton-bearing aquatic invertebrates used as food, including molluscs and crustaceans. Furthermore, unlike crustacean, mollusc allergy is clinically underreported and their allergens are ill-defined. To date, only five mollusc allergens are listed in the WHO/International Union of Immunological Societies (IUIS) Allergen Nomenclature SubCommittee database, all of which are only different tropomyosins (http://www.allergen.org/index.php). Additional mollusc allergens have been reported, but not yet fully characterised.

The work outlined in this thesis was aimed to improve current approaches on the identification of novel allergens from Pacific oyster (*Crassostrea gigas*), to characterise the structural and immunological properties of the newly registered-major allergen, tropomyosin Cra g 1, and to evaluate the available shellfish allergen IgE-binding epitopes for their potential as a novel strategy to predict cross-reactivity between crustacean and mollusc species.

A comprehensive methodology for the identification of unreported allergens was developed in Chapter 2. This methodology combines the technological advances of omics science with the traditional IgE-reactivity techniques and utilises advanced of bioinformatics and computational tools for the integration, analysis and interpretation of data sets produced. Using this approach, 24 unreported allergens were identified from over 25,000 proteins of the Pacific oyster. Of those unreported allergens, only four allergens were discovered in the heated extracts suggesting that many allergens from oysters were overlooked in previous studies due to the omission of raw extract in the analysis. Not only is this is the first time that these IgE binding allergens were

identified in the Pacific oyster, but these allergens also showed high identity with known allergens from previously reported cross-reactive species. For example, triosephosphate isomerase and 78 kDa glucose regulated protein share 74% and 65% amino acid identity with dust-mite allergens, possibly responsible for the clinical mollusc-mite cross-reactivity, previously reported during mite immunotherapy. The application of omics science for the identification of unreported allergens can accelerate the process of knowledge gain on the offending allergens. From the transcriptomic data, the sequence and structure of the allergens will be easily elucidated helping further study on the production of recombinant proteins and the prediction of potential cross-reactivity with other allergen sources. Furthermore, proteomics, in particular 2D-electrophoresis coupled with mass-spectrometry provide high-resolution separation of proteins from the allergen sources and give better dynamic ranges as compared to the commonly used 1D electrophoresis, which can improve the detection of low abundance allergens.

The abundance of particular proteins also can be improved by modifying the buffers used in the extraction procedures, often used for producing *in vitro* diagnostic tests for molluscs and crustacean species. In Chapter 3, eight buffers used to extract proteins from Pacific oyster and their extract efficiency were compared with the commonly used buffer, Phosphate-buffered saline (PBS) and Tris-buffered saline (TBS). Significant differences in the recovered proteins were observed between PBS and other buffers. While low pH buffers had a very poor extractability, high pH buffers showed better recovery of proteins. Alternatively, increasing the ionic strength of the PBS and TBS also significantly improved the protein content. This improvement resulted in the identification of IgE-reactive proteins previously undetected due to low concentration.

After identifying several unreported Pacific oyster allergens, this project then focused on the development of better diagnostic tools for mollusc allergy. It has been known that mollusc allergy is underreported due to lack of specific

and sensitive diagnostic tools. Current diagnosis of mollusc allergy relies on the use of commercial preparations derived from whole protein extracts with unknown protein composition rather than allergen components. However, developing component-resolved diagnosis for mollusc allergy may require knowledge on the natural abundance of the allergens, its localisation in the organism, as well as its cross-reactive potential with other related allergen sources. This development of better diagnostic tools was dedicated in Chapter 4 and Chapter 5. Tropomyosin is believed to be the major allergen of Pacific oyster; however, the studies only used a very limited number of patients to determine the IgE-reactivity of the protein, and the cross-reactive nature of tropomyosin was determined using pooled serum-IgE. Therefore, in Chapter 4 a comprehensive evaluation of the structural and immunological properties of the Pacific oyster tropomyosin (Cra g 1) were carried out, using 21 patients with confirmed oyster allergy. The complete oyster tropomyosin was successfully cloned and recombinantly expressed.. The recombinant tropomyosin had identical structural and immunological properties with the natural counterpart. Screening of IgE-reactivity of this tropomyosin using immunoblotting against 21 oyster-sensitised patients showed more than 80% of the patients demonstrated IgE-reactivity to tropomyosin, confirming previous findings that tropomyosin is the major allergen. These tropomyosin-reactive patients also demonstrated IgE-reactivity to related invertebrate tropomyosins from Black tiger prawn and House dust mite although different degrees of reactivity were observed in each patient. Sequence alignment of these three tropomyosins revealed some region-specific similarities in the IgE-binding epitopes, however whether the patient multi-reactivity was due to cross-reactivity or co-sensitisation need to be confirmed through further investigation.

In Chapter 5, a decision tree was proposed to identify cross-reactivity between crustacean and mollusc species. This decision tree was developed based on the conservation analysis of the available IgE-binding epitopes of tropomyosin and arginine kinase. Conserved IgE-binding epitopes have been shown to be

responsible for the cross-reactivity between not only closely related allergens but also between very different allergens. Two peptide sequences are defined as conserved when they share similar amino acid sequences with maximum of two amino acid mismatches. Although IgE-binding epitope data are available for four shellfish allergens, conservation analysis demonstrated that only tropomyosin and arginine kinase shared considerable portion of conserved IgE-binding epitopes in crustacean and mollusc species. More interestingly, class-specific conservation was observed within mollusc species where cephalopods are most likely to cross-react with crustaceans as compared to gastropods or bivalves to predict cross-reactivity between shrimp and molluscs based on the major allergens, TM and AK. These fundamental findings could simplify the diagnosis of cross-reactivity among shellfish-allergic patients, thereby avoiding complete avoidance of all shellfish species, which may otherwise have an impact on their nutrition and diet.

Future directions

a. Evaluation of allergenicity of newly identified putative allergens - Bioinformatics analysis of the Pacific oyster genome predicted 95 potential allergens, however only 44 of those were present in the extracts and further analysis showed only 24 proteins were IgE-reactive. Expression of these proteins in the adult Pacific oyster as well as solubility issues are thought to contribute to the less IgE-reactive proteins detected as compared to the number of potential allergens predicted. Future studies involving production of recombinant proteins of those potential allergens and analysing their IgE-binding against oyster allergic patients may provide an insight, confirming the allergenicity of these proteins.

b. Investigation of clinical relevance of the unreported Pacific oyster allergens

The work conducted in this thesis revealed several unreported allergens from Pacific oyster. The IgE-reactivity of these unreported allergens was confirmed

using a serum pool from five shellfish allergic patients. Utilisation of these allergens for component resolved diagnosis requires information on the clinical relevance of each allergen. Therefore, future research evaluating clinical reactivity of these allergens in individual patients are needed. Purification by physicochemical means or by affinity chromatography followed up by IgE-reactivity assay to each allergen should be performed.

c. Epitope mapping of Pacific oyster tropomyosin

Prediction of immunological cross-reactivity between an allergen and closely related proteins based on similarity of the IgE-binding epitopes was demonstrated to be more accurate than the prediction based on similarity of the complete amino acid sequence of the allergenic proteins. Food allergens including shellfish allergens tend to have sequential IgE-binding epitopes due to digestion stability in the gastrointestinal tract. Thus, epitope sequence comparison is more relevant and conceivable for assessing the potential cross-reactivity of allergenic proteins, than comparing the whole protein sequence. Currently, only one epitope has been discovered from Pacific oyster, determined by using protease digestion followed by a competitive ELISA inhibition assay. Further studies utilising more sophisticated methodologies, including the use of overlapping synthetic peptides need to be carried out.

d. Development of component-resolved diagnosis for shellfish allergy based on IgE-binding epitopes

Diagnosis of allergy has gradually integrated the allergen components to support allergen extracts due to their high accuracy. Purified allergens from shellfish species, particularly shrimp, are quite well covered; however their sensitivity to correctly diagnose shrimp allergy is still below 95% due to highly cross-reactive nature of their major allergen tropomyosin. In Chapter 5, it was demonstrated that IgE-binding epitopes of shrimp tropomyosin and arginine kinase showed group-specific conservation, which could be used to resolve the questions about clinical cross-reactivity. Using advanced technologies for

peptide synthesis and microarray platform, hundreds of IgE-binding peptides could be attached to a single panel. These technologies could significantly improve the accuracy of current diagnosis for shellfish allergy.

The experimental work and the findings generated in this study were intended to improve the diagnostics for mollusc allergy and provide a foundation for future peptide and protein-based immunotherapy for mollusc allergy.

APPENDIX A

BUFFERS AND SOLUTIONS

General buffer

20 x Phosphate buffered saline (PBS)

Na_2HPO_4 ---------- 28.8 g

KH_2PO_4 ---------- 4.8 g

NaCl ---------- 160 g

KCl ---------- 4 g

Milli-Q H_2O ---------- 1 l

Mix to dissolve and adjust pH to 7.2. Autoclave or filter through 0.45µm membrane for long-time storage. Dilute 1:20 with Milli-Q water before use and adjust pH if necessary.

ELISA buffers

PBS/0.05% Tween 20 (wash buffer)

Tween 20 ---------- 2.5 ml

1 x PBS ---------- 5 l

1M HCl (stop buffer)

38% HCl ---------- 8.3 ml

Milli-Q H_2O ---------- up to 100 ml

SDS-PAGE solutions

Solution B

Tris-HCl (2 M, pH 8.8) ---------- 75 ml

10% SDS in Milli-Q H_2O ---------- 4 ml

Milli-Q H_2O ----------21 ml

Solution C

Tris-HCl (1 M, pH 6.8) ---------- 50 ml

10% SDS in Milli-Q H_2O ---------- 4 ml

Milli-Q H_2O ----------46 ml

5 x Protein sample loading buffer

Tris-HCl (1 M, pH 6.8) ---------- 0.6 ml

50% Glycerol ---------- 5ml

10% SDS ---------- 2 ml

Dithiothreitol (1 M) ---------- 1 ml

1% Bromophenol blue ---------- 1 ml

Milli-Q H_2O ---------- up to 10 ml

12% SDS-PAGE gel recipe

Resolving gel

40% 29:1 Acrylamide ---------- 6 ml

Solution B ---------- 5 ml

Milli-Q H_2O ---------- 8.9 ml

10% Ammonium persulphate ---------- 100 µl

TEMED ---------- 10 µl

Stacking gel

40% 29:1 Acrylamide ---------- 0.93 ml

Solution C ---------- 2.5 ml

Milli-Q H_2O ---------- 6.5 ml

10% Ammonium persulphate ---------- 100 µl

TEMED ---------- 10 µl

1 x Gel Electrophoresis running buffer

Tris ---------- 3 g/l

Glycine ---------- 14.4 g/l

SDS ---------- 1 g/l

Milli-Q H_2O ---------- up to 1 l

SDS-PAGE gel destaining solution

Methanol (AR grade) ---------- 500 ml

Glacial acetic acid ---------- 100 ml

Milli-Q H_2O ---------- 400 ml

Immunoblotting Buffers

Transfer buffer

Tris ---------- 1.164 g

Glycine ---------- 0.58 g

10% SDS ---------- 750 µl

Methanol ---------- 40 ml

Milli-Q H_2O ---------- up to 200 ml

1.7 DNA clone buffers

LB broth

Tryptone ---------- 10 g/l

NaCl ---------- 10 g/l

Yeast extract ---------- 5 g/l

Milli-Q H_2O ---------- up to 1 l

Mix to dissolve and autoclave before use for bacterial culture.

LB plates

Tryptone ---------- 10 g/l

NaCl ---------- 10 g/l

Yeast extract ---------- 5 g/l

Agar ---------- 15 g/l

Milli-Q H_2O ---------- up to 1 l

Ampicillin stock

Ampicillin ---------- 1 g

70% Ethanol ---------- 10 ml

Filter sterilised before use.

Glycerol stocks

Fresh overnight culture ---------- 700 µl

50% sterile glycerol in Milli-Q H_2O ------ 300 µl

Protein Purification Buffers

Phosphate buffer

Na_2HPO_4 ---------- 1.44 g

KH_2PO_4 ---------- 0.24 g

NaCl ---------- 17.5 g

KCl ---------- 0.2 g

Milli-Q H_2O ---------- 1 l

Mix to dissolve and adjust pH to 7.4.

Stock solution

Imidazole ---------- 0.068 g

Phosphate buffer ---------- 50 ml

Equilibration buffer

Stock solution ---------- 1 ml

Milli-Q H_2O ---------- up to 50 ml

Wash buffer

Stock solution ---------- 5 ml

Milli-Q H_2O ---------- up to 50 ml

Elution buffer

Stock solution ---------- 30 ml

Milli-Q H_2O ---------- up to 50 ml

Two dimensional SDS-PAGE buffers

Sample Buffer

Urea 8M

CHAPS 2%

Dithiothreitol (DTT) 50mM

Bio-Lyte ® 2/10 ampholytes 0.2%

Bromophenol Blue (trace)

Equilibration Buffer I

Urea 6M

SDS 2%

Tris-HCl (pH 8.8) 0.375 M

Glycerol 20%

DTT 2%

Equilibration Buffer II

Urea 6M

SDS 2%

Tris-HCl (pH 8.8) 0.375 M

Glycerol 20%

Iodoacetamide 2.5%

APPENDIX II

SUPPLEMENTARY TABLES

Supplementary Table 1 Identified potential allergens of Pacific Oyster from the *in silico* analysis.

Protein	Gene name	Best matched allergen	Amino acid identity (%)
Very likely allergenic (amino acid identity ≥70%)			
Tropomyosin	CGI_10013163	tropomyosin [Crassostrea gigas]	92.94
Tropomyosin	CGI_10013164	tropomyosin, partial [Crassostrea virginica]	86.67
Tubulin alpha chain	CGI_10002456	Der f 33 allergen [Mite]	85.71
Tubulin alpha-1C chain	CGI_10002455	Der f 33 allergen [Mite]	83.2
Tubulin alpha-3 chain	CGI_10018930	Der f 33 allergen [Mite]	82.43
Tubulin alpha-1C chain	CGI_10024998	Der f 33 allergen [Mite]	81.8
Tubulin alpha-1C chain	CGI_10007570	Der f 33 allergen [Mite]	81.53
Tubulin alpha-1C chain	CGI_10024999	Der f 33 allergen [Mite]	81.35
78 kDa glucose-regulated protein	CGI_10015492	Aed a 8 [Mosquito]	81
Tubulin alpha-1C chain (Fragment)	CGI_10002454	Der f 33 allergen [Mite]	80
Tubulin alpha-1C chain	CGI_10008247	Der f 33 allergen [Mite]	77.7
Tubulin alpha-1A chain	CGI_10007571	Der f 33 allergen [Mite]	77.63
Fructose-bisphosphate aldolase (EC 4.1.2.13)	CGI_10019801	Thu a 3 [Tuna]	74.29
Heat shock protein 70 B2	CGI_10010646	Der f 28 allergen [Mite]	74.27
Heat shock protein 70 B2	CGI_10010647	Der f 28 allergen [Mite]	74.27
Triosephosphate isomerase (EC 5.3.1.1)	CGI_10003538	triosephosphate isomerase [shrimp]	74.03
Heat shock protein 68	CGI_10002594	Der f 28 allergen [Mite]	73.96
Triosephosphate isomerase (EC 5.3.1.1)	CGI_10003539	Der f 25 allergen [Mite]	73.37
Heat shock protein 70 B2	CGI_10003417	Der f 28 allergen [Mite]	73.13
Enolase	CGI_10022154	enolase [Tunas]	72.83
Fructose-bisphosphate aldolase (EC 4.1.2.13)	CGI_10025556	Thu a 3 [Tuna]	72.22

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	CGI_10010974	Glyceraldehyde-3-phosphate dehydrogenase [Wheat]	71.21
Peptidyl-prolyl cis-trans isomerase E (PPIase E) (EC 5.2.1.8)	CGI_10026365	Der f 6 [Mite]	70.19
Likely allergenic (amino acid identity ≥50%, but <70%			
Ferritin (EC 1.16.3.1)	CGI_10016317	Ferritin [*Mite*]	69.92
Paramyosin	CGI_10001653	Paramyosin [*Abalone*]	68.59
Arginine kinase	CGI_10021480	Arginine kinase [*Octopus*]	66.96
Arginine kinase	CGI_10021483	Arginine kinase [*Octopus*]	66.67
Heat shock protein HSP 90-alpha 1	CGI_10017621	Asp f 12 [*Fungus*]	66.26
Ferritin (EC 1.16.3.1)	CGI_10021660	Ferritin [*Mite*]	66.06
Cytochrome c	CGI_10012574	Cur l 3 [*Fungus*]	66.02
Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	CGI_10017958	Ole e 3 [*Olive tree*]	66
Peptidyl-prolyl cis-trans isomerase 6	CGI_10022249	Cyclophilin [*Carrot*]	65.96
Ferritin (EC 1.16.3.1)	CGI_10027591	Ferritin [*Mite*]	65.58
78 kDa glucose-regulated protein	CGI_10008834	Der f 28 [*Mite*]	65.3
78 kDa glucose-regulated protein	CGI_10027395	Der f 28 [*Mite*]	65.25
Arginine kinase	CGI_10024056	Arginine kinase [*Octopus*]	64.65
Inorganic pyrophosphatase	CGI_10027722	Der f 32 [*Mite*]	64.24
Fructose-bisphosphate aldolase	CGI_10000078	Sal s 3 *[Salmon*]	64.12
60S ribosomal protein L3 (Fragment)	CGI_10010529	Asp f 23 [*Fungus*]	63.96
Arginine kinase	CGI_10021482	Arginine kinase [*Octopus*]	63.83
Heat shock protein 68	CGI_10002823	Der f 28 [*Mite*]	63.34
40 kDa peptidyl-prolyl cis-trans isomerase	CGI_10015504	Cyclophilin [*Carrot*]	62.72
Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	CGI_10013880	Cyclophilin [*Carrot*]	61.76
Plasma kallikrein	CGI_10016607	Der f 3 [*Mite*]	61.7
60S ribosomal protein L3	CGI_10012282	Asp f 23 [*Fungus*]	60.76
Arginine kinase	CGI_10021481	Arginine kinase [*Octopus*]	60.6

Tubulin alpha chain	CGI_10018903	Der f 33 [*Mite*]	60.49
78 kDa glucose-regulated protein	CGI_10018425	Der f 28 [*Mite*]	59.9
Eukaryotic translation initiation factor 3 subunit I (Fragment)	CGI_10025943	For t 1 [*Midges*]	59.88
Calcium-binding atopy-related autoantigen 1	CGI_10026057	Hom s 4 [*Human*]	59.3
Transaldolase	CGI_10002311	Fus p 4 [*Fungus*]	59.2
Peptidyl-prolyl cis-trans isomerase B	CGI_10023851	Cyclophilin [*Carrot*]	58.7
Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	CGI_10023850	Asp f 27 [*Fungus*]	58.33
Aldehyde dehydrogenase, mitochondrial	CGI_10012671	Cla h 10 [*Fungus*]	58.02
Thioredoxin	CGI_10021611	Mala s 13 [*Yeast*]	58
Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	CGI_10024975	Cyclophilin [*Carrot*]	57.63
Thioredoxin domain-containing protein 5	CGI_10009327	Alt a 4 [*Fungus*]	57.45
60S acidic ribosomal protein P1	CGI_10009326	Alt a 12 [*Fungus*]	57.14
NK-tumor recognition protein	CGI_10007438	Asp f 27 [*Fungus*]	56.8
Thaumatin-like protein 1a	CGI_10012508	Pathogenesis related protein 5 [*Apple*]	55.62
Superoxide dismutase (EC 1.15.1.1)	CGI_10017307	Pis v 4 [*Pistachio*]	55.61
Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	CGI_10006179	Asp f 11 [*Fungus*]	55.56
Peptidylprolyl isomerase domain and WD repeat-containing protein 1	CGI_10011521	Mala s 6 [*Yeast*]	55.47
Stress-70 protein, mitochondrial	CGI_10016162	Pen c 19 [*Fungus*]	55.22
Peptidyl-prolyl cis-trans isomerase-like 6	CGI_10024382	Cat r 1 [*Periwinkle*]	55.22
U4/U6.U5 tri-snRNP-associated protein 1	CGI_10021218	Hom s 1 [*Human*]	54.96
Alpha-amylase (EC 3.2.1.1)	CGI_10022190	Bla g 11 [*Cockroach*]	54.37
Alpha-amylase (EC 3.2.1.1)	CGI_10022189	Bla g 11 [*Cockroach*]	54.18
Endoplasmin	CGI_10025730	Asp f 12 [*Fungus*]	54.04
Calmodulin	CGI_10006247	Amb a 9 [*Ragweed*]	53.7

Calmodulin	CGI_10014525	B1 protein allergen [*Bermuda grass*]	53.57
Aldehyde dehydrogenase	CGI_10021688	Cla h 10 [*Fungus*]	52.82
Retinal dehydrogenase 1	CGI_10026868	Cla h 10 [*Fungus*]	52.33
Protein disulfide-isomerase (EC 5.3.4.1)	CGI_10011652	Alt a 4 [*Fungus*]	52.27
Collagen alpha-3(VI) chain	CGI_10015798	collagen alpha [*Bovine*]	52.27
Peptidyl-prolyl cis-trans isomerase B	CGI_10027458	Cat r 1 [*Periwinkle*]	52.17
78 kDa glucose-regulated protein	CGI_10011272	Der f 28 [*Mite*]	52.1
Thioredoxin	CGI_10003765	Asp f 28 [*Fungus*]	51.85
Uncharacterized protein	CGI_10016401	Der f 3 [*Mite*]	51.85
Transcription elongation factor 1-like protein	CGI_10026699	Tri a 45 [*Wheat*]	51.81
Alpha-amylase (EC 3.2.1.1)	CGI_10023778	Bla g 11 [*Cockroach*]	51.75
Malate dehydrogenase, mitochondrial	CGI_10015004	Mala f 4 [*Yeast*]	51.43
Calmodulin	CGI_10022491	Tyr p 24 [*Mite*]	51.32
Retinal dehydrogenase 1	CGI_10026867	Cla h 10 [*Fungus*]	51.27
Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	CGI_10005876	Mala s 6 [*Yeast*]	51.16
Calmodulin	CGI_10011301	Par j 4 [*Weed*]	50.94
Calmodulin	CGI_10006481	Syr v 3 [*Common lilac*]	50.85
Alpha-amylase (EC 3.2.1.1)	CGI_10023781	Bla g 11 [*Cockroach*]	50.51
Elongation factor 1-beta	CGI_10021397	Pen c 22 [*Fungus*]	50.44
Calmodulin	CGI_10002924	B1 protein allergen [*Bermuda grass*]	50
Calmodulin-like protein 12	CGI_10004114	putative Cup a 4 allergen [*Cypress*]	50
Calmodulin	CGI_10011293	polcalcin [Artemisia vulgaris]	50
Supervillin	CGI_10014153	villin 2 [*Tobacco*]	50
Calmodulin	CGI_10017056	Bla g 6 [*Cockroach*]	50
Protein disulfide-isomerase (EC 5.3.4.1)	CGI_10026048	Alt a 4 [*Fungus*]	50

Supplementary Table 2 Proteins identified in the IgE-reactive spots of the raw extract of Pacific Oyster.

Spot No.	Protein name	Protein accession numbers	Protein molecular weight (KDa)	Exclusive unique peptide count	Exclusive unique spectrum count	Total spectrum count	Percentage of total spectra	Percentage sequence coverage
Spot 1	Tropomyosin	B7XC66_CRAGI	33.02	2	5	103	1.41%	54.60%
Spot 1	Tropomyosin (Fragment)	Q95WY0_CRAGI	26.87	0	0	66	0.91%	54.90%
Spot 1	Filamin-C	K1PW06_CRAGI	323.73	25	32	34	0.47%	11.30%
Spot 1	Tropomyosin	K1QNV6_CRAGI	39.01	0	0	34	0.47%	23.60%
Spot 1	Uncharacterized protein	K1PV81_CRAGI	39.24	13	23	25	0.34%	32.90%
Spot 1	Actin, cytoplasmic	K1RWD4_CRAGI	41.90	1	1	18	0.25%	33.80%
Spot 1	Protein disulfide-isomerase	K1Q6X5_CRAGI	55.50	14	18	18	0.25%	30.30%
Spot 1	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	7	7	12	0.17%	19.80%
Spot 1	Muscle M-line assembly protein unc-89 (Fragment)	K1QPH2_CRAGI	50.98	8	11	11	0.15%	24.30%
Spot 1	Arginine kinase	K1PLF9_CRAGI	39.63	8	9	9	0.12%	22.90%
Spot 1	Calumenin	K1PH89_CRAGI	47.66	9	9	9	0.12%	23.60%
Spot 1	Uncharacterized protein	K1PU04_CRAGI	38.43	4	4	8	0.11%	29.80%
Spot 1	Severin	K1PE57_CRAGI	37.21	5	7	7	0.10%	16.70%
Spot 1	Heat shock protein HSP 90-alpha 1	K1PNQ5_CRAGI	83.32	0	0	7	0.10%	10.10%
Spot 1	Vitellogenin-6	K1QNA2_CRAGI	273.31	0	0	7	0.10%	2.49%
Spot 1	Fructose-bisphosphate aldolase	K1R8R6_CRAGI	43.48	3	3	7	0.10%	21.60%

Spot 1	Uncharacterized protein	K1PKQ3_CRAGI	38.10	6	6	6	0.08%	17.40%
Spot 1	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	1	1	6	0.08%	11.80%
Spot 1	Collagen alpha-6(VI) chain	K1QEB9_CRAGI	46.30	2	2	6	0.08%	11.60%
Spot 1	Calreticulin	A5LGG9_CRAGI	48.19	1	2	6	0.08%	15.00%
Spot 1	Uncharacterized protein	K1Q0U6_CRAGI	27.04	6	6	6	0.08%	23.30%
Spot 1	S-adenosylmethionine synthase	K1QZB1_CRAGI	45.38	4	4	5	0.07%	13.60%
Spot 1	Collagen alpha-2(I) chain	K1PT11_CRAGI	168.96	4	5	5	0.07%	2.53%
Spot 1	Uncharacterized protein	K1QWT8_CRAGI	45.15	5	5	5	0.07%	15.20%
Spot 1	Fructose-bisphosphate aldolase	K1RTQ6_CRAGI	38.99	3	3	5	0.07%	14.00%
Spot 1	14-3-3 protein epsilon	K1R5F2_CRAGI	29.07	5	5	5	0.07%	21.10%
Spot 1	Small glutamine-rich tetratricopeptide repeat-containing protein beta	K1PRK2_CRAGI	29.25	4	5	5	0.07%	18.10%
Spot 1	78kDa glucose regulated protein	Q75W49_CRAGI	73.08	0	0	5	0.07%	7.11%
Spot 1	Enolase	K1QX37_CRAGI	127.42	3	4	4	0.05%	2.80%
Spot 1	Protein BCCIP homolog	K1R3C2_CRAGI	32.24	3	3	3	0.04%	10.20%
Spot 1	ATP synthase subunit alpha	K1R6Z7_CRAGI	59.87	2	3	3	0.04%	4.16%
Spot 1	Phosphotriesterase-related protein	K1QFI9_CRAGI	38.54	2	3	3	0.04%	5.76%
Spot 1	Actin Cyl, cytoplasmic	K1PZQ5_CRAGI	12.90	1	1	3	0.04%	20.90%
Spot 1	Alpha-actinin, sarcomeric	K1RH58_CRAGI	102.20	3	3	3	0.04%	3.41%
Spot 1	40S ribosomal protein SA	K1R4D4_CRAGI	33.30	2	3	3	0.04%	7.57%
Spot 1	Protocadherin Fat 4	K1QB61_CRAGI	723.24	3	3	3	0.04%	0.53%

Spot 1	Glycerol-3-phosphate dehydrogenase [NAD(+)]	K1QMD3_CRAGI	38.40	3	3	3	0.04%	9.69%
Spot 1	Fructose-1,6-bisphosphatase 1	K1QSB0_CRAGI	36.79	3	3	3	0.04%	10.40%
Spot 1	71kDa heat shock connate protein	Q9XZJ2_CRAGI	72.04	0	0	3	0.04%	4.70%
Spot 1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	K1RDG2_CRAGI	45.34	3	3	3	0.04%	11.10%
Spot 1	Uncharacterized protein	K1R7R5_CRAGI	21.43	3	3	3	0.04%	14.80%
Spot 1	Heterogeneous nuclear ribonucleoprotein A/B	K1PNI6_CRAGI	53.89	3	3	3	0.04%	6.11%
Spot 1	Plectin-1	K1R091_CRAGI	100.61	2	2	2	0.03%	2.51%
Spot 1	Sulfotransferase family cytosolic 1B member 1	K1QLP7_CRAGI	69.89	2	2	2	0.03%	3.48%
Spot 1	Spectrin alpha chain	K1R401_CRAGI	287.01	2	2	2	0.03%	0.93%
Spot 1	Protein SET	K1PWR0_CRAGI	28.11	2	2	2	0.03%	9.76%
Spot 1	ATP synthase subunit beta	K1RWW5_CRAGI	44.93	2	2	2	0.03%	5.56%
Spot 1	Uncharacterized protein	K1R3U2_CRAGI	35.42	2	2	2	0.03%	5.48%
Spot 1	Adipophilin	K1PJC1_CRAGI	54.04	2	2	2	0.03%	5.71%
Spot 1	Nuclear autoantigenic sperm protein	K1RN77_CRAGI	72.95	2	2	2	0.03%	3.53%
Spot 1	Hsc70-interacting protein	K1PZU1_CRAGI	31.95	2	2	2	0.03%	9.57%
Spot 1	Uncharacterized protein	K1QFW9_CRAGI	94.86	1	1	2	0.03%	2.23%
Spot 1	Uncharacterized protein	K1R5R9_CRAGI	111.24	2	2	2	0.03%	1.97%
Spot 1	Titin	K1R2G8_CRAGI	1,228.82	2	2	2	0.03%	0.18%

Spot 1	Ubiquitin-conjugating enzyme E2 Z	K1PJ71_CRAGI	36.60	2	2	2	0.03%	6.21%
Spot 1	Protocadherin Fat 4	K1PTY5_CRAGI	1,111.10	2	2	2	0.03%	0.19%
Spot 1	Uncharacterized protein	K1R7Q9_CRAGI	19.63	2	2	2	0.03%	13.10%
Spot 1	Uncharacterized protein	K1PM19_CRAGI	22.11	2	2	2	0.03%	10.30%
Spot 1	Uncharacterized protein	K1QMD2_CRAGI	55.68	2	2	2	0.03%	3.62%
Spot 1	Sarcoplasmic calcium-binding protein	K1PY28_CRAGI	21.14	2	2	2	0.03%	11.70%
Spot 1	Low-density lipoprotein receptor-related protein 6	K1QY50_CRAGI	74.81	2	2	2	0.03%	3.81%
Spot 1	Enolase-phosphatase E1	K1PQI4_CRAGI	34.67	2	2	2	0.03%	6.45%
Spot 1	14-3-3 protein gamma	K1PPQ1_CRAGI	28.48	1	1	2	0.03%	8.76%
Spot 2	Actin 2	Q8TA69_CRAGI	41.74	0	0	44	0.65%	32.70%
Spot 2	Actin	K1R6J7_CRAGI	41.81	1	3	42	0.62%	34.80%
Spot 2	Actin	C4NY64_CRAGI	41.81	2	2	36	0.53%	34.80%
Spot 2	Filamin-C	K1PW06_CRAGI	323.73	20	26	27	0.40%	7.13%
Spot 2	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	8	10	19	0.28%	23.30%
Spot 2	Actin	K1RA57_CRAGI	42.06	1	2	17	0.25%	18.00%
Spot 2	Actin-1/3	K1RBG6_CRAGI	59.45	1	3	15	0.22%	8.87%
Spot 2	Glyceraldehyde-3-phosphate dehydrogenase	K1Q350_CRAGI	36.08	9	13	13	0.19%	29.60%
Spot 2	Regucalcin	K1P756_CRAGI	28.14	1	1	11	0.16%	29.50%
Spot 2	Regucalcin	K1PMC8_CRAGI	35.78	1	1	11	0.16%	22.40%
Spot 2	Serine-threonine kinase receptor-associated protein	K1QAG0_CRAGI	139.75	3	3	10	0.15%	6.98%

Spot 2	Heat shock protein HSP 90-alpha 1	K1PNQ5_CRAGI	83.32	1	2	10	0.15%	14.70%
Spot 2	Arginine kinase	K1PLF9_CRAGI	39.63	7	10	10	0.15%	19.40%
Spot 2	Fascin	K1QEZ3_CRAGI	55.60	10	10	10	0.15%	21.40%
Spot 2	Malate dehydrogenase (Fragment)	K1PU26_CRAGI	36.27	8	9	9	0.13%	25.70%
Spot 2	Serine-threonine kinase receptor-associated protein	K1QTK2_CRAGI	22.24	2	3	9	0.13%	34.30%
Spot 2	Uncharacterized protein	K1QFW9_CRAGI	94.86	8	8	9	0.13%	11.60%
Spot 2	Transitional endoplasmic reticulum ATPase	K1PVA1_CRAGI	88.70	6	7	8	0.12%	7.76%
Spot 2	Malate dehydrogenase, mitochondrial	K1R4Z3_CRAGI	29.84	6	7	8	0.12%	32.90%
Spot 2	Formin-binding protein 1-like protein	K1PYZ6_CRAGI	42.89	6	8	8	0.12%	17.20%
Spot 2	Enolase	K1QX37_CRAGI	127.42	7	8	8	0.12%	6.56%
Spot 2	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	1	1	8	0.12%	16.40%
Spot 2	Inorganic pyrophosphatase	K1Q3F4_CRAGI	48.34	6	6	8	0.12%	16.90%
Spot 2	Tubulin beta chain	K1PN21_CRAGI	50.04	1	1	7	0.10%	17.80%
Spot 2	Aldehyde dehydrogenase	K1Q9Z4_CRAGI	25.35	5	7	7	0.10%	24.50%
Spot 2	Actin	K1RHJ4_CRAGI	42.31	1	1	7	0.10%	7.92%
Spot 2	Guanine nucleotide-binding protein subunit beta	K1PWZ3_CRAGI	37.33	5	7	7	0.10%	18.80%
Spot 2	Twinfilin-2	K1QHG0_CRAGI	32.09	5	6	6	0.09%	18.40%

Spot 2	Apoptosis-inducing factor 3	K1QFI3_CRAGI	97.02	6	6	6	0.09%	7.08%
Spot 2	Citrate synthase	K1RM80_CRAGI	49.44	6	6	6	0.09%	15.00%
Spot 2	Actin CyI, cytoplasmic	K1PZQ5_CRAGI	12.90	1	2	6	0.09%	20.90%
Spot 2	Dynactin subunit 2	K1PZS6_CRAGI	44.63	6	6	6	0.09%	17.20%
Spot 2	Putative pyridoxine biosynthesis SNZERR	K1QMG8_CRAGI	32.94	6	6	6	0.09%	17.80%
Spot 2	3-hydroxyisobutyrate dehydrogenase	K1PR93_CRAGI	42.83	6	6	6	0.09%	19.00%
Spot 2	Tropomyosin	B7XC66_CRAGI	33.02	1	1	6	0.09%	22.20%
Spot 2	Glyoxalase domain-containing protein 4	K1P979_CRAGI	32.57	5	5	5	0.07%	19.40%
Spot 2	Betaine--homocysteine S-methyltransferase 2	K1QTS9_CRAGI	41.54	3	3	5	0.07%	13.80%
Spot 2	V-type proton ATPase catalytic subunit A	K1Q9V3_CRAGI	70.85	5	5	5	0.07%	8.81%
Spot 2	Uncharacterized protein	K1QE98_CRAGI	38.17	4	5	5	0.07%	13.40%
Spot 2	Vitellogenin	Q8IU34_CRAGI	179.21	1	2	5	0.07%	2.59%
Spot 2	Heterogeneous nuclear ribonucleoprotein Q	K1R7I9_CRAGI	69.23	4	4	4	0.06%	7.01%
Spot 2	Phosphoglucomutase-1	K1PQD4_CRAGI	64.80	1	1	4	0.06%	6.58%
Spot 2	Uncharacterized protein	K1PU04_CRAGI	38.43	2	2	4	0.06%	13.70%
Spot 2	Protein phosphatase 1 regulatory subunit 7	K1RMV1_CRAGI	38.02	4	4	4	0.06%	10.80%
Spot 2	Cathepsin Z	K1P5K4_CRAGI	76.00	4	4	4	0.06%	5.99%

Spot 2	Aldehyde dehydrogenase, mitochondrial	K1QNT7_CRAGI	56.97	4	4	4	0.06%	7.32%
Spot 2	Leucine zipper transcription factor-like protein 1	K1QMM4_CRAGI	34.58	4	4	4	0.06%	12.80%
Spot 2	Caspase-7	K1QSW8_CRAGI	34.52	2	2	4	0.06%	13.70%
Spot 2	Glycerol-3-phosphate dehydrogenase [NAD(+)]	K1QMD3_CRAGI	38.40	3	3	4	0.06%	12.00%
Spot 2	Ubiquitin-like modifier-activating enzyme 1	K1R3M4_CRAGI	65.99	2	2	4	0.06%	6.60%
Spot 2	Fructose-bisphosphate aldolase	K1RTQ6_CRAGI	38.99	2	2	4	0.06%	11.80%
Spot 2	Alpha-aminoadipic semialdehyde dehydrogenase	K1RNB6_CRAGI	55.22	4	4	4	0.06%	9.78%
Spot 2	Heterogeneous nuclear ribonucleoprotein A/B	K1PNI6_CRAGI	53.89	4	4	4	0.06%	7.79%
Spot 2	3-demethylubiquinone-9 3-methyltransferase	K1Q7I3_CRAGI	38.57	4	4	4	0.06%	14.80%
Spot 2	Aldo-keto reductase family 1 member B10	K1Q6D1_CRAGI	35.38	3	3	3	0.04%	8.04%
Spot 2	Dual specificity mitogen-activated protein kinase kinase 3	K1Q3Q7_CRAGI	41.67	3	3	3	0.04%	8.15%
Spot 2	Sulfotransferase family cytosolic 1B member 1	K1QLP7_CRAGI	69.89	3	3	3	0.04%	4.81%
Spot 2	Uncharacterized protein	K1QQI8_CRAGI	21.22	2	2	3	0.04%	17.40%

Spot 2	Phytanoyl-CoA dioxygenase domain-containing protein 1	K1PUP5_CRAGI	46.87	3	3	3	0.04%	7.28%
Spot 2	T-complex protein 1 subunit gamma	K1R466_CRAGI	63.91	3	3	3	0.04%	4.66%
Spot 2	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	1	1	3	0.04%	8.11%
Spot 2	Uncharacterized protein yfeX	K1RBF6_CRAGI	39.59	3	3	3	0.04%	7.63%
Spot 2	Thymidine phosphorylase	K1R192_CRAGI	48.20	2	2	3	0.04%	8.44%
Spot 2	Vitellogenin-6	K1QNA2_CRAGI	273.31	0	0	3	0.04%	1.12%
Spot 2	Splicing factor, proline-and glutamine-rich	K1PNY5_CRAGI	62.32	3	3	3	0.04%	4.80%
Spot 2	Uncharacterized protein	K1RLJ5_CRAGI	31.43	3	3	3	0.04%	6.81%
Spot 2	Protein-serine/threonine phosphatase	K1PXG6_CRAGI	37.32	3	3	3	0.04%	8.23%
Spot 2	26S protease regulatory subunit 6B	K1QSB2_CRAGI	47.46	3	3	3	0.04%	7.86%
Spot 2	Troponin T, skeletal muscle	K1QPC9_CRAGI	20.67	2	2	2	0.03%	10.70%
Spot 2	Lambda-crystallin-like protein	K1PK94_CRAGI	35.45	2	2	2	0.03%	6.33%
Spot 2	Uncharacterized protein	K1QT31_CRAGI	49.72	2	2	2	0.03%	3.60%
Spot 2	Eukaryotic translation initiation factor 2 subunit 1	K1Q435_CRAGI	29.39	2	2	2	0.03%	7.00%
Spot 2	Putative proline racemase	K1P4P6_CRAGI	35.90	2	2	2	0.03%	6.44%

Spot 2	Succinate-semialdehyde dehydrogenase, mitochondrial	K1PV02_CRAGI	74.22	2	2	2	0.03%	3.28%
Spot 2	Putative methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	K1R252_CRAGI	57.24	2	2	2	0.03%	6.48%
Spot 2	Mannose-6-phosphate isomerase	K1QR42_CRAGI	53.64	2	2	2	0.03%	4.12%
Spot 2	Alpha-actinin, sarcomeric	K1RH58_CRAGI	102.20	2	2	2	0.03%	2.16%
Spot 2	Uncharacterized protein	K1R3U2_CRAGI	35.42	2	2	2	0.03%	5.48%
Spot 2	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	K1S6V7_CRAGI	117.54	2	2	2	0.03%	2.41%
Spot 2	Uncharacterized protein	K1QM61_CRAGI	40.41	2	2	2	0.03%	6.59%
Spot 2	Multifunctional protein ADE2	K1RJ97_CRAGI	46.77	2	2	2	0.03%	4.98%
Spot 2	Uncharacterized protein	K1PKQ3_CRAGI	38.10	2	2	2	0.03%	5.23%
Spot 2	Glutamine synthetase	K1R0H0_CRAGI	40.96	2	2	2	0.03%	4.71%
Spot 2	Glycerol-3-phosphate dehydrogenase [NAD(+)]	K1P8T5_CRAGI	38.81	1	1	2	0.03%	5.43%
Spot 2	Proteasome subunit alpha type-1	K1PCR9_CRAGI	27.74	2	2	2	0.03%	8.33%
Spot 2	Fumarylacetoacetase	K1Q4C3_CRAGI	46.29	2	2	2	0.03%	5.49%
Spot 2	Pyruvate kinase	K1QRW8_CRAGI	58.46	2	2	2	0.03%	3.58%
Spot 2	Malate dehydrogenase	K1PWW1_CRAGI	70.65	2	2	2	0.03%	3.00%
Spot 2	Xaa-Pro dipeptidase	K1PBR3_CRAGI	43.51	2	2	2	0.03%	5.26%

Spot 2	Nudix hydrolase 20	K1Q851_CRAGI	29.86	2	2	2	0.03%	8.20%
Spot 2	Sarcoplasmic calcium-binding protein	K1PY28_CRAGI	21.14	2	2	2	0.03%	11.70%
Spot 2	UDP-N-acetylhexosamine pyrophosphorylase	K1R4X9_CRAGI	57.26	2	2	2	0.03%	4.90%
Spot 2	5'-nucleotidase	K1Q4F6_CRAGI	33.61	2	2	2	0.03%	8.90%
Spot 2	Uncharacterized protein	K1RF40_CRAGI	38.45	2	2	2	0.03%	6.41%
Spot 2	Uncharacterized protein	K1PYT9_CRAGI	42.80	2	2	2	0.03%	5.01%
Spot 2	Uncharacterized protein	K1PTP3_CRAGI	34.46	2	2	2	0.03%	9.06%
Spot 2	Uncharacterized protein	K1R6U0_CRAGI	27.18	1	1	2	0.03%	9.09%
Spot 2	Eosinophil peroxidase	K1QHY1_CRAGI	78.24	2	2	2	0.03%	3.11%
Spot 2	Betaine--homocysteine S-methyltransferase 2	K1QFK7_CRAGI	40.57	2	2	2	0.03%	5.22%
Spot 2	Dehydrogenase/reductase SDR family member 13	K1QSX2_CRAGI	50.76	3	3	3	0.04%	6.91%
Spot 2	Dihydropyrimidinase	K1PGY5_CRAGI	71.26	2	2	2	0.03%	3.55%
Spot 2	Transaldolase	K1QVK0_CRAGI	62.89	9	9	11	0.16%	17.30%
Spot 2	Uncharacterized protein	K1QZX9_CRAGI	50.75	7	7	7	0.10%	18.00%
Spot 2	Nitrile-specifier protein 5 (Fragment)	K1S4K7_CRAGI	34.92	4	4	4	0.06%	13.80%
Spot 3	Severin	K1PE57_CRAGI	37.21	4	6	6	0.09%	13.60%
Spot 3	Hypoxanthine-guanine phosphoribosyltransferase	K1RH42_CRAGI	25.33	3	3	3	0.05%	17.00%
Spot 3	Non-selenium glutathione peroxidase	A7M7T7_CRAGI	24.45	5	5	5	0.08%	24.00%

Spot 3	Sarcoplasmic calcium-binding protein	K1PY28_CRAGI	21.14	2	2	2	0.03%	12.30%
Spot 3	Uncharacterized protein	K1PU04_CRAGI	38.43	1	1	2	0.03%	8.48%
Spot 3	Non-neuronal cytoplasmic intermediate filament protein	K1PBC0_CRAGI	69.29	4	4	4	0.06%	6.15%
Spot 3	Tubulin beta chain	K1PN21_CRAGI	50.04	0	0	2	0.03%	6.07%
Spot 3	Protein SET	K1PWR0_CRAGI	28.11	2	2	2	0.03%	8.54%
Spot 3	Eukaryotic peptide chain release factor subunit 1	K1PX23_CRAGI	49.52	4	4	5	0.08%	11.50%
Spot 3	T-complex protein 1 subunit theta	K1R0S3_CRAGI	83.14	5	7	7	0.11%	8.38%
Spot 3	Uncharacterized protein	K1PUI4_CRAGI	27.33	5	5	5	0.08%	21.40%
Spot 3	14-3-3 protein zeta	K1PHM8_CRAGI	28.64	1	1	2	0.03%	7.63%
Spot 3	Uncharacterized protein	K1RRY6_CRAGI	22.02	1	1	4	0.06%	20.30%
Spot 3	Complement component 1 Q subcomponent-binding protein, mitochondrial	K1PX73_CRAGI	33.67	3	5	5	0.08%	10.20%
Spot 3	Mammalian ependymin-related protein 1 (Fragment)	K1QAN0_CRAGI	20.58	2	2	2	0.03%	11.30%
Spot 3	Filamin-C	K1PW06_CRAGI	323.73	8	8	9	0.14%	3.15%
Spot 3	Nuclear autoantigenic sperm protein	K1RN77_CRAGI	72.95	2	2	2	0.03%	3.53%
Spot 3	Uncharacterized protein	K1QFW9_CRAGI	94.86	2	2	2	0.03%	2.23%
Spot 3	Ubiquitin-like modifier-activating enzyme 1	K1R3M4_CRAGI	65.99	3	3	5	0.08%	6.60%

Spot 3	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	0	0	3	0.05%	5.48%
Spot 3	Uncharacterized protein	K1Q1K3_CRAGI	18.14	2	2	2	0.03%	10.30%
Spot 3	Metalloproteinase inhibitor 3	K1QDB0_CRAGI	20.63	3	5	6	0.09%	14.50%
Spot 3	Sarcoplasmic calcium-binding protein	K1QVZ9_CRAGI	22.17	1	1	4	0.06%	20.20%
Spot 3	Uncharacterized protein	K1R7B8_CRAGI	35.11	2	2	2	0.03%	5.92%
Spot 3	Triosephosphate isomerase	K1PJ59_CRAGI	18.67	2	2	2	0.03%	15.30%
Spot 3	Mammalian ependymin-related protein 1	K1QJ28_CRAGI	40.53	2	2	2	0.03%	5.01%
Spot 3	Arginine kinase	K1PLF9_CRAGI	39.63	3	5	5	0.08%	9.71%
Spot 3	Uncharacterized protein	K1R7Q9_CRAGI	19.63	4	5	6	0.09%	19.40%
Spot 3	Alpha-actinin, sarcomeric	K1RH58_CRAGI	102.20	5	6	7	0.11%	5.91%
Spot 3	Serine protease inhibitor dipetalogastin	K1PRF2_CRAGI	17.60	2	2	2	0.03%	14.50%
Spot 3	Glutathione S-transferase A	K1QJ85_CRAGI	23.06	1	1	3	0.05%	13.50%
Spot 3	Tudor domain-containing protein 1	K1QBW6_CRAGI	278.65	2	2	2	0.03%	0.74%
Spot 3	Uncharacterized protein	K1PV81_CRAGI	39.24	5	5	5	0.08%	13.30%
Spot 3	Carbonic anhydrase 2	K1QSG0_CRAGI	36.53	2	2	2	0.03%	6.42%
Spot 3	Tropomyosin	K1QNV6_CRAGI	39.01	0	0	12	0.18%	21.20%
Spot 3	Enolase-phosphatase E1	K1PQI4_CRAGI	34.67	2	2	2	0.03%	6.45%
Spot 3	14-3-3 protein epsilon	K1R5F2_CRAGI	29.07	3	5	5	0.08%	14.10%
Spot 3	14-3-3 protein gamma	K1PPQ1_CRAGI	28.48	4	4	5	0.08%	19.50%

Spot 3	Counting factor associated protein D	K1S185_CRAGI	38.87	2	3	4	0.06%	6.59%
Spot 3	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	5	5	8	0.12%	12.10%
Spot 3	Protein disulfide-isomerase	K1Q6X5_CRAGI	55.50	2	2	2	0.03%	4.24%
Spot 3	Muscle M-line assembly protein unc-89 (Fragment)	K1QPH2_CRAGI	50.98	2	2	2	0.03%	3.91%
Spot 3	14-3-3 protein zeta	K1P9N7_CRAGI	35.15	4	4	5	0.08%	14.80%
Spot 3	Small glutamine-rich tetratricopeptide repeat-containing protein beta	K1PRK2_CRAGI	29.25	3	3	3	0.05%	13.20%
Spot 3	Dihydropteridine reductase	K1PFL3_CRAGI	24.87	3	3	3	0.05%	12.70%
Spot 3	Tropomyosin	B7XC66_CRAGI	33.02	1	2	24	0.37%	43.30%
Spot 3	Rho GDP-dissociation inhibitor 1	K1QCM0_CRAGI	23.57	6	7	7	0.11%	26.20%
Spot 3	Calcium-dependent protein kinase isoform 2	K1QQK6_CRAGI	27.31	10	12	13	0.20%	31.50%
Spot 3	Eukaryotic translation initiation factor 6	K1R7N6_CRAGI	51.33	1	2	3	0.05%	6.02%
Spot 4	Tubulin alpha-1C chain	K1QII6_CRAGI	50.14	2	2	2	0.06%	7.76%
Spot 4	Filamin-C	K1PW06_CRAGI	323.73	3	3	3	0.09%	1.46%
Spot 4	Putative aminopeptidase W07G4.4	K1PVV6_CRAGI	55.71	2	2	4	0.12%	10.00%
Spot 4	Uncharacterized protein	K1Q9G9_CRAGI	42.96	2	2	3	0.09%	11.90%
Spot 4	Plastin-3	K1R2D6_CRAGI	79.69	1	1	2	0.06%	3.26%
Spot 4	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	1	1	3	0.09%	7.17%

Spot 4	60 kDa heat shock protein, mitochondrial	K1Q5G6_CRAGI	59.71	10	22	28	0.84%	24.60%
Spot 4	Uncharacterized protein	K1QT31_CRAGI	49.72	2	2	2	0.06%	6.08%
Spot 4	Leucine-rich repeats and immunoglobulin-like domains protein 3	K1QHG2_CRAGI	52.21	2	2	2	0.06%	5.32%
Spot 4	Actin 2	Q8TA69_CRAGI	41.74	0	0	4	0.12%	12.50%
Spot 5	Transitional endoplasmic reticulum ATPase	K1PVA1_CRAGI	88.70	4	4	4	0.06%	4.26%
Spot 5	Protocadherin Fat 1 (Fragment)	K1RE12_CRAGI	32.50	2	2	2	0.03%	7.09%
Spot 5	Apoptosis-inducing factor 3	K1QFI3_CRAGI	97.02	7	7	7	0.11%	8.43%
Spot 5	Severin	K1PE57_CRAGI	37.21	2	3	3	0.05%	6.97%
Spot 5	Uncharacterized protein	K1PU04_CRAGI	38.43	2	2	3	0.05%	10.50%
Spot 5	Sarcoplasmic calcium-binding protein	K1PY28_CRAGI	21.14	2	2	2	0.03%	11.70%
Spot 5	Spectrin beta chain	K1QFR9_CRAGI	280.21	11	11	12	0.19%	4.26%
Spot 5	Neural cell adhesion molecule 1	K1R7L4_CRAGI	96.79	6	7	8	0.12%	8.32%
Spot 5	Plastin-3	K1R2D6_CRAGI	79.69	3	3	3	0.05%	4.67%
Spot 5	Uncharacterized protein	K1QT31_CRAGI	49.72	4	4	4	0.06%	6.31%
Spot 5	Radixin	K1PUJ1_CRAGI	69.54	2	2	2	0.03%	3.40%
Spot 5	Neural cell adhesion molecule L1	K1PQF1_CRAGI	75.37	2	2	6	0.09%	8.38%
Spot 5	Non-neuronal cytoplasmic intermediate filament protein	K1PBC0_CRAGI	69.29	8	8	8	0.12%	11.20%

Spot 5	Spectrin alpha chain	K1R401_CRAGI	287.01	15	15	15	0.23%	6.87%
Spot 5	Putative aminopeptidase W07G4.4	K1PVV6_CRAGI	55.71	2	2	6	0.09%	11.80%
Spot 5	Tubulin beta chain	K1PN21_CRAGI	50.04	0	0	2	0.03%	4.49%
Spot 5	Metalloendopeptidase	K1RGT5_CRAGI	118.98	2	2	2	0.03%	1.58%
Spot 5	ATP synthase subunit beta	K1RWW5_CRAGI	44.93	3	3	3	0.05%	7.97%
Spot 5	Uncharacterized protein	K1R3U2_CRAGI	35.42	3	4	4	0.06%	8.39%
Spot 5	Ubiquitin-like modifier-activating enzyme 1	K1R1M7_CRAGI	97.95	4	4	7	0.11%	8.54%
Spot 5	Fibrinolytic enzyme, isozyme C	K1PH66_CRAGI	74.60	2	2	2	0.03%	2.87%
Spot 5	Stress-70 protein, mitochondrial	K1P9D0_CRAGI	76.37	2	2	2	0.03%	2.70%
Spot 5	Filamin-C	K1PW06_CRAGI	323.73	36	43	49	0.76%	15.00%
Spot 5	Uncharacterized protein	K1R871_CRAGI	37.44	4	5	5	0.08%	14.10%
Spot 5	Heat shock protein HSP 90-alpha 1	K1PNQ5_CRAGI	83.32	3	3	13	0.20%	19.50%
Spot 5	V-type proton ATPase catalytic subunit A	K1Q9V3_CRAGI	70.85	6	6	6	0.09%	9.12%
Spot 5	Ubiquitin-like modifier-activating enzyme 1	K1R3M4_CRAGI	65.99	4	4	7	0.11%	11.30%
Spot 5	71kDa heat shock connate protein	Q9XZJ2_CRAGI	72.04	0	0	14	0.22%	17.00%
Spot 5	Vitellogenin-6	K1QNA2_CRAGI	273.31	3	3	6	0.09%	2.20%
Spot 5	78 kDa glucose-regulated protein	K1QIR8_CRAGI	73.03	0	0	61	0.94%	40.20%
Spot 5	Uncharacterized protein	K1PYT9_CRAGI	42.80	2	2	2	0.03%	5.01%

Spot 5	ATP-dependent DNA helicase II subunit 2	K1Q7H0_CRAGI	81.43	3	3	3	0.05%	4.53%
Spot 5	Kyphoscoliosis peptidase	K1PV35_CRAGI	77.79	2	3	4	0.06%	4.79%
Spot 5	Titin	K1RVK9_CRAGI	335.75	7	8	8	0.12%	2.99%
Spot 5	Kyphoscoliosis peptidase	K1PEZ6_CRAGI	32.96	4	4	4	0.06%	15.80%
Spot 5	14-3-3 protein gamma	K1PPQ1_CRAGI	28.48	1	1	2	0.03%	7.17%
Spot 5	Alpha-actinin, sarcomeric	K1RH58_CRAGI	102.20	6	6	6	0.09%	7.50%
Spot 5	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	4	4	5	0.08%	8.22%
Spot 5	Protein disulfide-isomerase	K1Q6X5_CRAGI	55.50	5	5	5	0.08%	11.50%
Spot 5	78kDa glucose regulated protein	Q75W49_CRAGI	73.08	2	7	69	1.06%	43.10%
Spot 5	Paramyosin	K1QTC1_CRAGI	97.88	2	2	2	0.03%	2.59%
Spot 5	Eosinophil peroxidase	K1QHY1_CRAGI	78.24	6	7	7	0.11%	10.60%
Spot 5	Tropomyosin	B7XC66_CRAGI	33.02	0	0	3	0.05%	9.86%
Spot 5	Glucose-regulated protein 94	A5LGG7_CRAGI	91.63	1	1	9	0.14%	12.40%
Spot 5	Dihydropyrimidinase	K1PGY5_CRAGI	71.26	3	3	3	0.05%	5.26%
Spot 5	Endoplasmin	K1QX26_CRAGI	125.42	1	1	9	0.14%	9.52%
Spot 6	Tubulin alpha-1C chain	K1QII6_CRAGI	50.14	2	2	4	0.12%	11.80%
Spot 6	Major vault protein	K1QQR1_CRAGI	96.31	4	4	4	0.12%	5.48%
Spot 6	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	4	6	13	0.39%	25.20%
Spot 6	Fascin	K1QEZ3_CRAGI	55.60	2	2	2	0.06%	5.01%
Spot 6	Enolase	K1QX37_CRAGI	127.42	9	14	20	0.60%	12.50%
Spot 6	Aldehyde dehydrogenase	K1Q9Z4_CRAGI	25.35	1	1	3	0.09%	10.90%
Spot 6	Heterogeneous nuclear ribonucleoprotein Q	K1R7I9_CRAGI	69.23	2	2	2	0.06%	3.50%

Spot 6	Aldehyde dehydrogenase, mitochondrial	K1QNT7_CRAGI	56.97	3	3	3	0.09%	8.67%
Spot 7	Putative ATP-dependent RNA helicase DDX4	K1Q923_CRAGI	81.51	0	0	3	0.08%	5.78%
Spot 7	Tubulin alpha-1C chain	K1QII6_CRAGI	50.14	3	3	5	0.14%	16.20%
Spot 7	Major vault protein	K1QQR1_CRAGI	96.31	4	4	4	0.11%	6.53%
Spot 7	Alpha-aminoadipic semialdehyde dehydrogenase	K1RNB6_CRAGI	55.22	2	2	2	0.05%	6.26%
Spot 7	Cytosolic non-specific dipeptidase	K1RJ70_CRAGI	58.73	3	3	3	0.08%	9.85%
Spot 7	Adenosylhomocysteinase	K1RW85_CRAGI	47.50	2	2	2	0.05%	4.62%
Spot 7	Aldehyde dehydrogenase	K1Q9Z4_CRAGI	25.35	1	1	5	0.14%	10.90%
Spot 7	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	6	8	18	0.49%	28.70%
Spot 7	Tetratricopeptide repeat protein 38	K1QWZ0_CRAGI	50.50	2	2	2	0.05%	7.96%
Spot 7	Enolase	K1QX37_CRAGI	127.42	9	16	36	0.98%	12.50%
Spot 7	Coronin	K1QRW4_CRAGI	61.69	3	4	4	0.11%	10.00%
Spot 7	Heterogeneous nuclear ribonucleoprotein Q	K1R7I9_CRAGI	69.23	2	2	2	0.05%	3.66%
Spot 7	T-complex protein 1 subunit beta	K1R294_CRAGI	57.49	3	3	3	0.08%	8.32%
Spot 7	Aldehyde dehydrogenase, mitochondrial	K1QNT7_CRAGI	56.97	3	3	3	0.08%	8.86%
Spot 7	Protein hu-li tai shao	K1PEX5_CRAGI	85.92	2	2	2	0.05%	3.62%

Spot 7	Selenium-binding protein 1 (Fragment)	K1QI40_CRAGI	53.35	4	4	4	0.11%	9.19%
Spot 8	Propionyl-CoA carboxylase beta chain, mitochondrial	K1RNB5_CRAGI	59.11	5	5	5	0.07%	10.70%
Spot 8	Leucine aminopeptidase-like protein	B6V956_CRAGI	57.40	0	0	2	0.03%	4.36%
Spot 8	Heterogeneous nuclear ribonucleoprotein Q	K1R7I9_CRAGI	69.23	4	4	4	0.06%	6.69%
Spot 8	ATP synthase subunit alpha	K1R6Z7_CRAGI	59.87	4	5	5	0.07%	8.32%
Spot 8	Protein disulfide-isomerase	K1Q7T5_CRAGI	55.52	9	10	10	0.14%	18.90%
Spot 8	Non-selenium glutathione peroxidase	A7M7T7_CRAGI	24.45	3	3	3	0.04%	13.60%
Spot 8	S-adenosylmethionine synthase	K1QZB1_CRAGI	45.38	1	1	3	0.04%	9.73%
Spot 8	T-complex protein 1 subunit beta	K1R294_CRAGI	57.49	2	2	2	0.03%	3.40%
Spot 8	N-acetylgalactosamine kinase	K1RY94_CRAGI	50.86	3	3	3	0.04%	6.64%
Spot 8	Legumain	K1QB32_CRAGI	90.07	2	2	2	0.03%	2.57%
Spot 8	T-complex protein 1 subunit zeta	K1PXN5_CRAGI	58.33	2	2	2	0.03%	3.39%
Spot 8	Plasma alpha-L-fucosidase	K1QZW1_CRAGI	53.44	1	1	2	0.03%	4.81%
Spot 8	Omega-crystallin	K1QUX5_CRAGI	19.69	4	7	14	0.20%	29.80%
Spot 8	Succinate-semialdehyde dehydrogenase, mitochondrial	K1PV02_CRAGI	74.22	6	7	7	0.10%	11.00%

Spot 8	Putative ATP-dependent RNA helicase DDX4	K1Q923_CRAGI	81.51	0	0	8	0.11%	8.99%
Spot 8	Leucine-rich repeat-containing G-protein coupled receptor 6	K1PGK1_CRAGI	46.64	3	3	3	0.04%	10.20%
Spot 8	Alpha-actinin, sarcomeric	K1RH58_CRAGI	102.20	3	3	3	0.04%	3.98%
Spot 8	Plasma alpha-L-fucosidase	K1QWY7_CRAGI	62.84	2	2	2	0.03%	4.39%
Spot 8	Beta-lactamase	K1QVA0_CRAGI	27.70	2	2	2	0.03%	8.37%
Spot 8	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	15	26	121	1.72%	48.60%
Spot 8	Beta-hexosaminidase subunit beta	K1Q6V0_CRAGI	51.16	0	0	8	0.11%	13.70%
Spot 8	Aldehyde dehydrogenase, mitochondrial	K1QNT7_CRAGI	56.97	18	20	21	0.30%	36.60%
Spot 8	Dihydrolipoyl dehydrogenase	K1Q330_CRAGI	173.92	7	9	9	0.13%	5.87%
Spot 8	Major vault protein	K1QQR1_CRAGI	96.31	2	2	2	0.03%	2.33%
Spot 8	Histidyl-tRNA synthetase, cytoplasmic	K1P8Z2_CRAGI	58.07	1	1	2	0.03%	3.69%
Spot 8	Plasma alpha-L-fucosidase	K1QC19_CRAGI	53.81	1	1	2	0.03%	5.22%
Spot 8	Filamin-C	K1PW06_CRAGI	323.73	27	34	34	0.49%	10.80%
Spot 8	Immunoglobulin superfamily containing leucine-rich repeat protein 2	K1RUQ7_CRAGI	16.56	2	2	2	0.03%	11.30%
Spot 8	4-aminobutyrate aminotransferase, mitochondrial	K1QXS3_CRAGI	50.07	3	3	3	0.04%	9.71%

Spot 8	T-complex protein 1 subunit eta	K1Q9W5_CRAGI	46.55	4	4	8	0.11%	17.00%
Spot 8	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	8	12	62	0.88%	54.60%
Spot 8	Septin-7	K1PCA0_CRAGI	70.81	2	2	2	0.03%	3.04%
Spot 8	Adenosylhomocysteinase	K1RW85_CRAGI	47.50	2	2	2	0.03%	4.16%
Spot 8	Arginine kinase	K1PLF9_CRAGI	39.63	4	4	4	0.06%	12.60%
Spot 8	Vitellogenin-6	K1QNA2_CRAGI	273.31	0	0	4	0.06%	1.82%
Spot 8	Amidase	K1QX82_CRAGI	13.46	2	3	6	0.09%	37.00%
Spot 8	Amidase	K1QFU8_CRAGI	75.36	1	1	5	0.07%	6.37%
Spot 8	Aldehyde dehydrogenase	K1Q9Z4_CRAGI	25.35	9	15	21	0.30%	53.30%
Spot 8	Dipeptidyl-peptidase 1 (Fragment)	K1QAY3_CRAGI	44.67	2	2	2	0.03%	5.87%
Spot 8	Aldehyde dehydrogenase family 8 member A1	K1QIL8_CRAGI	53.30	6	6	6	0.09%	12.00%
Spot 8	Vitellogenin	Q8IU34_CRAGI	179.21	1	1	5	0.07%	3.66%
Spot 8	Selenium-binding protein 1 (Fragment)	K1QI40_CRAGI	53.35	22	38	45	0.64%	54.90%
Spot 8	Alpha-aminoadipic semialdehyde dehydrogenase	K1RNB6_CRAGI	55.22	19	27	28	0.40%	46.40%
Spot 8	Putative methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	K1R252_CRAGI	57.24	14	22	22	0.31%	38.70%
Spot 8	Guanine deaminase	K1QRG3_CRAGI	50.05	3	3	3	0.04%	5.39%
Spot 8	Carboxypeptidase E	K1P9W2_CRAGI	57.11	2	2	2	0.03%	3.58%
Spot 8	Fascin	K1QEZ3_CRAGI	55.60	16	22	23	0.33%	32.90%
Spot 8	Amidase	K1Q2A1_CRAGI	59.11	7	7	13	0.19%	24.80%

Spot 8	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B	K1QPJ9_CRAGI	51.51	4	4	4	0.06%	9.44%
Spot 8	Beta-hexosaminidase subunit beta	K1QHI3_CRAGI	47.22	1	1	9	0.13%	18.10%
Spot 8	Enolase	K1QX37_CRAGI	127.42	7	7	7	0.10%	7.34%
Spot 8	Glutathione synthetase	K1P746_CRAGI	62.49	2	2	2	0.03%	3.77%
Spot 8	Dihydropyrimidinase	K1PGY5_CRAGI	71.26	10	13	16	0.23%	21.90%
Spot 8	Filamin-A	K1RZ99_CRAGI	90.77	15	19	20	0.29%	27.30%
Spot 9	Propionyl-CoA carboxylase beta chain, mitochondrial	K1RNB5_CRAGI	59.11	4	4	4	0.05%	8.46%
Spot 9	Beta-hexosaminidase subunit beta	K1Q6V0_CRAGI	51.16	1	1	9	0.12%	15.50%
Spot 9	Protein disulfide-isomerase	K1Q7T5_CRAGI	55.52	8	8	8	0.11%	17.50%
Spot 9	T-complex protein 1 subunit eta	K1Q9W5_CRAGI	46.55	6	6	9	0.12%	23.40%
Spot 9	Non-selenium glutathione peroxidase	A7M7T7_CRAGI	24.45	6	6	6	0.08%	28.50%
Spot 9	Legumain	K1QB32_CRAGI	90.07	3	3	3	0.04%	4.90%
Spot 9	Coronin	K1QRW4_CRAGI	61.69	2	2	2	0.03%	4.12%
Spot 9	Plasma alpha-L-fucosidase	K1QWY7_CRAGI	62.84	2	2	2	0.03%	3.84%
Spot 9	ATP synthase subunit alpha	K1R6Z7_CRAGI	59.87	4	5	5	0.07%	8.86%
Spot 9	Omega-crystallin	K1QUX5_CRAGI	19.69	5	7	14	0.19%	33.10%

Spot 9	Putative ATP-dependent RNA helicase DDX4	K1Q923_CRAGI	81.51	0	0	11	0.15%	12.60%
Spot 9	Beta-lactamase	K1QVA0_CRAGI	27.70	2	2	3	0.04%	12.70%
Spot 9	Amidase	K1QFU8_CRAGI	75.36	1	1	4	0.05%	6.37%
Spot 9	ATP synthase subunit beta	K1RWW5_CRAGI	44.93	2	2	2	0.03%	5.80%
Spot 9	Aldehyde dehydrogenase, mitochondrial	K1QNT7_CRAGI	56.97	9	11	11	0.15%	21.60%
Spot 9	Dihydrolipoyl dehydrogenase	K1Q330_CRAGI	173.92	6	7	7	0.10%	4.77%
Spot 9	Filamin-C	K1PW06_CRAGI	323.73	24	30	31	0.42%	10.30%
Spot 9	4-aminobutyrate aminotransferase, mitochondrial	K1QXS3_CRAGI	50.07	2	2	2	0.03%	4.74%
Spot 9	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	8	11	60	0.82%	43.20%
Spot 9	T-complex protein 1 subunit zeta	K1PXN5_CRAGI	58.33	2	2	2	0.03%	3.39%
Spot 9	T-complex protein 1 subunit eta	K1QVN9_CRAGI	25.51	1	1	4	0.05%	18.20%
Spot 9	71kDa heat shock connate protein	Q9XZJ2_CRAGI	72.04	0	0	2	0.03%	3.79%
Spot 9	Enolase	K1QX37_CRAGI	127.42	13	21	22	0.30%	15.50%
Spot 9	Arginine kinase	K1PLF9_CRAGI	39.63	6	6	6	0.08%	20.90%
Spot 9	Vitellogenin-6	K1QNA2_CRAGI	273.31	0	0	3	0.04%	1.29%
Spot 9	Amidase	K1QX82_CRAGI	13.46	2	3	6	0.08%	37.00%
Spot 9	Plasma alpha-L-fucosidase	K1QC19_CRAGI	53.81	1	1	2	0.03%	4.78%

Spot 9	Echinoderm microtubule-associated protein-like 1	K1PWP8_CRAGI	80.69	2	2	2	0.03%	5.59%
Spot 9	Aldehyde dehydrogenase	K1Q9Z4_CRAGI	25.35	8	13	20	0.27%	53.30%
Spot 9	Dipeptidyl-peptidase 1 (Fragment)	K1QAY3_CRAGI	44.67	2	2	2	0.03%	5.61%
Spot 9	Alpha-actinin, sarcomeric	K1RH58_CRAGI	102.20	2	2	2	0.03%	2.50%
Spot 9	Aldehyde dehydrogenase family 8 member A1	K1QIL8_CRAGI	53.30	6	6	6	0.08%	12.00%
Spot 9	N-acetylgalactosamine kinase	K1RY94_CRAGI	50.86	5	5	5	0.07%	9.85%
Spot 9	Succinate-semialdehyde dehydrogenase, mitochondrial	K1PV02_CRAGI	74.22	4	4	4	0.05%	6.55%
Spot 9	Alpha-aminoadipic semialdehyde dehydrogenase	K1RNB6_CRAGI	55.22	18	23	23	0.31%	45.00%
Spot 9	Putative methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	K1R252_CRAGI	57.24	14	20	20	0.27%	37.00%
Spot 9	Guanine deaminase	K1QRG3_CRAGI	50.05	6	6	6	0.08%	12.80%
Spot 9	Fascin	K1QEZ3_CRAGI	55.60	14	25	29	0.39%	31.90%
Spot 9	Protein henna	K1QHH0_CRAGI	52.07	1	1	3	0.04%	5.65%
Spot 9	Selenium-binding protein 1 (Fragment)	K1QI40_CRAGI	53.35	23	43	51	0.69%	55.90%
Spot 9	Glutamate decarboxylase-like protein 1	K1P7P6_CRAGI	41.82	2	2	2	0.03%	6.52%
Spot 9	Amidase	K1Q2A1_CRAGI	59.11	6	7	12	0.16%	23.50%

Spot 9	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B	K1QPJ9_CRAGI	51.51	3	3	3	0.04%	7.42%
Spot 9	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	14	28	118	1.61%	43.90%
Spot 9	Leucine-rich repeat-containing G-protein coupled receptor 6	K1PGK1_CRAGI	46.64	6	7	7	0.10%	21.40%
Spot 9	Beta-hexosaminidase subunit beta	K1QHI3_CRAGI	47.22	2	2	10	0.14%	21.00%
Spot 9	Alanine aminotransferase 2	K1RGF4_CRAGI	51.34	2	2	2	0.03%	7.33%
Spot 9	Glutathione synthetase	K1P746_CRAGI	62.49	6	6	6	0.08%	12.00%
Spot 9	Dihydropyrimidinase	K1PGY5_CRAGI	71.26	13	14	16	0.22%	27.50%
Spot 9	Filamin-A	K1RZ99_CRAGI	90.77	18	26	27	0.37%	32.90%
Spot 9	Uncharacterized protein	K1QBB5_CRAGI	38.46	2	2	2	0.03%	5.64%
Spot 10	Propionyl-CoA carboxylase beta chain, mitochondrial	K1RNB5_CRAGI	59.11	2	2	2	0.03%	3.31%
Spot 10	Protein disulfide-isomerase	K1Q7T5_CRAGI	55.52	4	4	4	0.06%	8.74%
Spot 10	Non-selenium glutathione peroxidase	A7M7T7_CRAGI	24.45	4	4	4	0.06%	19.50%
Spot 10	T-complex protein 1 subunit beta	K1R294_CRAGI	57.49	3	3	3	0.04%	5.86%
Spot 10	Uncharacterized protein	K1QBB5_CRAGI	38.46	2	2	2	0.03%	5.93%
Spot 10	Legumain	K1QB32_CRAGI	90.07	4	4	4	0.06%	6.13%
Spot 10	T-complex protein 1 subunit zeta	K1PXN5_CRAGI	58.33	3	3	3	0.04%	5.65%

Spot 10	Plasma alpha-L-fucosidase	K1QZW1_CRAGI	53.44	1	1	2	0.03%	4.81%
Spot 10	ATP synthase subunit alpha	K1R6Z7_CRAGI	59.87	5	6	6	0.09%	10.30%
Spot 10	Omega-crystallin	K1QUX5_CRAGI	19.69	4	8	14	0.20%	29.80%
Spot 10	Putative ATP-dependent RNA helicase DDX4	K1Q923_CRAGI	81.51	0	0	7	0.10%	7.57%
Spot 10	Leucine-rich repeat-containing G-protein coupled receptor 6	K1PGK1_CRAGI	46.64	6	7	7	0.10%	21.40%
Spot 10	Beta-lactamase	K1QVA0_CRAGI	27.70	4	4	6	0.09%	27.50%
Spot 10	Beta-hexosaminidase subunit beta	K1Q6V0_CRAGI	51.16	0	0	9	0.13%	15.80%
Spot 10	Aldehyde dehydrogenase, mitochondrial	K1QNT7_CRAGI	56.97	4	4	4	0.06%	8.86%
Spot 10	Dihydrolipoyl dehydrogenase	K1Q330_CRAGI	173.92	7	7	7	0.10%	5.42%
Spot 10	Filamin-C	K1PW06_CRAGI	323.73	14	16	16	0.23%	6.10%
Spot 10	4-aminobutyrate aminotransferase, mitochondrial	K1QXS3_CRAGI	50.07	4	4	4	0.06%	13.80%
Spot 10	T-complex protein 1 subunit eta	K1Q9W5_CRAGI	46.55	1	1	3	0.04%	7.33%
Spot 10	Uncharacterized protein	K1QFW9_CRAGI	94.86	1	1	2	0.03%	2.23%
Spot 10	Uncharacterized protein	K1RCM9_CRAGI	52.17	2	3	3	0.04%	3.90%
Spot 10	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	6	9	60	0.85%	51.90%
Spot 10	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	0	0	2	0.03%	5.18%

Spot 10	71kDa heat shock connate protein	Q9XZJ2_CRAGI	72.04	0	0	3	0.04%	5.31%
Spot 10	Enolase	K1QX37_CRAGI	127.42	3	3	3	0.04%	2.97%
Spot 10	Arginine kinase	K1PLF9_CRAGI	39.63	3	3	3	0.04%	9.71%
Spot 10	Vitellogenin-6	K1QNA2_CRAGI	273.31	0	0	3	0.04%	1.29%
Spot 10	Amidase	K1QX82_CRAGI	13.46	2	4	5	0.07%	26.10%
Spot 10	Aldehyde dehydrogenase	K1Q9Z4_CRAGI	25.35	6	11	13	0.19%	43.70%
Spot 10	Aldehyde dehydrogenase family 8 member A1	K1QIL8_CRAGI	53.30	7	7	7	0.10%	14.10%
Spot 10	Succinate-semialdehyde dehydrogenase, mitochondrial	K1PV02_CRAGI	74.22	2	2	2	0.03%	3.28%
Spot 10	Alpha-aminoadipic semialdehyde dehydrogenase	K1RNB6_CRAGI	55.22	16	21	21	0.30%	37.20%
Spot 10	Putative methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	K1R252_CRAGI	57.24	3	3	3	0.04%	9.71%
Spot 10	Guanine deaminase	K1QRG3_CRAGI	50.05	7	7	7	0.10%	13.50%
Spot 10	Plasma alpha-L-fucosidase	K1QC19_CRAGI	53.81	1	1	2	0.03%	5.22%
Spot 10	Fascin	K1QEZ3_CRAGI	55.60	14	18	20	0.28%	31.30%
Spot 10	Selenium-binding protein 1 (Fragment)	K1QI40_CRAGI	53.35	22	36	41	0.58%	54.90%
Spot 10	Glutamate decarboxylase-like protein 1	K1P7P6_CRAGI	41.82	2	2	2	0.03%	4.89%
Spot 10	Amidase	K1Q2A1_CRAGI	59.11	3	3	6	0.09%	12.90%

Spot 10	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	16	29	124	1.76%	49.70%
Spot 10	Beta-hexosaminidase subunit beta	K1QHI3_CRAGI	47.22	1	1	10	0.14%	20.30%
Spot 10	Glutathione synthetase	K1P746_CRAGI	62.49	5	5	5	0.07%	9.87%
Spot 10	Protein hu-li tai shao	K1PEX5_CRAGI	85.92	2	2	2	0.03%	2.72%
Spot 10	Dihydropyrimidinase	K1PGY5_CRAGI	71.26	11	12	14	0.20%	24.00%
Spot 10	Filamin-A	K1RZ99_CRAGI	90.77	13	16	16	0.23%	22.90%
Spot 11	Isocitrate dehydrogenase [NADP]	K1R7T2_CRAGI	46.31	4	4	4	0.13%	13.20%
Spot 11	Filamin-C	K1PW06_CRAGI	323.73	5	6	6	0.20%	3.05%
Spot 11	Actin, cytoplasmic	K1RWD4_CRAGI	41.90	1	1	6	0.20%	15.20%
Spot 11	Actin	C4NY64_CRAGI	41.81	1	1	6	0.20%	16.20%
Spot 11	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	1	1	4	0.13%	11.20%
Spot 11	71kDa heat shock connate protein	Q9XZJ2_CRAGI	72.04	0	0	2	0.07%	2.88%
Spot 11	Actin	K1Q0U8_CRAGI	41.76	1	1	10	0.33%	24.20%
Spot 11	Enolase	K1QX37_CRAGI	127.42	4	5	5	0.17%	6.21%
Spot 11	Filamin-A	K1RZ99_CRAGI	90.77	2	2	2	0.07%	4.28%
Spot 11	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	K1RDF6_CRAGI	52.16	3	3	3	0.10%	11.00%
Spot 11	Actin	K1R6J7_CRAGI	41.81	1	1	4	0.13%	10.40%
Spot 11	Actin 2	Q8TA69_CRAGI	41.74	1	3	9	0.30%	16.00%
Spot 11	Phosphoenolpyruvate carboxykinase [GTP]	K1QEA6_CRAGI	71.59	2	2	2	0.07%	4.42%
Spot 11	Aldose 1-epimerase	K1QL49_CRAGI	39.57	2	2	2	0.07%	7.10%
Spot 11	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	1	1	5	0.17%	14.90%

Spot 12	Enolase	K1QX37_CRAGI	127.42	2	2	2	0.07%	2.10%
Spot 12	Actin, cytoplasmic	K1RWD4_CRAGI	41.90	0	0	4	0.14%	12.50%
Spot 12	Apoptosis-inducing factor 3	K1QFI3_CRAGI	97.02	2	2	2	0.07%	2.36%
Spot 12	Actin 2	Q8TA69_CRAGI	41.74	2	3	5	0.18%	12.80%
Spot 12	Isocitrate dehydrogenase [NADP]	K1R7T2_CRAGI	46.31	2	2	2	0.07%	5.61%
Spot 12	Lysosomal aspartic protease	K1P5Z3_CRAGI	47.64	4	4	4	0.14%	13.70%
Spot 12	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	K1RDF6_CRAGI	52.16	4	4	4	0.14%	13.80%
Spot 12	Actin-1/3	K1RBG6_CRAGI	59.45	1	1	2	0.07%	6.42%
Spot 13	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	1	1	2	0.08%	5.86%
Spot 13	71kDa heat shock connate protein	Q9XZJ2_CRAGI	72.04	0	0	2	0.08%	3.19%
Spot 13	Multifunctional protein ADE2	K1RJ97_CRAGI	46.77	2	2	2	0.08%	4.74%
Spot 13	Actin, cytoplasmic	K1RWD4_CRAGI	41.90	0	0	9	0.34%	20.20%
Spot 13	Apoptosis-inducing factor 3	K1QFI3_CRAGI	97.02	2	2	2	0.08%	4.16%
Spot 13	Actin 2	Q8TA69_CRAGI	41.74	1	1	6	0.23%	18.10%
Spot 13	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	K1RDF6_CRAGI	52.16	3	3	3	0.11%	8.41%
Spot 13	Isocitrate dehydrogenase [NADP]	K1R7T2_CRAGI	46.31	2	2	2	0.08%	7.07%
Spot 14	Citrate synthase	K1RM80_CRAGI	49.44	3	3	3	0.11%	8.66%
Spot 14	Phosphoglycerate kinase	K1QCC1_CRAGI	43.03	3	3	3	0.11%	12.80%

Spot 14	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	3	4	8	0.30%	19.60%
Spot 14	Obg-like ATPase 1	K1R8Y1_CRAGI	46.48	2	2	2	0.07%	4.87%
Spot 14	Multifunctional protein ADE2	K1RJ97_CRAGI	46.77	3	5	9	0.34%	8.77%
Spot 14	Enolase	K1QX37_CRAGI	127.42	2	2	2	0.07%	2.53%
Spot 14	Actin-1/3	K1RBG6_CRAGI	59.45	2	2	2	0.07%	7.74%
Spot 14	Phosphoenolpyruvate carboxykinase [GTP]	K1QEA6_CRAGI	71.59	3	3	3	0.11%	7.10%
Spot 14	Ornithine aminotransferase, mitochondrial	K1Q1I3_CRAGI	47.45	2	3	3	0.11%	5.09%
Spot 15	Malate dehydrogenase (Fragment)	K1PU26_CRAGI	36.27	4	4	4	0.07%	13.30%
Spot 15	26S proteasome non-ATPase regulatory subunit 9	K1RB21_CRAGI	21.93	4	4	4	0.07%	23.10%
Spot 15	Transketolase-like protein 2	K1RBC9_CRAGI	74.87	2	2	2	0.03%	2.30%
Spot 15	Adenosylhomocysteinase	K1RW85_CRAGI	47.50	6	6	6	0.10%	10.60%
Spot 15	ES1-like protein, mitochondrial	K1RPZ2_CRAGI	99.96	2	2	2	0.03%	2.55%
Spot 15	Malate dehydrogenase, mitochondrial	K1R4Z3_CRAGI	29.84	1	1	2	0.03%	8.57%
Spot 15	Non-selenium glutathione peroxidase	A7M7T7_CRAGI	24.45	13	15	15	0.25%	42.50%
Spot 15	Carbonyl reductase [NADPH] 1	K1RCR8_CRAGI	30.58	2	2	4	0.07%	16.50%
Spot 15	Cathepsin B	K1QS40_CRAGI	37.69	3	4	4	0.07%	10.90%

Spot 15	Phosphoglucomutase-1	K1PQD4_CRAGI	64.80	0	0	3	0.05%	5.40%
Spot 15	Calcyclin-binding protein	K1Q337_CRAGI	26.04	5	5	5	0.08%	17.90%
Spot 15	T-complex protein 1 subunit theta	K1R0S3_CRAGI	83.14	2	2	2	0.03%	2.49%
Spot 15	Alpha-actinin, sarcomeric	K1RH58_CRAGI	102.20	2	2	2	0.03%	2.50%
Spot 15	D-erythrulose reductase	K1RLT0_CRAGI	25.95	5	5	5	0.08%	23.00%
Spot 15	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	0	0	6	0.10%	14.90%
Spot 15	Aldehyde dehydrogenase, mitochondrial	K1QNT7_CRAGI	56.97	2	2	2	0.03%	4.24%
Spot 15	Translin	K1Q888_CRAGI	26.57	3	3	3	0.05%	11.70%
Spot 15	Dihydropteridine reductase	K1PFL3_CRAGI	24.87	3	3	3	0.05%	16.00%
Spot 15	60S ribosomal protein L4	K1P8W6_CRAGI	43.11	3	3	3	0.05%	9.66%
Spot 15	Filamin-C	K1PW06_CRAGI	323.73	7	7	7	0.12%	2.72%
Spot 15	Multifunctional protein ADE2	K1RJ97_CRAGI	46.77	2	2	2	0.03%	4.50%
Spot 15	Natterin-3	K1QRB6_CRAGI	15.52	3	5	5	0.08%	19.60%
Spot 15	Methylmalonyl-CoA mutase, mitochondrial	K1QE55_CRAGI	81.73	2	2	2	0.03%	2.97%
Spot 15	Uncharacterized protein	K1QFW9_CRAGI	94.86	3	4	4	0.07%	3.64%
Spot 15	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	1	1	5	0.08%	10.10%
Spot 15	Periostin	K1QVV5_CRAGI, K1R2K2_CRAGI	30.39	2	2	2	0.03%	6.94%
Spot 15	Phosphoenolpyruvate carboxykinase [GTP]	K1QEA6_CRAGI	71.59	2	2	2	0.03%	2.52%

Spot 15	Prolyl 4-hydroxylase subunit alpha-1	K1QQ59_CRAGI	71.18	3	3	3	0.05%	4.54%
Spot 15	Triosephosphate isomerase	K1PJ59_CRAGI	18.67	4	6	6	0.10%	29.40%
Spot 15	Arginine kinase	K1PLF9_CRAGI	39.63	3	3	3	0.05%	7.43%
Spot 15	Vitellogenin-6	K1QNA2_CRAGI	273.31	2	2	6	0.10%	2.20%
Spot 15	60S ribosomal protein L6	K1QW36_CRAGI	25.95	3	3	3	0.05%	12.30%
Spot 15	Peroxiredoxin-1	K1Q615_CRAGI	28.85	2	2	5	0.08%	12.00%
Spot 15	3-oxoacyl-[acyl-carrier-protein] reductase	K1RIS2_CRAGI	28.00	3	3	3	0.05%	12.40%
Spot 15	Fibrinolytic enzyme, isozyme C	K1PH66_CRAGI	74.60	2	2	2	0.03%	3.02%
Spot 15	StAR-related lipid transfer protein 5	K1PIK9_CRAGI	28.21	2	2	2	0.03%	7.32%
Spot 15	Transgelin	K1R1X5_CRAGI	43.38	2	2	2	0.03%	6.14%
Spot 15	Glutathione S-transferase A	K1QJ85_CRAGI	23.06	1	1	4	0.07%	19.00%
Spot 15	Fructose-bisphosphate aldolase	K1R8R6_CRAGI	43.48	0	0	4	0.07%	9.55%
Spot 15	Uncharacterized protein C11D3.13	K1PH24_CRAGI	39.86	3	3	3	0.05%	9.89%
Spot 15	Triosephosphate isomerase	K1PCV6_CRAGI	11.53	2	2	2	0.03%	17.80%
Spot 15	Kyphoscoliosis peptidase	K1RL06_CRAGI	24.03	3	3	3	0.05%	15.80%
Spot 15	Guanine nucleotide-binding protein subunit beta-2-like 1	K1RV41_CRAGI	35.01	3	3	3	0.05%	9.15%
Spot 15	Fascin	K1QEZ3_CRAGI	55.60	2	2	2	0.03%	5.81%
Spot 15	Cathepsin F	K1QYP7_CRAGI	78.59	4	4	4	0.07%	6.43%

Spot 15	Glyceraldehyde-3-phosphate dehydrogenase	K1Q350_CRAGI	36.08	6	6	6	0.10%	17.60%
Spot 15	Carbonic anhydrase 2	K1QSG0_CRAGI	36.53	5	7	11	0.19%	14.40%
Spot 15	Prostaglandin reductase 1	K1RGE2_CRAGI	36.01	2	2	2	0.03%	7.01%
Spot 15	Kyphoscoliosis peptidase	K1PEZ6_CRAGI	32.96	2	2	2	0.03%	7.88%
Spot 15	Putative phosphoglycerate mutase	K1QBL3_CRAGI	28.58	2	2	2	0.03%	8.40%
Spot 15	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	5	5	10	0.17%	13.10%
Spot 15	Eukaryotic peptide chain release factor GTP-binding subunit ERF3B	K1RCY7_CRAGI	61.96	2	2	2	0.03%	3.24%
Spot 15	Protein-L-isoaspartate O-methyltransferase	K1R7V8_CRAGI	27.27	2	3	3	0.05%	7.57%
Spot 15	Uncharacterized protein (Fragment)	K1QSS1_CRAGI	39.32	2	2	2	0.03%	5.03%
Spot 15	Elongation factor 1-alpha	K1QGS8_CRAGI,Q75W48_CRAGI	50.47	6	6	6	0.10%	13.20%
Spot 15	GTP-binding nuclear protein	K1R5V4_CRAGI	24.12	5	5	5	0.08%	20.20%
Spot 15	Proteasome subunit alpha type	K1R008_CRAGI	28.00	5	6	6	0.10%	17.30%
Spot 15	Deoxycytidylate deaminase	K1PIP9_CRAGI	18.86	5	5	5	0.08%	27.80%
Spot 15	Adenylyl cyclase-associated protein	K1QI97_CRAGI	67.29	4	4	4	0.07%	6.63%

Spot 16	Malate dehydrogenase (Fragment)	K1PU26_CRAGI	36.27	2	2	2	0.03%	5.44%
Spot 16	Transketolase-like protein 2	K1RBC9_CRAGI	74.87	2	2	2	0.03%	3.74%
Spot 16	Cathepsin L	K1RCD5_CRAGI	36.55	1	1	7	0.11%	26.40%
Spot 16	Malate dehydrogenase, mitochondrial	K1R4Z3_CRAGI	29.84	3	3	3	0.05%	12.10%
Spot 16	Non-selenium glutathione peroxidase	A7M7T7_CRAGI	24.45	8	9	9	0.14%	37.60%
Spot 16	T-complex protein 1 subunit beta	K1R294_CRAGI	57.49	2	2	2	0.03%	4.35%
Spot 16	Carbonyl reductase [NADPH] 1	K1RCR8_CRAGI	30.58	2	2	3	0.05%	13.30%
Spot 16	Acetyltransferase component of pyruvate dehydrogenase complex	K1R8I8_CRAGI	52.06	4	4	4	0.06%	10.30%
Spot 16	Cathepsin B	K1QS40_CRAGI	37.69	4	5	5	0.08%	14.40%
Spot 16	Calcyclin-binding protein	K1Q337_CRAGI	26.04	2	2	2	0.03%	10.30%
Spot 16	Annexin	K1RAI3_CRAGI	36.30	1	1	2	0.03%	5.88%
Spot 16	Omega-crystallin	K1QUX5_CRAGI	19.69	3	3	5	0.08%	21.90%
Spot 16	T-complex protein 1 subunit alpha	K1RAJ1_CRAGI	75.32	2	2	2	0.03%	2.86%
Spot 16	Putative aminopeptidase W07G4.4	K1PVV6_CRAGI	55.71	0	0	4	0.06%	8.69%
Spot 16	Carboxypeptidase B	K1RCT9_CRAGI	45.61	3	3	3	0.05%	8.27%
Spot 16	Fascin	K1QEZ3_CRAGI	55.60	5	5	5	0.08%	12.80%
Spot 16	Peptidyl-prolyl cis-trans isomerase	K1Q5P7_CRAGI	23.74	4	5	5	0.08%	18.60%
Spot 16	Translin	K1Q888_CRAGI	26.57	5	5	5	0.08%	23.00%

Spot 16	Uncharacterized protein	K1QKU3_CRAGI	24.60	2	2	2	0.03%	13.80%
Spot 16	Dihydroorotase	K1RCK9_CRAGI	47.82	4	4	4	0.06%	11.80%
Spot 16	Dihydropteridine reductase	K1PFL3_CRAGI	24.87	2	2	2	0.03%	7.59%
Spot 16	Uncharacterized protein	K1QHA4_CRAGI	35.38	3	4	4	0.06%	13.30%
Spot 16	T-complex protein 1 subunit gamma	K1R466_CRAGI	63.91	2	2	2	0.03%	3.62%
Spot 16	60S ribosomal protein L4	K1P8W6_CRAGI	43.11	3	3	3	0.05%	9.40%
Spot 16	Filamin-C	K1PW06_CRAGI	323.73	6	6	6	0.09%	2.35%
Spot 16	Multifunctional protein ADE2	K1RJ97_CRAGI	46.77	3	3	3	0.05%	7.58%
Spot 16	Heat shock protein HSP 90-alpha 1	K1PNQ5_CRAGI	83.32	1	1	2	0.03%	2.63%
Spot 16	ES1-like protein, mitochondrial	K1RPZ2_CRAGI	99.96	5	6	6	0.09%	6.64%
Spot 16	Aspartate aminotransferase	K1R2Q9_CRAGI	43.19	0	0	4	0.06%	11.20%
Spot 16	Uncharacterized protein	K1QZX9_CRAGI	50.75	4	4	4	0.06%	9.23%
Spot 16	Uncharacterized protein	K1QFW9_CRAGI	94.86	4	4	5	0.08%	5.88%
Spot 16	Peptide methionine sulfoxide reductase msrA 2	K1RR10_CRAGI	24.82	1	2	3	0.05%	11.80%
Spot 16	3-hydroxyacyl-CoA dehydrogenase type-2	K1QDS1_CRAGI	27.90	4	4	4	0.06%	18.20%
Spot 16	Phosphoenolpyruvate carboxykinase [GTP]	K1QEA6_CRAGI	71.59	4	4	4	0.06%	5.36%
Spot 16	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	1	1	11	0.17%	19.10%
Spot 16	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	1	1	8	0.12%	19.40%

Spot 16	Periostin	K1QVV5_CRAGI, K1R2K2_CRAGI	30.39	5	5	5	0.08%	16.30%
Spot 16	4-hydroxyphenylpyruvate dioxygenase	K1QX22_CRAGI	211.58	2	2	2	0.03%	1.12%
Spot 16	Putative phosphoglycerate mutase	K1QBL3_CRAGI	28.58	7	10	10	0.15%	29.20%
Spot 16	Proteasome subunit alpha type-1	K1PCR9_CRAGI	27.74	2	2	2	0.03%	7.94%
Spot 16	Protein ETHE1, mitochondrial	K1PVX0_CRAGI	27.77	2	2	2	0.03%	10.50%
Spot 16	Adenosylhomocysteinase	K1RW85_CRAGI	47.50	6	6	6	0.09%	10.60%
Spot 16	71kDa heat shock connate protein	Q9XZJ2_CRAGI	72.04	0	0	12	0.18%	16.10%
Spot 16	Guanine nucleotide-binding protein subunit beta-2-like 1	K1RV41_CRAGI	35.01	4	4	4	0.06%	14.20%
Spot 16	Vitellogenin-6	K1QNA2_CRAGI	273.31	1	1	4	0.06%	1.45%
Spot 16	Peroxiredoxin-1	K1Q615_CRAGI	28.85	2	2	3	0.05%	12.00%
Spot 16	3-oxoacyl-[acyl-carrier-protein] reductase	K1RIS2_CRAGI	28.00	11	13	13	0.20%	45.90%
Spot 16	StAR-related lipid transfer protein 5	K1PIK9_CRAGI	28.21	6	6	6	0.09%	18.70%
Spot 16	Fructose-bisphosphate aldolase	K1RTQ6_CRAGI	38.99	1	1	2	0.03%	4.41%
Spot 16	Methylmalonyl-CoA mutase, mitochondrial	K1QE55_CRAGI	81.73	2	2	2	0.03%	2.97%
Spot 16	15-hydroxyprostaglandin dehydrogenase [NAD+]	K1QLT1_CRAGI	27.71	2	2	2	0.03%	6.72%

Spot 16	Transgelin	K1R1X5_CRAGI	43.38	4	4	4	0.06%	12.50%
Spot 16	Glutathione S-transferase A	K1QJ85_CRAGI	23.06	2	2	4	0.06%	19.00%
Spot 16	Dehydrogenase/reductase SDR family member 11	K1RIX4_CRAGI	31.01	6	8	8	0.12%	20.20%
Spot 16	Glutathione S-transferase A	K1R6D4_CRAGI	26.51	1	1	3	0.05%	12.60%
Spot 16	Proteasome subunit alpha type-4	K1R7N8_CRAGI	21.25	4	7	7	0.11%	19.90%
Spot 16	Acyl-protein thioesterase 2	K1QXF9_CRAGI	23.68	2	2	2	0.03%	8.76%
Spot 16	Aldehyde dehydrogenase, mitochondrial	K1QNT7_CRAGI	56.97	3	3	3	0.05%	5.78%
Spot 16	Triosephosphate isomerase	K1PCV6_CRAGI	11.53	3	6	7	0.11%	17.80%
Spot 16	Kyphoscoliosis peptidase	K1RL06_CRAGI	24.03	2	2	2	0.03%	11.60%
Spot 16	Cathepsin F	K1QYP7_CRAGI	78.59	2	2	2	0.03%	2.80%
Spot 16	Carbonic anhydrase 2	K1QSG0_CRAGI	36.53	8	12	19	0.29%	29.40%
Spot 16	Glyceraldehyde-3-phosphate dehydrogenase	K1Q350_CRAGI	36.08	5	5	5	0.08%	15.20%
Spot 16	Glutathione S-transferase P 2	K1PV52_CRAGI, K1RQZ1_CRAGI	27.93	2	2	2	0.03%	9.58%
Spot 16	Selenium-binding protein 1 (Fragment)	K1QI40_CRAGI	53.35	2	2	2	0.03%	4.59%
Spot 16	Cathepsin L	K1Q7M2_CRAGI	36.53	1	1	7	0.11%	26.40%
Spot 16	Kyphoscoliosis peptidase	K1PEZ6_CRAGI	32.96	2	2	2	0.03%	7.88%

Spot 16	Cysteine-rich secretory protein LCCL domain-containing 2	K1RAD3_CRAGI	38.17	2	2	2	0.03%	5.60%
Spot 16	Heterogeneous nuclear ribonucleoprotein L	K1QHI2_CRAGI	60.35	2	2	2	0.03%	4.54%
Spot 16	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	5	5	16	0.24%	20.80%
Spot 16	Elongation factor 1-alpha	K1QGS8_CRAGI,Q75W48_CRAGI	50.47	6	7	7	0.11%	13.20%
Spot 16	GTP-binding nuclear protein	K1R5V4_CRAGI	24.12	3	3	3	0.05%	16.00%
Spot 16	Proteasome subunit alpha type	K1R008_CRAGI	28.00	6	7	7	0.11%	24.30%
Spot 16	Calcium-dependent protein kinase isoform 2	K1QQK6_CRAGI	27.31	5	6	6	0.09%	20.20%
Spot 16	3-oxoacyl-[acyl-carrier-protein] reductase	K1QNK4_CRAGI	27.39	2	2	3	0.05%	14.00%
Spot 16	Triosephosphate isomerase	K1PJ59_CRAGI	18.67	8	13	18	0.27%	49.40%
Spot 16	Filamin-A	K1RZ99_CRAGI	90.77	3	3	3	0.05%	4.16%
Spot 16	Adenylyl cyclase-associated protein	K1QI97_CRAGI	67.29	6	7	7	0.11%	9.71%
Spot 17	Malate dehydrogenase (Fragment)	K1PU26_CRAGI	36.27	5	5	5	0.10%	14.80%
Spot 17	Non-selenium glutathione peroxidase	A7M7T7_CRAGI	24.45	13	14	16	0.31%	42.10%
Spot 17	Citrate synthase	K1RM80_CRAGI	49.44	2	2	2	0.04%	4.78%

Spot 17	Glyceraldehyde-3-phosphate dehydrogenase	K1Q350_CRAGI	36.08	5	5	6	0.11%	14.90%
Spot 17	D-erythrulose reductase	K1RLT0_CRAGI	25.95	3	3	3	0.06%	11.50%
Spot 17	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	0	0	2	0.04%	5.18%
Spot 17	Filamin-C	K1PW06_CRAGI	323.73	3	3	3	0.06%	1.26%
Spot 17	S-(hydroxymethyl)glutathione dehydrogenase	K1QKF8_CRAGI	39.73	2	2	2	0.04%	4.83%
Spot 17	Aspartate aminotransferase	K1R2Q9_CRAGI	43.19	0	0	3	0.06%	9.67%
Spot 17	Methylmalonyl-CoA mutase, mitochondrial	K1QE55_CRAGI	81.73	2	2	2	0.04%	2.97%
Spot 17	Uncharacterized protein	K1QFW9_CRAGI	94.86	3	3	4	0.08%	4.82%
Spot 17	S-methyl-5'-thioadenosine phosphorylase	K1R867_CRAGI	62.75	2	2	2	0.04%	3.35%
Spot 17	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	1	1	4	0.08%	7.51%
Spot 17	Electron transfer flavoprotein subunit beta	K1PB82_CRAGI	28.02	2	2	2	0.04%	7.06%
Spot 17	Uncharacterized protein (Fragment)	K1QSS1_CRAGI	39.32	3	4	4	0.08%	8.94%
Spot 17	Adenosylhomocysteinase	K1RW85_CRAGI	47.50	2	2	2	0.04%	4.16%
Spot 17	Triosephosphate isomerase	K1PJ59_CRAGI	18.67	2	3	3	0.06%	14.10%
Spot 17	Vitellogenin-6	K1QNA2_CRAGI	273.31	3	3	4	0.08%	1.66%

Spot 17	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	K1PLM3_CRAGI	33.67	2	2	2	0.04%	7.12%
Spot 17	60S ribosomal protein L6	K1QW36_CRAGI	25.95	2	2	2	0.04%	7.89%
Spot 17	Fructose-bisphosphate aldolase	K1RTQ6_CRAGI	38.99	1	1	2	0.04%	4.41%
Spot 17	Ornithine aminotransferase, mitochondrial	K1Q1I3_CRAGI	47.45	2	2	2	0.04%	6.94%
Spot 17	Fructose-bisphosphate aldolase	K1R8R6_CRAGI	43.48	0	0	3	0.06%	6.28%
Spot 17	Acyl-protein thioesterase 2	K1QXF9_CRAGI	23.68	2	2	2	0.04%	8.76%
Spot 17	Uncharacterized protein C11D3.13	K1PH24_CRAGI	39.86	3	3	3	0.06%	9.89%
Spot 17	Carbonic anhydrase 2	K1QSG0_CRAGI	36.53	7	8	10	0.19%	21.70%
Spot 17	Cathepsin F	K1QYP7_CRAGI	78.59	2	2	2	0.04%	2.80%
Spot 17	Cystathionine gamma-lyase	K1R7F6_CRAGI	46.93	2	2	2	0.04%	4.24%
Spot 17	Uncharacterized protein	K1QPJ5_CRAGI	21.68	2	2	2	0.04%	5.76%
Spot 17	Putative phosphoglycerate mutase	K1QBL3_CRAGI	28.58	2	2	2	0.04%	8.40%
Spot 17	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	5	5	8	0.15%	10.80%
Spot 17	Elongation factor 1-alpha	K1QGS8_CRAGI,Q75W48_CRAGI	50.47	3	3	3	0.06%	6.06%
Spot 17	GTP-binding nuclear protein	K1R5V4_CRAGI	24.12	4	4	5	0.10%	19.70%

Spot 17	Proteasome subunit alpha type	K1R008_CRAGI	28.00	4	4	4	0.08%	12.50%
Spot 17	Deoxycytidylate deaminase	K1PIP9_CRAGI	18.86	2	2	2	0.04%	10.10%
Spot 17	Kyphoscoliosis peptidase	K1RL06_CRAGI	24.03	3	3	3	0.06%	15.80%
Spot 17	Puromycin-sensitive aminopeptidase	K1R866_CRAGI	116.13	4	4	4	0.08%	2.91%
Spot 17	Adenylyl cyclase-associated protein	K1QI97_CRAGI	67.29	4	4	4	0.08%	5.66%
Spot 18	Malate dehydrogenase (Fragment)	K1PU26_CRAGI	36.27	4	4	4	0.07%	13.30%
Spot 18	Adenosylhomocysteinase	K1RW85_CRAGI	47.50	2	2	2	0.04%	4.16%
Spot 18	Malate dehydrogenase, mitochondrial	K1R4Z3_CRAGI	29.84	2	2	3	0.05%	11.80%
Spot 18	Non-selenium glutathione peroxidase	A7M7T7_CRAGI	24.45	9	10	10	0.18%	37.60%
Spot 18	Carbonyl reductase [NADPH] 1	K1RCR8_CRAGI	30.58	3	3	4	0.07%	17.60%
Spot 18	Cathepsin B	K1QS40_CRAGI	37.69	3	4	4	0.07%	12.00%
Spot 18	PDZ and LIM domain protein 5	K1PQ23_CRAGI	55.25	3	3	3	0.05%	6.32%
Spot 18	D-erythrulose reductase	K1RLT0_CRAGI	25.95	4	4	4	0.07%	18.00%
Spot 18	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	1	1	4	0.07%	9.68%
Spot 18	Dihydropteridine reductase	K1PFL3_CRAGI	24.87	2	2	2	0.04%	8.44%
Spot 18	Filamin-C	K1PW06_CRAGI	323.73	3	3	3	0.05%	1.26%
Spot 18	Uncharacterized protein	K1PF62_CRAGI	40.43	2	2	2	0.04%	7.28%

Spot 18	S-(hydroxymethyl)glutathione dehydrogenase	K1QKF8_CRAGI	39.73	2	2	2	0.04%	4.83%
Spot 18	Uncharacterized protein	K1QFW9_CRAGI	94.86	3	3	3	0.05%	3.64%
Spot 18	3-hydroxyacyl-CoA dehydrogenase type-2	K1QDS1_CRAGI	27.90	2	2	2	0.04%	9.30%
Spot 18	S-methyl-5'-thioadenosine phosphorylase	K1R867_CRAGI	62.75	2	2	2	0.04%	3.35%
Spot 18	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	1	1	6	0.11%	11.80%
Spot 18	Protein quaking-B	K1QD80_CRAGI	52.00	2	2	2	0.04%	3.63%
Spot 18	Arginine kinase	K1PLF9_CRAGI	39.63	2	2	2	0.04%	4.57%
Spot 18	Vitellogenin-6	K1QNA2_CRAGI	273.31	1	1	3	0.05%	1.16%
Spot 18	Fructose-bisphosphate aldolase	K1RTQ6_CRAGI	38.99	1	1	3	0.05%	7.44%
Spot 18	Fructose-bisphosphate aldolase	K1R8R6_CRAGI	43.48	0	0	4	0.07%	9.05%
Spot 18	Acyl-protein thioesterase 2	K1QXF9_CRAGI	23.68	2	2	2	0.04%	8.76%
Spot 18	Kyphoscoliosis peptidase	K1RL06_CRAGI	24.03	5	5	5	0.09%	27.90%
Spot 18	Uncharacterized protein	K1QPJ5_CRAGI	21.68	2	2	2	0.04%	5.76%
Spot 18	Glyceraldehyde-3-phosphate dehydrogenase	K1Q350_CRAGI	36.08	4	4	4	0.07%	12.50%
Spot 18	Carbonic anhydrase 2	K1QSG0_CRAGI	36.53	5	6	10	0.18%	14.40%
Spot 18	Putative phosphoglycerate mutase	K1QBL3_CRAGI	28.58	2	2	2	0.04%	8.40%

Spot 18	AP-3 complex subunit beta	K1QJN8_CRAGI	119.85	2	2	2	0.04%	1.76%
Spot 18	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	5	5	10	0.18%	14.50%
Spot 18	Uncharacterized protein (Fragment)	K1QSS1_CRAGI	39.32	4	5	5	0.09%	8.94%
Spot 18	Elongation factor 1-alpha	K1QGS8_CRAGI,Q75W48_CRAGI	50.47	5	5	5	0.09%	11.00%
Spot 18	GTP-binding nuclear protein	K1R5V4_CRAGI	24.12	3	3	3	0.05%	13.60%
Spot 18	Deoxycytidylate deaminase	K1PIP9_CRAGI	18.86	3	3	3	0.05%	15.40%
Spot 18	Triosephosphate isomerase	K1PJ59_CRAGI	18.67	3	4	4	0.07%	21.80%
Spot 18	Filamin-A	K1RZ99_CRAGI	90.77	2	2	2	0.04%	2.73%
Spot 18	Adenylyl cyclase-associated protein	K1QI97_CRAGI	67.29	5	5	5	0.09%	7.93%
Spot 19	Malate dehydrogenase (Fragment)	K1PU26_CRAGI	36.27	4	4	4	0.06%	13.00%
Spot 19	Transketolase-like protein 2	K1RBC9_CRAGI	74.87	2	2	2	0.03%	3.74%
Spot 19	Malate dehydrogenase, mitochondrial	K1R4Z3_CRAGI	29.84	6	10	13	0.19%	34.60%
Spot 19	Glyoxylate reductase/hydroxypyruvate reductase	K1P6I1_CRAGI	35.02	3	3	3	0.04%	8.05%
Spot 19	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	0	0	3	0.04%	7.43%
Spot 19	Filamin-C	K1PW06_CRAGI	323.73	6	6	6	0.09%	2.32%
Spot 19	Arginine kinase	K1PLF9_CRAGI	39.63	4	4	4	0.06%	12.60%

Spot 19	Vitellogenin-6	K1QNA2_CRAGI	273.31	8	8	14	0.21%	5.72%
Spot 19	Uncharacterized protein	K1R512_CRAGI	121.72	2	2	2	0.03%	2.13%
Spot 19	Fructose-bisphosphate aldolase	K1RTQ6_CRAGI	38.99	5	6	8	0.12%	24.20%
Spot 19	Ornithine aminotransferase, mitochondrial	K1Q1I3_CRAGI	47.45	2	2	2	0.03%	6.94%
Spot 19	Vitellogenin	Q8IU34_CRAGI	179.21	1	2	8	0.12%	4.49%
Spot 19	Glyceraldehyde-3-phosphate dehydrogenase	K1Q350_CRAGI	36.08	17	35	58	0.87%	62.40%
Spot 19	3-hydroxyisobutyrate dehydrogenase	K1PR93_CRAGI	42.83	6	7	7	0.10%	17.30%
Spot 19	Prostaglandin reductase 1	K1RGE2_CRAGI	36.01	2	2	2	0.03%	7.01%
Spot 19	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	4	4	7	0.10%	12.60%
Spot 19	Elongation factor 1-alpha	K1QGS8_CRAGI,Q75W48_CRAGI	50.47	4	4	4	0.06%	8.87%
Spot 19	Fructose-bisphosphate aldolase	K1R8R6_CRAGI	43.48	4	4	8	0.12%	22.60%
Spot 20	Transketolase-like protein 2	K1RBC9_CRAGI	74.87	3	3	3	0.05%	4.89%
Spot 20	Malate dehydrogenase, mitochondrial	K1R4Z3_CRAGI	29.84	6	7	9	0.15%	31.40%
Spot 20	Glyoxylate reductase/hydroxypyruvate reductase	K1P6I1_CRAGI	35.02	7	8	8	0.13%	19.50%

Spot 20	Ribosomal protein L24 (Fragment)	Q70MN8_CRAGI	18.10	2	2	2	0.03%	10.80%
Spot 20	Uncharacterized protein	K1R512_CRAGI	121.72	2	2	2	0.03%	2.13%
Spot 20	Aspartate aminotransferase	K1R2Q9_CRAGI	43.19	2	2	4	0.06%	10.90%
Spot 20	Filamin-C	K1PW06_CRAGI	323.73	8	8	8	0.13%	3.12%
Spot 20	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	1	1	3	0.05%	7.30%
Spot 20	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	0	0	2	0.03%	5.18%
Spot 20	Vitellogenin-6	K1QNA2_CRAGI	273.31	7	7	19	0.31%	7.80%
Spot 20	Fructose-bisphosphate aldolase	K1RTQ6_CRAGI	38.99	4	4	7	0.11%	17.90%
Spot 20	Vitellogenin	Q8IU34_CRAGI	179.21	2	2	14	0.23%	9.03%
Spot 20	Fructose-bisphosphate aldolase	K1R8R6_CRAGI	43.48	1	1	3	0.05%	8.04%
Spot 20	Glyceraldehyde-3-phosphate dehydrogenase	K1Q350_CRAGI	36.08	12	22	35	0.56%	41.50%
Spot 20	3-hydroxyisobutyrate dehydrogenase	K1PR93_CRAGI	42.83	5	5	5	0.08%	13.70%
Spot 20	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	3	3	6	0.10%	9.27%
Spot 20	Elongation factor 1-alpha	K1QGS8_CRAGI,Q75W48_CRAGI	50.47	5	5	5	0.08%	11.50%
Spot 21	Transketolase-like protein 2	K1RBC9_CRAGI	74.87	3	4	4	0.06%	5.04%
Spot 21	71kDa heat shock connate protein	Q9XZJ2_CRAGI	72.04	0	0	9	0.14%	16.50%
Spot 21	Transgelin	K1R1X5_CRAGI	43.38	2	2	2	0.03%	4.86%

Spot 21	Aspartate aminotransferase, mitochondrial	K1R083_CRAGI	32.52	1	1	13	0.20%	24.90%
Spot 21	ATP synthase subunit alpha	K1R6Z7_CRAGI	59.87	5	5	5	0.07%	10.10%
Spot 21	Isocitrate dehydrogenase [NADP]	K1RZE2_CRAGI	50.26	1	1	3	0.04%	8.33%
Spot 21	Filamin-C	K1PW06_CRAGI	323.73	7	7	7	0.11%	2.59%
Spot 21	Aspartate aminotransferase	K1R2Q9_CRAGI	43.19	4	4	16	0.24%	26.00%
Spot 21	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	1	1	4	0.06%	9.74%
Spot 21	Protein unc-87	K1R832_CRAGI	56.98	2	2	2	0.03%	4.63%
Spot 21	Arginine kinase	K1PLF9_CRAGI	39.63	3	3	3	0.04%	9.71%
Spot 21	Vitellogenin-6	K1QNA2_CRAGI	273.31	14	17	41	0.61%	14.80%
Spot 21	Protein RCC2	K1R5P6_CRAGI	54.84	2	2	2	0.03%	4.58%
Spot 21	Fructose-bisphosphate aldolase	K1RTQ6_CRAGI	38.99	9	11	13	0.20%	32.80%
Spot 21	Vitellogenin	Q8IU34_CRAGI	179.21	1	2	26	0.39%	14.70%
Spot 21	Succinate-semialdehyde dehydrogenase, mitochondrial	K1PV02_CRAGI	74.22	3	3	3	0.04%	4.70%
Spot 21	Fructose-bisphosphate aldolase	K1R8R6_CRAGI	43.48	6	7	12	0.18%	24.10%
Spot 21	Uncharacterized protein	K1R512_CRAGI	121.72	9	11	12	0.18%	8.91%
Spot 21	Cystathionine gamma-lyase	K1R7F6_CRAGI	46.93	3	3	3	0.04%	6.59%
Spot 21	Glyceraldehyde-3-phosphate dehydrogenase	K1Q350_CRAGI	36.08	2	2	2	0.03%	4.78%

Spot 21	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	K1RGD1_CRAGI	63.68	4	4	4	0.06%	8.10%
Spot 21	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	2	2	5	0.07%	9.09%
Spot 21	Elongation factor 1-alpha	K1QGS8_CRAGI,Q75W48_CRAGI	50.47	5	5	6	0.09%	12.60%
Spot 21	UTP--glucose-1-phosphate uridylyltransferase	K1Q1D1_CRAGI	57.23	4	4	4	0.06%	6.88%
Spot 22	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	1	1	3	0.10%	7.34%
Spot 22	Fructose-bisphosphate aldolase	K1R8R6_CRAGI	43.48	4	7	9	0.30%	33.90%
Spot 22	Arginine kinase	K1PLF9_CRAGI	39.63	5	5	5	0.16%	26.90%
Spot 22	4-hydroxyphenylpyruvate dioxygenase	K1QX22_CRAGI	211.58	2	2	2	0.07%	1.34%

Supplementary Table 3 Proteins identified in the IgE-reactive spots of the heated extract of Pacific Oyster.

Spot No.	Protein name	Protein accession numbers	Molecular weight (Da)	Exclusive unique peptide count	Exclusive unique spectrum count	Total spectrum count	Percentage of total spectra	Percentage sequence coverage
Spot 23	Non-neuronal cytoplasmic intermediate filament protein	K1PBC0_CRAGI	69.29	9	9	9	0.14%	13.90%
Spot 23	Splicing factor 3B subunit 2	K1QGF1_CRAGI	119.73	2	2	2	0.03%	2.14%
Spot 23	Calreticulin	K1PXS8_CRAGI	39.12	0	0	11	0.17%	30.70%
Spot 23	Filamin-C	K1PW06_CRAGI	323.73	7	7	7	0.11%	2.85%
Spot 23	Integrin alpha-8	K1PAJ8_CRAGI	115.38	2	2	2	0.03%	1.56%
Spot 23	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	1	1	5	0.08%	10.30%
Spot 23	Myosin heavy chain, striated muscle	K1R1B3_CRAGI	79.95	6	6	7	0.11%	9.55%
Spot 23	Collagen alpha-6(VI) chain	K1QEB9_CRAGI	46.30	2	2	2	0.03%	2.38%
Spot 23	Calreticulin	A5LGG9_CRAGI	48.19	2	2	13	0.20%	33.10%
Spot 23	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	K1RDG2_CRAGI	45.34	3	3	3	0.05%	8.96%
Spot 23	Tropomyosin (Fragment)	Q95WY0_CRAGI	26.87	0	0	44	0.67%	52.40%
Spot 23	Tropomyosin	K1QNV6_CRAGI	39.01	0	0	26	0.40%	23.60%
Spot 23	Carboxylic ester hydrolase	K1S0A7_CRAGI	271.16	3	3	4	0.06%	1.10%
Spot 23	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	2	2	6	0.09%	9.79%
Spot 23	Tropomyosin	B7XC66_CRAGI	33.02	2	4	63	0.96%	52.50%
Spot 24	Severin	K1PE57_CRAGI	37.21	8	11	11	0.16%	28.50%
Spot 24	Troponin T, skeletal muscle	K1QPC9_CRAGI	20.67	3	3	3	0.04%	18.50%

Spot 24	Non-neuronal cytoplasmic intermediate filament protein	K1PBC0_CRAGI	69.29	5	5	5	0.07%	7.77%
Spot 24	Filamin-C	K1PW06_CRAGI	323.73	6	6	6	0.09%	2.49%
Spot 24	Retinal dehydrogenase 1	K1QVG5_CRAGI	53.15	1	1	3	0.04%	7.71%
Spot 24	Myosin heavy chain, striated muscle	K1R1B3_CRAGI	79.95	2	2	2	0.03%	2.81%
Spot 24	Collagen alpha-6(VI) chain	K1QEB9_CRAGI	46.30	1	1	2	0.03%	4.28%
Spot 24	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	K1RDG2_CRAGI	45.34	2	2	2	0.03%	9.44%
Spot 24	Tropomyosin (Fragment)	Q95WY0_CRAGI	26.87	0	0	116	1.66%	65.70%
Spot 24	Tropomyosin	K1QNV6_CRAGI	39.01	0	0	69	0.99%	31.00%
Spot 24	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	1	1	3	0.04%	6.12%
Spot 24	Calumenin	K1PH89_CRAGI	47.66	7	7	7	0.10%	19.00%
Spot 24	Tropomyosin	B7XC66_CRAGI	33.02	2	6	161	2.30%	70.10%
Spot 25	Non-neuronal cytoplasmic intermediate filament protein	K1PBC0_CRAGI	69.29	2	2	2	0.03%	2.75%
Spot 25	Filamin-C	K1PW06_CRAGI	323.73	4	4	4	0.06%	1.53%
Spot 25	Uncharacterized protein	K1R7Q9_CRAGI	19.63	3	3	3	0.05%	18.90%
Spot 25	Tropomyosin	K1QNV6_CRAGI	39.01	2	2	16	0.26%	23.60%
Spot 25	SAP domain-containing ribonucleoprotein	K1QWU5_CRAGI	26.34	2	2	2	0.03%	8.44%
Spot 25	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	2	2	5	0.08%	8.92%
Spot 25	Calcium-dependent protein kinase isoform 2	K1QQK6_CRAGI	27.31	5	6	6	0.10%	15.50%
Spot 26	Myosin regulatory light chain A, smooth adductor muscle	K1Q801_CRAGI	14.62	4	4	4	0.08%	35.90%
Spot 26	Uncharacterized protein	K1R5R9_CRAGI	111.24	2	2	2	0.04%	2.07%

Spot 26	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	0	0	2	0.04%	4.37%
Spot 26	Myosin essential light chain, striated adductor muscle	K1RH05_CRAGI	16.35	1	1	2	0.04%	16.70%
Spot 26	Troponin C	K1QFK6_CRAGI	25.65	3	3	5	0.10%	16.00%
Spot 27	Troponin T, skeletal muscle	K1QPC9_CRAGI	20.67	3	3	3	0.04%	18.50%
Spot 27	Tropomyosin	K1QNV6_CRAGI	39.01	0	0	18	0.26%	20.90%
Spot 27	Retinal dehydrogenase 1	K1R266_CRAGI	62.12	2	2	3	0.04%	4.90%
Spot 27	Glutathione synthetase	K1P746_CRAGI	62.49	2	2	2	0.03%	2.87%