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# Edible Insects as a Source of Food Allergens

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EDIBLE INSECTS AS A SOURCE OF FOOD ALLERGENS

by

Lee Palmer

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

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Under the Supervision of Professors Philip E. Johnson and Michael G. Zeece

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## EDIBLE INSECTS AS A SOURCE OF FOOD ALLERGENS

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University of Nebraska, 2016

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Increasing global population increasingly limited by resources has spurred interest in novel food sources. Insects may be an alternative food source in the near future, but consideration of insects as a food requires scrutiny due to risk of allergens. Currently, the insect *Dactylopius coccus*, known as cochineal, is used to produce carmine, a natural red pigment used in food, which has caused allergic reactions. This study investigated allergens of cochineal focusing on purification from the pigment. Mass spectrometry identified a previously described major allergen of cochineal and a tropomyosin, although further work is required.

Tropomyosin is a major cross-reactive allergen across invertebrates including insects and shellfish and has multiple isoforms per species of varying function, sequence, and expression. Extractions of diverse insects must be sufficiently representative to be comparable. This study used a mass spectrometry compatible buffer and a zwitterionic-chaotropic buffer with sequential extractions. Both buffers were found to be sufficiently representative via rabbit anti-shrimp tropomyosin IgG. These extractions were used for further immunoblotting with shrimp-allergic sera and sera from subjects with self-reported shellfish allergy or sensitization to shellfish. Tropomyosins were cloned from several samples and their sequences investigated for epitopes and semi-quantitative mass

spectrometry. A pattern of low reactivity was found for several samples not corroborated by quantitative data. Further cloning is necessary to align these data sets.

Resistance to digestion is a common test for potential allergenicity as epitopes may persist after digestion. Use of pepsin is standard, although this may not be as representative as a direct assay of the source food. Simulated gastric pepsinolysis was performed with defatted *Acheta domesticus* cricket powder and immunoblotted against shrimp-allergic sera and rabbit anti-tropomyosin IgG. Patterns of reactivity were similar against non-reduced samples with relatively lower reactivity with allergic sera against reduced samples. The allergic sera was predominantly cross-reactive with tropomyosin with lesser reactivity against reduced forms of cricket tropomyosins.

It was found that insect based foods pose potential risk to shellfish allergic patients due to homologous proteins including tropomyosin.

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# CHAPTER 1

## LITERATURE REVIEW

### 1.1 INTRODUCTION

Insects are a constant part of life predominantly as pests, but in the future they may be widely used as an alternative food source. Consideration of insects as food requires scrutiny due to potential risk of allergens. Allergens are receiving increasing interest as a risk to individual allergic patients but also as a matter of public health. Cross-reactivity among allergens drives concerns about insects as food due to the invertebrate allergen tropomyosin. The following will review food allergy and allergic cross-reactivity, lay out a historical and future basis for the use of insects as food, as well as give basic information about insects.

### 1.2 FOOD ALLERGY

#### 1.2.1 Overview

Food allergy has grown from being significant for allergic individuals to that of public health and safety [1]. Food allergies are caused by allergens, which are innocuous and otherwise benign compounds that elicit immunological responses ranging from mild urticaria to anaphylaxis [1, 2]. Some symptoms caused by food allergens are listed in table 1.1. Severity of symptoms vary per patient age, specific allergen, dose, and route of exposure [2].

In 1995, the Food and Agriculture Organization of the United Nations (FAO) consultation identified eight groups of foods are the most common causes of food allergy:

milk, egg, peanut, tree nuts, wheat, soy, fish, and crustacean shellfish. These are known as the big eight and are now included in regulatory lists for allergens among several countries around the world [3]. Since then, the U.S. passed legislation as of 2006 to require labeling of packaged foods that include any of the big eight. [4]. European legislation has since expanded their legislation to fourteen groups of foods to include sesame, mustard, celery, lupin, mollusks, sulfites, and gluten as opposed to only wheat [5].

There are several risk factors associated with the development of allergy separated into genetic or environmental. Genetic risk factors are grouped within atopy, which is an exaggerated genetic predisposition to mount IgE responses resulting in higher total IgE levels and more susceptible to developing allergic diseases including rhinoconjunctivitis, asthma, or eczema [2]. Environmental risk factors include the route, age, and dose of exposure to the allergen as well as any changes in diet, atmospheric pollution, and specific history of infections [2, 6].

### **1.2.2 Mechanisms**

Typically, exposure to dietary proteins results in induced tolerance to those proteins, however food allergy can occur when that tolerance has failed. IgE-mediated hypersensitivity reactions account for most food allergic reactions [6]. IgE-mediated reactions include both directly-mediated type I immediate hypersensitivity and indirectly-mediated type IV delayed hypersensitivity reactions to otherwise benign environmental antigens. Such reactions begin with a sensitization step for initial antigen-specific IgE production and subsequent exposure results in eliciting symptoms (Figure 1.1) [2].

Immune response during sensitization is driven by two signals: differentiation of naïve T cells into  $T_{H2}$  cells and cytokine and other signals from  $T_{H2}$  cells to stimulate B cells to switch to IgE production. Differentiation of T cells is driven by antigen presenting cells presenting a peptide to naïve T cells to result in T cell activation and differentiation into  $T_{H2}$  cells.  $T_{H2}$  cells then begin secreting cytokines, including IL-4 and IL-13, that both encourage continued  $T_{H2}$  differentiation as well as B cell IgE production. Plasma B cells persist to respond to future exposure to antigen and produce IgE long after sensitization, additionally IgE produced can strongly bind to  $Fc\epsilon RI$  on the surface of mast cells and basophils priming them [2].

Upon subsequent exposure to antigen, IgE will bind the antigens and cross-link  $Fc\epsilon RI$  resulting in activation and degranulation. These granules include several mediators including histamine, which causes immediate increases to local blood flow and blood vessel permeability. Nearer the skin, histamine release can result in vasodilation and reddening of the skin seen as a wheal-and-flare reaction. Activated mast cells also produce lipid mediators including leukotrienes and prostaglandins. Associated symptoms of mast cell degranulation can occur in seconds to minutes. Excessive activation of cells including mast and  $T_{H2}$  cells due to prolonged or high antigen dose can result in  $T_{H2}$  type IV hypersensitivity reactions hours after initial exposure [2].

Other forms of allergic reactivity can be seen in type II, III, and IV hypersensitivity reactions as well as in celiac disease. Type II hypersensitivity reactions are due to IgG binding cell surfaces modified with allergen resulting in the destruction of the cell such as in penicillin allergy. Type III reactions result from immune complexes forming with the potential for tissue damage as in serum sickness. Type IV reactions

include those from CD8 cells and  $T_H1$  and function similarly to the type IV  $T_H2$  reaction as a delayed response as in mosquito bites or contact with poison ivy [2]. Of food reactions resulting from non-IgE-mediated mechanisms, celiac disease is the best understood. Celiac disease is characterized by inflammation of the small bowel and atrophy of the villi. T cells and endogenous transglutaminase work in tandem with gluten to result in initial damage compounded by an autoantibody response to complexes of gluten and transglutaminase [7].

### **1.2.3 Diagnosis**

Diagnosing a potential food allergy begins with a medical history to resolve a history of causative foods, age of symptoms, specific symptoms, and time from consumption to symptoms [8]. A medical history is needed to whittle down if there is an allergy, likely mechanism, and severity to choose the best tests to minimize risk to the patient. In this, there are several reliable tests available to effectively diagnose food allergy including skin-prick test (SPT), specific IgE (sIgE), and oral food challenge (OFC) [9].

SPT is a quick and inexpensive means to assess sensitization, but positive results do not conclusively mean food allergy is present [8]. The test is performed by placing a small amount of test material into the subject's epidermis to induce a wheal-and-flare reaction. After 15 minutes, a raised bump and surrounding redness are measured as wheal and flare and compared with positive histamine and negative saline controls. Variations among operators and testing materials results in SPT being useful for high sensitivity but

with low specificity, therefore SPT should not be used as a screening tool due to false-positives leading to unneeded dietary restrictions [10].

Serum sIgE can be measured with several methods including enzyme linked immunosorbent assay (ELISA) capture systems such as ImmunoCAP®. ImmunoCAP® is a cellulose matrix housed in a capsule made specific for an allergenic source by extracting the source and irreversibly binding the allergens to the matrix. The subject's serum is run through the matrix and reactive antibodies will bind with present allergens. After washing, a second antibody bound to an enzymatic indicator which can be used to generate a colorimetric or fluorescent signal to measure the sIgE. Similarly, another system is the ImmunoCAP® Immuno solid-phase allergen chip (ISAC). As opposed to ImmunoCAP®, ISAC measures sIgE to individual allergens rather than whole samples and can measure across 112 allergen components across 46 major allergens simultaneously. Further, both ImmunoCAP® and ISAC values do correlate, but the values generated differ [11]. Levels of sIgE are more comparable between runs than SPT as they are less operator dependent, however alternative means to measure sIgE are not comparable with each other. Levels of sIgE can be used to predict reactions to specific food OFCs [10].

Due to SPT and sIgE testing markers of sensitization, the presence of sIgE or positive results with SPT do not correlate to clinical allergy or reaction severity and so must be understood in context [10]. Although both tests investigate sensitization, the two are not correlated to each other [9]. Fleischer et al investigated how sIgE and SPT correlated with oral food challenge results to assess how predictive these tests were. It

was found that without anaphylaxis these tests were not sufficient to predict allergy and oral challenge may be required to confirm allergy [12].

In cases where clinical history is not indicative of food allergy, OFC is the recommended starting point. OFC is an important means to confirm symptoms and identify causative as a negative reaction to OFC suggests no food allergy, however there is risk of anaphylaxis [8, 9]. As a means of reducing bias that can interfere with diagnosis, double blinded placebo controlled food challenge (DBPCFC) may be employed. [13]. DBPCFC is the highest standard of food allergy diagnosis, however single blinded or open OFC is more common due to greater costs and time investments [10, 13].

As Chafen et al has explained, OFC, SPT, and sIgE each have their roles in diagnosing food allergy but no test has the necessary sensitivity and specificity to be recommended over the other tests [14]. Each of these tests needs to be used along with a detailed clinical history to effectively diagnose food allergy.

#### **1.2.4 Prevalence**

The prevalence of food allergy is quite variable dependent on the country of study, the age of the patients, as well as the standard of diagnosis [15]. A meta-analysis of total prevalence estimated that more than 1-2% but less than 10% of the world population is effected by food allergies [14]. Another review by Sicherer and Sampson suggests that 5% of adults and 8% of children are likely affected by food allergy [9]. Many studies of the prevalence of food allergy rely on surveys and self-reporting of allergy or allergen-specific IgE, which may skew statistics [16].

Gupta et al performed a randomized survey of US households with children to estimate prevalence. It was found that the top 3 allergies were peanut, milk, and shellfish respectively. It was found that allergy varied with race, age, income, and region and further that diagnosis varied per race and income [17]. Liu et al used allergen-specific IgE to estimate clinical food allergy to peanut, milk, egg, and/or shrimp at 2.5% of the total US population with 4.2% in children under 5 [18].

Soller et al performed a telephone survey of Canadian households and estimated food allergy to all food to be between 6.58% for adults and 7.12% for children with children being most afflicted with milk, peanut, and tree nut allergies and adults afflicted with shellfish, milk, and fruit allergies. Statistics adjusted for self-reports of convincing history or physician diagnosis peanut was found to affect 1.68% of children and 0.71% of adults, tree nuts were found to affect 1.59% of children and 1.00% of adults, and shellfish was found to affect 0.50% of children and 1.69% of adults [19].

Osborne et al investigated Australian children at a population level using initial skin prick testing and secondary food challenge. SPT positive prevalence of peanut was 8.9%, raw egg white 16.5%, sesame 2.5%, cow's milk 5.6%, and shellfish 0.6%. Challenge proven prevalence was 3.0%, 8.9%, and 0.8% for peanut, raw egg, and sesame. Further it was found that approximately 80% of those with challenge proven egg allergy could tolerate baked egg. Therefore, they found that over 10% of 1 year old infants had challenge proven IgE-mediated food allergy [20].

Reviews of the changes in prevalence of food allergy are conflicted. Chafen et al was unable to find sufficient evidence that the prevalence of food allergies was increasing over three reviewed studies from the United States, Canada, and the United Kingdom

[14]. Prescott et al across a global survey of food allergy patterns found that food allergy appeared to be increasing although there was a lack of quality data [21]. The International Collaboration on Asthma, Allergy, and Immunology suggests that food allergy has increased in past decades based on increasing prevalence of food-induced anaphylaxis in Australia and food challenge in China [22]. Sicherer and Sampson suggested that food allergy data “generally support an increase in prevalence”. Increases of self-reported cases of food allergy could suggest an increase in prevalence, however this could be explained by increases in awareness of food allergy [9].

### **1.2.5 Detection of food allergens**

Methods to identify and quantify food allergens include antibody-based methods, polymerase chain reaction (PCR), and mass spectrometry (MS). Each method has their own strengths and weaknesses, as well as cost, time, and expertise needed (Table 1.2) [23].

Antibody-based methods include lateral flow devices (LFD) and ELISA. Both LFD and ELISA use antibodies raised against either a single target (monoclonal) or multiple targets (polyclonal) to detect allergens, which adds specificity or sensitivity respectively. LFD is a faster method than ELISA and can be used on the manufacturing floor as opposed to the laboratory as the case with ELISA, however LFD cannot be used for multiplexed detection whereas ELISA can be multiplexed [24, 25].

Antibody based methods are used to detect protein antigens and this can be beneficial for targeting clinically relevant targets. Likewise there have been a proliferation of different kits for different allergens, however there are a number of

problems with such methods: not all antibodies for the same targets are exactly the same resulting in differences between kits, a lack of universal standards between kits, protocols across kits are likewise not standardized, antibodies non-specifically binding as well as cross-reactivity, and interference with food matrices confound results to make comparisons between kits difficult and individual results less accurate [24-26].

Despite the flaws of antibody based methods, they are well-recognized and time tested tools. They can be semi-quantitative within the kit's range and are very sensitive. Differences between kit antibodies can be used as a positive to prioritize different targets. Such is the case with specifically targeting a clinically relevant target or any protein from a specific hazardous source [26].

Another method for the detection of allergens is PCR. PCR targets short segments of DNA of a targeted gene from an allergenic source to amplify and identify. Due to this method targeting DNA instead of protein, relevant information from the genome of the target is necessary to effectively select for only the desired target and further because this method only targets DNA it cannot directly correlate to the clinical relevance of results. Further, groups of organisms such as crustaceans are difficult to unilaterally detect with a single method due to number of species and genomic variability. PCR can also have interference due to the food itself and the DNA may also have variable extractabilities resulting in similarly variable results [27].

However, PCR detection methods can be qualitative and quantitative though testing must be done in a specialist laboratory to minimize contamination and is subsequently costlier and more time consuming than antibody-based methods. PCR methods can be developed in-house for specific demands, but there are many methods

already available on the market and such methods do include multiplexed methods from a single DNA extraction [23, 27].

Lastly, MS is the most specific and targeted of the methods. MS is used to directly detect and measure peptides derived from proteins. Sample preparation is done first by extracting the sample, using a protease such as trypsin to generate peptides from the proteins of that sample, and fractionating the peptides using high pressure liquid chromatography while feeding the peptides into the mass spectrometer. In this way, peptides can be identified and quantified using a database associated with the parent proteins. This allows for many proteins even from different sources to be handled simultaneously [23, 28].

A major caveat to the use of an MS is that there are high set up, development, and equipment costs along with the need for expertise to run and interpret the data generated. Further, extraction procedures and amino acid modifications can affect detection and quantification. Lastly, reliance on databases means that targets without previous data will require more investment of time and money to effectively differentiate and quantify [23, 28-32].

### **1.3 ALLERGIC CROSS-REACTIVITY**

The ability of antibodies to function within the body relies on their specificity for their target molecule, their antigen. The DNA specific for each antibody is modified repeatedly per B cell in a series of recombination events resulting in the generation of unique antibodies. The regions that share are in the hypervariable regions that come together to define the specific antigen-binding site called the paratope. An antibody's

paratope is the region that will bind the antigen called the antigen's epitope. Protein epitopes are typically 8-25 amino acids in length and are divided into linear and conformational epitopes. Linear epitopes are single segments of a polypeptide chain and conformational epitopes are determined by multiple polypeptide segments such as protein folds [33-35]. However, epitopes are not unique and can be shared across antigens [2]. Cross-reactivity can be defined as antibodies that bind and react with an antigen other than the one that had caused the formation of the antibody, in other words elicitation of symptoms without prior sensitization to that specific antigen [36]. Because mast cells require crosslinking of FcεRI via IgE, this then stipulates that degranulation of mast cells can only occur when an antigen has at least two epitopes with bound IgE present to result in clinical symptoms [2].

Antibody diversity has pushed more focus onto predicting and understanding how cross-reactivity occurs. Predicting allergenicity of a protein is based from characteristics shared by other allergens such as resistance to heat, acid, or proteolysis, but predicting cross-reactivity also incorporates sequence and structure [37]. Clinical cross-reactivity is increasingly common if the two antigens are at 70% or higher sequence identity [38, 39]. World Health Organization (WHO) guidelines for predicting allergenic cross-reactivity suggest a minimum 35% sequence similarity in 80 amino acids or complete identity within a 8 amino acid peptide [40]. Basic homology studies are a good start; however, it is not nearly the entire picture regarding cross-reactivity. Protein structural homology must also be considered as homology is based from shared function and therefore a shared protein fold. A specific fold can be stabilized and conserved with as little as 35%

sequence similarity [33]. Other structural features that can contribute to cross-reactivity are repeated amino acid motifs and multimeric proteins [37].

Cross-reactivity is common among food allergens [41]. Regarding cross-reactivity between food and non-food allergens, approximately 60% of food allergic reactions arise from cross-reactivity stemming from inhalant reactions [42]. Cross-reactivity between airborne and food allergens may induce food allergy in patients with respiratory allergies and so cross-reactivity may be an underestimated clinical problem. [43]. Most allergens can be grouped into structural families divested from their biological origin [44]. Accordingly, as species are more related there is a higher chance of cross-reactivity and further related species have less homologous proteins. The interrelationship between two suspected cross-reactive proteins as well as their protein structure and homology is important to understanding their potential cross-reactivity. Reactions caused by proteins that were highly conserved evolutionarily along with wide-spread presence are pan-allergens. [33]. Although such pan-allergens contain homologous epitopes across species, discovering clinical cross-reactivity to correlate with *in vitro* cross-reactivity can be difficult [45]. Several pan-allergens have been identified such as tropomyosin, arginine kinase, profilin, lipid transfer protein, and chitinase [39, 41, 45].

Of the pan-allergens, tropomyosin is a particularly pervasive and diverse protein. Tropomyosin is found across all of Animalia as part of the conserved mechanism for muscle contraction. In muscle, tropomyosin filaments function in concert with troponin to mediate the interactions of myosin and actin where calcium is the trigger for contraction. A signal-dependent increase in the concentration of calcium to 5  $\mu\text{M}$  is sufficient to bind to troponin causing a structural shift in the tropomyosin-troponin

complex such that thick and thin filament interaction is no longer blocked and contraction can occur [46, 47]. Structurally, tropomyosin is a dimeric coiled-coil weighing around 35-38 kDa that tends to align head to tail allowing for tropomyosin to better wrap around the actin light chain as well as perform other functions [41, 48]. The amino acid sequence of tropomyosins is periodic and forms seven amino acid repeats [49]. Tropomyosin has multiple isoforms in invertebrates and often also coincides with multiple genes. Although different tropomyosin isoforms have been identified to dimerize together, this has not been well investigated [48].

Invertebrate tropomyosin isoforms have been investigated through *Drosophila* to identify two muscle tropomyosin genes with several isoforms generated via alternative splicing of the mRNA as well as different promoter regions [48]. Work specifically on *Drosophila melanogaster* has demonstrated at least 8 isoforms with roughly 47% identity to vertebrate tropomyosins [50]. Other allergenic tropomyosins include those of mites and there has been much focus on mite tropomyosin cross-reactivity. Current consensus is that tropomyosin from mites, mollusks, crustaceans, and cockroaches have been identified as potentially cross-reactive. House dust mites are often a primary source of sensitization to tropomyosin [51]. Vertebrate tropomyosins are spared from the pan-allergenicity of invertebrate forms such that even with a 60% sequence identity between chicken and shrimp tropomyosins, they are not cross-reactive [41].

As a food allergen, tropomyosin is the major allergen for shrimp and a major source of cross-reactivity across mollusks and crustaceans. Specifically, it has been demonstrated to be an allergen across shrimp, lobsters, crabs, snails, whelks, squid,

oysters, and octopus. More than 80% of shrimp allergic patients react to tropomyosin and at least 5 epitopes have been described [41].

#### **1.4 BRIEF HISTORY OF ENTOMOPHAGY**

In ancient times, the consumption of insects as food, entomophagy, was not unheard of and records still exist from across the world. From the fourth century BCE in Greece, Aristotle wrote accounts of when cicadas are best consumed and described how females were best when laden with eggs. In the first century CE from Rome, Pliny the Elder described cossus, which is the larvae of longhorn beetles. Li Shizhen wrote a comprehensive book on medicine and food of the Chinese Ming Dynasty, which included many insects. In 1737 in France, René Antoine Ferchault de Réaumur wrote to point out the incongruence of how the frogs, snakes, and lizards were consumed across France, yet there was a repugnance toward entomophagy [52, 53].

In 1885 in England, Vincent Holt wrote “Why not eat Insects?” where he states that “it is hard... to overcome the feelings that have been instilled into us from our youth” and that “the general abhorrence of insects seems almost to have increased of late years, rather than diminished, owing, no doubt, to the fact of their being no longer familiar as medicines.” A growing taboo from childhood on is described but he goes further, “there is not such a very strong prejudice among the poorer classes against the swallowing of insects” and “there cannot be said to be any really strong objection, among the upper classes, to making any new departure in the direction of foods, if it once becomes the fashion to do so” [54].

Hypotheses to attempt to explain western views range from psychological associations between insects and disease, death, and dirt [55, 56], food contamination [57], as well as neophobia [58]. The impact of the western taboo is such that consumer acceptance has been implicated as the principal obstacle to entomophagy in western cultures [53].

To this end, there has been great focus into understanding how to better incorporate insects into western diets. Due to the lack of current placement of insects as a food, there has been much discussion about how exactly to place insects in the diet. Without a clear need or culinary goal using insects, there is no need to both endure disgust as well as innovative culinary work. Therefore, studies have been done that incorporate insects as a meat substitute with clear understanding that meat has a clear culinary path as well as specific sensory properties that are expected to be maintained when substituted [59, 60].

An example of entomophagy in the west comes from the Italian island of Sardinia where casu marzu is produced from sheep's milk pecorino cheese after it has been allowed to be infested by *Piophilidae casei*, the cheese fly. The cheese flies are lured into the fermenting cheese where they lay their eggs to hatch into hundreds of maggots that continue to break down the cheese with enzymes resulting in a soft gooey cheese. The cheese is considered edible only whilst the maggots are alive. The Sardinian government has since banned the product for allergic and parasitic concerns [61].

A further example of indirect entomophagy is carmine dye derived from the bodies of crushed female cochineal, *Dactylopius coccus*. Cochineal are insects native to South and Central America with a majority of production from Peru and the Canary

Islands. Cochineal produces carminic acid as 17-24% of female bodyweight and is processed by crushing, extraction, and mixing with aluminum or calcium salts to make carmine dye. Carmine has been historically used as a dye for textiles but has also found use for cosmetics and some foods such as processed meats, preserves, alcoholic beverages, yogurt, and baked goods as a substitute for beetroot [62, 63]. Regulations initially did not require carmine to be specifically labeled on products, but concerns about unintentional consumption and allergy led to current U.S. Food and Drug Administration (FDA) regulations requiring carmine or cochineal extracts to be listed by name on the label [4, 63].

Entomophagy is currently a globalizing phenomenon where 2 billion people are estimated to eat insects regularly for taste and nutrition, and 1,600 species of insects are documented as consumed by humans. Insects were a traditional source of the human diet in almost 100 countries of the world clustered in Asia, Africa, and South America [53, 64, 65]. To an outside observer it may seem that entomophagy is practiced when there is no alternative but in Botswana, South Africa, and Zimbabwe husbandry co-exists with entomophagy [66]. It can also be a misconception that those who practice entomophagy are indiscriminate in their views of edible insects, however Thai consumers are picky about their insects similar to Western consumers [59].

Thailand is a leading focus regarding entomophagy. Consumption of insects has increased in Thailand over the years and continues to grow. Entomophagy moved from the realm of the poor and rural to include the rich and the urban. Almost 200 species of edible insects have been reported in Thailand. Rearing of edible insects has focused on crickets and palm weevil larvae wherein between the years of 1996 and 2011 cricket

production averaged around 7,500 metric tons. Insects such as weaver ants, bamboo caterpillars, and grasshoppers are popular and are captured from the wild seasonally [64].

## **1.5 FOOD INSECURITY**

### **1.5.1 Current Food Insecurity**

Ensuring that people have access to enough nutritious food is important to the health and wellbeing of people around the world, but as it stands there are many people that are food insecure. Food insecurity is the lack of access to nutritionally adequate, safe, and socially acceptable food. Being food insecure can lead to undernourishment, lack of food to meet energy needs, as well as malnutrition, lack of food to meet biological requirements. These conditions can result in chronic disease, which include examples such as kwashiorkor and iron deficiency anemia respectively [67].

Kwashiorkor is a chronic malnutrition attributable to insufficient dietary protein along with sufficient dietary calories. Symptoms include in edema of the extremities giving the appearance of being well-nourished, skin breakdown, and changes in hair color [68]. Kwashiorkor is seldom seen outside of developing countries. Rates of incidence range from 6% in regions of food insecurity to 25% in regions of famine. Diets of those affected focus on starches such as corn, cassava, or rice. Children of both sexes are typically affected between 1 to 3 years of age [69].

Iron deficiency anemia is the result of a lack of iron in the diet and can cause impeded cognitive development in children. At the turn of the millennium, 40-60% of children between 6 months and 2 years of age in developing countries had iron deficiency anemia and more than 60,000 women would die annually during pregnancy and

childbirth as a consequence of the disease [70]. In the year 2010, anemia accounted for 8.8% of total disability conditions and of that children under 5 and women account for a majority [71].

Undernourishment and malnourishment of mothers and children is a major concern. Half of the world's mothers and children are affected by undernutrition and malnutrition. Of children under 5 in developing countries, 54% of all deaths are attributable to some form of undernutrition or malnutrition. Of these, 12% of deaths are due to a lack of either iron, iodine, vitamin A, or zinc, 19% due to being underweight, and 14.6% due to wasting [72].

As of 2015, there are 795 million people undernourished across the globe with a majority of those living in developing regions. The total number of people undernourished has decreased 216 million from 1990 [73]. Between the years of 2012 and 2015 it was found that most regions of the world had seen decreasing food insecurity due to diet diversity, availability of high-quality protein, and improved government programs to spread and collect information [74].

A 2015 USDA study of world food insecurity found that food insecurity was estimated to drop between the years of 2014 and 2015 from 521 million to 475 million individuals (14.8% to 13.4%). Sub-Saharan Africa is projected to remain the most food insecure region of the world although Asia also greatly contributes to this projection [75].

### **1.5.2 Future Food Insecurity**

As of 2015, the current projection for the world population in the year 2050 is 9.7 billion with the majority originating from Africa and Asia [76]. Average calories per

capita per day are expected to rise from 2770 to 3070 by the year 2050 [77]. It is expected that global production will meet global demand for food, although these increases in demand are expected to be met with intensification of land use at the cost to the ecosystem and increased water scarcity [77-79]. Agriculture accounts for 70% of water withdrawals from lakes, rivers, and aquifers and both changes in climate and excessive use can drastically alter projected availability of water [79]. For example, in 2015 it was estimated that 15-27% of China's crop production was due to mining for ground water [79].

Despite projections suggesting that world crop production could meet goals in 2050, it is likewise projected that 5% of the populations of developing countries will remain undernourished [78]. Further, the share of the global population that is food insecure is projected to increase 1.7% by the year 2025 [75]. Some developing countries that depend on their own production rather than importing may not see increases in global production result in decreases in food insecurity or undernourishment [77].

### **1.5.3 Solutions to Food Insecurity**

Future projections of food insecurity point to several areas of concern if there is to be enough food to feed the world. It is not good enough that there is enough food, but also that there is sufficient water to drink, land to live upon, and both an ecosystem and climate that is livable and so there is great room for improvement [77-79]. Key points of improvement to mitigate food insecurity are efficiency of food production and production of safe, unspoiled food.

An example that hits many of these points would be the use of insects as alternative animal protein sources. Crickets have a greater food conversion ratio allowing for more cricket to be produced with less input of feed. Insects such as mealworms and crickets both generate fewer greenhouse gasses and ammonia than conventional livestock. Mealworms further generate less carbon dioxide compared to traditional meat sources such as pigs and cows as well as requiring less land. When raised in a year-round climate-controlled facility in the Netherlands, mealworms required an energy input greater than chicken or milk but less than beef and comparable to pork due to heating and ventilation of the areas containing the mealworms [53, 80]. Raising the mealworms in a climate without the need for heating may result in an energy efficiency within literature values for pork and chicken [80, 81].

Management of food spoilage needs to be applied through the supply chain to identify spoilage hazards and control them. Examples of spoilage include chemical and microbiological impairments to organoleptic properties of the food such as taste, odor, color but also safety in the form of toxins. Decreasing endogenous microbial loads of food and food ingredients coupled with improvements to food packaging to minimize exogenous microbiota tainting food can improve the quality and quantity of safe food for the world market [82].

## **1.6 INSECTS**

### **1.6.1 Classification and Development**

Insects are invertebrates with a chitinous exoskeleton and their body is segmented into three parts comprising a head, thorax, and abdomen and three pairs of legs. Common

additional features include compound eyes, antennae, and wings [83, 84]. More than 1 million living insect species have been described and so 58-67% of described eukaryotes are insects. The class Insecta is comprised of 29 orders wherein Coleoptera (beetles), Diptera (flies), Hymenoptera (ants, bees, and wasps), and Lepidoptera (butterflies and moths) account for 81% of all living insect species with Coleoptera comprising the plurality [85, 86].

Insects as a group are divided in two as apterygota whose adults do not have wings and pterygota whose adults have wings. These differences are also reflected in their development as apterygotes do not undergo a metamorphosis in their lifespan and so develop from nymphs and grow to be adults. On the other hand, pterygotes have a division into either hemimetabolous or homometabolous development. Hemimetaboly is a type of development where wing buds will successively grow with molting and therefore will grow from nymphs into adults. Holometabolic development is one where abrupt metamorphosis from wingless to winged occurs through a pupal stage and therefore these develop from larvae into pupae then adults [83].

The phylum Arthropoda is divided into four subphyla: Chelicerata, Myriapoda, Crustacea, Hexapoda and therein Hexapoda is divided into the classes Entognatha and Insecta. Insecta is principally formed of Dicondylia, which is formed of Zygentoma and Pterygota. Pterygota is formed of Odonata, Ephemeroptera, and Neoptera [87]. Neoptera is formed of many orders including Coleoptera, Diptera, Hymenoptera, and Lepidoptera as mentioned before and seen in Figure 1.2 [87-91]. However, it is of note that much of entomologic phylogeny is continuously being revised as new evidence and hypotheses arise. More recently this is due to utilizing both comparative anatomy with molecular

approaches including single nuclear and mitochondrial gene comparisons or the more recent use of genomic and transcriptome comparisons. [87].

### **1.6.2 Insects as food and feed alternatives**

Insects are an alternative animal source of protein to traditional livestock such as chickens, pigs, and cows, but also a source of nutrition to feed those same traditional livestock. A push for less conventional livestock comes from the gap in animal protein availability between the developed and developing world coupled with quickly approaching the limits of global animal protein production [92]. Conventional livestock are also stressful to environments in terms of feed, water, and land [53]. It has been suggested that insects can be an alternative via their greater diversity, energy efficiency, and cleaner production to result in a greater quantity of animal protein produced at lesser environmental costs [92]. Although most insect species that are consumed are wild, but some have been domesticated or farmed commercially including silkworms, cochineal, house crickets, palm weevils, giant water bugs, and water beetles [93].

An important aspect of insects as an alternative is their efficiency as compared to conventional livestock. For example, the metric of how well animals grow against how much feed they consume is known as a feed conversion ratio (FCR). Crickets have been found to have an FCR of 1.7 whereas chickens, pigs, and cows are 2.5, 5, and 10 respectively. Other metrics of efficiency include land usage where drugstore beetles have been investigated to recycle waste material and serve to provide 100 people with animal protein in only a space of 40 m<sup>3</sup>. Water usage has also been a point of contention, however there needs to be more studies done to better understand how much water

insects need; however, estimates suggest that they may be far lower than conventional livestock [53]. However, an opposing example holds that mealworms require equivalent or more energy input per equivalent production of conventional animal products including pork, chicken, and milk, but less than that required to produce beef [53]. Most commercially reared edible insect species including yellow mealworm, house cricket, and migratory locust compare well to conventional livestock such as cows, pigs, and chickens in terms of direct greenhouse gas and ammonia production [93].

Concerns about food waste have driven interest in insects. Composting using earthworms and microorganisms is a well-known practice, but there are also insects including house flies, black soldier flies, and some mealworms that can be used for this purpose. Black soldier fly has been investigated in this regard to consume manure of traditional livestock and could consume a majority, but also reduce bacterial counts of *E. coli* and *Salmonella* from chicken manure [93].

Insects are a potential source for food and feed because of their solid nutritional profile, amino acid compositions, and polyunsaturated fats, vitamins, and minerals and so may be an alternative for fishmeal animal feed [94]. Fish meal and oil comprise approximately a fifth of global fish use and has pushed increases in the use of aquaculture but also overexploitation of fish. This has also been complicated with increases in price to push for interest in alternatives. House fly maggots are a potential solution as poultry feed because they consume poultry manure to produce a balanced high protein source for the poultry. It was found that diets of 10-15% of these maggots resulted in improved quality and growth in chickens and further the larvae have compared favorably to soybean meal for turkeys [93].

### 1.6.3 Composition and nutrition

Although there are many edible insects in the world and many people around the world actively consuming insects, there is still a limited amount of consistent and complete composition data on many insects. Factors that can influence these include specific species, development stage, location, season, feed, and gut content. A review of insect nutritive data focusing on mealworm found that much of the data on insect nutrition was variable although it was found that regardless of these differences mealworms were high in protein and polyunsaturated fats. Nutrition varies with life cycle for example mealworm larvae are sources of calcium, zinc, and magnesium, pupae are a source of only magnesium, while adults are a source of iron, iodine, magnesium, and zinc. Mealworms were also found to be a source of several B vitamins including 2, 3, 6, 9, and 12. More generally, insects tend to be low in calcium due to lack of a skeleton. Some insects are more investigated than others, but a level of consistency is still required for future work [91].

Other investigations of insects have likewise found that high variation and gaps in data suggests need for more information in volume and standardization. Despite this, insects typically had high levels of iron and zinc, which supports the use of insects to combat micronutrient deficiencies. Some insects are high in saturated fats. Honey bees, termites, weaver ants, and palm weevils are all high in iron. Crickets and mealworms are high in zinc. Termites and palm weevils are both high in saturated fat [95].

Analysis of lipids from several insects including *Tenebrio molitor*, *Alphitobius diaperinus*, *Acheta domesticus*, and *Blaptica dubia* found that of *T. molitor* had the

highest lipid content at 13%. Triacylglycerol distributions was found to be similar across these species. In some samples, trans isomers C16:1 and C18:1 were also found.

However, lipid profiles were found to be unlike vegetable or animal lipid sources. These insects had high omega-6 fats and ratios of omega-6 to omega-3 ranging from 27 to 17, which is greater than FAO recommendations of 10 [96]. Another study of insect lipids found that total lipid contents of insect vary widely from cicadas at 24% to june beetles at 0.3% although all insects were found to predominantly have polyunsaturated fats and all had omega-6, but only some had omega-3 [97].

Gut content becomes a concern regarding proximate composition of insects as the guts of the insects are consumed with the insect. This results in the edible weight of insects to be greater than traditional livestock as crickets have been found to be approximately 80% edible weight whereas chickens and pigs are 58% and cows 40% [93]. However, this results in gut content and gut loading to change insect nutritional content. Gut loading is a technique of selectively increasing specific nutrient contents of insects through including those nutrients in the diet for a period so the gut contains additional nutrients. This has been a means to influence calcium, vitamin A, and vitamin D contents. The purpose is to improve the nutritional content of the product, but could also skew potential results of compositional proximate values [98, 99].

#### **1.6.4 Safety and risk factors**

Insects can act opposed to food in the form of storage pests and infesting stored food and products. Annual losses of food to storage pests have been estimated between 2-9% as infestation of stored food products can cause physical loss, spoilage,

contamination, as well as risks to health. The three orders of Insecta that contain major storage pests are Coleoptera, Lepidoptera, and Psocoptera (booklice) [84]. Foods that are at risk of storage pests include grains, pet food, and food with biodegradable packaging [100]. However, it is not feasible to have food entirely free of such contaminants and so in the United States, the FDA utilizes their Defect Levels Handbook as a standard of what levels of naturally occurring or otherwise unavoidable defects occur in some foods that do not act as a risk for food. Of these, insects are commonly listed as contaminants and sources of infestation in forms such as insect fragments, whole or equivalent insects, eggs, or larvae [101].

Insects cannot be ignored as a potential source of microbiological hazards. Although insects do carry pathogenic bacteria, they are often not pathogenic to humans and so many bacterial hazards to humans originate from rearing, handling, processing, and preservation of the insects. Despite this, farmed insects have been found to have high levels of aerobic and anaerobic bacteria. Insects may be a vector for both *Campylobacter* and *Salmonella*, but insects must be re-exposed to continuously transmit these pathogens. They also may carry viruses but much like their bacteria are principally problematic for other insects. Of note is the potential for mycotoxigenic fungal growth, although this is not of concern to humans if properly processed and stored. With this, the European Food Safety Authority found that food-grade insects are unlikely to be of significant safety risk [102]. Edible insects have also been investigated as carriers of bacteria with transferrable antibiotic resistance genes and it has been found that despite a high variability among samples there was a high frequency of a tetracycline resistant gene. It is suggested that

these genes may find their way into human microbiomes through consuming such insects [103].

Allergies to insects can be divided into stinging, biting, and inhalant allergies. Of these, stings most commonly cause anaphylaxis where systemic reactions are estimated to occur in 3% of adults and 1% of children but 10% of adults are estimated to have local reactions. Bites may result in anaphylactic reactions but more rarely than stings and so more likely cause local reactions. Inhalant allergies are more associated with symptoms including asthma, rhinitis, and atopic dermatitis [104].

Stinging insects belong to the order Hymenoptera and examples include honeybees, yellow jackets, and fire ants. The venom of the sting carries allergenic proteins and other compounds into the blood. One example of a stinging allergen is Phospholipase A from honeybee venom. Stinging allergies are typically only cross-reactive across genera [104].

Common sources of reactions to biting allergies result from kissing bugs and mosquitos. Kissing bugs are of focus because they are a common cause of bite-related anaphylaxis via feeding from blood. The bite causes minimal pain but salivary gland allergens are injected resulting in symptoms. Despite the amount of reported mosquito bites, systematic reactions are particularly rare [104].

Inhalant insect allergies can be the result of either outdoor or indoor exposure to insects including caddisflies and midges outdoors or cockroaches indoors [104]. Occupational exposure and sensitization to insects have also been reported for honeybees, silkworms, and mealworms. Airborne insect-derived particles include shed hair, scales, excreta, and disintegrated body parts that all become part of amorphous dust [51].

German cockroaches are common in inner cities of the United States. Cockroach allergens come from feces, saliva, or bodily debris and may remain in homes for years post-infestation. Cockroach allergens tend to be airborne only if disturbed. Cockroach allergens tend to accumulate most in kitchens but are most associated with hospitalization in children's bedrooms. A number of cockroach allergens have been characterized including tropomyosin and arginine kinase [51, 104]. Entomophagy may demand the view of traditionally airborne allergens as potentially oral allergens [43].

Another molecule of potential risk associated with insects is chitin. Chitin is a polysaccharide that comprises the insect exoskeleton, but is also found in fungi, parasites, and crustaceans [105, 106]. Chitin has been demonstrated to function as an adjuvant, which is a substance that assist in eliciting adaptive immunity, although how it functions as an adjuvant is not well understood. Different studies have demonstrated different effects of chitin immunomodulation. De Silva et al found that chitin functioned to induce Th2, Th1, and Th17 immune and innate inflammatory responses in mouse lungs and was comparable to the common adjuvant alum [106]. Dubey et al found that compared to alum, the use of chitin as an adjuvant with mice results in a lesser inflammatory response and similar total and specific IgE and IgG1 levels. However, it was found that pre-treatment with chitin, and not alum, depresses Th2 cytokines and enhances Th1 cytokines [107]. Ghotloo et al also found that in mice small chitin particles downregulate Th2 responses and upregulate Th1 responses [108]. Further, Bae et al found that in mouse models the oral administration of chitin was protective against IgE-mediated anaphylaxis and could inhibit food allergy [109]. Sigsgaard et al investigated effects of chitin administered by inhalation in a double-blinded experiment and found that while

chitin particles were weakly inflammatory it also enhanced cytokines associated with the downregulation of Th2 responses [110].

Other hazards associated with insects can come from how they are grown rather than intrinsic factors. Raising mealworms can be done through feeding of low quality or inedible agricultural products and those products will often have pesticides applied to them for productivity, but these pesticides may accumulate in the mealworms consuming them. Different pesticides have been found to both accumulate and dissipate more slowly from the mealworm with increasingly hydrophobic pesticides [111].

## **1.7 SUMMARY**

Insects represent a high-quality alternative food source for the world, provided that risks from allergenic reactions and other food safety concerns can be defined. Of the known allergens, tropomyosin stands out as significant. It is suggested that research focused on structural differences of tropomyosin across crustaceans and insects represents a promising approach to defining allergenic risk.

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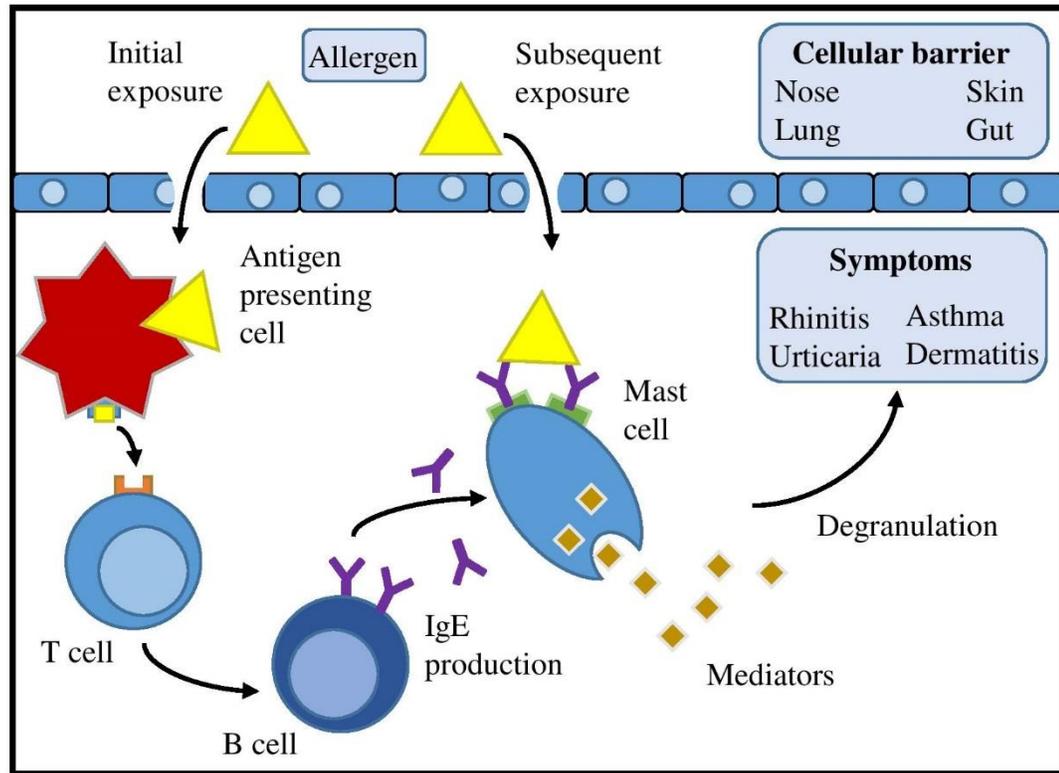
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**Table 1.1 Routes of exposure and corresponding symptoms of food-allergic reactions** (adapted from [107])

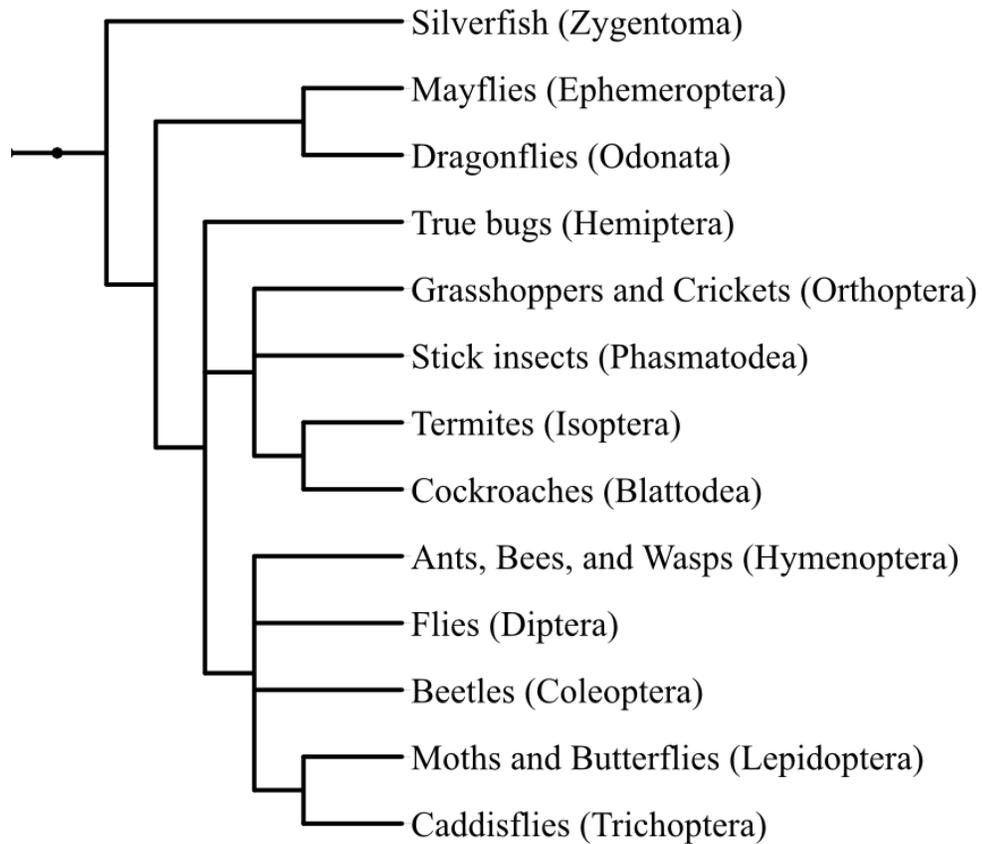
<b>Route of exposure</b>	<b>Symptoms</b>	
Cutaneous	Pruritis Urticaria	Erythema/flushing Angioedema
Ocular	Pruritis Tearing	Conjunctival injection Periorbital edema
Upper respiratory	Pruritis Sneezing Hoarseness	Nasal congestion Rhinorrhea Laryngeal edema
Lower respiratory	Cough Wheezing	Dyspnea Chest tightness
Gastrointestinal	Oral pruritus Vomiting Nausea Diarrhea	Oral angioedema Colicky abdominal pain Pharyngeal pruritus
Cardiovascular	Tachycardia Hypotension	Dizziness Loss of consciousness
Miscellaneous	Metallic taste Uterine cramping	Sense of impending doom



**Figure 1.1 Mechanisms involved in sensitization and elicitation of allergy** (Adapted from [108].)

**Table 1.2 Comparison of various allergen detection methods**

<b>Method</b>	<b>Pro</b>	<b>Con</b>
<b>Antibody-based</b>	Fast Qualitative and quantitative Industry standard High throughput	Requires antibodies Antibodies may cross-react Depends on antibody target presence Depends on food matrix Lack of reference materials
<b>Mass spectrometry</b>	High sensitivity Absolute identification Quantitative Multiplex potential	Expensive High level of expertise Time consuming
<b>PCR</b>	High throughput Multiplex potential	Detects DNA not protein Cross-contamination Requires training and equipment



**Figure 1.2 Abridged phylogeny of the class Insecta**

No information is to be inferred from branch length. Phylogenetic tree of Insecta is divided according to Dicondylia separating Zygentoma, Pterygota separating Ephemeroptera and Odonata, and Neoptera with the remainder. Tree was generated using Phylot per NCBI taxonomy [83-86].

## CHAPTER 2

### METHODOLOGIES FOR THE IDENTIFICATION OF PROTEINS OF DACTYLOPIUS COCCUS AND CARMINES

#### 2.1 ABSTRACT

*Dactylopius coccus*, commonly known as cochineal, is an insect used to produce carmine, a natural red pigment used for numerous purposes ranging from food to pharmaceuticals. Carmine has been found in rare cases to be the cause of allergic reactions eliciting symptoms from occupational asthma to anaphylaxis. This work seeks to identify allergen homologues in cochineal and carmine products using protein separation techniques and mass spectrometry. Methods were evaluated on the basis of purification of protein from the dye. Techniques investigated include ultrafiltration, disposable C18 solid-phase extraction, ion exchange chromatography, and trichloroacetic acid (TCA) precipitation. Precipitation of cochineal and carmine using trichloroacetic acid with SDS-PAGE and in-gel tryptic digestion minimized contaminant dye. Mass spectrometry of these separated samples identified several proteins including a previously described cochineal major allergen, but no proteins were identified from carmine products. Further work to identify contaminant proteins in carmine products require a more complete database of proteins from *D. coccus* and related species.

## 2.2 INTRODUCTION

Growing interest in natural dye alternatives has spurred interest in several dyes including cochineal and carmine. *Dactylopius coccus*, the American cochineal, is a parasitic insect of prickly pear cacti used as the source of carminic acid and refined with calcium or aluminum salts to yield the lake pigment carmine. Cochineal, carminic acid, and carmines are labeled in food in Europe under E120 and have been regarded as safe except with regard to allergy. Despite widespread consumption of carmine, it is rarely implicated in allergic reactions, however incorporation of less refined cochineal dyes can allow for protein contaminants to remain and sensitize individuals to an IgE-mediated allergy. Sensitization is more likely to occur through cosmetic or occupational exposure whereas subsequent reactions may also result from oral exposure [1, 2].

Allergic reactions to cochineal products have been segregated into either occupational asthma or oral ingestion. Reports of occupational asthma have implicated several proteins as characterized by SDS-PAGE and Immunoblotting including a 30 kDa band from a cochineal extract and diffuse banding from 40-97 kDa from a carmine extract using sera from a non-atopic butcher [3]. Other reports of occupational asthma have implicated proteins of 17 kDa from raw cochineal, 28 kDa from carmine, and 50kDa in boiled cochineal [4]. Oral allergy reports have described symptoms ranging from rhinitis and urticaria to anaphylaxis and have implicated bands from 23-88 kDa from raw cochineal [5], 39-45 kDa from cochineal extract [6], as well as bands at 16-18 and 38-40 kDa from cochineal extract and 38-45 kDa in carmine extract [7]. A major allergen of cochineal has been identified through 3' and 5' RACE and Immunoblotting of both native and recombinant protein. Full-length cDNA codes for a 38 kDa protein

encoding a single pre-pro secretory protein resulting in four variants of the protein via secondary modifications and further found to have homology to hornet PLA<sub>1</sub>, a major venom allergen [8].

Detection and quantification of cochineal allergens is complicated by interference from carminic acid and derivative compounds. Carminic acid has a number of absorption maxima ranging from 222 to 531 nm and both derivative carmines and increasing pH result in a red shift. Carmines can result from numerous cations such as aluminum and calcium but also zinc, gadmium, thorium, and copper [9, 10]. Cupric carmines are known to result in insoluble dark precipitates [11]. Further, carminic acid can form strong bonds with proteins and resist separation [12]. These properties make spectrophotometric protein determinations of cochineal solutions difficult.

The primary method for protein determination in these cases is suspension in 1% phosphoric acid with repeated separation with HPLC [13]. Early work suggested that many membrane filter compositions adsorb carminic acid, but aqueous cochineal products were effectively filtered using to cellulose membrane filters [14], although more recent work has identified that protein contaminants in carmine can be purified using ultrafiltration across a polyethersulfone membrane [12]. Identification of allergens of cochineal is stymied by ineffective separation from endogenous dyes.

## **2.3 METHODS AND MATERIALS**

### **2.3.1 Materials and Sample preparation**

Whole cochineal insects, Carmine 40-202, and CC-5000-WS-P were a kind gift from Chr. Hansen (Milwaukee, Wi). Total nitrogen determination was performed by the Natural Product and Food Analysis Facility of the University of Nebraska-Lincoln. In brief, samples were weighed at 0.100 +/- 0.05 g in triplicate and analyzed on a LECO Corp. FP-528 Nitrogen Determinator to determine total percent nitrogen in the samples. Percent protein was determined by a 6.25 conversion factor. Whole cochineal, Carmine 40-202, and CC-5000-WS-P were  $49.07 \pm 0.91\%$ ,  $24.49\%$ , and  $19.92 \pm 0.68\%$  crude protein respectively.

Hexane was added to whole insects 10% w/v and the mixture homogenized with a Polytron instrument in three 30 second bursts at 15,000 RPM. Hexane was added to 5% w/v and stirred at 100 rpm for 2 hours, filtered through filter paper, and the retentate was dried in a hood overnight. These solids were used as defatted cochineal powder (Figure 2.1).

### **2.3.2 Carmine extractions**

CC-5000-WS-P and Carmine 40-202 were extracted in 30% and 10% w/v respectively with several buffers, extracted for 30 minutes with rocking, centrifuged at  $16,000 \times g$  for 10 minutes, and pellets discarded. Buffers included 25 mM Tris, pH 8 with 1% w/v N-lauroylsarcosine (sarcosyl) or 1% w/v sodium dodecyl sulfate (SDS). Extractions were optionally filtered through a 0.45  $\mu\text{m}$  nitrocellulose membrane (Millipore, Billerica, Massachusetts). Samples were stored at  $-20\text{ }^{\circ}\text{C}$  until use.

### **2.3.3 Spin ultrafiltration**

Defatted cochineal powder was extracted in 25% w/v of different buffers for 30 minutes with rocking, centrifuged at 16,000 x g for 10 minutes, and the pellets discarded. Buffers and variations of the extraction included 50 mM Tris, pH 8 with 2% w/v beta-mercaptoethanol (BME), 2% w/v polyvinylpyrrolidone (PVPP), 0.2% SDS, 0.2% sarcosyl, 60°C heated extraction, or 3 minute extraction time. 200 µl of supernatant were applied to a Vivacon 500 hydrosart® 2,000 molecular weight cut-off (MWCO; Sartorius Stedim Biotech GmbH, Goettingen, Germany), Nanosep Omega™ 3,000 MWCO (Pall, Port Washington, New York), or Amicon Ultra-0.5 hydrosart® 3,000 MWCO (Millipore, Billerica, Massachusetts) ultrafiltration device. Samples applied to the Vivacon 500 were filtered through a 0.45 µm nitrocellulose membrane before ultrafiltration. Samples were washed with 5 volumes of 50 mM Tris, pH 8 and returned to their original volume. Filtrates were discarded and the retentate stored at -20 °C until use.

### **2.3.4 C18 solid-phase extraction**

Defatted cochineal powder was extracted in 25% w/v 50 mM Tris pH 8, extracted for 30m with rocking, centrifuged at 16,000 x g for 10 minutes, and the pellet discarded. Sep-pak C18 plus short cartridges (Waters, Milford, Massachusetts) were prepared with 4 ml of methanol followed by 4 ml of deionized water before application of 2 ml of sample and washing with 1.5 ml of water three times. The cartridge was then eluted five times with 1 ml of methanol, pH 4. Fractions were washed with 50 mM Tris, pH 8 and

concentrated to 200 $\mu$ l using a Nanosep Omega<sup>TM</sup> 3,000 MWCO ultrafiltration device.

Filtrates were discarded and the retentate stored at -20°C until use.

### **2.3.5 Ion exchange chromatography**

Defatted cochineal powder was extracted in 25% w/v 100 mM Tris, pH 8 or 100 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6, extracted for 30 minutes with rocking, centrifuged at 16,000 x g for 10 minutes, and pellets were discarded.

Supernatants were filtered using a 0.45  $\mu$ m nitrocellulose membrane. DEAE-Sephadex A-25 and CM-Sephadex C-50 (Sigma, St. Louis, Missouri) were equilibrated in Tris or MES buffers respectively overnight before use. Disposable Filter Columns (Fisherbrand, Cat No 11-387-50) were used as gravity columns and filled with 1 ml of equilibrated resin with a flow rate of approximately 500  $\mu$ l per minute. Columns loaded with between 50 to 200  $\mu$ l of sample and washed with 5 bed volumes of respective buffer. DEAE-Sephadex columns were eluted with one of 0.15, 0.25, 0.35, or 0.5 M NaCl in 100 mM Tris, pH 8. CM-Sephadex columns were eluted with 0.5 M NaCl in 100 mM MES, pH 6. Fractions taken were approximately 10ml or smaller avoiding the dye front. Fractions were concentrated and desalted using Amicon Ultra-15 hydrosart® 3,000 MWCO ultrafiltration devices. Filtrates were discarded and the retentate stored at -20°C until use.

### **2.3.6 Trichloroacetic acid precipitation**

Defatted cochineal powder and Carmine 40-202 were extracted in 25% w/v of different buffers for 30 minutes with rocking, centrifuged at 16,000 x g for 10 minutes, and the pellets discarded. Buffers investigated were 50 mM maleic acid, pH 2, 50 mM

citric acid, pH 4, 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7, 50 mM Tris, pH 8, 50 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), pH 10, 1x PBS, pH 7.8, and a 50 mM Tris, pH 8 solution with 50% Methanol v/v. 150  $\mu$ l of cochineal supernatants and 300  $\mu$ l were subjected to trichloroacetic acid (TCA) or TCA-Acetone precipitation. TCA precipitation was performed through addition of 100% TCA w/v to a final concentration of 10% TCA v/v and TCA-Acetone precipitation was performed with 1:1:8 sample, 100% TCA, and acetone. Precipitations were kept at -20 °C for 1 or 24 hours at -20 °C. Samples were centrifuged at 16,000 x g for 10 minutes at 4 °C, decanted, and washed with acetone repeated twice. Pellets were resuspended in 200  $\mu$ l 0.1 M NaOH with 4% SDS and shaken overnight. Samples were stored at -20 °C until use.

### **2.3.8 SDS-PAGE**

Samples were prepared for SDS-PAGE by adding 2% beta-mercaptoethanol v/v and diluting the sample 1:2 with 2x Laemmli sample buffer. Samples were heated at 95°C for 5 m, cooled, centrifuged down, 20  $\mu$ l of samples loaded, and run using Criterion 4-12% Tris-HCl gels (Biorad, Hercules, California). Gels were run at 200 V until the tracking dye reached the bottom of the gel. Gels were stained with a Coomassie R-250 staining mixture overnight and subsequently destained.

### **2.3.9 Mass Spectrometry**

Mass spectrometry was performed on excised gel bands and sent to the Nebraska Center for Mass Spectrometry. Briefly, the samples were washed with 100 mM ammonium bicarbonate, reduced with 10 mM dithiothreitol (DTT), alkylated with 55

mM iodoacetamide, washed twice with 100 mM ammonium bicarbonate, and digested *in situ* with 10 ng/ $\mu$ l trypsin (Promega, Madison, Wisconsin). Peptides were extracted with two 60  $\mu$ l aliquots of 1:1 acetonitrile:water containing 1% formic acid. The extracts were dried using a SpeedVac and then reconstituted in 12  $\mu$ l of 0.1% formic acid in water.

The formic-acid reconstituted extract solution (4  $\mu$ l) was injected onto a trapping column (300 micron x 1 mm) in line with a 75 micron x 150 mm 1.7  $\mu$ m BEH130 C18 reversed phase LC column (Waters). Peptides were eluted from the column using a water + 0.1% formic acid (A) / 95% acetonitrile:5% water + 0.1% formic acid (B) gradient at a flow rate of 270 nl/min. The gradient was developed with the following time profile: 0 min 0% B, 5 min 5% B, 35 min 35% B, 40 min 45% B, 42 min 60% B, 45 min 90% B, 48 min 95% B, and 50 min 5% B. Eluted peptides were analyzed using a quadrupole time-of-flight (Q-TOF) Synapt G2-S tandem mass (MS/MS) spectrometer (Micromass Waters), with electrospray ionization. Analyses were performed using data-dependent acquisition with lock mass correction. The MS/MS data were processed with Distiller software (Matrix Science, Boston, Massachusetts) to produce peak lists for database searching using MASCOT (Matrix Science). Data were searched against the Uniprot database with the search restricted to proteins in Arthropoda (accessed June 11<sup>th</sup>, 2015). The following search parameters were used: peptide mass tolerance 0.1 Da, enzyme specificity trypsin, fixed modification carbamidomethylation of cysteine, and variable modification oxidized methionine. Protein identifications were based on a protein significance threshold of  $p < 0.001$  using an ion score threshold of 20, minimum 2 peptides matched, and protein matches requiring at least one peptide that is both highest scoring peptide match listed under the highest scoring protein containing that match (bold red).

## **2.4 RESULTS AND DISCUSSION**

### **2.4.1 Preliminary SDS-PAGE of carmine products**

Preliminary extractions of carmine products indicate that Carmine 40-202 did not contain protein whereas CC-5000-WS-P may have proteins present in the extraction but presence of dye in the gel post-destaining makes confident identification tenuous (Figure 2.2). Minor potential banding appears in CC-5000-WS-P extractions around 200 kDa, although heavy distortion of the gel due to both dye and high ionic strength of the extracts causing bloating of the lanes as well as disappearance of the 6 and 14 kDa molecular weight marker bands. Differences between extraction methods or the effect of filtration cannot be assessed. Kjeldahl total nitrogen coupled with the lack of appreciable protein bands suggests either highly insoluble proteins predominate in these samples or presence of non-protein nitrogen such as 4-aminocarminic acid in the case of acid-stable carmine [15, 16]. Follow-up methodologies focused toward optimization of dye and protein contents for downstream analysis. Thereafter, cochineal was used as a surrogate for carmine products to improve qualitative and quantitative assessments of purification methodologies.

### **2.4.2 Cochineal spin ultrafiltration**

Ultrafiltration using different membrane compositions and MWCO did not alter banding patterns. Extraction with BME resulted in a reduction in bands below 21 kDa indicating that much of the present banding could be the result of breakdown products due to the handling and preparation of the dried cochineal (Figure 2.3.A). Stringent extraction conditions including heat or detergents resulted in increases in streaking in

lanes whereas minimal extraction times or PVPP minimized both the number of bands and their vibrancy (Figure 2.3.B). Resultant supernatants were all similarly dyed with no retentate being free from dye. Similar techniques have been used for the quantification of carminic acid in food as well as purification of protein contaminants from carmine, however methods were either using a far lower load of carminic acid or were highly time intensive without entirely removing dye [12, 14].

### **2.4.3 Cochineal C18 solid-phase extraction and fractionation**

Fractionation of cochineal using a disposable C18 syringe column show a majority of extracted proteins washing through the column rather than eluting (Figure 2.4.A). Washes starting with wash 2 included dye, with most the dye eluting from the column later. This indicates that proteins extracted were predominantly hydrophilic and that proteins are co-eluting with contaminant dye. During washes of the column the dye visibly permeates the resin (Figure 2.4.B), but lower sample loads into the column result in imperceptible protein banding on the resultant SDS-PAGE (Not shown). Sep-Pak c18 cartridges had been used for the quantification of carminic acid spiked into mixtures of simulated fruit beverages prior to HPLC, although this was used as a means to quantify minute amounts of carminic acid from a mixture rather than remove predominant carminic acid from a mixture [14].

### **2.4.4 Anion and cation exchange chromatography**

Use of ion exchange chromatography to purify cochineal proteins was relatively ineffective as a means of maintaining protein bands when compared to other techniques

(Figure 2.5). Initial investigations sought whether washing protein from dye or dye from protein was more effective (Figure 2.5.A). Use of cation exchange sought to minimize dye remaining in the sample and elute protein thereafter whereas anion exchange was used to quickly allow protein to elute from the column and minimizing dye migration. It was more effective to use anion exchange chromatography in this manner as it is likely that more protein was extracted with a higher pH and minimization of dye in the eluent did not maximize protein recovery. As minimization of dye migration was more favorable, optimizing elution buffers was investigated (Figure 2.5.B). Minimal differences were found using different eluting salt concentrations suggesting that protein is flowing without interacting with the resin, however lower concentrations of salt resulted in a similar reduction in elution of dye. Effectiveness of ion exchange for purification of cochineal and carmine proteins stems from precipitation of dye into the resin requiring the use of fresh resin per purification thereby limiting long-term use of the technique (Figure 2.5.C).

#### **2.4.5 Trichloroacetic acid precipitation of cochineal and carmine**

Precipitative methods of protein purification were found to be more effective than filtration, solid-phase extraction, or ion exchange. Use of TCA precipitation as well as TCA-acetone precipitation indicate that extraction of proteins at high pH result in more representative banding patterns on SDS-PAGE (Figure 2.6.A). Aqueous alcoholic extracts were effective at extracting dye from cochineal, but failed to extract protein. Addition of acetone in the precipitation allowed for nearly complete removal of dye from the resultant pellet coupled with minimal banding below 14 kDa. TCA precipitation was

then applied to a similar set of extractions of either cochineal or Carmine 40-202 with precipitation over 1 or 24 hours, however appreciable banding from Carmine 40-202 was only seen in 24 hour precipitations and only at bands of 14 kDa or lower (Figure 2.6.B). Lack of higher weight bands from Carmine 40-202 restricted the addition of acetone to the TCA precipitation despite improved removal of dye.

#### **2.4.6 Identifications of proteins from cochineal and carmines**

Bands taken from representative SDS-PAGE gels were organized to investigate bands that had previous indications of reactivity as well as to compare identifications across extractions and purification methods (Figure 2.7). Protein bands were compared across gels according to molecular weight of the band taken: 1, 7, and 10; 2, 8, and 12; 6 and 9. Comparisons of the proteins putatively identified lack any identifications from Carmine 40-202 bands 1 and 2 (Table 2.1). Likewise, there was a lack of identifications from band 7 disallowing comparisons between bands 1, 7, and 10. Bands 8 and 12 similarly identified ubiquitins, however these are not known as allergens. Bands 6 and 9 did not identify proteins similarly as band 6 identified a putative thioredoxin peroxidase as the potentially closest functional hit to band 9's peroxiredoxin-2, however the organism sources were diverse and band 6 lacks protein-level data to confirm any potential functional homology. Beyond identifications based from agreement between bands, band 4 was identified as a previously described major allergen of cochineal, which has homology to vespid venom phospholipase [8]. Band 10 identified a tropomyosin, an invertebrate cross-reactive allergen [17], however there was no comparable identification in either bands 1 or 7 and with band 10 being taken at approximately 14 kDa if

appropriately identified then it is likely a breakdown product rather than a whole protein.

Other bands including 3, 5, and 11 had identified hits but none were known allergens.

Comparisons between SDS-PAGE bands can only be done sparingly as identifications

shared were minimal and the identifications that were made were weak with the

exclusion of the cochineal major allergen. SDS-PAGE served as a valuable step to purify

proteins from present dye. A greater database of sequences from *D. coccus* and related

species would allow for better protein identifications.

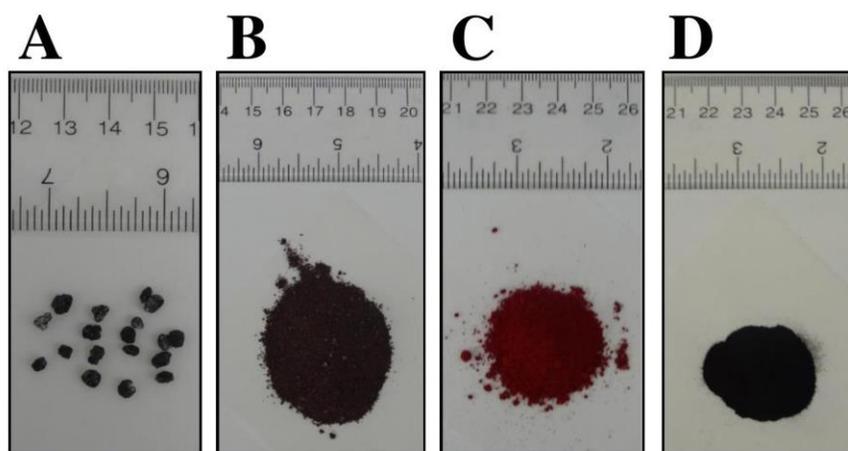
## 2.5 CONCLUSION

Multiple methodologies for purification of proteins from *Dactylopius coccus* and carmine products were investigated. Trichloroacetic acid precipitation resulted in minimization of final dye content with relatively representative protein banding on SDS-PAGE. Several proteins were identified using LC-MS/MS including the cochineal major allergen, however a low amount of sequences available for *D. coccus* and related species hinders confident identifications. Follow-up should first rest upon an annotated genome of *D. coccus* or a closely related species with subsequent techniques for purification of proteins focused on extraction with a zwitterionic-chaotropic buffer such as 2-D buffer and short gel fractionation with in-gel tryptic digestion for LC-MS/MS analysis.

## 2.6 REFERENCES

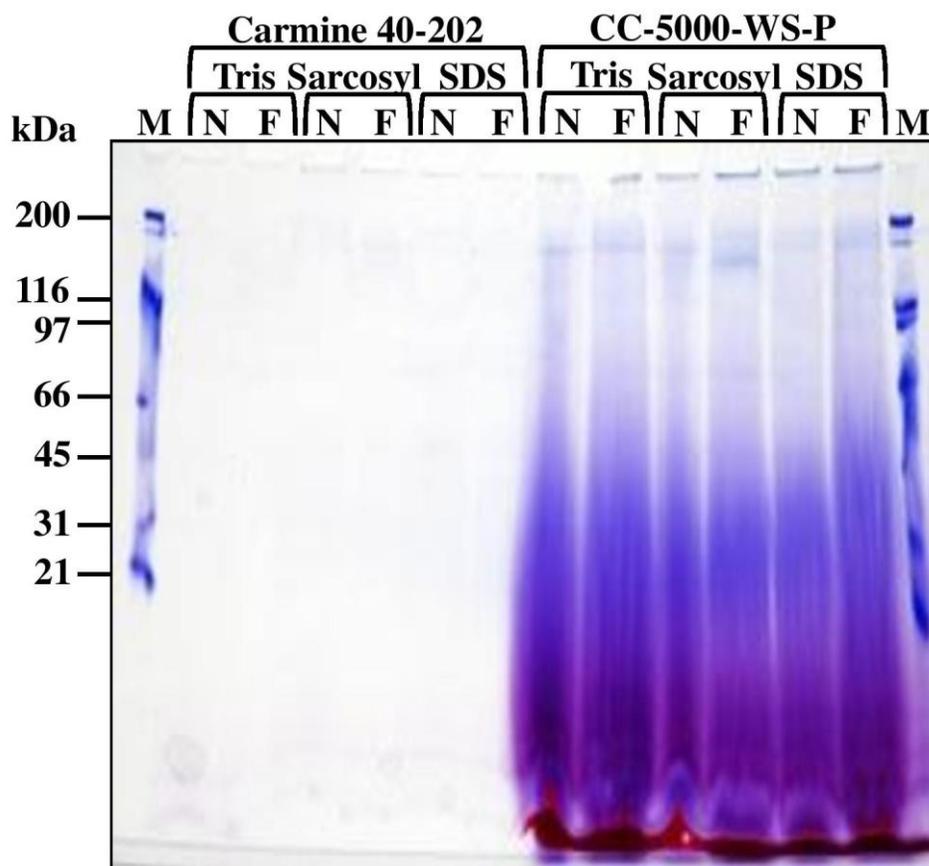
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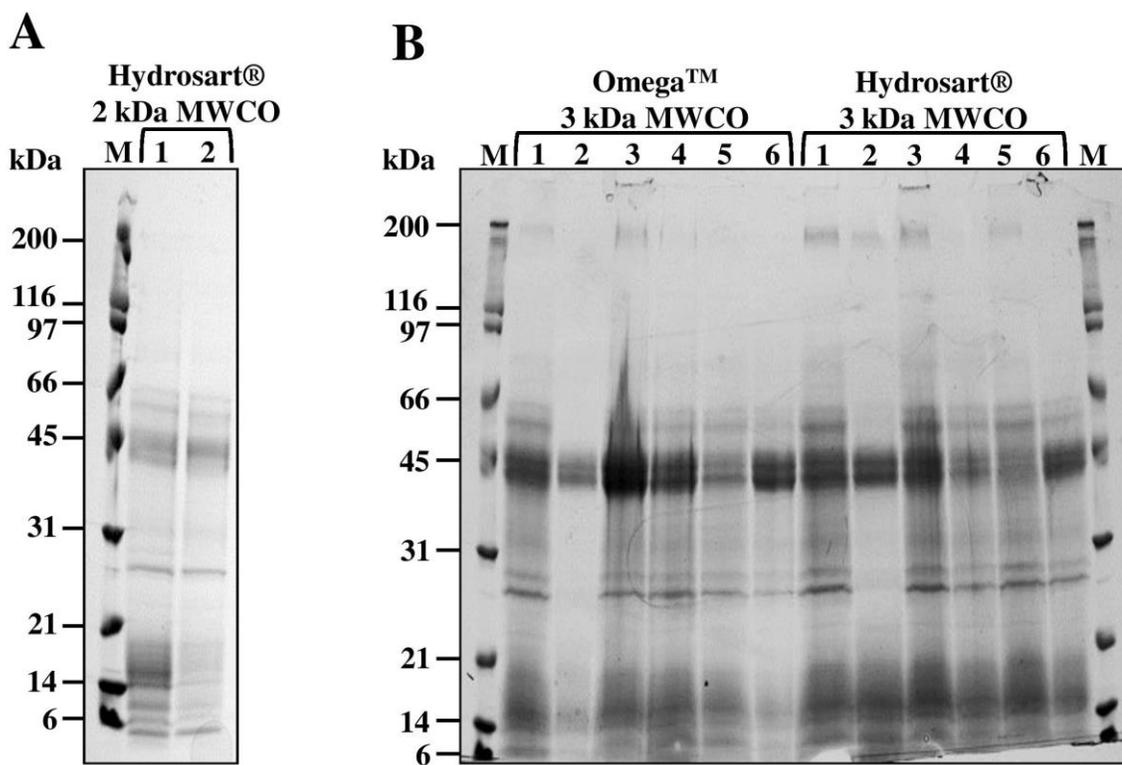
**Figure 2.1. Cochineal and carmine samples**

Samples used in this study: whole cochineal (A), defatted cochineal powder (B), Carmine 40-202 milled (C), and CC-5000-WS-P (D).



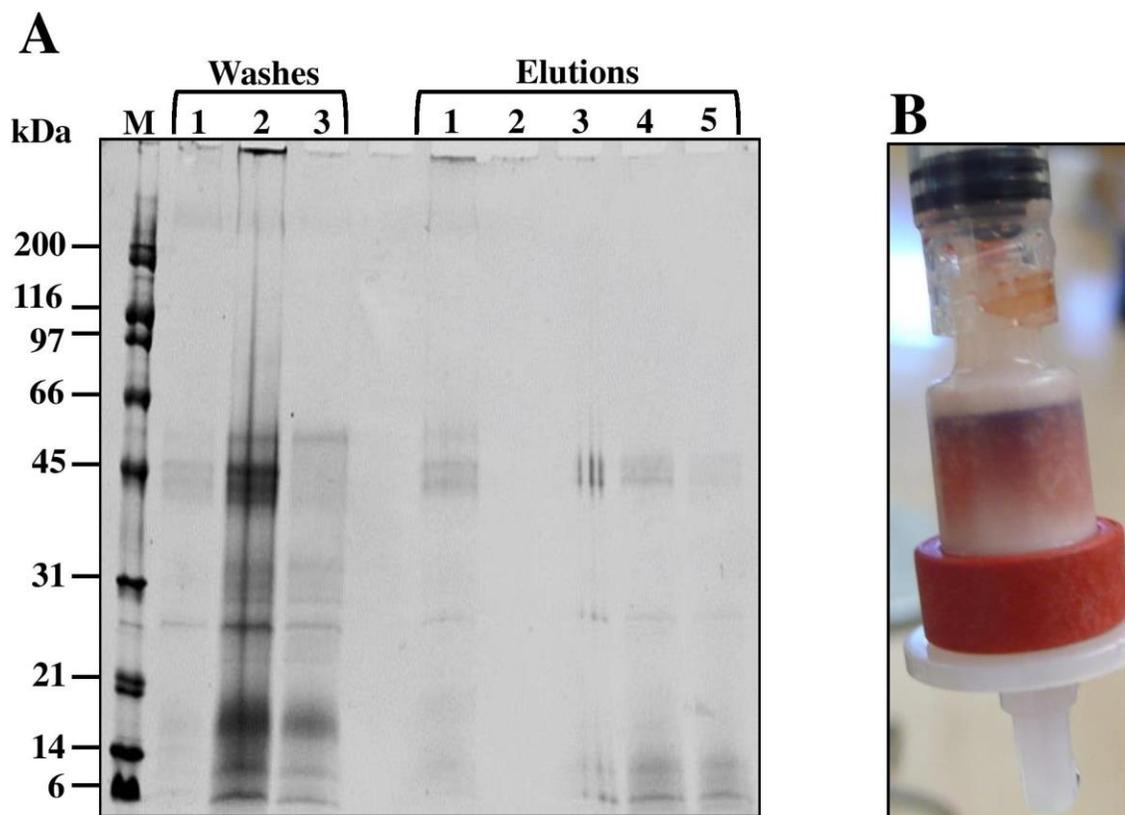
**Figure 2.2. Stained SDS-PAGE gel of Carmine 40-202 and CC-5000-WS-P extractions**

Extractions of carmine products using 25 mM Tris, pH 8 (Tris), 25 mM Tris, pH 8, with 1% N-lauroylsarcosine w/v (Sarcosyl), and 25 mM Tris, pH 8, with 1% SDS w/v (SDS). Extractions were either filtered (F) or not (N) through a 0.45  $\mu$ m nitrocellulose membrane. No appreciable protein bands were found from Carmine 40-202. Potential protein bands around 200 kDa can be seen in extractions of CC-5000-WS-P. Extraction method nor filtration altered results on SDS-PAGE. Coloration of CC-5000-WS-P lanes result from remnant carmine dye within the gel after destaining.



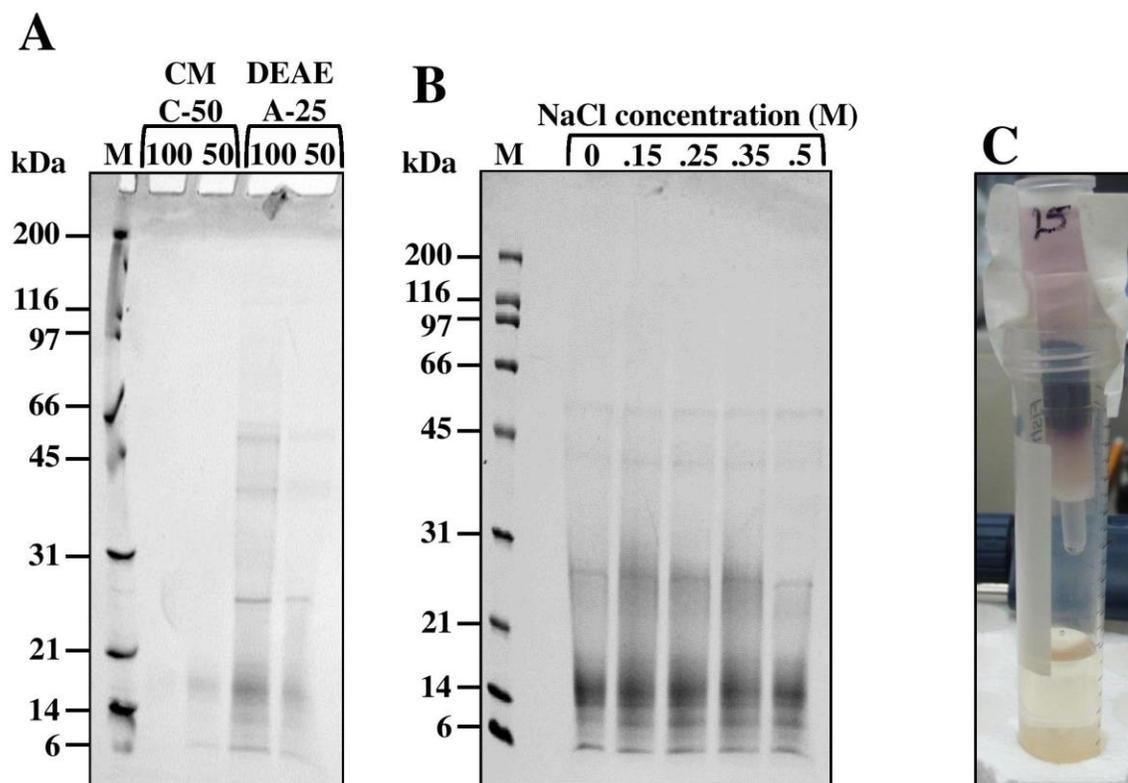
**Figure 2.3. Stained SDS-PAGE gels of ultrafiltered extractions of defatted cochineal powder**

A) Lane 1) 25 mM Tris, pH 8, 2) 25 mM Tris, pH 8, 2% BME. Banding patterns are similar above 20 kDa with clearer bands using BME during extraction. B) Lane 1) 50 mM Tris, pH 8, 2) 50 mM Tris, pH 8, 2% PVPP, 3) 50 mM Tris, pH 8, heated 60°C extraction, 4) 50 mM Tris, pH 8, 0.2% SDS, 5) 50 mM Tris, pH 8, 0.2% sarcosyl, 6) 50 mM Tris, pH 8, 3 minute extraction time. Membrane composition did not significantly alter banding across extractions. Compared to Tris alone, extraction conditions including detergents or heat resulted in streaking of the lanes whereas lower extraction time or PVPP lowered the vibrancy and number of bands present.



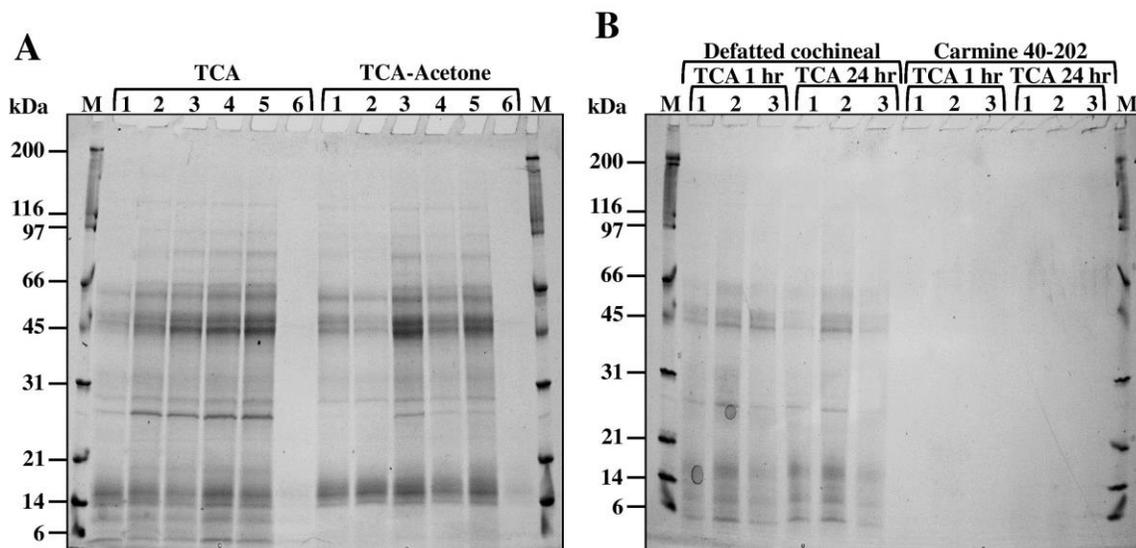
**Figure 2.4. Stained SDS-PAGE gel of a C18 fractionated extract of defatted cochineal powder and representative column example**

A) An extraction of defatted cochineal powder using 50 mM Tris, pH 8 with subsequent load of 200  $\mu$ l on a prepared Waters Sep-pak C18 plus short column fractionated by washes with water and elution with acidified methanol. Extracted protein principally present in wash 2 with similar banding in following washes and elution. Dye was present in collected fractions following wash 1. B) An example of the Waters Sep-pak C18 plus short after washes and prior to elution. Dye forms both solid and mobile phases to stick to the resin and overflow into the wash.



**Figure 2.5. Stained SDS-PAGE gels of defatted cochineal powder fractionated using cation and anion exchange chromatography with representative apparatus**

A) Extractions of defatted cochineal powder using 100 mM MES, pH 6 and 100 mM Tris, pH 8 applied to CM-Sepharose C-50 and DEAE-Sepharose A-25 respectively in 50 and 100  $\mu$ l loads. Cation exchange using CM-Sepharose C-50 allowed dye and protein to likewise flow through the resin resulting in ineffective purification. Anion exchange using DEAE-Sepharose A-25 was more effective at purifying protein. B) Extractions of defatted cochineal powder using 100 mM Tris, pH 8 and applied to individual DEAE-Sepharose A-25 columns and eluted with 100 mM Tris, pH 8 with NaCl concentrations as listed. Use of different concentrations of NaCl as eluent indicates proteins are flowing through the column without interaction with the resin. C) An example of the ion exchange chromatography gravity column apparatus used.



**Figure 2.6. Stained SDS-PAGE gels of resolubilized trichloroacetic acid precipitations of defatted cochineal powder and Carmine 40-202**

A) Comparison of 10% TCA precipitations with and without acetone during extraction: Lane 1) 50 mM maleic acid, pH 2, 2) 50 mM citric acid, pH 4, 3) 50 mM Tris, pH 8, 4) 50 mM CAPS, pH 10, 5) 1x PBS, pH 7.8, 6) 50 mM Tris, pH 8 in 50% methanol v/v. Precipitations with acetone lack banding below 14 kDa present without acetone. Extractions favor higher pH without addition of alcohol. B) TCA precipitations comparing defatted cochineal and Carmine 40-202 over 1 or 24 hours. Lane 1) 50 mM MOPS, pH 7, 2) 50 mM Tris, pH 8, 3) 1x PBS, pH 7.8. Carmine 40-202 extractions demonstrate minimal banding to the exclusion of faint bands at 6 and 14 kDa.

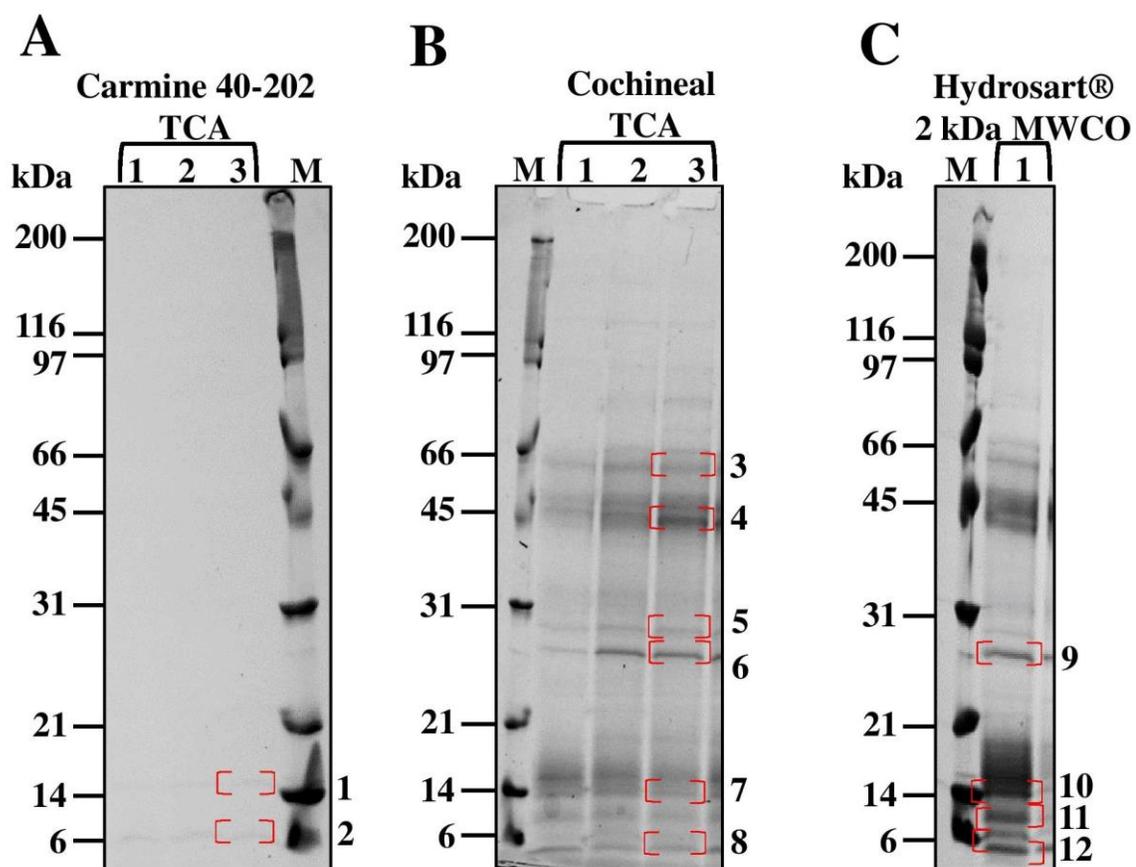


Figure 2.7. Gel bands removed from SDS-PAGE gels for in-gel trypsin digestion and LC-MS/MS

**Table 2.1. Identified proteins of Carmine 40-202 and defatted cochineal powder identified by LC-MS/MS**

Band no. <sup>1</sup>	Protein candidates	Accession #	Source Organism	MW (kDa)	Protein MOWSE Score	% Coverage (# peptides)
1	NI <sup>2</sup>					
2	NI					
3	Uncharacterized protein	A0A087ZS68	<i>Apis mellifera</i>	62.0	140	3 (3)
	Catalase (fragment)	D3J8W2	<i>Limatus durhamii</i>	28.9	78	9 (2)
4	Cochineal major allergen	C4B4W5	<i>Protortonia cacti</i>	38.4	454	29 (10)
	Putative actin-related protein	A0A023EPU9	<i>Aedes albopictus</i>	42.1	144	6 (2)
	Fructose-bisphosphate aldolase	A0A069DYS3	<i>Panstrongylus megistus</i>	40.0	119	9 (3)
5	Heat shock protein	A0A097PHM0	<i>Phenacoccus solenopsis</i>	73.0	154	7 (3)
	Putative heat shock 70 kDa protein cognate 4	A0A023F9S2	<i>Triatoma infestans</i>	71.6	128	4 (2)
	heat shock protein 70-4	A0A077EU92	<i>Phenacoccus solenopsis</i>	72.0	120	5 (2)
6	GJ22855	B4LXH6	<i>Drosophila virilis</i>	23.0	103	15 (2)
	Heat shock protein 70-4	A0A077EU92	<i>Phenacoccus solenopsis</i>	72.0	78	5 (2)
	Putative thioredoxin peroxidase	A0A023FGK2	<i>Amblyomma cajennense</i>	22.3	64	12 (2)
7	NI					
8	Ubiquitin/ribosomal protein S27Ae fusion protein	Q4LAY8	<i>Eucinetus sp.</i>	18.1	408	35 (13)
9	Peroxiredoxin-2	C1C2D2	<i>Caligus clemensi</i>	22.8	75	7 (2)
10	Tropomyosin-1	A0A026WJC8	<i>Cerapachys biroi</i>	29.3	96	10 (2)
	Putative heat shock cognate 70 (fragment)	A0A023ETI6	<i>Aedes albopictus</i>	57.9	76	4 (2)
11	Putative ubiquitin/60s ribosomal protein 140 fusion (Fragment)	A0A023EDL7	<i>Aedes albopictus</i>	15.0	107	19 (2)
	Thioredoxin	A2I3V9	<i>Maconellicoccus hirsutus</i>	12.1	97	14 (3)
	Putative heat shock cognate 70 (fragment)	A0A023ETI6	<i>Aedes albopictus</i>	57.9	65	4 (2)
12	Ubiquitin/ribosomal protein S27Ae fusion protein	Q4LAY8	<i>Eucinetus sp.</i>	18.1	317	29 (17)
	Putative polyubiquitin (fragment)	T1DQT8	<i>Anopheles aquasalis</i>	18.5	281	29 (15)
	Polyubiquitin-B (fragment)	M4GY12	<i>Antricola delacruzii</i>	17.4	274	36 (15)
	Ubiquitin-40S ribosomal protein S27a (fragment)	A0A0A1XE86	<i>Bacterocera cucurbitae</i>	18.9	256	26 (14)

<sup>1</sup>Protein band numbers correspond to numbers labeled on the SDS-PAGE gels in Figure 2.6.

<sup>2</sup>NI: None identified

## CHAPTER 3

### SEQUENTIAL EXTRACTIONS OF DIVERSE INSECTS

#### 3.1 ABSTRACT

Extraction is a key step in analysis of allergens, and improper extractions can negatively impact observed IgE-binding profiles. An extraction needs to contain sufficiently representative proteins from the food but also be simple to transition for downstream processes. A mass spectrometry compatible buffer (MS buffer) and zwitterionic-chaotropic buffer (2D-gel extraction buffer) were investigated to extract and compare proteins from varied insect and control arthropod samples. MS buffer was found to be unable to extract a wide array of proteins that were extractible using 2D-gel extraction buffer. Immunoblotting of all samples with 2D-gel extraction buffer found that all samples had detected tropomyosin while extractions with MS buffer did not detect tropomyosin in German cockroach samples. Each set of extractions may be separately representative for mass spectrometry and immunoblotting.

### 3.2 INTRODUCTION

Extraction of allergens is a principal step in their analysis and likewise it is important that the extraction represents all of the allergens present. Extractions are dependent on the variable solubility of proteins. Extraction conditions can alter discrepancies between extracted proteins and proteins present in the food matrix. Optimal extraction conditions vary according to protein properties and the buffers used [1]. Extraction procedures must be adequately assessed for their representativeness and use for downstream processes.

Extractions can vary dependent on buffers and additives used as well as the physical conditions of extraction. Use of high or low pH, salts, detergents, reductants, or heat can serve to solubilize proteins [2]. Many extraction additives e.g., detergents, are unsuitable for downstream processes including mass spectrometry but subsequent removal may result in protein precipitation [3, 4]. Repetitive extraction using a mass spectrometry compatible buffer may enhance extraction of all representative proteins and allow ease of use. Zhou *et al.* assessed the use of sequentially extracting defatted, reduced peanut powder using a Tris buffer up to 5 total times and found that total protein yields improved as well as the extraction relatively insoluble proteins [5].

Extractions of varied insects can be expected to produce a variety of opposing optimal extraction conditions not unlike tree nuts, but sequential extractions may allow for more accurate representations of protein profiles across varied samples [1, 5]. Assessment of the representativeness of extractions incorporates comparisons among extraction buffers such as zwitterionic-chaotropic buffers, which are often used to

maximize solubility of proteins [6, 7]. Here, use of different buffers are examined using immunoblotting and a cross-reactive anti-shrimp tropomyosin antibody.

### 3.3 METHODS AND MATERIALS

#### 3.3.1 Materials and sample preparation

Adult *Acheta domesticus* (crickets), *Tenebrio molitor* larvae (mealworm), *Zophobas morio* larvae (superworm), *Galleria mellonella* larvae (waxworm), and *Hermetia illucens* larvae (black soldier fly) were obtained from a commercial supplier. Adult *Dactylopius coccus* (cochineal) were obtained from Chr. Hansen. *Blattella germanica* (German cockroach) and pork-medium-raised *Dermatophagoides pteronyssinus* powder (dust mite) were obtained from Greer Laboratories. Adult *Gromphadorhina portentosa* (Madagascar hissing cockroach) were a gift from the University of Nebraska-Lincoln entomology department. Headless, shell-less, deveined frozen shrimp were obtained from a local supermarket labeled as “Natural wild EZ peel gulf shrimp, 21-30 ct”. Partially purified *Litopenaeus setiferus* (shrimp) tropomyosin and purified *Argopecten irradians* (scallop) tropomyosin were gifts from Dr. Mei Lu, University of Nebraska-Lincoln. Samples were stored at -20 °C until used.

The commercially sourced shrimp samples were sent to Applied Food Technologies (AFT, Alachua, FL, U.S.) for species identification. The AFT laboratory followed the U.S. Food and Drug Administration DNA barcoding method (<http://www.fda.gov/Food/FoodScienceResearch/DNASeafoodIdentification/ucm237391.htm>). Briefly, DNA was extracted from muscle tissues and the amplified using polymerase chain reaction. Two primers used were (5'-CACGACGTTGTAAAACGACTCAACYAATCAYAAAGATATYGGCAC) and (5'-GGATAACAATTTACACAGGACTTCYGGGTGRCCRAARAATCA) [8]. The PCE products were sequenced at the University of Chicago, Cancer Research Center,

DNA Sequencing Facility. The DNA sequences of the samples were compared against the FDA Reference Standard Sequence Library for Seafood Identification database ([http://www.accessdata.fda.gov/scripts/fdcc/?set=seafood\\_barcode\\_data](http://www.accessdata.fda.gov/scripts/fdcc/?set=seafood_barcode_data)). Shrimp were identified as *Litopenaeus setiferus*.

Samples were homogenized using a mortar and pestle, the homogenate filtered through cheesecloth, and washed with 100% acetone. The mixture was stirred for 1 hour at room temperature in 5% w/v acetone, dried, then stirred for 2 hours at room temperature in 5% w/v hexane and dried resulting in a defatted powder.

### **3.3.2 Sequential extractions of powders**

Defatted powders were extracted with two protocols that were modified as described by Zhou *et al.* [5]. First, 50 mg of defatted sample with 40 mg of polyvinylpyrrolidone (PVPP) were extracted using 1 ml of a mass spectrometry compatible buffer (MS buffer; 50 mM Tris-HCl, pH 8.8, 2% v/v beta-mercaptoethanol) for 20 minutes in a heated (60 °C) sonicating water bath. Extracts were centrifuged (16 k x g, 10 minutes) and the supernatant (500 µl) decanted. Pellets were re-extracted with an equal volume of MS buffer. Extracts were stored at -20 °C until used. Each sample was extracted three sequential times and pooled samples were prepared by adding equal volumes of each of the three sequential extracts per sample (10 extracts of cochineal). Protein content was determined using a GE 2D Quant-Kit™ per manufacturer's instructions (GE Healthcare Life Sciences).

Secondly, 50 mg of defatted sample with 40 mg of PVPP was extracted using 1 ml of zwitterionic-chaotropic buffer (2D-gel extraction buffer; 50 mM Tris-HCl, pH 8.8,

10 mM ethylenediaminetetraacetic acid (EDTA), 5 M urea, 2 M thiourea, 2% w/v 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 67 mM Dithiothreitol (DTT)) for 20 minutes in a sonicating water bath (4 °C). Extracts were centrifuged and decanted as before with re-extraction by adding an equal volume of 2D-gel extraction buffer. Extracts were stored at -20 °C until used.

MS extracted cochineal supernatants optionally underwent trichloroacetic acid (TCA) precipitation by adding 15 µl of 100% fresh TCA solution to 150 µl of sequential supernatant (-20 °C, 1 hour). Samples were then centrifuged (16 k x g, 30 minutes, 4 °C), decanted, washed with chilled (-20 °C) acetone, and centrifuged (16 k x g, 10 minutes, 4 °C). TCA pellets were decanted, washed, and centrifuged a total of three times. Washed pellets were resolubilized with 2D-gel extraction buffer (120 µl, overnight, 4 °C). Samples were stored at -20 °C until used.

Remnant pellets sequentially extracted using MS buffer were re-extracted by adding 2D-gel extraction buffer (500 µl), shaken (2 hours, 25 °C), and centrifuged (16 k x g, 10 minutes). Resultant supernatants (500 µl) decanted and stored at -20 °C until used.

### **3.3.3 SDS-PAGE**

Samples were prepared with 4x sample buffer (NuPAGE, Invitrogen, Carlsbad, California, U.S.) and dithiothreitol (DTT; 175 mM) at a 9:3:2 ratio respectively, heated (95 °C, 10 minutes), cooled (4 °C, 5 minutes), and centrifuged (16 k x g, 15 seconds). Prepared samples were either used immediately or stored at -20 °C until used. Precision Plus Protein™ Dual Xtra Protstained Protein Standards (Biorad, Hercules, CA) were

used as molecular weight standards. Samples were loaded onto 4-12% gradient gels (Bis-Tris, NuPAGE), run with MES SDS running buffer (NuPAGE), and run at 200 V until the tracking dye reached the bottom (approximately 40 minutes). Gels were used for blotting or stained with Coomassie R-250 overnight with destaining overnight.

### **3.3.4 Immunoblotting**

Samples were diluted to 0.8  $\mu\text{g}/\mu\text{l}$  with the same buffer used to extract and samples were prepared for SDS-PAGE per a ratio of 50:23:20 diluted sample, 4x sample buffer, and DTT (175 mM) and 9.3  $\mu\text{l}$  loaded per lane for 4  $\mu\text{g}$  per lane. Cochineal sample concentrations were estimated at 1  $\mu\text{g}/\mu\text{l}$  and 4  $\mu\text{g}/\mu\text{l}$  for MS buffer and 2D-gel extraction buffer extracts respectively and were prepared as before. SDS-PAGE was performed as described before.

Following SDS-PAGE, immunoblotting of samples was performed as described elsewhere [9]. Rabbit anti-shrimp tropomyosin IgG (1:10,000; Indoor Biotechnologies) was used as the primary antibody with subsequent addition of goat anti-rabbit IgG conjugated with horseradish peroxidase (1:50,000; Thermo scientific, Wilmington, DE) as the secondary antibody. Shrimp allergic sera (see below) (1:10) were used as the primary antibody with subsequent addition of mouse anti-human IgE conjugated with horseradish peroxidase (1:1000; Southern Biotech, Birmingham, AL) as the secondary antibody. IgG blots were visualized after addition of DAB substrate (DAB substrate kit; Thermo) and IgE Blots were visualized after addition of chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate Kit; Thermo) both according to manufacturer's instructions and using a Kodak Gel Logic 440 image station (Eastman

Kodak, Rochester, NY) equipped with Kodak 1D v. 3.6.5 software (Kodak Scientific Imaging Systems, New Haven, CT).

Sera was from an atopic 61-year-old male with positive SPT (12 mm wheal), 3 iCAP to shrimp, and a case history of angioedema, nausea, nasal congestion, laryngeal edema, and sweating upon consumption of shrimp. This Individual was orally challenged with approximately 1 gram of cooked shrimp and found to be allergic. This Patient is also allergic to milk and other crustacean shellfish.

### 3.4 RESULTS AND DISCUSSION

#### 3.4.1 Samples and sequential extractions using MS buffer

Insects included in this study were based from both availability and reports of consumption [10-13], whereas shrimp, German cockroaches, and dust mites were included as controls based from their documented reactivity and cross-reactivity with shrimp-allergic patients (Fig 3.1) [14]. Dust mites included in this study were obtained pre-processed and powdered to minimize contamination from pork medium the mites were grown upon, potentially skewing its representativeness compared to whole samples. However, inclusion of this sample allows for perspective into extraction effects upon processed samples and were included regardless. Phylogeny of these samples suggests potential cross-reactivity is more likely among more closely related samples such as among German cockroach and Madagascar hissing cockroach (Fig 3.2) [15], however cross-reactivity between other samples are unclear. Effective investigation across varying samples requires an appropriate method for extraction of all allergens present in the sample, but methods such as sequential extraction may allow representative extraction across samples [1, 5].

Cochineal is a difficult insect to effectively extract as discussed before (Chapter 2), however the depth of the problem can be better understood using sequential extractions (Fig 3.3). Extractions of cochineal did not produce a perceptible amount of protein as judged by SDS-PAGE until repeated 6 times (Fig 3.3.A). Use of TCA precipitation to allow for greater loads in the gel without distortion indicated that there are multiple populations of proteins that differ in predominance along the extractions (Fig 3.3.B). These phenomena present with cochineal are used as rationale for the use of

sequential extractions. Sequential extractions served to decrease dye content in the supernatants, but did not eliminate presence of the dye even after a tenth extraction (Fig 3.4).

Use of sequential extractions among remaining samples demonstrates an expected dilution series across repeated extractions (Fig 3.5). Removal of supernatants and application of fresh buffer with theoretically perfectly soluble proteins will result in a strict dilution series across all proteins present in the sample, therefore any observed increases in intensity on SDS-PAGE is a result of increases of specific solubility of a protein or proteins of similar relative molecular mass. The third extraction of superworm and second extraction of dust mite demonstrate increased intensity in higher molecular weight bands. Analysis based on uniform load volumes allows for quick identification of underrepresented proteins in initial extractions and with the presence of bands of increasing intensity in later extractions. This analysis suggests that there is at least one protein less represented in initial extractions but it is more likely there are many proteins that follow trends of increasing or decreasing presence in earlier and later extractions. Sequential extraction of a single pellet thereby can more accurately average the total present soluble proteins when pooled.

### **3.4.2 Immunoblotting of MS buffer extracted samples**

Investigation of cricket and cochineal samples as further validation of the method were performed using anti-shrimp tropomyosin IgG and shrimp-allergic sera (Fig 3.6). SDS-PAGE indicates no banding present for cricket extractions and only the 45 kDa band is present in cochineal samples (Fig 3.6.A). Anti-tropomyosin antibody identifies

the presence of a band at 37 kDa across all lanes including the molecular weight markers, the identity of this reactive protein in the markers is unknown (Fig 3.6.B). Reactivity across lanes with more varied reactivity across control tropomyosins indicates the presence of reactive tropomyosins in insect samples. Immunoblotting with shrimp-allergic sera did not product any evidence of binding indicating that this patient is not reactive to tropomyosin; however, reactivity can be seen at 25 kDa in shrimp tropomyosin owing to the crude purification (Fig 3.6.C). Reactivity can also be seen in cochineal with similar patterns across preparations and higher reactivity in the first extraction compared to later extractions. Reactive cochineal bands in first extracts can be seen at approximately 150, 120, 100, 60, 45, 34, and 28 kDa with banding at 60 and 45 kDa maintained across extractions. The presence of reactive tropomyosins in all tested samples of cochineal, with shifting reactivity using shrimp-allergic sera, indicates that single extractions are inappropriate. Pooling sequential extractions will result in dilution of some reactive proteins, but also be more representative.

### **3.4.3 Re-extraction and sequential extractions using 2D-gel extraction buffer**

More representative extractions required focus on the extraction buffer itself as although a mass spectrometry compatible buffer would simplify subsequent work, it may not appropriately extract many proteins as would a zwitterionic-chaotropic buffer. Using 2D-gel extraction buffer to extract pellets previously extracted with MS buffer gives an indication of proteins that were insoluble using MS buffer (Fig 3.7). Use of MS buffer failed to appropriately extract many proteins, particularly those of higher molecular weight, and did not allow extraction of all representative proteins.

Extraction using 2D-gel extraction buffer was performed in a similar manner as MS buffer to identify any notable anomalies of intensity on SDS-PAGE (Fig 3.8). Dust mite was notably more intense as a pooled sample compared to its first extraction in opposition to other samples, which may stem from pre-processing. Use of a zwitterionic-chaotropic buffer is expected to solubilize a majority of proteins present and therefore pooling of sequential extractions will simply dilute initial extractions and result in decreased concentration as seen in samples excluding dust mite [6]. Use of equal protein loads will minimize dilution issues, but not concerns of representativeness and therefore use of sequential extractions is prudent even when used with 2D-gel extraction buffer.

#### **3.4.4 Immunoblot and comparison of buffers**

Side-by-side comparison of each buffer was carried out using anti-shrimp tropomyosin IgG (Fig 3.9). Gels show a weaker overall banding pattern using 2D-gel extraction buffer compared to MS buffer (Fig 3.9.A and C), although this is more of an effect of extracting a greater profile of proteins resulting in fewer proteins rising above the detection limit of the Coomassie stain. The lack of reactivity of MS extracted German cockroach compared to 2D extract indicates that MS buffer extractions may not as accurately represent the insect as 2D-gel extraction buffer, however it may be the case that tropomyosins extracted using MS buffer were not reactive with this antibody or no tropomyosins were extracted (Fig 3.9.B and D). Differences in the intensity of reactivity between MS and 2D-gel extraction buffers can be expected using anti-shrimp tropomyosin IgG according to the cross-reactivity of the antibody to both the present and extracted tropomyosins.

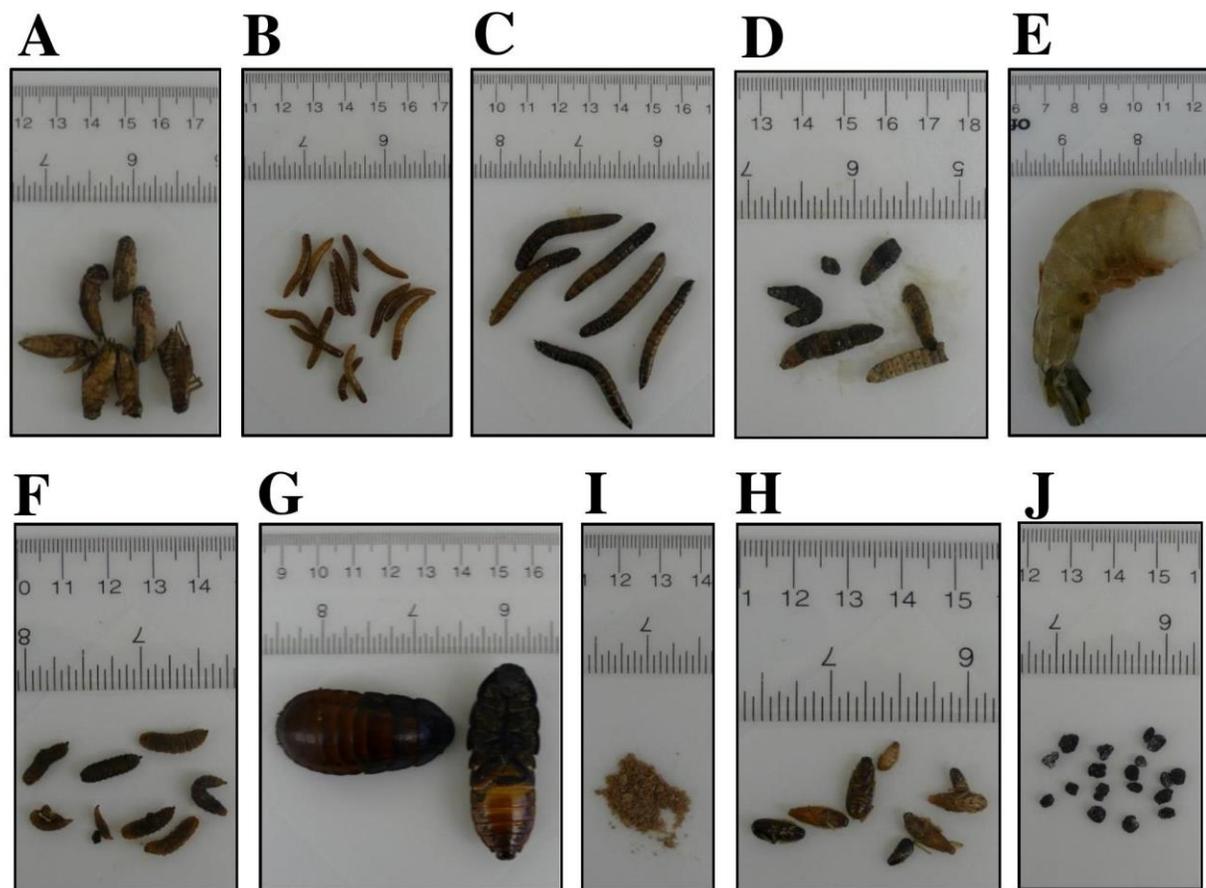
### 3.5 CONCLUSIONS

Extraction buffers were investigated with sequential extractions for representativeness of protein profiles using SDS-PAGE and immunoblot using anti-shrimp tropomyosin IgG or shrimp-allergic sera. MS buffer failed to extract many proteins, particularly those of higher molecular weight, that were visible during re-extraction using 2D-gel extraction buffer. Immunoblotting indicated that each buffer detected a 37 kDa band across all samples except German cockroach when extracted with MS buffer, which may result from either the antibody not reacting with an isoform that was extracted or no reactive isoforms were extracted. 2D-gel extraction buffer allowed enhanced extraction of all representative proteins in the samples and is applicable to future immunoblotting.

### 3.6 REFERENCES

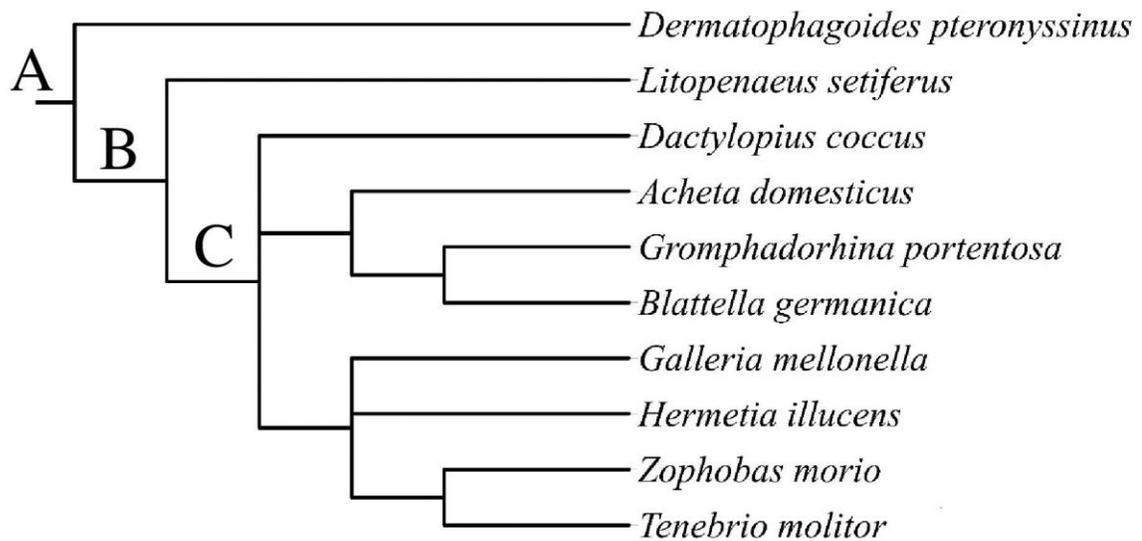
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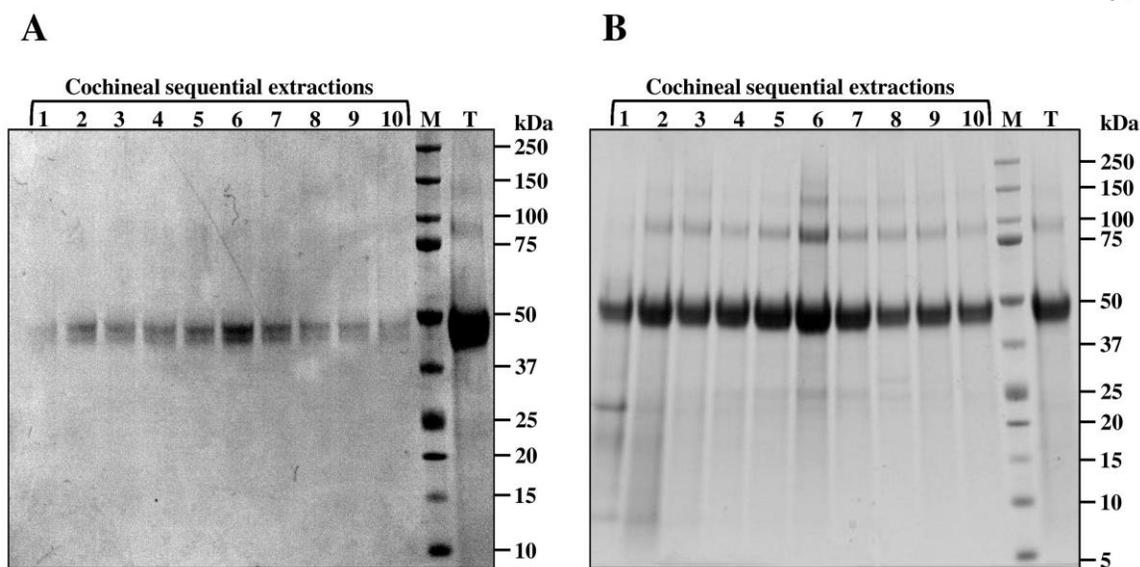
**Figure 3.1 Size and appearance of tested insects and arthropods**

Representative examples of samples tested. (A) *Acheta domesticus* cricket, (B) *Tenebrio molitor* mealworm, (C) *Zophobas morio* superworm, (D) *Galleria melonella* waxworm, (E) *Litopenaeus setiferus* shrimp, (F) *Hermetia illucens* black soldier fly, (G) *Gromphadorhina portentosa* Madagascar hissing cockroach, (H) *Dermatophagoides pteronyssinus* dust mites, (I) *Blattella germanica* German cockroach, and (J) *Dactylopius coccus* cochineal.



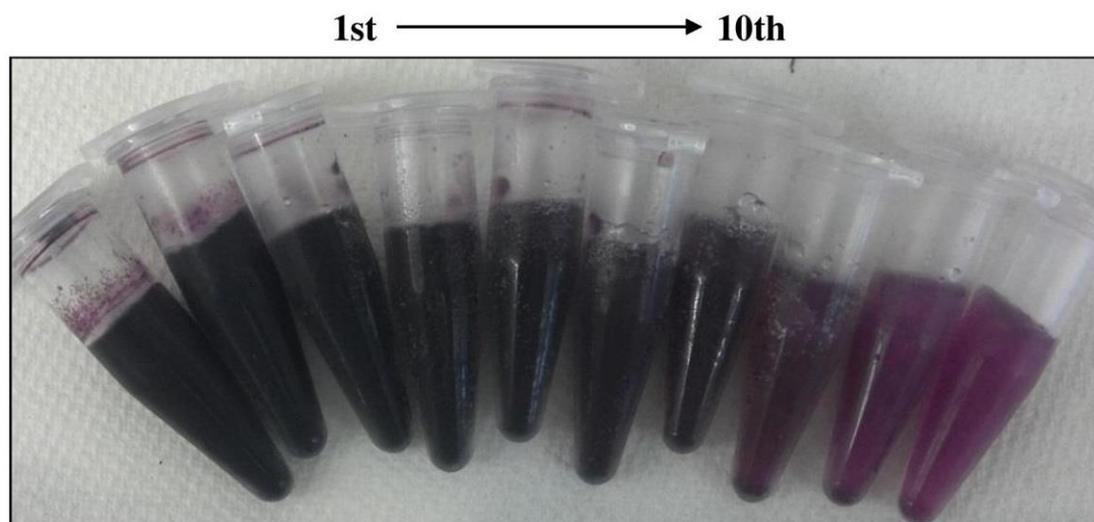
**Figure 3.2 Phylogenetic tree of samples**

Phylogeny of tested insects and arthropods. No information is to be inferred from branch length. (A) Phylum Arthropoda with *D. pteronyssinus* and separately divided (B) subphylum Crustacea with *L. setiferus* and subphylum Hexapoda and (C) class Insecta represented in the remainder. Tree was generated using Phylot according to NCBI taxonomy [16].



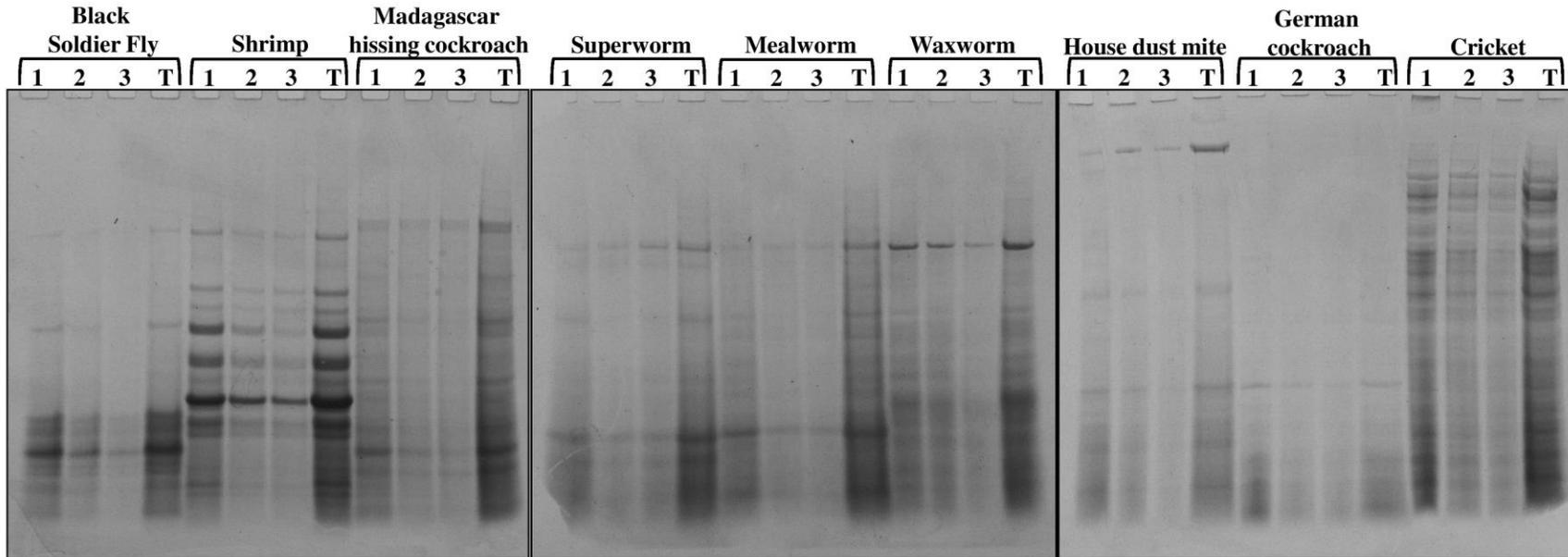
**Figure 3.3 Sequential extractions of cochineal and TCA cochineal**

First (1) to tenth (10) and pooled total (T) sequential extractions from cochineal using MS buffer. (A) 2  $\mu$ l of supernatants loaded per lane and 20  $\mu$ l of the pooled sample. Major band present peaks in the 6<sup>th</sup> extraction and falls off in subsequent extracts. (B) 20  $\mu$ l of TCA precipitated supernatants loaded per lane and 20  $\mu$ l pooled sample. Present banding appearing and disappearing across extractions as a 20 kDa band in 1<sup>st</sup> disappears by the 3<sup>rd</sup>, 25 kDa band from the 4<sup>th</sup> extraction disappears by the 9<sup>th</sup>, and 28 kDa band from the 8<sup>th</sup> is only appearing in that extraction.



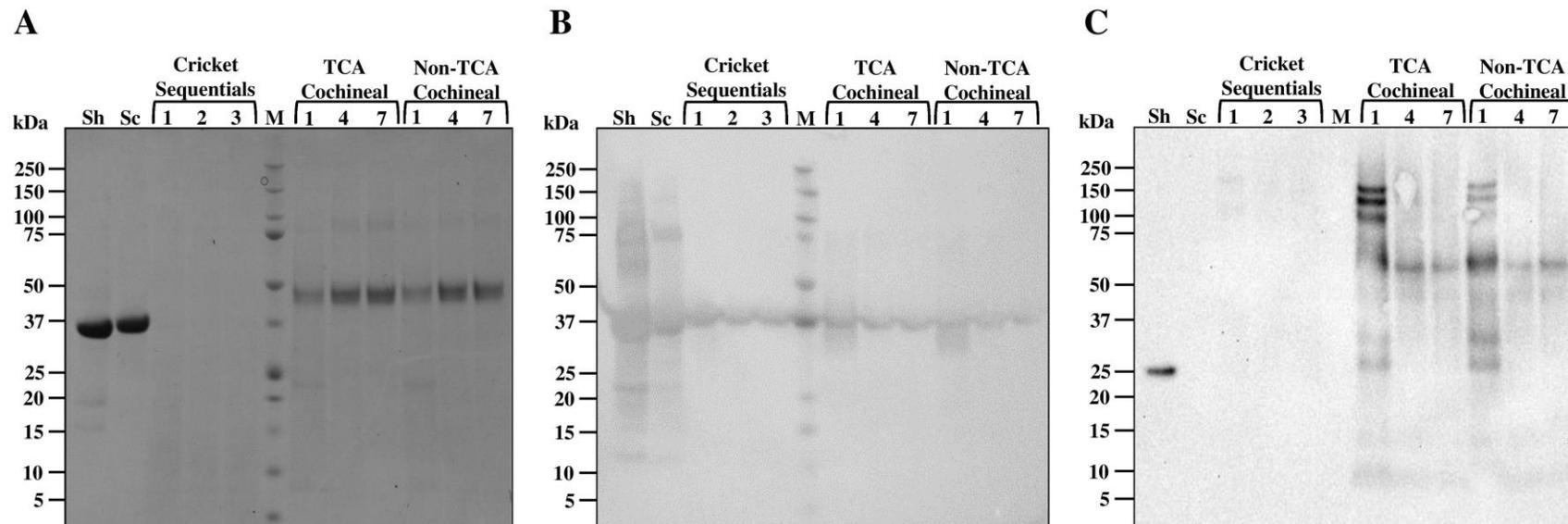
**Figure 3.4 Appearance of sequentially extracted supernatants of cochineal.**

Sequential extractions of cochineal were performed and the supernatants are shown. Supernatants show incremental decreases in the amount of dye present signified by the change in color from purple to magenta, however dye is still visibly present after 10 sequential extractions.



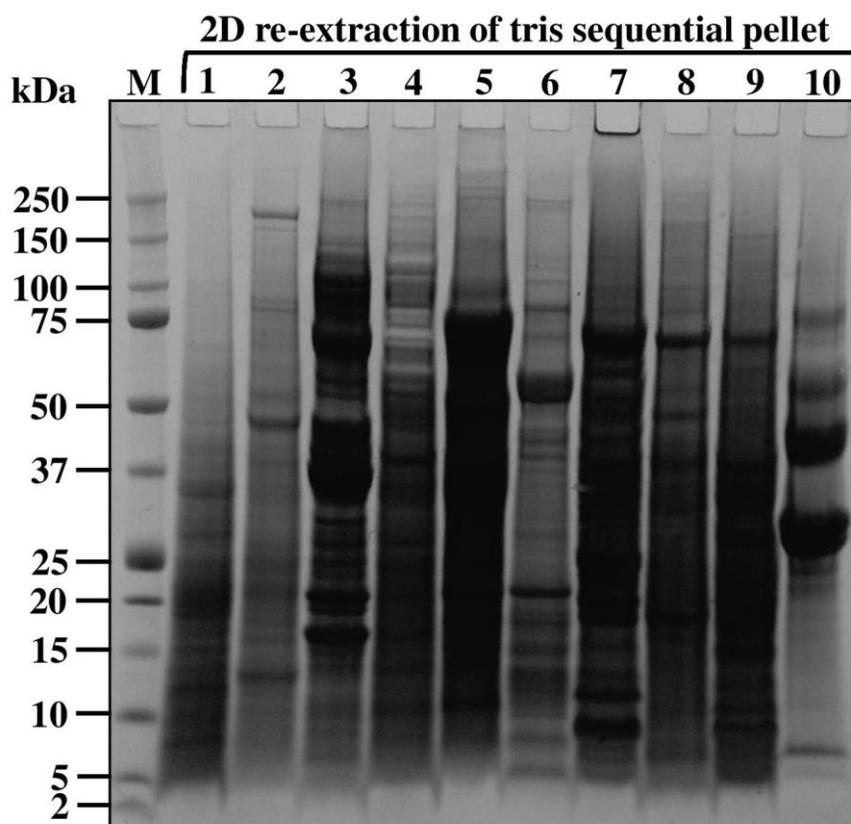
**Figure 3.5 Sequential extractions from insect and arthropod samples using MS buffer**

First to third (1, 2, and 3) sequentially extracted defatted insect and arthropod samples with representative pooled sample (T) using MS buffer. Lanes loaded with 7  $\mu$ l per sequential extraction and 21  $\mu$ l per pooled sample. Sequential extractions indicate that higher molecular weight bands of dust mite peak in intensity in the second extraction and similarly in superworm in the third extraction.



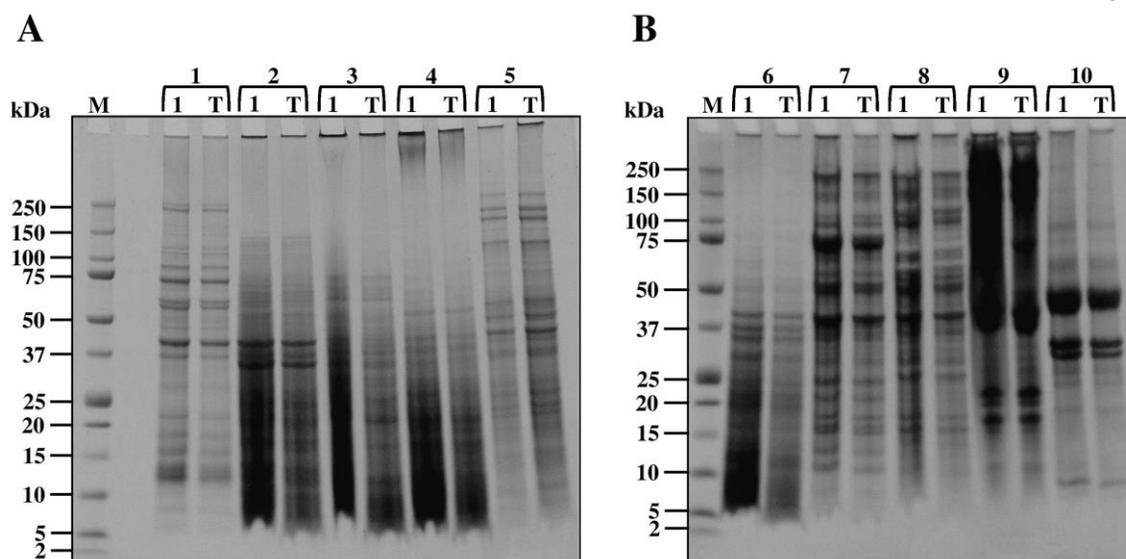
**Figure 3.6 SDS-PAGE and immunoblotting of cricket and cochineal.**

Shrimp tropomyosin (Sh), scallop tropomyosin (Sc), first to third cricket sequential extractions (1, 2, and 3), as well as first to seventh cochineal sequential extractions (1, 4, and 7) with and without TCA precipitation were used with 4  $\mu$ g per lane. (A) SDS-PAGE has imperceptible banding for all cricket extractions and similar patterns across cochineal preparations. (B) Anti-shrimp tropomyosin IgG immunoblot has reactivity across all control and insect lanes. (C) Shrimp allergic IgE immunoblot does not have reactivity similar to control anti-shrimp tropomyosin IgG and has little reactivity to cricket. Sera was highly reactive to cochineal with reactivity maintained across the 7<sup>th</sup> extraction.



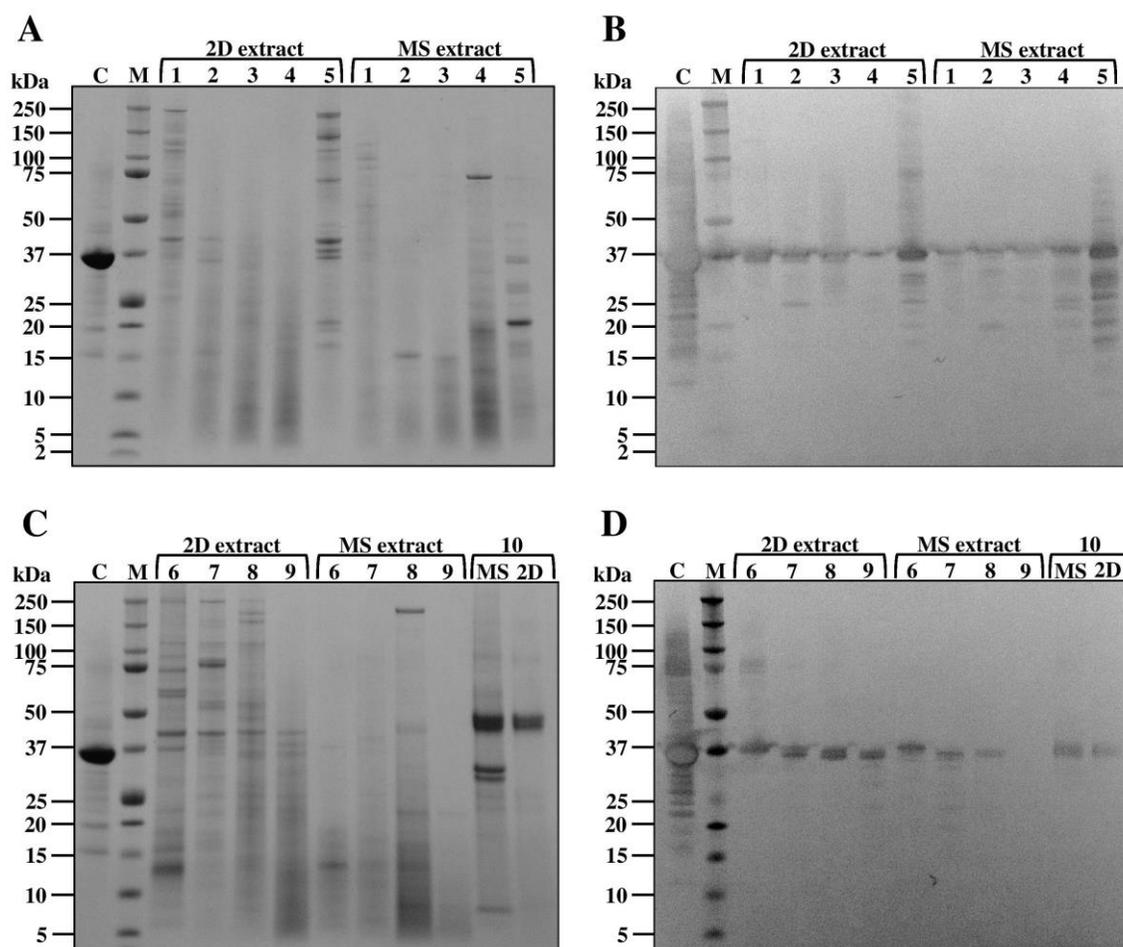
**Figure 3.7 Re-extraction of MS extracted pellets using 2D-gel extraction buffer**

Re-extraction of pellets post-MS buffer sequential extraction using 2D-gel extraction buffer with 10  $\mu$ l loads per lane with SDS-PAGE. Samples: (1) German cockroach, (2) dust mite, (3) shrimp, (4) cricket, (5) Madagascar hissing cockroach, (6) black soldier fly, (7) superworm, (8) waxworm, (9) mealworm, (10) cochineal. Re-extraction shows a preponderance of proteins that were not extracted using MS buffer, but were extracted using 2D-gel extraction buffer.



**Figure 3.8 Sequential extractions from insect and arthropod samples using 2D-gel extraction buffer**

Per sample are the (1) first sequentially extracted defatted insect and arthropod using 2D-gel extraction buffer and (T) representative pooled extract split across two gels (A and B). Extracts 2 and 3 not shown. Samples were diluted 1:4 prior to preparation for SDS-PAGE. Each lane 1 was loaded with 5  $\mu$ l and lane T loaded with 15  $\mu$ l. Samples were as follows: (1) black soldier fly, (2) mealworm, (3) superworm, (4) waxworm, (5) dust mite, (6) German cockroach, (7) Madagascar hissing cockroach, (8) cricket, (9) shrimp, (10) cochineal. Samples predominantly more intense across entire profile in first extracts as opposed to total pooled extracts. Dust mite demonstrated an increase in total pooling indicating a higher amount of total protein despite dilution effects of sequential extractions.



**Figure 3.9 SDS-PAGE and immunoblot comparison of MS and 2D-gel extraction buffers**

MS and 2D-gel extraction buffer pooled extracts visualized using SDS-PAGE (A, C) and immunoblot (B, D) using rabbit anti-shrimp tropomyosin IgG with 4  $\mu$ g of protein per lane. Samples were as follows: (C) crude purified shrimp tropomyosin, (1) cricket, (2) mealworm, (3) superworm, (4) waxworm, (5) shrimp, (6) black soldier fly, (7) Madagascar hissing cockroach, (8) dust mite, (9) German cockroach, (10) cochineal. Sample differences are apparent across extraction buffer with extractions using 2D-gel extraction buffer generally demonstrating a weaker total banding pattern compared to MS buffer. Immunoblot shows reactivity was maintained across buffers excluding MS buffer extraction of German cockroach.

## CHAPTER 4

### SHRIMP-ALLERGIC IGE CROSS-REACTIVITY WITH DIVERSE INSECTS FOCUSING ON TROPOMYOSIN

#### 4.1 ABSTRACT

Insects are a potential source of future nutrition as an alternative to conventional protein sources, but may carry risks of consumption. Shellfish is a major source of food allergy and has known cross-reactivity with cockroach and dust mite tropomyosins. Tropomyosin is an invertebrate cross-reactive allergen that may result in adverse reactions in shrimp allergic individuals. Here, immunoblotting was performed with shrimp allergic and self-reported shrimp allergic sera. Insect tropomyosins were cloned and aligned against known epitopes and the sequences used for analysis of data from liquid chromatography tandem mass spectrometry (LC-MS/MS). Among those reactive to shrimp tropomyosin there was a pattern of low reactivity to *Tenebrio molitor* (mealworm), *Zophobas morio* (superworm), and *Galleria mellonella* (waxworm). Sequence alignment of cloned partially sequenced tropomyosins found a region of high homology across a previously described epitope. LC-MS/MS indicates that waxworm contained a higher amount of homologous tropomyosin and *Acheta domesticus* (cricket) contained less, contrary to immunoblotting. The identified region of homology is not a major reactive epitope and further sequencing of tropomyosins is necessary to explain the low reactivity of mealworm, superworm, and waxworm to shrimp allergic and sensitized sera.

## 4.2 INTRODUCTION

The prevalence of shellfish allergy has been estimated at 1.91% of adults and 0.55% of children and tends to be a lifelong allergy [1, 2]. Studies have shown that children tend to have greater levels of shrimp specific IgE with a wider diversity of epitopes identified [3], and further children exposed to cockroach allergens who are sensitized to cockroach and shrimp show increased shrimp IgE [4]. Cross-reactive epitopes between shrimp, cockroach, and dust mite can contribute to higher levels of shrimp IgE [5]. However, shrimp specific IgE and positive shrimp skin prick tests can result from populations that have not been exposed to shrimp [6]. Investigation of shrimp in context of arthropod cross-reactivity is important to understand the allergy.

Tropomyosin is an allergen across invertebrates and has multiple isoforms [7, 8], which may or may not share epitopes across isoforms. Likewise, expression of isoforms may change and thereby change cross-reactive profiles [9]. Therefore, both the present epitopes, but also, the quantity of tropomyosins that carry those epitopes determine the potential cross-reactivity. A key aspect of allergy compared to sensitization is the presence of multiple epitopes that can cross-link IgE [10]. Here, immunoblotting, cloning, and semi-quantitative mass spectrometry were used to identify potential cross-reactive epitopes as well as quantify tropomyosins in a series of diverse edible insects in context of known allergenic arthropods and tropomyosins.

## 4.3 METHODS AND MATERIALS

### 4.3.1 Sample preparation and sequential extractions

Adult *Acheta domesticus* (crickets), *Tenebrio molitor* larvae (mealworm), *Zophobas morio* larvae (superworm), *Galleria mellonella* larvae (waxworm), *Litopenaeus setiferus* (shrimp), *Hermetia illucens* larvae (black soldier fly), *Gromphadorhina portentosa* (Madagascar hissing cockroach), pork-medium-raised *Dermatophagoides pteronyssinus* powder (dust mite), *Blattella germanica* (German cockroach), and adult *Dactylopius coccus* (cochineal) were obtained and prepared as previously (see 3.3.1). Purified *Litopenaeus setiferus* (shrimp) tropomyosin was a kind gift from Dr. Mei Lu, University of Nebraska-Lincoln. Sequential extractions using mass spectrometry compatible buffer (MS buffer) and zwitterionic-chaotropic buffer (2D-gel extraction buffer) were carried out as previously (see 3.3.2). Samples were stored at -20 °C until used.

### 4.3.2 SDS-PAGE and Immunoblotting

SDS-PAGE was performed as previously (see 3.3.3). Pooled 2D-gel extraction buffer sequential extractions were used for immunoblotting and were prepared as before (see 3.3.4). Immunoblotting was performed as described elsewhere [11]. Rabbit anti-shrimp tropomyosin IgG (1:4,000; Indoor Biotechnologies) was used as the primary antibody with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:4,000; Thermo scientific, Wilmington, DE) as the secondary antibody. Sera (see below) (1:10) were used as the primary antibody with mouse anti-human IgE conjugated with horseradish peroxidase (1:1,000; Southern Biotech, Birmingham, AL) used as the

secondary antibody. IgE blots were visualized after addition of chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate Kit; Thermo) according to manufacturer's instructions and using a Kodak Gel Logic 440 image station (Eastman Kodak, Rochester, NY) equipped with Kodak 1D v. 3.6.5 software (Kodak Scientific Imaging Systems, New Haven, CT).

#### **4.3.3 Human sera for immunoblotting**

A total of 16 sera were used and serological data for all subjects are summarized in Appendix 4.1. Seven sera were kindly provided by the Food Allergy Research and Resource Program at the University of Nebraska-Lincoln of which five were from subjects with clinically-confirmed shrimp allergy and two that were a control atopic and control non-atopic. A further nine sera were kindly provided by Dr. Rick Goodman of the University of Nebraska-Lincoln, which were obtained from a U.S. FDA licensed facility (PlasmaLab International, Everett, WA) from subjects who either presented with self-reported fish allergy, shellfish allergy, or positive shellfish or arthropod specific IgE ImmunoCAP<sup>®</sup> scores. Other allergies listed include other shellfish, corn, fish, eggs, "nuts", "raw vegetables", and "legumes". All individual sera were kept at -20 °C and were collected either with voluntary consent or from a U.S. FDA licensed facility.

Clinically-confirmed shrimp allergic subjects had been orally challenged with cooked shrimp and whose histories include skin-prick tests (SPT), ImmunoCAP<sup>®</sup> specific IgE values to shrimp, symptoms from consumption of shrimp, and other allergies. Specific IgE ranged from 2.55 to 81.1 kU/L and symptom history ranged from sweating or wheezing to cardiovascular dysfunction. Other allergies listed include other shellfish,

apple, banana, beans, carrot, peas, strawberry, tree nuts, milk, and peanut. Subjects were atopic adult males diagnosed allergic at 18 years or older.

Control subjects report no adverse reaction to shellfish and sera were subjected to ImmunoCAP<sup>®</sup> ISAC and the results structured by species. Atopic control (C1) reports inhalant allergies and had non-zero specific IgE to: timothy grass Phl p 1 (0.590), ragweed Amb a 1 (0.920), alternaria mold Alt a 1 (1.240), birch Bet v 1 (0.960), and apple Mal d 1 (0.460). Non-atopic control (C2) reports no allergies and had negative (<0.1 ISU) specific IgE to all tested allergens.

#### **4.3.4 RNA extractions**

Extraction and verification of quality and integrity of total RNA from all samples was performed with the assistance of Dr. Justin Marsh of the University of Nebraska-Lincoln. Samples were frozen in liquid nitrogen and immediately homogenized, using a prechilled mortar and pestle into a fine powder. RNA was isolated from tissues by incubating 100 mg of each powder with 1 ml of TRIzol<sup>®</sup> per manufacturer's instructions. RNA were resuspended in 20 µl Nuclease-free water (HyClone Laboratories, Logan, UT). Concentrations and quality of RNA were assessed using a NanoDrop 2000c (Thermo Scientific). RNA quality was assessed using a minimum 1.7  $A_{260}/A_{280}$  ratio as a criterion for further use.

RNA integrity was assessed using a Formaldehyde-MOPS gel. A 1.2% v/v Formaldehyde-MOPS gel was made by melting 1.2 g agarose in deionized water (81 ml) and adding 5.8 ml formaldehyde (Fisher, Fair Lawn, NJ) and 10 ml 10x MOPS buffer (10 mM Ethylenediaminetetraacetic acid (EDTA), 50 mM sodium acetate, 0.2 M MOPS, pH

7) in a hood. The solution and allowed to set (1 hour, room temperature) in the gel apparatus. Samples were prepared with approximately 5  $\mu\text{g}$  (in 5  $\mu\text{l}$ ) of total RNA per lane and adding a mixture of: 10  $\mu\text{l}$  of formamide (Fisher), 4  $\mu\text{l}$  of formaldehyde, 2  $\mu\text{l}$  of 10x MOPS buffer, 2  $\mu\text{l}$  0.4 mg/ml ethidium bromide, 1  $\mu\text{l}$  of bromophenol blue dye mix (0.2% w/v bromophenol blue, 10 mM EDTA, 50% v/v glycerol). Samples were heated (65 °C, 10 minutes), centrifuged (16 k x g, 5 minutes), and cooled (4 °C, 5 minutes). Samples were loaded, 1x MOPS buffer added as running buffer, and the gel run at 70 V until the tracking dye front moved through half of the gel length, and visualized using a UV transilluminator. RNA had sufficient quality if rRNA bands were distinct and lack smearing signifying RNA integrity.

#### **4.3.5 Degenerate primer design**

Degenerate primers were designed by Dr. Justin Marsh of the University of Nebraska-Lincoln. In summary, degenerate primers were designed by the following strategy: *Litopenaeus vannamei* tropomyosin (Lit v 1; NCBI ACB38288.1) was searched using tblastn (<https://blast.ncbi.nlm.nih.gov>) against individual sample organisms. Where DNA sequences are not present, searches were expanded up the taxonomic hierarchy until a match was found (Appendix 4.2). From matched queries, degenerate primers were generated targeting regions of high homology across tropomyosin (Appendix 4.3). A representative example of overall primer design strategy is provided (Figure 4.1)

#### 4.3.6 Cloning tropomyosins

Cloning of tropomyosins was principally performed by Dr. Justin Marsh of the University of Nebraska-Lincoln. Superscript<sup>®</sup> IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA) was used to obtain complimentary DNA (cDNA) from extracted total RNA per manufacturer's instructions using 1  $\mu$ g of total RNA and oligo dT primers. cDNA was used for degenerate polymerase chain reaction (PCR) using GoTaq DNA polymerase (Promega, Madison, WA) per manufacturer's instructions using 1  $\mu$ l of cDNA, 0.1  $\mu$ M up and downstream degenerate primers, PCR Nucleotide Mix (Promega), and Nuclease-free water. PCR conditions are listed in Appendix 4.4.

PCR products (20  $\mu$ l) and a DNA ladder (100 bp ladder, New England Biolabs) were visualized by adding 4  $\mu$ l of 6x DNA gel loading buffer (Novagen, Billerica, MA) and separated on a 1% w/v agarose gel. The gel was prepared by melting 0.65 g of agarose in 65 ml of 1x TAE buffer. After cooling, 2  $\mu$ l of 0.3  $\mu$ g/ml ethidium bromide was added and was allowed to set (1 hour, room temperature) in the gel apparatus. The agarose gel was run with 1x TAE buffer at 200 V until tracking dye neared the bottom of the gel. Gels were visualized using a UV transilluminator, discrete and intense bands were excised, and amplified DNA extracted using a QIAquick gel extraction kit (Qiagen, Valencia, CA). Extracted cDNA concentration and quality was assessed using a NanoDrop 2000c and underwent a second round of PCR to verify identity and quality.

Amplified DNA were ligated into a pGEM vector using a pGEM<sup>®</sup>-T Easy Vector System I per manufacturer's instructions (Promega). Ligated vectors were transformed into JM109 *E. coli* cells using heat shock transformation. Transformation proceeded by placing JM109/ligated vector-inserts cells on ice for 20 minutes, placing them in a 42  $^{\circ}$ C

water bath for 45 seconds, and returning to ice. Transformed cells were added to sterile SOC medium (Fisher) and incubated for 90 minutes at 37 °C shaking at 150 rpm. The media/cells (200 and 20  $\mu$ l) were plated onto sterile LB-agar (Fisher) with 100  $\mu$ g/ml ampicillin (IBI scientific, Peosta, Iowa), 120  $\mu$ g/ml Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG; Fisher), and 80  $\mu$ g/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal; IBI scientific) and incubated overnight at 37 °C. White colonies were picked, inoculated into sterile LB broth (Fisher) and ampicillin (100  $\mu$ g/ml), and incubated overnight at 37 °C at 220 rpm. Plasmids were purified using a Wizard<sup>®</sup> *plus* SV Minipreps DNA purification system (Promega) per manufacturer's instructions. PCR and visualization (previously described) were carried out on the plasmids to confirm orientation and successful ligation. After assessment, plasmids were sent to Genewiz (South Plainfield, NJ) for sequencing using the T7 and SP6 promoters within the vector. Obtained sequences were used for generating a new set of gene specific primers for use with 5'/3' rapid amplification of cDNA ends (RACE) (Appendix 4.5).

To obtain full-length cDNAs from total RNA (see 4.3.4), a SMARTer<sup>®</sup> RACE 5'/3' Kit (Clontech, Mountain View, CA) was used per manufacturer's instructions. Full-length cDNA underwent PCR using gene specific primers, generated from obtained sequences, in combination with the SMART primer. Nested PCR was performed in successive rounds using a nested gene specific primer and the nested SMART primer (Appendix 4.6). PCR products amplified, via 5' and 3' RACE, were ligated into pGEM<sup>®</sup>-T Easy vectors and sequenced as before. Nucleotide sequences for partial and full-length tropomyosin sequences are listed in Appendix 4.7 and listed as translated sequences in Appendix 4.8. An overlapping region of translated amino acids among sequenced

tropomyosins is listed in Appendix 4.9. Sequences were first aligned once using Clustal Omega (1.2.3) and again incorporating *Litopenaeus vannamei* tropomyosin (Lit v 1; Uniprot B4YAH6), *Blattella germanica* tropomyosin (Bla g 7; Uniprot Q9NG56), and *Dermatophagoides pteronyssinus* tropomyosin (Der p 10; Uniprot O18416) for analysis of pairwise identity.

#### 4.3.7 Mass spectrometry

Samples sequentially extracted with MS buffer, excluding cochineal, were pooled, quantified using a 2D Quant-Kit<sup>TM</sup> (GE Healthcare Life Sciences), and prepared for LC-MS/MS. Three  $\mu\text{g}$  of protein was diluted to 10.5  $\mu\text{l}$  in water (Optima<sup>TM</sup> LC-MS grade; Fisher), added 15  $\mu\text{l}$  of 50 mM ammonium bicarbonate, and reduced with 1.5  $\mu\text{l}$  of 100 mM dithiothreitol (DTT; ACROS, Fair Lawn, NJ). The mixture was centrifuged (16 k x g, 5 minutes), heated (95 °C, 5 minutes), and put on ice (30 seconds). Sample was alkylated with 3  $\mu\text{l}$  of 100 mM iodoacetamide (Sigma, St. Louis, MO) for 20 minutes at room temperature in the dark. One  $\mu\text{l}$  of 100 ng/ $\mu\text{l}$  trypsin (Promega) was added and the mixture kept at 37 °C for 3 hours and an additional 1  $\mu\text{l}$  of trypsin added maintained at 30 °C overnight. Supernants were frozen at -20°C prior to analysis. SDS-PAGE was used to verify digestion by fractionating 0.75  $\mu\text{g}$  of sample protein before and after digestion.

Following digestion, 12.8  $\mu\text{l}$  of the protein digest (93.75 ng/ $\mu\text{l}$ ) were added to 14.2  $\mu\text{l}$  of Optima<sup>TM</sup> water and 3  $\mu\text{l}$  of acidified glycogen phosphorylase (600 fmol rabbit glycogen phosphorylase B (Uniprot P00489, Sigma) in 1% v/v formic acid) to yield 100 fmol rabbit glycogen phosphorylase B per 200 ng tryptic peptides. 1D liquid chromatography separation a 5  $\mu\text{l}$  injection of tryptic peptides was performed with an

Ultimate 3000RSL® liquid chromatography (UPLC) system (Thermo Scientific), equipped with a Hypersil Gold C18 1.9  $\mu\text{m}$ , 100 x 1 mm analytical reversed phase column (Thermo Scientific).

Mass spectrometry was performed using a Thermo Fisher Q-exactive plus<sup>TM</sup> with the following MS settings: scan range 200-2000 m/z, resolution 70,000, min AGC target  $1.5 \times 10^3$ , intensity threshold  $2.5 \times 10^4$  with MS2 acquisition of the 10 most abundant targets of each MS1 scan and a 3s dynamic exclusion window. MS2 spectra were acquired using a resolution of 70,000 with an AGC target of  $1 \times 10^6$ , maximum fill time of 60ms and a normalized collision energy of 27mV.

Data analysis was performed using Proteome Discover ver. 2.1 interrogated by SEQUEST HT and parameters listed in Table 4.1 with workflow listed in Figure 4.2. In separate analyses a database of the sequenced tropomyosins and rabbit glycogen phosphorylase B were searched with all injected samples and this database was also used with a database of Arthropoda (Uniprot, accessed 21-06-2016) to search against cricket samples.

## 4.4 RESULTS AND DISCUSSION

### 4.4.1 SDS-PAGE and Immunoblotting

Representative SDS-PAGE indicates that 2D pooled extractions of diverse insects and arthropods have similarly diverse protein content (Figure 4.3.A). Representative post-transfer gel indicates that there are proteins that were not entirely transferred including some control shrimp tropomyosin, higher molecular weight bands of cricket, and a cochineal protein profile comparable to the original SDS-PAGE (Figure 4.3.B). Rabbit anti-shrimp tropomyosin IgG indicates reactivity to a 37 kDa band across all samples including a 70 kDa band in shrimp, black soldier fly, and cochineal with lesser reactivity at 70 kDa in madagascar hissing cockroach, dust mite, and german cockroach (Figure 4.4.A). Control shrimp tropomyosin has reactivity between 15 and 150 kDa with intense bands at 37, 70, and 120 kDa and diffuse banding elsewhere. Although representative SDS-PAGE indicates that all samples had broad protein content represented, anti-tropomyosin IgG shows that all samples had reactive tropomyosins and therefore to some degree share reactive epitopes. Further, remaining proteins in the gel post-transfer, particularly of control shrimp tropomyosin, suggests that these immunoblots are primarily qualitative rather than quantitative [12]. IgG also had reactivity to the molecular standards at 37 kDa and was investigated, however the identity of the markers is proprietary. Despite this, control sera indicated no reactivity to any protein (Figure 4.4.B, Figure 4.4.C).

Allergic sera indicate differential reactivity to tropomyosin as well as reactivity to samples. Non-tropomyosin reactive sera demonstrate reactivity to other proteins in shrimp, but do not similarly react with purified tropomyosin (Figure 4.5). Allergic sera

with reactivity to control tropomyosin demonstrate reactivity to all samples including the molecular standard at 37 kDa (Figure 4.6). Sera A4 has reactivity to shrimp at 25, 32, 37 and 75 kDa with discrete banding at 37 kDa with all samples excluding superworm (Figure 4.6.A). Sera A5 has lower reactivity to mealworm, superworm, waxworm, and cochineal at 37 kDa with discrete banding at 70 and 120 kDa with cricket, and 70 kDa with shrimp and black soldier fly (Figure 4.6.B). Tropomyosin is a coiled-coil dimer explaining the 75 kDa in context of the 37 kDa band [13]. Likewise, the 32 kDa bands present may also be whole tropomyosin [14].

Self-reported allergic and sensitized sera included in this study were all reactive to control tropomyosin at 37 kDa (Figure 4.7, Figure 4.8, and Figure 4.9), although minimally reactive in the case of sera S5 and S6 (Figure 4.8.B, Figure 4.8.C). Reactivity to control tropomyosin at 45 and 60 kDa present may be impurities in the control tropomyosin, tropomyosin proteolytic products, or other tropomyosin isoforms present. Although these sera were predominantly given with data of specific IgE to shellfish, sera S2 and S5 also included high specific IgE to dust mites and S7 to cockroach (Figure 4.7.B, Figure 4.8.B, and Figure 4.9.A). Serum S2 demonstrated a similar pattern to other sera with a higher reactivity to dust mite, and likewise serum S5 displayed reactivity to a 37 kDa protein of dust mite along with cochineal and cricket although with minimal reactivity to shrimp. Serum S7 demonstrates cockroach reactivity only to 37 and 55 kDa bands with strong reactivity to both shrimp and control shrimp tropomyosin.

Self-reported allergic and sensitized sera were all reactive to control tropomyosin at 37 kDa and tended to a pattern of lower reactivity to mealworm, superworm, and waxworm tropomyosins as compared to rabbit anti-shrimp tropomyosin IgG. Shrimp-

allergic sera were less in agreement with this trend, however A5 demonstrated a similar pattern. Tropomyosins are present in all samples, but there are significant differences in reactivity according to sera reactive to shrimp tropomyosin suggesting fewer reactive epitopes present either due to strictly fewer cross-reactive epitopes or less tropomyosin containing the reactive epitopes.

#### **4.4.2 Tropomyosin cloning**

Translated sequences of cloned and sequenced insect tropomyosins were aligned in Figure 4.10. These sequences were also incorporated with other tropomyosins of known allergenicity: Lit v 1, Bla g 7, Der p 10 (Figure 4.11). These pairwise identities were calculated from regions common to pairs of sequences and therefore will not reflect pairwise identity of the whole sequences in some pairings (e.g. partial waxworm tropomyosin 2 (pWW2) and partial superworm tropomyosin (pSW)). Tropomyosins cloned from the same organism were diverse: cricket 66% identity, waxworm 66% identity, mealworm 78% identity indicating that different isoforms were successfully identified. Tropomyosins from taxonomically related samples had high pairwise identities: Bla g 7 and hissing cockroach (HC) 99%, partial waxworm tropomyosin 1 (pWW1) and pSW 98%, pWW1 and partial mealworm tropomyosin 2 (pMW2) 98%, pMW2 and pSW 99%, partial shrimp tropomyosin (pSH) and Lit v 1 100%. Der p 10 diverges from other tropomyosins as being about 80% identical to others, however partial mealworm tropomyosin 1 (pMW1), pWW2, and partial cricket tropomyosin 1 (pCR1) deviate from other tropomyosins even further indicating that these are entirely separate isoforms of tropomyosin.

A region of 36 common amino acids are present in these cloned tropomyosins (Figure 4.12), and these sequences were similarly aligned using known allergenic tropomyosins (Figure 4.13). For effective comparisons of these proteins it is important for all proteins to be approximately the same length, however the disparities between clones missing either N-terminal or C-terminal ends make the use of the overlapping region prudent. Using this overlapping region, partial cricket tropomyosin 2 (pCR2), pMW2, and pSW are identical as are pWW1 and HC, and finally Lit v 1, Bla g 7, Der p 10, and pSH. Across these, excluding pCR1, pWW2, and pMW1; tropomyosins were at least 97% identical. These tropomyosins therefore had similar exons in this region [8]. Additionally, pCR1 and pWW2 were 94% identical but deviated from pMW1 suggesting that three different isoforms of tropomyosins were cloned.

The identified common region has also been previously identified as a shrimp tropomyosin epitope by peptide studies and epitope prediction, however peptides may not necessarily represent purified, native full-length proteins [3, 5, 15]. This region is shared among known allergenic tropomyosins. The lower immunoblot reactivity of waxworm, mealworm, and superworms is contrary to this theoretically shared epitope. However, it cannot be disregarded that the presence of different tropomyosins may be a function of development as waxworms, mealworms, and superworms were all larval compared to remaining samples as adults [9]. Use of this region as a proxy for full length sequences is not optimal, but serves to question if the tropomyosins containing this region are less expressed in these larval samples.

### 4.4.3 LC-MS/MS

Prior to LC-MC/MS, tryptic digests were compared to undigested protein to assess for failed trypsinolysis (Figure 4.14). Due to a low amount of protein added to the gel, only a 16 kDa band present in both mealworm and superworm were found post-digest. As the bands were not found to be reactive to any sera, they were not used as justification to alter the digestion protocol.

Alignment of shrimp and cricket cloned tropomyosin sequences along with identified peptides from an analysis of cricket shows that there are no peptides present that are unique to shrimp and that the peptides identified in shrimp were also belonging to pCR2 (Figure 4.15). The cricket tropomyosin pCR2 also contained identified peptides not shared with partial shrimp tropomyosin. Similarly, pCR1 had unique peptides identified separately from pCR2. Therefore, the cloned tropomyosins from cricket were not only identified as present through RNA but also at the protein level.

Proteins quantified according to peak area quantification were similarly normalized according to glycogen phosphorylase both according to species as well as by the normalized area of shrimp tropomyosin in Figure 4.16 and tabulated in Appendix 4.10. It is notable that glycogen phosphorylase is not a protein unique to rabbits and therefore may interfere with the quantification via variable expression of homologues across samples (Appendix 4.11), although use of all peptides instead of the top 3 quantified would somewhat mitigate the potential of common peptides artificially increasing the perceived amount of glycogen phosphorylase present. Despite this, the quantified glycogen phosphorylases were similar between samples, quantification of tropomyosins and comparisons were carried out. Shrimp contained the greatest amount of

its tropomyosin as well as the total overall of species aggregated tropomyosin, however waxworm contained was notably high for both of its tropomyosins. Cricket tropomyosins were quantified as equal or lower to superworm and mealworm, which undermines the hypothesis that the sequences as cloned contained the cross-reactive epitope and the quantification of these tropomyosins would reflect the reactivity seen by immunoblot. Additional investigation would be required to explain the disparity between immunoblots and mass spectrometry. Full-length sequences for each of the tropomyosins here would allow for a better insight into the nature of the potential cross-reactivity investigated here.

Further studies are needed to explain potential clinical cross-reactivity such as basophil assays or rat basophil leukemia cells to identify if insect tropomyosins can cross-link IgE and result in degranulation. Similarly, studies must be undertaken to identify if sensitization to insect tropomyosin could elicit allergy when subjects are challenged using shrimp tropomyosin. Such a study could be performed using a mouse model as sensitized to a purified cricket tropomyosin separately investigated for intravenous, intranasal, and oral exposure and challenged using purified shrimp tropomyosin. This would allow for routes of exposure to be investigated as well as if shrimp allergy could be induced in a non-exposed mouse.

## 4.5 CONCLUSIONS

Immunoblotting indicates a majority of sera that are minimally reactive to mealworm, superworm, and waxworm. These species share a tropomyosin isoform between 98-99% identity across their pairwise matched lengths. However, semi-quantitative LC-MS/MS suggests that waxworm contains a high amount of this shared tropomyosin, whereas cricket contains less despite cricket being similarly reactive as shrimp. The sera were largely unreactive to the identified homologous region and so may have other regions of homology across the reactive tropomyosins. Quantification of tropomyosins is contrary to the limited sequencing, therefore further sequencing of the tropomyosins is needed to identify if this shared tropomyosin isoform diverges in other known epitopes.

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DNA: atggacgccatcaagaagaagatgcaggcgatgaagctggagaaggataacgccatggac  
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DNA: agggcgataccctcgaacagcagaacaaggaggccaacaacagggctgagaagagcgag  
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DNA: ctgtctggctac  
 +1fr: ·L·S·G·Y·

**Figure 4.1 Representative overall PCR primer strategy for *L. setiferus***

As *L. setiferus* lacks DNA sequences for its tropomyosin, *Litopenaeus vannamei* tropomyosin (Lit v 1; NCBI EU410072.1) was identified via tblastn as a close taxonomic stand-in. Yellow regions were targeted for degenerate PCR as highly homologous among related tropomyosins. After sequencing, nested PCR proceeded in two rounds first green, underlined regions and second the teal region out towards 5' and 3' untranslated regions.

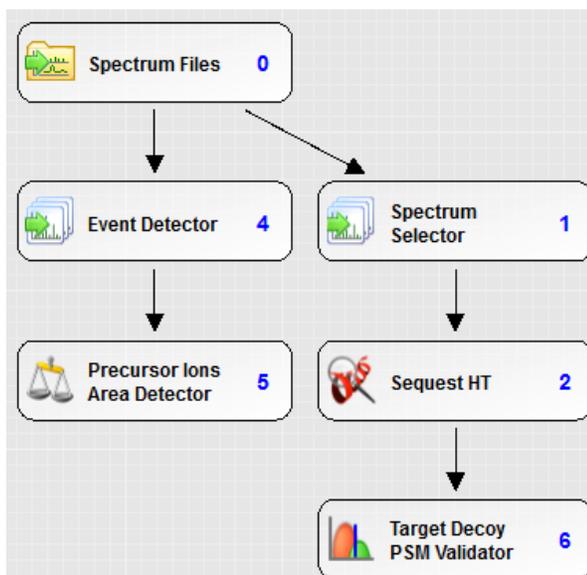
**Table 4.1 Mass spectrometry data analysis parameter settings**

<b>Analysis software</b>	Proteome discoverer 2.1
Search engine	Sequest HT
Databases	Sequenced tropomyosins, Uniprot P00489 Arthropoda (Uniprot; accessed 21-06-2016)
Enzyme	Trypsin (Full)
Maximum number of missed cleavages	0
Peptide length (min-max)	6-144
Precursor mass tolerance	5
Fragment mass tolerance	0.02 Da
Maximum Delta Cn	0.05
Target false discovery rate	0.01 strict/0.05 relaxed
Modifications	Static: carbamidomethyl cysteines (C) Dynamic: oxidized methionines (M)
Spectrum selection conditions	Total intensity threshold: 0 Minimum peak count: 1 S/N threshold: 1.5 Precursor mass range: 350-5,000 Da

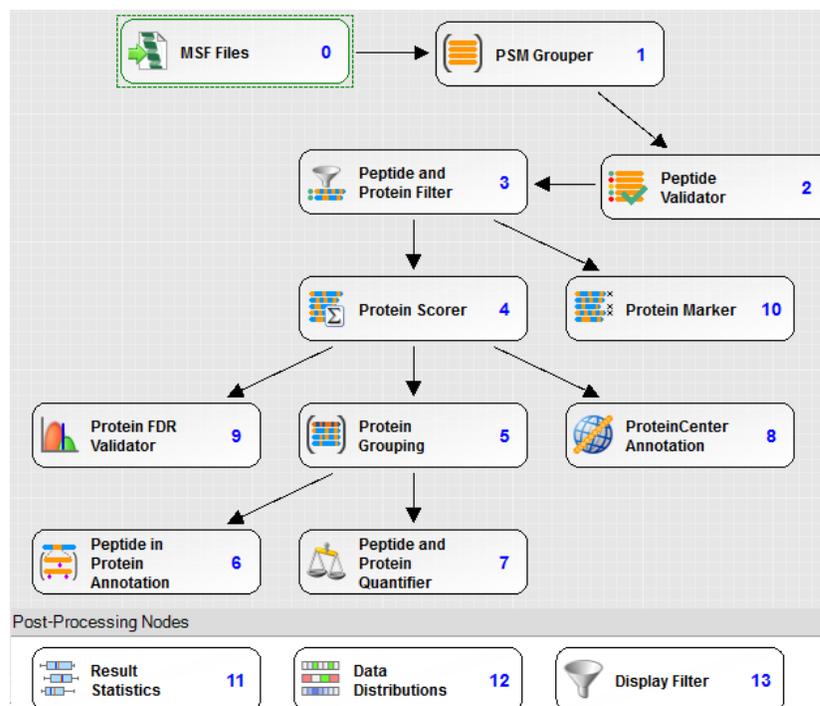
**Result Filters**

Peptide confidence	Low
Target protein false discovery rate	0.01 strict/0.05 relaxed
Top N quantification	All

A

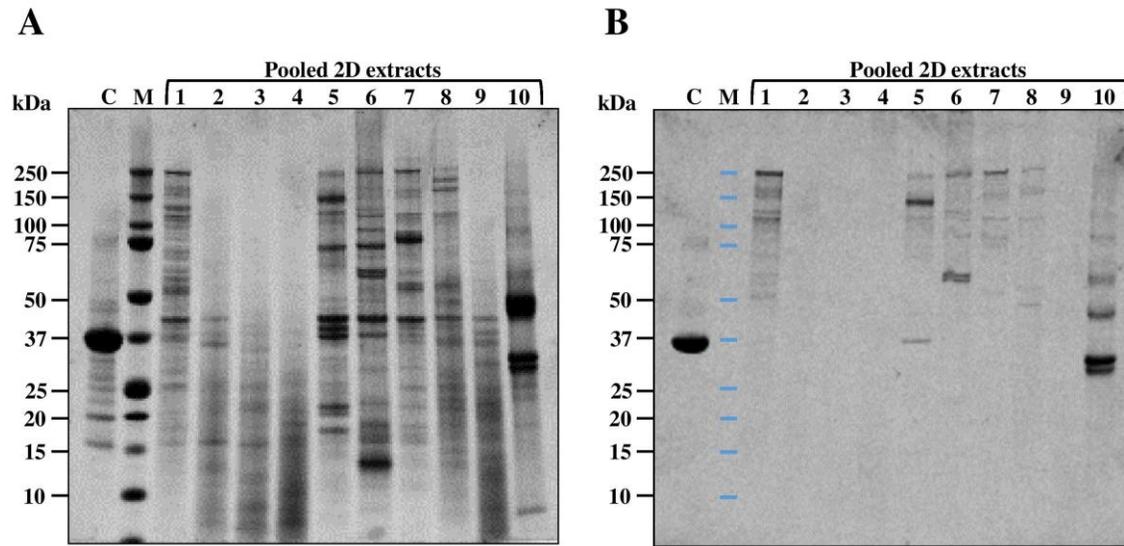


B



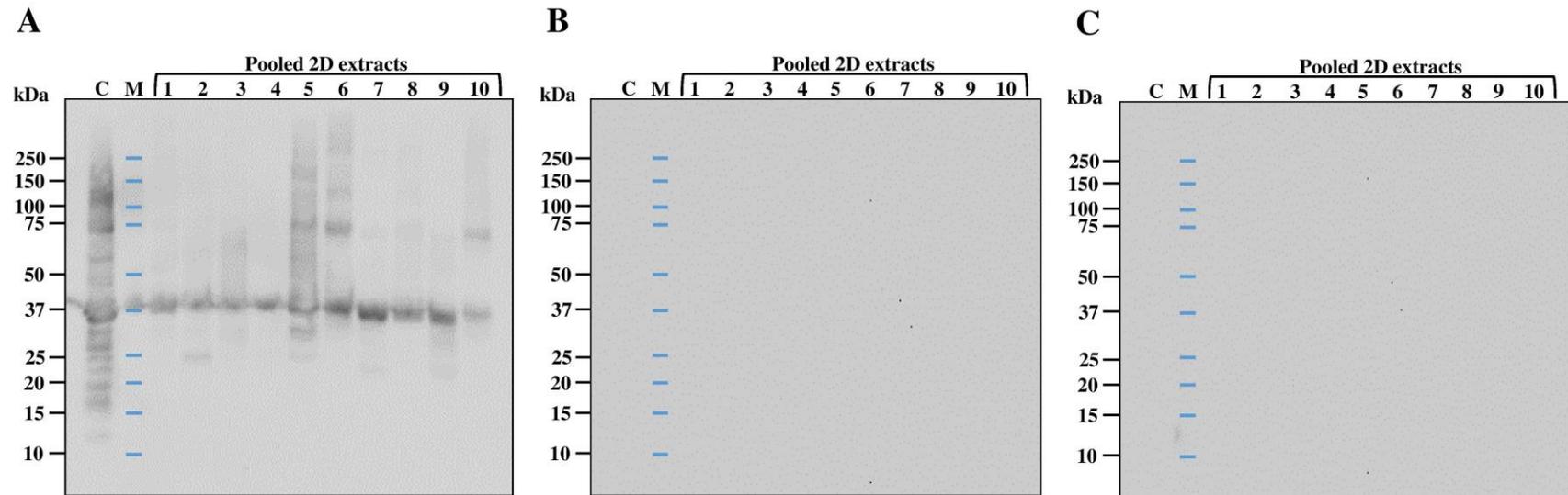
**Figure 4.2** Mass spectrometry data analysis processing and consensus work flows in Proteome Discoverer 2.1

Data analysis processing (A) and consensus (B) workflows in Proteome discoverer 2.1.



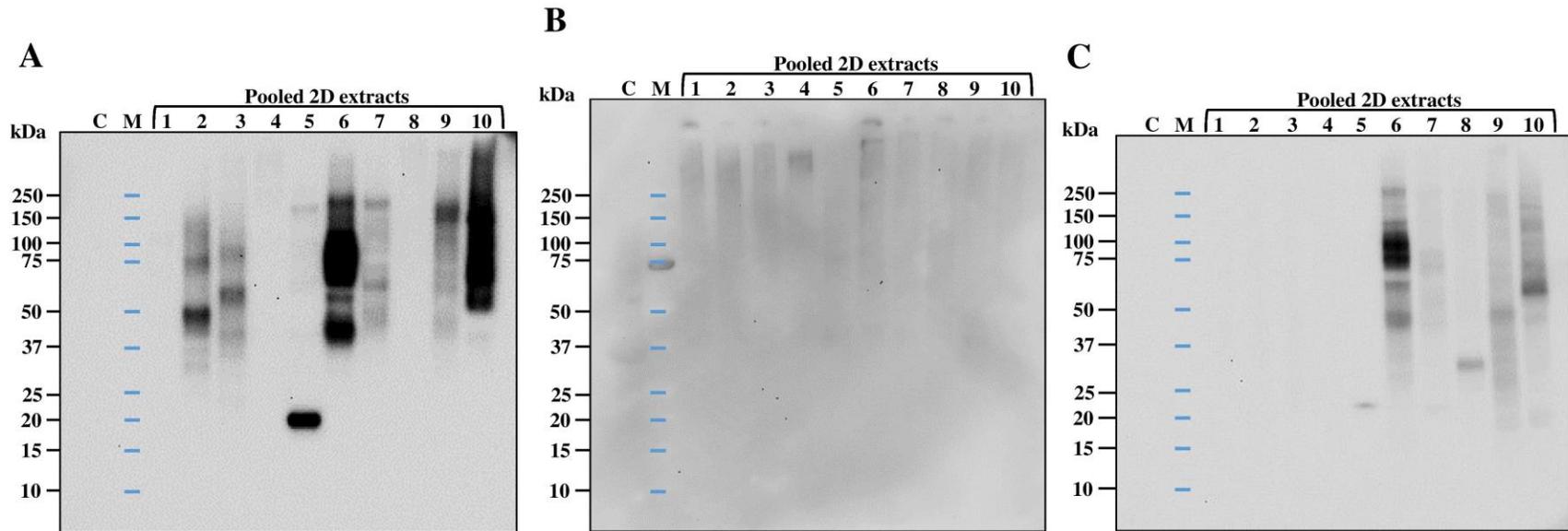
**Figure 4.3 Representative SDS-PAGE and post-transfer gel**

Four  $\mu\text{g}$  of sample protein were separated on SDS-PAGE (A) and run on SDS-PAGE, transferred onto PVDF membrane for immunoblot, and proteins remaining on the gel stained (B). Samples per lane are: control purified shrimp tropomyosin (C), molecular markers (M) and pooled 2D extractions of cricket (1), mealworm (2), superworm (3), waxworm (4), shrimp (5), black soldier fly (6), madagascar hissing cockroach (7), dust mite (8), german cockroach (9), cochineal (10).



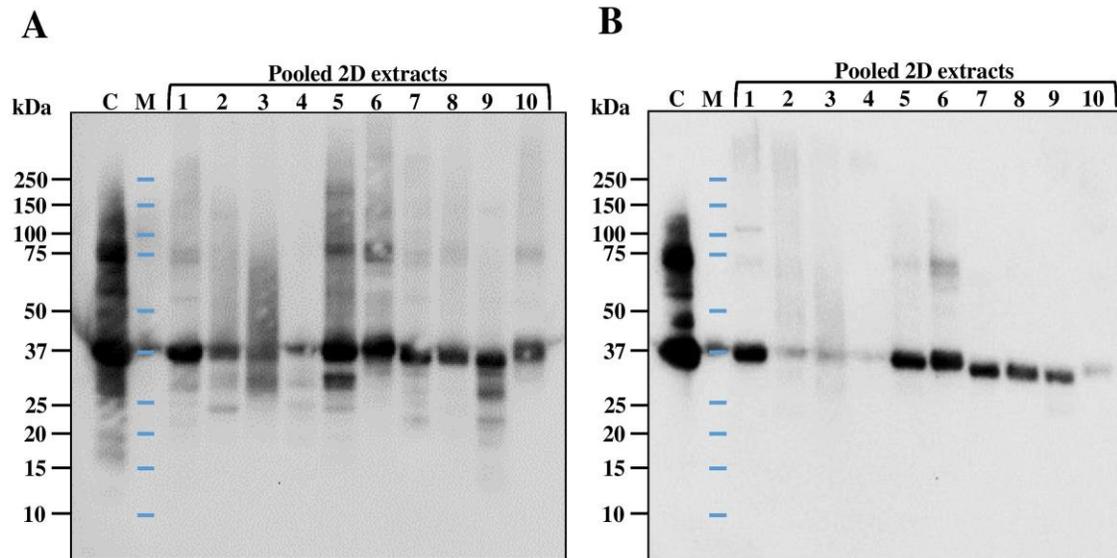
**Figure 4.4 Anti-tropomyosin and control immunoblots**

Four  $\mu\text{g}$  of sample protein were separated by SDS-PAGE, transferred to polyvinylidene fluoride and subjected to immunoblotting as follows: control immunoblots using rabbit anti-shrimp tropomyosin IgG (A), atopic control sera C1 (B), and non-atopic control sera C2 (C). Exposure times were 2 minutes each. Samples per lane are: control purified shrimp tropomyosin (C), molecular markers (M), cricket (1), mealworm (2), superworm (3), waxworm (4), shrimp (5), black soldier fly (6), madagascar hissing cockroach (7), dust mite (8), german cockroach (9), cochineal (10). Anti-tropomyosin IgG shows reactivity to a 37 kDa band across all samples including higher and lower molecular weights. Control sera have no reactivity to any protein.



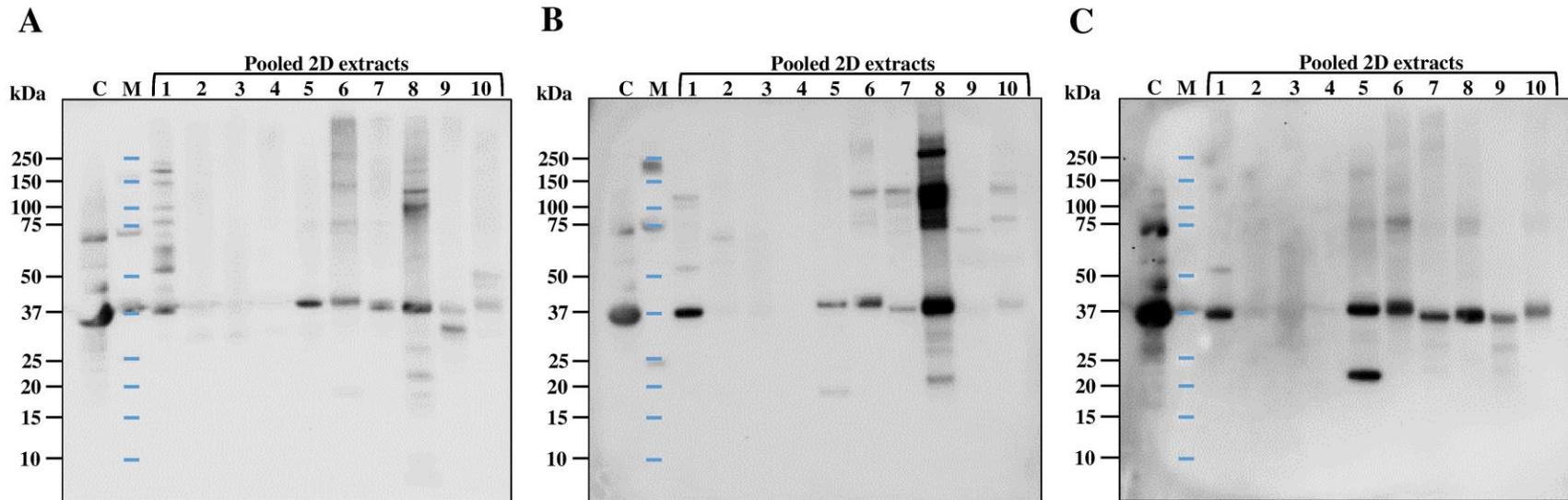
**Figure 4.5 Non-tropomyosin reactive shrimp allergic sera**

Four  $\mu\text{g}$  of sample protein were separated by SDS-PAGE, transferred to polyvinylidene fluoride and subjected to immunoblotting as follows: immunoblots using shrimp-allergic sera A1 (A), A2 (B), and A3 (C). Exposure times were 2, 5, and 2 minutes respectively. Samples per lane are: control purified shrimp tropomyosin (C), molecular markers (M), and pooled 2D extractions of cricket (1), mealworm (2), superworm (3), waxworm (4), shrimp (5), black soldier fly (6), madagascar hissing cockroach (7), dust mite (8), german cockroach (9), cochineal (10). No sera were reactive to control shrimp tropomyosin, but A1 and A3 were reactive to shrimp at 20 kDa and 22 kDa respectively.



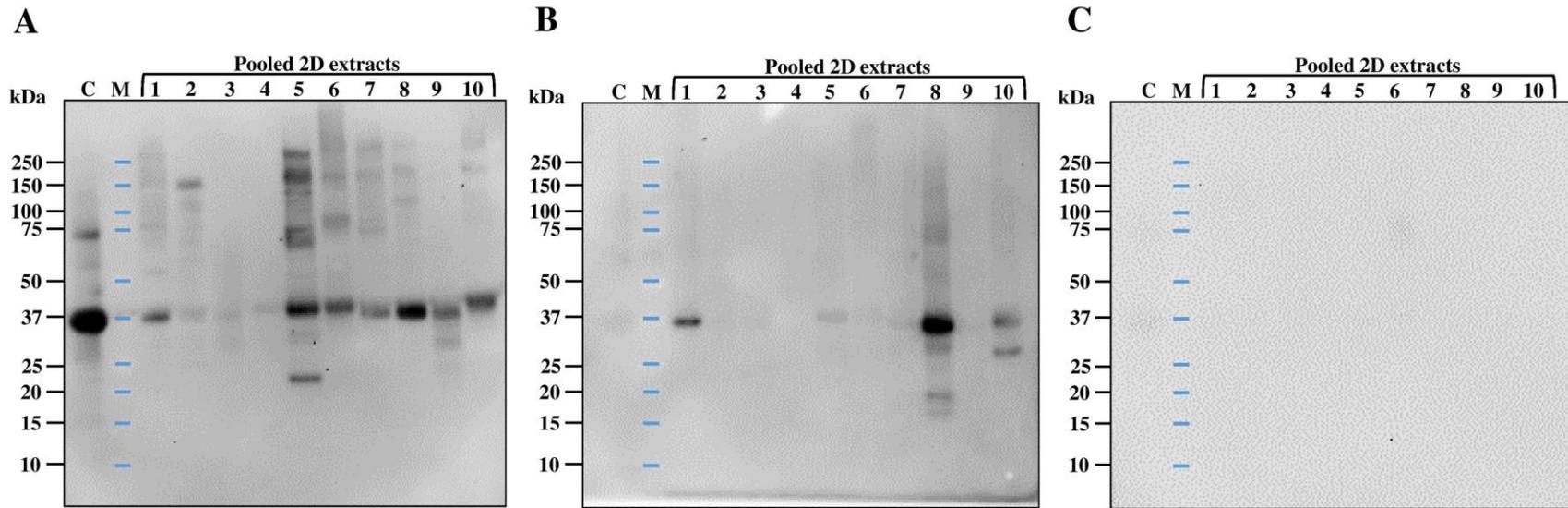
**Figure 4.6 Tropomyosin reactive shrimp allergic sera**

Four  $\mu\text{g}$  of sample protein were separated by SDS-PAGE, transferred to polyvinylidene fluoride and subjected to immunoblotting as follows: immunoblots using shrimp-allergic sera A4 (A) and A5 (B). Exposure times were 1 and 2 minutes respectively. Samples per lane are: control purified shrimp tropomyosin (C), molecular markers (M), and pooled 2D extractions of cricket (1), mealworm (2), superworm (3), waxworm (4), shrimp (5), black soldier fly (6), madagascar hissing cockroach (7), dust mite (8), german cockroach (9), cochineal (10). Both sera were reactive to control shrimp tropomyosin with sera A5 showing lesser reactivity to mealworm, superworm, waxworm, and cochineal.



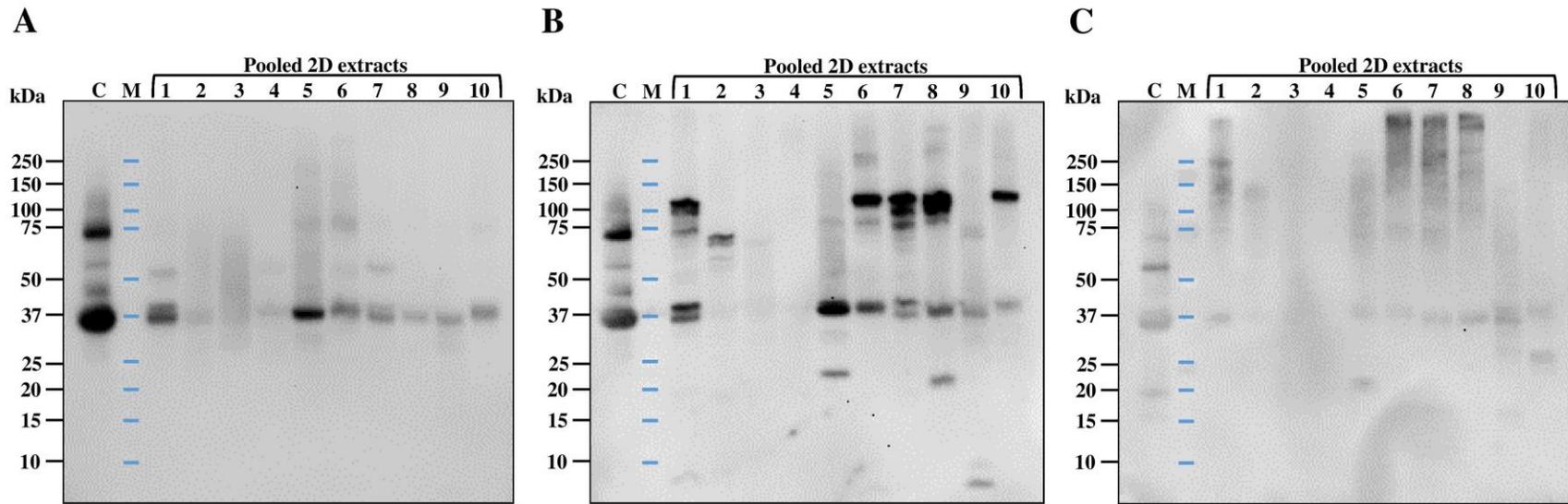
**Figure 4.7 Self-reported shrimp reactive sera 1**

Four  $\mu$ g of sample protein were separated by SDS-PAGE, transferred to polyvinylidene fluoride and subjected to immunoblotting as follows: immunoblots using shrimp-allergic sera S1 (A), S2 (B), and S3 (C). Exposure times were 2, 2, and 10 minutes respectively. Samples per lane are: control purified shrimp tropomyosin (C), molecular markers (M), and pooled 2D extractions of cricket (1), mealworm (2), superworm (3), waxworm (4), shrimp (5), black soldier fly (6), madagascar hissing cockroach (7), dust mite (8), german cockroach (9), cochineal (10). Sera all reactive to control shrimp tropomyosin with all showing lower reactivity to mealworm, superworm, and waxworm.



**Figure 4.8 Self-reported shrimp reactive sera 2**

Four  $\mu$ g of sample protein were separated by SDS-PAGE, transferred to polyvinylidene fluoride and subjected to immunoblotting as follows: immunoblots using shrimp-allergic sera S4 (A), S5 (B), and S6 (C). Exposure times were 5, 10, and 10 minutes respectively. Samples per lane are: control purified shrimp tropomyosin (C), molecular markers (M), and pooled 2D extractions of cricket (1), mealworm (2), superworm (3), waxworm (4), shrimp (5), black soldier fly (6), madagascar hissing cockroach (7), dust mite (8), german cockroach (9), cochineal (10). Sera S4 reactive to samples at 37 kDa excluding mealworm, superworm, and waxworm. Sera S5 minimally reactive to control tropomyosin, but more reactive to cricket, dust mite, and cochineal at 37 kDa. Sera S6 minimally reactive to control tropomyosin with only other banding to black soldier fly above 75 kDa.



**Figure 4.9 Self-reported shrimp reactive sera 3**

Four  $\mu\text{g}$  of sample protein were separated by SDS-PAGE, transferred to polyvinylidene fluoride and subjected to immunoblotting as follows: immunoblots using shrimp-allergic sera S7 (A), S8 (B), and S9 (C). Exposure times were 10, 5, and 5 minutes respectively. Samples per lane are: control purified shrimp tropomyosin (C), molecular markers (M), and pooled 2D extractions of cricket (1), mealworm (2), superworm (3), waxworm (4), shrimp (5), black soldier fly (6), madagascar hissing cockroach (7), dust mite (8), german cockroach (9), cochineal (10). Sera each reactive to control shrimp tropomyosin with even reactivity at 37 kDa across all samples excluding mealworm, superworm, and waxworm.

Partial cricket TM1	MDAIKKKMQAMKLEKDNAMDKADTCEGQAKDANNKADKINEDVQELTKKLAQVENDLITT	60
Partial cricket TM2	MDAIKKKMQAMKLEKDNAMDRALLCEQQARDANLRAEKAESEEARGLQKKIQTIENELDQT	60
Hissing cockroach TM	MDAIKKKMQAMKLEKDNAMDRALLCEQQARDANLRAEKAESEEARSLQKKIQQIENDLDQT	60
Partial waxworm TM1	MDAIKKKMQAMKLEKDNALDRAAMCEQQAKDANLRAEKAESEEARQLQKKIQTIENDLDQT	60
Partial waxworm TM2	MDAIKKKMQAMKLEKDNAMDKADTCEQQARDANLRAEKVNEEVRELQKKLAQVEEDLTLN	60
Partial shrimp TM	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV	60
Partial mealworm Tm1	-----	
Partial mealworm Tm2	-----	
Partial superworm TM	-----	
Partial cricket TM1	KANLEQANKDLEDKEKALQAAESEMAALNRKVQLVEEDLERSEERAATAATKLQEASEAA	120
Partial cricket TM2	QEQLMQVNAKLEEKDKALQTAEGETIAALNRRIQLLEEDLERSEERLATATAKLAEASQAA	120
Hissing cockroach TM	LEQLMQVNAKLEEKDKAFQNAESEVAALNRRIQLLEEDLERSEERLATATAKLAEASQAA	120
Partial waxworm TM1	QESLMQVNGKLEEKEKALQNAESEVAALNRRIQLLEEDLERSEERLATATAKLSEASQAA	120
Partial waxworm TM2	KNKLDQANKDLEEREKQLAATEAEVASLNRKVQQIEEDLEKSEERSGTAQQKLLAEASQAA	120
Partial shrimp TM	QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAEASQAA	120
Partial mealworm Tm1	-----	
Partial mealworm Tm2	-----	
Partial superworm TM	-----	
Partial cricket TM1	DEAQRMCVKLENRSQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRKLAFVEDELEVAE	180
Partial cricket TM2	DESERARKILENRSLADEERMDALENQLKEARFLAEEADKKYDEVARKLAMVEADLERAE	180
Hissing cockroach TM	DESERARKILESGLADEERMDALENQLKEARFMAEEADKKYDEVARKLAMVEADLERAE	180
Partial waxworm TM1	DESERARKVLENRSGLADEERMDALENQLKEARFLAEEADKKYDEVARKLAMVEADLERAE	180
Partial waxworm TM2	DENNRMCVKLENRAQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRKLAFVEDELEVAE	180
Partial shrimp TM	DESERMVKVLENRSLSDEERMDALENQLKEARFLAEEADRKYDEVARKLAMVEADLERAE	180
Partial mealworm Tm1	-----AKLIAEEADKKYEEVARKLVLMEQDLERAE	30
Partial mealworm Tm2	-----RKLAMVEADLERAE	14
Partial superworm TM	-----EARFLAEEADKKYDEVARKLAMVEADLERAE	31
	***.:*:** **	
Partial cricket TM1	DRVKSGDSKIMELEEEELKVVGNSLK-----	205



Pairwise Percent Identity

1	2	3	4	5	6	7	8	9	10	11	12		
-	80	60	63	68	68	66	66	69	70	66	69	1	pCR1
	-	58	64	65	65	65	64	67	68	64	66	2	pWW2
		-	72	75	74	76	79	78	79	79	79	3	pMW1
			-	80	80	81	80	81	81	79	81	4	Der p 10
				-	100	82	83	92	92	83	85	5	pSH
					-	82	82	85	87	82	85	6	Lit v 1
						-	92	92	95	91	91	7	pCR2
							-	88	90	99	91	8	Bla g 7
								-	99	89	98	9	pMW2
									-	90	98	10	pSW
										-	92	11	HC
											-	12	pWW1

**Figure 4.11 Full-length pairwise identities of sequenced tropomyosins and known allergenic tropomyosins**

Full-length pairwise percent identity alignments using Clustal O (version 1.2.3) of sequenced tropomyosins and allergenic tropomyosins. Numbers on the top row and right side relate to the designations on the right. Remaining numbers in colored boxes correspond to the pairwise percent identity. Boxes are colored according to percent identity on a gradient from 60 to 100% identity from white to red. Samples are as follows: *Litopenaeus vannamei* tropomyosin Lit v 1 (Uniprot B4YAH6), *Blattella germanica* tropomyosin Bla g 7 (Uniprot Q9NG56), and *Dermatophagoides pteronyssinus* tropomyosin Der p 10 (Uniprot O18416), partial cricket tropomyosin 1 (pCR1), partial waxworm tropomyosin 2 (pWW2), partial mealworm tropomyosin 1 (pMW1), partial shrimp tropomyosin (pSH), partial cricket tropomyosin 2 (pCR2), partial mealworm tropomyosin 2 (pCR2), partial superworm tropomyosin (pSW), hissing cockroach tropomyosin (HC), and partial waxworm tropomyosin 1 (pWW1).

Partial cricket tropomyosin 1 trimmed	RKLAFVEDELEVAEDRVKSGDSKIMELEEEELKVVGN	36
Partial waxworm tropomyosin 2 trimmed	RKLAFVEDELEVAEDRVKSGDAKISELEEEELKVVGN	36
Partial cricket tropomyosin 2 trimmed	RKLAMVEADLERAEERAEAGESKIVELEEEELRVVGN	36
Partial mealworm tropomyosin 2 trimmed	RKLAMVEADLERAEERAEAGESKIVELEEEELRVVGN	36
Partial superworm tropomyosin trimmed	RKLAMVEADLERAEERAEAGESKIVELEEEELRVVGN	36
Hissing cockroach tropomyosin trimmed	RKLAMVEADLERAEERAESGESKIVELEEEELRVVGN	36
Partial waxworm tropomyosin 1 trimmed	RKLAMVEADLERAEERAESGESKIVELEEEELRVVGN	36
Partial shrimp tropomyosin trimmed	RKLAMVEADLERAEERAETGESKIVELEEEELRVVGN	36
Partial mealworm tropomyosin 1 trimmed	RKLVLMEQDLERAEERAEQSESKIVELEEEELRVVGN	36
	***.::* :** **:*.: .::** *****:****	

**Figure 4.12 Alignment of 36 amino acid overlap of sequenced tropomyosins**

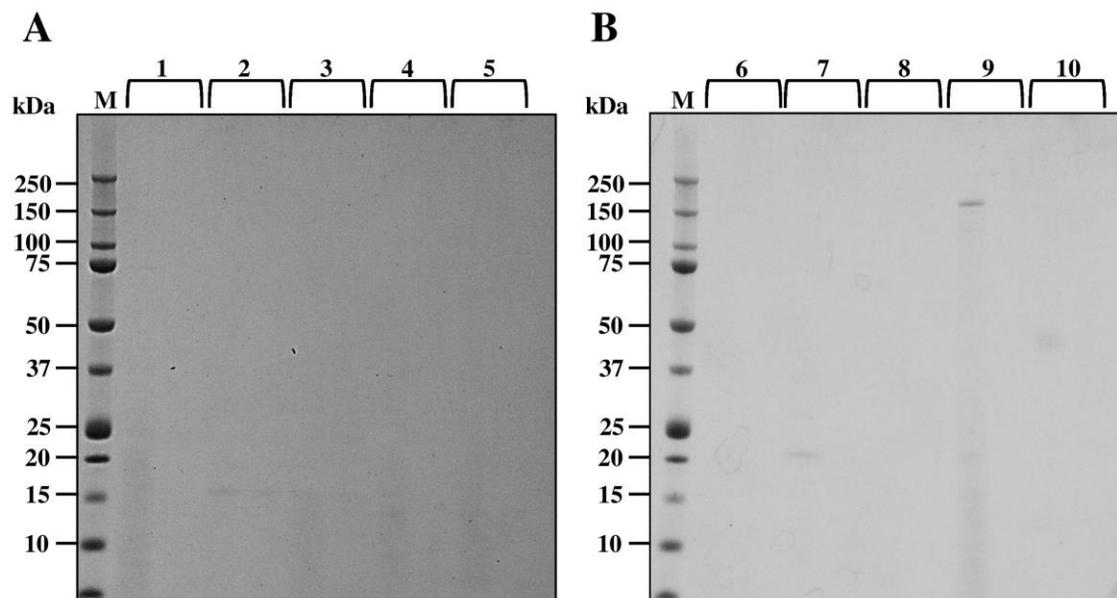
Alignment of 36 amino acid overlap of sequenced tropomyosins from cricket, madagascar hissing cockroach, waxworm, shrimp, mealworm, and superworm using Clustal O (version 1.2.3). Sequence overlaps for tropomyosins cricket 2, mealworm 2, and superworm were identical as were tropomyosins waxworm 1 and hissing cockroach.

Pairwise Percent Identity

1	2	3	4	5	6	7	8	9	10	11	12		
-	94	69	69	69	72	72	69	69	69	69	61	1	pCR1
	-	67	67	67	69	69	67	67	67	67	58	2	pWW2
		-	100	100	97	97	97	97	97	97	83	3	pCR2
			-	100	97	97	97	97	97	97	83	4	pMW2
				-	97	97	97	97	97	97	83	5	pSW
					-	100	97	97	97	97	83	6	HC
						-	97	97	97	97	83	7	pWW1
							-	100	100	100	83	8	pSH
								-	100	100	83	9	Lit v 1
									-	100	83	10	Der p 10
										-	83	11	Bla g 7
											-	12	pMW1

**Figure 4.13 Common 36 amino acid pairwise identities of sequences tropomyosins and allergenic tropomyosins**

Common 36 amino acid pairwise percent identity alignments using Clustal O (version 1.2.3) of sequenced tropomyosins and allergenic tropomyosins. Numbers on the top row and right side relate to the designations on the right. Remaining numbers in colored boxes correspond to the pairwise percent identity. Boxes are colored according to percent identity on a gradient from 60 to 100% identity from white to red. Samples are as follows: *Litopenaeus vannamei* tropomyosin Lit v 1 (Uniprot B4YAH6), *Blattella germanica* tropomyosin Bla g 7 (Uniprot Q9NG56), and *Dermatophagoides pteronyssinus* tropomyosin Der p 10 (Uniprot O18416), partial cricket tropomyosin 1 (pCR1), partial waxworm tropomyosin 2 (pWW2), partial mealworm tropomyosin 1 (pMW1), partial shrimp tropomyosin (pSH), partial cricket tropomyosin 2 (pCR2), partial mealworm tropomyosin 2 (pCR2), partial superworm tropomyosin (pSW), hissing cockroach tropomyosin (HC), and partial waxworm tropomyosin 1 (pWW1).



**Figure 4.14 Tryptic digestion control**

0.75  $\mu\text{g}$  sample protein before (sample left) and after (sample right) digestion were separated on SDS-PAGE separated on two gels (A and B). Samples per lane are: molecular markers (M), and pooled MS extractions of cricket (1), mealworm (2), superworm (3), waxworm (4), shrimp (5), black soldier fly (6), madagascar hissing cockroach (7), dust mite (8), german cockroach (9), cochineal (10). Mealworm and superworm had a 16 kDa band that was resistant to tryptic digestion.

```

pCr1_TM      MDAIKKKMQAMKLEKDNAMDKADTCEGQAKDANNKADKINEDVQELTKKLAQVENDLIT 60
pCr2_TM      MDAIKKKMQAMKLEKDNAMDRALLCEQQARDANLRAEKAE EEEARGLQKKIQTIENELDQT 60
pSh_TM       MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV 60
              *****:* * * ::* * ::* * :*:. * *:. :*:* * .

pCr1_TM      KANLEQANKDLEDKEKALQAAESEMAALNRKVQVVEEDLERSEERAATAATKLEQASEEA 120
pCr2_TM      QEQLMQVNAKLEEKDKALQTAEGEIAALNRRIQLLEEDLERSEERLATATAKLAEASQAA 120
pSh_TM       QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAEASQAA 120
              : . * :. * . * :*:***. **.*:*****:~*:~***** **.:** ***:**

pCr1_TM      DEAQRMCKVLENRSQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRKLAFVEDELEVAE 180
pCr2_TM      DESERARKILENRSLADEERMDALENQLKEARFLAEEADKKYDEVARKLAMVEADLERAE 180
pSh_TM       DESERMCKVLENRSLSDEERMDALENQLKEARFLAEEADRKYDEVARKLAMVEADLERAE 180
              **.:* * :***** ***** * *****:~*:~** * ***:~*:~** :** **

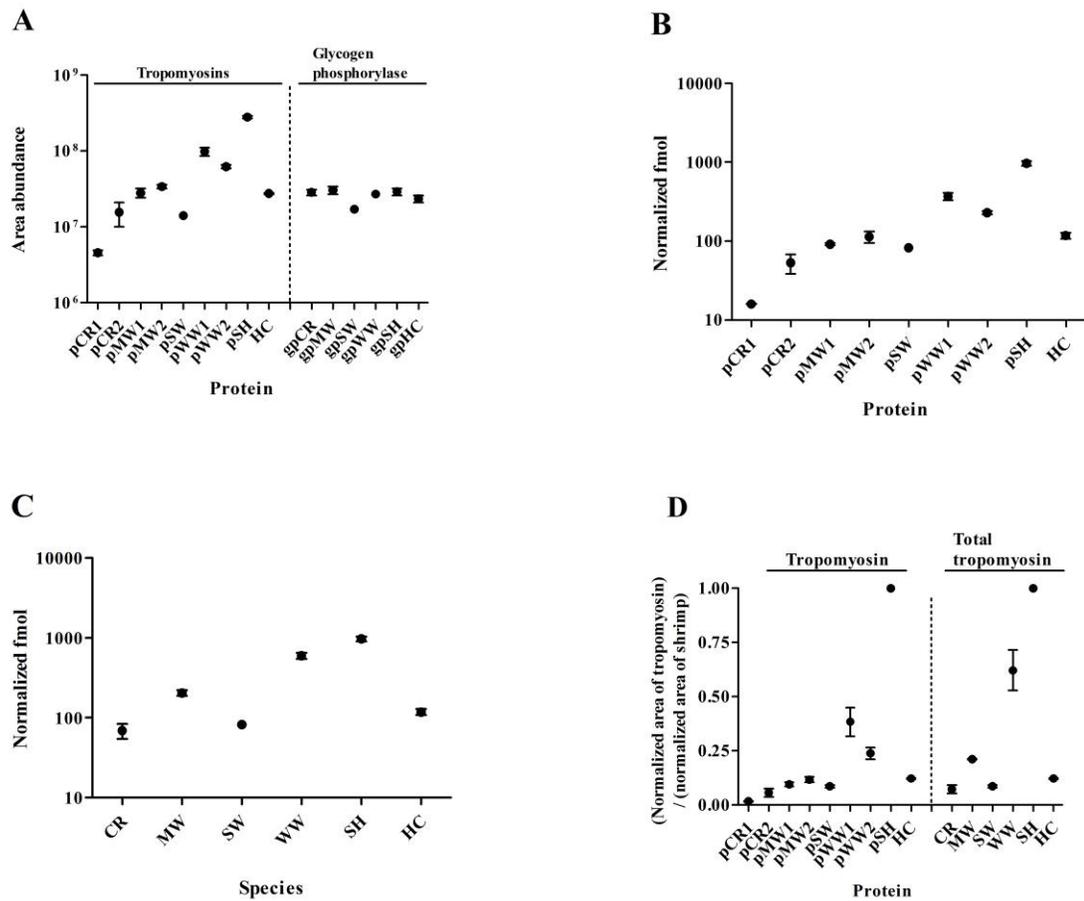
pCr1_TM      DRVKS GDSKIMELEEEELKVVGNLK----- 205
pCr2_TM      ERAEAGESKIVELEEEELRVVNXPE----- 205
pSh_TM       ERAETGESKIVELEEEELRVVGNNLKSLEVSEEKANQREEAYKEQIKTLTNKLKAAEARAE 240
              :* . :*:~*:~*****:***** :

pCr1_TM      -----
pCr2_TM      -----
pSh_TM       FAERSVQKLQKEVDRLEDELVN 262

```

**Figure 4.15 Identified peptides from cloned cricket tropomyosins against shrimp**

Alignment of partially sequenced cricket and shrimp tropomyosins as generated by Proteome Discoverer 2.1. Mass spectrometry of cricket queried simultaneously against an Arthropoda database (accessed June 21<sup>st</sup>, 2016), sequenced tropomyosins and rabbit glycogen phosphorylase B. Identified peptides are highlighted in green.



**Figure 4.16 Quantification of cloned tropomyosins**

Quantification of cloned tropomyosin as raw data (A), normalized by glycogen phosphorylase (B), summed according to each species (C), and as a ratio of normalized area of tropomyosins to normalized area of shrimp (D). Glycogen phosphorylase quantifications were relatively similar allowing for normalization per run and tropomyosin. Shrimp contains the greatest amount of its single tropomyosin as well as total with waxworm following. Other tropomyosins were of comparable quantity with the exception of pCR1.

**Appendix 4.1 Serological data from clinically confirmed, self-reported, and control shrimp allergic or sensitive subjects**

Serum ID	Sex(age)	Specific IgE via ImmunoCAP (kU/L)					SPT* (mm)	Symptoms	Other Allergies	
		Shrimp	Crab	Lobster	Cockroach	Der p				Der f
<b>Shrimp allergic sera with clinically confirmed diagnosis</b>										
A1	M (23)	16.3					10/25	AE, LS, OAS	ap, p	
A2	M (51)	2.55					13/35	AE, NC, L, DY, CD	ap, b, bn, c, ca, pe, st, tn	
A3	M (61)	3					12/31	AE, N, NC, L, SW	m, c	
A4	M (27)	81.1					5/?	P, AE, E, W	tn	
A5	M (33)	25.3					14/50	P, U, N, AP, E	c	
<b>Self-reported shellfish allergic or sensitized subjects</b>										
S1	M (45)		>100	>100				Itchy throat, rash	co, nuts, raw vegetables	
S2	M (64)				11.4	>100	>100	Respiratory/cutaneous	e, f, legumes, nuts	
S3	M (45)	26.7			8.31	5.39	7.67	Itchy/swollen throat	c, f	
S4	M (49)	27.3	23.9					DY	c	
S5	M (28)	6.21			2.65	>100	>100	Anaphylaxis to peanut	c, f, nuts	
S6	M (27)		6.4	0.56	6.52	2.12		Nose and lung	c, f	
S7	M (38)	84.3	76.8	77.6	66.5			NI	c, f	
S8	F (35)	71.5	33.9			6.94		Oral itch	f	
S9	M (67)	21.9	13	9.29	9.75	6.57	5.9	Respiratory/cutaneous	c, f	
<b>Shellfish non-reactive control sera</b>										
		Specific IgE via ImmunoCAP ISAC (ISU)								
C1	F (46)	0 √	0 √	0 √	0 √	0 √	0 √	None	Inhalant	
C2	F (55)	0 √	0 √	0 √	0 √	0 √	0 √	None	None	

\*Wheal and flare; NI, no information; SPT, skin prick test; √, for all allergenic proteins per species;

AE, angioedema; AP, abdominal pain; CD, cardiovascular dysfunction; DY, dyspnea; E, emesis; L, laryngeal edema; LS, lip swelling; N, nausea; NC, nasal congestion; OAS, oral allergy symptoms; P, pruritus; SW, sweating; U, urticaria; W, wheezing;

ap, apple; b, banana; bn, bean; c, other shellfish; ca, carrot; co, corn; f, fish; e, eggs; m, milk; p, peanut; pe, pea; st, strawberry; tn, tree nuts

Appendix 4.2 Summary of NCBI tblastn hits queried with *Litopenaeus vannahii* tropomyosin (Lit v 1)

Target species	Taxonomic rank and name of hits	NCBI GenBank tblastn hits against				
		<i>Litopenaeus vannahii</i> tropomyosin (GenBank ACB38288.1)				
<i>Gromphadorhina portentosa</i>	Order	AF260897.1	FJ976895.1	AF106961.1		
<i>Blattella germanica</i>	Blattodea	AF454866.1	Y14854.1			
<i>Tenebrio molitor</i> <i>Zophobas morio</i>	Family Tenebrionidae	XM_962035.3	XM_008200693.2	XM_008200701.2		
		XM_015984156.1	XM_008200697.2	XM_008200699.2		
		XM_015984155.1	XM_008200695.2	XM_008200705.2		
		XM_008200704.2	XM_015984154.1	XM_008200694.2		
		XM_008200706.2	XM_008200702.2			
<i>Acheta domestica</i>	Family	AK284720.1	AK285122.1	AK281198.1		
	Gryllidae	AK281119.1				
<i>Dactylopius coccus</i>	Superfamily Coccoidea	EF091948.1	EF091971.1			
<i>Litopenaeus setiferus</i>	Family Penaeidae	JX171685.1	EU410072.1	AB270629.1		
		U08008.1	GU233303.1	DQ151457.1		
		AB270630.1	HM486525.1	AY827100.1		
		GU369817.5				
		XM_005181012.2	XM_011293193.1	XM_005181025.2		
<i>Hermetia illucens</i>	Species <i>Musca domestica</i>	XM_005181011.2	XM_005181021.2	XM_005181026.2		
		XM_005181029.2	XM_005181022.2	KF974800.1		
		XM_005181031.2	XM_011293198.1	XM_011293195.1		
		XM_005181030.2	XM_005181018.2	XM_011293196.1		
		XM_011293203.1	XM_011293199.1	XM_011293194.1		
		XM_011293202.1	XM_011293200.1	XM_011293197.1		
		XM_005181023.2	XM_005181028.2	XM_005181027.2		
		XM_011293201.1				
		<i>Dermatophagoides pteronyssinus</i>	Species	FJ204409.1	AF016278.1	DQ247970.1
			<i>Dermatophagoides pteronyssinus</i>	Y14906.1		
		<i>Galleria mellonella</i>	Family Pyralidae	XM_013327567.1	XM_013327527.1	XM_013327533.1
XM_013327543.1	XM_013327528.1			XM_013327532.1		
XM_013327536.1	XM_013327541.1			XM_013327535.1		
XM_013327542.1	XM_013327538.1			XM_013327529.1		
XM_013327540.1	XM_013327534.1			XM_013327531.1		

### Appendix 4.3 Degenerate primers for cloning tropomyosins

No.	Acronym	Target(s)	Primer Sequence (5'-3')	Direction (F/R)	Species (isoform) specific	Melting temp (°C)
1	CR Start For	Cricket	ATGGACGCGATCAAGAAGAAGATGCAG	F	N (N)	60.7
2	C0 R1 For	Cochineal	ATGGATGCGTTGGAAAATCAGTTGAAA	F	Y (Y)	57.8
3	CR R1a For	Cricket	ATGGACCAGCTCACCAACCAACTGAAG	F	Y (Y)	62.5
4	CR R1b For	Cricket	ATGGACGCTCTGGAGAACCAGCTCAAG	F	Y(Y)	63.7
5	HC R1 For	Hissing cockroach	ATGGACGCACTGGAGAACCAGCTGAAG	F	Y (N)	64.1
6	MW/SW R1a For	Mealworm / Superworm	ATGGACGCCCTCGAGAATCAGCTGAAG	F	Y (N)	63.7
7	MW/SW R1b For	Mealworm / Superworm	GTAGGCATCTTGGAACCCAAGTCT	F	Y (N)	60.5
8	MW/SW/WW F1c For	Mealworm / Superworm / Waxworm	ATGGACCAGCTCACCAACCAGCTGAAG	F	N (N)	64.2
9	SH R1 For	Shrimp	ATGGACGCCCTGGAGAACCAGCTCAAG	F	Y (N)	65.7
10	SF R1a For	Soldier fly	ATGGACGCTCTTGAGAATCAATTGAAG	F	Y (N)	57.3
11	SF R1b For	Soldier fly	GTAGCTGTTTTGGAAGCCCAATTAGCA	F	Y (N)	59.6
12	WW R1a For	Waxworm	GTGGCGATACTGGAGGCGCAACTAGTG	F	Y (N)	64.4
13	WW R1b For	Waxworm	ATGGACGCCCTCGAGAACCAGCTGAAG	F	Y (N)	65.5
14	CO R2 Rev	Cochineal	GCGTAATTCTTCTTCTAATTC	R	Y (Y)	46.8
15	CR R2a Rev	Cricket	TCGCAGCTCTTCCTCAAGCTC	R	Y (Y)	59.3
16	CR R2b Rev	Cricket	CTTCAACTCTTCCTCAAGCTC	R	Y (Y)	53
17	HC R2 Rev	Hissing cockroach	ACGGAGCTCCTCCTCAAGCTC	R	Y (N)	61
18	MW/SW R2 Rev	Mealworm / Superworm	GCGAAGTTCCTCCTCAAGCTC	R	Y (N)	57.9
19	SF R2 Rev	Soldier fly	GCGGAGTTCCTTCTTCAAGTTC	R	Y (N)	53.7
20	WW R2 Rev	Waxworm	GCGCAGTTCCTTCTTCAAGCTC	R	Y (N)	56.6
21	Co R3 Rev	Cochineal	CTTTTCTTCTGATACTTCCAATG	R	Y (Y)	49.5
22	CR/HC/SH R3a Rev	Cricket / Shrimp / Hissing cockroach	CTTCTCCTCAGACACCTCAAGGG	R	N (N)	58.7
23	CR R3b Rev	Cricket	CTTCTCCTCGGACACTTCCAAGG	R	Y (Y)	59.1
24	HC R3 Rev	Hissing cockroach	CTTCTCTTCAGACACCTCAAGGG	R	Y (N)	56.9
25	MW/SW R3 Rev	Mealworm / Superworm	CTTTTCTTCAGACACTTCAAGGG	R	Y (N)	53.8
26	SF R3 Rev	Soldier fly	CTTTTCTTCTGAAACTTCAAGGG	R	Y (N)	52.1
27	WW R3 Rev	Waxworm	CTTCTCCTCAGAGACTTCCAGGG	R	Y (N)	58.3

#### Appendix 4.4 PCR methods for degenerate PCR

No.	Acronym	Initial denaturation (°C)	30 cycles			Final Elongation (°C)
			Denaturation temp (°C)	Annealing temp (°C)	Elongation temp (°C)	
1	CR Start For	95	95	55.7	72	72
2	C0 R1 For	95	95	52.8	72	72
3	CR R1a For	95	95	57.5	72	72
4	CR R1b For	95	95	58.7	72	72
5	HC R1 For	95	95	59.1	72	72
6	MW/SW R1a For	95	95	58.7	72	72
7	MW/SW R1b For	95	95	55.5	72	72
8	MW/SW/WW F1c For	95	95	59.2	72	72
9	SH R1 For	95	95	60.7	72	72
10	SF R1a For	95	95	52.3	72	72
11	SF R1b For	95	95	54.6	72	72
12	WW R1a For	95	95	59.4	72	72
13	WW R1b For	95	95	60.5	72	72
14	CO R2 Rev	95	95	41.8	72	72
15	CR R2a Rev	95	95	54.3	72	72
16	CR R2b Rev	95	95	49	72	72
17	HC R2 Rev	95	95	56	72	72
18	MW/SW R2 Rev	95	95	52.9	72	72
19	SF R2 Rev	95	95	48.7	72	72
20	WW R2 Rev	95	95	51.6	72	72
21	Co R3 Rev	95	95	44.5	72	72
22	CR/HC/SH R3a Rev	95	95	53.7	72	72
23	CR R3b Rev	95	95	54.1	72	72
24	HC R3 Rev	95	95	51.9	72	72
25	MW/SW R3 Rev	95	95	48.8	72	72
26	SF R3 Rev	95	95	47.1	72	72
27	WW R3 Rev	95	95	53.3	72	72

#### Appendix 4.5 Gene specific primers for cloning tropomyosins

No.	Acronym	Target(s)	Sequence (5'-3')	Direction(F/R)	Start(stop) position	T <sub>m</sub> (°C)
1	HC GSP1_3'	Hissing cockroach	GCCAGGTTTCATGGCTGAAGAGGCA	F	4 (27)	68.5
2	HC GSP2_3'	Hissing cockroach	GCAGAAGAACGTGCCGAGAGTGGT	F	88 (111)	68.5
3	HC GSP1_5'	Hissing cockroach	GCCAACAACACGAAGTTCCTCCTCA	R	156 (132)	67.4
4	HC GSP2_5'	Hissing cockroach	CGGCACGTTCTTCTGCTCTTTCCAA	R	103 (79)	67.4
5	CR1 GSP1_3'	Cricket	GAGGCGAGGCTGCTGGCTGAAGA	F	2 (23)	69.9
6	CR1 GSP2_3'	Cricket	CGCTGACGGCAAGTCCGACGAGG	F	24 (46)	71.9
7	CR1 GSP1_5'	Cricket	AGCTGTTGCCGACGACCTTCAACT	R	163 (140)	66.9
8	CR1 GSP2_5'	Cricket	CGATCCTCAGCCACTTCCAGCTCGT	R	98 (74)	70.7
9	CR2 GSP1_3'	Cricket	GGATCGCGCCCTTCTCTGCGAGC	F	30 (52)	71.9
10	CR2 GSP2_3'	Cricket	GCCCCGATGCCAACTTGCGGGC	F	58 (78)	73.6
11	CR2 GSP1_5'	Cricket	CAGCAGCCTGGGAGGCTTCGGCCA	R	330 (311)	73.8
12	CR2 GSP2_5'	Cricket	TGGATGCGGCGGTTTCAGGGCAGC	R	248 (229)	71.9
13	SH GSP1_3'	Shrimp	CGACGAGGTTGCCCGTAAGCTGGC	F	39 (62)	72.2
14	SH GSP2_3'	Shrimp	CGTGCTGAGGAGCGTGCCGAGAC	F	85 (106)	71.9
15	SH GSP1_5'	Shrimp	GCCAACGACACGCAGCTCTTCCT	R	156 (134)	68.3
16	SH GSP2_5'	Shrimp	CTCGGCACGTCCTCAGCACGCTC	R	103 (84)	73.8
17	WW 1GSP1_3'	Waxworm	TCCTCGCTGAGGAAGCCGACAAGA	F	5 (28)	68.5
18	WW 1GSP2_3'	Waxworm	CGATGAGGTGGCTCGTAAGCTGGC	F	33 (56)	70.6
19	WW 1GSP1_5'	Waxworm	ACCAGATTCGGCACGTTCCCTCCGC	R	105 (82)	70.6
20	WW 1GSP2_5'	Waxworm	GTCAGCCTCGACCATGGCCAGCT	R	71 (50)	69.9
21	WW 2GSP1_3'	Waxworm	GAGGCCCGTCTCCTGGCTGAGGA	F	2 (21)	71.9
22	WW 2GSP2_3'	Waxworm	CGCTGACGGCAAATCTGATGAGGT	F	23 (47)	66.9
23	WW 2GSP1_5'	Waxworm	CACGATCCTCAGCGACTTCGAGC	R	100 (78)	68.3
24	WW 2GSP2_5'	Waxworm	AACGAAGGCCAGCTTACGTGAAACC	R	69 (45)	67.4
25	SW GSP1_3'	Superworm	AGGCCCGCTTCTTGGCTGAAGAAG	F	2 (22)	68.5
26	SW GSP2_3'	Superworm	CTCGTAAATTGGCCATGGTGGAAGCC	F	48 (72)	69.5
27	SW GSP1_5'	Superworm	CCTGCCTCGGCGGCTCTTCGGC	R	109 (90)	67.8
28	SW GSP2_5'	Superworm	GGCTTCCACCATGGCCAATTTACGA	R	75 (51)	67.4
29	MW GSP1_3'	Mealworm	GCGAAACTAATAGCCGAAGAGGCCG	F	2 (26)	69.1
30	MW GSP2_3'	Mealworm	CGAAGAGGTTGCCCGAAAATTGGTTC	F	36 (60)	67.9
31	MW GSP1_5'	Mealworm	GCTCTCGCTCTGTTTCAGCGCGTTC	R	115 (92)	70.6
32	MW GSP2_5'	Mealworm	CCAATTTTCGGGCAACCTCTTCG	R	60 (36)	64.6

## Appendix 4.6 PCR conditions for RACE PCR

No.	Acronym	PCR round	Denaturation (°C)	Cycles	Denaturation (°C)	Anneal (°C)	Elongation (°C)	Elongation (°C)
1	HC GSP1_3'	1	95	30	95	63.5	72	72
2	HC GSP2_3'	2	95	30	95	63.5	72	72
3	HC GSP1_5'	1	95	30	95	62.4	72	72
4	HC GSP2_5'	2	95	30	95	62.4	72	72
5	CR1 GSP1_3'	1	95	30	95	64.9	72	72
6	CR1 GSP2_3'	2	95	30	95	66.9	72	72
7	CR1 GSP1_5'	1	95	30	95	61.9	72	72
8	CR1 GSP2_5'	2	95	30	95	65.7	72	72
9	CR2 GSP1_3'	1	95	30	95	66.9	72	72
10	CR2 GSP2_3'	2	95	30	95	68.6	72	72
11	CR2 GSP1_5'	1	95	30	95	68.8	72	72
12	CR2 GSP2_5'	2	95	30	95	66.9	72	72
13	SH GSP1_3'	1	95	30	95	67.2	72	72
14	SH GSP2_3'	2	95	30	95	66.9	72	72
15	SH GSP1_5'	1	95	30	95	63.3	72	72
16	SH GSP2_5'	2	95	30	95	68.8	72	72
17	WW 1GSP1_3'	1	95	30	95	63.5	72	72
18	WW 1GSP2_3'	2	95	30	95	65.6	72	72
19	WW 1GSP1_5'	1	95	30	95	65.6	72	72
20	WW 1GSP2_5'	2	95	30	95	64.9	72	72
21	WW 2GSP1_3'	1	95	30	95	66.9	72	72
22	WW 2GSP2_3'	2	95	30	95	61.9	72	72
23	WW 2GSP1_5'	1	95	30	95	63.3	72	72
24	WW 2GSP2_5'	2	95	30	95	62.4	72	72
25	SW GSP1_3'	1	95	30	95	63.5	72	72
26	SW GSP2_3'	2	95	30	95	64.5	72	72
27	SW GSP1_5'	1	95	30	95	62.8	72	72
28	SW GSP2_5'	2	95	30	95	62.4	72	72
29	MW GSP1_3'	1	95	30	95	64.1	72	72
30	MW GSP2_3'	2	95	30	95	62.9	72	72
31	MW GSP1_5'	1	95	30	95	65.6	72	72
32	MW GSP2_5'	2	95	30	95	59.6	72	72

## Appendix 4.7 Nucleotide sequences of fully and partially cloned tropomyosins with overlapping targeted PCR region

### Partial Cricket tropomyosin 1

ATGGACGCGATCAAGAAGAAGATGCAGGCGATGAAGCTGGAGAAGGACAATGCCATGGACAAGGCAGACAC  
CTGCGAAGGGCAGGCTAAGGACGCAAACAACAAGGCGGACAAGATCAATGAGGATGTCCAGGAGCTGACCA  
AGAAGCTCGCTCAGGTTGAGAATGACCTGATCACCACCAAGGCTAACCTGGAGCAGGCCAACAAGGACCTG  
GAAGACAAGGAAAAGGCCCTCCAGGCCGCTGAGAGCGAGATGGCCGCCCTGAACAGGAAGGTGCAGCTCGT  
TGAGGAGGACCTGGAGCGCTCTGAGGAGCGCGCCGCCACC GCCACCAAGCTGCAGGAGGCCTCCGAGG  
CTGCTGATGAGGCCAGCGTATGTGCAAGGTATTGGAGAACCGCAGCCAGCAGGATGAGGAGAGGATGGAC  
CAGCTCACCACCAACTGAAGGAGGCGAGGCTGCTGGCTGAAGACGCTGACGGCAAGTCCGACGAGGTGTC  
ACGTAAGCTGGCCTTCGTTGAAGACGAGCTGGAAGTGGCTGAGGATCGTGTGAAGTCCGGAGACTCCAAGA  
TCATGGAGCTTGAGGAAGAGTTGAAGGTCGTCGGCAACAGCTTGAAGT

### Partial Cricket tropomyosin 2

ATGGACGCGATCAAGAAGAAGATGCAGGCGATGAAGCTGGAGAAGGATAACGCGATGGATCGCGCCCTTCT  
CTGCGAGCAGCAGGCCCGCGATGCCAACTTGC GGCGGAGAAGGCTGAGGAGGAGGCCCGTGGCCTGCAGA  
AGAAGATCCAGACCATTGAGAACGAGCTCGACCAGACCCAGGAGCAGCTGATGCAGGTCAACGCCAAGCTT  
GAGGAGAAGGACAAGGCTCTGCAGACCGCTGAGGGTGAAATTGCTGCCCTGAACCGCCGCATCCA ACTGTT  
GGAGGAGGACTTGGAGCGCTCTGAGGAGCGTCTGGCCACTGCCACCCTAAGTTGGCCGAAGCCTCCCAGG  
CTGCTGATGAGTCTGAGCGAGCTCGTAAGATTCTTGAGAACCGCAGCTTGGCAGATGAAGAGCGCATGGAC  
GCTCTGGAGAACCAGCTCAAGGAAGCCAGATTCTTGGCAGAAGAGGCTGACAAGAAGTATGATGAGGTTGC  
CCGTAAGTTGGCCATGGTTGAGGCAGACTTGGAGAGAGCAGAAGAGCGTGTGAGGCTGGTGAATCTAAGA  
TTGTGGAGCTTGAGGAAGAGCTGCGAGTTGTTGGTAACANCCCTGAAAT

### Partial mealworm tropomyosin 1

GCGAAACTAATAGCCGAAGAGGCCGACAAAAAATACGAAGAGGTTGCCCGAAAATTGGTTCTTATGGAACA  
AGACTTAGAACGAGCCGAAGAACGCGCTGAACAGAGCGAGAGCAAAATCGTAGAGCTTGAGGAGGAACTTC  
GCGTCGTTGGCAACAACCTTGAAGTCCCTAGAAGTGTCCGAGGAAAAGGCTACGTTAAAAGAGGAGGAATAT  
TCGGTTACCCTGAAACAGGTGGATCAACGATTGCAAGAGGCTGAGGCTCGCGCCGAGTTTGGCCGACGTT  
GGTACAGAACTCCAGAAGGAGGTCGACAGACTAGAAGACGATCTCTTGGCAG

### Partial mealworm tropomyosin 2

CTCGTAAATTGGCCATGGTGGAAGCCGACTTGGAGAGAGCAGAAGAACGCGCCGAAGCAGGAGAATCCAAA  
ATCGTAGAGCTTGAGGAGGAACTTCGCGTCGTTGGCAACAACCTTGAAGTCCCTAGAAGTGTCCGAGGAAAA  
GGCCAACCAACGCGAAGAAGAGTACAAAAATCAAATTAAGAACTTGACCACCCGCTAAAGGAGGCTGAGG  
CTCGCGCCGAGTTTGGCCGACGCTCGGTACAGAACTCCAGAAGGAGGTCGACAGACTAGAAGAAGAGCTC  
GTCGCCGAAAAGGAGCGTTACAAGGAAATCGGCGACGACTTGGACACGGCTTTCGTCGAACTCATCTTGTA  
A

### Partial superworm tropomyosin

GAGGCCCGCTTCTTGGCTGAAGAAGCCGATAAAAAATACGATGAGGTAGCTCGTAAATTGGCCATGGTGGA  
AGCCGACTTGGAGAGAGCCGAAGAGCGCGCCGAGGCAGGAGAATCCAAAATCGTAGAGCTTGAGGAGGAAAC  
TTCGCGTCGTTGGCAACAACCTTGAAGTTCGCTTGAAGTGTCTGAGGAGAAGGCCAACCAACGCGAAGAAGAG  
TACAAAAATCAAATTAAGAACTTGACAACCCGCTAAAGGAGGCTGAGGCTCGCGCTGAGTTTGCTGAACG  
TTCCGTACAGAACTGCAAAAGGAGGTCGACAGATTAGAAGATGAGCTCGTCGCCGAAAAGGAACGCTACA  
AGGAAATCGGCGACGACTTGGACACCGCTTTCGTCGAACTCATCTTGTA

### Partial waxworm tropomyosin 1

ATGGACGCGATCAAGAAGAAGATGCAGGCGATGAAGCTGGAGAAGGACAACGCCCTTGACCGCGCCGCTAT  
GTGCGAGCAACAGGCCAAGGACGCCAACCTTCGTGCCGAGAAGGCGGAAGAGGAGGCCAGACAGCTCCAGA  
AGAAAATCCAGACCATTGAGAATGATCTGGACCAGACACAAGAGTCTCTAATGCAGGTCAACGGTAAACTT  
GAGGAGAAGGAGAAGGCTCTTCAGAACGCGGAGTCCGAAGTGGCTGCACTCAACCGACGTATTCAACTGTT

GGAGGAAGATCTGGAGAGATCCGAGGAGCGTCTTGCTACTGCCACAGCCAAACTGTCTGAGGCCAGCCAGG  
 CAGCTGATGAGTCTGAACGTGCCCCGTAAGGTGCTCGAGAACAGGTCGTTGGCCGATGAAGAGCGCATGGAC  
 GCCCTGGAGAACCAGCTGAAGGAAGCCAGGTTCCCTCGCTGAGGAAGCCGACAAGAAATACGATGAGGTGGC  
TCGTAAGCTGGCCATGGTTCGAGGCTGACTTGGAGCGCGCGGAGGAACGTGCCGAATCTGGTGAATCTAAAA  
 TCGTTCGAGCTTGAGGAAGAACTCCGCGTAGTTGGTAACAACCTGAAGTCCCTCGAGGTCTCCGAGGAGAAG  
 GCCAACCAACGTGAGGAGGAGTACAAAAATCAGATCAAAACACTCACCACCCGTCTAAAGGAGGCTGAGGC  
 GCGCGCCGAGTTCGCCGAGCGTTCGGTGCAGAACTGCAGAAGGAGGTCG

### **Partial waxworm tropomyosin 2**

ATGGACGCGATCAAGAAGAAGATGCAGGCGATGAAGCTGGAGAAGGACAATGCCATGGACAAGGCAGACAC  
 CTGCGAACAGCAGGCCAGGGATGCCAACCTCCGTGCTGAGAAGGTCAATGAGGAAGTCCGTGAACTCCAGA  
 AAAAATCGCACAAAGTGAAGAGGATCTCACTCTTAACAAAAACAACTGGACCAGGCCAACAAAGACTTG  
 GAGGAGAGGGAAAAGCAGCTCGCCGCCACCGAGGCTGAAGTCGCCTCCCTCAACAGGAAAGTCCAACAGAT  
 TGAGGAGGATTTGGAGAAATCTGAGGAAAGGTCTGGCACAGCCCAGCAAAAATCTTGAAGCCCAGCAGT  
 CGGTGACGAGAAACAACCGTATGTGCAAAGTATTGGAGAACAGAGCCAGCAGGACGAGGAGCGCATGGAC  
 CAGCTCACAAACCAGCTGAAGGAGGCCCGTCTCCTGGCTGAGGACGCTGACGGCAAATCTGATGAGGTTTC  
ACGTAAGCTGGCCTTCGTTGAAGACGAGCTCGAAGTCGCTGAGGATCGTGTCAAGTCTGGTGACGCTAAGA  
 TCTCAGAACTTGAGGAGGAATTAAGGTAGTAGGTAAC

### **Partial shrimp tropomyosin**

ATGGACGCCATCAAGAAGAAGATGCAGGCGATGAAGCTGGAGAAGGATAACGCCATGGACAGGGCGGATAC  
 CCTCGAACAGCAGAACAAAGGAGGCCAACAAACAGGGCTGAGAAGAGCGAGGAGGAGGTTACAACCTTCAGA  
 AGAGGATGCAGCAACTTGAGAACGACCTTGACCAGGTGCAGGAATCCTTGCTGAAGGCTAACATCCAGCTT  
 GTGGAGAAGGACAAGGCCCTCTCTAACGCTGAGGGCGAGGTGGCCGCTCTGAACCGCCGCATCCAGCTGCT  
 CGAGGAGGACCTGGAGCGCTCTGAGGAGCGCTCAACACCGCCACCACCAAGCTGGCCGAGGCCTCCCAGG  
 CCGCCGACGAGTCCGAGCGCATGCGCAAGGTGCTCGAGAACCCTCCCTGTCCGACGAGGAGCGCATGGAC  
 GCCCTGGAGAACCAGCTCAAGGAGGCTCGATTCTGGCTGAGGAAGCCGACAGGAAATACGACGAGGTTGC  
 CCGTAAGCTGGCCATGGTTGAGGCCGACCTTGAGCGTGCTGAGGAGCGTGCCGAGACTGGTGAATCAAAGA  
 TCGTTCGAGCTTGAGGAAGAGCTGCGTGTCTGTTGGCAACAACCTGAAGTCCCTTGAGGTGTCTGAGGAGAAG  
 GCCAACCGAGCGGAGGAAGCCTACAAGGAGCAGATTAAGACACTTACCAACAAGCTGAAGGCGGCTGAGGC  
 CCGTGCCGAGTTCGCCGAGAGGTCTGTGCAGAAGCTCCAGAAGGAGGTCGACAGGCTTGAAGACGAAGTGG  
 TTAACGA

### **Hissing cockroach tropomyosin**

ATGGATGCGATCAAGAAGAAGATGCAGGCGATGAAGCTGGAGAAGGACAACGCGATGGATCGCGCCCTGCT  
 CTGCGAACAGCAGGCCCGAGATGCCAACATTCCGGCTGAGAAGGCTGAGGAGGAGGCCAGATCCCTGCAGA  
 AGAAGATCCAGCAGATTGAGAATGATCTGGACCAGACCTTGGAACAGTTAATGCAGGTCAACGCCAAACTG  
 GACGAGAAAGACAAGGCCCTTCCAGAACGCTGAGAGTGAAGTCGCTGCCCTGAACCGCCGAATCCAGTTGTT  
 GGAAGAGGATCTCGAGAGGTCTGAGGAGCGTTTAGCCACAGCCACTGCTAAGCTGGCTGAAGCTTACAAG  
 CTGCTGATGAGTCTGAACGAGCTCGTAAGATTCTTGAATCGAAAGGCCTGGCAGATGAAGAGCGTATGGAT  
 GCTCTGGAGAATCAGCTGAAGGAAGCCAGGTTTATGGCTGAAGAGGCAGACAAGAAATATGATGAGGTTGC  
 ACGTAAGTTGGCTATGGTTGAAGCTGACTTGGAAAGAGCAGAAGAACGTGCCGAGAGTGGTGAATCAAAAA  
 TTGTAGAGCTTGAGGAGGAACCTTCGTGTTGTTGGCAACAACCTGAAGTCCCTTGAGGTTTTCAGAAGAGAAG  
 GCCAACCTGCGTGAAGGAGTACAAGCAGCAGATTAAGACCCTGACAACCAGGCTAAAGGAGGCTGAAGC  
 TCGTGTGAGTTTCGCTGAAAGATCGGTGCAGAAATTGCAGAAGGAGGTCGACAGGCTTGAAGGATGAATTGG  
 TACACGAGAAGGAGAAGTACAAGTACATTTGTGACGATCTAGATATGACTTTCACCGAACTTATTGGCAAC  
 TAA

## Appendix 4.8 Amino acid sequences of fully and partially cloned tropomyosins

### Partial cricket tropomyosin 1

MDAIKKKMAMKLEKDNAMDKADTCEGQAKDANNKADKINEDVQELTKKLAQVENDLITTKA  
NLEQANKDLEDKEKALQAAESEMAALNRKVQLVEEDLERSEERAATAATKLEASEAADEAQR  
MCKVLENRSQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRKLAFFVEDELEVAEDRVKSGDS  
KIMELEEEELKVVGNLKL

### Partial cricket tropomyosin 2

MDAIKKKMAMKLEKDNAMDRALLCEQQARDANLRAEKAEERARGLQKKIQTIENELDQTQEQ  
LMQVNAKLEEKDKALQTAEGEIAALNRRIQLLEEDLERSEERLATATAKLAEASQAADERARKI  
LENRSLADEERMDALENQLKEARFLAEEADKKYDEVARKLAMVEADLERAEERAEAGESKIVEL  
EEELRVVGNXPPE

### Partial mealworm tropomyosin 1

AKLIAEEADKKYEEVARKLVLMEQDLERAEEAEQSESKIVELEEEELRVVGNLKSLEVSEEKATL  
KEEYESVTLKQVDQRLQEAEEARAEEFAERSVQKLQKEVDRLEDDLLAE

### Partial mealworm tropomyosin 2

RKLAMVEADLERAEERAEAGESKIVELEEEELRVVGNLKSLEVSEEKANQREEEYKNQIKNLTTTR  
LKEAEARAEEFAERSVQKLQKEVDRLEEEELVAEKERYKEIGDDLDTAFVELIL

### Partial superworm tropomyosin

EARFLAEEADKKYDEVARKLAMVEADLERAEERAEAGESKIVELEEEELRVVGNLKSLEVSEEKA  
NQREEEYKNQIKNLTTTRLKEAEARAEEFAERSVQKLQKEVDRLEDELVAEKERYKEIGDDLDTAFV  
ELIL

### Partial waxworm tropomyosin 1

MDAIKKKMAMKLEKDNALDRAAMCEQQAKDANLRAEKAEERARQLQKKIQTIENDLDQTQES  
LMQVNGKLEEKALQNAESEVAALNRRIQLLEEDLERSEERLATATAKLSEASQAADERARK  
VLENRSLADEERMDALENQLKEARFLAEEADKKYDEVARKLAMVEADLERAEERAEAGESKIVE  
LEEEELRVVGNLKSLEVSEEKANQREEEYKNQIKTLTTRLKEAEARAEEFAERSVQKLQKEV

### Partial waxworm tropomyosin 2

MDAIKKKMAMKLEKDNAMDKADTCEQQARDANLRAEKVNEEVRELQKKLAQVEEDLTLNKN  
KLDQANKDLEEREKQLAATEAEVASLNRKVQIEEDLEKSEERSGTAQQKLEAQQSADENNRM  
CKVLENRAQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRKLAFFVEDELEVAEDRVKSGDAKI  
SELEEEELKVVGN

### Partial shrimp tropomyosin

MDAIKKKMAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQVQ  
ESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAEASQAADERMR  
KVLENRSLADEERMDALENQLKEARFLAEEADRKYDEVARKLAMVEADLERAEERAEAGESKIV  
ELEEEELRVVGNLKSLEVSEEKANQREEA YKEQIKTLTNKLKAAEARAEEFAERSVQKLQKEVDRL  
EDELVN

### Hissing cockroach tropomyosin

MDAIKKKMAMKLEKDNAMDRALLCEQQARDANLRAEKAEERARSLQKKIQQIENDLDQTLEQL  
MQVNAKLDEKDKAFQNAESEVAALNRRIQLLEEDLERSEERLATATAKLAEASQAADERARKI  
LESKGLADEERMDALENQLKEARFMAEEADKKYDEVARKLAMVEADLERAEERAEAGESKIVEL  
EEELRVVGNLKSLEVSEEKANLREEEYKQIKTLTTRLKEAEARAEEFAERSVQKLQKEVDRLED  
ELVHEKEYKYICDDLDMTFTLIGN

## **Appendix 4.9 Amino acid sequences of cloned tropomyosins trimmed to shared 36 amino acid overlap**

### **Partial cricket tropomyosin 1 trimmed**

RKLAFVEDELEVAEDRVKSGDSKIMELEEEELKVVGN

### **Partial cricket tropomyosin 2 trimmed**

RKLAMVEADLERAEERAEAGESKIVELEEEELRVVGN

### **Partial mealworm tropomyosin 1 trimmed**

RKLVLMEQDLERAEEERAEQSESKIVELEEEELRVVGN

### **Partial mealworm tropomyosin 2 trimmed**

RKLAMVEADLERAEERAEAGESKIVELEEEELRVVGN

### **Partial superworm tropomyosin trimmed**

RKLAMVEADLERAEERAEAGESKIVELEEEELRVVGN

### **Partial waxworm tropomyosin 1 trimmed**

RKLAMVEADLERAEERAESGESKIVELEEEELRVVGN

### **Partial waxworm tropomyosin 2 trimmed**

RKLAFVEDELEVAEDRVKSGDAKISELEEEELKVVGN

### **Partial shrimp tropomyosin trimmed**

RKLAMVEADLERAEERAETGESKIVELEEEELRVVGN

### **Madagascar hissing cockroach tropomyosin trimmed**

RKLAMVEADLERAEERAESGESKIVELEEEELRVVGN

**Appendix 4.10 Areas and raw values for mass spectrometry quantification of Figure 4.16**

Fig. A	pCR1	pCR2	pMW1	pMW2	pSW	pWW1	pWW2	pSH	HC	gpCR	gpMW	gpSW	gpWW	gpSH	gpHC
	4.20E+06	1.00E+07	2.40E+07	3.60E+07	1.40E+07	8.60E+07	5.90E+07	2.70E+08	2.70E+07	2.60E+07	2.70E+07	1.70E+07	2.70E+07	2.60E+07	2.10E+07
	4.90E+06	2.10E+07	3.20E+07	3.20E+07	1.40E+07	1.10E+08	6.50E+07	2.90E+08	2.80E+07	3.10E+07	3.40E+07	1.70E+07	2.70E+07	3.20E+07	2.60E+07
Fig B	pCR1	pCR2	pMW1	pMW2	pSW	pWW1	pWW2	pSH	HC						
	16.154	38.462	88.889	133.33	82.353	329.63	218.52	1038.5	128.57						
	15.806	67.742	94.118	94.118	82.353	407.41	240.74	906.25	107.69						
Fig C	CR	MW	SW	WW	SH	HC									
	54.615	222.22	82.353	548.15	1038.5	128.57									
	83.548	188.24	82.353	648.15	906.3	107.69									
Fig D	pCR1	pCR2	pMW1	pMW2	pSW	pWW1	pWW2	pSH	HC	CR	MW	SW	WW	SH	HC
	0.0156	0.0370	0.0856	0.1284	0.0793	0.3174	0.2104	1.0000	0.1238	0.0526	0.2140	0.0793	0.5278	1.0000	0.1238
	0.0174	0.0747	0.1039	0.1039	0.0909	0.4496	0.2656	1.0000	0.1188	0.0922	0.2077	0.0909	0.7152	1.0000	0.1188

#### Appendix 4.11 Common tryptic peptides between rabbit and fruit fly glycogen phosphorylase

Peptide	Length (amino acids)	Peptide mass
TNGITPR	7	757.845
YGNPWEK	7	892.966
NLAENISR	8	916.002
VIFLENYR	8	1053.226
TQQHYYEK	8	1096.164
VLYPNDNFFEGK	12	1442.591
VAIQLNDTHPSLAIPELMR	19	2118.479
LAACFLDSMATLGLAAYGYGIR	22	2277.681

Tryptic peptides predicted by PeptideCutter ([http://web.expasy.org/peptide\\_cutter/](http://web.expasy.org/peptide_cutter/)) common between rabbit glycogen phosphorylase B (Uniprot P00489) and *Drosophila melanogaster* glycogen phosphorylase (Uniprot Q9XTL9). Peptides listed as potentially used for quantification according to parameters listed in Table 4.1.

## CHAPTER 5

### **SIMULATED GASTRIC PEPSINOLYSIS OF DEFATTED ACHETA DOMESTICUS AND CROSS-REACTIVITY WITH SHRIMP-ALLERGIC IGE**

#### **5.1 ABSTRACT**

*In vitro* pepsinolysis has been used as a guide to infer potential allergenicity of proteins. Tropomyosins are ubiquitous proteins of diverse function, sequence, and expression as well as an often represented invertebrate allergen. *In vitro* assays against purified proteins or extracts may not be accurate representations of whole foods as opposed to a direct assay of the food itself. Defatted powder of *Acheta domesticus* underwent simulated gastric pepsinolysis and blotting using an anti-shrimp tropomyosin IgG and shrimp-allergic IgE under reducing and non-reducing conditions. Patterns of reactivity were similar between control and immuno-blots suggesting tropomyosin was the predominant cross-reactive allergen. This patient was minimally reactive to reduced forms of cricket tropomyosins.

## 5.2 INTRODUCTION

*In vitro* assays are used in-part to assess the allergenicity of foods and proteins [1]. Pepsin is often used in *in vitro* assessments stemming from Astwood et al. where a link between resistance to pepsin digestion and the allergenicity of the proteins was proposed [2], although follow-up studies found that such a link is more tenuous [3]. Interpretation of these assays for novel foods relies on the guidance given for assessments of genetically modified plants and a “weight-of-evidence approach” incorporating pepsin resistance as a criterion [1, 4].

The original pepsin resistance assay focused on individual purified proteins whereas simulated gastric pepsinolysis incorporates proteins of non-interest and non-proteins as a matrix to better represent digestion. Incorporation of whole matrices can then use pepsin as a means to investigate interactions between food components that can positively or negatively alter digestibility [5]. This distinction is useful as extracts of foods can result in sub-optimal representation of allergens present and misrepresent the food [6, 7].

Tropomyosin is a cross-reactive invertebrate allergen identified as with multiple isoforms of varying function and size [8]. Tropomyosin has also been identified in *Drosophila*, a model organism for insects, as having an atypical isoform that forms filaments without co-localization with actin or other tropomyosins [9]. These alternative interactions may alter both extractability and digestibility to misrepresent the extent of cross-reactivity. Simulated gastric pepsinolysis may serve to mitigate these effects.

## 5.3 METHODS AND MATERIALS

### 5.3.1 Materials and sample preparation

*Acheta domesticus* crickets were obtained from a commercial source. Crickets were homogenized using a mortar and pestle, the homogenate filtered through cheesecloth, and washed with 100% acetone. The mixture was stirred for 1 hour at room temperature in 5% w/v acetone, dried, then stirred for 2 hours at room temperature in 5% w/v hexane and dried resulting in a defatted cricket powder (Defat-Cr).

### 5.3.2 Direct pepsinolysis assay

Digestions were performed with a 3:1 w/w ratio of pepsin to predicted Defat-Cr protein (875 mg/g defatted powder) at 37 °C in an orbital shaker (150 rpm). The powder (3 mg) was incubated for 30 min, in 11.4 ml of pre-heated (37 °C) simulated gastric fluid (35mM NaCl, 0.084N HCl, 0.002% BSA w/v, pH 1.2). The assay began by adding 600 µl of pre-heated (37°C) pepsin in water (16.06 mg/ml; Sigma, 3280 U/mg). Pepsin only controls (E0 and E60) (no Defat-Cr); Digestion controls (DC) (Inactivated pepsin added to Defat-Cr) and Defat-Cr only controls (P0 and P60) (no pepsin added) were also included. Sampling (105 µl) of the digestion occurred at time points over 60 min. Digestion assay samples were inactivated by adding 35 µl of stop solution (200mM NH<sub>4</sub>CO<sub>3</sub>, pH 11) and 35 µl of 6x Laemmli buffer (20% v/v glycerol, 10% w/v SDS, 0.05% bromophenol blue, 200 mM Tris pH 6.8) or 5x Laemmli reducing buffer (6x Laemmli buffer, 16.7% v/v beta-mercaptoethanol). The samples were immediately heated to 95 °C for 10 minutes, cooled to room temperature, and centrifuged (16 k x g, 5 minutes).

### **5.3.3 SDS-PAGE**

Digestion assay controls and samples were loaded (20  $\mu$ l SYPRO SDS-PAGE and non-reducing blots, 16  $\mu$ l reducing blots) onto 4-12% gradient SDS-PAGE gels (NuPAGE Bis-Tris, Invitrogen), using MES SDS running buffer (NuPAGE Bis-Tris, Invitrogen), and run at 200 V until the tracking dye reached the bottom. Gels were used for blotting or stained with SYPRO (SYPRO ruby protein gel stain, Bio-Rad) according to the manufacturer's instructions. Stained gels were visualized using a Kodak Gel logic 440 image station with a 590 nm filter.

### **5.3.4 Western and immuno-blot**

Western blotting and immunoblotting of the digestion assay samples was performed as described elsewhere [10]. For western blotting the primary antibody was rabbit anti-shrimp tropomyosin IgG (Indoor Biotechnologies) (1:4,000 dilution) and the secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (Thermo) (1:4000 dilution). For immunoblotting the primary antibody was shrimp allergic serum (see below) (1:10 dilution) and the secondary antibody was mouse anti-human IgE conjugated with horseradish peroxidase (Southern Biotech). Blots were visualized after addition of substrate (SuperSignal West Dura Extended Duration Substrate Kit, Thermo) using a Kodak Gel Logic 440 image station.

Serum was from an atopic 27 year old male with positive SPT (5 mm wheal), 81.1 iCAP to shrimp, and a case history of pruritus, angioedema, emesis, and wheezing upon

consumption of shrimp. The individual was orally challenged and found to be allergic.

Patient is also allergic to tree nuts (Appendix 4.1, Serum A4).

## **5.4 RESULTS AND DISCUSSION**

### **5.4.1 SDS-PAGE analysis of digestion assay**

As whole defatted powder was used as the substrate for pepsinolysis, a holistic view of all proteins present in the sample is required (Fig 5.1). Gel indicates minimal banding present in the digestion control and both enzyme and protein controls indicate no changes after 60 minutes. Banding present in digestion controls is not present in any digestion time point indicating the bands were labile. The low concentration of the assay coupled with use of a non-optimal filter for visualization may have negatively impacted resolution.

### **5.4.2 Western and immuno-blotting**

As a means to improve the resolution of the assay, rabbit anti-shrimp tropomyosin IgG (A) and shrimp-allergic serum (B) were used (Fig 5.2). Non-reducing western blotting demonstrated similar patterns of reactivity using both antibodies at 120, 75, and 37 kDa in controls. Digestion time points indicate at 30 seconds that 120 and 75 kDa reacting bands are slowly degraded. Bands originating from 37 kDa disappear and bands of approximately 34 and 30 kDa appear with the 34 kDa band disappearing by 60 minutes, but in both blots the 30 kDa band increases through 60 minutes. However, reactivity to the 34 and 30 kDa bands were less using the shrimp-allergic serum. Shrimp-allergic serum additionally indicated reactivity to a 150 kDa band.

Patterns of reactivity are largely shared in non-reduced samples using anti-tropomyosin IgG and shrimp-allergic serum. A band at 150 kDa is visible at 30 seconds

using shrimp-allergic serum, not demonstrated using anti-tropomyosin, indicating either a non-reactive tropomyosin or another protein such as a cross-reactive myosin-heavy chain. Banding at 37, 75, and 120 kDa in controls of each blot can be explained as polymerized tropomyosins [9, 11, 12]. Banding at 34 and 30 kDa are likely fragmentation products of extracted proteins, although it cannot be disregarded that these could be products of pepsinolysis upon insoluble proteins from the defatted flour. In this modified pepsinolysis, there is no difference between a fragmentation product of an extracted protein and generation of new banding from insoluble protein, however this is a feature of the method to better represent the whole proteome present.

Further western blotting to focus on individual tropomyosins without the effects of disulfide bonds was also investigated (Fig 5.3). Reducing western blotting was conducted as before with rabbit anti-shrimp tropomyosin IgG (A) and shrimp-allergic serum (B). Patterns of reactivity are similar across blots with reactivity at 120, 75, and 37 kDa in controls, although reactivity using shrimp-allergic serum is markedly lower than with anti-tropomyosin IgG. Digestion time points lose reactivity to the 120 kDa band in both blots after 30 seconds, the 75 kDa band is faintly visible in the anti-tropomyosin blot after 60 minutes but imperceptible at 5 minutes using shrimp-allergic serum. Trimming of the 37 kDa band using anti-tropomyosin IgG results in a smear until 20 minutes where it is resolved as a 34 kDa band and a lower 30 kDa band with the 34 kDa band lost by 30 minutes and the 30 kDa band present through 60 minutes. Shrimp-allergic serum has the 37 kDa band similarly resolve into 34 and 30 kDa bands where they disappear by 20 minutes and remain through 60 minutes respectively.

Highly reactive bands at 37 kDa are seen in both the digestion control and 30 second time-point, but no other controls suggesting this is a sampling error whereby solid sample was taken with the supernatant. This also indicates that the higher reactivity of bands above 37 kDa in the 30 second time-point can be explained in-part by sampling error. Anti-tropomyosin IgG had greater reactivity across all controls and time points compared to shrimp-allergic serum, but with a similar pattern of reactivity within the controls and through the 30 second point.

Reducing conditions result in a preponderance of monomeric tropomyosins that are less immunoreactive compared to non-reducing conditions. Increases in reactivity seen using anti-tropomyosin antibody on reduced samples can be explained by exposure of additional epitopes. While reduction of the samples increased reactivity to anti-tropomyosin IgG, it decreased reactivity with the shrimp-allergic serum. This suggests that this serum is particularly sensitive to conformational rather than linear epitopes. Bands were present to indicate other reactive allergens, however predominant patterns of reactivity between conditions and antibodies were shared indicating that tropomyosin is the major cross-reactive protein for this patient. It should be expected that different shrimp-allergic sera would not all behave in a similar pattern as demonstrated here as patients reactive to linear epitopes may show patterns of reactivity closer to those seen using the anti-tropomyosin IgG.

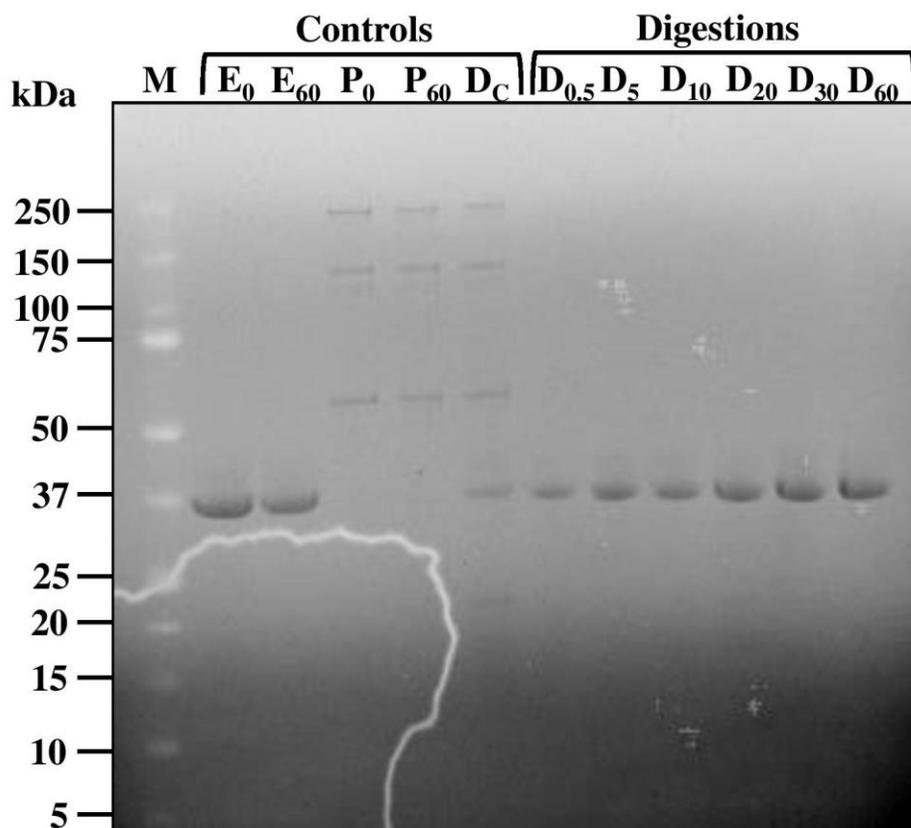
## 5.5 CONCLUSION

Tropomyosin was found in polymeric immunoreactive forms with decreased reactivity under reducing conditions indicating that these polymers are significant sources of potential allergy. A 37 kDa band to represent tropomyosin resisted digestion for up to 20 minutes, however a 75 kDa band and 30 kDa fragment persisted through digestion in non-reducing conditions. Patterns of reactivity were similar between control and immunoblots suggesting tropomyosin was the predominant cross-reactive allergen and this patient is minimally reactive to reduced forms of allergenic insect proteins.

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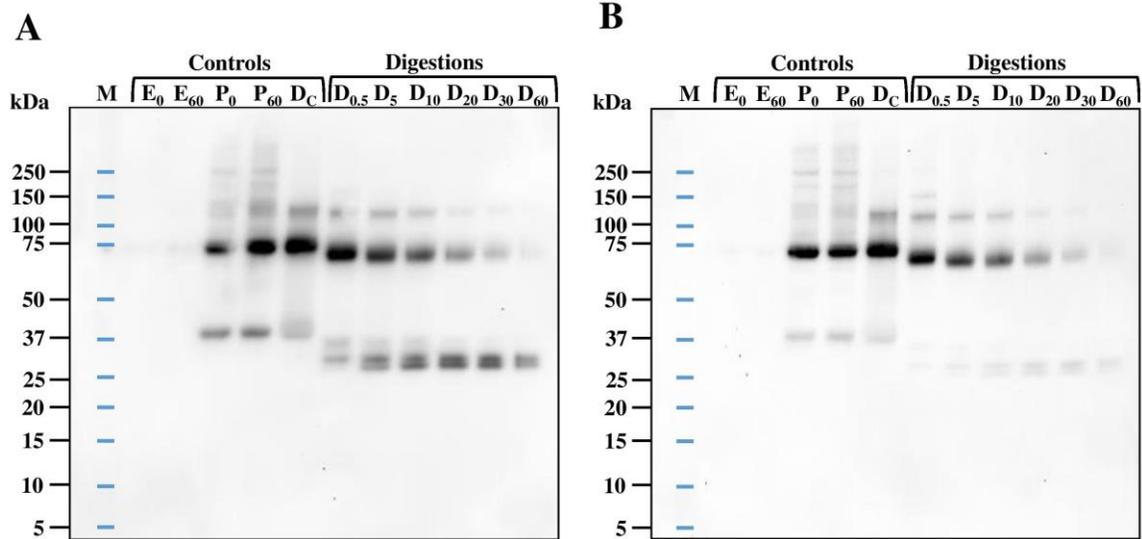
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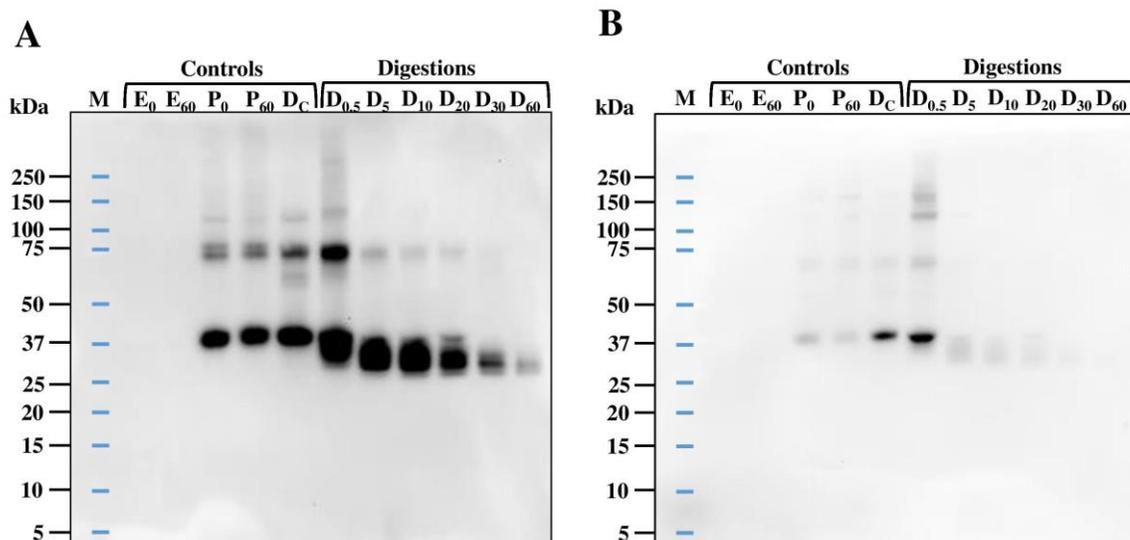
**Figure 5.1 Stimulated gastric digestion of defatted *A. domesticus* powder analyzed by SDS-PAGE and stained with SYPRO ruby**

Image taken with a Kodak Gel Logic 440 image station and filtered using a 590 nm lens. Lanes are as follows: molecular markers (M), Enzyme controls at 0 and 60 minutes ( $E_0$  and  $E_{60}$ ), Protein controls at 0 and 60 minutes ( $P_0$  and  $P_{60}$ ), Digestion control at 60 minutes (DC), and digestion time points from 0.5 to 60 minutes ( $D_{0.5}$  –  $D_{60}$ ). Banding present in protein and digestion controls indicates that proteins extracted in SGF are resistant to precipitation through heating. Digestion time points beginning with 30 seconds have no perceptible bands present to the exclusion of pepsin.



**Figure 5.2** Non-reducing immunoblots of stimulated gastric digestion of defatted *A. domesticus* powder

Probed using either anti-shrimp tropomyosin IgG (A) or shrimp-allergic IgE (B). Lanes are as follows: molecular markers (M), Enzyme controls at 0 and 60 minutes (E<sub>0</sub> and E<sub>60</sub>), Protein controls at 0 and 60 minutes (P<sub>0</sub> and P<sub>60</sub>), Digestion control at 60 minutes (DC), and digestion time points from 0.5 to 60 minutes (D<sub>0.5</sub> – D<sub>60</sub>). (A) Digestion demonstrates a trimming of reactive bands at 37, 75, and 120 kDa along with the formation of fragment bands at 30 and 34 kDa. Fragment bands increase in reactivity through the duration of the digestion. (B) A similar pattern of reactivity is evident excluding a decreased reactivity to fragment bands. Similar reactivity is seen to 75 and 120 kDa bands between blots.



**Figure 5.3 Reducing immunoblots of simulated gastric digestion of defatted *A. domesticus* powder.**

Loaded with 80% of standard protein load and probed using either anti-shrimp tropomyosin IgG (A) or shrimp-allergic IgE (B). Lanes are as follows: molecular markers (M), Enzyme controls at 0 and 60 minutes (E<sub>0</sub> and E<sub>60</sub>), Protein controls at 0 and 60 minutes (P<sub>0</sub> and P<sub>60</sub>), Digestion control at 60 minutes (D<sub>C</sub>), and digestion time points from 0.5 to 60 minutes (D<sub>0.5</sub> – D<sub>60</sub>). (A) Digestion demonstrates initial reactivity to 37, 75, and 120 kDa bands with disappearance at 0.5, 20, and 30 minutes respectively. Fragment bands resolve into 30 and 34 kDa with persistence through digestion and disappearance at 30 minutes respectively. (B) A similar pattern of reactivity is seen with a lesser degree of total reactivity at all points. Reactivity is seen at 120, 75, and 37 kDa with disappearance of each after 0.5 minutes and fragment bands at 30 and 34 kDa disappearing at 30 and 60 minutes respectively.