RESEARCH ARTICLE

Molecular Nutrition & Food Research
www.mnf-journal.com

The Allergen Profile of Two Edible Insect Species—*Acheta domesticus* and *Hermetia illucens*

*Shaymaviswanathan Karnaneedi, Elecia B. Johnston, Utpal Bose, Angéla Juhász, James A. Broadbent, Thimo Ruethers, Emily M. Jerry, Sandip D. Kamath, Vachiranee Limviphuvadh, Sally Stockwell, Keren Byrne, Dean Clarke, Michelle L. Colgrave, Sebastian Maurer-Stroh, and Andreas L. Lopata**

Scope: Edible insect proteins are increasingly introduced as an alternative sustainable food source to address the world's need to feed the growing population. Tropomyosin is the main insect allergen; however, additional potential allergens are not well characterized and the impact of extraction procedures on immunological reactivity is unknown.

Methods and results: Proteins from different commercial food products derived from cricket (*Acheta domesticus*) and black soldier fly (BSF) (*Hermetia illucens*) are extracted using five different extraction buffers. The proteins are analyzed by SDS-PAGE and immunoblotting using allergen-specific antibodies and crustacean allergic patient sera. IgE binding bands are analyzed by mass spectrometry as well as the complete allergen profile of all 30 extracts. Urea-based buffers are most efficient in extracting insect allergens. Shrimp-specific antibody cross-reactivity to tropomyosin from cricket and BSF indicates high sequence and structural similarity between shrimp and insects. Additional unique allergens are identified in both species, including hemocyanin, vitellogenin, HSP20, apolipophorin-III, and chitin-binding protein.

Conclusions: Identifying potential allergenic proteins and their isoforms in cricket and BSF requires specific extraction approaches using urea-based methods. While tropomyosin is the most abundant and immunoreactive allergen, seven unique allergens are identified, highlighting the need for insect species-specific allergen detection in food products.

1. Introduction

The world's increasing population will eventually lead to a shortage in our mainstream food supply.[1] To supplement the global dietary needs, various alternative and innovative solutions have been identified in the past decade including insects, algae, and culture-grown foods.[1,2] Among these, edible insects such as silkworm (*Bombyx mori*), yellow mealworm (*Tenebrio molitor*), field and house cricket (Family: Gryllidae), locust and grasshopper (Family: Acrididae) seem very promising due to their high nutritional values and reduced land and resource use.[1,3] Insects contain proportionally higher levels of protein, vitamins, and minerals, in part due to their high feed-conversion ratio.[4,5]

However, introducing a new food source to the general population requires safety assessments including detection of toxins, pathogen load, and particularly allergens.[6] Allergenic proteins are complicated to analyze as they are generally

S. Karnaneedi, E. B. Johnston, T. Ruethers, E. M. Jerry, S. D. Kamath, A. L. Lopata
Molecular Allergy Research Laboratory
College of Public Health
Medical and Veterinary Sciences
James Cook University
Townsville, Queensland 4811, Australia
E-mail: andreas.lopata@jcu.edu.au

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202300811

DOI: 10.1002/mnfr.202300811

S. Karnaneedi, E. B. Johnston, T. Ruethers, E. M. Jerry, S. D. Kamath, A. L. Lopata
Australian Institute of Tropical Health and Medicine
James Cook University
Townsville, Queensland 4811, Australia

S. Karnaneedi, E. B. Johnston, T. Ruethers, E. M. Jerry, S. D. Kamath, A. L. Lopata
Centre for Food and Allergy Research
Murdoch Children's Research Institute
Melbourne, Victoria 3052, Australia

U. Bose, J. A. Broadbent, S. Stockwell, K. Byrne, M. L. Colgrave
CSIRO Agriculture and Food
306 Carmody Rd, St Lucia, Queensland 4067, Australia

U. Bose, A. Juhász, M. L. Colgrave
Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science
School of Science
Edith Cowan University
Joondalup, Western Australia 6027, Australia

harmless proteins to the non-allergic community. Several studies have attempted to address this major health issue in different edible insect species; specifically in house cricket (*Acheta domesticus*) but these studies mainly focussed on two allergens, tropomyosin (TM) and arginine kinase (AK), comparing Immunoglobulin type E (IgE) binding by shrimp-allergic patients.[7–11] However a recent study clearly demonstrated that different extraction methods affect the proteomic profile of the house cricket and that additional allergens might be of importance for clinical cross-reactivity and for appropriate food labeling.[12]

Unlike yellow mealworm (*T. molitor*), which is a mainstream human food product from the larval stage of an insect, black soldier fly (BSF; *Hermetia illucens*) larvae have yet to become a major source of food protein for humans.[13,14] This can be primarily attributed to the growing conditions of BSF larvae which are usually on food waste material, and thus raises concerns of microbial and heavy metal contamination.[15] Nonetheless, BSF larvae are a widely utilized protein source for animal feed, particularly in aquaculture, livestock, and companion animals due to their high protein content and efficiency in bio-converting waste biomass.[16]

While it is known that some edible insects may affect consumers with crustacean allergy,[8,9] little is known about the complete allergen repertoire that may result in this cross-reactivity. Furthermore, in vitro immunological investigations require proteins extracted optimally from insect food products, which has not been previously established. Therefore, in this study, we investigate the optimal chemical extraction method to investigate allergenic proteins from commercially available cricket and BSF food products to identify putative as well as potential clinical cross-reactive allergens affecting consumers with crustacean or dust-mite allergy. Subsequently, the lack of quantification of insect TM in commercial cricket and BSF products using commercial crustacean allergen test kits highlights the need for targeted insect species-specific allergen detection and measurement.

T. Ruethers, A. L. Lopata
Tropical Futures Institute
James Cook University Singapore
149 Sims Drive, Singapore 387380, Singapore

V. Limviphuvadh, S. Maurer-Stroh
Biomolecular Function Discovery Division
Bioinformatics Institute, Agency for Science, Technology and Research (A*STAR)
Singapore 138671, Singapore

V. Limviphuvadh, S. Maurer-Stroh
IFCS Programme
Singapore Institute for Food and Biotechnology Innovation, Agency for Science, Technology and Research (A*STAR)
Singapore 138669, Singapore

D. Clarke
National Measurement Institute
Melbourne, Victoria 3207, Australia

S. Maurer-Stroh
YLL School of Medicine and Department of Biological Sciences
National University of Singapore
Singapore 117597, Singapore

2. Experimental Section

2.1. Cricket and BSF Protein Extraction

Crickets (*A. domesticus*) used in human food products and BSF (*H. illucens*) larvae for pet food were sourced from three different manufacturers in Australia. Analyses of insect samples, including extraction with different buffers, immunoblots, and mass spectrometry allergen identification and allergen abundance measurement were schematically represented in **Figure 1** (see Appendix for full details of materials and methods).

2.2. Protein Analysis by SDS-PAGE and Immunoblotting

The insect extracts were analyzed using one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) to separate and visualize proteins, as previously described,[17] with some modifications. Following electrophoresis, the separated proteins were transferred to a nitrocellulose membrane and analyzed using six antibodies specific to shrimp allergens as well as serum from five crustacean-allergic patients for IgE binding (Figure 1). Patient details were included in Table S1, Supporting Information.

2.3. Analysis of Cricket and BSF Extracts for Whole Proteome and IgE Antibody-Binding Proteins by Liquid Chromatography-Mass Spectrometry

Insect protein extracts were digested for subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis according to the methods described in Bose et al.[12,18] IgE antibody binding protein bands were excised from SDS-PAGE gels and processed for analysis. Mass spectrometry data of all insect extracts were analyzed using ProteinPilot software v5.0.3 (SCIEX). The 15 cricket extracts (three samples extracted using five protocols; Figure 1) were analyzed using an in-house generated Orthoptera protein database and BSF extracts using an in-house generated Diptera protein database. Both the in-house Orthoptera and Diptera databases also included reference allergens for crustaceans, house dust mites, and cockroaches from the World Health Organization International Union of Immunological Society (WHO/IUIS) Allergen Nomenclature database (www.allergen.org). The peptide peak area was measured for each isoform of TM, AK, myosin light chain (MLC), sarcoplasmic calcium-binding protein (SCP), hemocyanin (HC), and troponin C (TnC), which reflects the relative abundance. Multiple sequence alignment analysis for different tropomyosin allergens was conducted using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Published IgE epitopes of Pen m 1 allergen from black tiger shrimp were retrieved from the Immune Epitope Database and Analysis Resource (IEDB; https://www.iedb.org/) to observe similar epitopes.

2.4. Insect Allergen Detection Using Commercial Food Testing Kits for Crustacean Allergens

Seven cricket-based food products manufactured for human consumption, including whole cricket snacks, corn chips, and

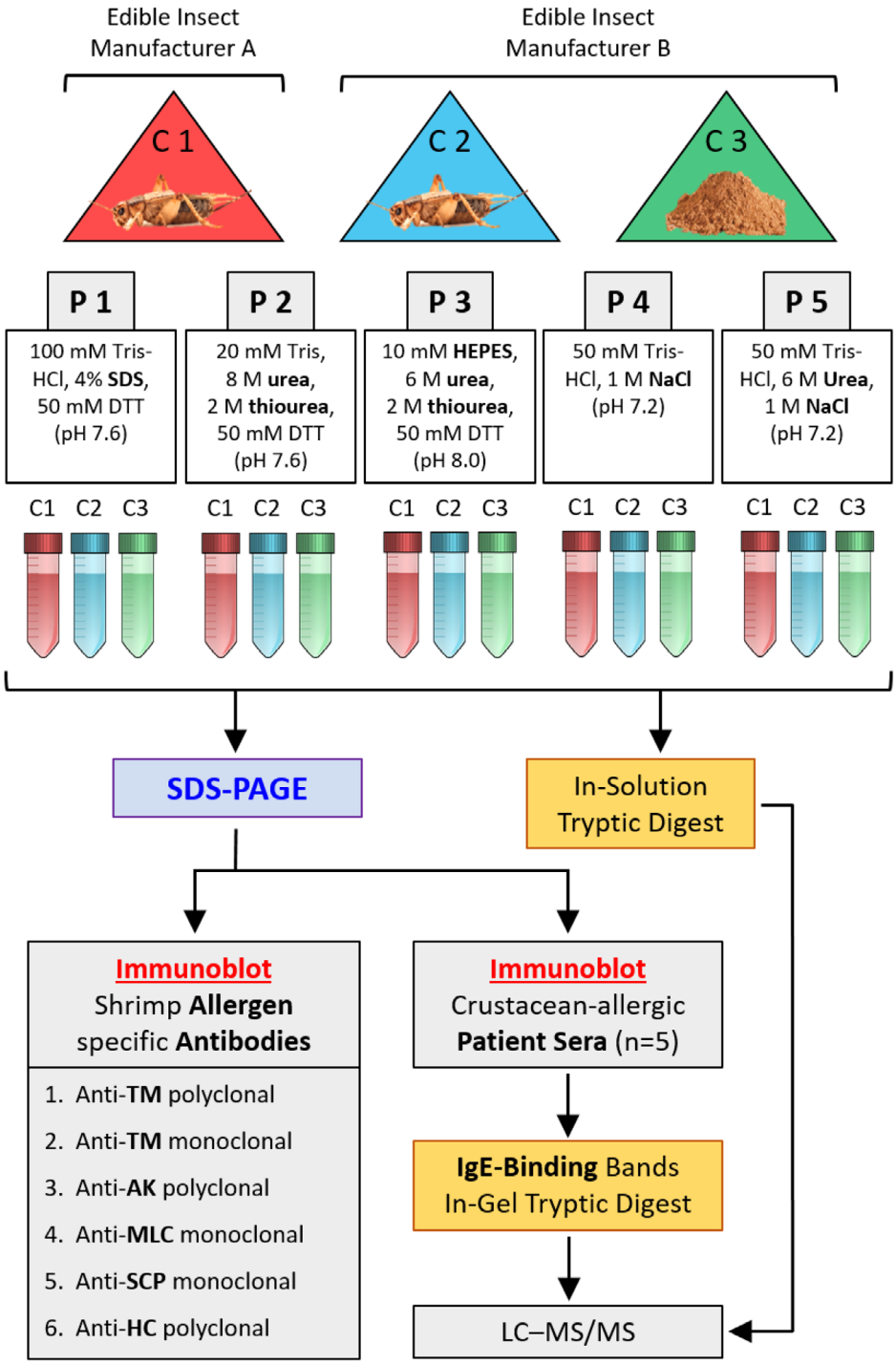

Figure 1. Schematic representation of cricket protein extraction followed by allergen identification and comparative analysis methods. Five extraction protocols (P1–P5) were used for extracting each cricket food product sample (C1–C3) resulting in 15 cricket extracts. Subsequent analysis by SDS-PAGE, Immunoblot, and LC-MS/MS. Note: AK, arginine kinase; HC, hemocyanin; MLC, myosin light chain; SCP, sarcoplasmic calcium-binding protein; TM, tropomyosin. The analysis of black soldier fly (BSF) samples follows the same methods as depicted in this schematic diagram.

energy bars; and two BSF-based pet food products, including sprinkles and biscuits, were analyzed. All nine insect-based food products were extracted and analyzed using two commercial test kits, ELISA Systems (Windsor, Queensland, AU) and R-Biopharm (Darmstadt, Germany), that detect the presence of crustacean allergens in food products using enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol.

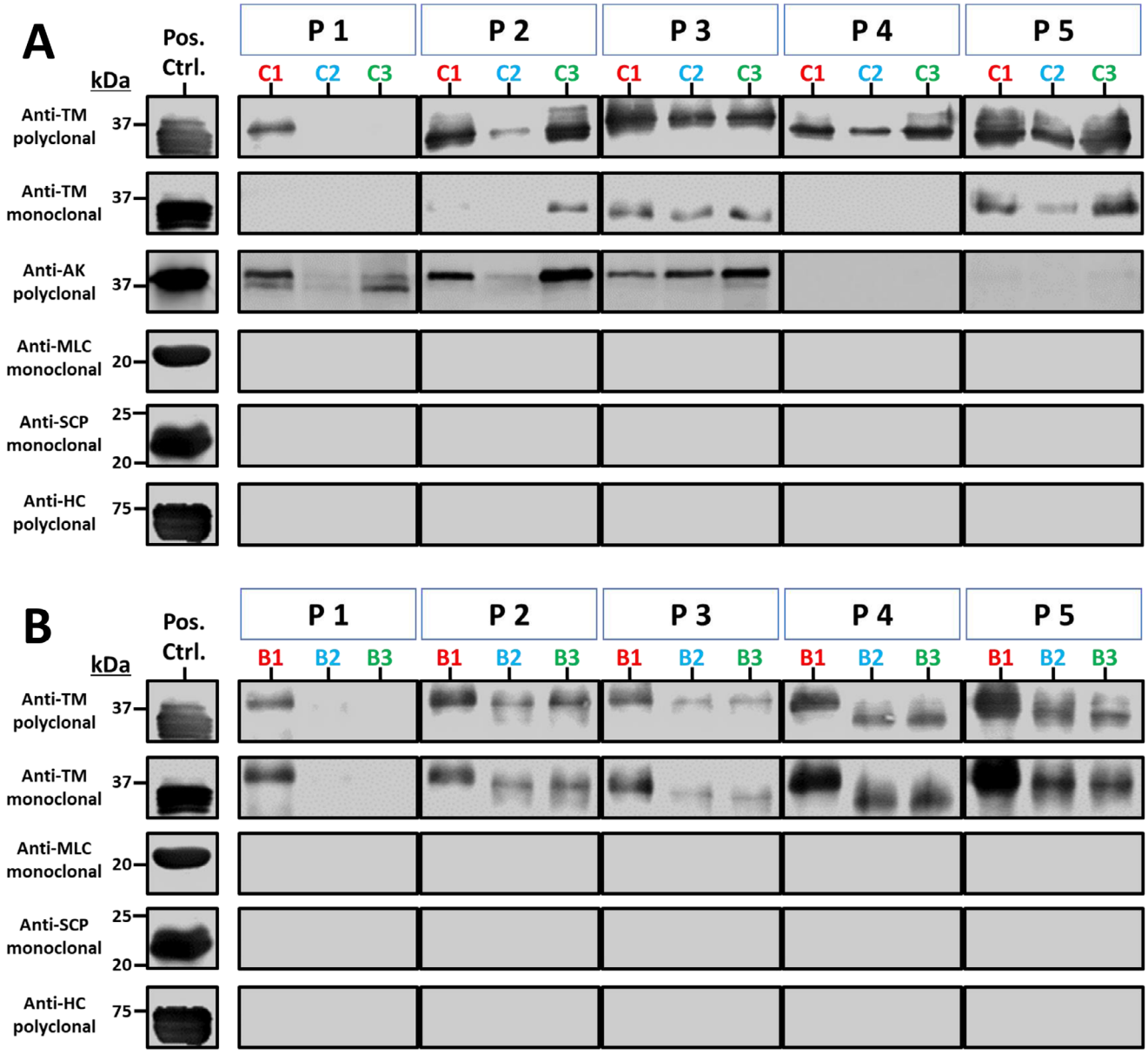

Figure 2. Immunoblot analysis of proteins from (A) cricket food products, C1-C3, and (B) black soldier fly (BSF), B1-B3, extracted using P1-P5 protocols, with shrimp allergen-specific antibodies. Antibodies were raised against shrimp allergens tropomyosin (TM), arginine kinase (AK), myosin light chain (MLC), sarcoplasmic calcium-binding protein (SCP), and hemocyanin (HC). Positive controls include raw extracts and purified allergens from shrimp.

3. Results

3.1. Allergen-Specific Antibody Binding to Cricket and BSF Protein Extracts

To evaluate the similarity of cricket and BSF homologues to shrimp allergens, the extracts generated from the insect-based products were analyzed with allergen-specific antibodies. Overall, among the five investigated allergenic proteins, the antibodies recognized TM and AK in the cricket extracts (**Figure** 2A). The anti-shrimp TM polyclonal antibody detected between one to four different bands of TM homologues in all the cricket extracts except P1-C2 and P1-C3. Although the commercial anti-TM monoclonal antibody was raised against an insect protein, the antibody binding to shrimp TM was previously reported by Kamath et al. and shown by the positive control (raw shrimp extract) used in this study.[17] The monoclonal anti-TM antibody recognized one protein band in all of the cricket samples extracted with P3 and P5 buffers, and two samples of P2 (C1 and C3). Overall, fewer TM bands were detected by the monoclonal antibody compared to the polyclonal antibody. The anti-AK antibody, raised against black tiger shrimp AK, recognized up to two bands of cricket AK in all samples extracted with P1, P2, and P3 buffers, but none in P4 and P5. The anti-MLC, -SCP, and -HC

antibodies did not recognize any bands in any cricket extracts (Figure 2A).

Among the BSF extracts, only anti-shrimp TM polyclonal and monoclonal antibodies bound to most extracts, except P1-B2 and P1-B3. The polyclonal anti-shrimp TM antibody detected up to three different bands, while the monoclonal antibody only detected one band (Figure 2B). Overall, the TM bands identified in the screw-pressed BSF samples (B2 and B3) seem to be lower in molecular weight on the SDS-PAGE gels as compared to the BSF larvae (B1) (Figure 2B). The anti-MLC, -SCP, and -HC antibodies did not recognize any bands in any BSF extracts; and anti-AK antibody was not conducted due to unavailability (Figure 2B).

3.2. Presence and Abundance of Cricket and BSF Homologues of Known Shrimp Allergens

The cricket and BSF extracts were digested with trypsin following an in-solution digestion protocol. The resulting peptides were analyzed using LC-MS/MS and searched against in-house generated Orthoptera and Diptera databases to identify the presence and abundance of known crustacean allergens. Cricket homologues of TM, AK, MLC, HC, and TnC were identified within the cricket extracts (**Figure 3**). The peptide peak area will be referred to as the relative abundance of the proteins from here on. The relative abundance was found to be highly variable across different extraction buffers used (P1–P5) for each protein. However, the abundance profile of TM, AK, MLC, HC, and TnC did not differ much between the different cricket preparations (C1–C3) (Figure 3). Across the buffers, P3 extracted the highest relative abundance of TM, while P4 had the least. AKs were well represented by extraction with P1–P3, but almost no AK was found in the P4 and P5 extracts (Figure 3). MLC, HC, and TnC were more abundant with P1, P2, P3, and P5 extraction protocols than in P4. Of the five proteins analyzed, TM had the highest abundance in cricket (Figure 3).

Within the BSF extracts, TM, HC, and TnC homologues were the only proteins identified (**Figure 4**). AK, MLC, and SCP were not detected in the extracts by tryptic-digested mass spectrometry analysis. Among the three identified proteins, HC was the most abundant, followed by TM and TnC (Figure 4). Between the different extraction buffers utilized in this study, HC was found to be highest in abundance in P1 in all three samples, followed by P2 and P3; and the lowest abundance in P4 and P5. The relative abundance of TM and TnC was similar in all extraction protocols except P4, which had the lowest abundance (Figure 4).

3.3. Protein Profile of Cricket Extracts Using Different Extraction Protocols

The 15 cricket extracts were analyzed using one-dimensional SDS-PAGE to visualize the protein profile. The stained and scanned gel image showed very few distinct protein bands (**Figure 5A**). The smearing of all samples shows that most proteins are degraded likely due to prolonged heat processing during the preparation of edible insect food products. The majority of distinct protein bands in the cricket extracts are visible between MW ranges of 20–50 kDa (Figure 5A). Overall, C2 had the least visible bands when compared to C1 and C3 across all protocols (P1–P5). Extraction using P1 showed very little distinguishable bands, followed by P4 and P3. P2 and P5 protocols, specifically for C1 and C3 samples, demonstrated the most visible bands between the 20 and 50 kDa MW range, including a 100 kDa MW band (Figure 5A).

3.4. Crustacean-Allergic Patient IgE Antibody Binding to Cricket Extracts

All cricket extracts were separated by SDS-PAGE before immunoblotting with a pool of five crustacean-allergic patient sera to detect all cricket proteins recognized by IgE antibodies. Up to six protein bands (A–F) in individual extracts had prominent binding to IgE antibodies. The MW of these protein bands on the SDS-PAGE gel were: A, 55 kDa; B, 36 kDa; C, 24 kDa; D, 23 kDa; E, 15 kDa; and F, 13 kDa (Figure 5B). Densitometric analyses confirmed the highest IgE binding to band-B, as visually observed on the immunoblot (Figure S1, Supporting Information).

IgE binding to B and C, the 36 and 24 kDa protein bands, was observed uniformly across all the cricket extracts (Figure 5B). Contrastingly, IgE binding to A and F, the 55 and 13 kDa bands, was observed only in P2, P3, and P5 extracts. Meanwhile, weak IgE binding to D and E, the 23 and 15 kDa bands were only detected in P5 extracts (Figure 5B). The variability of IgE binding to the extracts was primarily between the different extraction protocols used on the cricket samples. Although there were some differences in the relative intensity of IgE binding, within each extraction buffer, there were no significant differences in the IgE binding profile between the three cricket samples (C1–C3) (Figure 5B). All six positive IgE binding bands were detected from P5 cricket extracts, with an overall highest intensity observed in the P5-C1 extract. Therefore, IgE binding bands from P5-C1 were excised and subjected to discovery LC-MS/MS analysis and protein identification.

3.5. Protein Profile of BSF Extracts Using Different Extraction Protocols

Subsequent to the cricket analyses, all 15 BSF extracts were analyzed using one-dimensional SDS-PAGE gel to determine the protein profile. The stained gel showed fewer distinct bands than that found in the cricket extracts (**Figure 6A**). As with the cricket samples, the smearing profile of all the samples shows high degradation of proteins due to prolonged heat processing. Most bands were high molecular weight bands in the 50–100 kDa range (Figure 6A). Of all the samples, sample B1 extracted using P4 and P5 had the most distinct bands ranging between 10 and 150 kDa (Figure 6A). The band with the highest intensity is found in P4-extracted BSF samples at 75 kDa. The same protein band was also identified in all of the other extraction protocols but at a much lower intensity (Figure 6A).

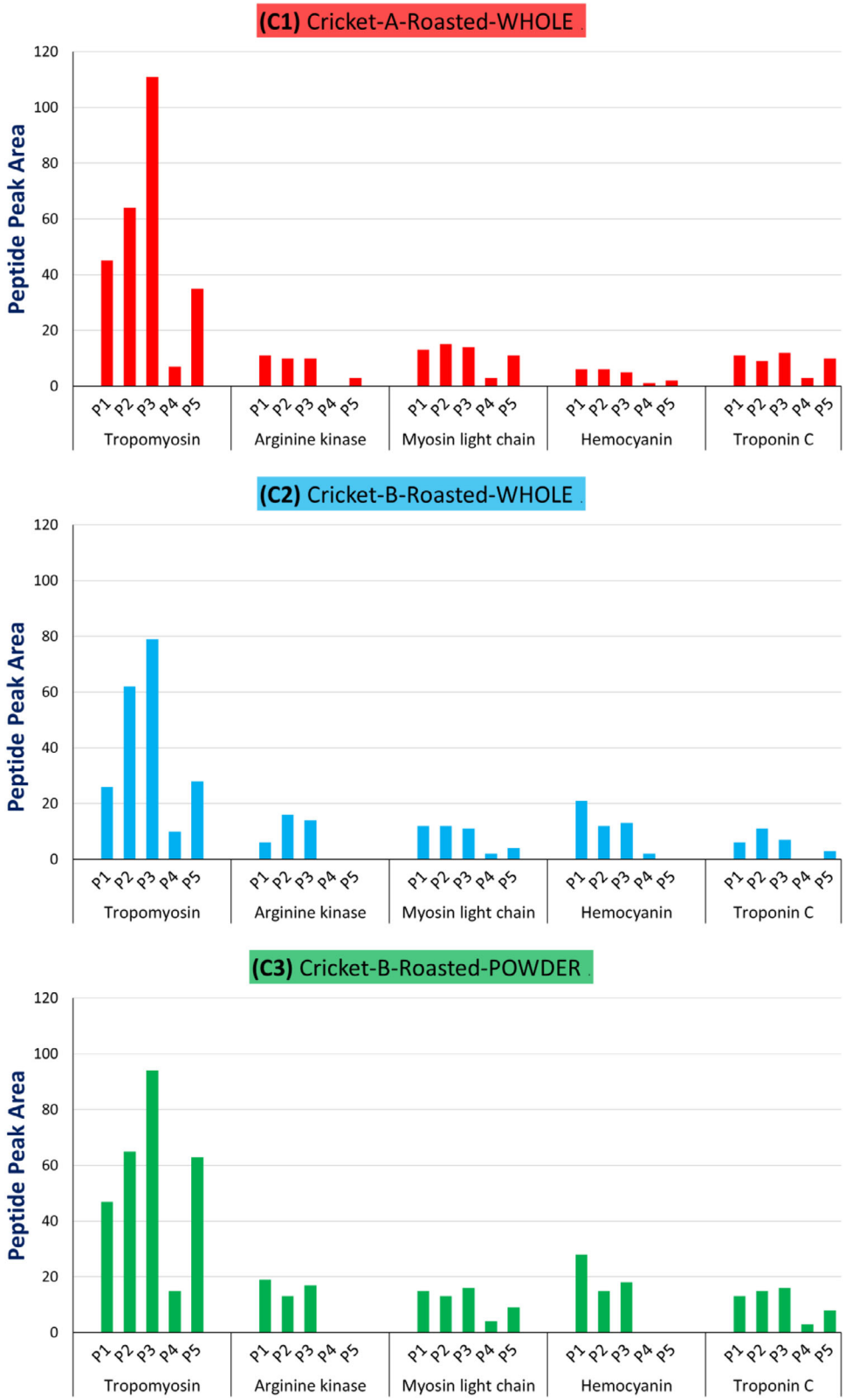

Figure 3. Mass spectrometric (LC-MS/MS) analysis of 15 cricket protein extracts (in-solution tryptic digest). Results shown for shrimp allergen homologues TM, AK, MLC, HC, and Troponin C (TNC). SCP was not detected. The peptide peak area reflects relative abundance. The LC-MS/MS search was conducted using an in-house generated Orthoptera database.

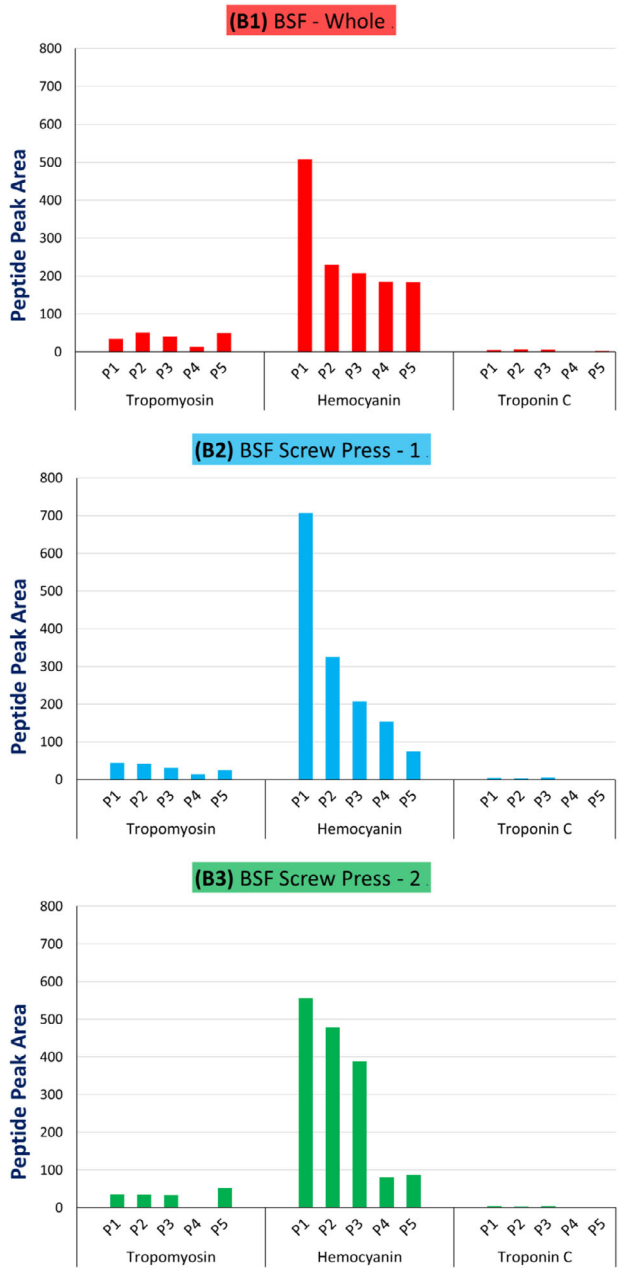

Figure 4. Mass spectrometric (LC-MS/MS) analysis of 15 black soldier fly protein extracts (in-solution tryptic digest). Results shown for shrimp allergen homologues TM, HC, and TNC. AK, MLC, and SCP were not detected. The peptide peak area reflects relative abundance. The LC-MS/MS search was conducted using an in-house generated Diptera database.

3.6. Crustacean-Allergic Patient IgE Antibody Binding to BSF Extracts

The BSF extracts separated by SDS-PAGE were immunoblotted with the same pool of five crustacean-allergic patient sera to detect IgE antibody-binding proteins in BSF. The sample with the highest number and most intense IgE-binding bands (P5-B1) was selected for further analysis (Figure 6B). Up to seven protein bands were identified (T–Z) that bound to IgE antibodies from the pool of patient sera. The MW of these protein bands on the SDS-PAGE gel were: T, 75 kDa; U, 60 kDa; V, 37 kDa; W, 25 kDa; X, 20 kDa; Y, 14 kDa; and Z, 12 kDa (Figure 6B). Band-V has the highest IgE-binding intensity across all samples and was confirmed by densitometric analyses conducted on the bands in P5-B1 (Figure S2, Supporting Information).

IgE antibody binding to the 37 kDa protein band (band-V) was detected in all the BSF samples (Figure 6B). Meanwhile, band-T (75 kDa) was only detected in P4 and P5 samples. Bands W, X, Y, and Z, were only identified in P4-B1 and all of P5 samples. Similar to the cricket extracts, the variability of IgE-binding was mainly observed between the different extraction protocols, but some variability between different samples (preparations) of BSF can be seen in the P4 protocol (Figure 6B). Furthermore, similar to the allergen-specific antibody analysis (see Section 3.2), IgE-binding protein bands in B2 and B3 (screw-pressed BSF samples) were observed to be at a slightly lower MW than the B1 sample (Figure 6B). All seven protein bands (T–Z) were analyzed by discovery LC-MS/MS for protein identification, identical to the analysis of cricket proteins.

3.7. IgE-Binding Protein Identification by Mass Spectrometry

IgE-binding protein bands from P5-C1 cricket extract and P5-B1 BSF extract were analyzed by LC-MS/MS to identify the proteins therein (**Tables 1** and **2**). Comprehensive protein identification was achieved by utilizing in-house databases that contained all Orthoptera (order of insects that comprises the crickets, grasshoppers, and locusts) proteins to analyze the cricket in this study, and Diptera (order of single-pair winged insects commonly known as flies) proteins to analyze BSF (see Supplementary Methods), a widely applied proteomics approach to identify proteins in under-investigated species.[12,18] Therefore, our results are reliable for protein identity but not necessarily amino acid sequence if the protein is unknown in cricket or BSF.

Among the IgE-binding protein bands from cricket, protein band A (55 kDa) was identified to contain two isoforms of vitellogenin protein (57 kDa), both matching vitellogenin from the cricket species, *Teleogryllus occipitalis*, one with an amino acid sequence coverage of 26% and the other 6% (Table 1). In protein band B (36 kDa), three isoforms of TM were identified, one matching with TM from the *Teleogryllus emma* cricket and the other two from *A. domesticus* (Table 1). The approximate MW of published TM in crickets (NCBI and UniProt database) is 33 kDa. The three identified TM isoforms had sequence coverage between 62% and 78%. Two proteins were identified within protein band C (24 kDa), Troponin I matching with the homologue from *T. emma* and MLC matching *T. occipitalis*. Both proteins had similar ProteinPilot unused peptide scores (≈7), reflecting very high confidence in detection; and sequence coverage of 32% and 26%, respectively (Table 1). Two isoforms of MLC were detected in protein band D (23 kDa), one matching with MLC from *Gryllotalpa orientalis* and one from *T. occipitalis* (Table 1). The approximate MW of MLC is about 23 kDa (NCBI/UniProt). However, the MLC protein matching the MLC from *T. occipitalis* was also detected in protein bands E (15 kDa) and F (13 kDa), likely representing a fragment of this protein. Another protein, apolipophorin-III was identified in protein bands E and F,

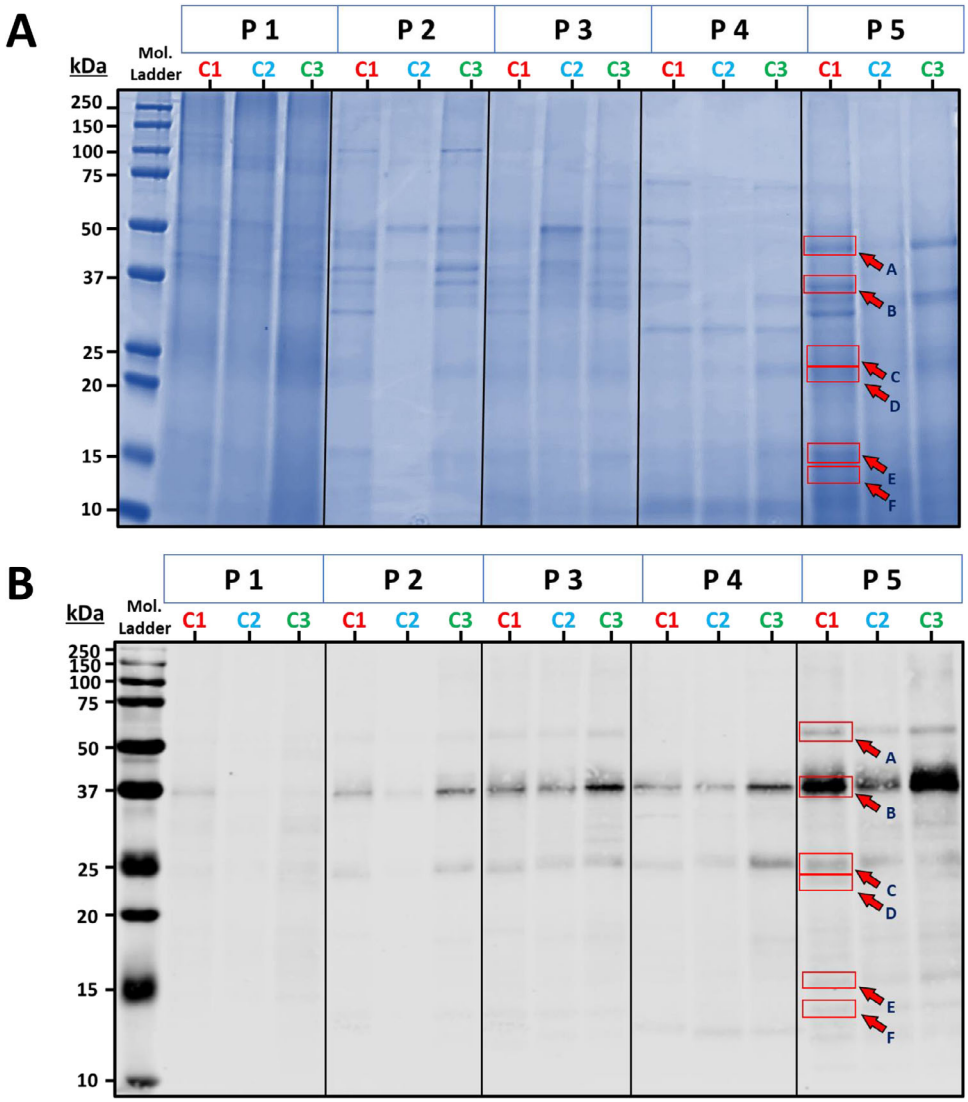

Figure 5. A) SDS-PAGE analysis of cricket proteins. 15 cricket extracts from three cricket food products (C1–C3) were extracted with five protocols (P1–P5). Proteins were stained with Coomassie blue. B) Immunoblot analysis using serum pool from five crustacean-allergic patients (see Table S1, Supporting Information). Six IgE-binding bands in C1/P5 (A–F; red boxed) analyzed by LC-MS/MS.

matching with the published apolipophorin-III protein sequence from *A. domesticus* with a known MW of 20 kDa (NCBI/UniProt). The % coverage was, however, higher in band E (68%) than in band F (15%) (Table 1).

Despite the relatively low peptide coverage of some of the proteins, the identified proteins have the highest % coverage among all the other MS-identified proteins. This may be due to an underrepresentation of the MS database utilized, even though the database utilized is highly comprehensive. Nonetheless, the results demonstrate the possibility of isoforms of some of the identified proteins as they were also identified with a higher % coverage in other IgE-binding protein bands such as Apolipophorin-III and Myosin light chain-2 (Bands D and E).

Analyzing the IgE binding protein bands in BSF, the 75 kDa band T contained three isoforms of hemocyanin (HC) from *H. illucens* (inferred from the annotated BSF genome within the in-house generated Diptera database). All three HC isoforms had high % sequence coverage (42–70%); while two of the three isoforms had high ProteinPilot unused peptide scores of 30 and 76 (Table 2). The same HC isoforms were also identified in protein band-U, which are likely fragmented proteins due to the lower MW of this band (60 kDa). Band-U also contained ATP synthase protein with a high unused peptide score (25.7) and 42% sequence coverage (Table 2). The band with the most intense IgE-binding profile which was found across all extractions, band-V, contains four isoforms of TM from various species including *H. illucens*. All four TM isoforms detected had high % sequence coverage (43–67%) (Table 2). TM was also found in band-W but with a lower % coverage (16.5%), indicating a fragment of TM. Bands W and X also contained HSP20 from *Drosophila*

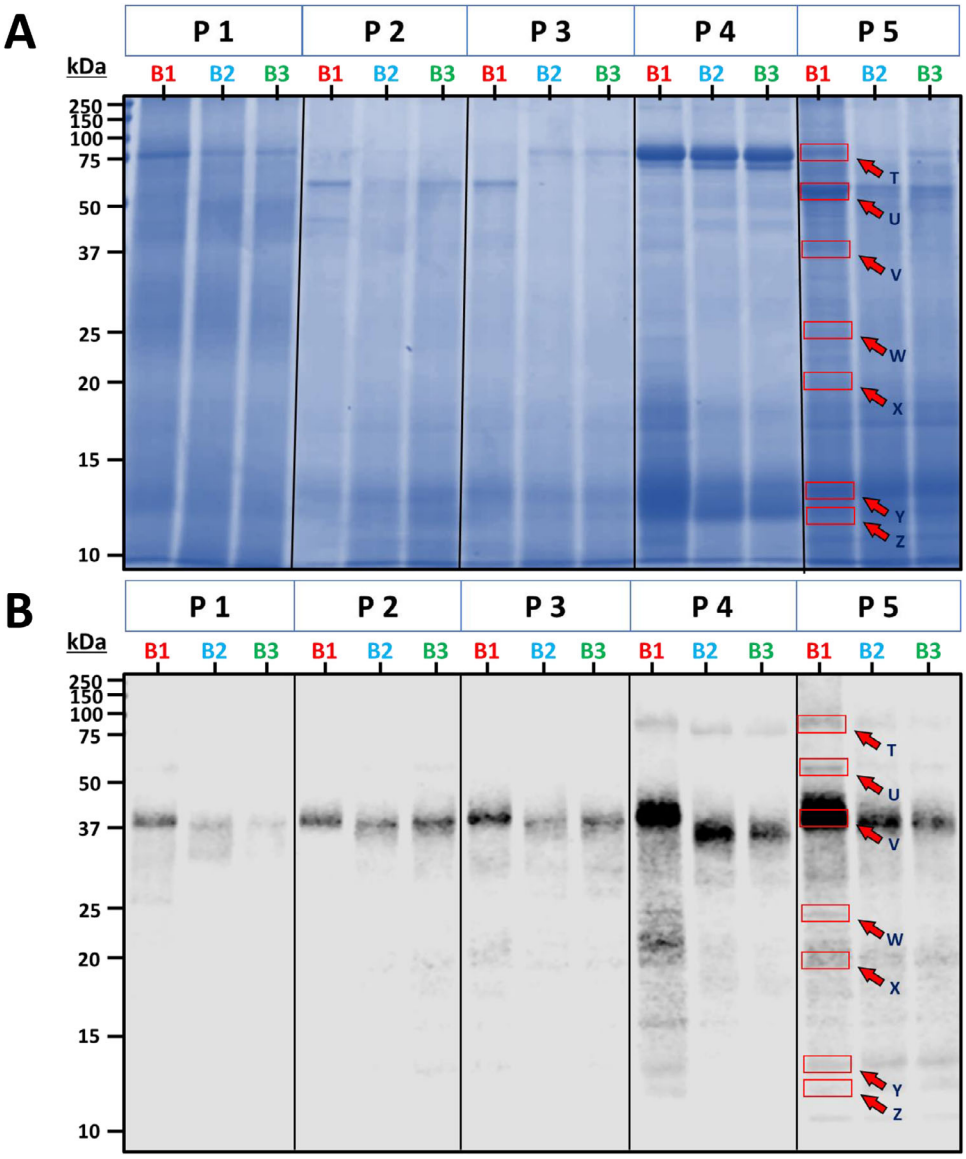

Figure 6. A) SDS-PAGE analysis of black soldier fly (BSF) proteins. Fifteen BSF extracts from whole (dehydrated), screw press-1, and screw press-2 BSF samples (B1–B3) were extracted with five protocols (P1–P5). Proteins were stained with Coomassie blue. B) Immunoblot analysis using serum pool from five crustacean-allergic patients (see Table S1, Supporting Information). Seven IgE-binding bands in B1/P5 (T–Z; red boxed) were analyzed by LC-MS/MS.

mojavensis and *H. illucens* (Table 2). The known MW of HSP20 is ≈20 kDa (NCBI/UniProt). IgE-binding bands X, Y, and Z also contained chitin-binding (cuticle) protein and odorant-binding protein (OBP), all of which had high % sequence coverage when analyzed by mass spectrometry (>53%) (Table 2).

3.8. Conservation and IgE Epitope Analysis of Tropomyosin from House Cricket, Shrimp, and Mite

Multiple sequence alignment of the well-studied Pen m 1 TM allergen from black tiger shrimp and Der p 10 TM allergen from house dust mite with the two published *A. domesticus* house cricket TM sequences demonstrated a pairwise sequence similarity between 68% and 83% (**Figure 7**). Consensus sequences or conserved regions were found throughout the cricket TM sequence. When aligning with published Pen m 1 allergen IgE epitopes,[19] based on amino acid sequence similarity, 1 out of 10 published epitopes was identified to be 100% identical in *A. domesticus* TM (Figure 7). Nine peptides identified by LC-MS/MS analysis fully covered this specific epitope region demonstrating strong evidence of the presence of this particular epitope in the *A. domesticus* samples analyzed in this study. Furthermore, two additional Pen m 1 IgE epitopes were also identified in the cricket TM, with one or two amino acid substitutions and less than 100% coverage from the peptides generated from *A. domesticus*.

Table 1. Proteins identified by LC-MS/MS in IgE-binding bands (see Figure 5) of cricket (*A. domesticus*) extract utilizing an Orthoptera protein database.

Protein band	MW on SDS-PAGE	In-gel tryptic digest mass spectrometry (LC-MS/MS) results of cricket					
		Identified proteins	Accession number	Species	Known MW of protein	Score[a)]	% Coverage
A	55	Vitellogenin	Tocci_0124176	*Teleogryllus occipitalis*	57	4.00	25.83
		Vitellogenin	Tocci_0549816	*Teleogryllus occipitalis*	57	4.00	6.38
B	36	Tropomyosin	AVI26879.1	*Teleogryllus emma*	33	50.02	61.62
		Tropomyosin	QCI56568.1	*Acheta domesticus*	33	31.21	71.71
		Tropomyosin	QCI56569.1	*Acheta domesticus*	33	8.05	78.22
C	24	Troponin I	A0A2P1ANL5	*Teleogryllus emma*	24	7.58	32.32
		Myosin light chain-2	Tocci_0215051	*Teleogryllus occipitalis*	23	7.28	25.78
D	23	Myosin light chain-2	AAW22542.1	*Gryllotalpa orientalis*	23	13.64	57.56
		Myosin light chain-2	Tocci_0215051	*Teleogryllus occipitalis*	23	6.00	32.81
E	15	Apolipophorin-III	Q16989	*Acheta domesticus*	20	15.51	67.93
		Myosin light chain-2	Tocci_0215051	*Teleogryllus occipitalis*	23	6.68	25.78
F	13	Myosin light chain-2	Tocci_0215051	*Teleogryllus occipitalis*	23	5.62	25.78
		Apolipophorin-III	Q16989	*Acheta domesticus*	20	2.86	14.67

Protein hit results are known allergens in insects and crustaceans, and filtered according to matching molecular weight (MW) on SDS-PAGE and MW of protein identified.
[a)] ProteinPilot unused peptide score (score ≥ 4 = confidence score ≥ 99.99%).

3.9. Insect Allergen Detection in Food Products Using Commercial Test Kits for Crustacean Allergens

With the increased consumption of insects as food, it is important to be able to detect insect allergens for appropriate food labeling and consumer safety.[20] Due to the similarity of some of the allergens between cricket and shrimp, commercial kits designed for detecting crustacean ingredients and contaminants were tested for their viability for insect allergen detection in food products. The results from the two crustacean test kits showed

Table 2. Proteins identified by LC-MS/MS in IgE-binding bands (see Figure 6) of black soldier fly (*H. illucens*) extract utilizing a Diptera protein database.

Protein band	MW on SDS-PAGE	In-gel tryptic digest mass spectrometry (LC-MS/MS) results of BSF					
		Identified proteins	Accession number	Species	Known MW of protein	Score[a)]	% Coverage
T	75	Hemocyanin	Hil_G2_018919	*Hermetia illucens*	75	76.23	69.48
		Hemocyanin	Hil_G2_073508	*Hermetia illucens*	75	30.17	41.81
		Hemocyanin	Hil_G1_010218	*Hermetia illucens*	75	6.43	63.74
U	60	Hemocyanin	Hil_G1_010218	*Hermetia illucens*	75	34.83	43.48
		ATP synthase subunit beta	B4L7D0	*Drosophila mojavensis*	54	25.7	42.29
		Hemocyanin	Hil_G2_018919	*Hermetia illucens*	75	4.68	42.62
V	37	Tropomyosin	A0A1Q3FNG3	*Culex tarsalis*	33	49.44	53.68
		Tropomyosin	D3TRT1	*Glossina morsitans*	33	37.18	42.61
		Tropomyosin	Hil_G1_014894	*Hermetia illucens*	39	18	67.20
		Tropomyosin	W8GQX8	*Musca domestica*	32	10.08	52.36
W	25	HSP20	A0A0Q9XQN6	*Drosophila mojavensis*	21	5.51	45.36
		Tropomyosin	A0A1L8E5L4	*Nyssomyia neivai*	32	4.55	16.49
X	20	HSP20	Hil_G2_027465	*Hermetia illucens*	20	3.73	32.20
		Chitin-binding (cuticle) protein	Hil_G2_068406	*Hermetia illucens*	24	2.06	62.04
Y	14	Chitin-binding (cuticle) protein	Hil_G2_068439	*Hermetia illucens*	24	11.82	74.52
		Odorant-binding protein (OBP)	Hil_G1_008909	*Hermetia illucens*	14	8.52	53.27
Z	12	Odorant-binding protein (OBP)	Hil_G2_046639	*Hermetia illucens*	14	14.14	62.70
		Odorant-binding protein (OBP)	Hil_G1_008909	*Hermetia illucens*	14	6.07	53.27

Protein hit results are filtered according to matching molecular weight (MW) on SDS-PAGE and MW of protein identified. [a)] ProteinPilot unused peptide score (score ≥ 4 = confidence score ≥ 99.99%).

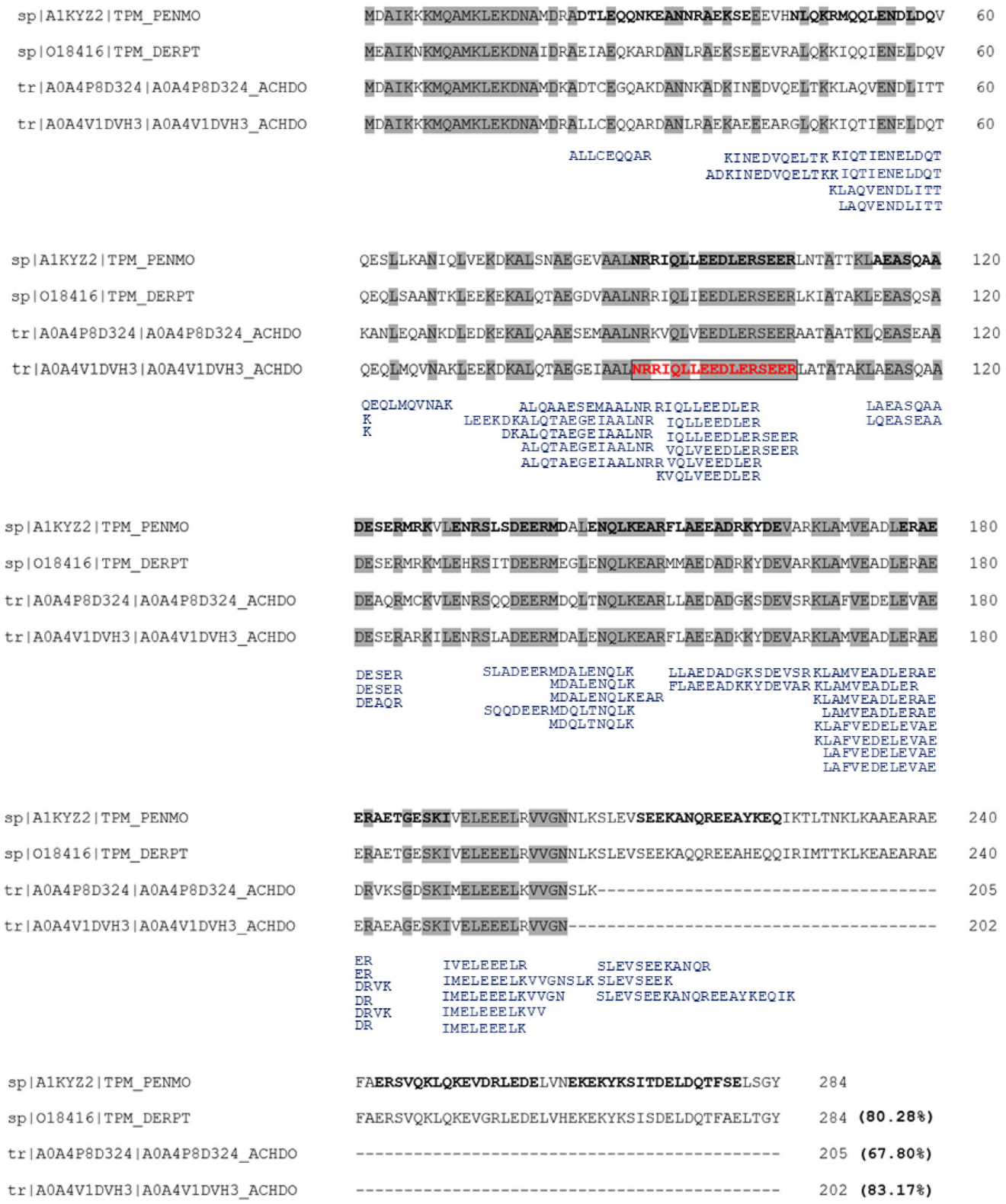

Figure 7. Amino acid multiple sequence alignment of tropomyosin allergen from black tiger shrimp (*Penaeus monodon*; Pen m 1; A1KYZ2), house dust mite (*Dermatophagoides pteronyssinus*; Der p 10; O18416), and house cricket (*Acheta domesticus*; A0A4P8D324, A0A4V1DVH3). Consensus sequences are highlighted in grey. Pen m 1 IgE-binding epitopes (sourced from IEDB) are in bold and in black. Pen m 1 IgE-binding epitope found in cricket is boxed, in bold and in red (100% identity). Amino acid sequences of peptides identified by mass spectrometry are aligned in blue. Percentage sequence similarity to Pen m 1 is noted for house dust mite tropomyosin (80.28%) and cricket tropomyosin (67.8% and 83.17%).

differences from each other but were consistent across all food products. One test kit detected crustacean allergens in all nine products, with the highest detected in BSF sprinkle (17 206 ppm) (Table S2, Supporting Information). Amongst the cricket-based products, the highest crustacean allergens were detected in plain organic roasted crickets by Grilo (610.8 ppm) and the lowest detected in cricket corn chips by Grilo (12.2 ppm). In contrast, the other test kit detected crustacean allergens in the BSF sprinkle (0.078 ppm), while crustacean allergens were not quantifiable in the two cricket products, and none were detected in the six other products (Table S2, Supporting Information).

4. Discussion

Evolutionarily, insects are closely related to crustaceans such as shrimps, crabs, and lobsters, as they belong to the same phyla: Arthropoda. Therefore, many proteins from crustaceans and insects but also mites contain similar conserved regions of amino acid sequences.[19,21] The mechanism of allergy hypersensitivity involves the recognition of immunoglobulin type E (IgE) antibodies to specific epitopes on a protein's amino acid sequence. Due to high conservation, epitopes are shared between the same protein families in crustaceans and insects.[19] This increases the likelihood of IgE antibodies, which originally recognized a crustacean protein (allergen), to also bind to a similar allergen in insects and cause an allergic reaction, a phenomenon known as cross-reactivity in allergy.[22,23] Crustacean food allergy affects up to 4% of the population in different regions of the world.[24] Given the high prevalence of crustacean food allergy, this poses a significant threat to individuals already allergic to crustaceans who may also react upon consuming novel insect protein-based foods.

Optimal protein extraction from potentially allergenic food sources is crucial and determines the detectability and accuracy of allergen measurement. The effect of extraction protocols in mass spectrometry proteomics analysis especially on *A. domesticus* and *H. illucens* has been recently published reporting that buffer selection has a major impact on proteomic profile and the detection of allergens.[12,18] The current study continues this investigation by focusing on identifying appropriate extraction buffers for immunological investigations including allergen-specific antibody and patient IgE antibody reactivity. The findings of this study demonstrated that urea-based buffers (P2, P3, and P5) successfully extracted the important crustacean allergen homologues in both insect species analyzed, which would otherwise be missed as was observed in P1 and P4. The addition of dithiothreitol (DTT) in P2 and P3 extraction protocols allowed the identification of AK in cricket; however, overall, P5 (without DTT) showed the highest intensity of IgE antibody binding. DTT primarily reduces disulphide bonds within the protein and is commonly used in SDS-PAGE prior to immunoblotting to assess IgE antibody binding; but not during protein extraction. This indicates that there is a potential cause for DTT, when used during extraction, to affect the IgE epitopes within the protein. The combinatorial use of urea and DTT in extraction buffers has been previously shown to yield the highest number of proteins and peptides overall, but also in detecting crustacean allergen homologues in insects when using mass spectrometry-based quantitative analysis.[12,18] However, mass spectrometric analysis examines digested peptides of proteins, while IgE antibody reactivity analysis requires intact epitopes for IgE antibody binding. Therefore, care should be taken when selecting extraction buffers depending on the downstream analysis of allergens in novel food products.

Insect species within the Orthoptera order, including crickets, grasshoppers, and locusts, have been used as a protein source in different regions for centuries, but also recently by many food product companies investing in alternative protein sources.[1,25] Among these, crickets—belonging to the Gryllidae family—are the most utilized and thus, have been widely studied for their allergenic properties.[7–10] In our study, IgE immunoblotting demonstrated that the muscle protein TM in cricket is the most IgE-reactive protein. TM has been the most frequently implicated allergenic protein in the house cricket *A. domesticus* by various allergenicity assessment studies.[8,9,11] While cross-reactivity between cricket and shrimp TM has been somewhat evident, the implicating IgE-binding epitopes have not been previously identified. Our study provides novel evidence for the presence of one epitope that is 100% identical between shrimp TM and cricket.

Another protein that was previously reported as an allergen in this cricket species is AK.[10] However, in our study, the IgE-reactive protein bands from cricket extract (P5-C1) selected in our study and subjected to mass spectrometry did not identify AK to be IgE-reactive. Evidently, we also did not observe binding to AK in the P5-C1 extract when using the AK-specific antibody, which was otherwise identified in P1, 2, and 3. Hence, we recommend using multiple buffers when identifying allergenic proteins as this may be one of the reasons for the identification of different allergenic proteins in the same species by different studies.

Our study provides the first immunological evidence of four additional proteins in crickets that are reactive to IgE antibodies from crustacean-allergic individuals. These IgE-reactive proteins include vitellogenin, troponin I (TnI), myosin light chain-2 (MLC), and apolipophorin-III. Vitellogenin, a yolk protein found in many oviparous animals, has previously been identified as an allergen in various arthropod species, including mites, cockroaches, and even shrimps, but has only been registered within the WHO/IUIS Allergen Nomenclature database as an arthropod allergen in bee/wasp venom.[26–29] The molecular weight varies across different species; however, evidently, they all seem to share similar IgE-reactive characteristics. Apolipophorin proteins have been previously registered as allergens in mites, but not apolipophorin-III in particular, which is a transport protein of the second messenger diacylglycerol. However, a recent study by Barre et al. identified apolipophorin-III in *A. domesticus* and proposed this protein to be an IgE-binding cross-reactive allergen based on computer modeling.[30] Our study demonstrated the IgE reactivity of crustacean allergic patients. The other two IgE-binding allergens identified in our study, TnI and MLC are both muscle proteins and importantly, they are both registered food allergens in crustaceans within the WHO/IUIS allergen database.[31] MLC has also been reported as an inhalant allergen in cockroaches.[32–34]

While many studies on edible insect allergens focus on other larvae-based food products such as yellow mealworm (*Tenebrio molitor*) and silkworm (*Bombyx mori*), few studies have thus far analyzed larvae from the BSF and report that TM is the allergen of concern in BSF.[11,18,35] The current study complements these findings as TM was also found to be highly IgE-reactive

in BSF larvae. TM is a widely known invertebrate pan-allergen that is also highly conserved, extremely heat-stable, and easily aerosolized.[36] Therefore, this allergen would pose a high risk to workers handling animal feed containing BSF larvae protein.

Furthermore, our study is the first to demonstrate IgE binding to additional proteins in BSF larvae including HC, HSP20, chitin-binding protein, and odorant-binding protein. Of particular interest is the HC protein, which is also a registered crustacean allergen in the WHO/IUIS allergen database. Unlike TM, HC is partially heat-stable but is known to be in high numbers of isoforms—up to 12 identified in black tiger shrimp.[37] Our study confirmed that HC is more abundant than TM within the BSF proteome in the P1, P2, and P3 protocols. However, IgE-binding was higher in P4 and P5, again stressing the importance of extraction buffer selection based on downstream analysis. Another contrasting finding was the absence of HC recognition by an HC-specific antibody raised against HC from black tiger shrimp—indicating a lack of similarity—despite IgE antibody recognition by crustacean-allergic individuals. This is likely due to the difference in epitopes recognized by these two types of antibodies; however, amino acid sequence similarity was not possible due to the lack of publicly available TM or HC sequences for BSF. Odorant binding proteins identified to be IgE-binding in BSF larvae in this study were also noted in yellow mealworm (*T. molitor*) and the silkworm (*B. mori*) in the multiomics study by Barre et al.[30] The same study also identified a larval cuticle protein as an insect allergen; however, the similarity of this protein to the chitin-binding cuticle protein identified in our study is unknown.

Only one of the two commercial crustacean allergen test kits detected "crustacean allergens" in all nine cricket- and BSF-based food products, while the other test kit utilized in this study detected no allergens in these products. Inconsistencies and limited capacities of ELISA-based food allergen test kits have been previously demonstrated, especially in a complex food source such as fish, where species-specific variation plays a major role in the diversity of allergens.[38] While crustaceans and insects have been demonstrated by current and past studies to possess similarities in their shared allergens, such as tropomyosin, they may still be missed by some crustacean-based food allergen test kits depending on their target proteins. Furthermore, our study shows additional allergens exist in insects not previously recorded in crustaceans. These conflicting results demonstrated by the two test kits compared in the current study indicate the need for insect-specific food allergen test kits, preferably targeting the most clinically relevant insect allergens.

In conclusion, our study demonstrates that buffer selection during insect protein extraction has a critical impact on the identification of allergens within different insect species. While urea-based buffers were observed to be the optimal extraction for in vitro immunological analysis of allergens, the presence or absence of other buffer compositions, such as DTT, will exclude/include certain proteins. Whole extract proteomic analysis suggested that TM and HC were the most abundant allergens in cricket and BSF respectively, and both were also identified to be IgE antibody-binding. Aside from TM, four unreported IgE-binding putative allergens in cricket and another four unreported IgE-binding putative allergens in BSF were identified in our study. However, TM was observed to be the most immunoreactive allergen in both species. Furthermore, conservation and IgE epitope comparative analysis between Pen m 1 allergen and cricket TM demonstrated a single IgE epitope that was 100% identical between the two species. The overall findings from this study suggest that edible insects have several cross-reactive allergens that could be of clinical relevance, impacting exposed crustacean-allergic consumers. These findings are complicated as several additional IgE-binding proteins were identified, which do not have homologues among published crustacean allergens. The identified IgE-binding proteins should be further investigated for their exact amino acid sequence, expressed recombinantly and analyzed in vitro for their clinical relevance as insect allergens and their potential to aid diagnostics and dietary management for respective allergic consumers. In addition, insect-based food products cannot be analyzed for the presence of food allergens using commercial crustacean allergen test kits, as demonstrated in this study. The combination of these findings implies that diagnostic tools, allergen test kits, and food allergen labeling should take into account these unique allergens in edible insects to prevent and improve clinical management of accidental allergic reactions to edible insects, which could be one of the primary sources of protein for the human population in the future.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The project was funded by the 1st Singapore-Australia Bilateral Program on Innovations in Food for Precision Health, 2019 (Grant number: SG-AUS2019_191D4) granted to M.C., A.L.L., and S.M.S. encompassing a partnership between The Commonwealth Scientific and Industrial Research Organisation (Australia), James Cook University (Australia) and Agency for Science, Technology and Research (A*STAR, Singapore). The patient sera was obtained as part of a project funded by the National Health and Medical Research Council (NHMRC) Australia (Grant number: GNT1086656) granted to A.L.L.. S.K. is a holder of a PhD scholarship from the Centre for Food and Allergy Research, an NHMRC Centre of Research Excellence, Australia. The authors thank Paula Pownall (Grubs Up, Australia) for supplying the whole cricket sample and the company Grilo for supplying roasted whole cricket and roasted cricket powder samples. They thank Olympia Yarger (GOTERRA, Australia) for supplying three types of BSF samples.

Open access publishing facilitated by James Cook University, as part of the Wiley - James Cook University agreement via the Council of Australian University Librarians.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

S.K. and E.B.J. contributed equally to this work. M.C., A.L.L., and S.M.S. acquired research funding and conceived the project idea. U.B., S.K., E.B.J., and S.S. prepared the samples. S.K., E.B.J., and T.R. performed the SDS-PAGE and immunoblot analysis. S.D.K. and A.L.L. acquired the patient sera. U.B. J.B., K.B., and M.C. performed the in-gel tryptic digest, in-solution tryptic digest, and conducted the LC-MS/MS analysis. A.J. created the database. S.K., A.J., and V.L. conducted the bioinformatics analysis to identify putative allergens. S.K., E.B.J., and A.L.L. determined the

relevant putative cross-reactive allergens. S.K. and E.M.J. conducted the multiple sequence alignment and, peptide and IgE-epitope alignment. S.K. and A.L.L. determined potential cross-reactive IgE-binding epitopes. D.C. conducted the allergen detection by commercial allergen detection test kits. All authors reviewed and approved the manuscript.

Data Availability Statement

Data has not been published in the Public Repository.

Keywords

allergen, black soldier fly, cricket, edible insect, food allergy

Received: November 15, 2023
Revised: May 31, 2024
Published online: July 18, 2024

[1] FAO, A. Van Huis, J. Van Itterbeeck, H. Klunder, E. Mertens, A. Halloran, G. Muir, P. Vantomme, *Edible Insects: Future Prospects for Food and Feed Security*, Food and Agriculture Organization of the United Nations, **2013**.
[2] J. L. García, M. De Vicente, B. Galán, *Microb. Biotechnol.* **2017**, *10*, 1017.
[3] B. A. Rumpold, O. K. Schlüter, *Innovative Food Sci. Emerging Technol.* **2013**, *17*, 1.
[4] V. J. Stull, E. Finer, R. S. Bergmans, H. P. Febvre, C. Longhurst, D. K. Manter, J. A. Patz, T. L. Weir, *Sci. Rep.* **2018**, *8*, 10762.
[5] M. Montowska, P. Ł. Kowalczewski, I. Rybicka, E. Fornal, *Food Chem.* **2019**, *289*, 130.
[6] X. Fernandez-Cassi, A. Supeanu, A. Jansson, S. Boqvist, I. Vagsholm, *EFSA J.* **2018**, *16*, e16082.
[7] L. De Marchi, F. Mainente, M. Leonardi, S. Scheurer, A. Wangorsch, V. Mahler, R. Pilolli, D. Sorio, G. Zoccatelli, *Food Chem.* **2021**, *359*, 129878.
[8] L. K. Palmer, J. T. Marsh, M. Lu, R. E. Goodman, M. G. Zeece, P. E. Johnson, *Mol. Nutr. Food Res.* **2020**, *64*, 1900923.
[9] I. Pali-Schöll, P. Meinlschmidt, D. Larenas-Linnemann, B. Purschke, G. Hofstetter, F. A. Rodríguez-Monroy, L. Einhorn, N. Mothes-Luksch, E. Jensen-Jarolim, H. Jäger, *World Allergy Organ. J.* **2019**, *12*, 100006.
[10] F. Francis, V. Doyen, F. Debaugnies, G. Mazzucchelli, R. Caparros, T. Alabi, C. Blecker, E. Haubruge, F. Corazza, *Food Chem.* **2019**, *276*, 714.
[11] H. C. H. P. Broekman, A. C. Knulst, G. De Jong, M. Gaspari, C. F. Den Hartog Jager, G. F. Houben, K. C. M. Verhoeckx, *Mol. Nutr. Food Res.* **2017**, *61*, 1601061.
[12] U. Bose, J. A. Broadbent, A. Juhász, S. Karnaneedi, E. B. Johnston, S. Stockwell, K. Byrne, V. Limviphuvadh, S. Maurer-Stroh, A. L. Lopata, M. L. Colgrave, *Food Chem.* **2021**, *348*, 129110.
[13] S. C. EFSA, *EFSA J.* **2015**, *13*, 4257.
[14] L. W. Bessa, E. Pieterse, J. Marais, L. C. Hoffman, *Compr. Rev. Food Sci. Food Saf.* **2020**, *19*, 2747.
[15] L. W. Bessa, E. Pieterse, J. Marais, K. Dhanani, L. C. Hoffman, *Foods* **2021**, *10*, 1934.
[16] M. Kaczor, P. Bulak, K. Proc-Pietrycha, M. Kirichenko-Babko, A. Bieganowski, *Biology* **2022**, *12*, 25.
[17] S. D. Kamath, A. M. Abdel Rahman, T. Komoda, A. L. Lopata, *Food Chem.* **2013**, *141*, 4031.
[18] U. Bose, J. A. Broadbent, A. Juhász, S. Karnaneedi, E. B. Johnston, S. Stockwell, K. Byrne, V. Limviphuvadh, S. Maurer-Stroh, A. L. Lopata, M. L. Colgrave, *J. Proteomics* **2022**, *269*, 104724.
[19] R. Nugraha, S. D. Kamath, E. Johnston, S. Karnaneedi, T. Ruethers, A. L. Lopata, *Front. Immunol.* **2019**, *10*, 2676.
[20] A. Wangorsch, A. Jamin, J. Spiric, S. Vieths, S. Scheurer, V. Mahler, S. C. Hofmann, *Mol. Nutr. Food Res.* **2024**, *68*, 2300420.
[21] R. Ayuso, G. Reese, S. Leong-Kee, M. Plante, S. B. Lehrer, *Int. Arch. Allergy Immunol.* **2002**, *129*, 38.
[22] A. L. Lopata, J. Kleine-Tebbe, S. D. Kamath, *Allergo J. Int.* **2016**, *25*, 24.
[23] S. D. Kamath, M. Bublin, K. Kitamura, T. Matsui, K. Ito, A. L. Lopata, *J. Allergy Clin. Immunol.* **2023**, *151*, 1178.
[24] H. Moonesinghe, H. Mackenzie, C. Venter, S. Kilburn, P. Turner, K. Weir, T. Dean, *Ann. Allergy, Asthma, Immunol.* **2016**, *117*, 264e264.
[25] S. Srivastava, N. Babu, H. Pandey, *Indian J. Trad. Knowledge* **2009**, *8*, 485.
[26] S. Blank, H. Seismann, M. Mcintyre, M. Ollert, S. Wolf, F. I. Bantleon, E. Spillner, *Plos One* **2013**, *8*, e62009.
[27] S. Khanaruksombat, C. Srisomsap, D. Chokchaichamnankit, P. Punyarit, P. Phiriyangkul, *Ann. Allergy, Asthma, Immunol.* **2014**, *113*, 301.
[28] J.-G. Chuang, S.-N. Su, B.-L. Chiang, H.-J. Lee, Lu-P Chow, *Proteomics* **2010**, *10*, 3854.
[29] J. F. Huntley, J. Machell, A. J. Nisbet, A. Van Den Broek, K. Y. Chua, N. Cheong, B. J. Hales, W. R. Thomas, *Parasite Immunol.* **2004**, *26*, 335.
[30] A. Barre, C. Pichereaux, M. Simplicien, O. Burlet-Schiltz, H. Benoist, P. Rougé, *Foods* **2021**, *10*, 280.
[31] R. Ayuso, G. Grishina, L. Bardina, T. Carrillo, C. Blanco, M. D. Ibáñez, H. A. Sampson, K. Beyer, *J. Allergy Clin. Immunol.* **2008**, *122*, 795.
[32] A. Wangorsch, A. Jamin, S. Eichhorn, I. Pablos, S. Sharma, B. Schweidler, B. Kastner, S. Wildner, J. Saloga, F. Führer, R. R. Reyna Orozco, R. Sherkat, S. Sadeghi, F. Teifoori, J.-W. Park, P. Briza, S. Vieths, F. Ferreira, N. Arora, J. Lidholm, G. Gadermaier, S. Scheurer, *Front. Allergy* **2021**, *2*, 691627.
[33] A. Pomés, L. K. Arruda, *Methods* **2014**, *66*, 75.
[34] A. Pomes, S. Wunschmann, J. Hindley, L. Vailes, M. Chapman, *Protein Pept. Lett.* **2007**, *14*, 960.
[35] G. Leni, T. Tedeschi, A. Faccini, F. Pratesi, C. Folli, I. Puxeddu, P. Migliorini, N. Gianotten, J. Jacobs, S. Depraetere, A. Caligiani, S. Sforza, *Sci. Rep.* **2020**, *10*, 1228.
[36] T. Ruethers, A. C. Taki, E. B. Johnston, R. Nugraha, T. T. K. Le, T. Kalic, T. R. Mclean, S. D. Kamath, A. L. Lopata, *Mol. Immunol.* **2018**, *100*, 28.
[37] O. Mendoza-Porras, S. Kamath, J. O. Harris, M. L. Colgrave, R. Huerlimann, A. L. Lopata, N. M. Wade, *J. Proteomics* **2020**, *218*, 103689.
[38] T. Ruethers, A. C. Taki, J. Khangurha, J. Roberts, S. Buddhadasa, D. Clarke, C. E. Hedges, D. E. Campbell, S. D. Kamath, A. L. Lopata, M. Koeberl, *J. Sci. Food Agric.* **2020**, *100*, 4353.