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Isolation and proteomic characterization of tropomyosin extracted from edible insect protein

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ABSTRACT

Edible insects are considered promising sustainable protein sources. Thermal treatments and proteolysis are commonly used to improve their safety and quality. However, their allergenicity remains mostly unexplored. Tropomyosin, a major insect pan-allergen, can be used to study processing effects on its immunoreactivity. In this study, selective precipitation was used to extract tropomyosin from heated and protease-treated crickets. Immunoinformatics predicted 31 epitope regions, while proteomic analysis suggested decreased amounts of intact epitope regions in microwave-heated/protease-treated crickets. Tropomyosin peptide sequences were identified in higher abundance in convection-heated samples. Finally, tropomyosin immunoreactivity by immunoblotting and ELISA, revealed that protease treatments under microwave heating had lower ($p < 0.05$) IgE and IgG reactivity. Based on results, processing insects using proteolysis and microwave-heating could be effective for generating hypoallergenic cricket protein ingredients. The use of proteomics and bioinformatics proved to be useful tools in understanding the impact of processing on allergenic reactivity of insect proteins.

1. Introduction

There is an increased interest worldwide to adopt alternative protein sources as we prepare to meet global food demand by the year 2050. Alternative proteins are mainly sought for their heighten sustainability compared to traditional protein sources. In this context, edible insects have become a novel source of alternative protein due to their high nutritional value and low environmental impact (Salter, 2019). As with any novel protein, food allergens are a major safety concern surrounding edible insects. Several investigations have identified potential antigens and IgE binding proteins in various insect species, which may correlate to an allergic reaction after consumption (Feng, Chen, Zhao, He, Sun, Wang, et al., 2018; Ribeiro, Cunha, Sousa-Pinto, & Fonseca, 2018). Consequently, a growing body of literature agrees that a high degree of cross–reactivity exists between homologous proteins found in crustaceans and other arthropods (Leoni, Volpicella, Dileo, Gattulli, & Ceci, 2019; Pali-Schöll, Meinlschmidt, Larenas-Linnemann, Purschke, Hofstetter, Rodríguez-Monroy, et al., 2019; Volpicella, Leoni, Dileo, & Ceci, 2019). Specifically in crickets, tropomyosin, arginine kinase, and glyceraldehyde 3-phosphate dehydrogenase are identified as highly allergenic (Hossny, Ebisawa, El-Gamal, Arasi, Dahdah, El-Owaidy, et al., 2019). In 2019, Kamemura et al. (2019) identified tropomyosin as the main reactive allergen in field crickets against shrimp-specific IgE. Likewise, tropomyosin was recognized as the major reactive allergen in unprocessed cricket species such as house (*Acheta domesticus*) (Abdel-moteleb, Palmer, Pavlovikj, Marsh, Johnson, & Goodman, 2018) and tropical banded crickets (*Gryllodes sigillatus*) (Hall, Johnson, & Liceaga, 2018).

Currently, insect-based foods are customized for western palates by developing new food products via traditional (e.g. roasting, defatting) insect flours and novel (e.g. enzymatic proteolysis, sonication) processing techniques that allow for insects to be used as protein-rich ingredients in food formulation (Gravel & Doyen, 2019; Hall, Jones, O'Haire, & Liceaga, 2017; Luna, Martin-Gonzalez, Mauer, & Liceaga, 2021; Melgar-Lalanne, Hernández-Álvarez, & Salinas-Castro, 2019). Following processing, allergenicity can remain the same, increase, or decrease, depending on the processing conditions. Most studies report retained IgE binding capacity after thermal treatments such as baking or frying (Broekman, Knulst, den Hartog Jager, Monteleone, Gaspari, De Jong, et al., 2015; Jeong, Son, Lee, Park, Lee, & Park, 2016; Phiriyangkul, Srinroch, Srisomsap, Chokchaichamnankit, & Punyarit, 2015; Van Broekhoven, Bastiaan-Net, de Jong, & Wichers, 2016), while others observed altered reactivity after extensive heating (e.g. boiling), protease treatment, or newer technologies such as high-pressure processing,

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microwave heating, and food irradiation (Hall, Johnson, & Liceaga, 2018; Leni, Tedeschi, Faccini, Pratesi, Folli, Puxeddu, et al., 2020; Mills & Mackie, 2008; Phiriyangkul, Srinroch, Srisomsap, Chokchaichamnankit, & Punyarit, 2015). Other scientists also reported depleted IgE binding activity in black soldier fly (*Hermetia illucens*) and lesser mealworm (*Alphitobius diaperinus*) after protease treatment (Leni, et al., 2020). Broekman and their team (Broekman, et al., 2015) explained these contradicting observations by demonstrating, *in vitro,* that mealworm IgE-binding antigen changes were mainly due to altered protein solubility. However, there is strong *in vivo* evidence of altered protein allergenicity after processing (de Gier & Verhoeckx, 2018; De Martinis, Sirufo, Suppa, & Ginaldi, 2020). To further explain this phenomenon, investigators have focused on assessing the effects of processing on purified/isolated antigens. For instance, thermal-treated oyster tropomyosin (*Cra g* 1) showed higher IgE reactivity than the raw form as a result of protein denaturation and polymer formation (Fang, Li, Gu, Cai, & Lu, 2018). Similar analyses have also correlated protein structural changes to altered reactivity of individual allergens such as egg white ovalbumin heated under electrostatic conditions or peanut Ara h 1 after roasting (Claude, Bouchaud, Lupi, Castan, Tranquet, Denery-Papini, et al., 2017; Tian, Rao, Zhang, Tao, & Xue, 2018). These studies elucidate structure-specific modifications that may occur under processing to help explain the change or lack thereof in allergenicity.

In our own work, we observed the impact of enzymatic proteolysis on tropomyosin reactivity in crickets (*G. sigillatus*); our SDS-PAGE and IgE-immunoblots results showed a prominent band at ~ 37 kDa, indicating the presence of intact tropomyosin after some thermal and proteolysis treatments (Hall, Johnson, & Liceaga, 2018; Hall & Liceaga, 2019). Based on these observations, the aim of our study was to use proteomic analysis and bioinformatics to determine the effects of heat treatments and enzymatic proteolysis methods on the insect allergen tropomyosin. We hypothesize that proteolysis and heating methods have a differential effect on the epitope region and immunoreactivity of tropomyosin. To test our hypothesis, tropomyosin was extracted from a commonly reared edible cricket species in the USA, after they were processed rather than processing the purified antigen in order to simulate realistic conditions. Proteomic analysis was applied to compare abundance of tropomyosin and its peptides present in the samples, while immunoinformatic tools were used to predict potential cricket epitope sequences. Since insects and their protein will predominantly be consumed in a processed state, we should consider the impact of various protein-processing technologies on these known insect allergens.

2. Materials and methods

2.1. Materials

Frozen, adult (> 6-weeks) food-grade tropical banded crickets (*G. Sigillatus*) were purchased from 3 Cricketeers (Hopkins, MN, USA) and stored at −20 °C until used. All chemicals were reagent grade and purchased from either Sigma Aldrich-Millipore (St. Louis, MO, USA) or Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise stated. Rabbit anti-shrimp tropomyosin, Mab 1A6, and shrimp tropomyosin standard were sourced from Indoor Biotechnologies (Charlottesville, VA, USA).

2.2. Tropomyosin extraction from crickets, verification, and identification

Tropomyosin was extracted from crickets using a method for fish tropomyosin (Huang and Ochiai (2005) with modifications. Frozen, food-grade crickets were homogenized with 25 mM Tris-HCl buffer with 0.1 M KCl, pH 8.0 (Buffer 1). After centrifugation (10,000 × g for 15 min), the supernatant was stored and pellet re-suspended in Buffer 1. Washing, homogenization with Buffer 1, and centrifugation was repeated three times to remove sarcoplasmic proteins. Washings from buffer 1 were pooled and designated as sarcoplasmic extracts (SE). The final precipitate was then washed another three times with four volumes of absolute acetone. After the final wash, residues were placed on a filter paper and dried overnight at 4 °C. The dried powder was re-suspended in 5–fold (w/v) of Buffer 2 (1 M KCl, 0.5 mM DTT, 25 mM Tris-HCl, pH 8.0, 0.1 mM $CaCl_2$), and stirred at 4 °C overnight. Aliquots of buffer 2 represented myofibrillar extracts (ME). The solution was centrifuged (15,000 × g for 15 min), then subjected to isoelectric precipitation at pH 4.5 with 1 N HCl. After another centrifugation, the pellet was resuspended in 1 M KCl/0.5 mM DTT and isoelectric precipitation was repeated once more. The precipitated protein was dissolved in 50 mM Tris buffer; pH adjusted to 7.6 with 1 N NaOH, then clarified by centrifugation (designated as the extract after isoelectric precipitation: IP). Supernatant was then fractionated with $(NH_4)_2SO_4$ to 40–60% (v/v) saturation, stirred at 4 °C for 4 h, and centrifuged (15,000 × g, 15 min). The precipitate was reconstituted and dialyzed with a Slide-A-Lyzer Dialysis Cassette (Thermo Scientific, Waltham, MA, USA) following manufacturer's protocol. Extracts of the final stage were designated as IP-AS. Efficiency of the extraction conditions was assessed using frozen crickets and frozen shrimp (as positive control) and aliquot removed at each stage of the extraction process as follows: SE = sarcoplasmic proteins extracted with Buffer 1, ME = myofibrillar proteins extracted with Buffer 2, IP = Isoelectric precipitate at pH 4.6 of myofibrillar extract, and IP-AS = extract after ammonium sulfate fractionation of the isoelectric precipitate, also designates the final extraction step. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used as verification of the tropomyosin extraction process above. Tropomyosin samples were prepared for electrophoresis as previously described in detail (Hall, Johnson, & Liceaga, 2018) and tropomyosin identified by a prominent band near 37 kDa.

2.2.1. In-gel digestion and LC-MS/MS identification

Electrophoresis was performed as described above. Tropomyosin bands at ~ 37 kDa were excised and processed at the Purdue Proteomics Core Facility in the Bindley Biosciences Center (West Lafayette, IN, USA) for in-gel extraction and LS-MS/MS analysis following standard procedures (Mittal, Aryal, Camarillo, Ferreira, & Sundararajan, 2019; Sooreshjani, Gursoy, Aryal, & Sintim, 2018). Detailed procedures are shown in "Supplementary Materials". Briefly, bands of interest were cut into cubes for in-gel digestion. MaxQuant software (v. 1.6.0.16)17–19 with its built-in Andromeda search engine was used for analysis of the LC-MS/MS data. The MS/MS spectra were searched against the Polyneoptera database from Uniprot (downloaded on April 16, 2019), for protein identification and relative quantification. Peptides were considered identified if the Mascot score was over 95% confidence limit. Label-free quantification intensities were used to calculate relative protein abundance. Normalized spectral counting quantification-exponentially modified protein abundance index (emPAI) were derived from Mascot search results and used for comparison of the relative abundance of proteins (Ishihama, Oda, Tabata, Sato, Nagasu, Rappsilber, et al., 2005).

2.2.2. Tropomyosin extraction from cricket protein treatments

To investigate the effect of heat treatments and enzymatic proteolysis on tropomyosin immunoreactivity, cricket protein hydrolysates (CPH) were first prepared following a previously described protocol (Hall & Liceaga, 2019). Briefly, CPH were prepared by either convection heating proteolysis in a water bath (WB-CPH) or microwave-assisted proteolysis (MW-CPH). In both cases, proteolysis was conducted using Alcalase (*Bacillus licheniformis* ≥ 2.4 U/g) at pH 8.0 and 55 °C. Alcalase was used to replicate tropomyosin reactivity observed in a previous study (Hall et al., 2018). Following proteolysis, CPH were pasteurized, centrifuged, and the supernatant collected. Control treatments consisted of cricket protein heated in a water bath (WB-Con) or microwave (MW-Con) with no protease added. CPH and control samples were lyophilized and stored frozen (−20 °C) until use. Tropomyosin was extracted from these treatments (WB-Con, MW–Con, WB-CPH, and MW-CPH) as

described above, stored at −80 °C, and used within 48 h.

2.3. Relative quantification of tropomyosin extracted from cricket protein treatments

Proteomic analysis was used to compare tropomyosin protein and peptides abundance extracted from WB-CPH and MW-CPH. Sample preparation, mass spectrometry analysis, bioinformatics and data evaluation were performed in collaboration with the Proteomics Core Facility at the Indiana University School of Medicine (Indianapolis, IN, USA). Methods used were from vendor provided protocols and from literature reports published elsewhere, including instruments used and data analysis (Mosley, Sardiu, Pattenden, Workman, Florens, & Washburn, 2011; Smith-Kinnaman, Berna, Hunter, True, Hsu, Cabello, et al., 2014). Tropomyosin extracts from WB-CPH and MW-CPH were suspended in 8 M Urea and quantified by Bradford assay before injection. Proteins were reduced with 5 mM TCEP at room temperature for 30 min, then alkylated with 10 mM chloroacetamide for 30 min in the dark at room temperature. Samples were diluted to 1 M Urea with 50 mM Tris pH 8.5, and digestions were carried out using Trypsin/Lys-C Mass spectrometry grade protease mix (Promega™, Madison, WI, USA) at a 1:100 protease to substrate ratio, overnight at 37 °C, reaction quenched then samples desalted using C18 desalting tips (Thermo Scientific, Waltham, MA, USA) following manufacturer instructions. Samples were analyzed using a trap column (2 μm particle size, 50 μm diameter) EasySpray (801A) column on an UltiMate 3000 HPLC and Q-Exactive Plus-Orbitrap™ (Thermo Scientific, Waltham, MA, USA) mass spectrometer. A data dependent top 20 method acquisition method was used with MS scan range of 350–1600 *m/z*, resolution of 70,000, AGC target 3e6, maximum IT of 50 ms. MS2 settings of fixed first mass 100 *m/z*, normalized collision energy of 36, isolation window of 1.5 *m/z*, resolution of 35,000, target AGC of 1e5, and maximum IT of 250 ms. For dd acquisition a minimum AGC of 2e3 and charge exclusion of 1, and ≥ 7 were used. Data analysis, including de novo and database searches were performed using PEAKS software (Bioinformatics Solutions Inc., Waterloo, ON, Canada), with Q-Exactive Plus parameters and search database of all TrEMBL/Swissprot *Gryllidae* proteins and common contaminants.

2.4. Allergenic potential of cricket tropomyosin and epitope region prediction

The allergic potential and cross-reactivity of cricket tropomyosin was verified by Allermatch (http://www.allermatch.org) according to the current recommendations of the FAO/WHO Expert Consultation. Comparison in Allermatch was based on UniProt and WHO–IUIS database. An 80 amino acid sliding window alignment was performed with a 35% cut-off percentage (Fiers, Kleter, Nijland, Peijnenburg, Nap, & Van Ham, 2004). Results are given as percent sequence identity, which indicates the extent to which two sequences have the same residues at the same positions in an alignment (Table 3). Allermatch further calculates the Expect value (E), which describes the number of hits one can expect to see by chance when searching a database of a particular size. An E-value closer to zero indicates a match is more significant. The sequence alignment was carried out by comparing the amino acid sequence of tropomyosin belonging to A0A4P8D324_ACHDO = house cricket and A0A2P1ANK0_9ORTH = field cricket, respectively. Additional tools, AlgPred (https://webs.iiitd.edu.in/raghava/algpred), ABCPred (http://www.imtech.res.in/ragh/abcpred/), and Bepipred (http://tools.iedb.org/bcell) were used to predict the location of linear epitopes.

2.5. Immunoreactivity of cricket protein treatments and their tropomyosin extracts

IgE-binding was used to corroborate if heating and proteolysis had an effect on tropomyosin allergenicity. Immunoreactivity was assessed on the treated crickets (WB-Con, MW-Con, WB–CPH, and MW-CPH) and their respective tropomyosin extracts to ensure that the observed immunoreactivity was not influenced by the extraction conditions.

2.5.1. Patient sera

Study protocols were approved by the institutional review board (IRB) and Institutional Biosafety Committee (IBC) of Purdue University (West Lafayette, USA). Human serum samples were kindly donated by The Jaffe Food Allergy Institute at the Icahn School of Medicine (New York, NY, USA) containing no identifiable information. Sera (#1–4) were from patients with a shellfish allergy positive for shrimp-specific IgE by ImmunoCAP (> 70 S-IgE) and history of immediate-type reaction to shrimp. A pool was prepared of the four sera and used in the indirect ELISA study.

2.5.2. Immunoblotting using IgG and IgE

Cricket treatments and tropomyosin extracts were electrophorized by SDS-PAGE as already described (Hall, Johnson, & Liceaga, 2018). SDS-PAGE protein bands were transferred to a PVDF membrane (30 V, 1 h). After washing with PBS-T, membranes were blocked using 5% BSA (w/v) for 2 h and incubated with pooled sera (1:10 dilution) overnight. Goat anti-human IgE conjugated with horseradish peroxidase (1:4,000; 1 hr) was used as the secondary antibody. Immunoblots were visualized after development with TMB-Blotting solution. Blots were washed between each step for 10 min four times with PBS-T (Phosphate-Buffered Saline, 0.1% Tween 20), unless otherwise stated.

2.5.3. Enzyme-linked immunosorbent assay (ELISA)

Binding to anti-tropomyosin IgG was quantified by sandwich ELISA using Monoclonal antibody 1A6 (mAb), tropomyosin standard and rabbit anti-shrimp tropomyosin packaged as a kit by Indoor Biotechnologies (Hall & Liceaga, 2019). IgE binding capacity was measured by indirect ELISA. The detailed procedures are shown in the "Supplementary Materials".

3. Results and discussion

3.1. Tropomyosin extraction and identification

The pan-allergen, tropomyosin, was extracted from food-grade frozen crickets and store-bought shrimp (used as reference) and then quantified to determine the efficacy of the extraction conditions. Bands in the ~ 37 kDa region were excised from each stage of the extraction process. Following the extraction steps, some expected differences are apparent in their protein profile (Fig. S1). The sarcoplasmic extract, myofibrillar extract and isoelectric precipitate show a wide range of bands, with a consistent band observed in the target region of 37 kDa. The electrophoretogram also confirms that several proteins were removed after washing with the salt-containing buffer and isoelectric precipitation, verifying the water-soluble nature of sarcoplasmic proteins. A prominent double band near 37 kDa was clear after the isoelectric precipitate was further clarified by ammonium sulfate fractionation (IP-AS). Bands at similar molecular weight (~37 kDa) have been established as the major reactive protein, tropomyosin, in crickets (Hall, Johnson, & Liceaga, 2018; Kamemura, et al., 2019) and other insects (Pali-Schöll, et al., 2019). Hence, tropomyosin bands at ~ 37 kDa were targeted for extraction and characterization in the present study. LC-MS/MS analysis revealed matches to tropomyosin (Table 1) in the isoelectric and ammonium sulphate precipitates from cricket (Cricket IP-AS) and shrimp (Shrimp IP-AS). Bands excised from the myofibrillar extract (Cricket-ME) and isoelectric precipitates (Cricket-IP) also matched to tropomyosin, as expected, but also to other structural proteins such as paramyosin and myosin heavy chain. Shrimp tropomyosin (Shrimp IP-AS) showed higher matches to termite (*Cryptotermes secundus*) and cockroach (*Blattella germanica*) tropomyosin. In contrast,

Table 1
Proteins identified in excised bands using LC-MS/MS and Polyneoptera database.

Protein			Spectral Count			
Accession	**Name**	**Species**	**Cricket- ME[a]**	**Cricket- IP[b]**	**CricketIP-AS[c]**	**ShrimpIP-AS[d]**
A0A2P0XJ16	Putative Per a allergen*	*Periplaneta americana*	671	354	44	
A0A2J7RDH4	Paramyosin, short form	*Cryptoterms secundus*	241	133	22	
A0A2P8ZN67	Paramyosin (Fragment)	*Blattella germanica*	286	142	16	
A0A109ZYM7	Arginine kinase (Fragment)	*Aglaothorax diminutiva*	142	114	105	
A0A109ZYQ8	Arginine kinase (Fragment)	*Aglaothorax gurneyi*	136	110	97	
A0A109ZYT6	Arginine kinase (Fragment)	*Aglaothorax ovata gigantea*	110	86	89	
A0A385MDB2	Calcium-transporting ATPase	*Teleogryllus emma*	120	94	114	
A0A1P8BJZ4	Myosin heavy chain isoform A	*Locusta migratoria*	120			
A0A2P9A976	Myosin Heavy Chain (Fragment)	*Blattella germanica*	101			
A0A067R416	Actin, clone 403	*Zootermopsis nevadensis*	66	89	103	18
A0A067QL86	Actin, muscle	*Zootermopsis nevadensis*	80	104		
A0A2P1ANK0	Tropomyosin isoform 2*	*Teleogryllus emma*	26	222	337	42
A0A2P1ANK6	Tropomyosin isoform 1*	*Teleogryllus emma*		210	349	943
Q9UB83	Tropomyosin*	*Periplaneta americana*				797
A0A067QXJ4	Tropomyosin*	*Zootermopsis nevadensis*		116	172	
A0A2J7PK46	Tropomyosin*	*Cryptotermes secundus*		127	219	
A0A2J7PK53	Tropomyosin*	*Cryptotermes secundus*				530
Q9NG56	Tropomyosin*	*Blattella germanica*				901

*Allergens belonging to the family of Tropomyosin proteins.
[a] ME: Extract from crickets with buffer 2 after removal of sarcoplasmic proteins in buffer 1
[b] IP: Isoelectric precipitate at pH 4.6 of myofibrillar extracts;
[c] Cricket IP-AS: Extract from cricket after isoelectric precipitation and ammonium sulfate fractionation;
[d] Shrimp IP-AS: Extract from shrimp after isoelectric precipitation and ammonium sulfate fractionation.

cricket tropomyosin (Cricket IP-AS) had no matches to cockroach tropomyosin but did show higher matches with field cricket (*Teleogryllus emma*) tropomyosin isoforms (Table 1). These observations are limited by the lack of databases and characterized insect proteome. Nonetheless, the main goal of this analysis was to identify protein near 37 kDa, validate the proposed tropomyosin extraction method, and does not serve as absolute quantification.

3.2. Relative quantification of tropomyosin extracts using proteomics

The relative quantification of tropomyosin extracts helped determine the extent to which the heating method used during proteolysis impacted tropomyosin detection by mass spectrometry. Tropomyosin extracted from crickets treated with protease and convection heated (WB-CPH) or microwave heated (MW-CPH) was subjected to proteomic analysis. Peptides were closely matched to tropomyosin from two cricket species *Telegryllus emma* and *Acheta domesticus* (Fig. 1A). In addition, for WB-CPH and MW-CPH, peptides matched to tropomyosin had the highest average area, representing the most abundant protein, amongst a few other structural proteins (Supplementary materials, Table S1). WB–CPH had more matched peptides to each cricket species than MW–CPH. This could be a result of a greater abundance of detectable tropomyosin peptides due to limited extractability or modified peptides. These are common observations in processed food proteins and proteomic analysis of their allergens. For example, in roasted peanuts, extractability of Ara h allergens was decreased and consequently their peptide abundance detected by mass spectrometry (Tian, Rao, Zhang, Tao, & Xue, 2018). Van Broekhoven, Bastiaan-Net, de Jong, & Wichers (2016) also demonstrated lower extractability of edible mealworm allergens after various thermal treatments. Therefore, the use of detergents and chaotropic agents are suggested to improve antigen extraction and accurately reflect IgE-binding characteristics and protein/peptide abundance. Nevertheless, tropomyosin extraction conditions used in the present study were effective for microwave (MW-Con) and convection heated (WB-Con) controls, as well as protease-treated with convection heating (WB–CPH). Hence, contrasting changes in allergenicity and lower peptide abundance observed in MW-CPH are probably more so attributed to specific protein cleavage during enzymatic proteolysis with microwave heating.

The number of tryptic peptides generated from the tropomyosin

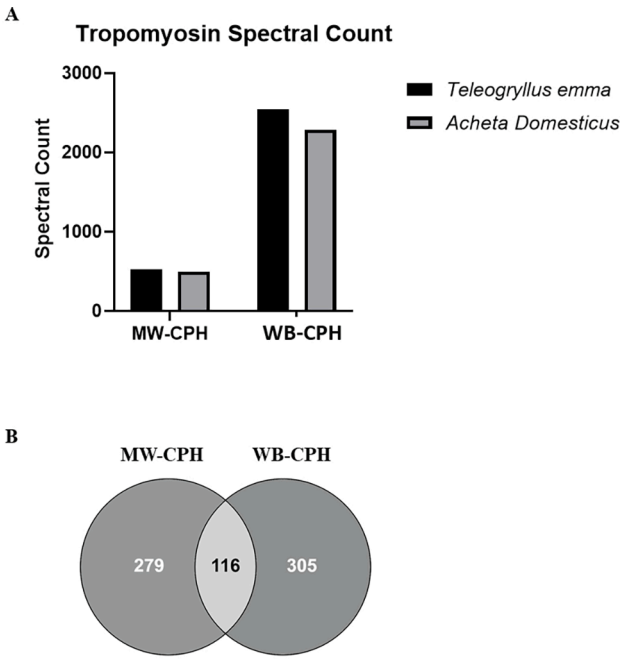

Fig. 1. Relative quantification of tropomyosin proteins in extracts from WB-CPH and MW-CPH (A) and number of identified peptides (B).

extracts are displayed in Fig. 1B and their relative abundance available in the supplementary material (Table S2). A list of de novo only peptides, which takes account of non-tryptic products, is provided in the supplementary materials (Table S3). As expected, the number of peptides is relatively large due to the proteolytic fragmentation which already occurred during the enzymatic proteolysis treatment. Thus, in the event that protease-treated cricket protein produced under different heating methods gave different peptide profiles, their tryptic peptide products would also significantly differ. A total of 395 peptides were detected in MW-CPH and 421 in WB-CPH; only a fraction of peptides (116) were detected in both samples with similar abundance (Fig. 1B). Sequence tags ADKINEDVQELTK and KVQLVEEDLERSEER, predicted

epitope regions, can be highlighted as an example of a peptide detected in both processed samples (Table S2). Conversely, some peptides were unique to MW-CPH or their abundances were higher than in WB-CPH. Peptides specific to either treatment and/or the relative abundance of shared peptides serve as markers of enzyme cleavage-site variation. For example, tropomyosin peptides LLAEDADGKSDEVSR and LAMVEADLER were identified in both samples, but with a higher abundance present in WB-CPH (Table S2).

Comparing tropomyosin tryptic peptides from these extracts obtained from crickets treated with different processing methods (e.g., microwave heating, enzymatic proteolysis, etc.) can provide a comprehensive analysis to differentiate cleavage changes caused by enzymatic proteolysis and the heating type (convection versus microwave) used. We have identified a series of peptides corresponding to tropomyosin that were found to be specific for either WB-CPH or MW-CPH, and their detection might therefore be indicative of difference in trypsin/alcalase cleavage sites, availability, and protein structure changes induced by the heating method used.

3.3. Immunoinformatics of cricket tropomyosin

Tropomyosin sequences of field cricket [*Teleogryllus emma* (A0A2P1ANK0)] and house cricket [*Acheta domesticus* (A0A4P8D324)] were acquired from UniProt (Fig. 2) and used to search against other known allergens in Allermatch. Their sequence alignment demonstrates the high shared homology between the two-cricket species. The results of allergenicity assessment by Allermatch using the full-length, 80-aa sliding window are shown in Table 2. Significance is assumed when the expected score is below 1.0 or a > 50% identity match (www.allermatch.org). Allergens from various species of shellfish, insects, and nematodes showed > 60% identity to cricket tropomyosin. The top two matches were tropomyosin from Lep s 1 silverfish (*Lepisma saccharina,)* and Pan b 1.0101 northern shrimp (*Pandalus borealis*). There were also matches to other insect allergens such as mite (*Dermatophagoides farina*) paramyosin (Der f 11), American cockroach (*Periplaneta americana*) tropomyosin (Per a 7), and German cockroach (*Blattella germanica*) tropomyosin (Bla g 7). Combined, the three immunoinformatic tools predicted a total of 31 peptides as potential linear epitopes within cricket tropomyosin allergen (Table 3), with some overlap in the predicted sequences. For example, the peptides RSQQDEERMDQ (IEDB), RSQQDEERMDQLTNQ (Algpred), and NRSQQDEERMDQLTNQ (ABCpred) shared the same 'RSQQDEERM' sequence. From these predicted epitope regions, the peptide ADKINEDVQELTKK was identified in both enzymatically-treated water bath (WB-CPH) and microwave (MW-CPH) heated samples, with a higher abundance in WB-CPH (Table S2). Portions of other predicted epitope regions (VQLVEEDLER, VQLVEEDLERSEER, ANLEQANKDLEDKEK) were also identified in tropomyosin extracted from both cricket protein treatments. These predicted active regions remained intact after alcalase and trypsin digestion, regardless of the heating method used. However, the relative abundance was higher in the WB–CPH, which suggests that more epitope regions remained intact after enzymatic proteolysis under convection (water bath) heating. Whereas, microwave heating is known to increase the rate of protein unfolding, enhancing epitope region exposure to the protease that was not accessible under convection heating (El Mecherfi, Saidi, Kheroua, Boudraa, Touhami, Rouaud, et al., 2011; Ketnawa & Liceaga, 2017). Confirmatory assays involving IgE-binding assays with a sequenced version of these epitope regions would further confirm their involvement in the cricket tropomyosin cross-reactivity observed in this study.

3.4. Immunoreactivity of cricket protein treatments and their tropomyosin extracts

Immunoblotting and ELISA were used to evaluate the allergenic response from cricket protein as well as their corresponding tropomyosin extracts following heat and proteolysis treatments (Supplementary Materials Fig. S2). The anti-shrimp tropomyosin IgG-binding activity of cricket protein samples, which were heated but not treated with enzymatic proteolysis (Fig. S2A, lanes 1 and 2), had similar reactivity with predominant bands near 37 kDa and above 60 kDa. There were some differences observed between convection and microwave heating of the samples. Microwave heating is known to impart spatial structural change in proteins compared with convection heating (Ketnawa & Liceaga, 2017). Additionally, previous studies show that proteolysis of cricket protein was more noticeable under microwave heating (Hall & Liceaga, 2020) even if no proteases were added to the substrate. The enzymatically treated cricket protein under convection heating (WB-CPH) retained some reactive response, while there was no apparent IgG-binding in the sample enzymatically treated under microwave (MW-CPH) heating (Fig. S2 A, lanes 3 and 4). When probed with IgE from shrimp-allergic patient sera (Fig. S2 B), reactivity remained in the heat-treated controls (lanes 1–2) and WB-CPH (lane 3), but was not visible in the microwave/protease-treated sample MW-CPH (lane 4). The tropomyosin extracts obtained from each sample had similar IgE-repsonses (Fig. S2 C and D) as those observed for the treated cricket protein, before isolating the antigen. These observations verify that IgE-binding is primarily occurring with tropomyosin as opposed to other antigens.

The overall trend demonstrates that both IgG and IgE reactivity decreased in cricket protein treated with microwave heating and enzymatic proteolysis (MW-CPH). The same results were observed in the tropomyosin extracts obtained from this sample. Both ELISA assays corroborate this pattern, where MW-CPH (protein sample and its tropomyosin extract) had significantly lower ($p < 0.05$) IgE and IgG

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A0A4P8D324_ACHDO     MDAIKKKMQAMKLEKDNAMDKADTCEGQAKDANNKADKINEDVQELTKKLAQVENDLITT
A0A2P1ANK0_9ORTH     MDAIKKKMQAMKLEKDNAMDKADTCEGQAKDANNKADKINEDVQELTKKLAQVENDLITT

A0A4P8D324_ACHDO     KANLEQANKDLEDKEKALQAAESEMAALNRKVQLVEEDLERSEERAATAATKLQEASEAA
A0A2P1ANK0_9ORTH     KANLEQANKDLEDKEKALQAAESEMAALNRKVQLVEEDLERSEERAATAATKLHEASEAA

A0A4P8D324_ACHDO     DEAQRMCKVLENRSQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRKLAFVEDELEVAE
A0A2P1ANK0_9ORTH     DEAQRMCKVLENRSQQDEERMDQLTNQLKEARLLAEDADGKSDEVSRKLAFVEDELEVAE

A0A4P8D324_ACHDO     DRVKSGDSKIMELEEELKVVGNSLK----------------------------------
A0A2P1ANK0_9ORTH     DRVKSGDSKIMELEEELKVVGNSLKSLEVSEEKANQRVEEYKRQIKTLSVKLKEAEARAE

A0A4P8D324_ACHDO     -------------------------------------------
A0A2P1ANK0_9ORTH     YAEKTVKKLQKEVDRLEDNLFSDKEKYKSITDDLDSTFAELTGY
```

Fig. 2. Sequence alignment of tropomyosin isoforms from *Telegryllus emma.* (A0A2P1ANK0_9ORTH) and *Acheta domesticus* (A0A4P8D324_ACHDO).

Table 2
Cricket tropomyosin predicted sequence homology with reported allergens derived from insects, shellfish and nematodes.

Species	Allergen	Sequence Link inSwissProt/NCBI	Full Alignment[a]	
			E-val	%ID
Lepisma saccharina	Lep s 1	CAC84590	1.7e-050	81.50%
Pandalus borealis	Pan b 1.0101	CBY17558	4.8e-049	78.50%
Penaeus monodon	Pen m 1	AAX37288	2.2e-040	67.30%
Penaeus aztecus	Pen a 1	11,893,851	1.2e-039	65.40%
Homarus americanus	Hom a 1.0102	AAC48288	9.5e-042	69.30%
Litopenaeus vannamei	Lit v 1.0101	EU410072	1.1e-040	67.30%
Homarus americanus	Hom a 1.0101	O44119	3.3e-041	67.80%
Periplaneta americana	Per a 7.0102	AAD19606	2.9e-041	68.30%
Blattella germanica	Bla g 7.0101	AAF72534	4.5e-041	68.30%
Dermatophagoides farinae	Der f 10.0101	BAA04557	3.7e-041	67.80%
Chironomus kiiensis	Chi k 10	CAA09938	7.4e-042	68.80%
Tyrophagus putrescentiae	Tyr p 10.0101	AAT40866	9.8e-038	65.60%
Blomia tropicalis	Blo t 10.0101	ABU97466	1.4e-041	68.30%
Metapenaeus ensis	Met e 1	Q25456	3.6e-039	66.80%
Panulirus stimpsoni	Pan s 1	O61379	4.7e-041	67.30%
Lepidoglyphus destructor	Lep d 10	Q9NFZ4	5.3e-038	66.20%
Dermatophagoides farinae	Der p 10	O18416	6.5e-042	68.30%
Charybdis feriatus	Cha f 1	Q9N2R3	2.1e-040	67.30%
Ascaris lumbricoides	Asc l 3.0101	ACN32322	3.5e-042	68.80%
Anisakis simplex	Ani s 3	Q9NAS5	5.8e-042	69.30%
Helix aspersa	Hel as 1	CAB38044	3.7e-041	67.80%
Haliotis diversicolor	Hal d 1	AAG08987	1e-039	65.40%
Mimachlamys nobilis	Mim n 1	AAG08989	6e-041	67.80%
Perna viridis	Per v 1	AAG08988	2e-041	68.30%
Crassostrea gigas	Cra g 1	AAK96889	1.1e-040	67.30%
Dermatophagoides farinae	Der p 11	AAO73464	3.7e-041	67.80%
Blomia tropicalis	Blo t 11	AAM83103	3.5e-042	69.30%
Dermatophagoides farina	Der f 11.0101	AAK39511	4.2e-041	68.30%

[a] Parameters assessed are % Identity and E-score. Duplicates were not included on the list.

reactivity, compared with the other treatments (Fig. 3 A and B). In contrast, there was no significant difference ($p > 0.05$) in quantifiable tropomyosin between WB–Con, MW-Con and WB-CPH, for either protein samples or their extracted tropomyosin (Fig. 3 A and B). However, WB-CPH demonstrated slightly decreased ($p < 0.05$) IgE response compared with shrimp tropomyosin, WB-Con, and MW-Con.

Binding to other allergens (not tropomyosin) is also possible given the polyclonal nature of sera antibodies. However, given our collective analyses (monoclonal IgG anti-tropomyosin binding, comparison before and after extraction, and MS identification) along with reported literature, we believe that the immunoreactivity demonstrated in our study is likely due to tropomyosin and/or its fragments. Nevertheless, the current results demonstrate that the overall IgE reactivity was suppressed after treating crickets with a combination of enzymatic proteolysis and microwave heating.

Table 3
Epitope prediction analysis of cricket tropomyosin.

Epitope Sequence	Position	Prediction Tool
AMKLEKDNAMDKADTC	10–25	ABCPred (http://www.imtech.res.in/ragh/abcpred/)
AMDKADTCEGQAKDAN	18–33	
TCEGQAKDANNKADKI	24–39	
KDANNKADKINEDVQE	30–45	
ADKINEDVQELTKKLA	36–57	
VQELTKKLAQVENDLI	43–59	
AQVENDLITTKANLEQ	51–66	
LEQANKDLEDKEKALQ	64–79	
EKALQAAESEMAALNR	75–90	
AESEMAALNRKVQLVE	81–96	
VQLVEEDLERSEERAA	92–107	
ERSEERAATAATKLQE	100–116	
AATAATKLQEASEAAD	106–121	
SEAADEAQRMCKVLEN	117–132	
AQRMCKVLENRSQQDE	123–135	
NRSQQDEERMDQLTNQ	132–147	
DADGKSDEVSRKLAFV	157–172	
DELEVAEDRVKSGDSK	174–189	
DSKIMELEEELKVVGN	187–202	
DNAMDKADTCEGQAKDANNKADKINEDVQE	16–45	IEDB (http://tools.iedb.org/bcell/)
NLEQANKDLEDKEKALQAAES	63–83	
LERSEERAATAATKLQEASEAADEA	99–123	
RSQQDEERMDQ	133–143	
EDADGKSDEV	156–165	
LEVA	176–179	
DRVKSGDS	181–188	
MAALNRKVQLVEEDL	85–99	Algpred (https://webs.iiitd.edu.in/raghava/algpred)
KVQLVEEDLERSEER	91–105	
RSQQDEERMDQLTNQ	133–144	
ARLLAEDADGKSD	151–163	
DSKIMELEEELKVVG	187–201	

4. Conclusion

Food allergenicity depends on various structural components, within a food matrix, which can be altered during processing treatments. The conditions of the heat treatment (e.g. source, intensity, length, and temperature) and the use of proteases may also impact the structure of allergenic proteins, their interactions with other constituents within the matrix, and finally their immunoreactivity. Our aim was to use proteomics and bioinformatics to evaluate the influence of heat treatment (convection or microwave) and enzymatic proteolysis on edible insects' tropomyosin antigen reactivity. Our hypothesis was confirmed as the results suggest that the decrease in allergenicity observed in the protease-treated with microwave heating (MW-CPH) sample was associated with increased cleavage of the epitope region. More importantly, new allergenic peptide fragments were not formed during enzymatic proteolysis when microwave heating (MW-CPH) was applied. Protein folding or cross-linking reactions during convection heating likely masked the epitope region, which resulted in retained tropomyosin reactivity observed in WB-CPH. These findings correlate with the proteomics analysis, where a higher abundance of detectable tropomyosin was observed in the convection heated protein (WB-CPH) compared with the microwave heated (MW–CPH) treatment.

Finally, microwave heating along with enzymatic proteolysis could be effective methods for lowering the concentration of active tropomyosin regions when formulating insect-based food products or

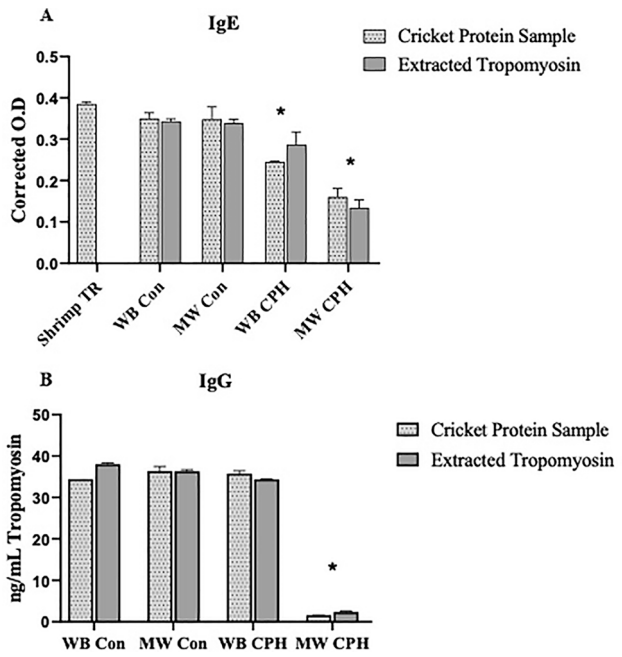

Fig. 3. IgE indirect (A) and IgG sandwich (B) ELISA of treated cricket protein and their tropomyosin extracts. Results are means of at least triplicate determinations. Samples denoted with '*' indicate significant difference ($p < 0.05$) from other samples tested. Purified shrimp tropomyosin was used as a positive reference for the IgE ELISA and used to create the standard curve for IgG ELISA quantification. 'Cricket protein samples' indicates the state before any tropomyosin extraction. Sample descriptions are as follows: WB-Con = cricket protein convection heated in a water bath with no enzyme, MW-Con = cricket protein heated in a microwave with no enzyme, WB-CPH = cricket protein hydrolysates produced under convection heating using a water bath, and MW-CPH = cricket protein hydrolysates produced using microwave heating.

developing bioactive peptides for therapeutic applications. However, evaluation and/or quantification will need to be performed for each individual insect species and processing conditions.

Although our aim was not to decrease cross–reactivity of cricket protein, our results underline the impact of different heat treatments and proteolysis on tropomyosin immunoreactivity. As we move forward with these novel proteins, we will need to establish specific concentration levels that trigger an allergenic response, similar to what has been established for fish proteins. Further work is required to confirm the reactivity of predicated epitope regions, the role of commonly used processing methods on tropomyosin solubility, and subsequent IgE response. This information will support continued allergenic risk assessment of emerging protein sources such as edible insects as more novel processing techniques are explored.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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