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Thermal processing of insect allergens and IgE cross-recognition in Italian patients allergic to shrimp, house dust mite and mealworm

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Highlights

- Insect tropomyosin is a notable cross-allergen for house dust mite and shrimp allergic patients.
- Thermal processing affected insect protein cross-allergenicity.
- The processing effect on immunoreactivity is protein-, species- and treatment-specific.
- Larval cuticle protein accounts for cross-reactivity of mealworm-sensitized patients.

Keywords

Edible insectsThermal processingCross-reactivityHouse dust mitesShrimpsTropomyosinLarval cuticle protein

Abstract

Edible insects are considered as a promising and sustainable alternative protein source for humans, although risk assessments, with particular reference to the allergic potential of insect proteins, are required. Considering that insects are likely to be consumed after processing, it is crucial to assess how processing can influence allergenicity. In our study, we investigated how boiling and frying affect the IgE cross-recognition of proteins from five edible insects (mealworm, buffalo worm, silkworm, cricket and grasshopper). We considered three groups of Italian patients allergic to shrimps and to house dust mites, who had never consumed insects before and two subjects with occupational allergy and food sensitization to mealworm. Our data suggest that thermal processing may change the solubility of proteins, thereby resulting in a protein shift from water-soluble fractions to water-insoluble fractions. Immunoblot and LC-MS/MS analyses have shown that tropomyosin may play an important role as a cross-allergen for house dust mite and shrimp allergic

patients, while larval cuticle protein seems to play a major role in the cross-reactivity of patients primarily sensitized to mealworm.
On the basis of our results, the effects of processing appear to be protein-, species- and treatment-specific. Therefore, house dust mite, shrimp and mealworm allergic patients should consume insects with caution, even after thermal processing.

1. Introduction

The global population is steadily growing and, as a consequence, food production needs to be increased in order to meet the world's nutritional needs, especially in terms of protein sources. In this context, insects could be promising candidates for human nutrition, since they may provide high quality proteins, with low CO_2 emissions, limit waste production, and lower water and feed consumption for their breeding (Elhassan et al., 2019).
Over 2000 insect species are currently included in the traditional diets of two billion people, mostly in Asia, Africa and Latin America (Tao & Li, 2018). Although entomophagy is not yet a common practice in Western countries, the use of insects as food in these regions has recently started to capture public attention. In view of the possibility of insect farming and commercialization in Europe, the European Food Safety Authority (EFSA) has requested scientific risk assessments on the use of insects as food, with particular focus on allergenicity (EFSA, 2015). Many researches on patients in Western countries have reported anaphylaxis following the ingestion of insects in patients who had not had previous allergic reactions toward insects. For example, seven cases of carmine (*Dactylopius coccus*) allergy have been described in Europe and three in the USA (de Gier & Verhoeckx, 2018). This insect, used as a color additive (E120) in many food products (yoghurt, sweets and juice), is able to elicit adverse reactions and cause symptoms such as nausea, urticaria and rhinitis, and even severe anaphylaxis. However, none of the patients involved in the studies had reported previous insect allergies (Beaudouin et al., 1995, De Pasquale et al., 2015, Kägi et al., 1994, Wüthrich et al., 1997). In Italy, entomophagy is still rare, and no studies related to the risk of cross-reactivity in allergic subjects have been reported so far. In Europe, the primary allergies to insects that have been described so far concern adverse work-related health issues in subjects who work with insects (Broekman et al., 2017, Harris-Roberts et al., 2011, Nebbia et al., 2019).
A crucial aspect of insect allergies in Western countries that needs to be elucidated is the cross-reactivity between insects, crustaceous and HDM allergens. These allergens, defined as pan-allergens, are ubiquitously distributed proteins with highly conserved sequences and structures (Pfaar et al., 2014). Tropomyosin, for instance, is a ubiquitous protein that is widely distributed among invertebrates, including house dust mites (HDM), crustaceans and insects. It is considered a major allergen in shrimp (SH) allergic patients and it is responsible for cross-reactivity with HDM, due to its high sequence homology (Barre et al., 2018, Leni et al., 2020). An Italian study, aimed at distinguishing shrimp-allergic subjects from subjects that are sensitized but tolerant, showed that the majority of shrimp IgE-positive but tolerant patients were also HDM-positive. In the Mediterranean Italian area, shrimp allergy is generally associated with and is almost always dependent upon HDM sensitization, and in particular upon HDM-induced allergic asthma (Farioli et al., 2017).
Furthermore, it should be taken into consideration that the mite species differ according to geographic regions. A Spanish study showed that Mediterranean mite-allergic patients showed a different prevalence of allergens involved in HDM allergy from Atlantic mite-allergic patients, and that this depended on the mite species toward which they were sensitized (Barber et al., 2012).
IgE cross-reactivity between HDM, SH and insects has already been demonstrated. In studies focused on HDM and crustacean allergic patients, tropomyosin was identified as the major cross-reactive allergen in mealworm (Broekman et al., 2015, Broekman et al., 2017a, Verhoeckx et al., 2014), grasshopper (Leung et al., 1996, Sokol et al., 2017) and cricket (Hall et al., 2018, Srinroch et al.,

2015). However, no studies on cross-reactivity of crustacean- and house dust mite-allergic patients against buffalo worm have been performed so far.
Edible insects are generally consumed after processing in order to improve their palatability and microbiological safety. These processes (industrial and domestic) may alter the protein structure, and may affect cross-reactivity through the masking/unmasking of pre-existing epitopes or even through the generation of new epitopes, previously not accessible to the patient's IgE (Wal, 2003). In a recent review, de Gier and Verhoeckx (2018) reported that thermal processing can affect insect allergenicity in three different ways: decreasing, increasing or not affecting the IgE immunoreactivity of allergic patients. Different thermal processes (baking, blanching, boiling, frying and freeze-drying) have been assessed, by means of immunoblotting experiments, on silkworm, mealworm and Bombay locust, to establish how they affect insect allergenicity (Broekman et al., 2015, Jeong et al., 2016, Phiriyangkul et al., 2015, Van Broekhoven et al., 2016). Pali-Schöll et al. (2019) have recently shown that certain food processing methods, such as enzymatic hydrolysis or autoclaving, are able to reduce the IgE binding of crustacean- and HDM-allergic patients to migratory locust proteins. It is worth noting that the cross-reactivity reduction was confirmed by means of *in vivo* skin prick tests. Contradictory results have often been found, probably because the processing conditions were not always comparable and also because the solubility of proteins changes as a result of processing, an issue that should be addressed by acting on the extraction protocols, as already suggested by Broekman et al. (2015).
The aim of the present study has been to investigate how thermal processing (boiling and frying) affects the IgE cross-recognition of allergens extracted from five edible insects (including buffalo worm, for the first time) in Italian patients allergic to shrimp, HDM and mealworm. The final goal has been to point out whether the sensitization to shrimp and to different HDM species of patients that had never eaten insects before has any effect on the cross-reactivity toward insects.

2. Materials and methods

2.1. Chemicals

The Complete™ tablet, HCl, NaCl, KCl, Na_2HPO_4, KH_2PO_4, hexane, urea, Coomassie Blue, Tween 20, Tris, vegetal gelatin used in the experiment were obtained from Sigma-Aldrich S.r.l. (St. Louis, MO, USA). The ReadyPrep™ 2-D Cleanup Kit and Alkaline Phosphatase Substrate Kit were from Biorad (Hercules, California, USA). The 2D-Quant-kit was from (GE Healthcare, Chicago, IL, USA). The methanol, chloroform, ethanol and ortophosphoric acid were from Merck KGaA (Darmstadt, Germany). The goat anti-Human IgE antibody was from SeraCare Life Sciences Inc. (Milford, Massachusetts, USA). The Lithium dodecyl sulfate (LDS) Sample Buffer, Low molecular weight (LMW) standards, NuPAGE Sample Reducing Agent, 10% NuPAGE mini gels, 4-Morpholineethanesulfonic acid, 2-(N-Morpholino) ethanesulfonic acid hydrate (MES) Running Buffer, Transfer buffer and Nitrocellulose Membranes were from Invitrogen, Life Technologies Ltd. (Paisley, UK). Sequence grade modified trypsin was acquired from Promega (Madison, WI, USA).

2.2. Study population

The sera of 38 adult patients were retrospectively collected from the Allergy and Immunology Unit of the Mauriziano Umberto I Hospital (The University of Turin, Italy) and grouped as follows:
(a) patients with a convincing history of house dust mite allergy (HDM, n = 28), with all the patients suffering from rhinitis and 13 of them suffering from asthma (HDM1, 3, 6–11, 14, 15, 22, 23 and 27);
(b) patients with a convincing history of shrimp allergy (SH, n = 8), with 1 patient (SH5) suffering from urticaria, and 5 patients (SH1, 3, 6, 7 and 8) suffering from both urticaria and angioedema; 1 patient (SH8) suffering from urticaria associated with gastrointestinal symptoms, and 2 patients (SH2

and SH4) suffering from urticaria associated with an oral allergy syndrome (starting within one hour from the ingestion of shrimps);
(c) patients with primary respiratory and food sensitization to mealworm (*Tenebrio molitor*) (TM, n = 2) with symptoms of rhinoconjunctivitis, itching and contact erythema, as previously described by Nebbia et al. (2019).
All the patients resulted positive to specific IgE CAP-FluorEnzymeImmunoAssay (CAP-FEIA) and/or skin prick tests. The demographic and clinical characteristics of the patients are reported in Table 1. The sera of three subjects who were not allergic to either shrimps or house dust mites were pooled and used as the negative control. All the patients gave written informed consent. The study was approved by the local ethics committee of the Città della Salute e della Scienza (Turin, Italy) with protocol number 0053278 and carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).
Table 1. Clinical characterization of the patient cohort (N = 38): 28 house dust mite (HDM) allergic patients, 8 Shrimp (SH) allergic patients and 2 mealworm (*Tenebrio molitor*, TM) allergic patients. DP: *Dermatophagoides pteronyssinus* protein extract, DF: *Dermatophagoides farinae* protein extract, SH: shrimp protein extract. Der p1 and Der p2: HDM allergens; Pen a1: shrimp allergen.

2.3. Insects

Lyophilized adults of buffalo worm (*Alphitobius diaperinus*), mealworm larvae (*Tenebrio molitor*), cricket (*Gryllodes sigillatus*) and grasshopper (*Locusta migratoria)* were bought from Eat Grub (London, UK). Silkworm larvae (*Bombyx mori*), belonging to the germplasm collection of the CREA Research Center for Agriculture and Environment (Padua, Italy) were grown on an artificial diet, as previously described by Lamberti et al. (2019), and lyophilized (5Pascal, Trezzano sul Naviglio, Italy). The insects were considered a) raw, b) boiled for 5 min at 100 °C, or c) fried for 3 min at 180 °C in sunflower oil. Each insect sample was ground before protein extraction.

2.4. Insect protein extraction

One gram of raw or boiled chopped insects was extracted with 5 mL of Phosphate Buffered Saline (PBS, 0.1 M pH 7.4) and a Complete^{TM} tablet in 50 mL of buffer as a protease inhibitor. Each sample was sonicated (40 MHz) for 30 sec on ice; seven sonication cycles were conducted with an interval of 30 min between cycles.
A different protocol was used for the fried samples. One gram of chopped insects was mixed with 10 mL of hexane in order to remove the residual frying oil. After 30 min of shaking in ice, the hexane was removed (this step was repeated three times) and each sample was dried in a Speedvac device for 30 min at room temperature. One gram of defatted fried insects was extracted with 5 mL of PBS (0.1 M pH 7.4, with a protease inhibitor), using a Polytron tissue homogenizer (Type PT 10–35; Kinematica GmbH, Luzern, Switzerland) (10 sec ON and 10 sec OFF), and 4 cycles were conducted. After sonication (of both the raw and boiled samples) or the Polytron step (for the fried samples), the extracts were centrifuged at 16300*g* for 30 min at 4 °C. The upper phase was recovered and centrifuged again, in order to remove the impurities, and the water-soluble protein extract (W) was thus obtained. The pellet was washed twice with PBS and extracted overnight at 4 °C with 1 mL of Urea (6 M). The urea soluble protein extract (U) was collected after centrifugation (16300*g*, 30 min, 4 °C). The W and U fractions of the raw insects were subjected to a precipitation step, using methanol/chloroform, as previously described by Wessel and Flügge (1984), while the W and U fractions of the fried insects were cleaned with a ReadyPrep™ 2-D Cleanup Kit . The protein contents were determined using a 2D-Quant-kit. Each modification of the extraction protocols, based on the different forms of applied processing, was verified by protein quantification and LDS PAGE (data not shown). All the experimental procedures are summarized in Fig. 1, Fig. 2.

2.5. LDS - PAGE of the insect proteins

Five µg of protein sample was diluted in an LDS Sample Buffer, under reducing conditions (with 2% of a NuPAGE Sample Reducing Agent), and separated with 10% NuPAGE mini gels in an MES Running Buffer, according to the manufacturer's protocol. The gel was then fixed in 30% ethanol and 10% orthophosphoric acid for 2 h, stained with Colloidal Coomassie Blue (Candiano et al., 2004) and scanned using a ChemiDoc MP System densitometer (Bio-Rad) at a resolution of 600 dpi.

2.6. Patient screening by means of a dot blot immunoassay and an immunoblottin assay

Dot blot was used to screen the patients' sera for the immunorecognition of the proteins extracted from insects. Dot-blot was performed in triplicate. One µL of a mixed (1:1) W and U fraction was spotted, at a final protein concentration of 0.5 µg/µL, on a Nitrocellulose Membrane (0.2 µm) and left to dry for 30 min at room temperature (RT, 25 °C). The membranes were blocked with Tris-buffered saline (TBS) with 0.3% Tween 20 for 30 min and incubated overnight (ON), at 4 °C, with the patients' sera diluted 1:10 in the incubation buffer (TBS, 0.05% Tween 20, 0.05% vegetal gelatin). The sera were removed and the membranes were washed three times with the washing solution (TBS, 0.05% Tween 20) for 10 min each step. The membranes were incubated for 1 h at RT with a goat anti-Human IgE antibody and diluted 1:5000 in the incubation buffer. The membranes were then washed three times with the washing solution. Immunoreacting spots were developed with an Alkaline Phosphatase Substrate Kit.

The membranes were scanned using a ChemiDoc MP System densitometer (Bio-Rad), at a resolution of 600 dpi, and the color intensity of the reactive droplets was quantified using Imagelab 4.1 software (Bio-Rad). The value obtained for the spot intensity of each allergic patient was divided by the value of the spot intensity obtained for the negative control pool. The sera of the allergic patients that recognized at least two of the three replicates, with a 1.8-fold higher spot intensity than the negative control pool, were used for the immunoblotting experiment on the insect extracts. The data from the dot blot experiments were analyzed, by means of a chi-square test, in order to verify whether the differences in dot blot positivity were statistically significant. The analyses were performed using GraphPad Prism software, version 8.4.3.

The W and U fractions of each sample were 1:1 mixed, at a final protein concentration of 2 µg/µL, for the Immunoblotting analysis and, after LDS-PAGE, the proteins were electro-transferred onto a Nitrocellulose Membrane (0.2 µm) using an XCell II Blot Module (Invitrogen) with a Transfer buffer to which 10% methanol (v/v) had been added. The immunoblot protocol and reagents were almost the same as those reported for the dot blot assay. The main difference was that the membranes were incubated with patient serum pools diluted 1:4 in the incubation buffer. The pools consisted of the sera of all the dot blot positive patients for each specific insect extract.

2.7. Protein identification

Immuno-reactive bands were cut from the gels and digested with sequencing grade trypsin, and the resulting peptides were then identified by means of LC-MS/MS, using a micro-LC system (Eksigent technologies Dublin, California, USA) interfaced with a 5600 + TripleTOFTM system (AB Sciex, Concord, Canada), as previously described by Nebbia et al. (2019). The DDA files were searched with Mascot v. 2.4 (Matrix Science Inc., Boston, USA), using the following NCBI databases: *Tenebrionoidea* for mealworm and buffalo worm, *Bombyx mori* for silkworm, and *Polyneoptera* for cricket and grasshopper. The following parameters were set for the searches: trypsin as the digestion enzyme with three missed cleavages, an S-carbamidomethyl derivate on cysteine as a fixed modification and oxidation on methionine, with Acetyl (N-term) and Met-loss (Protein N-term M) as variable modifications. Peptide mass tolerance was set at 50 ppm and MS/MS tolerance was set at 0.1 Da. The peptide charges (on a monoisotopic mass) were set at 2+, 3+ and

4+. Only proteins identified with i) at least three peptides with a peptide score > peptide identity, and ii) a protein score greater than or equal to 15% of the sum of the protein score identified in the band were considered for identification purposes.

2.8. Determination of the allergenic potential

The allergic potential of the proteins identified in the reactive bands as cross-reactive allergens was verified using Allermatch™ (http://www.allermatch.org). The comparison in Allermatch™ is based on the UniProtKB, WHO–IUIS and COMPARE databases (Fiers et al., 2004). The search was only performed on proteins with more than 35% of identity in a sliding window of 80 amino acids. The percent sequence identity, which indicates the extent to which two sequences have the same residues at the same position, and the expected value, which describes the number of hits that can be expected when searching a database of a specific size, were given for each of the resulting allergens. The allergen list provided by Allermatch™ was further processed by selecting the five allergens with the highest % sequence identity among the organisms belonging to the *Acariformes* superorder, the *Decapoda* order and the *Insecta* class as cross-allergens for patients allergic to HDM, shrimp and mealworm, respectively.

3. Results

3.1. Processing affects protein solubility

The protein profiles of the raw, boiled (100 °C) and fried (180 °C) insects (mealworm (*Tenebrio molitor*), buffalo worm (*Alphitobius diaperinus*), silkworm (*Bombyx mori*), cricket (*Gryllodes sigillatus*) and grasshopper (*Locusta migratoria*)) are shown in Fig. 3. Two different protocols were applied to optimize the extraction of both the water-soluble (W) and urea-soluble (U) proteins (Fig. 1). As shown in Fig. 3, the mealworm and buffalo worm were characterized by similar protein profiles, in both the raw and boiled samples. The W extract from the fried mealworm showed fewer bands than the raw and boiled samples, while the U protein profile was comparable with the boiled one. A similar, albeit more marked trend, was observed in the fried buffalo worm.
Silkworm showed an increase in the low molecular weight bands in the boiled W extracts, compared to raw and fried ones, thus suggesting a greater protein degradation after boiling than after frying. Moreover, the boiling procedure seemed to increase the number of U protein bands, although no differences were observed across the whole range of molecular weights.
A gradual decrease in the number of high molecular weight W bands was observed in cricket as the processing temperature increased. The same trend was observed for U proteins, but only after frying. This phenomenon is evident for the two major bands of the W fraction at around 55–65 kDa, which completely disappeared after both forms of processing.
The shift in the proteins from the W to U fraction was more marked for the grasshopper protein extracts when moving from mild to high processing temperatures, although the band separation was more resolved in the boiled samples than in the fried U ones.
Overall, the fried protein profiles differed to a great extent, compared to the raw and boiled ones. The presence of sunflower oil and the high temperature of processing (180 °C) were responsible for a reduction in protein water-solubility. In most cases, this phenomenon in the W fraction was accompanied by the appearance of some smearing in the U extracts (especially in the cricket and grasshopper ones), which likely indicate the presence of both oil residues and insoluble protein complexes generated by the denaturation/degradation of the proteins due to the high processing temperature. In order to overcome this issue, a hexane extraction step was included to remove any remaining oil from the protein extracts, even though different levels of effectiveness were achieved, depending on the considered insect.

3.2. Serum screening by means of dot-blotting

The sera of the patients that were able to bind insect proteins (derived from the 1:1 mixing of the W and U fractions) were selected from the dot-blot results (Table 2 and Fig. 2). Seventy-one percent of the HDM allergic patients and 87% of the shrimp allergic patients recognized at least one insect extract in dot-blot. The HDM allergic patients tested against buffalo worm is the only case in which a statistically significant difference in dot blot positivity, in relation to processing, can be demonstrated ($p < 0.01$). In details, the raw buffalo worm extract was the most immunoreactive for the HDM allergic patients (reaching 57% of positivity to dot-blot), while the remaining 4 raw insect extracts showed a positivity that ranged from 18 to 32%. Although not statistically significant, an overall decrease in the positivity of the HDM allergic patients to dot-blot was found after processing (both boiling and frying) for mealworm, buffalo worm and cricket, even though a percentage of patients remained positive (between 11 and 36%). The same behavior was observed for grasshopper, but only after frying. On the other hand, the silkworm immunoreactivity was unchanged after boiling and only slightly affected after frying.
As far as the SH allergic patients are concerned, after taking into account the small number of patients ($n = 8$), their positivity to dot-blot appeared lower than for the HDM allergic patients. No shrimp allergic patient recognized raw cricket, fried grasshopper or either raw or processed silkworm blotted proteins, and these protein extracts were therefore not tested in the subsequent immunoblotting assay. Only for fried buffalo worm, boiled cricket and raw and boiled grasshopper, was it possible to pool the patient's sera, with a number of sera that varied between 2 and 4. The grasshopper protein extracts resulted to be the most cross-reactive, with around half of the patients showing a positive dot blot.
Both TM allergic patients showed positivity to silkworm and to the raw mealworm extracts, and one of them (patient TM1) lost his ability to recognize mealworm after processing. Both patients were negative to buffalo worm. One of them (patient TM2) was positive to fried cricket and processed grasshopper extracts, while the other one (patient TM1) was only positive to the raw grasshopper extract.

3.3. Effect of processing on cross-reactive proteins

After testing each serum individually, by means of dot blot, against each sample (each of the 5 insects after each of the processing procedures), all of the patients' sera characterized by the same primary allergy and which resulted positive to dot blot against the same sample were pooled, thus generating three groups: HDM, SH and TM allergic patients. Immunoblotting was performed by incubating the three groups of pooled sera with the protein extracts from the 5 insects, each of which was considered raw, boiled and fried (1:1 mixing of the proteins from the W and U fractions) (Fig. 4 and Table 3). The immunoreactive proteins were identified by means of mass spectrometry (Table 3 and Supplementary Table 1).
The cross-reactivity and the effect of processing on cross-reactivity appeared to be variable over the insect species, over the reactive proteins and for each of the three groups of considered patients. The consideration about the fried samples could partially be biased by the lower quality of the protein extract, which led to a less resolved LDS-PAGE separation as a consequence of processing, mainly in the cricket and grasshopper samples.
Tropomyosin and larval cuticle protein (LCP) resulted to be the most cross-reactive proteins, as the HDM, SH and TM allergic patients reacted to them in the immunoblotting experiment in most of the considered extracts. Tropomyosin was identified in a single band, at around 36 kDa, in mealworm, buffalo worm, silkworm and cricket (bands M3, B3, S4 and C3). LCP was identified in mealworm, buffalo worm, silkworm and grasshopper in multiple bands, ranging from 20 to 70 kDa (M1, M2, M4, M5, B1, B4, B5, B6, S2, S3, S5 and G6). LCP was identified as a single protein or together with other potentially cross-reactive proteins (myosin light chain, troponin, 56 kDa early-stage encapsulation-

inducing protein, serpin 5 and muscle-specific protein 20). It was found, mainly by considering the HDM patient sera (the largest pool), that the cross-reactivity of these proteins was not affected by processing, that is, by boiling or frying.
Unlike tropomyosin and the cuticle protein, some insect-specific allergenic proteins, such as the cockroach allergen-like protein (only identified in mealworm), the 30 kDa protein and the vitellogenin precursor (only identified in silkworm) were identified. These three proteins were found in single bands: at 14, 27 and 190 kDa, respectively. The cockroach allergen-like protein cannot be considered a cross-reactive protein, since it was only recognized by the TM allergic patients in the mealworm extract, and its immunoreactivity disappeared after processing. The 30 kDa protein was only cross-reactive for the HDM allergic patients, and it retained its cross-reactivity after processing. Vitellogenin was found to be a cross-reactive protein for the HDM and TM patients; its cross-reactivity was not affected by the thermal treatments.
The myosin of mealworm, buffalo worm and grasshopper was found to be a cross-reactive protein, but it was only identified as a single protein in grasshopper, while it co-migrated with the cuticle protein in the other extracts. When a cross-reactive band contained more than one potentially allergenic protein, it was not possible to determine which protein cross-reactivity it was due to. As far as grasshopper is concerned, myosin cross-reactivity (bands G1, G3 and G5) appeared to be resistant to boiling- but not to frying - for both the HDM and TM allergic patients.
Lastly, troponin and β-actin were identified as single proteins in cricket (bands C1 and C2) and grasshopper (band G4), respectively. They were both cross-reactive for the HDM allergic patients, and β-actin was also cross-reactive for one of the two TM allergic patients. Their cross-reactivity was affected by both boiling and frying.

3.4. Protein allergenicity potential

The allergenic potential of the identified cross-reactive proteins was predicted using Allermatch™. A protein was considered potentially allergenic when it showed more than 35% identity with a known allergen within a window of 80 amino acids or more (Van Broekhoven et al., 2016). The identified proteins that resulted to be potentially allergenic are listed in Table 4 on the basis of the above-mentioned criteria. The five best hits that show a higher sequence identity than 40% for the HDM allergic patients, higher than 58% for the shrimp allergic patients and higher than 70% for the TM allergic patients are reported. Tropomyosin, one of the most cross-reactive proteins, matches the corresponding HDM, shrimp and different insect tropomyosin isoallergens. Myosin heavy chains E and A, myosin light chain and myosin regulatory light chain 2 match the paramyosin and myosin allergens found in three HDM species, in two shrimp species and in *Blattella germanica,* with a sequence identity of between 40% and 80%. No isoallergen with a higher identity percentage than 35% was found for the cross-reactive proteins that are not reported in Table 4.

4. Discussion

In the present study, we have investigated the effect of boiling and frying on the IgE cross-recognition of patients allergic to shrimp, house dust mite and mealworm toward five edible insects. Our results confirm that the IgEs of these patients are able to bind proteins from mealworm (*Tenebrio molitor*), buffalo worm (*Alphitobius diaperinus*), silkworm (*Bombyx mori*), cricket (*Gryllodes sigillatus*) and grasshopper (*Locusta migratoria*). Depending on the nature of the cross-reactive protein and on the considered thermal treatment, the observed cross-reactivity resulted to be affected in different ways.
Our data confirm that thermal processing may change the solubility of insect proteins, as already demonstrated by Broekman et al. (2015), shifting a protein from a water-soluble fraction to water-insoluble fraction, more so after frying than after boiling. It has been demonstrated that the alteration of protein solubility is caused by protein unfolding, with a consequent exposure of

hydrophobic residues, which may lead to the formation of insoluble aggregates (Lasekan & Nayak, 2016). This protein solubility alteration may influence the digestibility, the analytical detection, the quantification and the immunoreactivity of allergenic proteins (Broekman et al., 2015, Pali-Schöll et al., 2019, Sharma et al., 2016).

In our study, we used dot-blot screening to group the allergic patients according to their positivity to the different insect species and types of processing. From our data, it can be seen that most of the patients recognized at least one insect extract, according to those found by Van Broekhoven et al. (2016), who investigated the immunoreactivity of HDM and shrimp allergic patients toward three mealworm species. The HDM allergic patients showed a higher overall percentage of positivity to dot blot than the shrimp allergic patients, although cross-reactivity was often only observed in one patient. Raw buffalo worm and processed grasshopper were the most cross-reactive insects for the HDM allergic patients. The grasshopper protein extract was the most cross-reactive for the shrimp allergic patients, although its immunorecognition decreased after boiling and completely disappeared after frying. Broekman, Knulst, de Jong, et al. (2017), while investigating the cross-reactivity of the sera of 15 shrimp allergic patients toward seven insect extracts, instead found that almost all the patients were cross-reactive to the tested insects, but unlike our cohort of patients (who had never eaten insects), most of their allergic subjects (13/15) also suffered from mealworm food allergies.

The two mealworm sensitized patients' IgEs bound silkworm (both patients), mealworm (both patients for raw extracts but only one for processed extracts), and grasshopper (one patient) proteins. No reactivity was observed for buffalo worm and only the IgEs from one patient bound fried cricket proteins. As already published by Nebbia et al. (2019), the two patients did not report any symptom after ingestion of other insects, that is, cricket or greater wax moth. This may indicate that primary mealworm sensitization is not sufficient to predict the development of other edible insect allergies, thus suggesting that edible insect allergenicity is insect species-specific. Focke et al. (2003) also reported species-specific insect allergies for housefly (*Musca domestica*), while Siracusa et al. (1994), reported them for greenbottle (*Lucilia caesar*) Broekman, Knulst, den Hartog Jager, et al. (2017), investigating the cross-allergenicity of four primary mealworm allergic subjects to seven different insects, stated that a primary mealworm allergy does not mean that the subjects are likely to react to all insects, a result that is confirmed by our findings.

When dealing with cross reactivity at the molecular level, it is necessary to consider that different types of cross-reactive proteins are involved in sensitization. Some allergens are specific for a single allergen matrix, while others, like tropomyosin, are pan-allergen and are contained in multiple allergen sources. Furthermore, sensitization, as a result of cross-allergen recognition, may have different clinical relevance. It has been reported that the clinical manifestations of subjects sensitized to pan-allergens depend on the allergen source, the way of exposure, the geographical area and the patients' characteristics (age, gender, etc.) (McKenna et al., 2016).

The cockroach allergen-like protein, which was only identified in mealworm, behaved like a specific allergen for the patients primary sensitized to *T. molitor*, as previously described by Nebbia et al. (2019) and its immunorecognition seems to be heat-labile. Larval cuticle protein (LCP) was instead identified in the reactive bands of all three worms (buffalo worm, silkworm and mealworm), and was cross-recognized by the HDM, shrimp and mealworm allergic patients. Verhoeckx et al., 2014, Van Broekhoven et al., 2016 also reported LCP cross-allergenicity for mealworm, in both shrimp and HDM allergic patients, as did Broekman et al. (Broekman et al., 2015, Broekman et al., 2017a, Broekman et al., 2017), who defined LCP as a cross-reactive protein that played the main role in primary mealworm allergies.

In our experiments, Troponin T and β actin cross-recognition was found to be affected by processing. Troponin T has already been identified in cockroach as a minor allergen, and 16.7% of cockroach allergic patients in Thailand reacted to it (Khantisitthiporn et al., 2007). Broekman, Knulst, de Jong, et al. (2017) identified Troponin T in different insect allergenic extracts and Verhoeckx et al. (2014) cross-recognized it in both HDM and shrimp allergic patients. They found that myosin cross-

reactivity was resistant to boiling, but not to frying. Its cross-allergenicity potential has already been described in insects for both HDM and shrimp allergic patients (Broekman et al., 2017, Van Broekhoven et al., 2016, Verhoeckx et al., 2014). Tropomyosin was found to be the most cross-reactive protein for both HDM and shrimp allergic patients, as already demonstrated by Van Broekhoven et al., 2016, Broekman et al., 2016, Broekman et al., 2017a), Verhoeckx et al., 2014, Leni et al., 2020. It is considered a pan-allergen in crustaceans, mollusks, mites and also in insects, due to the high level of similarity of its amino acid sequences among species (Barre et al., 2018, Leni et al., 2020). Our patients cross-reacted to mealworm, buffalo worm, silkworm and cricket tropomyosin, and the cross-reactivity appeared to be heat-stable, as already found by Broekman et al. (2015) in both boiled (100 °C for 10 min) and fried (180 °C for 3 min) samples. Van Broekhoven et al. (2016) instead demonstrated a decrease in tropomyosin immunoreactivity after frying at 180 °C for 5 min, probably due to their longer processing time than in our experiments (5 *vs* 3 min). The two patients primarily sensitized to *T. molitor* showed a faint immunoblotting signal on the tropomyosin bands, compared to the patients in the other two groups. Tropomyosin has rarely been reported to have been recognized by the sera of patients primarily sensitized to insects (Broekman et al., 2017, Linares et al., 2008), as confirmed by our observation. These findings suggest that tropomyosin may play an important cross-allergenic role for HDM and shrimp allergic patients, while other proteins, such as LCP, seem to play a major role in the sensitization process of patients primarily sensitized to insects.

5. Conclusion

Considering the overall effect of processing on the IgE cross-recognition of allergens extracted from five edible insects in the sera of HDM, shrimp and mealworm allergic patients, it is possible to state that the effect is protein-, species- and treatment-specific. Despite the limitation of the preliminary nature of the work, it is possible to state that thermal processing partially reduces cross-allergenicity. According to our results, HDM, shrimp and mealworm allergic patients should be cautious about consuming insects, since different proteins, some of which are thermostable, are involved in cross-sensitization. Further studies are needed to verify the real risk for HDM and shrimp allergic patients, who have never tasted insects before, of developing allergic symptoms after insect ingestion. This could be established by performing an oral food challenge (OFC) in order to clarify the relationship between the patterns of primary sensitization and the OFC results.

CRediT authorship contribution statement

Cristina Lamberti: Conceptualization, Investigation, Methodology, Writing – original draft. **Stefano Nebbia:** Investigation, Methodology, Software. **Simona Cirrincione:** Investigation, Validation, Data curation, Writing – original draft. **Luisa Brussino:** Conceptualization, Investigation. **Veronica Giorgis:** Investigation, Methodology. **Alessandra Romito:** Investigation, Methodology. **Cristiana Marchese:** Writing – review & editing. **Marcello Manfredi:** Methodology, Investigation. **Emilio Marengo:** Investigation, Supervision. **Maria Gabriella Giuffrida:** Investigation, Supervision, Writing – review & editing. **Giovanni Rolla:** Conceptualization, Supervision, Writing - review & editing. **Laura Cavallarin:** Conceptualization, Writing – original draft, Resources, Project administration.
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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Table 1. Clinical characterization of the patient cohort (N = 38): 28 house dust mite (HDM) allergic patients, 8 Shrimp (SH) allergic patients and 2 mealworm (*Tenebrio molitor*, TM) allergic patients. DP: *Dermatophagoides pteronyssinus* protein extract, DF: *Dermatophagoides farinae* protein extract, SH: shrimp protein extract. Der p1 and Der p2: HDM allergens; Pen a1: shrimp allergen.

	Patient ID	Age (years)	Sex	Skin prick test positivity	Specific IgEs (ImmunoCAP), KUA/L	Clinical symptoms
HDM allergic patients	HDM1	52	M	DP, DF	N/A	rhinitis, asthma
	HDM2	42	M	DP, DF	N/A	rhinitis
	HDM3	31	F	DP, DF	N/A	rhinitis, conjunctivitis, asthma
	HDM4	30	F	DP, DF	N/A	rhinitis
	HDM5	36	M	DP, DF	N/A	rhinitis
	HDM6	40	M	DP, DF	N/A	rhinitis, asthma
	HDM7	67	F	DP, DF	N/A	rhinitis, asthma
	HDM8	34	F	DP, DF	N/A	rhinitis, asthma
	HDM9	22	F	DP, DF	Der p1 26.80 Der p2 51.00	rhinitis, asthma
	HDM10	61	M	DP, DF	DP 3.24 DF 3.00	rhinitis, asthma
	HDM11	64	F	DP, DF	DP 1.05 DF 1.01	rhinitis, asthma
	HDM12	20	M	DP, DF	DP 19.60 DF 16.80	rhinitis
	HDM13	80	M	DP, DF	DP 0.31 DF 1.97	rhinitis
	HDM14	50	M	DP, DF	Der p1 2.58 Der p2 2.57	rhinitis, asthma
	HDM15	70	M	DP, DF	Der p1 0.01 Der p2 7.97	rhinitis, asthma
	HDM16	18	M	DP, DF	Der p1 > 100 Der p2 > 100	rhinitis
	HDM17	25	M	DP, DF	Der p1 22.70 Der p2 46.20 Der p23 6.27	rhinitis
	HDM18	33	M	DP, DF	Der p1 6.78 Der p2 18.10	rhinitis

	Patient ID	Age (years)	Sex	Skin prick test positivity	Specific IgEs (ImmunoCAP), KUA/L	Clinical symptoms
	HDM19	40	F	DP, DF	DP 2.67 DF 2.35	rhinitis
	HDM20	23	F	DP, DF	Der p1 0.01 Der p2 46.50	rhinitis, conjunctivitis
	HDM21	49	F	DP, DF	DP 16.50 DF 23.30	rhinitis
	HDM22	26	M	DP, DF	Der p1 9.66 Der p2 12.70	rhinitis, asthma
	HDM23	27	F	DP, DF	Der p1 0.00 Der p2 1.80	rhinitis, asthma
	HDM24	38	M	DP, DF	DP 7.59 DF 7.74	rhinitis
	HDM25	27	M	DP, DF	Der p1 0.00 Der p2 2.48	rhinitis
	HDM26	35	F	DP, DF	DP 34.70 DF 32.10	rhinitis
	HDM27	42	F	DP, DF	DP 0.55 DF 0.55 Der p2 0.45	rhinitis, asthma
	HDM28	19	M	DP, DF	Der p1 6.18 Der p2 19.20	rhinitis
SH allergic patients	SH1	42	M	SH	SH 0.58	urticaria, angioedema
	SH2	45	M	SH	SH 3.92	urticaria, oral allergy syndrome
	SH3	76	M	SH	SH 0.14 Pen a1 0.13	urticaria, angioedema
	SH4	26	M	SH	SH 0.25	oral allergy syndrome
	SH5	35	F	SH	SH 0.23	urticaria
	SH6	72	M	SH	SH 1.24	urticaria, angioedema
	SH7	37	F	SH	SH 1.36 Pen a 1 1.30	urticaria, angioedema
	SH8	35	M	SH	SH 0.35	urticaria, angioedema, diarrhea
TM allergic patients	TM1	27	M	SH/DP/DF : negative	N/A	oral allergy syndrome after eating a TM hamburger

	Patient ID	Age (years)	Sex	Skin prick test positivity	Specific IgEs (ImmunoCAP), KUA/L	Clinical symptoms
	TM2	30	M	SH/DP/DF : negative	N/A	oral allergy syndrome after eating a TM hamburger

HDM: House dust mites; DP: Dermatophagoides pteronyssinus; DF: Dermatophagoides farinae; SH: Shrimp; TM: *Tenebrio molitor*.

Table 2. Screening of the HDM (HDM1-HDM28), shrimp (SH1-SH8) and primary sensitized mealworm (TM1-TM2) patients by means of dot-blot. The percentage of positive patients (+) for each insect protein extract was calculated by grouping the patients according to the primary allergy/sensitization (HDM, SH or TM).

		Mealworm			**Buffalo worm**			**Silkworm**			**Cricket**			**Grasshopper**		
	N° of patients	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**
HDM allergic patients	**HDM 1**	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	HDM 2	+	–	+	–	+	–	–	–	–	–	–	–	+	–	–
	HDM 3	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+
	HDM 4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	HDM 5	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
	HDM 6	–	–	–	+	–	–	–	–	–	+	+	–	+	+	+

	Mealworm			Buffalo worm			Silkworm			Cricket			Grasshopper		
N° of patients	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**
HDM 7	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+
HDM 8	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−
HDM 9	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
HDM 10	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
HDM 11	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
HDM 12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
HDM 13	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
HDM 14	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
HDM 15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
HDM 16	−	−	−	+	+	+	+	+	+	+	−	−	+	+	+
HDM 17	+	+	−	+	+	−	+	+	−	−	−	−	−	+	−
HDM 18	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−
HDM 19	−	−	−	+	+	+	−	−	−	+	+	−	+	+	−
HDM 20	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
HDM 21	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
HDM 22	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
HDM 23	−	−	+	+	+	+	−	−	−	+	+	+	+	+	+

		Mealworm			Buffalo worm			Silkworm			Cricket			Grasshopper		
	N° of patients	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**
	HDM 24	–	–	–	–	–	–	–	–	+	–	–	–	+	+	–
	HDM 25	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
	HDM 26	–	–	+	+	+	–	+	–	+	–	–	–	–	+	+
	HDM 27	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+
	HDM 28	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Total	6	3	5	16	10	5	5	5	4	7	6	3	9	12	8
		21%	11%	18%	57%	36%	18%	18%	18%	14%	25%	21%	11%	32%	43%	29%
SH allergic patients	**SH1**	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–
	SH2	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
	SH3	–	–	–	+	+	+	–	–	–	–	+	–	+	+	–
	SH4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	SH5	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
	SH6	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–
	SH7	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
	SH8	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
	Total	1	1	1	1	1	2	0	0	0	0	3	1	4	3	0
		12.5%	12.5%	12.5%	12.5%	12.5%	25%	0%	0%	0%	0%	37.5%	12.5%	50%	37.5%	0%
TM allergic patients	**TM1**	+	–	–	–	–	–	+	+	+	–	–	–	+	–	–
	TM2	+	+	+	–	–	–	+	+	+	–	–	+	–	+	+

	Mealworm			Buffalo worm			Silkworm			Cricket			Grasshopper		
N° of patients	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**	**Raw**	**Boiled**	**Fried**
Total	2	1	1	0	0	0	2	2	2	0	0	1	1	1	1
	100%	50%	50%	0%	0%	0%	100%	100%	100%	0%	0%	50%	50%	50%	50%

Table 3. List of the identified proteins contained in the cross-reactive bands. The name of each band corresponds to those indicated in LDS-PAGE (M = mealworm; B = buffalo worm; S = silkworm; C = cricket and G = grasshopper). The pools of the patients whose IgE recognized the corresponding LDS-PAGE bands are listed in the last three columns.

	Band	ID (NCBI)	Description	RAW extract	BOILED extract	FRIED extract
MEALWORM	M1	P80681.1	Larval cuticle protein A1A	HDM, SH	HDM	–
	M2	XP_008201464.1	Troponin T isoform X2	TM	SH	–
		P80681.1	Larval cuticle protein A1A			
		1TMQ_A	Alpha-amylase			
	M3	XP_008198924.1	Tropomyosin-2 isoform X6	HDM, SH, TM	HDM, SH, TM	HDM
		XP_015839642.1	Tropomyosin-1, isoforms 9A/A/B isoform X13			
	M4	BAA78480.1	56 kDa early-staged encapsulation-inducing protein	–	SH	–
		P80681.1	Larval cuticle protein A1A			
	M5	XP_008198303.1	Myosin regulatory light chain 2	HDM, SH	HDM	HDM, SH
		BAA78480.1	56 kDa early-staged encapsulation-inducing protein			
		P80681.1	Larval cuticle protein A1A			

	Band	ID (NCBI)	Description	RAW extract	BOILED extract	FRIED extract
	M6	AAP92419	Cockroach allergen-like protein	TM	–	–
BUFFALO WORM	B1	EEZ98281.1	Larval cuticle protein A3A-like Protein	HDM	HDM	HDM
	B2	not identified	–	–	HDM	–
	B3	XP_008198924.1	Tropomyosin-2 isoform X6	HDM, SH	HDM, SH	–
		XP_015839642.1	Tropomyosin-1, isoforms 9A/A/B isoform X13			
	B4	XP_015837065.1	Uncharacterized protein LOC664580	HDM	HDM	HDM
		EEZ98281.1	Larval cuticle protein A3A-like Protein			
		XP_015837064.1	Uncharacterized protein LOC664584			
	B5	XP_008198303.1	Myosin regulatory light chain 2	SH	SH	SH
		EEZ98281.1	Larval cuticle protein A3A-like Protein			
		EEZ98387.1	Hypothetical protein TcasGA2_TC000851			
	B6	RZC33111.1	Larval cuticle protein A3A-like	–	–	HDM, SH
		XP_970301.1	Pupal cuticle protein C1B			
		EEZ98387.1	Hypothetical protein TcasGA2_TC000851			
SILKWORM	S1	BAA02444.1	Vitellogenin precursor	HDM, TM	HDM, TM	HDM, TM
	S2	FAA00462.1	Putative cuticle protein	HDM, TM	HDM	HDM
		BAE06190.1	Glycine rich protein			
	S3	FAA00450.1	TPA: putative cuticle protein	–	TM	–
		AAS68506.1	Serpin-5			
	S4	P80034.1	Antichymotrypsin-2	–	HDM	HDM
		ABF51441.1	Tropomyosin isoform 1			
	S5	FAA00470.1	Putative cuticle protein	–	HDM	HDM

	Band	ID (NCBI)	Description	RAW extract	BOILED extract	FRIED extract
	S6	CAA38531	30 kDa protein	HDM	HDM	HDM
CRICKET	C1	AVI26881.1	Troponin T	HDM	–	–
	C2	AVI26881.1	Troponin T	HDM	–	–
	C3	AVI26879.1	Tropomyosin isoform 1	–	HDM, SH	HDM, SH, TM
		QCI56569.1	Tropomyosin 2, partial			
GRASSHOPPER	G1	ANS83649.1	Myosin heavy chain isoform E	HDM, TM	HDM, TM	–
		ANS83645.1	Myosin heavy chain isoform A			
		BBE27867.1	C-type lysozyme			
	G2	AQE30075.1	Mitochondrial F0F1-ATP synthase subunit beta	HDM, SH, TM	HDM, SH, TM	–
		AVI26881.1	Troponin T			
	G3	ANS83649.1	Myosin heavy chain isoform E	–	HDM	–
		ANS83645.1	Myosin heavy chain isoform A			
	G4	ACV32627.1	Beta-actin	HDM, TM	TM	–
	G5	AAW22542.1	Myosin light chain	HDM, TM	HDM, TM	–
	G6	P82167.1	Cuticle protein 21.3	HDM, TM	HDM,TM	HDM
		PNF35287.1	Muscle-specific protein 20			

Table 4. Cross-allergens analyzed by means of Allermatch™. Tyr p (*Tyrophagus putrescentiae*); Der f (*Dermatofagoides farinae*); Cho a (*Chortoglyphus arcuatus*); Blo t (*Blomia tropicalis*); Der p (*Dermatophagoides pteronyssinus*); Pen m (*Penaeus monodon*); Pen a (*Penaeus aztecus*); Pan b (*Pandalus borealis*); Lit v (*Litopenaeus vannamei*); Chi k (*Chironomus kiiensis*); Lep s (*Lepisma saccharina*); Per f (*Periplaneta fuliginosa*); Bla g (*Blattella germanica*).

Identified proteins				**Cross-allergen by Allermatch™**				
Band	**Proteins**	**Allergen**	**Organism**	**Full sequence length alignment**			**80 AA sliding window analysis**	
				% Sequence Identity	**E[a]**	**Overlap (AA)**	**# hits > 35% identity**	**% hits > 35% identity**
	Recognized by HDM patients							
M3/B3	Tropomyosin-2 isoform X6 (XP_008198924.1)	Tyr p 10	Tyrophagus putrescentiae	81.10	2e-88	281	204	100
		Der f 10	Dermatophagoides farinae	81.50	1.2e-90	281	204	100
		Cho a 10	Chortoglyphus arcuatus	81.10	3.2e-90	281	204	100
		Der p 10	Dermatophagoides pteronyssinus	80.40	4e-89	281	204	100
		Blo t 10	Blomia tropicalis	80.10	2.9e-89	281	204	100
S4	Tropomyosin isoform 1 (ABF51441.1)	Tyr p 10	Tyrophagus putrescentiae	82.60	4.2e-91	281	206	100
		Der f 10	Dermatophagoides farinae	81.50	4.2e-91	281	206	100
		Cho a 10	Chortoglyphus arcuatus	81.10	4.2e-91	281	206	100
		Der p 10	Dermatophagoides pteronyssinus	80.40	1.3e-89	281	206	100
		Blo t 10	Blomia tropicalis	80.10	5.2e-90	281	206	100

Identified proteins				Cross-allergen by Allermatch™				
Band	Proteins	Allergen	Organism	Full sequence length alignment			80 AA sliding window analysis	
				% Sequence Identity	E[a]	Overlap (AA)	# hits > 35% identity	% hits > 35% identity
C3	Tropomyosin isoform 1 (AVI26879.1)	Tyr p 10	Tyrophagus putrescentiae	80.60	3.5e-91	284	205	100
		Der f 10	Dermatophagoides farinae	82.00	3.1e-93	284	205	100
		Cho a 10	Chortoglyphus arcuatus	81.30	5e-93	284	205	100
		Der p 10	Dermatophagoides pteronyssinus	81.00	1e-91	284	205	100
		Blo t 10	Blomia tropicalis	79.90	2.6e-91	284	205	100
G1/G3	Myosin heavy chain isoform E (ANS83649.1)	Blo t 11	Blomia tropicalis	41.70	3.9e-89	820	693	36.98
		Der f 11	Dermatophagoides farinae	42.20	2.8e-90	820	670	35.75
		Der p 11	Dermatophagoides pteronyssinus	41.50	2.8e-89	819	645	34.42
	Recognized by shrimp allergic patients							
M3/B3	Tropomyosin-2 isoform X6 (XP_008198924.1)	Pen m 1	Penaeus monodon	82.60	4.4e-90	281	204	100
		Pen a 1	Penaeus aztecus	82.60	4.4e-90	281	204	100
		Pan b 1	Pandalus borealis	82.90	5.2e-90	281	204	100
		Met e 1	Metapenaeus ensis	81.90	4e-86	271	204	100
		Mel l 1	Melicertus latisulcatus	81.50	5.5e-89	281	204	100

Identified proteins				Cross-allergen by Allermatch™				
Band	Proteins	Allergen	Organism	Full sequence length alignment			80 AA sliding window analysis	
				% Sequence Identity	E[a]	Overlap (AA)	# hits > 35% identity	% hits > 35% identity
C3	Tropomyosin isoform 1 (AVI26879.1)	Pen m 1	Penaeus monodon	82.40	4.2e-93	284	205	100
		Pen a 1	Penaeus aztecus	82.40	4.2e-93	284	205	100
		Pan b 1	Pandalus borealis	83.10	1.4e-93	284	205	100
		Met e 1	Metapenaeus ensis	81.80	7.3e-89	274	205	100
		Mel l 1	Melicertus latisulcatus	81.70	4.5e-92	284	205	100
M5/B5	Myosin regulatory light chain 2 (XP_008198303.1)	Pen m 3	Penaeus monodon	58.00	1.7e-33	176	120	100
		Lit v 3	Litopenaeus vannamei	58.00	2.2e-33	176	120	100
	Recognized by primary sensitized mealworm patients							
M3	Tropomyosin-2 isoform X6 (XP_008198924.1)	Chi k 10	Chironomus kiiensis	91.90	5.1e-102	283	204	100
		Lep s 1	Lepisma saccharina	92.90		239	204	100
		Per f 7	Periplaneta fuliginosa	87.90	2.7e-85	282	204	100
		Bla g 7	Blattella germanica	87.60	1.7e-97	282	204	100
		Per a 7	Periplaneta americana	87.20	4.4e-97	282	204	100

Identified proteins				Cross-allergen by Allermatch™				
Band	Proteins	Allergen	Organism	Full sequence length alignment			80 AA sliding window analysis	
				% Sequence Identity	E[a]	Overlap (AA)	# hits > 35% identity	% hits > 35% identity
C3	Tropomyosin isoform 1 (AVI26879.1)	Per f 7	Periplaneta fuliginosa	91.90	1e-102	283	205	100
		Bla g 7	Blattella germanica	92.20	2.5e-103	283	205	100
		Per a 7	Periplaneta americana	91.50	7.7e-103	284	205	100
		Copt f 7	Coptotermes formosanus	93.60	1.3e-92	251	205	100
		Aed a 10	Aedes aegypti	91.80	5.6e-103	281	205	100
M5	Myosin regulatory light chain 2 (XP_008198303.1)	Bla g 8	Blattella germanica	71.20	2.1e-43	198	120	100
G5	Myosin light chain (AAW22542.1)	Bla g 8	Blattella germanica	83.60	5.9e-48	195	120	100

Fig. 1. Scheme of the protocol applied for insect protein extraction.

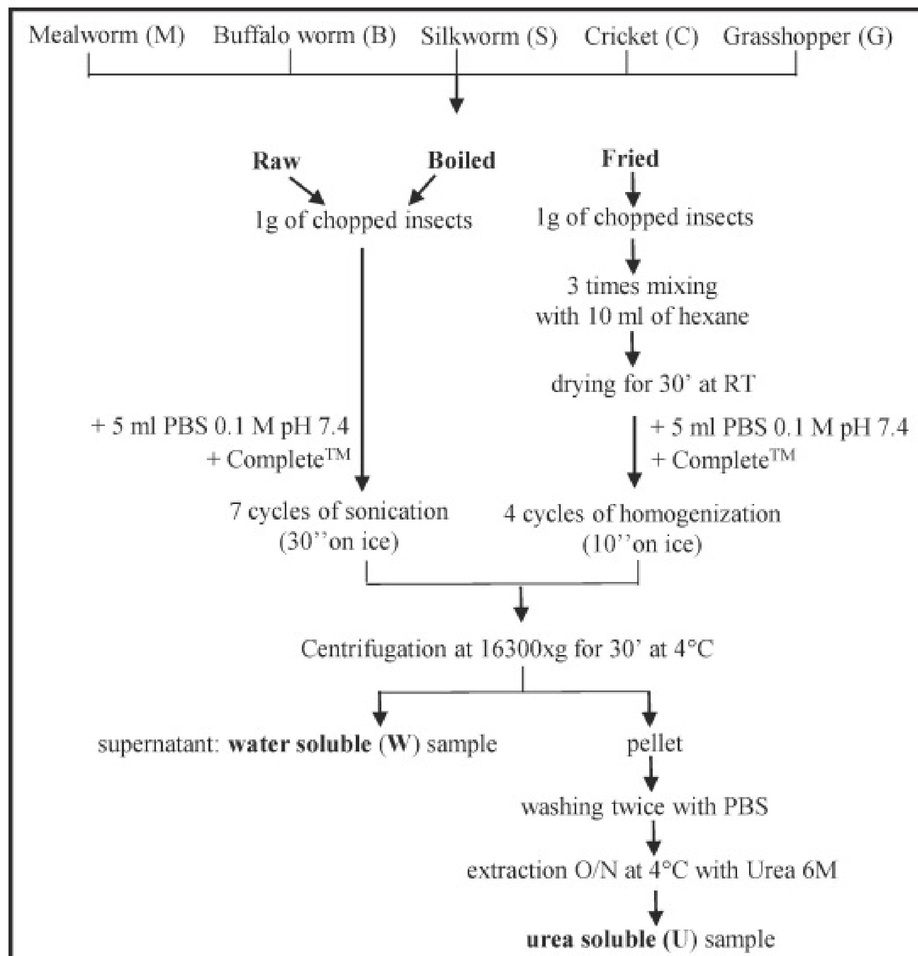
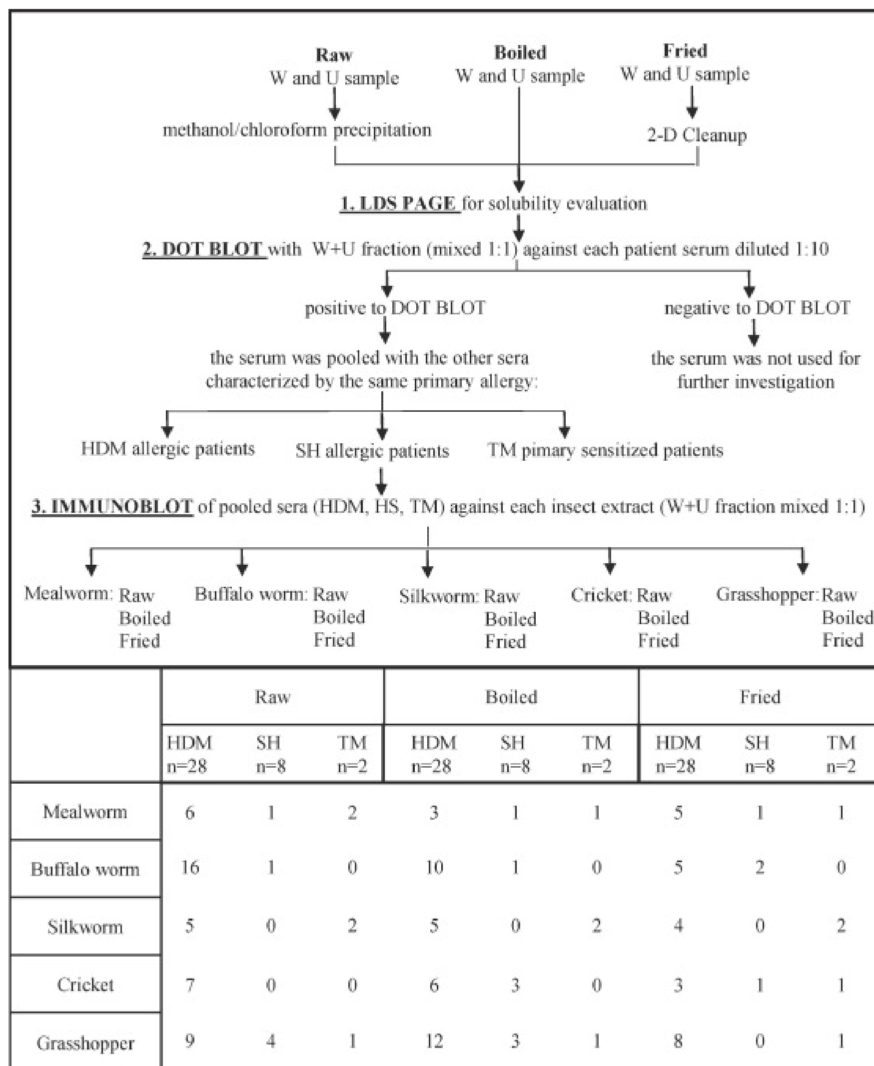


Fig. 2. Experimental workflow. In the lower part of the figure, the numbers (reported for each extract) indicate the sum of the patients who resulted to be positive after dot blot screening and who were thus considered to generate the pools for the immunoblotting analysis. When the number of positive patients was zero, the corresponding insect extract was not tested by means of immunoblotting. W: water-soluble protein extract, U: urea-soluble protein extract, HDM: house dust mite, SH: shrimp, TM: *Tenebrio molitor*.



	Raw			Boiled			Fried		
	HDM n=28	SH n=8	TM n=2	HDM n=28	SH n=8	TM n=2	HDM n=28	SH n=8	TM n=2
Mealworm	6	1	2	3	1	1	5	1	1
Buffalo worm	16	1	0	10	1	0	5	2	0
Silkworm	5	0	2	5	0	2	4	0	2
Cricket	7	0	0	6	3	0	3	1	1
Grasshopper	9	4	1	12	3	1	8	0	1

Fig. 3. LDS-PAGE of the water-soluble (W) and urea-soluble (U) protein fractions for each of the five insects under the three tested conditions: raw, boiled and fried. MW: molecular weight.

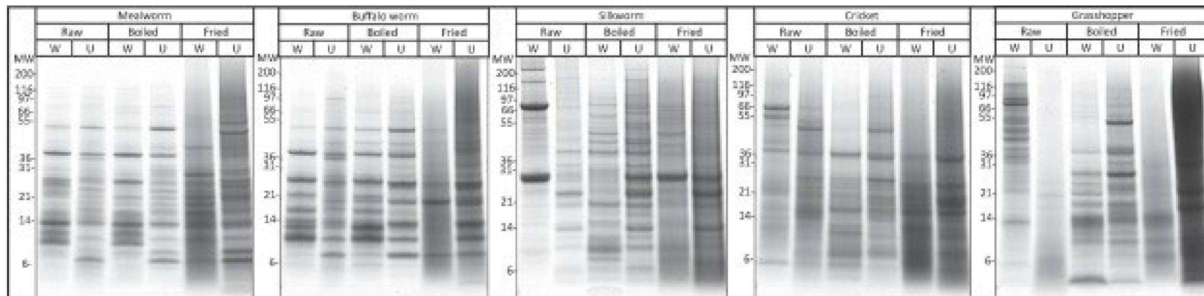
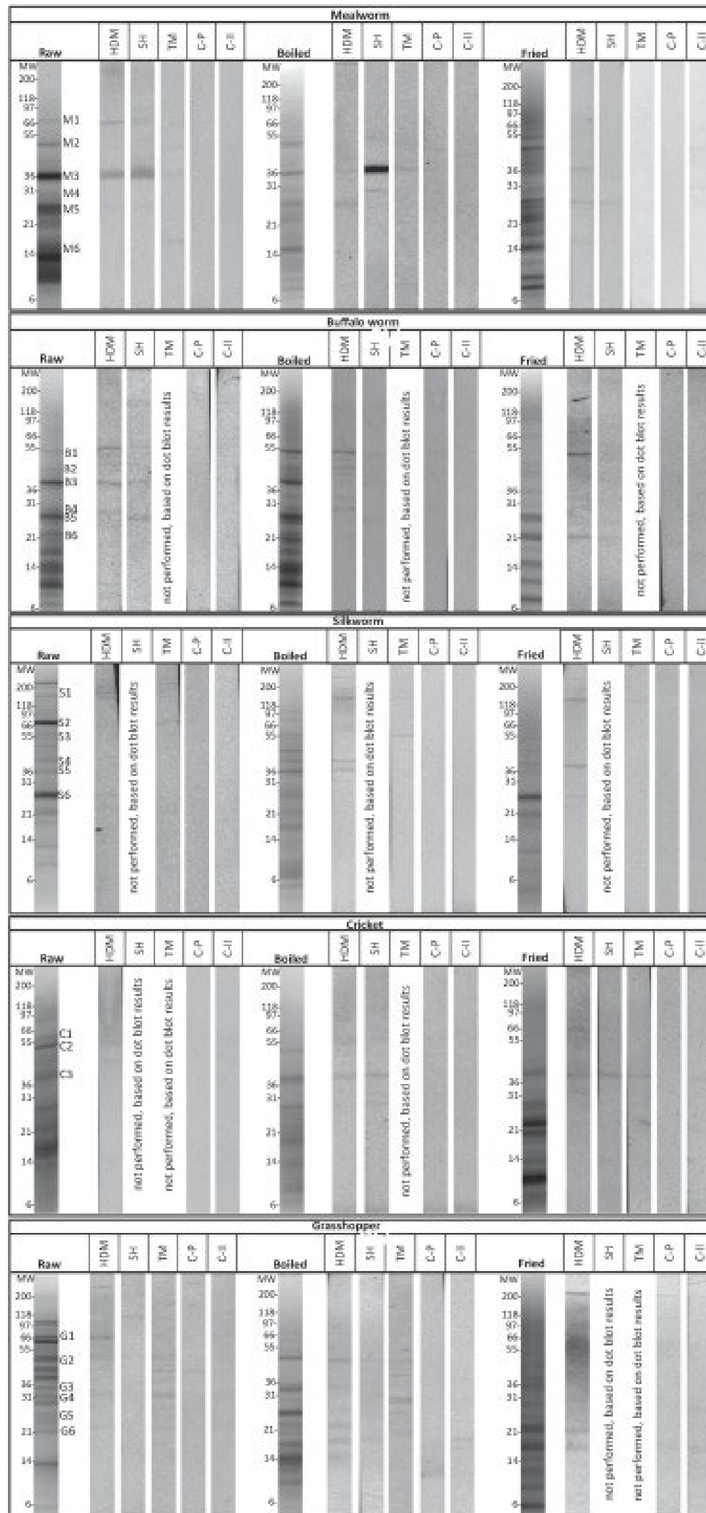


Fig. 4. Cross-reactivity of house dust mite (HDM), shrimp (SH) and primary sensitized mealworm (TM) patient sera toward water-soluble and urea-soluble (mixed 1:1) protein extracts of the five edible insects. The letters indicate the reactive bands analyzed by means of mass spectrometry (M for bands reactive to mealworm (*Tenebrio molitor*), B for those reactive to buffalo worm (*Alphitobius diaperinus*), S for those reactive to silkworm (*Bombyx mori*), C for those reactive to cricket (*Gryllodes sigillatus*) and G for those reactive to grasshopper (*Locusta migratoria*). MW: molecular weight; C-P: non-allergic patient pool; C-II: secondary antibody control.


Mealworm
Buffalo worm
Silkworm
Cricket
Grasshopper
Raw
Boiled
Fried
HDM
SH
TM
C-P
C-II
MW
200
118
97
66
55
36
31
21
14
6
M1
M2
M3
M4
M5
M6
B1
B2
B3
B4
B5
B6
S1
S2
S3
S4
S5
S6
C1
C2
C3
G1
G2
G3
G4
G5
G6
not performed, based on dot blot results