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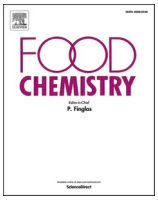

Allergenicity of tropomyosin variants identified in the edible insect *Hermetia illucens* (black soldier fly)

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ABSTRACT

Insect consumption could address the increasing protein demand in compliance with environmental sustainability. *Hermetia illucens* (black soldier fly, BSF) is a promising insect for human diet and it is essential to assess the related allergenic risk, meant as primary sensitization or cross-reactivity with known allergens. In this work, we investigate the allergenicity of two tropomyosin variants identified in the BSF genome and produced as recombinant proteins. Immunoblot experiments showed that both proteins were recognized by sera of patients allergic to shrimp or mites highlighting the cross-reactivity risk. CD spectroscopy, cross-linking assays and size-exclusion chromatography showed a structure composed of alpha-helices oligomers for both variants. These proteins were quite stable to pH but sensitive to increasing temperatures. In vitro simulated digestion associated to mass-spectrometry allowed the identification of peptides resistant to gastrointestinal conditions which were compared with epitopes of Arthropoda and Mollusca allergens to predict the persistence of allergenicity upon digestion.

1. Introduction

It is estimated that the constant growth in the world population will lead to an increase of food demand by 60 % and to unsustainable levels of meat production by 2050. These predictions induce the scientific community to evaluate protein sources alternative to traditional foods of animal origin. In this context, insects could represent an excellent source of proteins of high nutritional quality, as well as of fats, vitamins, and minerals (Liceaga et al., 2021).

Insects are widespread in the diet of many populations, predominantly in parts of Asia, Africa and Latin America and over 2000 species are consumed in different ways (Hlongwane et al., 2020). Conversely, entomophagy is refused in Western countries due to cultural bias and numerous studies are focused on processing methodologies capable to make insects more acceptable (Liceaga, 2021). The production of edible insects is very advantageous in terms of water, soil and energy consumption and greenhouse gas emissions if compared with traditional livestock rearing (Jantzen da Silva Lucas et al., 2020). In addition, insects can use different agricultural wastes as nutrient source promoting environmental sustainability (Gold et al., 2018).

To date, based on EFSA evaluation, four different insects, yellow mealworm (*Tenebrio molitor*), migratory locust (*Locusta migratoria*), house cricket (*Acheta domesticus*) and lesser mealworm (*Alphitobius diaperinus*) received the authorization from the Commission as novel foods for human consuming in EU (Mancini et al., 2022; Regulation (EU) 2023/58 of 5 January 2023). Among the other applications submitted to EFSA, one dealt with larvae of black soldier fly (*Hermetia illucens*, BSF) which have great potential as sustainable source of nutrients for human food (Bessa et al., 2020) and have been authorized for animal feed since 2017 (Mouithys-Mickalad et al., 2020).

One of the risks that should not be underestimated in the possible use of BSF larvae or their components in human nutrition is the presence of allergenic molecules. Primary sensitization deriving from allergens from edible insects has been described in Asia (Ji et al., 2009) and in Africa

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(Kung and Steenhoff, 2013), where entomophagy is a habit. Conversely, numerous studies describe cross-reactivity between insect proteins and shellfish allergens or insect inhalant allergens highlighting potential risks deriving from the consumption of insects (Lamberti et al., 2021). Among the allergenic proteins identified in arthropods and molluscs, tropomyosin (TPM) and arginine kinase are considered the main causes of allergic reactions in the population (de Gier and Verhoeckx, 2018).

Tropomyosin is a highly conserved protein located in both muscle and non-muscle cells of all species of vertebrates and invertebrates, it is usually encoded by multiple genes and characterized by different splicing variants (Karlik and Fyrberg, 1986; Goins and Mullins, 2015; Schevzov et al., 2011). Its structure consists of two parallel alpha-helical molecules forming a coiled-coil dimer that can associate to form polymers (Stafford et al., 2012). Invertebrates TPMs are pan-allergens whereas, vertebrate TPM were long been considered non-allergenic and incapable of cross-reactivity with invertebrate TPMs due to their low sequence identity (i.e., about 50 % with shrimp TPM; Reese et al., 1999), but recently TPMs from three different fish species have been included in the WHO/IUIS Allergen database (http://allergen.org/) and evidence of cross-reactivity between fish and crustacean TPMs have been reported (Ruethers et al., 2021; Peixoto et al., 2018).

Recently, studies combining mass-spectrometry approaches and bioinformatics analysis have identified peptide sequences related to BSF TPM (Bessa et al., 2021; Leni et al., 2020) and immunoblotting experiments have shown that TPM in BSF protein extracts is recognized by sera of patients allergic to crustaceans, suggesting a cross-reactivity between TPM from BSF and crustaceans (Leni et al., 2020). In this work, we report the production as recombinant proteins of two TPM variants identified in BSF genome by bioinformatics analysis, their structural characterization and experiments showing their allergenic potential.

2. Materials and methods

2.1. Reagents and solvents

Media, buffer components and standard reagents were obtained from Merck (Milan, Italy).

2.2. Bioinformatics analyses

H. illucens sequences encoding for tropomyosin variants were identified by searching the complete genome of BSF deposited in the NCBI database (08.11.2021) by using the tBlastn algorithm and the tropomyosin sequence of *Drosophila melanogaster* NP_001163619 as a query.

Nucleotide or amino acid multiple sequence alignments were performed by using ClustalW (Larkin et al., 2007)' and visualized with ESPript 3.0 (Robert and Gouet, 2014). The search in the Sequence Read Archive derived from RNAseq of the midgut of BSF larvae adapt to different food substrates (BioProject PRJEB39181) was performed by using the tBlastn algorithm and the RNA sequences reported in Table S1 as queries.

The three-dimensional homology modeling of TPM2-X16 and TPM1-X1 was performed using online server SWISS-MODEL (https://swissmodel.expasy.org). The analysis of the resulting structures was carried out by PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.).

For the in silico linear epitope mining, the whole set of linear epitopes already discovered and made available so far belonging to insects, crustaceans, arachnida and molluscs were retrieved from the Immune Epitope Database and Analysis Resource (IEDB; last database access 25th January 2023; https://www.iedb.org/)(Vita et al., 2019), a gold benchmark database for epitope sequences. Specifically, 2974 non-redundant epitopes were retrieved from IEDB and their inclusion in the sequences of *H. illucens* TPM1-X1 and TPM2-X16 was checked both in native proteins and in their hydrolysates (after simulated gastrointestinal digestion) through an *ad hoc* python script.

2.3. Cloning, expression, and purification of tropomyosin variants from BSF

The sequences encoding for the tropomyosin-2 isoform X16 (TPM2-X16) and for the tropomyosin-1 isoform X1 (TPM1-X1) from BSF (NCBI protein id XP_037922235 and XP_037922243, respectively) added of the *Nde*I restriction site at 5′ end, optimized for expression in *E. coli* and cloned in the pEX-A128 vector were purchased from Eurofins (Milan, Italy). The TPM cDNAs were sub-cloned into the *Nde*I and *Bam*HI restriction sites of the expression vector pET28b (Merck). The resulting plasmids pET28b-TPM2-X16 and pET28b-TPM1-X1 were sequenced on both strands and used to transform *E. coli* BL21(DE3) cells (Merck) by electroporation. Cells were grown in LB medium at 37 °C under gentle shaking until OD 600 nm reached 0.6–0.8, the expression of TPM variants was then induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After 2 h of incubation at 37 °C, the cells were harvested by centrifugation at 4500 × g for 15 min. The pellet from 1L of culture was resuspended in 12 mL of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 1 mM PMSF and 10 % glycerol, protease inhibitor cocktail powder 0.85 μg/ml, pH 7.5) containing lysozyme 1 mg/ml. After incubation for 45 min at 4 °C, cells were sonicated on ice for 30 min (at 1 s pulse on and 3 s pulse off) and then centrifuged at 14000 × g for 30 min. The recombinant proteins were purified from supernatants by affinity chromatography on a His-Trap column connected to an ÄKTA Pure FPLC System (GE Healthcare, Chicago, IL, USA). The column was equilibrated with 50 mM sodium phosphate, 300 mM NaCl pH 7.5 containing imidazole 15 mM and, after sample loading, it was washed with the same buffer containing 2 M NaCl to remove nucleic acid contaminants. The recombinant proteins were eluted by raising the imidazole concentration to 500 mM. Protein fractions (2 mL each) were detected by UV280, analyzed by 12 % SDS–PAGE, pooled and concentrated using centrifugal concentrator with a 30 KDa cut-off (Merck). In the case of TPM1-X1, the protease inhibitor cocktail powder 0.85 μg/ml was added to the purified protein to avoid degradation. As both proteins are lacking tryptophan residues, their final concentration was estimated after SDS-PAGE by densitometric analysis by using a ChemiDoc MP imager (Biorad, Hercules, CA, USA) and the Image Lab software (Biorad). Aliquots of purified proteins were snap-frozen in liquid nitrogen and stored at −80 °C.

2.4. Analytical size exclusion chromatography and cross-linking assays

Analytical size-exclusion chromatography was performed on a Superdex200 increase 5/150 GL column connected to an Äkta FPLC system (GE Healthcare). The column was equilibrated with 50 mM sodium phosphate, 150 mM NaCl pH 7.5 and calibrated with proteins of known molecular weight.

Cross-linking reactions were performed in buffer 50 mM sodium phosphate, 150 mM NaCl pH 7.5 by incubating about 1.8 ug of TPM in the presence of 0.05, 0.1, 0.2 and 0.5 % glutaraldehyde for 5 min at room temperature. The reactions were stopped by adding Tris-HCl pH 8 to reach a final concentration of 20 mM. The reaction products were analyzed by 12 % SDS-PAGE.

2.5. Mass spectrometry analysis

TPM2 X16 (approximatively 1 mg/mL in water) was analyzed using a UPLC ACQUITY system coupled with an ACQUITY SQ ESI-MS system (Waters, Milford, MA, USA). The analysis was performed with an RP ACQUITY UPLC BEH C4 (300 Å, 1.7 μm, 2.1 mm × 150 mm) column (Waters) with an ACQUITY UPLC BEH C4 VanGuard™ (300 Å, 1.7 μm, 2.1 mm × 5 mm) pre-column (Waters). The mobile phase was made by Milli-Q water with 0.2 % of ACN and 0.1 % of Formic acid (eluent A) and ACN with 0.1 % of Formic acid (eluent B). Analysis conditions were as follows: column temperature: 35 °C, injection volume: 6 μL, flow: 0.2 mL/min, gradient: 0–2 min isocratic 90 % A, 2–10 min linear from 90 %

A to 60 % A, 10–13 min 60 % A, 13–23 min linear from 60 % A to 0 % A, 23–25 min 0 % A, 25–40 min linear from 0 % A to 90 % A, 40–45 min 90 % A. Detection conditions were as follows: polarity: ES+, capillary: 3.2 kV, cone: 30 V, extractor: 4 V; RF: 0.2 V, source temperature: 150 °C, desolvation temperature: 300 °C, cone gas flow: 100 L/h, desolvation gas flow: 650 L/h, LM 1 resolution: 14.6, HM 1 resolution: 15.2, ion energy 1: 0.3; gain: 1.00, acquisition mode: Full Scan (*m/z* 100–2000), scan duration: 1 s. The software used for data acquisition and data processing was MassLynxTM V4.0 (Waters).

2.6. Characterization of human sera samples

Four sera samples collected from allergic patients, indicated by numbers 1–4 and two control sera from non-allergic individuals (number 5 and 6), were analyzed. The samples were collected by the Allergy and Clinical Immunology Unit of the University of Parma. Patients' ages ranged from 23 to 61 years. The allergic sera have been selected based on evidence of food allergy to shrimp and/or of airway allergy to mites verified by in-vivo skin tests (ThermoFisher Scientific, Waltham, MA, USA) performed according to the international guidelines. The experimental protocol was approved by the Ethical Committee of the University of Parma (Approval No 19448). Informed consent was obtained from all subjects.

2.7. Immunoblotting assays

In IgG and IgE-immunoblotting assays, 0.8 μg of TPM were spotted on a nitrocellulose membrane (GE Healthcare) and allowed to dry completely at room temperature. The membrane was incubated with blocking solution containing 5 % Skim Milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.05 % Tween 20, pH 7.5) at room temperature for 45 min and then washed by using TBS-T buffer.

IgG-immunodetection of TPM variants was performed by using rabbit anti-human TPM1 polyclonal antibodies (PA5-29846, ThermoFisher Scientific) diluted 1: 250 as primary, and goat anti-rabbit IgG antibodies labeled with StarBright Blue 700 (12004162, Bio-Rad) diluted 1:5000, as secondary.

For IgE –immunodetection of TPM variants, diluted (1:15) sera from patients with food allergy to shrimp and/or with inhalant allergy to mites or from non-allergic patients were used for the first incubation (1 h at room temperature) and goat anti-human IgE antibodies labeled with DyLight 680 (Microtech, Napoli, Italy) diluted 1: 1000 were used in the second incubation (1 h at room temperature). Negative controls were performed by incubating 0.8 μg of bovine seroalbumin spotted on the membrane with the same aliquot of diluted IgG or diluted patient serum used for TPM detection. Dot blot images were recorded by using ChemiDoc MP imaging system (Bio-Rad) and analyzed with Image Lab software (Bio-Rad).

2.8. Stability of TPM to temperature and pH

The stability of TPM variants to temperature and pH was analyzed by SDS-PAGE and by circular dichroism (CD) spectroscopy. To evaluate the stability to increasing temperature 1 ug of protein was analyzed on 12 % SDS-PAGE after incubation at different temperatures (4, 25, 37, 45, 60, 80 °C) in buffer 50 mM sodium phosphate pH 7.5 for 45 min. The effect of pH on protein stability was investigated using 12 % SDS-PAGE after incubation of 1ug of protein in 10 mM sodium acetate (pH 3, 4 and 5) or in 10 mM sodium phosphate (pH 6, 7, 8 or 9) for 1 h at room temperature.

Far UV CD spectra (190–240 nm) were recorded on a Jasco J1500 spectropolarimeter (Applied Photophysics Ltd., Surrey, UK) using a 0.1 cm path length cuvette, a scanning rate of 50 nm/min, a bandwidth of 1.0 nm and TPM concentrations of 3 μM in buffer 50 mM sodium phosphate pH 7.5. Spectra were recorded after 5 min incubation at different temperatures from 20 to 55 °C at intervals of 5 °C, followed by the cooling to 20 °C. CD spectra of TPM variants were also recorded at pH 3, 7 and 9 after 5 min incubation in buffer 10 mM sodium acetate (pH 3) and 10 mM sodium phosphate (pH 7 and pH 9) at 20 °C.

CD data were reported as mean residual ellipticity (θ) in deg cm^2 $dmol^{-1}$. Data from three spectra were accumulated to obtain mean spectra, which was analyzed by using the K2D3 online CD server (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/; Louis-Jeune et al., 2012). CD spectroscopy experiments were carried out in duplicate.

2.9. Simulated gastrointestinal digestion

Forty microliters of TPM solution (corresponding to 80 μg of protein) were dried under nitrogen flow and stored at −20 °C until simulated gastrointestinal digestion. The sample was reconstituted with 50 μL of 1 mM HCl (pH 2) and 1 μL of pepsin solution (1 mg/mL, 727 U/mg) was added; the simulated gastric phase was conducted at 37 °C for 3 h. Then, 30 μL of 100 mM NH_4HCO_3 (pH 7.8), 1 μL of trypsin solution (1 mg/mL, 1749 BAEE units/mg solid), and 1 μL of chymotrypsin solution (1 mg/mL, 55 units/mg of protein) were added; the simulated intestinal phase was conducted at 37 °C for 4 h. At the end of the digestion, the sample was stored at −20 °C until analysis. The samples were digested in duplicate, and two control samples were subjected to the same procedure, but without the addition of digestive enzymes, to check for the presence of peptides deriving from autolytic cleavage.

2.10. Liquid chromatography coupled to high resolution mass spectrometry analysis (LC-HRMS)

To identify the peptides generated during *in vitro* digestion, each sample was analysed directly by the Vion IMS QTof mass spectrometer (Waters). For analysis, chromatographic separation was achieved using a reversed-phase column (ACQUITY UPLC Peptide BEH C18 column, 300 Å, 1.7 μm, 2.1 mm × 150 mm, Waters). Eluent A was ultrapure water and 0.1 % HCOOH (grade MS). Eluent B was CH_3CN and 0.1 % HCOOH (MS grade). The injection volume was 1 μL and the flow rate was maintained at 0.25 mL/min; total run time was 36 min and applied gradient was 0–3.5 min, 100 % A; 3.5–25 min, 100 % A to 50 % A; 25–26.3 mins, 50 % AL; 26.3–26.5 min, 50 % A to 0 % A; 26.5–29.1 min, 0 % A; 29.1–29.5, 0 % A to 100 %; 29.5–36 min, 100 %. The column temperature was fixed at 35 °C; the autosampler temperature was set to 10 °C. The acquisition parameters were set as follows: positive ion mode (ESI +); MS acquisition time 3.5–29.1 min; analyser mode, sensitivity; mode, standard transmission; capillary, 3 kV; sample cone voltage, 40 V; source offset voltage, 80 V; source temperature, 120 °C; desolvation temperature, 450 °C; gas cone, 50 L/h; desolvation gas, 800 L/h; MS^e mode, high definition MS^e; scan range, 100–2000 *m/z*; scan time, 0.4 s; low collision energy, 6 V; high collision energy ramp, 20 to 45 V; automatic block correction (leucine enkephalin). Data were processed using UNIFI software (Waters). The lists of identified peptides were filtered selecting the peptides as follows: % of corresponding ions, more than 20 %; first generation fragment ions, at least 2. Hence, the in-source fragments were dropped from the final peptide lists.

3. Results and discussion

3.1. Identification of TPM variants in BSF

Current knowledge regarding insect TPM is primarily based on *Drosophila melanogaster* TPM variants produced by alternative transcription and alternative splicing of two distinct genes (Karlik and Fyrberg, 1986);'(Goins and Mullins, 2015).

Accordingly, by searching BSF genome deposited in NCBI database, we identified two genes associated to TPM located on chromosome 6, LOC119658701 and LOC119658706. LOC119658701 encompasses nucleotide region 18209508–18303750, it contains 17 exons, and 21 splicing variants are predicted from its transcription (**Table S1** and

Figure S1). LOC119658706 spans about 19.7 Kb (between nucleotide 18,160,894 and nucleotide 18180590) and contains 6 exons leading to two distinct predicted TPM variants (Table S1 and Figure S1).

The 23 TPM isoforms predicted by bioinformatics analysis show a variable length, from 252 to 925 amino acids. Conversely, most tropomyosin isoforms recognized as allergens in various species of mites, crustaceans and molluscs are composed of 284 amino acids even if different apparent molecular weights have been observed on SDS-PAGE (Table S2). Based on this observation, we excluded all the TPM sequences of *H. illucens* with a molecular weight higher than 45 kDa from further analysis.

The protein sequences XP_037922235, XP_037922236, XP_037922237, XP_037922238, XP_037922240 XP_037922243 and XP_037922244, with molecular mass below 45 KDa, were aligned and compared with TPM from mites, crustaceans and molluscs classified as allergens (Figure S2, Table S3). The sequences XP_037922243 and XP_037922244, encoded by locus LOC119658706, share 95 % of sequence identity, while the proteins XP_037922235, XP_037922236, XP_037922237, XP_037922238 and XP_037922240 encoded by locus LOC119658706, share sequence identity ranging from 52 % to 92 %. The comparison with allergenic TPM from mites and crustaceans highlights high sequence identity (61 %-80 %), whereas lower sequence identities are observed with mollusc TPMs (50 %-60 %). An exception is the protein XP_037922240, which shows sequence identity in the range 49 %-52 % with mites and crustaceans and in the range 40 %-45 % with molluscs, (Figure S2, Table S3) and was then excluded from further analysis.

We then proceeded by investigating the conservation of the amino acid regions recognized as epitopes in the allergens Pen a 1 (R. Ayuso et al., 2012; Ayuso et al., 2002b) and Der p 10 (Ayuso et al., 2002a), the TPM of the edible shrimp *Penaeus aztecus* and of the mite *Dermatophagoides pteronyssinus,* respectively. In Fig. 1 is reported the multiple alignment comparing the sequences XP_037922235, XP_037922236, XP_037922237, XP_037922238, XP_037922243 and XP_037922244 from BSF with Pen a 1 and Der p 10. Considering the six epitopes individually, the sequence identity varies from 20 to 100 % for proteins encoded by LOC119658701 and from 46 to 100 % for proteins encoded by LOC119658706 (Fig. 1 **and** Table S4). Considering all the six epitopes the sequence XP_037922235 shows the highest identity among proteins encoded by LOC119658701, while the two sequences encoded by LOC119658706, XP_037922243 and XP_037922244, differ only for one amino acid replacement in a single epitope (Table S4).

To evaluate the expression of TPM, we also analyzed data reported in the literature regarding TPM peptides identified in total protein extracts of BSF larvae (Bessa et al., 2021; Leni et al., 2020). In Table S5 these peptides are associated with the TPM variants predicted in BSF. In particular, the sequence XP_037922235 and XP_037922237 contain all the peptides associated with the gene LOC119658701 and the sequence XP_037922243 contains all the peptides associated with the gene LOC119658706. However, to have more insights about the expression of the predicted TPM, we also performed a search in the Sequence Read Archive containing the results of RNAseq experiments conducted on midgut of BSF larvae adapted to different substrates (Bonelli et al., 2020). By using the mRNA associated to XP_037922235 and XP_037922243 as queries, the retrieved sequences covered the entire coding sequences, whereas the additional sequences at 5′ or 3′ end of

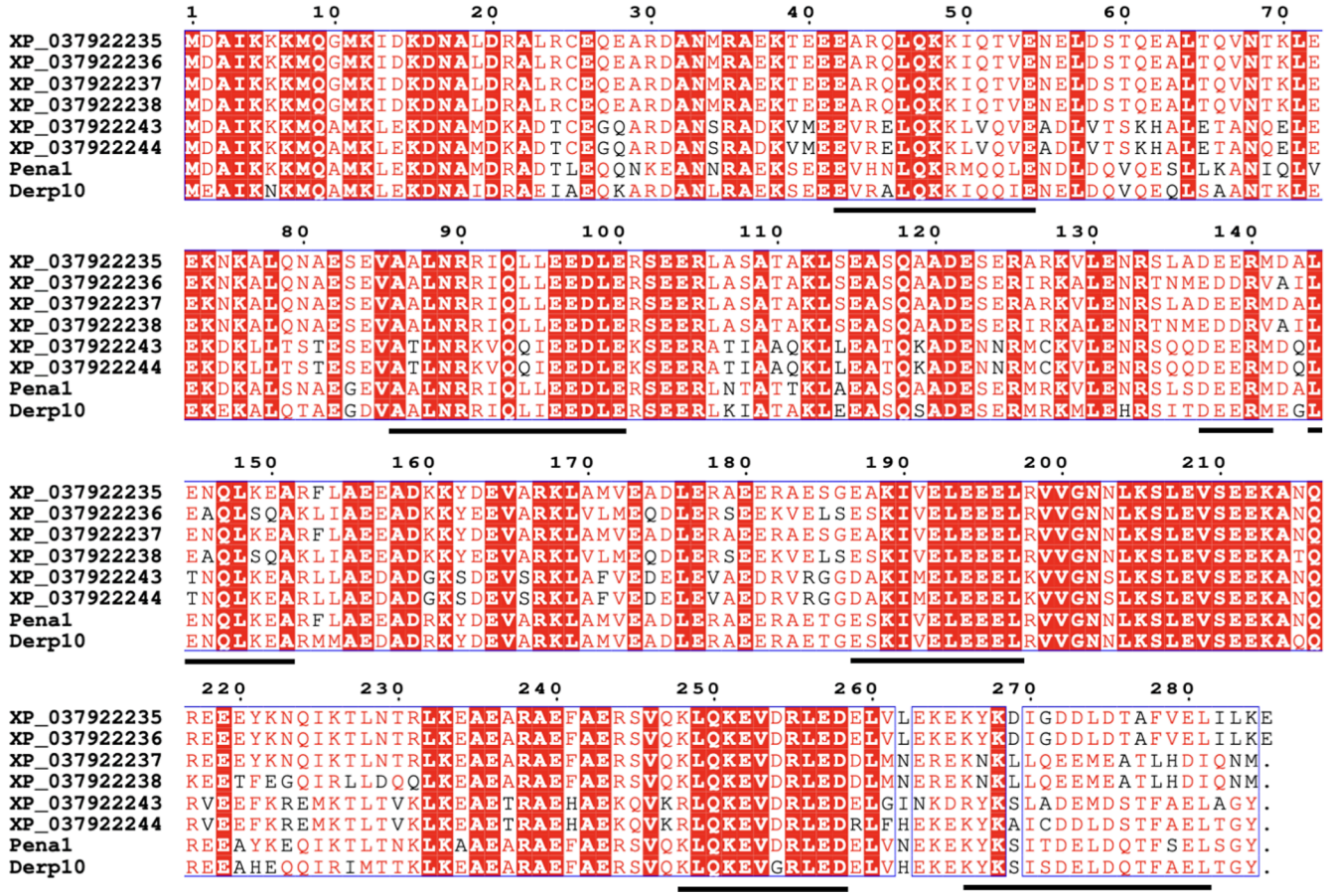


Fig. 1. Multiple sequence alignment of H. illucens TPM variants (XP_037922235, XP_037922236, XP_037922237, XP_037922238, XP_037922243 and XP_037922244) compared with the allergenic TPM Pen a 1 from the shrimp Penaeus aztecus and Der p 10 from the house dust mite Dermatophagoides pteronyssinus. Amino acids conserved in all sequences are reported with white letters, amino acids conserved in at least 50% of sequences are reported with red letters. The amino acid regions identified as epitopes in Pen a 1 and Der p 10 (Ayuso et al., 2002a) are indicated with black lines.

longer TPM variants were not present in the RNAseq database.

Based on all these observations, we finally selected the sequence XP_037922235 (TPM2-X16) located on LOC119658701 gene and the sequence XP_037922243 (TPM1-X1) located on LOC119658706 gene to evaluate the allergenicity for patients sensitized to mites or crustaceans.

3.2. Production of tropomyosin-2 isoform X16 and of tropomyosin-1 isoform X1 from BSF as recombinant proteins

TPM2-X16 and TPM1-X1 were produced as recombinant proteins in *E. coli* BL21 cells by using the pET expression system. The expression level was very high for both the TPM variants when *E. coli* cells were freshly transformed with the recombinant expression vector (Fig. 2**a** and **b**), but the synthesis of the exogenous proteins decreased very quickly during the time and completely disappeared after two weeks. Surprisingly, also the transformed *E. coli* cells stored at −80 °C in glycerol 50 % lost the ability to produce the proteins in the same time. Plasmids extracted from *E. coli* cells unable to produce recombinant TPM were sequenced but no mutations were identified both in the TPM coding sequence and in the promoter region. These observations suggest that TPM2-X16 and TPM1-X1 are toxic for bacterial cells and the loss of their expression could be attributed to an impaired production of functional T7 RNA polymerase (Vethanayagam and Flower, 2005).

TPM were purified from the supernatant fraction after cell lysis by using affinity chromatography exploiting a histidine tag fused to the protein *N*-terminus with final yields of approximately 3 mg/l culture for TPM2-X16 and 1 mg/l culture for TPM1-X1. The analysis by SDS-PAGE showed a slower electrophoretic mobility for TPM1-X1 and apparent MW between 37 and 50 KDa for both proteins (Fig. 2**a and b**). These apparent MW are higher than those calculated based on the amino acid sequences (35017.94 Da for TPM2-X16 and 34683.77 for TPM1-X1) probably due to the typical elongated structure of TPM proteins.

Both variants of TPM were subjected to degradation during the time when stored at room temperature or at 4 °C, whereas TPM1-X1 degraded to form discrete bands corresponding to lower molecular weights even at –20 °C. For this reason, freshly purified TPM were used for experiments.

3.3. Oligomeric state of tropomyosin-2 isoform X16 and of tropomyosin-1 isoform X1

The structural models of TPM2-X16 and TPM1-X1, reported in Fig. 3**a** and **b** respectively, show coiled-coil α-helices structures for both proteins. To investigate the oligomeric form in solution of recombinant TPM1-X1 and TPM2-X16, both proteins were analyzed by an analytical

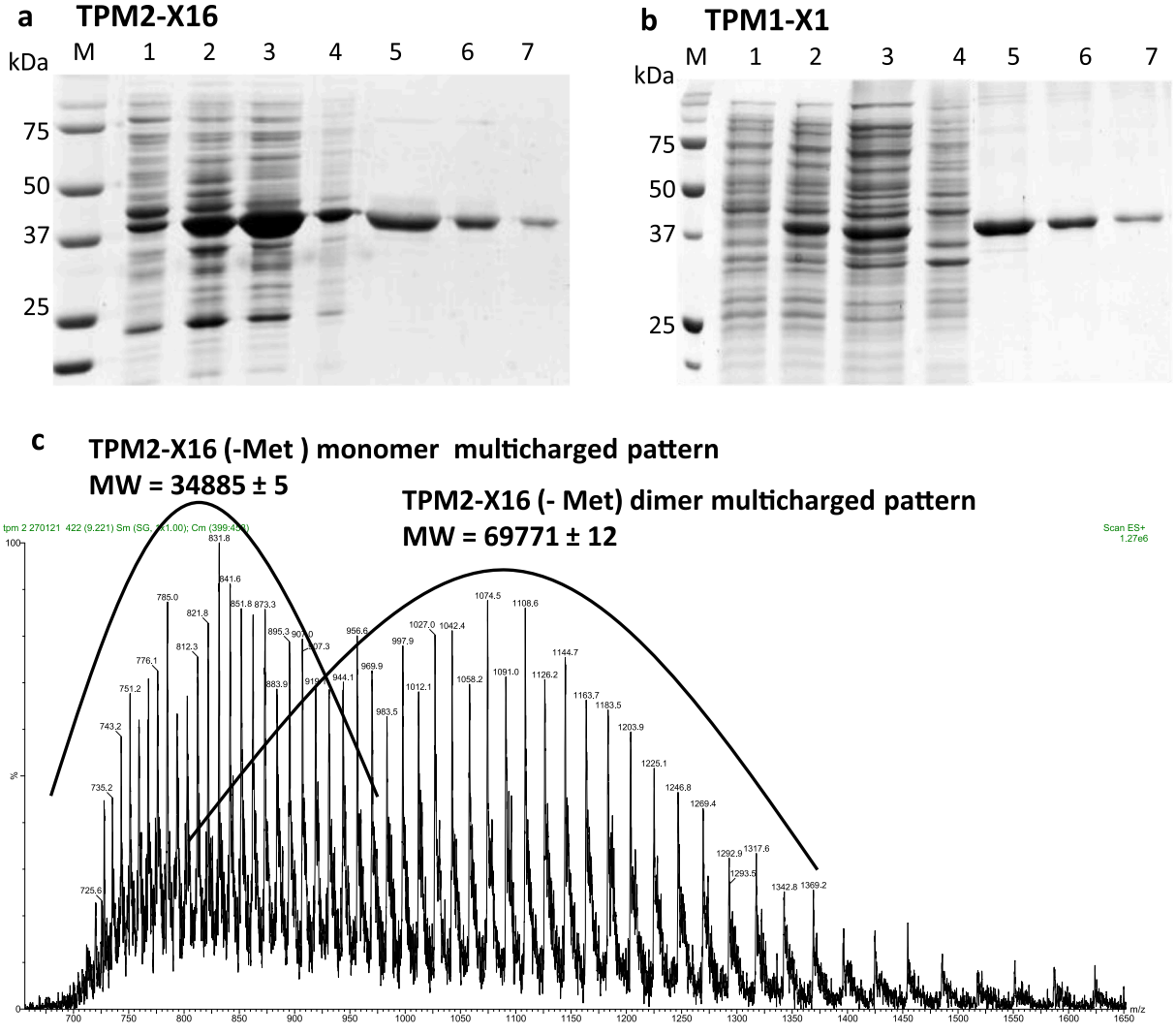


Fig. 2. Expression and purification of recombinant TPM2-X16 (a) and TPM1-X1 (b). M, protein molecular weight standards; 1, not-induced *E. coli* cell culture; 2, IPTG-induced *E. coli* cell culture; 3, soluble fraction after cell lysis; 4, not- soluble fraction after cell lysis; 5–7, purified protein eluted from the His-Trap column by applying the elution buffer. (c): ESI MS spectrum of TPM2-X16 variant. Multicharged pattern and calculated MW obtained by spectra deconvolution (experimental conditions are described in paragraph 2.5 of Materials and Methods).

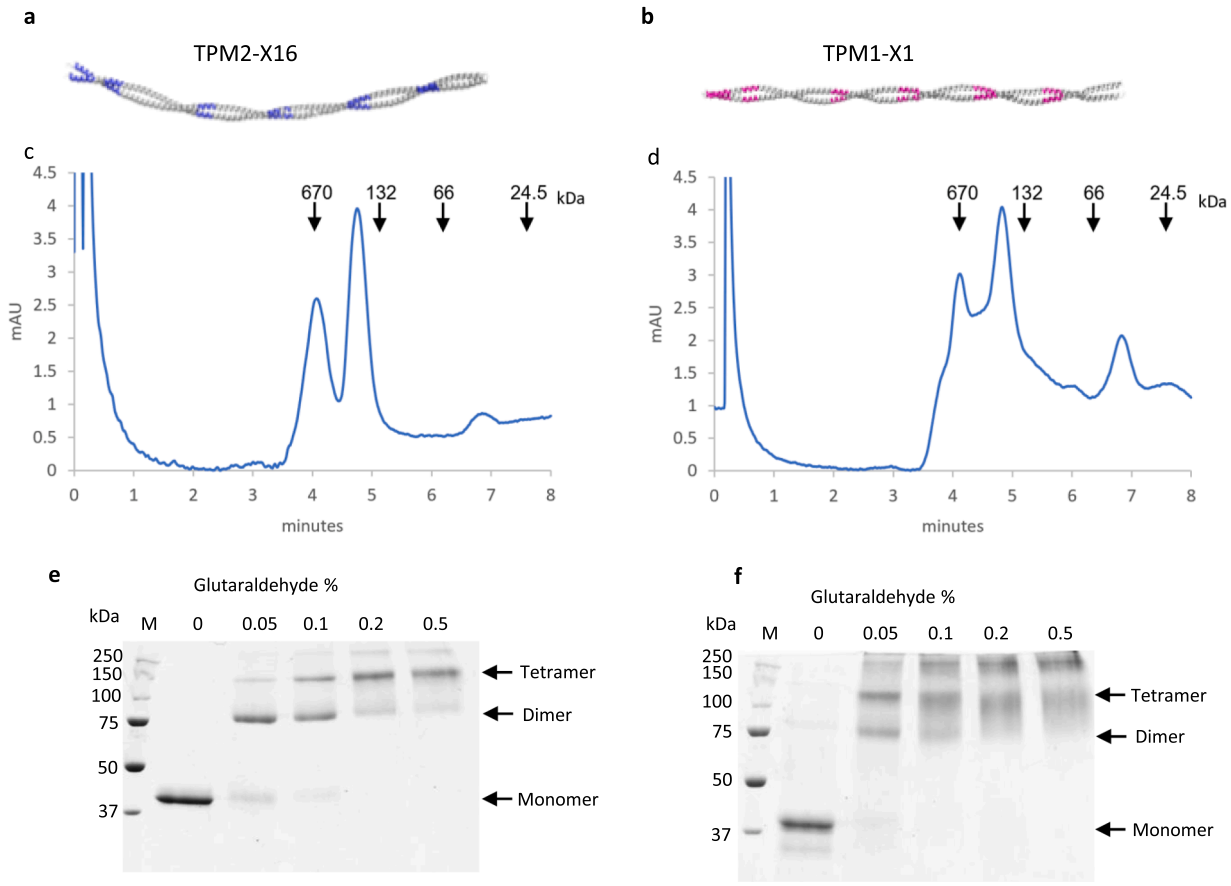


Fig. 3. Oligomeric state of TPM2-X16 and of TPM1-X1. 3D modelling of the coiled-coil α-helices structures of TPM2-X16 (a) and TPM1-X1 (b), the regions corresponding to Pen a 1 epitopes are highlighted by colors. Size-exclusion chromatography (Superdex 200 Increase 5/150 GL column) elution profile of TPM2-X16 (c) and TPM1-X1 (d); column calibration was performed by using Thyroglobulin (670 kDa), albumin dimer and monomer (132 kDa, 66 kDa), and trypsinogen (24 kDa). SDS-PAGE analysis of cross-linked TPM2-X16 (e) and TPM1-X1 (f) using increasing percentages of glutaraldehyde; M, protein molecular weight standards.

size-exclusion chromatography. The elution profiles of TPM2-X16 and of TPM1-X1 were similar and showed a main peak approximately corresponding to the tetrameric form of TPM (Fig. 3**c** and **d**). To further verify the oligomeric state of both TPM variants cross-linking reactions with glutaraldehyde were carried out. In both cases, the results showed the presence of two main bands corresponding to dimer and tetramer of TPM, this latter being prevalent in the presence of high concentration of glutaraldehyde (Fig. 3**c** and **d**). This behavior is particularly evident for TPM1-X1 that shows the total absence of dimeric form and the appearance of polymeric forms with higher molecular mass other than tetramers. These results suggest that the tetramer is the main form present in solution for TPM2-X16 and TPM1-X1 in accordance with data reported in the literature for tropomyosin from rabbit skeletal striated muscle (Stafford et al., 2012);(Lassing et al., 2010).

3.4. Mass spectrometry analysis

The two TPM isoforms were characterized by LC-MS. Chromatographic separation conditions allowed to identify TPM2-X16 both in the monomeric and in the dimeric forms (Fig. 2**c**). The MW calculated from the multicharged pattern, for the monomeric TPM2 X16 was 34885 ± 5, corresponding to the protein without the *N*-terminal methionine, the MW calculated for its dimeric form was 69771 ± 12. Instead, various attempts were carried out to analyze TPM1-X1 without any success. These results are in agreement with the cross-linking experiments (Fig. 3), which show that TPM1-X1 variant is more prone than TPM2-X16 to form tetramers and more complex polymeric structures with a very low ionization capacity to be analyzed by ESI mass-spectrometry.

3.5. Immunoreactivity of TPM2-X16 and TPM1-X1

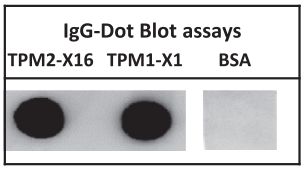
The IgG immunoreactivity of TPM2-X16 and TPM1-X1 was verified by using commercial polyclonal IgG produced against the amino acid region 92–273 of human TPM1 (UniProt P09493). The results of Dot-blot experiments performed by using recombinant TPM variants from BSF show that both proteins are recognized by the IgG antibodies suggesting the presence of IgG epitopes similar to those present in the region encompassing amino acid 92–273 of human TPM1 (Fig. 4**a**).

The allergenicity of TPM2-X16 and TPM1-X1 was then investigated by using Dot-blot experiments and four sera from patients with evidence of food allergy to shrimp and/or of airway allergy to mites verified by in-vivo skin tests (Fig. 4**c**). The results show that both TPM are recognized by all the analyzed sera even if with different IgE-binding capacity (Fig. 4**b**). The specificity of the IgE-recognition was verified by testing sera of allergic patients against bovine seroalbumin (BSA) and by incubating TPM2-X16 and TPM1-X1 with sera of patients with negative skin prick tests against shrimp and mites (Fig. 4**b**). IgE immunoreactivity data strongly indicate a cross-reactivity between TPM of shrimp and/or mites and TPM variants of BSF.

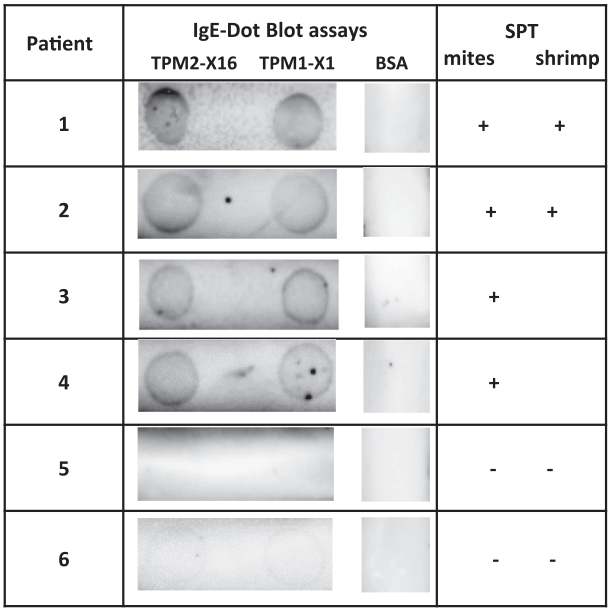
3.6. Stability to temperature and to pH of TPM2-X16 and TPM1-X1

The stability to increasing temperature of TPM2-X16 and TPM1-X1 from BSF was evaluated by CD spectroscopy and SDS-PAGE (Fig. 5). Far-UV spectra collected at 20 °C exhibit a maximum at around 193 nm and two minimums at 222 and 208 nm, which are typical for α-helical secondary structure in agreement with the known TPM structures

a



b



c

Patient	1	2	3	4	5	6
sex	M	M	M	F	M	F
age	23	59	31	55	61	31
total IgE (IU/ml)	329	99	1220	130	24	78
SPT *D. farinae*	+	+	+	+	-	-
SPT *D. pteronyssinus*	+	+	+	+	-	-
SPT shrimp	+	+			-	-

Fig. 4. Immunoreactivity of TPM2-X16 and TPM1-X1 from *H. illucens*. Dot-blot IgG-immunoassay of TPM2-X16 and TPM1-X1 (a) carried out by using commercial IgG anti human TPM. Dot-blot IgE-immunoassays carried out with sera of patients with a diagnosis of allergy to shrimp and/or to mites or with sera of non-allergic individuals (b). Characterization of patients is reported in (c). SPT, skin prick test; M, male; F, female.

(Hitchcock-DeGregori and Barua, 2017). In the case of TPM2-X16, the characteristic CD spectrum remained almost unchanged up to 35 °C; at 40 °C the protein began the unfolding process and at 55 °C the secondary structure was almost completely lost. Interestingly, when the TPM2-X16 incubated at 55 °C was subsequently cooled to 20 °C the α-helices content was restored for more than 90 % showing that the unfolding process of TPM2-X16 may be reversible (Fig. 5**c**). TPM1-X1 was more sensitive to temperature showing a significative alteration of the CD spectrum already at 30 °C. At 55 °C the native structure is completely abolished but, as observed for TPM2-X16, α-helical secondary structure is largely restored when the protein was cooled to 20 °C (Fig. 5**g**). These results agree with data reported in the literature for shrimp TPM showing the refolding to native structures of hea- denatured protein after chilling (Usui et al., 2013).

To evaluate the propensity to degradation at high temperatures, both TPM were also analyzed on SDS-PAGE after incubation at different temperatures from 25 to 80 °C for 45 min. TPM2-X16 was stable at all the tested temperatures, while the band corresponding to TPM1-X1 partially disappeared at 80 °C suggesting that part of the protein could be degraded (Fig. 5**a** and **e**).

Similar experiments were carried out to investigate the stability of both proteins at basic and acidic pH. In Fig. 5**d** and **h**, it is possible to note that the spectra collected at pH 3 and 9 were very little modified compared to the typical α–helical spectra recorded at pH7, however in the case of TPM1-X1 the spectra collected at pH 3 showed a red-shift of the maximum at 196 nm (Fig. 5**h**). The propensity to degradation of both TPM was investigated on SDS-PAGE after incubation for 1 h at different pH from 3 to 9 but no significant modifications of TPM bands were observed (Fig. 5**b and f**).

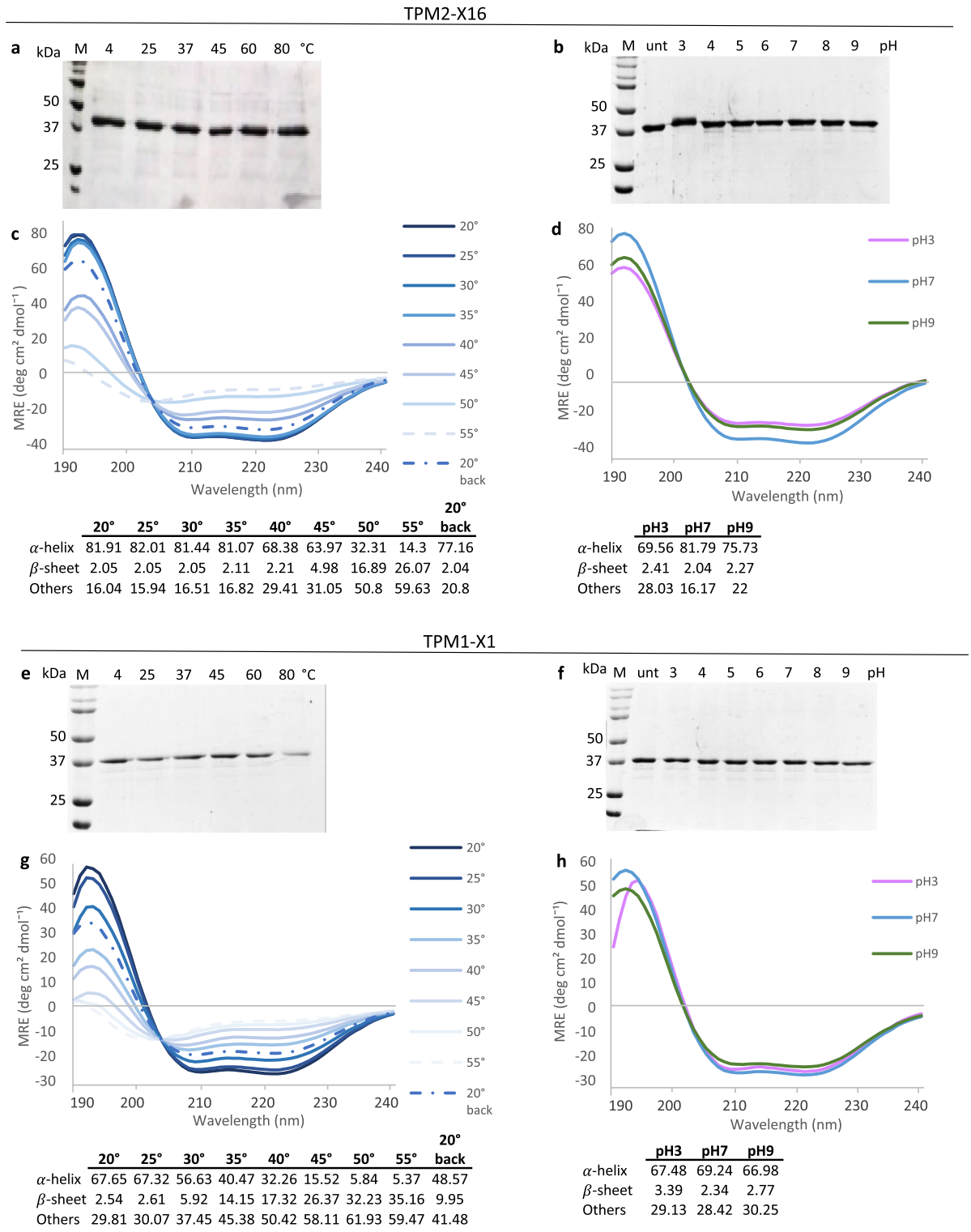
3.7. Peptide composition after simulated gastrointestinal digestion

Simulated gastrointestinal digestion was conducted as described previously (Prandi et al., 2012). Briefly, the method involves the simulation of stomach conditions by using hydrochloric acid at pH 2 and pepsin; then the pH is brought to that of the intestinal digestion phase (between 7 and 8) and the pancreatic endoproteases trypsin and chymotrypsin are added. At the end of the digestion, the possible presence of undigested proteins was checked by SDS-PAGE. As shown in Fig. 6**a** and **c**, at the end of the digestion there is no longer any intact protein, which means that TPM2-X16 and TPM1-X1 are completely cleaved into shorter peptides during digestion process. This can obviously influence the allergenicity of the protein, as some linear and conformational epitopes can be destroyed during digestion, or eventually some epitopes could also have been unmasked. To evaluate this aspect, it is necessary to identify the peptides present after digestion and to verify, within their sequences, the presence of residual epitopes. The peptides were thus identified by liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS). The resulting chromatograms for TPM2-X16 and TPM1-X1 are shown in Fig. 6**b** and **d**. Consistent with what observed on SDS-PAGE, TPM2-X16 and TPM1-X1 variants were completely digested, generating many peptides detectable with LC-HRMS (black traces). No autoproteolytic cleavages were observed (red traces), which means that the proteins are stable at the temperature and pH conditions of digestion and the formation of peptides is solely due to the action of digestive proteases. Respectively, 41 and 39 peptides were identified by mass spectrometry in replicates 1 and 2 of *in vitro* digested TPM2-X16 (**Table S6**), with a protein coverage of 52 %. Similar experiments performed on TPM1-X1 identified 41 and 43 peptides in the two digestion replicates (**Table S7**), with a protein coverage of 58 % for digestion replicate 1 and 61 % for digestion replicate 2.

3.8. Mining of linear epitopes

The IEDB (last database access May 2023)(Vita et al., 2019) is a gold benchmark database, funded by the National Institute of Allergy and Infectious Disease and promoted by the National Institute of Health (NIH), reporting the information for allergenic epitopes discovered and made available so far. According to curators' declaration, IEDB catalogues experimental data on antibody and *T*-cell epitopes in humans, primates, and other animal species in the context of infectious disease, allergy, autoimmunity, and transplantation. It collects more than 1.5 million of epitopes supported by experimental data and more than 4.000 epitope source organisms. For the sake of this project, all the linear experimentally verified epitopes (as per IEDB declaration) from crustaceans, insects, arachnids, and molluscs made available at the time of analysis (2974 epitopes) were mined out IEDB and systematically searched on the native sequence of TMP2-X16 and TPMP1-X1 to assess their allergenic potential thoroughly. In addition, those epitopes were also searched on the peptide mixtures released by the simulated gastrointestinal digestion to investigate whether digestion may impact the epitopes profile.

As shown in **Table S8**, both TPM sequences include several already characterised epitopes highlighting further their high allergenic potential. Specifically, 56 epitopes were identified on TPM2-X16 and 13 epitopes were identified on TPM1-X1. This difference agrees with the sequence identity found between the TPM isoforms from BSF and the



	20°	25°	30°	35°	40°	45°	50°	55°	20° back
α-helix	81.91	82.01	81.44	81.07	68.38	63.97	32.31	14.3	77.16
β-sheet	2.05	2.05	2.05	2.11	2.21	4.98	16.89	26.07	2.04
Others	16.04	15.94	16.51	16.82	29.41	31.05	50.8	59.63	20.8

	pH3	pH7	pH9
α-helix	69.56	81.79	75.73
β-sheet	2.41	2.04	2.27
Others	28.03	16.17	22

	20°	25°	30°	35°	40°	45°	50°	55°	20° back
α-helix	67.65	67.32	56.63	40.47	32.26	15.52	5.84	5.37	48.57
β-sheet	2.54	2.61	5.92	14.15	17.32	26.37	32.23	35.16	9.95
Others	29.81	30.07	37.45	45.38	50.42	58.11	61.93	59.47	41.48

	pH3	pH7	pH9
α-helix	67.48	69.24	66.98
β-sheet	3.39	2.34	2.77
Others	29.13	28.42	30.25

Fig. 5. Stability of TPM2-X16 and TPM1-X1 to increasing temperatures and to acidic and basic conditions. SDS-PAGE of TPM2-X16 (a) and TPM1-X1 (e) and far-UV CD spectra of TPM2-X16 (c) and TPM1-X1 (g) after incubation at increasing temperatures, spectra obtained for the samples cooled at 20 °C after incubation at 55 °C (20 back) are also reported. SDS-PAGE of TPM2-X16 (b) and TPM1-X1 (f) and CD spectra of TPM2-X16 (d) and TPM1-X1 (h) after incubation at different pH.

allergenic TPM from mites and crustaceans analyzed in Table S3. However, it is worth noting that the digestion process may reduce qualitatively the number of epitopes as many were not detected in TPMs hydrolysates (Table S8; Figure S3). In particular, the number of epitopes after the simulated gastrointestinal digestion reduced from 13 to 3 and from 56 to 5 for TPM1-X1 and TPM2-X16 respectively.

These results.

4. Conclusion

Since 2018 insects have been included in the category of Novel Foods highlighting the importance to investigate the presence of allergens

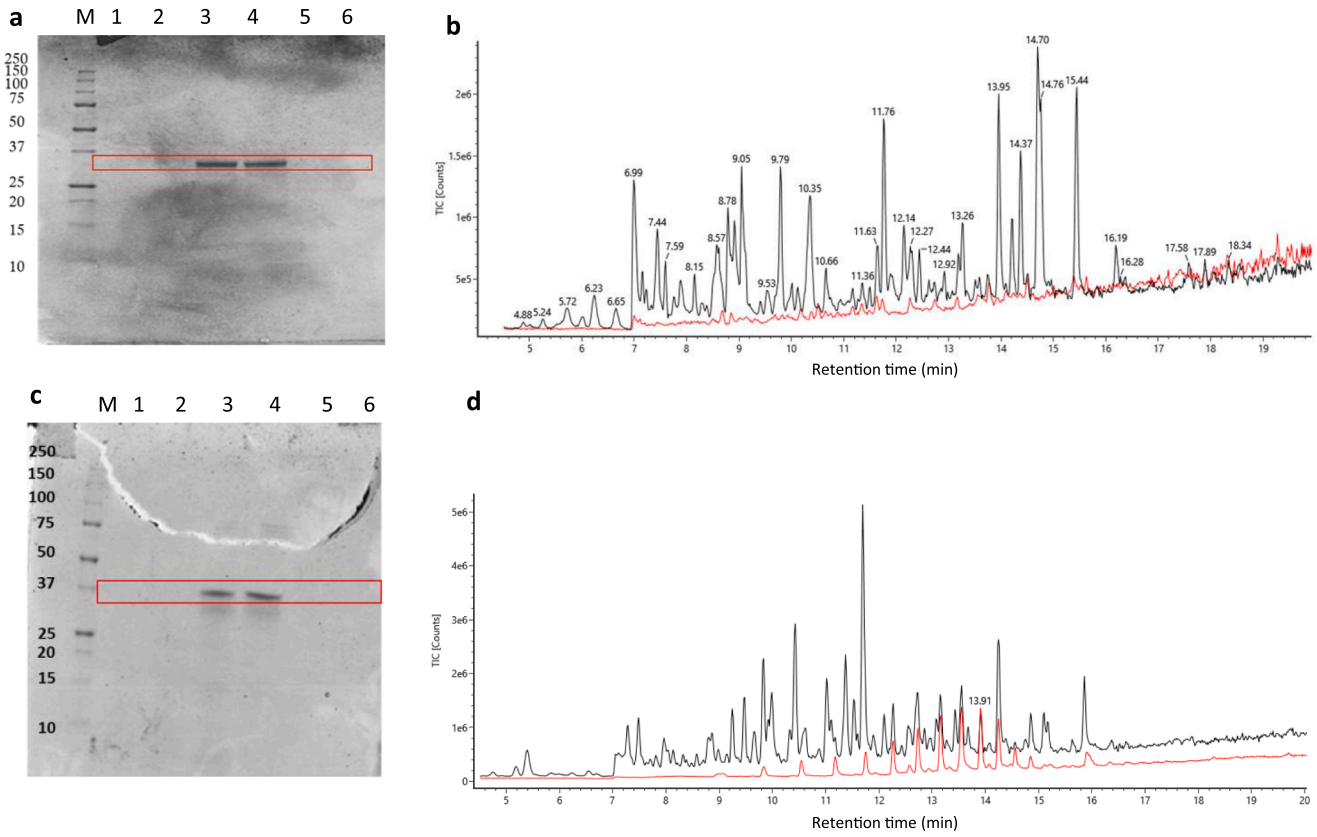


Fig. 6. Simulated gastrointestinal digestion oof TPM from BSF. SDS-PAGE of TPM2-X16 (a) and TPM1-X1 (c) subjected to digestion. M, protein standards. 1 and 2 control samples containing saline solutions and enzymes; 3,4 undigested control samples containing TPM2-X16 (a) or TPM1-X1 (c) and saline solutions; 5,6 digested samples containing TPM2-X16 (a) or TPM1-X1 (c), saline solutions and enzymes. LC-HRMS chromatogram of digested (black) and undigested (red) TPM2-X16 (b) or TPM1-X1 (d).

capable of primary sensitization or cross-reactivity with arthropod pan-allergens. TPM is one of the most important allergens in crustacea and mites and the possibility of cross-reactions with BSF homologous proteins has been proposed (De Marchi et al., 2021).

In the present work two TPM variants of the edible insect BSF have been characterized for their structure, stability and allergenicity. TPM variants were identified into the BSF genome by bioinformatics analysis and produced in *E. coli* as recombinant proteins. The main structure identified for both proteins in solution was a tetramer of α-helices as already shown for rabbit TPM (Lassing et al., 2010).

Cross-reactivity with arthropod pan-allergens has been investigated by IgE-immunoblotting experiments by using sera from patients with food allergy to shrimp and/or with inhalant allergy to mites. As a result, both TPM from BSF were recognized by sera of allergic patients, confirming the cross-reactivity between shrimp, mites and BSF.

To evaluate the stability to increasing temperature, the propensity of TPM variants to denaturation and to degradation was analyzed. TPM2-X16 appeared as a single band on SDS-PAGE after incubation at increasing temperatures up to 80 °C, while TPM1-X1 was only partially degraded at 80 °C. However, the secondary structure of TPM variants was affected by quite low temperatures, leading to significant modifications of CD-spectra as early as 40 °C for TPM2-X16 and 35 °C for TPM1-X1; both variants refolded to alpha-helical structures when the temperature was decreased to 20 °C. Conversely, the influence of acidic or basic pH on the α-helical CD-spectra was very limited.

In the context of food allergy, sequential IgE binding epitopes are particularly relevant as conformational epitopes are easily degraded in the gastrointestinal tract (Pekar et al., 2018). A method reported in the literature for the prediction of TPM cross-reactivity in invertebrate species based on the conservation of linear epitopes have identified 23 sequences defined pan-epitopes because shared by crustacean, cockroach, mites and molluscs (Nugraha et al., 2019); 12 of these epitopes are conserved in BSF TPM2-X16 and 3 are shared with TPM1-X1, supporting possible cross-reactivity with crustacean, cockroach, mites and molluscs. In this work, by screening the 2974 linear epitopes from crustaceans, insects, arachnids, and molluscs deposited in the IEDB, 56 sequences were identified in TPM2-X16 and 13 in TPM1-X1. In addition, by using simulated gastrointestinal digestion coupled with mass spectrometry analysis we identified the peptides generated after TPM2-X16 and TPM1-X1 digestion. The bulk mining of epitopes from crustaceans, insects, arachnidan and molluscs (as per IEDB; https://www.iedb.org) after simulated gastrointestinal digestion reduced from 13 to 3 and from 56 to 5 the epitopes found in digested TPM1-X1 and TPM2-X16, respectively (Table S8, Figure S1). This suggests the need of further investigations to assess the role of hydrolysis, either by chemical, enzymatic or microbiological means, to mitigate the allergenic potential of *H. illucens* TPMs and other insect proteins.

These results highlight that the allergenicity assessment of Novel Foods is crucial to correctly inform the potentially allergic consumer. The availability of recombinant TPM from BSF will also allow the inclusion of these allergens in molecular screening aimed to specifically identify reacting allergenic molecules in view of the use of this insect and its derivatives in the human diet.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137849.

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