



SWATH-MS-based proteomics reveals functional biomarkers of Th1/Th2 responses of tropomyosin allergy in mouse models

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ARTICLE INFO

Keywords:

Shellfish allergy
Tropomyosin
Intestine
Spleen
Proteomics
Biomarker

ABSTRACT

Type-I food allergies are hypersensitive reactions compromising the immune organs and epithelial barriers. To investigate the organ-specific proteomic alterations of the allergy responses, the spleen and intestine of mice sensitized with high (shrimp and clam) and weak (fish) allergenic tropomyosins were analyzed using sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH-MS)-based proteomics. The results showed that Th1 and Th2 tropomyosin-induced responses in the spleen are characterized by the unique upregulation of innate (cochlin) and adaptive (Ig κ chain V-III region PC 7175) immune regulators, respectively. In the intestine, tropomyosin allergy concurred with the downregulation of 35 differentiating proteins featuring the overall impairment of metabolic pathways, absorption processes and ammonium ion responses. These data provide new functional biomarkers of tropomyosin-induced immune responses as well as candidate targets for intervention.

1. Introduction

Type-I food allergies are pathological immune reactions triggered by otherwise innocuous food proteins (Ruethers et al., 2018; Sicherer & Sampson, 2018). Clinically, type I food allergies involve a spectrum of symptoms ranging from mild irritations to life-threatening anaphylaxis that are outgrown or persist throughout the life of the affected individuals (Matricardi, Kleine-Tebbe, Hoffmann, Valenta, & Hilger, 2016; Sicherer & Sampson, 2018). In the absence of a general current treatment beyond strict food avoidance, the discovery of novel allergic response biomarkers is essential for establishing yet unknown pathogenic pathways and targets of intervention.

Of the different food allergens, the tropomyosin family of proteins includes a large number of cross-reactive allergens, most of which are from invertebrate sources (Matricardi et al., 2016). Tropomyosin (TM), with a molecular mass of 35–38 kDa and a coiled-coil dimer, is considered the major allergen in shellfish and a very weak allergen in some fish species (Ruethers et al., 2018; Xu, Chen, et al., 2020; Xu, Sun, Lin, Ishfaq, & Li, 2020). Studies of shellfish allergies have allowed the generation of *Litopenaeus vannamei* TM (Lit v 1; shrimp-TM)-sensitized

mouse models (Ruethers et al., 2018; Xu et al., 2020). Shrimp-TM-sensitized BALB/c mice produce adequate levels of anti-TM IgE and Th2 cytokines and display histological alterations in both the duodenum and spleen (Xu et al., 2020). Studies of these mice have also shown that *Ruditapes philippinarum* TM (Ven ph 1; clam-TM,) provokes an elicitation similar to shrimp-TM, whereas *Larimichthys crocea* TM (fish-TM) displays a very weak stimulation, if any (Xu et al., 2020). Differences in allergy elicitation suggested that mice sensitized with fish-TM, shrimp-TM and clam-TM may display distinct immunological responses and marked proteome alterations, which may unveil novel aspects of the allergic responses to TM-containing foods.

Mass spectrometry proteomic-based methodologies have emerged as key tools for the study of protein biological networks (Altealar, Munoz, & Heck, 2013; Ludwig et al., 2018; Fung, Sugumar, Ren, & Kulasingam, 2020). Of these methods, sequential window acquisition of all theoretical fragment ion mass spectra (SWATH-MS) permits the simultaneous parallel quantitation of thousands of proteins and comprehensive overview of protein profiles with quantitative consistency and accuracy, summing the strengths of both shotgun and targeted proteomics (Altealar et al., 2013; Fung et al., 2020; Jiang et al., 2021; Ludwig et al., 2018;

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<https://doi.org/10.1016/j.foodchem.2022.132474>

Received 21 October 2021; Received in revised form 22 January 2022; Accepted 13 February 2022

Available online 16 February 2022

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Simburger, Dettmer, Oefner, & Reinders, 2016). SWATH-MS is a label-free method that uses data-independent acquisition (DIA) in combination with the spectral libraries created from data-dependent acquisition (DDA) methods used to align the fragment ion maps of the DIA method (Fung et al., 2020). Its major drawbacks are its up to 10-fold lower quantitative sensitivity compared to classical targeted approaches and the requirement of an accurate spectral library (Ludwig et al., 2018; Fung et al., 2020; Jiang et al., 2021). Despite these limitations, SWATH-MS is suited for experiments requiring accurate and reproducible quantification for the major fraction of the sample peptidome, as in the case of biomarker studies (Ortea, Rodriguez-Ariza, Chicano-Galvez, Arenas Vacas, & Jurado Gamez, 2016).

In this study, the proteome differences in the spleen and intestine of mice sensitized to high (shrimp and clam) and weak (fish) allergenic TMs were analyzed using SWATH-MS-based proteomics.

2. Materials and methods

2.1. Materials

Dithiothreitol (DTT) and iodoacetamide (IA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was purchased from Promega (Promega, Madison, WI, USA). LC–MS grade acetonitrile was purchased from Thermo Fisher Scientific (Waltham, MA, USA), formic acid was purchased from Fluka (Buchs, Switzerland), and ultra-pure water was obtained from an in-house Milli-Q system (EMD Millipore, Billerica, MA, USA). Ammonium bicarbonate and urea were supplied by Sinopharm Chemical Reagent Company (Shanghai, China). Ultrafiltration tubes (10 kDa) were obtained from Merck (Merck, Whitehouse Station, NJ, USA). RPMI-1640 medium, fetal bovine serum and penicillin–streptomycin were obtained from Gibco Life Technologies (Grand Island, NY, USA). All other reagents used were of analytical grade.

2.2. Ethics statement

All animal experiments were performed in strict accordance with the established guidelines and regulations of the Ethical Committee of Experimental Animal Welfare of the Ocean University of China (permit SPXY20200620001).

2.3. Sensitizing TMs

Fish (*Larimichthys crocea*), shrimp (*Litopenaeus vannamei*) and clam (*Ruditapes philippinarum*) TMs were isolated and purified as previously described (Xu, Lin et al., 2020). Sequences UniProtKB P58771 (mouse-TM), GenBank XP_019127831.1 (fish-TM), UniProtKB B4YAH6 (shrimp-TM) and B7XC71 (clam-TM) were retrieved from the UniProtKB and National Center for Biotechnology Information (NCBI) databases and analyzed using BioEdit tools (Supplementary Material, Figure S1).

2.4. Mouse model of TM allergy

Six-week-old BALB/c female mice were obtained from Jinan Laboratory at the Animal Center (Jinan, China), kept in a pathogen-free environment and fed fish-, clam- and shrimp-free food (LAD3001 M, Trophic Animal Feed High-tech Co., Ltd., Jiangsu, China). After one week, the mice were divided into four groups of 6 mice each (fish-TM allergy, clam-TM allergy, shrimp-TM allergy and PBS as the control) for their sensitization and challenge following the protocol described for the shrimp-TM allergy model (Supplementary Material, Figure S2) (Xu et al., 2020). Briefly, sensitization was initiated by intraperitoneal (i.p.) injection with 50 µg of fish-TM, shrimp-TM, and clam-TM adsorbed in 2 mg/mL alum adjuvant (Pierce, Rockford, IL, USA) on Days 1, 7 and 14, followed by i.p. injection of 100 µg TM (clam, fish, shrimp) in PBS or PBS alone on Days 22, 25, 31, 34, 37, 40, and 43. On Day 50, mice were

challenged with 1 mg TM (clam, fish, shrimp) in PBS, and after 30 min, their body weight and clinical symptoms were evaluated. The clinical symptoms were scored using the following scale: 0, absence of symptoms; 1, scratching and rubbing around nose and mouth; 2, puffiness and scratching around eyes or nose and mouth; 3, wheezing, labored respiration, or cyanosis around mouth and tail; 4, no activity after prodding; and 5, death (Xu et al., 2020). PBS was injected as a negative control. After overnight fasting, mice were anesthetized and killed by cervical dislocation, and their blood, intestine and spleen were collected under sterile conditions for further analysis. The sera were used to determine the IgG₁ and IgE levels using Bioss sandwich ELISA (Biosynthesis Biotechnology Co., Ltd., Beijing, China). Spleen and intestine samples from 3 mice selected randomly from each group were immediately frozen in liquid nitrogen and stored at −80 °C until mass spectrometry analysis was performed. The other samples were used for histopathology and cytokine production (see below).

2.5. Duodenal and splenic histopathology

Formalin-fixed duodenum and spleen samples were embedded in paraffin. Paraffin sections (5 µm thickness) were stained with hematoxylin/eosin (Sigma–Aldrich, St. Louis, MO, USA) and examined under a microscope (Olympus Corporation, Tokyo, Japan).

2.6. Splenic lymphocyte cultures and cytokine level determinations

Lymphocyte cells from mouse spleens were isolated and cultured as reported (Liu et al., 2017). Stimulation was performed in duplicate using 20 µg/mL of TM (clam, fish, shrimp) or PBS for 3 days. The supernatants were collected, and the levels of IL-4, IL-10, IL-12, IL-13 and IFN-γ were measured using ELISA kits from R&D Systems (Minneapolis, MN, USA).

2.7. Protein extraction and trypsin digestion

Spleen and intestine samples from each mouse group were pooled and placed at 10% w/v in 50 mM NH₄HCO₃ and 8 M urea, shaken for 30 min with vertical oscillation and centrifuged (12,000×g, 20 min). Typically, supernatants (400 µL) were mixed first with 8 µL of 1 M DTT at 37 °C for 1 h and then with 20 µL of 1 M IA for 1 h in darkness. Samples were centrifuged (12000×g, 20 min) using 10 kDa ultrafiltration tubes and diluted with 200 µL of 50 mM NH₄HCO₃. Samples were then digested with modified trypsin digestion (enzyme to substrate ratio, 1:50 w/w) at 37 °C for 16 h and centrifuged for 20 min at 12000×g using 10 kDa ultrafiltration tubes. The peptide filtrates were collected and used for further analysis.

2.8. Ultrahigh-performance liquid chromatography-quadrupole/time of flight (UHPLC-Q/TOF) mass spectrometry, protein identification and spectral library generation

Digested samples were analyzed by UHPLC-Q/TOF using a Nexera X2 30 Å ultrahigh-performance liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with an Agilent Advance Bio Peptide Map C18 column coupled to a Triple TOF™ 5600 mass spectrometer system (SCIEX, Framingham, MA, USA), as previously described (Jiang et al., 2021). Protein identification was performed using ProteinPilot™ software (version 5.0.1 SCIEX) with the *Mus musculus* UniProt protein database (downloaded in October 2020) containing 116,041 protein sequences (17,519 fully reviewed), specifying iodoacetamide as a cysteine alkylation agent and setting the false discovery rate (FDR) to 1%.

2.9. Label-free protein relative quantitation

For SWATH data-independent acquisition, 50 ms full mass scans (350–1,500 *m/z*) with all fragmented precursor ions were followed using

50 isolation windows of product ions (400–1,250 m/z); the variable window was predicted by an Excel calculation tool (version 1.0, SCIEX) according to the precursor m/z frequency of DDA data (Jiang et al., 2021). The SWATH-acquired data were loaded into Peak View software (version 2.1, SCIEX, Framingham, MA, USA) with MS/MS^{ALL} with SWATH acquisition MicroApp (version 2.0, SCIEX, Framingham, MA, USA) together with the generated library. The parameters set for quantitation were as follows: 5 min extraction window, 50 ppm mass tolerance for precursor ions, >95% peptide of confidence, and FDR < 1%, without share or modification. Measurements were performed on samples from three independent mice of each group, each of the samples being analyzed in triplicate.

2.10. Chemometric and statistical analysis

Data were normalized with MarkerView 1.2.1 software (SCIEX, Framingham, MA, USA) using the sample total peak areas. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) chemometric tools were employed for subsequent data mining using SIMCA14.0 software (Umetrics), as

described (Jiang et al., 2021). Other data are presented as the mean $\pm$ standard deviation SD ($n = 3$) using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) and Origin 8. Software (Origin Lab, Hampton, AK, USA). $p < 0.05$ was considered to indicate a significant difference. Differentiating protein sequences were first analyzed with Blastp (<https://blast.ncbi.nlm.nih.gov/>), choosing the *Mus musculus* protein hits with $E < 10^{-15}$ values. Candidate proteins were analyzed for functional enrichment using STRING (<https://string-db.org>).

3. Results

3.1. Phenotypic features of tropomyosin-sensitized mice

To gain insights into the proteomic alterations provoked by a TM allergy in immune relevant organs, the shrimp-TM allergy mouse model was used and extended to include both high and weak allergenic TMs (Xu et al., 2020). Based on their sequence similarities and known allergenicity and cross-reactivity, shrimp-TM and clam-TM were chosen as sensitizers to generate two groups of TM-allergic mice (Supplementary Material, Figure S1). As a model of weak allergenic TM, a

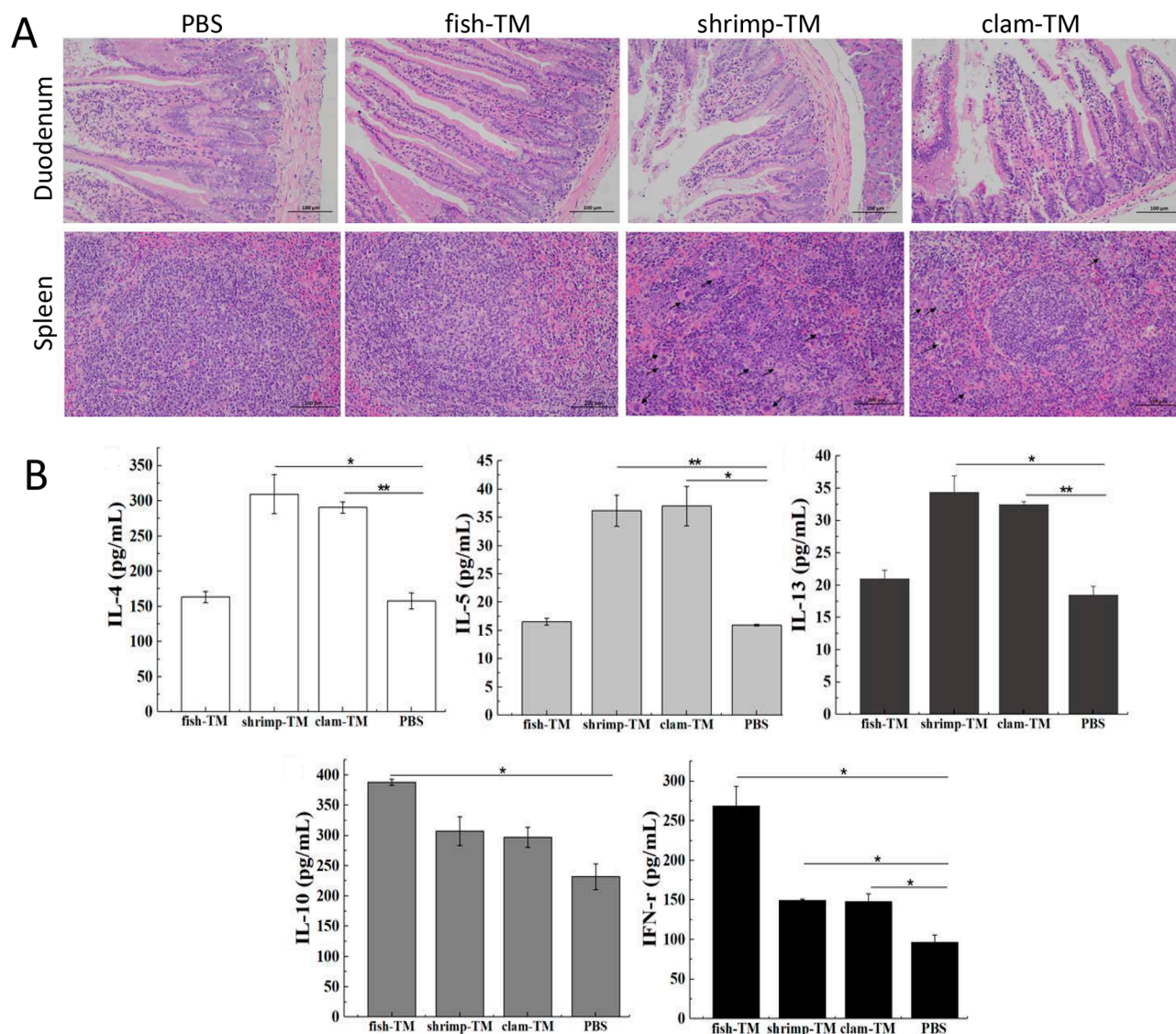


Fig. 1. Histopathology of duodenal and splenic tissues and cytokine production. (A) Hematoxylin and eosin staining of the duodenum and spleen of mice sensitized with fish-TM, shrimp-TM, clam-TM and PBS. Black arrows indicate detected macrophages. (B) IL-4, IL-5, IL-13, IL-10 and IFN- γ levels released from splenocytes isolated from the different mouse groups after stimulation with 20 $\mu\text{g/mL}$ sensitizing TM or PBS. Data are expressed as the mean $\pm$ SD. Symbols stand for “*” $p < 0.05$; “**” $p < 0.01$.

fish-TM sharing 93% sequence identity with its mouse homolog and <20% cross-reactivity with shrimp-TM was included in the study (Jenkins, Breiteneder, & Mills, 2007; Xu, Chen et al., 2020). Mice were divided into four groups, and each group was sensitized with fish-TM, shrimp-TM, clam-TM or PBS (Supplementary Material, Figure S2). As predicted, the mice sensitized with shrimp-TM and clam-TM displayed the clinical symptoms, weight alterations and levels of anti-TM IgG₁ and IgE described for allergic phenotypes (Supplementary Material, Figure S2). In contrast, mice treated with fish-TM and PBS lacked any of the previous characteristics of allergies (Supplementary Material, Figure S2).

Clam-TM- and shrimp-TM-sensitized mice compared to fish-TM- and PBS-sensitized mice also shared a pattern of duodenal lesions featuring villous atrophy, destruction of the mucosal lining and infiltration of inflammatory cells (Fig. 1A). Moreover, the spleen of clam-TM and shrimp-TM mice displayed lymphoid hyperplasia and macrophage infiltrations that were absent in the fish-TM and PBS groups (Fig. 1A).

Meanwhile, stimulation of the splenocytes isolated from shrimp-TM and clam-TM mouse groups released significantly higher levels ($p < 0.05$) of Th2 cytokines (IL-4, IL-5 and IL-13) compared to the lymphocytes isolated from the fish-TM and PBS groups (Fig. 1B). Conversely, the levels of Th1 cytokines IL-10 and IFN- γ were uniquely upregulated in the fish-TM group (Fig. 1B).

Taken together, these data showed that mice sensitized to either clam-TM or shrimp-TM displayed allergic phenotypes characterized by pronounced splenic and intestinal lesions and a Th2-shifted immune response, whereas mice treated with fish-TM developed an antigenic phenotype linked to an enhanced Th1 immune response.

3.2. Chemo-metric analysis of intestine and spleen proteomes

To analyze the variable differences in intestine and spleen proteomes caused by TM sensitizations, the OPLS-DA supervised chemometric method was employed. As shown in Fig. 2A, the intestine total protein data of each group were all inside of the Hotelling T^2 (95%) confidence ellipse and clearly clustered into four groups. Along the direction of $t[1]$, the allergic (shrimp-TM and clam-TM) groups were unambiguously separated from the others, revealing a similar proteome change. As with the intestine data, the spleen total protein samples were clustered into four groups (Fig. 2B). In this case, the allergic groups were clearly differentiated in the direction of $t[2]$.

To strengthen the differentiating pattern, the OPLS-DA data were analyzed using pairwise comparisons. The subsequent identification procedure is illustrated for the spleen data of the shrimp-TM and PBS mouse groups (Figure S2). The sample data were first divided into two groups in the $t[1]$ direction (Figure S2A) and then filtered with VIP > 2.5 (Figure S2B), s-plot variables with $p(\text{corr}) > 0.8$ (Figure S2C), and fold-change ratio > 1.5. This approach permitted the selection of 20 proteins out of >1,600 proteins (Figure S2D). Application of this procedure yielded 19, 36 and 39 differentiating proteins in the intestine and 18, 20 and 5 differentiating proteins in the spleen in the fish-TM, shrimp-TM and clam-TM groups compared to PBS, respectively (Table 1). Of the identified differentiating proteins, 11 in the intestine and 2 in the spleen were common to all TM-sensitized groups (Fig. 2C, D). Allergic phenotypes (clam-TM and shrimp-TM groups) had in common 24 intestine and 1 spleen differentiating proteins

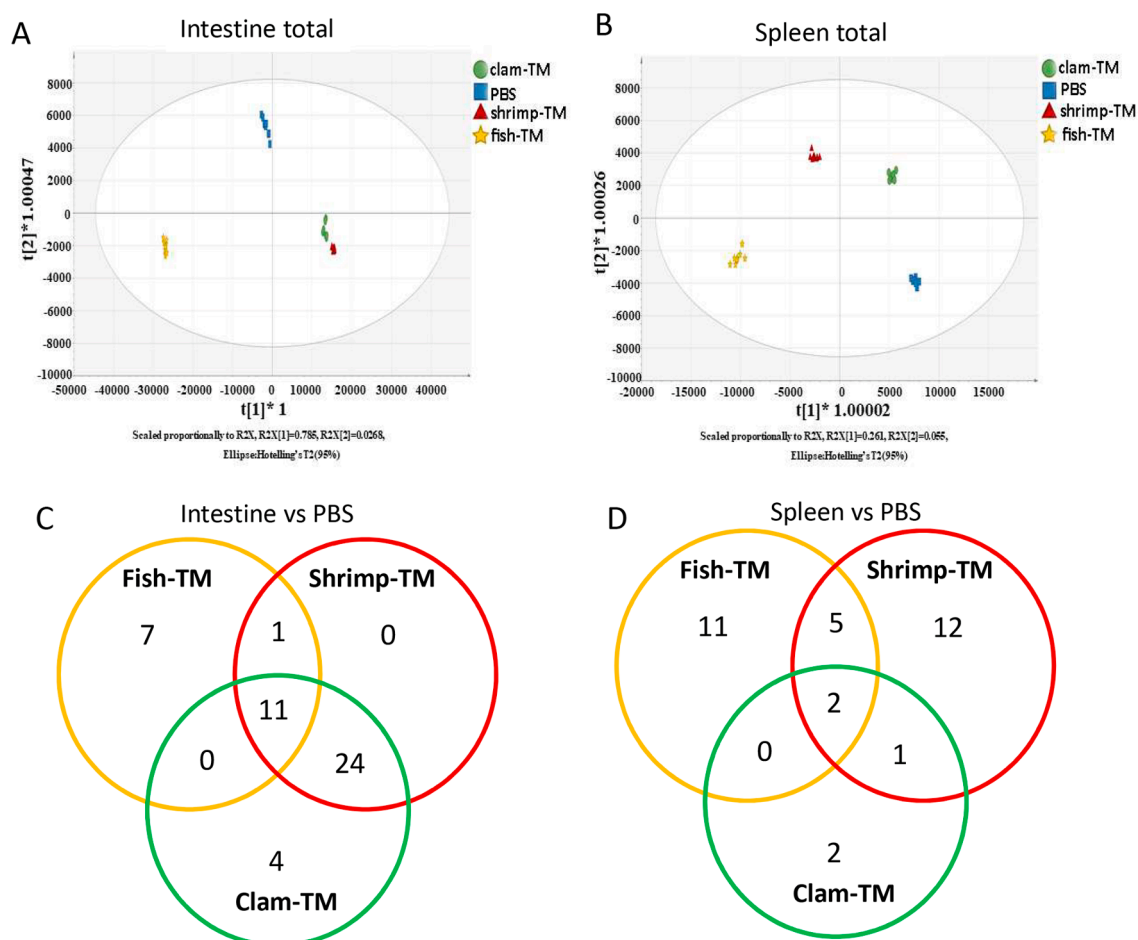


Fig. 2. OPLS-DA analysis of intestine and spleen proteomes of the distinct mouse groups. Total protein of the (A) intestine and (B) spleen. Venn diagram of the correlation between differentiating proteins in the (C) intestine and (D) spleen.

Table 1

Intestine and spleen differentiating proteins in mice sensitized with fish-TM, shrimp-TM and clam-TM compared to PBS.

Sensitizing TM	Differentiating proteins ^a			
	n	Intestine	n	Spleen
Fish	19	Q8VDN2, P52480, Q91V38, P27773, Q03265, Q564E2, O08601, Q921I1, Q62468, P40142, Q3TAN0, Q99K10, Q04447, Q542G9, P08249, Q3UER1, P14152, Q8R1Q7, Q8K1I1	18	Q7TPR4, Q3TJX0, Q01853, P14733, P14152, B2RXM2, Q61RU2, Q5SS40, Q9CVB6, Q3U0Z1, Q3UER8, Q9DCY1, Q544F6, Q3U5U6, B2RVP5, Q642L7, P10639, Q545D8
Shrimp	36	Q8VDN2, P56480, P52480, Q5FW97, P20029, Q58E64, P27773, Q03265, D2KHZ9, Q564E2, Q8C196, O08601, Q921I1, Q62468, B1AQ78, Q04447, P11679, Q3V0K9, O88428, Q3UER1, Q5SX50, P14152, Q5SVY2, Q545N7, P99027, B2RXM2, Q642K0, Q61425, Q52LA0, Q9DCY1, Q3V2F7, Q3U2X5, Q53YP5, Q9D881, P10639, Q8K1I1	20	Q3TJX0, Q5FWJ3, Q3TZQ2, P14733, A0A0R4J0Z1, P48036, P14152, B2RXM2, Q3U0Z1, Q544F6, Q5EBP8, Q3T9S3, Q542F1, Q3TKK3, Q3TVS6, Q5EBQ2, Q49713, Q4JF18, P01671, Q545D8
Clam	39	Q546G4, Q8VDN2, P56480, P52480, Q5FW97, P20029, Q58E64, P27773, Q03265, Q3UDR2, D2KHZ9, Q564E2, Q8C196, O08601, Q62468, B1AQ78, Q04447, P11679, Q3V0K9, O88428, B2M1R6, Q3UER1, Q5SX50, P14152, Q5SVY2, Q545N7, P99027, B2RXM2, Q642K0, Q61425, Q52LA0, Q9DCY1, Q8VC30, Q3V2F7, Q3U2X5, Q53YP5, Q9D881, P10639, Q8K1I1	5	Q3U0Z1, Q3UDY1, Q3U6T2, P01671, Q545D8
All	11	Q8VDN2, P52480, P27773, Q03265, Q564E2, O08601, Q62468, Q04447, Q3UER1, P14152, Q8K1I1	2	Q3U0Z1, Q545D8
Allergenic TMs ^b	24	B1AQ78, B2RXM2, D2KHZ9, O88428, P10639, P11679, P20029, P56480, P99027, Q3U2X5, Q3V0K9, Q3V2F7, Q52LA0, Q53YP5, Q545N7, Q58E64, Q5FW97, Q5SVY2, Q5SX50, Q61425, Q642K0, Q8C196, Q9D881, Q9DCY1	1	P01671

^aProteins are displayed using the UniProtKB database code. ^bCommon to clam-TM and shrimp-TM.

3.3. Relative quantities and functional enrichment of the splenic differentiating proteins

The relative quantities of the differentiating proteins common to both the three TM sensitizers and the two provoking allergic phenotypes (shrimp-TM and clam-TM) were determined by SWATH-based proteomics (Fig. 3). Two common differentiating proteins were found, which corresponded to the immune response regulators cochlin (Q3U0Z1) and bone marrow proteoglycan 2 (Q545D8). Of them, follicular dendritic cell-secreted cochlin appeared significantly upregulated in the fish-TM group compared with the shrimp-TM and clam-TM groups (Py et al., 2013). On the other hand, allergic phenotypes yielded as a unique feature the upregulation of Ig kappa chain V-III region PC 7175 (P01671), with roles in the adaptive immune response and Ig repertoire production (Kraneveld et al., 2005).

Taken together, these results supported the finding that the cochlin and Ig kappa chain V-III region PC 7175 are novel splenic biomarkers of the Th1 response, as observed in the fish-TM group, and of the Th2-shifted response in the allergic groups, respectively.

3.4. Relative quantities and functional enrichment of the differentially expressed proteins in intestine

The intestine samples of fish-TM, shrimp-TM and clam-TM mouse groups shared 11 differentiating proteins, all downregulated in both shrimp-TM and clam-TM groups and upregulated in the fish-TM group (Fig. 3). This group of proteins consisted of the α 1-subunit Na⁺/K⁺-transporting ATPase (Q8VDN2), pyruvate kinase (P52480), protein disulfide-isomerase A3 (P27773), α -subunit of the mitochondrial ATP synthase (Q03265), L-lactate dehydrogenase (Q564E2), large subunit of the microsomal triglyceride transfer protein (O08601), villin-1 (Q62468), creatine kinase B-type (Q04447), fructose-bisphosphate aldolase (Q3UER1), cytoplasmic malate dehydrogenase (P14152) and galectin-2 (Q8K1I1). The functional enrichment analysis of this protein group mostly revealed their involvement in several metabolic pathways (Fig. 4A). As judged from the protein level alterations and the observed histological lesions (Fig. 1A), the resulting pathways would be upregulated under the Th1 response caused by fish-TM and drastically impaired in the allergic phenotypes.

In addition to the previous alterations, the shrimp-TM and clam-TM groups shared 24 other differentiating proteins that appeared to be significantly downregulated compared with the fish-TM and PBS groups (Fig. 3). These proteins were keratin-8 (P11679), keratin-19 (B1AQ78), plastin-1 (Q3V0K9), Myl6 (Q642K0), profilin-1 (Q5SX50), hnRNPA3 (B2RXM2), 60S acidic ribosomal protein P2 (P99027), elongation Factor 1- α (Q58E64), glyceraldehyde-3-phosphate dehydrogenase (D2KHZ9), bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (O88428), thioredoxin (P10639), endoplasmic reticulum chaperone BiP (P20029), peptidyl-prolyl cis-trans isomerases a (Q5SVY2) and b (Q9DCY1), amine oxidase (Q3U2X5), fatty acid-binding protein 1 (Q3V2F7) and 2 (Q53YP5), galectin-3 (Q52LA0), creatine kinase (Q545N7), 2-phospho-D-glycerate dehydrogenase (Q5FW97), mitochondrial ATP synthase β -subunit (P56480), mitochondrial hydroxyacyl-coenzyme A dehydrogenase (Q61425), mitochondrial carbamoyl-phosphate synthase (Q8C196), and cytochrome c oxidase polypeptide Vb (Q9D881). The functional enrichment analysis highlighted their involvement in the intestinal response to ammonium ion and absorption pathways, among others (Fig. 4B). Since the differentiating proteins involved in these functions were found to be downregulated, such pathways are expected to be impaired. In this sense, the improper functioning of intestinal absorption processes might impact proper nutrient absorption beyond the known enhanced permeability (Odenwald & Turner, 2017; Sicherer & Sampson, 2018). Taken together, all the differentiating proteins downregulated in the allergic phenotypes (11 + 24) for the functional enrichment analysis clearly and additionally revealed the impairment of D-glucose absorption (Fig. 4C), a key process

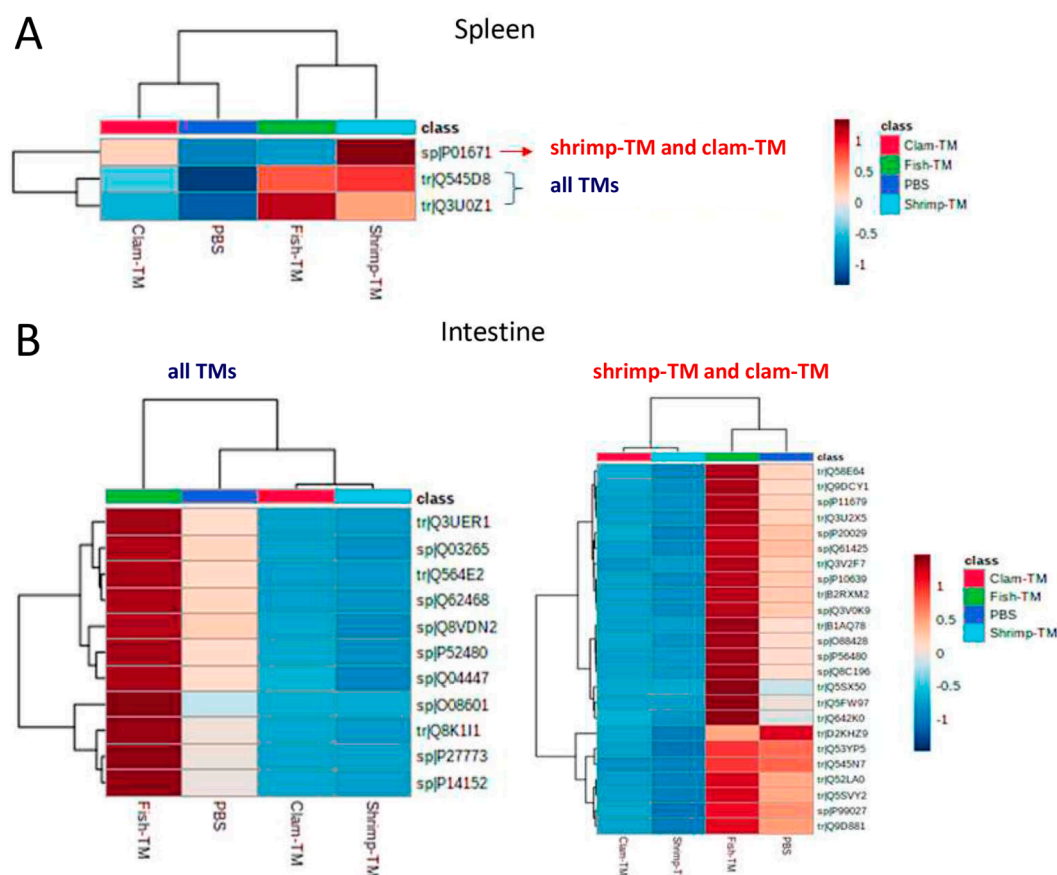


Fig. 3. Heatmaps of the relative quantitative analysis of differentially expressed proteins from the intestine and spleen of immunized mice. (A) Splenic differentiating proteins common to all sensitizing TMs (fish-TM, shrimp-TM and clam-TM) and to those causing allergic reactions (shrimp-TM and clam-TM). (B) Differentiating intestinal proteins.

involved in the appearance of exogenous glucose in the peripheral circulation, the release of gastrointestinal hormones and energy intake (Wu et al., 2020).

4. Discussion

Type-I food allergies are considered disorders of the epithelial barrier (gastrointestinal, lung, skin) in which food proteins are wrongly taken up and recognized as invaders in Th2 immune responses (Sicherer & Sampson, 2018; Eiwegger, Hung, San Diego, O'Mahony, & Upton, 2019). Despite the complexity of food proteins, only a few protein groups, such as the highly conserved muscle TMs, trigger such a response (Matricardi et al., 2016).

Vertebrate TMs, such as those of fish, have been regarded as weak and minor allergens in some fish species, whereas crustacean and mollusk TMs are considered major allergens (Ruethers et al., 2018; Xu, Chen, et al., 2020; Xu et al., 2020). As allergens, members of the TM family differ in their allergenicity degree and cross-reactivity capacity (Jenkins et al., 2007; Ruethers et al., 2018; Xu, Chen, et al., 2020; Xu et al., 2020). As predicted from the sequence distance of fish-TM, shrimp-TM and clam-TM from the homolog TM of the host mouse, only shrimp-TM and clam-TM provoked changes in weight, clinical symptoms, anti-TM IgG1 and IgE levels, duodenal and splenic histopathology patterns and cytokine production of allergic phenotypes. It merits comment that sensitization was achieved delivering the full-length TMs through the i.p. route together with alum, which avoids the conventional contributions from protein stability and epithelial barrier triggers (James, Pike, Khan, & Nanda, 2018; Kamath et al., 2020; Czolk et al., 2021). Indeed, the TM stability differences may be critical

for their escape from gastrointestinal proteases and access to the target epithelial barrier. Nonetheless, this parameter is further probed by dendritic cells through the uptake and endolysosomal processing of essential filters (Kamath et al., 2020). On the other hand, signals programming dendritic cells (DCs) to trigger Th2 cell-mediated immunity and boost ILC2, basophil and mast cell functions might not only be triggered by the intestinal epithelium but systemically, as in the case of i. p. allergen delivery (Czolk et al., 2021, and references therein).

As the largest immune organ, the spleen is significantly impacted by allergens (Matricardi et al., 2016; Sicherer & Sampson, 2018; Toyoshima et al., 2017; Wai, Leung, Chu, & Leung, 2021). Two immune response regulators, namely cochlin and Ig κ chain V-III region PC 7175, were found to be splenic differentiating proteins. Of them, cochlin appeared significantly upregulated only in the spleen of fish-TM mice, positioning it as a candidate biomarker for the Th1-immune responses provoked by the weakly allergenic or nonallergenic TMs. In this sense, cochlin is expressed and secreted from follicular dendritic cells within the spleen, selectively stored in the splenic extracellular network of conduits of the B cell zone and released after its aggrecanase processing into the blood circulation during inflammatory conditions involving TNF- α (Py et al., 2013). Despite the need for a deeper study, the differential regulation of cochlin levels suggests a role for TNF- α pleiotropy at the early stages of sensitization.

On the other hand, the Ig κ chain V-III region PC 7175 was upregulated only in shrimp-TM and clam-TM mice, both sharing allergic phenotypes. This result is in line with the increased levels of κ light chains in the sera of asthma patients compared with healthy controls (Kranefeld et al., 2005). Since Ig κ free light chains can mediate mast cell-dependent hypersensitivity-like reactions with antigen specificity, the obtained

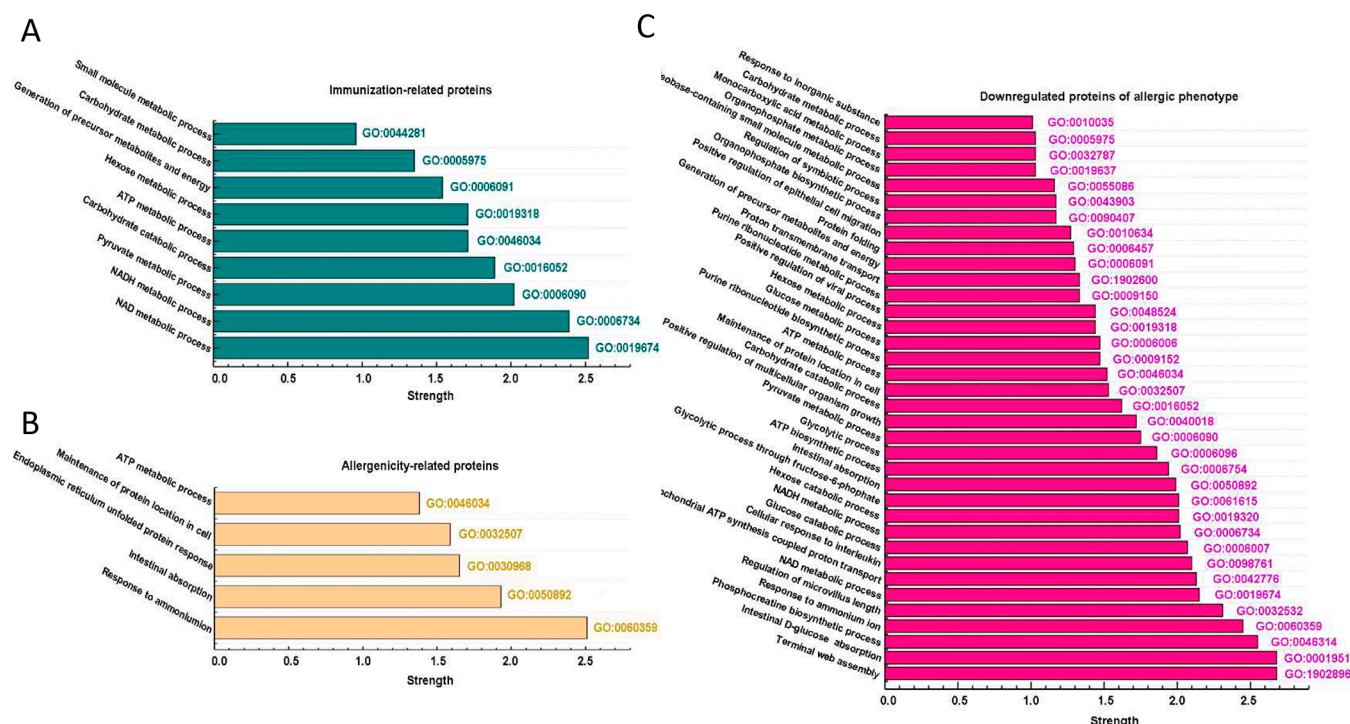


Fig. 4. Functional enrichment of the intestinal differentiating proteins. (A) Differentiating proteins common to fish-TM, shrimp-TM and clam-TM sensitizations. (B) Differentiating proteins uniquely common to shrimp-TM and clam-TM sensitizations. (C) All differentiating proteins (35) were downregulated in allergic phenotypes. Analysis was performed with STRING software. Displayed pathways correspond to those with strengths > 1.

results support the search for serum allergen-specific Ig κ chains and the study of their contribution to disease (Kranefeld et al., 2005).

Playing an essential immune role along with the spleen, the intestine constitutes a physical and dynamic barrier in coordination with the gut microbiota, which is drastically altered under food allergy conditions (Nowak-Węgrzyn, Szajewska, & Lack, 2017; Odenwald & Turner, 2017; Sampath et al., 2017). As judged from the histopathology analysis, TM allergy co-occurred with significant tissue lesions involving the remodeling of the intestine cellular organization (loss of some populations and gain of the invader ones). From the proteomics side, TM sensitization caused the dysregulation of 11 differentiating proteins with a major role in metabolism. In addition to this functional network, microsomal triglyceride transfer protein is an essential factor for the development of mucosal immunity-relevant NKT cells (Dougan, Rava, Hussain, & Blumberg, 2007), galectin-2 is an inducer of T cell apoptosis (Paclik et al., 2008), L-lactate dehydrogenase plays a key role in the conversion of microbiota-produced lactate for tissue regeneration (Kahlert et al., 2016; Lee et al., 2018), and actin-binding villin-1 is key for epithelial cell survival (Roy et al., 2018). Interestingly, these 11 proteins appeared to be upregulated in the fish-TM group and downregulated in the allergic groups. Taking together the tissue lesions and the roles such as those played by L-lactate dehydrogenase and villin-1, it is tempting to speculate that wound healing routes required for tissue regeneration were impeded in the TM allergic groups. In this sense, of the 24 downregulated differentiating proteins found uniquely in the shrimp-TM and clam-TM groups, keratins are known regulators of intestinal epithelial fragility and are receptors for microbiota metabolites, the moonlighting glyceraldehyde-3-phosphate dehydrogenase and galectin-3 are involved in the innate immune response regulating microbiota composition, and thioredoxin is key for intestinal redox homeostasis needed for maintaining mucus fluidity, absorption of nutrients, protection against chemical-induced oxidant injury and wound healing pathways (Wang, Srinivasan, Theiss, Merlin, & Sitaraman, 2007; Circu & Aw, 2011; Chauhan et al., 2015; Tsai et al., 2016; Plugis, Palanski, Weng, Albertelli, & Khosla, 2017; Hu et al., 2018). Therefore, the downregulation of

all 35 proteins underscores the impairment of intestinal epithelial self-renewal and microbiota synergic interactions (Nowak-Węgrzyn et al., 2017; Bunyavanich & Berin, 2019).

In addition to the improper proportions of the functions played by each of the intestinal differentiating proteins, functional enrichment analysis revealed their connection with several metabolic pathways, ammonium ion responses and intestinal absorption processes, among others. Intestinal ammonia is a waste product from urease-containing microbiota that buffers the short-chain fatty acids produced by the microbiota, and its levels correlate with gut motility (Fried, Watson, Robson, & Gulbransen, 2017). Consequently, the disruption of the response to the ammonium ion pathway mirrored the gut microbiota changes accompanying food allergies (Bunyavanich & Berin, 2019). As with the ammonium ion responses, D-glucose absorption appeared compromised, highlighting yet unexplored food-related features of allergies.

In summary, this work has shown that of the three studied TMs, shrimp-TM and clam-TM produced allergic reactions in mice, and fish-TM elicited a Th1 immune response. TM allergy co-occurred with splenic upregulation of cochlin and Ig κ chain V-III region PC 7175 and intestinal downregulation of a collection of proteins involved in metabolic routes, absorption processes, ammonium ion responses and tissue regeneration. Nevertheless, further investigations are needed to establish the relevance and exploitation capacity of the found patterns in the intervention of tropomyosin allergy as well as their parallelism in the human allergic population.

CRediT authorship contribution statement

Li Li Xu: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Hong Wei Zhang:** Data curation. **Hong Lin:** Supervision, Project administration. **Xiao Mei Zhang:** Investigation, Data curation. **Yun Qi Wen:** Investigation, Resources. **Jin Long Zhao:** Investigation, Resources. **Zhen Xing Li:** Conceptualization, Supervision, Writing – review & editing. **María Gasset:**

Conceptualization, Supervision, Writing – review & editing.

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.

Funding

This work was supported by the National Natural Science Foundation of China (grant number 31871719) and by the Spanish Ministry of Science and Innovation (Spanish AEI grant PID2019-103845RB-C21). L. L. Xu was supported by the China Scholarship Council (CSC).

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2022.132474>.

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Figure S1. TM allergens used for BALB/c mouse sensitization. (A) Sequence alignment of the TMs used as allergen sensitizers. **(B)** Homology of seafood TMs sequences compared to the mouse-TM. Sequence retrieval and analysis was performed as described in the material and method section.

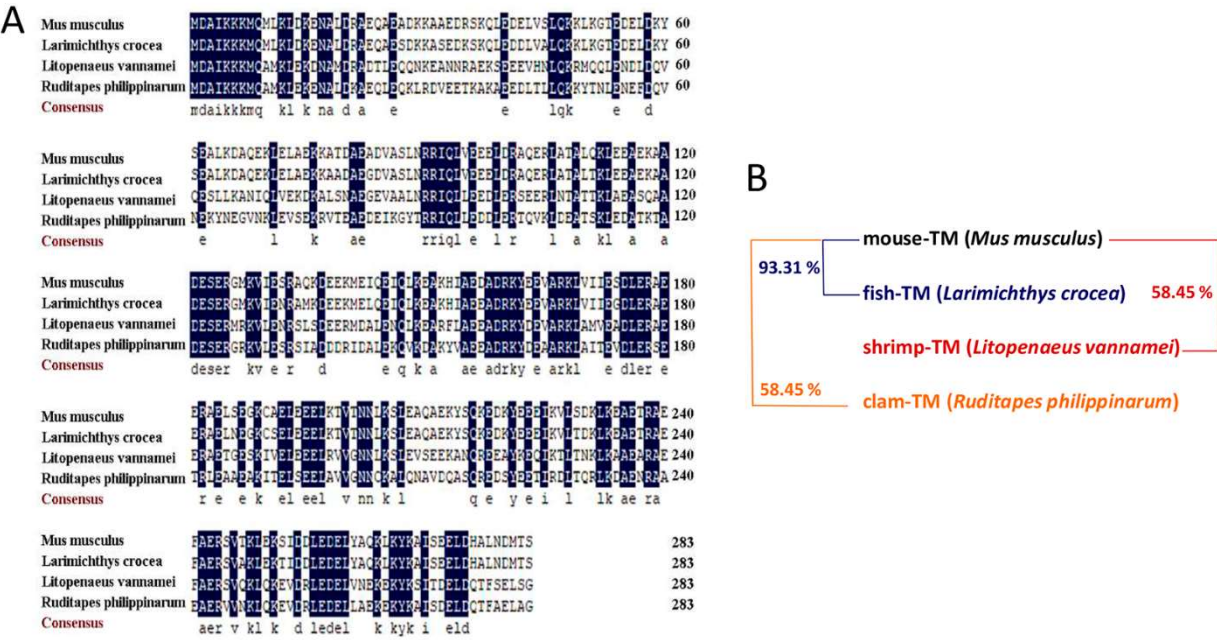


Figure S2. BALB/c mouse model of TM allergy. (A) Sensitization protocol. **(B)** Clinical symptoms score and **(C)** weight variations observed 30 min after challenge. **(D)** Anti-sensitizing TM IgG₁ and **(E)** IgE levels in mouse sera observed at day 49. PBS was used as control. Data are expressed as mean $\pm$ SD (n=6) and the symbol of “*” stand for $p < 0.05$, “**” stand for $p < 0.01$.

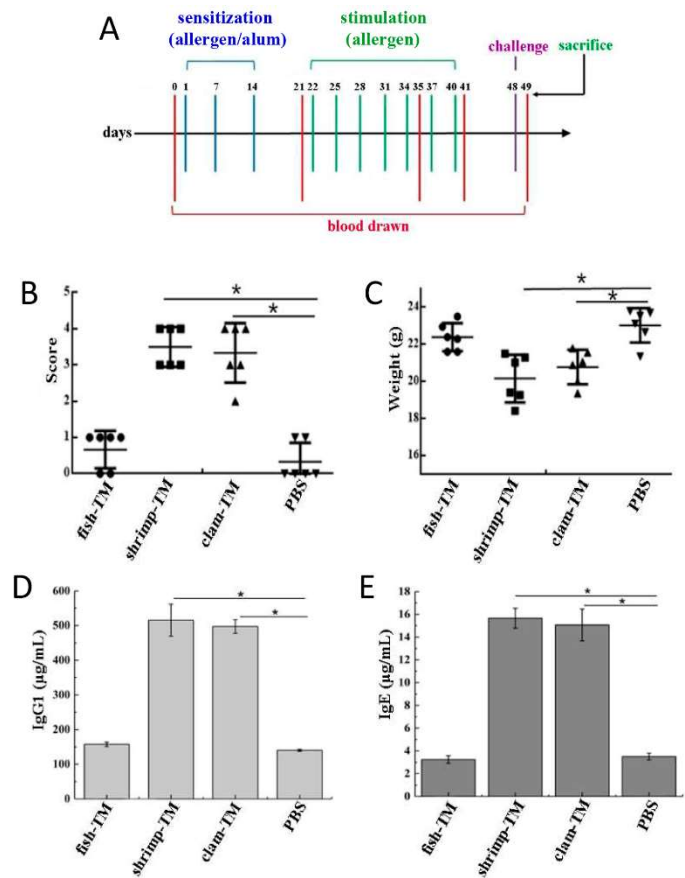


Figure S3. Strategy for differentiating protein discovery with pairwise comparison in spleen shrimp-TM vs spleen PBS. (A) Score plots. (B) VIP plot. (C) *s*-plot. (D) Use of jack-knifed confidence intervals for plotting.

