SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS.

If not otherwise stated, chemicals, reagents, and antibodies were from Sigma-Aldrich (St Louis, USA).

Basophil activation test (BAT)

The quantitative determination of in vitro basophil activation was performed using Flow CAST (Bühlmann Laboratories AG, Schönenbuch, Switzerland), as described before [1].
Briefly, venous blood of the patient and a healthy control (no history of food allergy and known tolerance to fish and crustaceans) was collected in EDTA tubes and further processed immediately. Cod extract was prepared based on the method described in [2] with slight modifications. 10 g of crude cod and 10 ml of phosphate-buffered saline (PBS) were blended. This mixture was then heated to boiling, followed by pureeing. The puree was centrifuged twice for 5 minutes each to eliminate particles, and the supernatant was subsequently collected to be used. The described cod extract and a cod prick test solution (Bencard Allergie GmbH, Munich, Germany) were used as allergen test solutions in different dilutions (undiluted, 1:10, 1:100, 1:1000). For each measurement, 50 µl of allergen test solution, 100 µl of stimulation buffer, 50 µl of blood, and 20 µl of staining reagent were mixed in polystyrene tubes. The staining reagent consisted of anti-CD63-fluorescein-isothiocyanate and anti-CCR3-pycoerythrin monoclonal antibodies. The tubes were incubated for 25 min at 37°C. The stimulation was stopped by adding 2 ml of lysis reagent and standing for 5 min in the dark at room temperature. The supernatant was decanted after a 5-minute centrifugation, and the cell pellet was resuspended in 300 µl of wash buffer. Anti-FcεRI-mAb and N-formyl-methionyl-leucyl-phenylalanine were used as positive controls, where both the patient and the control showed positive values to be confirmed as BAT-responders. Stimulation buffer alone was used to determine the background values. Basophils were gated as low side scatter CCR3/side scatter^{low}. CCR3 was used for basophil identification, and CD63 as basophil activation marker, both marked with fluorescence-dye-labeled monoclonal antibodies. The flow cytometry analysis was conducted using the BD FACSCanto II flow cytometer with 488 nm excitation wavelength (argon ion laser) and the BD FACSDiva-Software (Becton-Dickinson Biosciences GmbH, Heidelberg,

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Deutschland). Each measurement included the counting of ≥ 500 basophils. Basophil activation was expressed as the percentage of basophil granulocytes expressing CD63.

Enzyme-linked immunosorbent assay (ELISA)

Protein preparations, fish (cod, tuna, salmon) and shrimp extracts, as well as purified fish and shrimp allergens (cod Gad m 1, Gad m 2, Gad m 3; tuna Thu a 1, Thu a 2, Thu a 3; salmon Sal s 1, Sal s 2, Sal s 3; shrimp Pen m 1), were produced as reported earlier [3-5]. Briefly, fish and shrimp extracts were produced from muscle tissues using a lysis buffer (50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 150 mM NaCl, pH 8.0, 1% Triton X-100) in a metal bead-based extraction (Mixer Mill MM400; Retsch, Germany). Parvalbumins from cod, salmon and tuna were purified from fish extracts by column chromatography. In short, a separation by anion exchange chromatography (Resource Q; GE Healthcare, Uppsala, Sweden; 0–500 mM NaCl in 20 mM Tris-HCl, pH 8) was followed by a second ion exchange chromatography (Mono Q 5/50 GL; GE Healthcare) and a final separation using a high-performance gel filtration column (Superdex 75 10/300 GL; GE Healthcare; 50 mM NaH_2PO_4, pH 7.2, 150 mM NaCl). Fish enolases and aldolases were purified from fish muscle extracts by combinations of ion exchange chromatography. To summarize, pure cod Gad m 2 and Gad m 3 were obtained after an anion exchange chromatography (Resource Q, GE Healthcare, Uppsala, Sweden; 0–1 M NaCl in 20 mM Tris-HCl, pH 8), followed by several runs on another ion exchange column (Mono Q 5/50 GL; GE Healthcare). Salmon and tuna allergens were isolated through cation exchange chromatography (Resource S, GE Healthcare; 0–1 M NaCl in 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6) and several runs on a size exclusion column (Superdex 75 10/300 GL; GE Healthcare; 50 mM NaH_2PO_4, pH 7.2, 150 mM NaCl as running buffer. Shrimp Pen m 1 was purified by anion exchange chromatography (Resource Q, GE Healthcare; 0–1 M NaCl in 20 mM Tris-HCl, pH 8), followed by another cation exchange chromatography (Resource S, GE Healthcare; 0–1 M NaCl in 20 mM MES, 1 pH 6). Recombinant Pen m 1 (A1KYZ2) was expressed in E. coli M15. Briefly, recombinant protein expression was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) and following, the bacterial extract containing the recombinant affinity-tagged Pen m 1 was separated using an immobilized metal ion affinity chromatography. All protein quantifications were performed using the Bradford method, with bovine serum albumin (BSA) as protein standard protein. Protein purities were checked in analytical sodium dodecyl polyacrylamide

gel electrophoresis (SDS-PAGE) followed by Coomassie G-250 dye (Pierce, Erembodegem, Belgium) and silver staining (SilverSNAP kit; Pierce).96-well plates (Nunc MaxiSorp ELISA Plates, Thermofisher, Waltham, USA) were coated with 100 µL of protein extract or purified allergen at a final concentration of 5 µg protein/mL diluted in PBS. After incubation overnight at 4°C, free protein-binding sites were saturated with 3% BSA. After each incubation, plates were washed three times with Tris-buffered saline (50 mmol/L Tris, pH 7.4 with 0.05% Tween 20 detergent; TBST). The patient's serum was diluted (1/2, 1/5, 1/10) in blocking buffer containing 3% BSA and applied to the ELISA plate overnight at 4°C. After further plate washing, incubation with the secondary antibody was performed for 2 hours at room temperature (biotinylated anti-human IgE, Southern Biotech, Birmingham, USA; 1:1,000 diluted in blocking buffer). Following this, the reagent streptavidin-alkaline phosphatase was incubated for another hour on the ELISA plate. ELISA plate signals were visualized using the chromogen p-nitrophenyl phosphate. The absorption was quantified at a wavelength of 405 nm using a VersaMax ELISA Microplate Reader (Molecular Devices; San Jose, USA). IgE values obtained from ELISA measurements were expressed in optical density units (OD at 405 nm) [3]. Sera of three non-atopic individuals were used as negative controls, resulting in a cut-off value (0.1, OD_{405nm}) that was 10-fold lower than the mean background. As positive controls, commercial antibodies against fish parvalbumin and shrimp tropomyosin were used (monoclonal anti-fish parvalbumin IgE-antibody 235, Swant, Burgdorf, Switzerland; polyclonal anti-shrimp tropomyosin IgG-antibody PA-SHM, InBio, Charlottesville, USA). Control antibodies were diluted at 1:5,000, followed by secondary antibody incubation (1:10,000; anti-mouse or anti-rabbit IgG-antibody labelled with alkaline phosphatase).

Results are shown in Figure E1 (B.). As no IgE binding was found for tuna (Thu a 1, Thu a 2, Thu a 3) and salmon allergens (Sal s 1, Sal s 2, Sal s 3), those results are not provided. All positive/negative controls confirmed the assay performance (results not shown).

For confirming the predominant sensitization to shrimp tropomyosin, the patient serum was inhibited with Pen m 1 (100 µg/mL final concentration, pre-incubation for serum inhibition/overnight, 4°C) and subsequently tested for residual IgE binding capacity to coated shrimp extract (Figure E1, B.).

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Immunoblot analysis

Immunoblot analysis of cod extract was done as previously reported [3, 5]. Briefly, the cod protein was separated by SDS-PAGE (70 µg protein/lane) and subsequently blotted onto a polyvinylidene difluoride (PVDF) membrane. After blocking of the PVDF membrane with 3% BSA/TBST, patient's serum was diluted 1:2 and incubated with single membrane strips. Bound IgE was detected using a monoclonal mouse anti-human IgE antibody (1:1,000 diluted; Southern Biotech), conjugated with alkaline phosphatase, and using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as insoluble precipitating substrate.

For the detection of tropomyosin in cod extract, anti-tropomyosin antibodies were used in parallel assays. Two antibodies were applied: the polyclonal anti-shrimp tropomyosin IgG-antibody (PA-SHM, InBio) and the polyclonal anti-shrimp tropomyosin IgG-antibody (PA-SHM, InBio) and the polyclonal anti-tropomyosin antibody (PA5-88151, Invitrogen/Thermo Fisher Scientific), the latter produced against a conserved C-terminal tropomyosin peptide. Those recognized a main (double-) band at around 35 kDa (Figure E1, D.), corresponding to cod tropomyosin.

IgE macroarray analysis

The patient's serum was analyzed also with the MADx ALEX2 macroarray (MacroArrayDX, Wien, Austria), according to the manufacturer's instructions and as reported before [6, 7]. This array comprises a panel of 120 extracts and 178 allergen molecules. Data acquisition of specific IgE reactivities to 298 allergens/extracts was done using a charge-coupled device camera and the ImageXplorer, followed by data mining using the Raptor software. All tropomyosins on the array were highly positive above 50 kUA/L (Ani s 3, Blo t 10, Der p 10, Pen m 1, Per a 7) as well as all tropomyosin containing extracts were positive (Figure E1, C.). In addition to the routine assay, a research macroarray was run in collaboration with Macroarray Diagnostics, including two fish tropomyosins, salmon Sal s 4 (allergen.org) and the cod homolog Gad m 4.

Data visualization

Data resulting from IgE ELISA were visualized using GraphPad Prism 10 (GraphPad software, USA) and data from basophil activation test with Microsoft Excel (Microsoft Corporation, Redmond, USA). Data from IgE multiplex analysis were plotted using R (version 4.3.1) in R

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studio (version 2023.09.1), using the webr package (https://CRAN.R-project.org/package=webr) [8].

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