

ORIGINAL ARTICLE Allergens

Tropomyosin from tilapia (*Oreochromis mossambicus*) as an allergenR. Liu¹, A. L. Holck², E. Yang³, C. Liu¹ and W. Xue¹¹College of Food Science and Nutritional Engineering, China Agricultural University, Haidian, Beijing, 100083, P. R. China, ²Nofima AS, Osloveien 1, Aas, Norway and ³College of Public Health, University of California at Berkeley, Berkeley, CA, 74720, USAClinical
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Summary

Background Tilapia is among the most common fresh water fish species raised by fish farms and can cause allergic reactions upon ingestion.

Objective To investigate important allergens in Tilapia (*Oreochromis mossambicus*).

Methods Allergens were detected using immunoblotting. An important allergen was purified to homogeneity by reversed-phase High Pressure Liquid Chromatography and characterized by enzyme linked immunosorbent assay (ELISA), competitive ELISA, Mass spectrometry (MS), circular dichroism measurements and differential scanning calorimetry.

Results By immunoblotting using sera from 10 patients with confirmed tilapia allergy, we identified a number of allergens with apparent molecular weights 114 to 17 kD. All patients produced IgE against a 32 kD allergen, Ore m 4, which was identified by MS as tropomyosin (TM). IgE binding of the pure protein was confirmed by immunoblotting, ELISA and ELISA inhibition. cDNA from tilapia tropomyosin (TM) was sequenced and compared with TMs from other species. The tilapia TM showed 53.5% homology to TM from shrimp. Homology was much higher to human TM isoform 5 (87.7%).

Conclusion and Clinical Relevance TMs are the major allergens in allergy to crustaceans. Auto-antibodies against human TM isoform 5 have been implicated as a causative agent in inflammatory bowel disease (IBD). Intriguingly, six of the 10 tilapia allergic patients had also been diagnosed with IBD, corroborating a connection between allergy and IBD. To our knowledge, this is the first report of tropomyosin from vertebrates as an allergen.

Keywords allergen, inflammatory bowel disease, *Oreochromis mossambicus*, tilapia, tropomyosin, ulcerative colitis

Submitted 24 February 2012; revised 24 September 2012; accepted 24 October 2012

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Cite this as: R. Liu, A. L. Holck, E.
Yang, C. Liu and W. Xue, *Clinical &
Experimental Allergy*, 2013 (43) 365–
377.

Introduction

Tilapia is the common name for nearly a hundred species of cichlid fish. It is a popular fresh water fish, which is low in saturated and total fat [1], calories, carbohydrates and sodium [2]. Tilapia is an excellent source of protein [3], phosphorus, niacin, selenium, vitamin B12 and potassium. Because of their large size, rapid growth and popular taste, tilapia has become the focus of major farming efforts to meet increasing consumer demands. Mozambique tilapia, (*Oreochromis mossambicus*), is native to southern Africa, but is now found in tropical and subtropical habitats around the globe.

Fish and their derived products can act as both ingested, contact and inhalant allergens [4]. Most allergic reactions to fish are IgE mediated, though non-IgE

mediated reactions also occur [4]. Estimated food allergy rates vary with region and appear to depend on the amount of fish in the local diet [4]. The estimated rates for North America are 0.1% for infants/children and 0.4% for adults [5, 6]. Most studies have focussed on cod and salmon allergy, however, all fish are considered potential allergen sources. Many people suffer from allergy from consuming freshwater fish [7, 8]. Also tilapia has been reported to cause allergic reactions upon ingestion [9].

In 1999, the Codex Alimentarius Commission included fish among eight commonly allergenic foods and recommended that member countries ensured that ingredient statements on packaged foods declare the presence of these allergenic foods and of ingredient derived from these foods [10]. Legislation has since been implemented in many countries aimed at

achieving a high level of health protection for allergic consumers. The Food Allergen Labelling & Consumer Protection Act (FALCPA), implies as of 2006, mandatory labelling of products containing fish and fish derived ingredients [11]. The EU directives 2000/13/EC and 2003/89/EC require mandatory declaration of fish and several other allergenic foods [12, 13].

Fish allergens can trigger a wide range of allergic symptoms from the commonly reported itching of the mouth and emesis [14] through skin irritations, digestive disorders, respiratory symptoms and circulatory symptoms [15]. Even fatal cases have been described [16].

Allergens from a variety of different marine fish species have been identified. The main allergens of marine fish are parvalbumins [17]. Parvalbumins have been described from a wide variety of sources such as Alaska Pollack (*Theragra chalcogramma*) [17], tuna, trout, red-fish, herring [18] and sole [19]. Cross-reactivity is common between the parvalbumins [19, 20]. Parvalbumins are small (12 kD) high-affinity $\text{Ca}^{2+}/\text{Mg}^{2+}$ -binding proteins resistant to heat and chemical denaturation [21, 22]. In some species, allergens other than parvalbumin have been recognized in some mono-sensitized patients [23]. Major allergens of 51, 46 and 12 kD were detected in indian scad (*Decapterus russelli*) [24]. Also collagen has been identified as an allergen in bigeye tuna (*Thunnus obesus*) [25].

In contrast, much less is known about allergens of freshwater fish. Parvalbumins homologous to marine fish parvalbumins have been described from carp (*Cyprinus carpio*) [26] and silver carp [27]. Parvalbumin from carp (*C. carpio*) was identified as an allergen by Swoboda et al. [28]. A number of different allergens have also been detected in blunt snout bream (*Megalobrama amblycephala*) [29]. Two of these were identified as enolase and creatine kinase. Allergens have also been described from pangasius [9].

Some allergens from tilapia have been identified. Parvalbumin from tilapia was described by Lee et al. [30]. Allergens other than parvalbumin were identified by Ebo et al. [9]. Also, collagen from tilapia (*Tilapia zillii*) is considered a potential allergen [31]. Still other allergens have described by Liu et al., of which two were identified as fructose-bisphosphate aldolase and enolase 3 [32].

Tropomyosin is considered the major allergen in shellfish allergy, which comprises allergy to crustaceans and molluscs [33]. This allergen is also responsible for cross-reactivity to other inhaled allergens such as house dust mites and insects. Tropomyosins from vertebrate species are generally considered non-allergenic [34]. This may be due to the greater sequence similarity between human and other vertebrate tropomyosins. Antibodies against vertebrate tropomyosins may

cross-react with human endogenous tropomyosins. Autoimmunity to tropomyosins has been implicated in the pathogenesis of ulcerative colitis [35].

Characterization of allergens will lead to better diagnosis and subsequently to better advice to allergic patients. Knowledge about the allergens may be used in immunotherapy and for authentication purposes.

Here, we describe the purification, identification and characterization of tropomyosin as an important allergen of tilapia (*O. mossambicus*).

Methods

Materials

Live tilapia (*O. mossambicus*) about one-year old were purchased at a local supermarket. Specimens were killed at the time of use.

Patients and sera

Patients were chosen from the Peking University Health Science Center in Beijing, China on the basis of a convincing clinical history of tilapia allergy as well as a positive skin prick test (SPT) (weal diameter ≥ 3 mm) and a positive double-blind placebo-controlled food challenge. These criteria ensured the best quality of sera for testing. The food in the food challenges consisted of increasing doses of active and placebo puddings prepared by a hospital dietician. The puddings consisted of a milkshake made with vanilla and chocolate ice cream. Tilapia was added to the active meals. Sera from ten patients were employed either separately or pooled by mixing 10 μL of each of the patient's sera. The serological characterization of the patients is given in Table 1. The control sera were collected from non-atopic laboratory volunteers. All serum samples were frozen at -80°C until use. The study was approved by the Regional Committee for Medical Research Ethics in Beijing (20100302) and informed consent was obtained from all participants.

Tilapia extract preparation

Fish muscles (100 g) were homogenized in 200 mL of 20 mM Tris-HCl buffer (pH 7.5) for 90 s using a Waring blender. The homogenate was separated by centrifugation at 13 400 g for 20 min at 4°C and the supernatant obtained was collected and used as the crude extract. The tilapia crude extract was boiled for 5 min at 90°C to denature proteases and other heat-sensitive proteins. The extract was then cooled to 0°C in an ice-water bath. The suspension was then centrifuged at 10,000 g for 20 min at 4°C to sediment insoluble materials, brought to 80% $(\text{NH}_4)_2\text{SO}_4$ saturation, and agitated for 60 min. Following centrifugation at 20,000 g for

Table 1. Serological characterization of patients with tilapia allergy*

Serum	Age	Sex	Tilapia IgE(KU _A /L) [†]	Symptoms to tilapia [‡]	Other reported allergies
1	9	F	6.3	IBD	None
2	21	M	2.2	OAS, Ur	Shrimp
3	35	M	2.6	IBD, OAS	Egg
4	28	F	0.9	Vom	Shrimp
5	56	F	6.8	IBD, UR	Crab
6	6	M	1.2	OAS, QE	Shrimp
7	15	F	7.2	IBD, UR	Cod
8	21	F	4.1	IBD	None
9	13	M	3.2	OAS	Shrimp
10	17	F	3.7	Ur, IBD	Salmon

*Data provided by Peking University Health Science Center. All patients tested positive by skin prick testing.

[†]Results from ELISA testing as described in Materials and Methods using tilapia crude extract as the allergen preparation in the wells.

[‡]Symptoms as observed during challenge tests. IBD, inflammatory bowel disease; BA, bronchial asthma; OAS, oral allergy syndrome; QE, Quincke's edema; Ur, urticaria; Vom, vomit. IBD diagnosis was made according to radiological, endoscopic and histological criteria.

20 min at 4°C, the supernatant was carefully collected and dialysed overnight in distilled water, then filtered through a 90-mm membrane filter (0.45 µm pore size, Millipore Corp., Billerica, MA, USA). This preparation was designated 'heat treated crude extract'.

Purification by ion-exchange chromatography and reversed-phase HPLC

The Ore m 4 allergen was further purified by ion-exchange chromatography on a Sepharose-CL-6B equilibrated with 20 mM Tris-HCl (pH 7.5) buffer. The column was eluted with a linear gradient of 0.5–1.5 M NaCl at pH 7.5. Fractions containing the main allergen were pooled and concentrated by nitrogen sweeping. The samples were placed in a water bath at 30°C and were concentrated by evaporating buffer using a flow of nitrogen gas. The sample was further purified using preparative reversed-phase high-performance liquid chromatography (RP-HPLC, Agilent 1100 system, Agilent Technologies, Switzerland) equipped with a reversed-phase C4 column (Teknokroma, Barcelona, Spain, 250 × 4.6 mm id, 5 µm, pore size 300 Å) at 30°C. Allergen sample analysis was monitored at 220 nm. The mobile phase consisted of a water gradient (obtained through Millipore Milli-Q system, Milford, MA, USA) and acetonitrile, both containing 0.1% trifluoroacetic acid (v/v). The protein was eluted at a flow rate of 1.2 mL/min with a linear gradient of 0–45% acetonitrile over 70 min. All isolation processes were carried out at 30°C and had an injected volume of 80 µL. The fractions containing the desired peaks were pooled and concentrated by nitrogen sweeping before

allergen activity testing. Purified natural shrimp tropomyosin was purchased from Bial, Bilbao, Spain.

Identification of main allergen by on-line LC-ESI-MS/MS and N-terminal amino acid sequencing

Tryptic digests of Ore m 4 generated from in-gel trypsin digestion were prepared as described in <http://mitchison.med.harvard.edu/protocols/MSTrypticDigest.htm> based on [36]. A volume of 5 µL of the tryptic digest was loaded onto a 150 mm × 2.0 mm Luna 5 µm C18 column (Phenomenex, Torrance, CA, USA). The elution was performed using a mobile phase consisting of water-trifluoroacetic acid (1000 : 0.3, v/v) (eluent A) and methanol (eluent B). Proteins were separated using a linear gradient 0–30% of eluent A and B. The eluates at 100 µL/min were directed into a Q-TOF2 hybrid quadrupole/TOF mass spectrometer (Waters Micromass, Hertfordshire, UK). Drying and ESI nebulization was performed by high purity nitrogen in the positive ion mode, operating at a source temperature of 80°C. The peptide sequencing program MasSeq was used to deduce the amino acid sequences. Combined with other data processed through MaxEnt3 as well as manual calculation, a database search (NCBI nr 20080418 containing 6,441,864 sequences entries) was run utilizing the Mascot search engine (<http://www.matrixscience.co.uk>). The N-terminal amino acid sequence of the purified tilapia allergen was determined by Edman degradation (Proteome Factory, Berlin, Germany) using the 492 Pro-cise sequencer (Applied Biosystems, Foster City, CA, USA) in pulse-liquid mode essentially as described by [37]. The allergen database SDAP (<http://fermi.utmb.edu/SDAP/>) was used determine sequence similarities.

Isolation of RNA, cDNA cloning and sequencing

The muscles of the tilapia were cut out and immediately frozen in liquid nitrogen. All RNA was isolated by TRIzol reagent (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA), and first-strand cDNA was synthesized with a cDNA kit (Pharmacia, Freiburg, Germany) using the Not I-d(T)18 primer. As a result of the LC-ESI-MS/MS analysis, primers were designed according to the cDNA alignment of tropomyosin from *Thunnus thynnus* and *Theragra chalcogramma*: 5': (5'-ATAAGCAGCCATGGATGCCATYAAGAAGAA-3'), 3': (5'-CGTCTTTGATGAGGTGTAAAMWRTTTATAT-3' and 5'-GTCATGTCGTTGAGGGCGTGGTCCAGCT-3') respectively. These primers were used for PCR under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 20 s; and finally maintaining at 72°C for 5 min. The PCR products were analysed by agarose gel electrophoresis, and fragments of the correct size were excised from the gel and purified

using the Wizard SV Gel PCR Clean-Up System (Qiagen, USA). Sub-cloning was performed using the TOPO TA Cloning Kit for Sequencing (Invitrogen) with the pCR4-TOPO vector, while competent *Escherichia coli* Top10 cells (Invitrogen) were utilized for transformation. The SV Miniprep DNA Purification Kit (Qiagen, Hilden, Germany) was used to extract plasmid DNA from the cells according to the manufacturer's instructions. The presence of the correct inserted DNA was confirmed by a restriction enzyme digest or, if the concentration of plasmid DNA was low, PCR was carried out using the isolated plasmid DNA. Automated sequencing based on the dye-terminator sequencing method was performed by the Molecular and Cell Biology Department, UCT.¹ Results were analysed using the BlastX program and clones were aligned to assess homology using DNAMAN (Lynnon Corporation, Pointe-Claire, Quebec, Canada) and BioEdit (ver. 7.0.1; Ibis Biosciences, Carlsbad, CA, USA).

SDS-PAGE and immunoblot analysis

Five µL samples from the purification were examined on 12.5% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250. Proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes (Nitrocell; Pharmacia Biotech, San Francisco, CA, USA.) using the MiniTrans-blot cell (Bio-Rad Laboratories, Richmond, CA, USA.) for 90 min at 100 mA/gel. To reduce non-specific binding, blotted membranes were incubated in TBS blocking buffer for 2 h at room temperature (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 3% BSA, pH 7.4). Strips were then probed overnight at 4°C with patients' sera (1 : 10 dilution); control strips were incubated with non-atopic volunteers' sera. Bound IgE antibodies were detected with HRP-labelled goat anti-human IgE antibody (1 : 2000 dilutions, Sigma Chemical Co., St Louis, MO, USA). When stated, bound IgE was detected using monoclonal mouse anti-human IgE (Fc)-HRP from Southern Biotech, Birmingham, AL, USA. Lack of presence of IgG antibodies against tropomyosin was shown using mouse anti-human IgG (Fc)-HRP also from Southern Biotech. Blots were developed through the addition of 4-chloro-1-naphthol buffer (Sigma).

ELISA

ELISA was performed essentially as described previously [38, 39]. Ninety six-well plates (Nagle Nunc International) were coated with 0.1 µg purified allergen in 100 µL phosphate-buffered saline (PBS) buffer (10 mM

Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 9.6). Coating was performed overnight at 4°C, followed by 3 rounds of PBST buffer wash (PBS with 0.05% Tween-20, pH 7.4) for 2 min each. Residual binding sites were saturated with blocking buffer (PBS, 2% gelatine, 0.1% Tween-20), by incubating for 1 h at room temperature. Afterwards, patients' sera (diluted 1 : 50 v/v in blocking buffer) was added and incubated for 1.5 h at 37°C, unbound antibody was removed by four PBST washes. 100 µL of the HRP-labelled monoclonal mouse anti-human IgE antibody (Southern Biotech, Birmingham, AL, USA), diluted 1 : 5000 (v/v) in blocking buffer, was added to each well. The plates were incubated at 37°C for 30 min and then washed four times with PBST. Peroxidase substrate (KPL Inc., Gaithersburg, Maryland, USA) was added. Reactions were stopped after 10 min by adding 100 µL/well of 6 N H₂SO₄, and optical densities (OD_{492 nm}) were determined on a MRX microplate reader (Dynex Technologies). The controls were incubated with non-atopic laboratory volunteers' sera. The amounts of IgE were determined by comparing with a standard curve based on peanut allergen Ara h 1. All ELISA were performed in triplicate, and the data obtained were expressed as mean ± SD. Average control background OD₄₉₂ (= 0.2) was subtracted from the average value for each serum sample. Individual allergen sample readings were considered significant if the average tilapia-allergic serum reading (minus the background), was greater than the average control serum reading (minus the background) plus two standard deviations for that allergen sample. Competitive inhibition ELISA was performed as follows. Purified native Ore m 4 (1 µg/mL, 100 µL) was added to a 96-well plate, allowed to bind overnight at 4°C, washed three times with PBST, blocked with blocking buffer for 1 h at 37°C, and washed. Tilapia crude extract (0.001, 0.01, 0.1, 1 and 10 µg/mL in 100 µL) or shrimp tropomyosin (0.001, 0.01, 0.1, 1 and 10 µg/mL in 100 µL) was mixed with 100 µL of serum (diluted 1 : 10). The mixtures were rotated at 4°C for 2.5 h before being added to the Ore m 4 -bound plates for 1 h at 37°C. Plates were washed, incubated with HRP-labelled monoclonal anti-human IgE antibody (Southern Biotech, Birmingham, AL, USA) for 30 min at 37°C, washed and determined on a MRX microplate reader.

Circular dichroism (CD) measurements

Far-UV CD spectra were measured with a JASCO spectropolarimeter, model J-710 coupled with quartz cuvettes (JASCO International Co, Tokyo, Japan) [40–42]. To determine the CD spectra of the allergen protein, 350 µL of the allergen was placed in a 1 mm path-length cuvette and maintained at 30°C. Temperature regulation was carried out by a Peltier temperature

¹The sequence has been submitted to Genbank, Acc. No. JX193712

controller PTC423S thermocouple (JASCO Corp.) for thermal measurements. Thermal transition of allergen was monitored at 222 nm between 25 and 100°C with a temperature slope of 10°C/min (scan speed 50 nm/min; resolution 1 nm; bandwidth 1 nm; sensitivity 50 mdeg; response 0.5 s). Fifteen mM Tris-HCl buffer at pH 7.5 was used as control. Molar ellipticity was calculated according to the published method [43]. The cooling graph was recorded after a delay of 5 min.

Differential scanning calorimetry (DSC)

Thermal analysis of the allergen was performed using a differential scanning calorimeter model DSC 7 (Perkin Elmer, Norwalk, CT, USA) [44–46]. The purified allergen was placed in an aluminium pan and sealed. The sample was allowed to stand for 2 days at 4°C prior to analysis. The sample was then scanned at 5°C/min over the range of 0–100°C to determine the gelatinization enthalpy and temperatures. An empty pan was used as the control. The thermogram was used to estimate the maximum transition temperature (T_{max}). To prevent the Ore m 4 from engaging in head-to-tail polymerization, the examination was performed under non-physiological conditions. The solvent used was 10 mM sodium phosphate (pH 7.0) containing 0.1 M KCl, 1 mM EDTA, 0.01% NaN₃ and 1 mM dithiothreitol. Protein concentration was in the range from 1.0 to 1.5 mg/mL.

Results

Identification of the major tilapia allergens

IgE-binding proteins were identified by separating crude tilapia extract by SDS-PAGE and immunoblotting using sera from ten patients with confirmed tilapia allergy (Fig. 1). The SDS-PAGE profile of the crude tilapia extract showed multiple protein bands ranging from 10 to 200 kD. Three bands were classified as low molecular weight, between 10 and 20 kD. Many clear and dominant bands were found in the middle molecular weight category, ranging from 25 to 90 kD. One pronounced band appeared at 114 kD. IgE-binding proteins were identified at 114, 73, 64, 52, 47, 37, 32, 28, 26 and 17 kD (Fig. 1, lanes 1–10). The frequencies of allergenic responses generally varied between 20 and 80%. All sera reacted with the 32 kD protein, which was thus considered a potential important allergen of tilapia (Ore m 4) and therefore selected for purification. No IgE binding was observed when a mixed control serum was used.

Purification of the Ore m 4 allergen

The 32 kD allergen, Ore m 4, was purified from crude tilapia extract using a novel method as described in

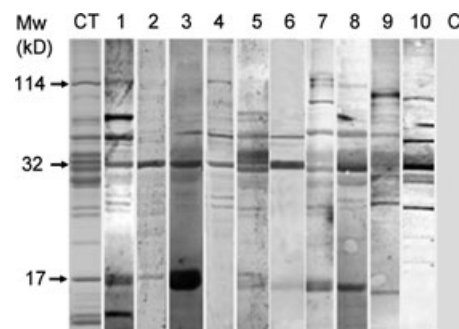


Fig. 1. SDS-PAGE and immunoblot analysis of tilapia allergens. IgE-binding proteins in a crude extract of tilapia were detected by immunoblotting. Lane CT, crude tilapia extract stained with Coomassie brilliant blue after SDS-PAGE. Lanes 1–10, immunoblots with individual sera from ten different tilapia allergic patients. Lane C, immunoblot with mixed serum from non-allergic volunteers.

Materials and Methods. Heat treated crude extract was subjected to ion-exchange chromatography. The Ore m 4 allergen eluted as a sharp single absorption peak (Fig. 2a). SDS-PAGE further resolved the purified main allergen as a single band around the estimated molecular weight 32 kD (Fig. 2b). Fractions containing the Ore m 4 were pooled and subjected to reversed-phase HPLC. Ten peaks eluting from 3.0 min to 48.5 min were collected and analysed by SDS-PAGE (results not shown). Fractions containing the 32 kD protein were pooled, concentrated and re-injected into RP-HPLC. The Ore m 4 protein eluted as a pure single peak without impurities (Fig. 2c). The purity was confirmed independently by SDS-PAGE and immunoblotting, showing single bands with a mw of 32 kD (Fig. 2d). The fractions containing the pure allergen were pooled and concentrated for further analyses.

LC-ESI-MS/MS and cDNA analysis of Ore m 4

Sequence determination of fragments of Ore m 4 generated from in-gel trypsin digestion was performed by LC-ESI-MS/MS analysis. The results are presented in Table 2. The purity of the isolated protein was confirmed by the quality of the sequence signals. The NCBI database was searched to identify sequences similar to Ore m 4. Ore m 4 showed extensive similarity to tropomyosin from the atlantic bluefin tuna (*Thunnus thynnus*). This similarity allowed us to synthesize primers and amplify and sequence Ore m 4 cDNA. The complete amino acid sequence of Ore m 4 according to the cDNA is shown in Fig. 3. The cDNA contained an 852 bp open reading frame, encoding a polypeptide of 284 amino acids. Forty six of 56 peptide mass queries were found in the reported tropomyosin as determined by cDNA. Together the MS generated peptides covered 84% of the total polypeptide sequence. Generally, the peptide sequences agreed well with the cDNA sequence.

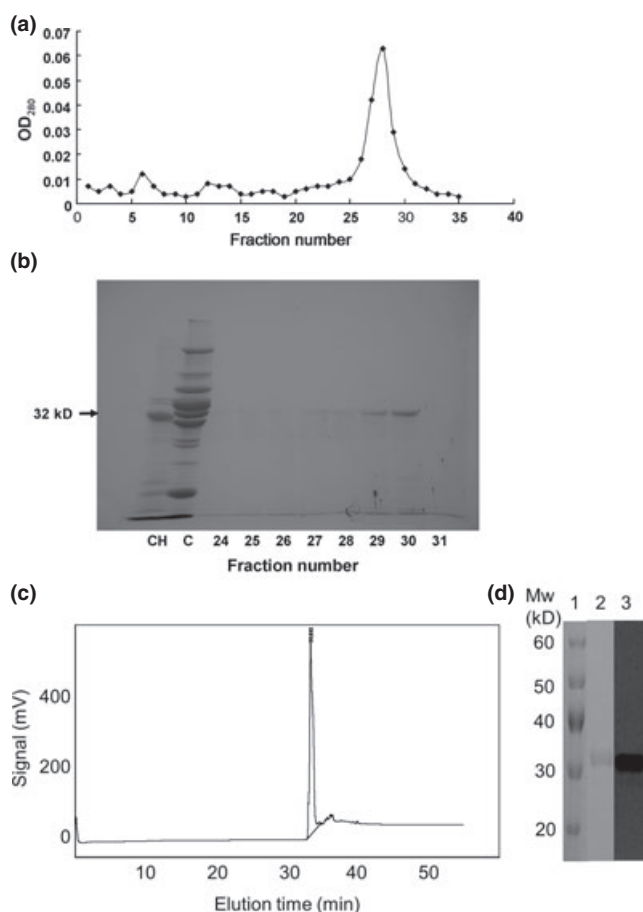


Fig. 2. Purification of the Ore m 4 allergen by ion-exchange chromatography and RP-HPLC. (a) Sepharose-CL-6B column chromatography purification of Ore m 4. (b) Analysis of eluted proteins by SDS-PAGE. Lane CH, heat treated crude extract after treatment at 90°C for 5 min. Lane C, tilapia crude extract. Lanes 24–31, fractions 24–31 from ion-exchange chromatography. The eluate from ion-exchange chromatography was subjected to RP-HPLC. Fractions containing Ore m 4 were pooled and subjected to a second round of RP-HPLC. (c) The eluate after the second round of RP-HPLC with the Ore m 4 eluting as a pure peak at 33.28 min. (d) The purified Ore m 4 analysed by SDS-PAGE and immunoblotting. Lane 1: Mw standard. Lane 2: Purified Ore m 4 stained with Coomassie brilliant blue. Lane 3: Immunoblot after incubating with pooled serum from the 10 tilapia allergic patients.

Three discrepancies were found at positions 77 (K→I), 84 (D→E) and 227 (V→A). The calculated pI value and nominal mass of Ore m 4 was 4.70 and 32 694 respectively. The N-terminal sequence as determined by Edman degradation was MDAIKKKMQM, which is typical for vertebrate tropomyosins and agreed with that obtained from the cDNA analysis.

Immunological analyses of purified Ore m 4 allergen

The presence of IgE antibodies against the pure tropomyosin was confirmed for all patients by immunoblotting with individual patient sera and monoclonal mouse anti-

IgE antibodies (Fig. 4). All sera displayed strong IgE-binding to Ore m 4, indicating that the allergen activity remains intact throughout all the purification steps. In a similar setup, we did not get any signals when probing the patient sera for IgG antibodies against purified tropomyosin using monoclonal anti-IgG antibodies. IgE-binding to shrimp tropomyosin allergen was also shown by immunoblotting using pooled serum from ten tilapia allergic patients (Fig. 4b). The sera were also tested using ELISA. Plates were coated with RP-HPLC purified Ore m 4 allergen or shrimp tropomyosin and incubated with individual sera from the ten tilapia allergic patients. Bound IgE was detected using monoclonal anti-IgE antibodies (Fig. 4c). All sera gave strong signals against both allergens, confirming that Ore m 4 could be considered an important allergen. Cross-reactivity between Ore m 4 and shrimp tropomyosins was analysed by ELISA inhibition using wells coated with purified Ore m 4 and probing with the pooled serum from the ten tilapia allergic patients mixed with increasing amounts of shrimp tropomyosin (Fig. 5). Peanut extract was used as a negative control indicating a low unspecific background in the ELISA. Shrimp tropomyosin turned out to be a strong competitor, inhibiting Ore m 4 binding by approx. 55% at 10 µg/mL. This showed a large cross-reactivity between Ore m 4 and shrimp tropomyosin IgE antibodies. On the other hand, the lack of full inhibition also indicated the presence of IgE antibodies binding to the Ore m 4 allergen, which do not cross-react with shrimp tropomyosin. ELISA inhibition using increasing amounts of purified Ore m 4 to inhibit binding to crude extract attached to the solid support, indicated that Ore m 4 anti-IgE comprises a large amount of the total tilapia anti-IgE in the pooled serum.

Structural properties of Ore m 4

The effects of temperature on the CD spectrum of Ore m 4 are shown in Fig. 6a. The CD spectrum of Ore m 4 at 30°C displayed two minima around 222 and 208 nm, which represents typical α -helical structure. The α -helical structure weakened with rising temperature, and nearly disappeared at 100°C. When the temperature cooled down to 30°C again, the α -helical structure was restored. The purified Ore m 4 was also subjected to differential scanning calorimetry analysis by heating in a calorimeter to 100°C (Fig. 6b). The results indicated a denaturation temperature of 46°C for Ore m 4.

Discussion

Allergy to fish can be a serious medical condition, especially in regions of the world where fish is an important protein source in the diet [47]. Allergens from marine fish have been widely studied. By far, most attention

Table 2. LC-ESI-MS/MS analysis of Ore m 4 digested by trypsin

Start-End	Observed	Mr (expt)	Mr (calc)	Sequence
13-21	537.2816	1072.5486	1072.5513	K.LDKENALDR.A
13-30	515.7521	2058.9793	2059.0181	K.LDKENALDRAEQAESDKK.A
13-30	687.3380	2058.9922	2059.0181	K.LDKENALDRAEQAESDKK.A
13-30	515.7555	2058.9929	2059.0181	K.LDKENALDRAEQAESDKK.A
13-30	687.3424	2059.0054	2059.0181	K.LDKENALDRAEQAESDKK.A
36-48	743.8213	1485.6280	1485.8038	R.SKQLEDDLVALQK.K
36-48	743.8826	1485.7506	1485.8038	R.SKQLEDDLVALQK.K
36-48	743.9090	1485.8034	1485.8038	R.SKQLEDDLVALQK.K
50-59	574.2874	1146.5602	1146.5768	K.LKGTEDLDK.Y
50-65	613.6311	1837.8715	1837.9309	K.LKGTEDLDKYSEALK.D
50-65	919.9686	1837.9226	1837.9309	K.LKGTEDLDKYSEALK.D
50-70	804.0724	2409.1954	2409.1911	K.LKGTEDLDKYSEALKDAQEK.L
50-76	774.1488	3092.5661	3092.5764	K.LKGTEDLDKYSEALKDAQEKLELAEK.K
52-65	799.3851	1596.7556	1596.7519	K.GTEDELDKYSEALK.D
52-70	723.6777	2168.0113	2168.0120	K.GTEDELDKYSEALKDAQEK.L
52-76	713.8493	2851.3681	2851.3974	K.GTEDELDKYSEALKDAQEKLELAEK.K
52-77	745.8765	2979.4769	2979.4924	K.GTEDELDKYSEALKDAQEKLELAEKK.A
66-76	637.3184	1272.6222	1272.6561	K.DAQEKLELAEK.K
71-76	702.3898	701.3825	701.3959	K.LELAEK.K
77-90	723.8540	1445.6934	1445.7110	K.KATDAEGDVASLNR.R
78-90	659.8135	1317.6124	1317.6161	K.ATDAEGDVASLNR.R
78-91	737.8604	1473.7062	1473.7172	K.ATDAEGDVASLNR.I
91-105	628.6572	1882.9498	1882.9860	R.RIQLVEELDRAQER.L
92-101	622.2968	1242.5790	1242.6456	R.IQLVEELDR.A
106-118	708.8614	1415.7082	1415.7871	R.LATALTKLEEAEK.A
106-125	725.7031	2174.0875	2174.1066	R.LATALTKLEEAEKAADESER.G
113-125	738.8291	1475.6436	1475.6739	K.LEEAEKAADESER.G
134-149	660.3287	1977.9643	1977.9751	R.AMKDEEKMELQEIQLK.E
141-149	566.3026	1130.5906	1130.6005	K.MELQEIQLK.E
153-167	605.9635	1814.8687	1814.8911	K.HIAEEADRKYEEVAR.K
168-182	600.6531	1798.9375	1798.9788	R.KLVIEGDLERTEER.A
169-178	578.8256	1155.6366	1155.6499	K.LVIEGDLER.T
169-182	836.3942	1670.7738	1670.8839	K.LVIEGDLERTEER.A
190-198	568.7539	1135.4932	1135.5067	K.CSELEELK.T
190-205	953.9782	1905.9418	1905.9353	K.CSELEELKTVTNL.K.S
206-217	691.3489	1380.6832	1380.6885	K.SLEAQAEKYSQK.E
206-226	849.0816	2544.2230	2544.2231	K.SLEAQAEKYSQKEDKYEEIK.V
214-226	844.9039	1687.7932	1687.7940	K.YSQKEDKYEEIK.V
214-226	844.9049	1687.7952	1687.7940	K.YSQKEDKYEEIK.V
214-231	749.0470	2244.1192	2244.1161	K.YSQKEDKYEEIKVLTDLK.L
214-233	829.4418	2485.3036	2485.2951	K.YSQKEDKYEEIKVLTDLKLK.E
218-226	591.7438	1181.4730	1181.5452	K.EDKYEEIK.V
227-238	701.8845	1401.7544	1401.7827	K.VLTDLKLKEATR.A
252-264	776.8154	1551.6162	1551.7304	K.TIDDELDLYAQK.L
269-284	879.8889	1757.7632	1757.8141	K.AISELDHALNDMTSL-
269-284	887.9108	1773.8070	1773.8091	K.AISELDHALNDMTSL-

has been paid to the main allergens which are the small (approximately 12 kD) parvalbumins. In contrast, much less is known about allergens from freshwater fish. Parvalbumin from tilapia was previously described by Lee et al. [30]. Ebo et al. detected specific IgE binding to 18 kD and 45 kD proteins from tilapia in immunoblot analysis with serum from a patient which did not react to parvalbumin [9]. In addition, both collagen from tilapia

(*T. zillii*) [31] and several other IgE-binding protein from tilapia (*O. mossambicus*) [32] are considered potential allergens. The present results from immunoblots using individual sera from ten patients with confirmed tilapia allergy corroborate the finding that a number of different allergens with varying molecular weights are present in *O. mossambicus*. The results also show that despite the many similarities observed, different patients

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1  ATGGATGCCATTAAGAAGAAGATGCAGATGCTCAAGCTCGACAAGGAAAATGCCTTGGACAGAGCTGAGCAGGCC
1  M D A I K K K M Q M L K L D K E N A L D R A E Q A

76  GAGTCAGACAAGAAAGCAGCAGAGGACAGAAGCAACAGCTTGAGGACGATTTGGTAGCTCTGCAGAAGAACTG
26  E S D K K A A E D R S K Q L E D D L V A L Q K K L

151 AAGGGAAGCTGAGGATGAGTTGGACAAGTACTCTGAGGCTCTTAAAGATGCCAGGAGAACTTGAGCTGGCTGAG
51  K G T E D E L D K Y S E A L K D A Q E K L E L A E

226 AAGATAGCCACCGATGCTGAGGAGAGGTCGCTTCCCTTAACAGACGTATCCAGCTGGTTGAGGAGGAGTTGGAT
76  K I A T D A E G E V A S L N R R I Q L V E E E L D

301 CGTGCTCAGGAGCGTCTGGCCACTGCTCTGACCAAGCTGGAGGAGGCTGAGAAGGCTGCTGATGAGAGTGAGAGA
101 R A Q E R L A T A L T K L E E A E K A A D E S E R

376 GGCATGAAGGTCATTGAGAACAGGGCCATGAAGGATGAGGAGAAGATGGAGCTGCAGGAGATCCAGCTGAAAGAG
126 G M K V I E N R A M K D E E K M E L Q E I Q L K E

451 GCCAAACACATCGCTGAGGAGGCTGACCGCAAATATGAGGAGGTTGCCCGTAAACTGGTCATCATTGAGGGTGAT
151 A K H I A E E A D R K Y E E V A R K L V I I E G D

526 CTGGAGCGTACAGAGGAGCGCCGAGCTGTCAGAAGGCAATGCTCTGAGCTTGAGGAAGAGTTGAAACTGTG
176 L E R T E E R A E L S E G K C S E L E E E L K T V

601 ACCAACAACCTGAAGTCACTGGAGGCACAGGCTGAGAAGTACTCACAGAAGGAGGACAAGTATGAGGAGGAGATC
201 T N N L K S L E A Q A E K Y S Q K E D K Y E E E I

676 AAGGCCCTCACTGACAAGCTGAAGGAGGCTGAGACTCGTGCTGAGTTCGCTGAGAGATCAGTAGCCAAGCTTGAG
226 K A L T D K L K E A E T R A E F A E R S V A K L E

751 AAGACCATTTGATGACCTGGAAGATGAGTTGTATGCCCAGAACTGAAGTACAAGGCCATCAGCGAGGAGCTGGAC
251 K T I D D L E D E L Y A Q K L K Y K A I S E E L D

826 CACGCCCTCAACGACATGACTTCCATATAA
276 H A L N D M T S I *

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Fig. 3. The nucleotide sequence and deduced amino acid sequence of the gene encoding the Ore m 4 as determined by sequencing of cDNA. The purple region represents the N-terminal amino acid sequence established by Edman degradation. Amino acid residues in red differ from those of *Thunnus thynnus* tropomyosin. Nucleotide numbers and amino acid residue numbers are indicated.

respond differently to these allergens. Some patient sera showed IgE binding to distinct bands not visible using other sera. The relative IgE titres of the different allergens also appeared to vary extensively between different patients. Conspicuously, parvalbumin, a low molecular weight polypeptide in the 10–14 kD region, appeared not to be significant for the majority of patients. Also for other fresh water fish allergens different from parvalbumins appear more important [27]. Commercially purchased parvalbumin from cod did not inhibit binding in a competitive ELISA test when using patient serum from a patient allergic to blunt snout bream [29]. This supports the finding that parvalbumins may be of lesser importance for freshwater fish allergic patients [27]. Proteins present at high concentration in the vertebrate (rabbit) muscle have been determined [48]. The main soluble proteins are glycolytic enzymes among them enolase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase and triosephosphate isomerase. In addition, high levels of

creatine kinase were found. The high levels of these enzymes are caused by the need to rapidly be able to create a large amount of energy. For tilapia, many of the allergens are still unidentified, though some have been recognized as fructose-bisphosphate aldolase and enolase 3 and thus being among the highly expressed proteins in the cell [32]. One should bear in mind that individuals may have IgE against allergens without showing clinical symptoms [6, 7]. This appears to be the case for several of the tilapia allergic patients. They all have positive ELISA reactions against shrimp tropomyosin (Fig. 4); however, only four of them were reported to have shrimp allergy (Table 1). Another complicating factor is that the IgE strength of signal does not necessarily correlate with severity of symptoms, even though strong signals increase the probability of eliciting a response. In our case, both strong and weak IgE signals were observed. Sometimes patients exhibited weak signals against important allergens. All the patients have documented tilapia allergy. We, however, do not know

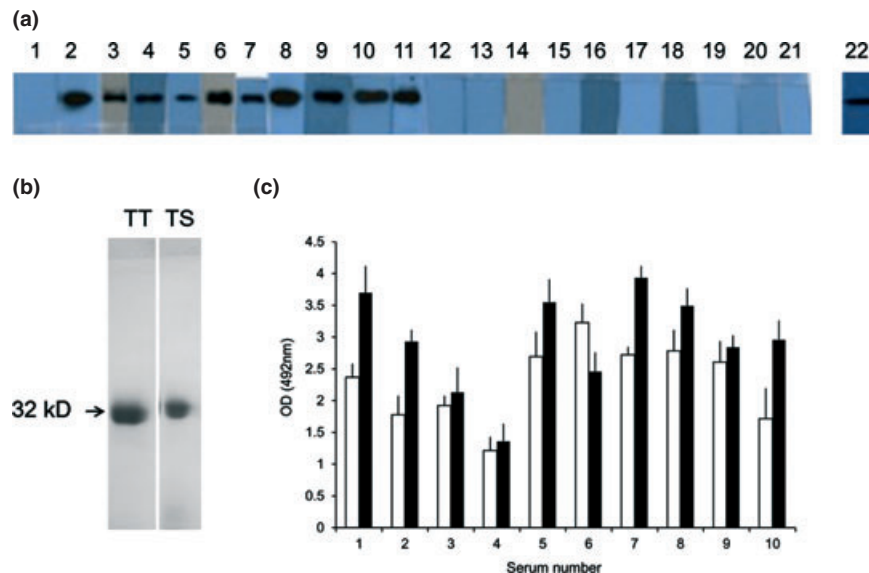


Fig. 4. Immunoblots and ELISA of Ore m 4 allergen purified by RP-HPLC. (a) Immunoblots of pure Ore m 4 allergen (approximately 10 ng/blot). Lane 1, blot incubated with negative control serum. Lanes 2–11, blots incubated with individual patient sera 1–10 respectively. In lanes 1–11, anti-tropomyosin IgE binding was detected using monoclonal anti-IgE antibodies. Lanes 12–21: blots incubated with individual patient sera 1–10 respectively. Lane 22 Human serum protein (IgG heavy chain, 53 kD) blotted as a positive anti-IgG control. In lanes 11–22, bound IgG was detected using monoclonal anti-IgG antibodies. (b) IgE immunoblot analysis of Ore m 4 purified by RP-HPLC (TT) and purified shrimp tropomyosin allergen (TS). The immunoblots were incubated with pooled serum from the 10 tilapia allergic patients. Approximately, 5 μ L of each protein was applied. (c) ELISA using purified Ore m 4 allergen (black columns) or shrimp tropomyosin (open columns) coated wells and incubating with individual sera from the 10 tilapia allergic patients. Bound IgE was quantified using monoclonal anti-IgE antibodies. Average background signals 0.2 from non-allergic individuals were subtracted. Values are averages of three parallels.

which of the allergens are responsible for eliciting reactions. Identifying the allergens responsible for the symptoms must be done by challenge tests using purified allergens.

All of the patients produced IgE against the 32 kD Ore m 4 allergen by immunoblotting (Fig. 4a). This protein was identified by LC-ESI-MS/MS analysis as tropomyosin. Tropomyosins are actin-binding proteins that regulate actin mechanics and are thus important for muscle contraction. They are also highly expressed in vertebrate muscle cells [48]. The nature of the Ore m 4 allergen was further corroborated using ELISA inhibition which showed that Ore m 4 IgE cross-reacted with tropomyosin from shrimp. Tropomyosins from shellfish are considered common major allergens [33]. The cDNA sequence of Ore m 4 was compared with sequences of other known vertebrates and invertebrates (Fig. 7). It is still an open question why the protein sequence deduced from the cDNA sequence differs at three positions from that obtained by mass spectrometry. Isoforms of tropomyosins have been described from other species, but these differ from each other at more than a few positions [35, 49]. As expected the Ore m 4 sequence was very similar to those of other fish tropomyosins. Tropomyosins from vertebrates are generally not considered as allergens [34], even though they share substantial similarity with invertebrate tropomyo-

sins. For example, Ore m 4 showed 58.8% identity with northern shrimp (*Pandalus borealis*) tropomyosin over the 284 the amino acid residues polypeptide chain. Likewise, Ore m 4 was 57.8% identical to the house dust mite *Dermatophagoides farinae* tropomyosin. Due to the conserved sequences, cross-reactions between different seafood tropomyosins and also extending to insects (cockroach) and arachnids (house dust mites) are widespread. Cross-reactions have been reviewed by Lopata and Lehrer [47]. According to FAO/WHO, cross-reactivity between a query protein and a known allergen has to be considered when there is more than 35% identity in the amino acid sequence of the expressed protein, using a window of 80 amino acids and a suitable gap penalty. The similarities between Ore m 4 and other invertebrates by far exceed this limit. The homology of Ore m 4 to human tropomyosin isoform 5 was 87.7%. The allergenicity of animal food proteins is dependent on the evolutionary distance from human homologues [50]. Proteins resembling human counterparts appear to lose their allergenic potential. Jenkins et al. found that proteins with a sequence identity to human homologues above approximately 62% were rarely allergenic. For tropomyosins, it has been suggested that vertebrate homologues are rapidly degraded during digestion, while homologues from crab, shrimp, cockroach and house dust mites will

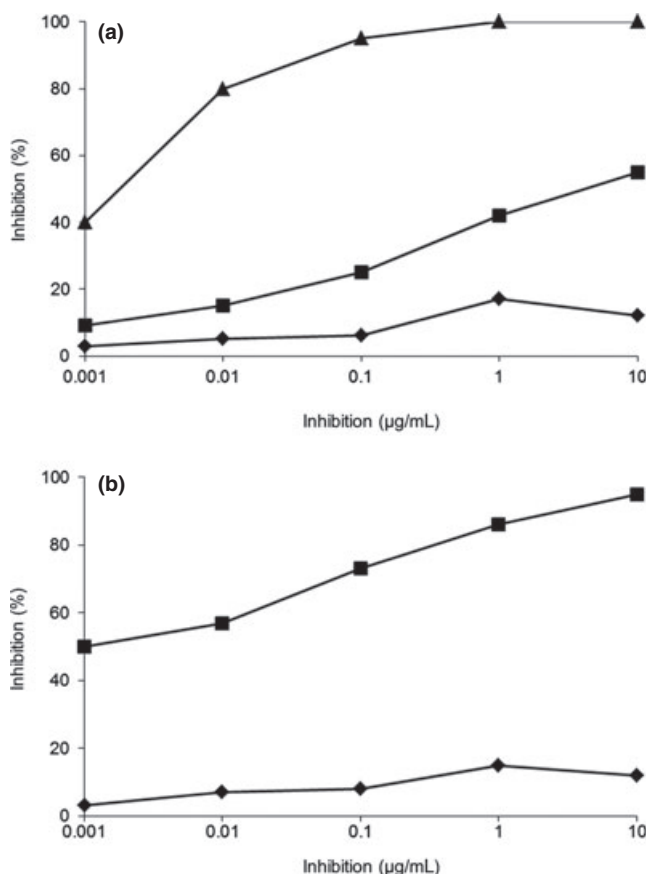


Fig. 5. Cross-reactivity between Ore m 4 and (a) shrimp tropomyosin allergens and (b) tilapia crude extract by competitive ELISA with pooled sera from ten tilapia allergic patients. Bound IgE was detected using monoclonal anti-IgE antibodies. (a) Purified Ore m 4 was attached to the solid support (approximately 0.1 µg Ore m 4/well) and serum binding was inhibited by addition of increasing amounts of purified Ore m 4 (▲) or shrimp tropomyosin (■). (b) Crude extract was attached to the solid support and serum binding was inhibited by addition of increasing amounts of purified Ore m 4. Peanut extract (●) was added as a negative control.

survive gastric digestion and may enter the bloodstream and trigger an immediate hypersensitivity response [34]. Vertebrate tropomyosins, being very similar to human tropomyosins, would challenge the capability of the human immune system to discriminate between foreign and self-protein. Such immune responses run close to becoming autoimmune responses [50]. Human tropomyosin isoform 5 is the predominant isoform expressed in epithelium of the colon and has been described as capable of inducing auto-antibodies and T cell response in ulcerative colitis (UC) [35]. UC and Crohn's disease constitute the two major forms of inflammatory bowel disease (IBD). Antibodies against human tropomyosin isoform 5 are found both in the sera and in the mucosa of patients with ulcerative UC, but not Crohn's disease. These specific IgG auto-antibodies in sera from UC patients induce destruction of colonic epithelial cells,

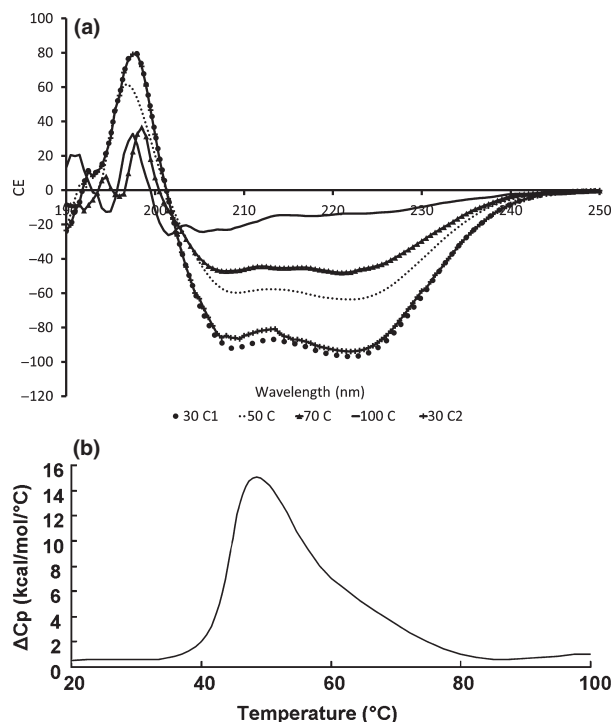


Fig. 6. Structural properties of Ore m 4. (a) CD spectrum of purified Ore m 4 at different temperatures. The heating rate was 10°C per min. 30°C1 and 30°C2, designate CD spectrum at 30°C before and after heating to 100°C respectively. (b) DSC scan of purified Ore m 4.

suggesting a direct pathogenic effect [51]. The C-terminal peptide of human tropomyosin isoform 5 has been implicated in the humoral and cellular immune response in UC [52]. Interestingly, the C-terminal peptide of human tropomyosin isoform 5 is virtually identical to that of Ore m 4 in contrast to those of shellfish tropomyosins. The pathogenic potential of the antibodies against human tropomyosin is however still debated [53]. The prevalence of IBD in the general population is less than 0.1% [54]. Intriguingly, six of the 10 Tilapia allergic patients were also diagnosed with IBD (Table 1). From a statistical point of view, since the patients were selected solely because of their tilapia allergy and given the low prevalence of IBD in the general population, the probability of this outcome purely by chance is exceedingly low. This high correlation corroborates a connection between allergy and IBD. Previous studies generally indicate a higher incidence of allergy in patients diagnosed with IBD (for a review, see [55]). However, further experiments are required to elucidate the exact reason for this high correlation, and whether anti-tropomyosin IgE antibodies can cross-react with human tropomyosin in the epithelium of the colon, bearing in mind that anti-tropomyosin IgG antibodies were not detected in the patient sera. In the literature databases, we found no hits on connections between fish allergy and UC.

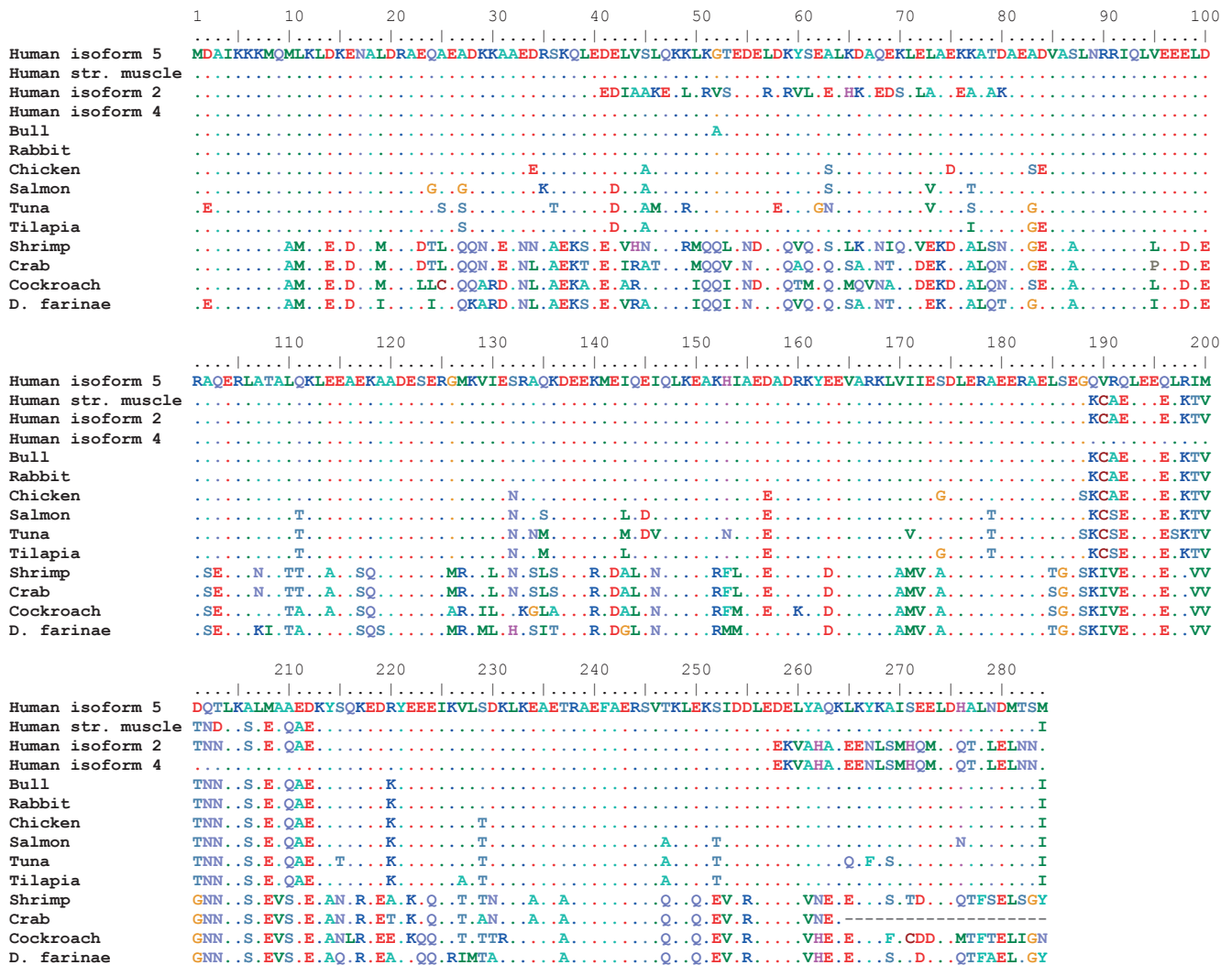


Fig. 7. Alignment from tropomyosins from different organisms. The Genbank accession numbers are given in brackets, human tropomyosin isoform 5 (gi27597085), human striated muscle (gi49660014), human tropomyosin isoform 2 (gi63252902), human tropomyosin isoform 4 (gi63252900), bull (gi73587005), rabbit (gi20178271), chicken (gi20522235), salmon (gi409468), tuna (AB538859), tilapia (this work), shrimp (gi73532979), crab (gi7024506), cockroach (gi4378573) and house dust mite (*D. farinae*, gi42559584).

Tropomyosin has been extensively studied. The crystal structure at 7 Å resolution was published by Whitby and Phillips [56]. Tropomyosin exists in striated muscle as a coiled coil composed of two identical or highly homologous α -helices polymerizing head-to-tail along the actin filament. The spectrum of tilapia tropomyosin also exhibited as expected a typical α -helical structure at 30°C similar to other known tropomyosins. The α -helical structure was gradually lost with increasing temperature, until it reached 100°C, when the α -helical structure essentially completely disappeared. As the temperature was lowered back to 30°C, the protein refolded back to its original secondary structure. These results indicate that the Ore m 4 allergen would regain allergenic activity also in heat treated fish.

Identification of allergens serves several purposes. It forms the basis for development of serum immunoassays to determine food-specific IgE antibodies and makes it possible to develop standardized reference materials for skin prick tests and challenge testing and for immunotherapeutic strategies [6]. Knowledge about the allergens may also be used for developing authentication tests. The traditional advice to fish allergies is to recommend that they avoid fish altogether. Some fish hypersensitive patients tolerate some fish species but not others. If one is allergic to a fish species, the approximate clinical reaction rate to other fish is 50% [6, 57]. Reliable authentication methods are therefore required to protect fish allergic consumers [58]. Such considerations may be of significance because of the

major importance of fish in human nutrition in large parts of the world.

To our knowledge, this is the first report of a tropomyosin allergen from vertebrates. The cross-reactivity with one of the most commonly studied and prevalent seafood allergens helps us better understand the tilapia allergy, and may also shed some light on clinical aspects of UC.

Acknowledgements

We thank National Natural Science Foundation of China NO. 31072248 for the fund support.

Conflict of interest: The authors declare no conflict of interest.

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