

Tilapia (*Oreochromis mossambicus*) allergens characterized by ELISA, SDS-PAGE, 2D gels, Western blotting and MALDI-TOF mass spectrometry

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Abstract

Parvalbumins have long been identified as major allergens in fish, but our research has found that parvalbumins are not the main cause of allergic reactions to tilapia. After homogenization, proteins were extracted from freshwater tilapia to react with a pooled sera sample taken from patients ($n = 20$) tested to be allergic to tilapia. Enzyme-linked immunosorbent assay revealed immunoglobulin E antibody activity, followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis, Western blot, 2D electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to carry out protein separation and analysis. Protein identification against different databases yielded three known high molecular weight proteins as tilapia allergens: chromosome undetermined SCAF7145, fructose-bisphosphate aldolase A and enolase 3 (beta muscle). A fourth, new, unidentified protein with two T-cell receptors was discovered.

Keywords: *fish, 2D SDS-PAGE, MALDI-TOF, ELISA*

Introduction

Fish, as a valuable source of protein, plays an important role in human nutrition (Poms et al. 2004; Lopata and Lehrer 2009). The market for fish products is growing in many countries (Jeebhay et al. 2001; Ebo et al. 2010). However, the increased consumption trend of fish has been accompanied by a rise in the report of clinical reactions. According to recent data, fish is the third most common food allergy in the USA, and can trigger a wide range of allergic symptoms (Sicherer and Sampson 2006). Since the first paper published on fish allergens came out in the 1930s, many following papers did research on the antigenic relationships between different fish species (De Besche 1937; Bernhiselbroadbent et al. 1992; Barletta et al. 2007; Jeebhay et al. 2008; Cai et al. 2010). Though many marine fish allergens have been extensively researched, very few studies have been published on freshwater fish allergens.

In recent years, freshwater fish have gained more and more popularity in markets around the world because of its high quality, texture and flavour. Tilapia (*Oreochromis mossambicus*) is one of the most highly consumed freshwater fish in the world. However, research on the tilapia allergy is scarce. Also, there are no tests commercially available for the detection of the immunoglobulin E (IgE) specific to tilapia.

Many techniques are utilized for the analysis of allergens. As far as the identification of allergens is concerned, enzyme-linked immunosorbent assay (ELISA) and Western blot are the main immunoassay-based methods currently employed (Yu et al. 2003; Chatterjee et al. 2006; Cabanillas et al. 2010; Suzuki et al. 2011). Recently, two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has been increasingly utilized for allergen analysis because of its accuracy (Lecoeur et al. 2010). Proteins are separated according to their isoelectric points in the

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first dimension, and according to their relative molecular masses (M_r) by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. In this study, we successfully applied 2D PAGE separation followed by immunoblotting, and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis identified chromosome undetermined SCAF7145, fructose-bisphosphate aldolase A and enolase 3 (beta muscle) as novel allergens of tilapia.

Materials and methods

Patients and sera

Patients at the Peking University Hospital with a clinical history of tilapia allergy were screened by a double-blind placebo-controlled food challenge and skin prick test. Sera from 20 tilapia-allergic patients were collected, as well as sera from non-atopic laboratory volunteers to use as controls. Samples were frozen at -80°C until use. Initial screening for the presence of specific IgE to tilapia was carried out using individual serums. Reactivity of each serum was tested, and samples with $\text{ABS} > 1$ (Figure 1) were deemed to be experimentally sound. For the remainder of the experiment, the sera were pooled to maximize the amount of antibodies present in each reaction.

Material

Live tilapia (*O. mossambicus*) were purchased at a local supermarket. Specimens were cultured in the laboratory until the time of use, at which they were euthanized.

Protein extraction

Fish muscles (100 g) were homogenized in 200 ml of 1.0 M Tris-HCl buffer (pH 7.5) for 90 s. The homogenate was separated through centrifugation at $12,000g$ for 20 min. The resulting supernatant was filtered through a $0.45\text{-}\mu\text{m}$ membrane filter (Millipore, Bedford, MA, USA).

Enzyme-linked immunosorbent assay

Ninety six-well plates were coated with $0.1\text{ }\mu\text{g}$ crude protein in $100\text{-}\mu\text{l}$ buffer, pH 9.6, then kept overnight at 4°C . This was followed by a wash (PBST buffer, pH 7.4), the addition of patients' sera and 90-min incubation at 37°C . After another wash, HRP-labelled goat anti-human immunoglobulin, IgE antibody (Sigma Chemical Co., St Louis, MO, USA), was used for incubation over 2 h. Finally, after another wash, the colour was developed through incubation with coloured solution. Absorbance read at 492 nm after 10 min. All ELISAs were carried out in

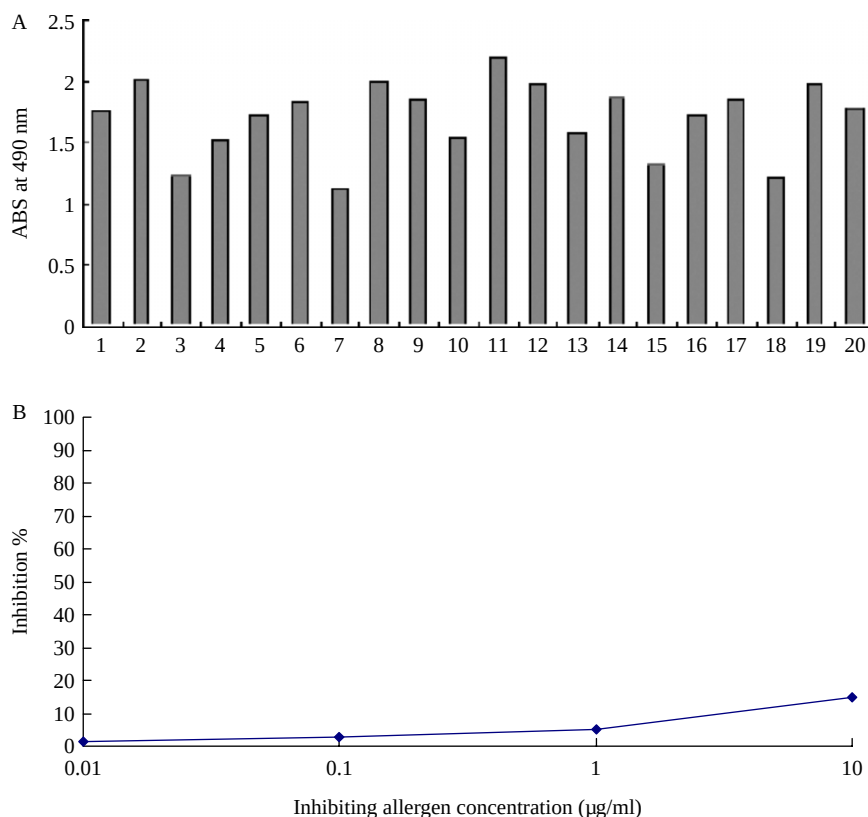


Figure 1. (a) Demonstration of IgE reactivity of patient sera against tilapia protein extract by ELISA. Sera from 20 individuals were incubated with the tilapia protein extract, and the specific binding was monitored with HRP-labelled goat anti-human IgE. (b) ELISA inhibition assay revealing the absence of IgE binding to cod parvalbumin.

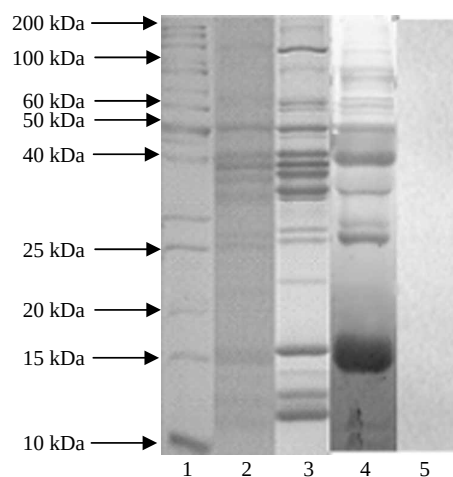


Figure 2. SDS-PAGE and IgE immunoblotting with tilapia proteins. Lane 1, ladder; Lane 2, tilapia proteins visualized with Ponceau S; Lane 3, crude tilapia protein extracts fractionated by SDS-PAGE; Lane 4, the reaction with pooled sera; Lane 5, the reaction with serum from an individual with no history of tilapia allergy.

triplicate, and the data obtained were expressed as mean $\pm$ SD.

Sulphate polyacrylamide gel electrophoresis

Fish extracts were run on 12.5% SDS-PAGE and visualized using Coomassie brilliant blue colloidal staining (Coomassie Brilliant Blue R-250, Bio-Rad Laboratories, Richmond, CA, USA). Molecular weights were determined by SDS-PAGE Standards, Broad Range (10–200 kDa).

Immunoblot analysis

Proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes (Nitrocell, Pharmacia Biotech, San Francisco, CA, USA) using the Mini Trans-Blot Cell (Bio-Rad Laboratories) for 2 h at 100 mA/gel. To reduce non-specific binding, blotted membranes were incubated in blocking buffer (TBS/Tween-20 0.05%, 2% BSA). Strips were then probed overnight at +4°C with patients' sera, while control strips were incubated in buffer alone. Bound IgE antibodies were detected using HRP-labelled goat anti-human immunoglobulin, IgE antibody (Sigma Chemical Co.). Blots were developed by the addition of 4-chloro-1-naphthol buffer (Sigma, St. Louis, USA).

2D PAGE and Western blotting

To carry out 2D electrophoresis experiments combined with Western blotting, 1.5 mg of fish protein were diluted in 120 μ l of IPG rehydration buffer (7.0 M urea, 2.0 M thiourea, 4% CHAPS, 0.5% Bio-Lyte, 10 mg/ml DTT). Samples were actively rehydrated into 17 cm, pH 3–10, IPG strips (Bio-Rad) at 17°C for 12 h using a Protean IEF cell (Bio-Rad).

Isoelectric focusing was carried out for a total of 80 kV-h (ramped to 250 V in 30 min, held at 1000 V after 1 h; ramped to 10,000 V after 5 h, held at 10,000 V for 60 kV-h). The IPG strips were equilibrated in equilibration buffer (6.0 M urea, 2% SDS, 50 mM Tris-HCl, pH 8.8, 30% glycerol) supplemented with 0.5% DTT for 15 min at room temperature followed by 4.5% iodoacetamide in equilibration buffer for another 15 min at room temperature.

A PowerPac 200 power supply (Bio-Rad Laboratories) was used for separation in the second dimension. Protein separation was carried out under a constant current of 60 mA for 6 h in SDS running buffer (2.5 mM MOPS, 2.5 mM Tris base, 0.005% SDS, 0.05 mM EDTA, pH 7.7). Each experiment was duplicated.

2D gel Western blotting was carried out by Bio-Rad Trans-Blot SD semi-dry transfer cells 170–3940 (Bio-

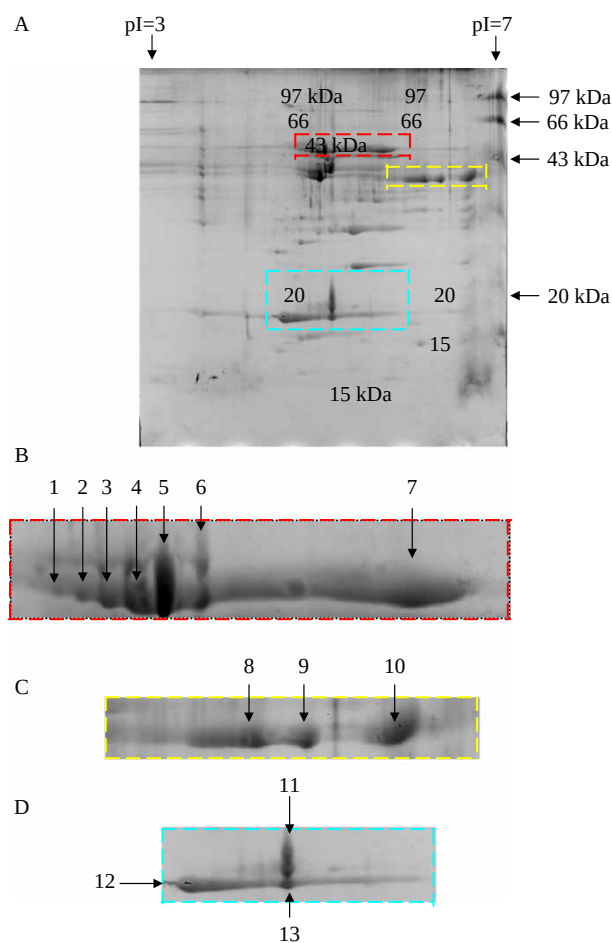


Figure 3. 2D DIGE images and Western blotting of tilapia proteins. (a) 2D gel of tilapia proteins; (b) annotated gel map of proteins of 52 kDa; spot number 1–7; (c) spots of the 37 kDa protein; spot numbers 8–10; (d) spots of the 17 kDa protein; spot numbers 11–13; (e) detection of the major tilapia allergens by 2D Western blotting analysis with the sera pool of 20 patients. Tilapia proteins were transferred to NC membranes. Binding IgE antibodies were detected with HRP-labelled goat anti-human immunoglobulin. Seven spots that responded strongly with the sera pool were selected (5, 7, 9, 10, 11, 12 and 13).

Table I. MALDI-TOF MS tryptic peptide ions matched from spot 5.

Start-end	Observed	M_r (expt)	M_r (calc)	ppm	Miss sequence	Sequence
10-15	732.3854	731.3782	731.3813	-4	0	R.EILDSR.G
16-28	1429.7083	1428.7010	1428.7249	-17	0	R.GNPTVEVDLWTAK.G
33-50		1763.9012	1763.9166	-9	0	R.AAVPSGASTGVHEALELR.D
81-89	1093.5476	1092.5403	1092.5815	-38	1	K.KFSVVEQEK.I
82-89	965.4728	964.4656	964.4866	-22	0	K.FSVVEQEK.I
82-103	2599.3176	2598.3104	2598.2523	22	1	K.FSVVEQEKIDQFMLELDGTENK.S
90-103	1652.7975	1651.7902	1651.7763	8	0	K.IDQFMLELDGTENK.S
133-162	3061.6724	3060.6651	3060.6257	13	1	R.HIADLAGHKDVLVPVAFNVINGGSHAGNK.L
142-162	2119.1233	2118.1160	2118.1222	-3	0	K.DVILPVPVAFNVINGGSHAGNK.L
163-183	2424.1453	2423.1380	2423.1436	-2	0	K.LAMQEFMILPVGAANFHEAMR.I
184-193	1143.5996	1142.5923	1142.6084	-14	0	R.IGAEVYHNLK.S
203-228	2743.3643	2742.3570	2742.3348	8	0	K.DATNVGDEGGFAPNILENNEALELLK.S
234-253	2216.0942	2215.0870	2215.0983	-5	1	K.AGYDPKIIIGMDVAASEFYR.S
257-269	1554.6926	1553.6854	1553.6998	-9	1	K.YDLDFKSPDDPSR.H
276-281	736.3920	735.3848	735.3915	-9	0	K.LGDLR.S
344-358	1633.7988	1632.7916	1632.8505	-36	1	K.VNQIGSVTESIKACK.L
359-372	1514.7416	1513.7343	1513.7460	-8	0	K.LAQSSGWGVMVSHR.S
395-400	661.2996	660.2923	660.3013	-14	0	K.TGAPCR.S
407-412	840.4004	839.3931	839.3959	-3	0	K.YNQLMR.I
413-422	1131.5703	1130.5630	1130.5819	-17	1	R.IEEELGDKAK.F

Rad Laboratories) at a voltage of 18 V for 40 min. The Ponceau S dye reagent was used after Western blotting to check transfer efficiency. Other steps were described under immunoblot analysis.

Protein in-gel digestion

Gels were stained with Coomassie Brilliant Blue. Protein spots of interest were excised and destained using 25 mM ammonium bicarbonate, 50% ACN. Gels were then completely dried by centrifugal lyophilization. In-gel digestion was carried out using 0.01 µg/µl trypsin (Promega, Madison, USA) in 25 mM ammonium bicarbonate for 15 h at 37°C. The supernatants were collected, and the tryptic peptides were sequentially extracted from the gel with 5% TFA at 40°C for 1 h and with 2.5% TFA, 50% ACN at 30°C for 1 h. The extracts were pooled and dried completely by centrifugal lyophilization.

Protein identification

Peptide mixtures were redissolved in 0.5% TFA, and 1 µl of peptide solution was mixed with 1 µl of matrix (4-hydroxy-cyanocinnamic acid in 30% ACN, 0.1%

TFA) before spotting on the target plate. MALDI-TOF mass spectrometry (MS) and tandem TOF/TOF MS were carried out on a 4700 Proteomics Analyzer (Applied Biosystems, Foster city, USA). Peptide mass maps were acquired using the positive reflection mode, averaging 1500 laser shots per MALDI-TOF spectrum and 3000 shots per TOF/TOF spectrum (the resolution was 20,000). The 4700 calibration mixtures (Applied Bio systems, Foster city, USA) were used to calibrate the spectrum to a mass tolerance within 0.1 Da. Parent mass peaks with a mass range of 600–4000 Da and a minimum signal-to-noise ratio of 15 were picked out for tandem TOF/TOF analysis. Mass database searches were carried out using Profound (<http://prowl.rockefeller.edu/prowl/cgi/profound.exe>), Mascot (<http://www.matrixscience.com>) and MS-FIT (<http://prospector.ucsf.edu>) online software. The comparisons between different database searches are a valuable check on protein identification through informatics analysis. Peptide tolerance was 500 ppm. All of the automatic data analysis and database searching were fulfilled by GPS Explorer TM software (version 3.6, Applied Biosystems). Known contaminant ions (keratin) were excluded. The confident identification had a statistically significant ($p \leq 0.05$)

Table II. MALDI-TOF MS tryptic peptide ions matched from spot 7.

Start-end	Observed	M_r (expt)	M_r (calc)	ppm	Miss sequence	Sequence
10-28	2120.1426	2119.1353	2119.0797	26	1	R.EILDSRGNPTVEVDLYTAK.G
106-126	2047.1400	2046.1327	2046.0932	19	1	K.FGANAILGVSLAVCKAGAAEK.G
163-183	2424.2222	2423.2149	2423.1436	29	0	K.LAMQEFMILPVGASNFHEAMR.I
184-193	1143.6206	1142.6133	1142.6084	4	0	R.IGAEVYHNLK.A
203-228	2743.4092	2742.4019	2742.3348	24	0	K.DATNVGDEGGFAPNILENNEALELLK.A
336-343	991.4962	990.4889	990.4990	-10	0	K.ACNCLLLK.V
344-358	1633.8282	1632.8210	1632.8141	4	0	K.VNQIGSVTESIQACK.L
373-394	2353.2036	2352.1963	2352.1519	19	0	R.SGETEDTFIADLVVGLCTGQIK.T
407-412	824.4268	823.4195	823.4010	22	0	K.YNQLMR.I

Table III. MALDI-TOF MS tryptic peptide ions matched from spot 9.

Start-End	Observed	M_r (expt)	M_r (calc)	ppm	Miss sequence	Sequence
2-13	1427.7148	1426.7076	1426.7245	-12	0	M.PHAYPFLTPEQK.K
29-42	1318.6417	1317.6344	1317.6776	-33	0	K.GILAADESTGSVAK.R
29-43	1474.8002	1473.7929	1473.7787	10	1	K.GILAADESTGSVAKR.F
33-50	1764.9084	1763.9012	1763.9166	-9	0	R.AAVPSGASTGVHEALELR.D
44-56	1551.6973	1550.6900	1550.6961	-4	0	R.FQSINAENTENRR.R
44-57	1707.7888	1706.7815	1706.7972	-9	1	R.FQSINAENTENRR.L
109-134	2690.3655	2689.3582	2689.3559	1	1	K.VDKGVVPLAGTNGETTTTQGLDGLYER.C
112-134	2348.1799	2347.1727	2347.1656	3	0	K.GVVPLAGTNGETTTTQGLDGLYER.C
141-147	723.3016	722.2943	722.3235	-40	0	K.DGADFAK.W
154-161	872.4899	871.4827	871.4763	7	0	K.ITPTTPSR.L
162-173	1296.7507	1295.7435	1295.7561	-10	0	R.LAIENANVLAR.Y
174-200	3046.5032	3045.4959	3045.4940	1	0	R.YASICQMHGIVPIVEPEILPDGDHDLK.R
174-201	3202.6045	3201.5972	3201.5951	1	1	R.YASICQMHGIVPIVEPEILPDGDHDLKR.C
216-243	3077.5891	3076.5818	3076.5222	19	0	K.ALSDDHHVYLEGTLTKPNMVTAGHACSQK.N
323-330	1024.4612	1023.4539	1023.4695	-15	0	K.ACQEEFIK.R
323-331	1180.5687	1179.5614	1179.5706	-8	0	K.ACQEEFIKR.A

protein score (based on combined mass). Protein redundancy, which occurred when proteins appeared in the database under different names and accession numbers, was eliminated. If more than one protein was identified in one spot, the single protein member with the highest protein score (top rank) was singled out from the multiprotein family. The molecular weight and pI values of most proteins were consistent with the gel regions from which the spots were excised.

Results

The ImmunoCAP system is a common way to test allergens. But this assay cannot be used to test for the tilapia IgE specifically. Consequently, ELISA assays were used to test the reactivity of IgE in 20 patients' sera against tilapia crude extract. The reactivity of each of the 20 sera samples was revealed to be at very high levels (Figure 1). Having all met the criteria of $ABS > 1$, the samples were pooled and further characterized by immunoblotting.

Protein components of the tilapia crude extract were analysed by SDS-PAGE and IgE immunoblotting using the sera pool from first step (results are shown in Figure 2). The SDS-PAGE profiles of the fish extracts showed multiple protein bands ranging from 10 kDa to 200 kDa to. IgE-binding proteins were identified at 114, 73, 64, 52, 47, 37, 32, 28, 26, and 17 kDa.

Parvalbumin is a calcium-binding, low molecular weight protein of an acidic nature. It has a remarkable resistance to heat and enzymatic degradation proteins, and is the best understood allergen within fish (Bugajska-Schretter et al. 1998, 1999, 2000; Beale et al. 2009). A prominent 17 kDa protein appears in the tilapia Western blot results. To see if there was any relationship between the 17 kDa protein and parvalbumin, we utilized the serum from the tilapia-allergic patients to see if it would recognize the commercially available cod parvalbumin. The result

showed no specific binding, which concludes that this parvalbumin protein is not responsible for the tilapia allergy (Figure 1).

2D electrophoresis analysis of proteins extracted from tilapia was carried out using isoelectrofocusing followed by SDS-PAGE. Coomassie blue was utilized to stain the 2D gel of tilapia protein extract, from Figure 3a we can see the visualization of more than 50 protein spots ranging in molecular masses from 10 to 100 kDa. The majority of these proteins, spots have pI values ranging from 3 to 7. Sera pools from the 20 patients were used for immunolabelling. To exclude artefacts caused by electrophoresis conditions, 2D gels were run in triplicate.

Panel A in Figure 3 shows the gel map of proteins from the raw tilapia extract.

The 52 kDa protein separated into seven distinct spots with isoelectric points ranging from 7.2 to 8.9, respectively (Figure 3b). The 37 kDa protein was resolved into three distinct spots with isoelectric points from 8.8 to 10 (Figure 3c). The 17 kDa protein was located in three spots having isoelectric points from 6 to 7 (Figure 3d).

Proteins separated by 2D gels were transferred onto a nitrocellulose membrane and incubated with pooled sera. One spot of 52 kDa protein, two spots of 37 kDa protein and all the spots of 17 kDa protein showed strong reaction with the patients' sera. Protein spots of interest were cut from the gel, and after in-gel digestion with trypsin, the extracted peptides were analysed by MALDI-TOF. CNBI databank was used. Tolerance of mass calibration on the TOF was set to 30 ppm. Potential modifications were also considered. The protein with the highest score was chosen.

Spot 5 (Table I) showed homology to chromosome undetermined SCAF7145 of *Tetraodon nigroviridis* (green puffer; Table II). Spot 9 (Table III) was identified as fructose-bisphosphate aldolase (Nakamura et al. 2009) A of *Danio rerio* (zebrafish). Spot 7 (Table II) was identified as enolase 3 (beta muscle) of

Table IV. Protein sequence of the three immunodominant spots identified with 2D SDS-PAGE and MALDI-TOF MS.

Spot no	Accession no	Sequence coverage	Number of mass values matched	Sequence
5	Q4TBD1_TETNG	57%	31	1 MSITKIQARE ILDSRGNPTV EVDLWTAKGL FRAAVPSGAS TGVHEALELR 51 DGDKSRYLK GTLKAVDHVN KDIAPKLIEK KFSVVEQEKI DQFMLELDGT 101 ENKSKFGANA ILGVSLAVCK AGAAEKGLPL YRHIADLAGH KDVLVPFAP 151 NVINGGSHAG NKLAMQEFMI LPVGAAFNHE AMRIGAEVYH NLKSVIKAKY 201 GKDATNVGDE GGFAPNILEN NEALELLKSA IEKAGYDPKI IIGMDVAASE 251 FYRSGKYDLD FKSPDDPSRH ISGEKLGDL RSFIQNYYPVQ SIEDPFDQDD 301 WENWAKFTAS TDIQIVGDDL TVTNPKRIQK AVDKKACNCL LLKVNQIGSV 351 TESIKACKLA QSSGWGVMVS HRSGETEDTF ISDLVVGLCT GQIKTGAPCR 401 SERLAKYNQL MRIEELGDK AKFAGKNFRH P 1 MSILKIHARE ILDSRGNPTV EVDLYTAKGL FRAAVPSGAS TGIYEALELR 51 DGDKSRYLK GVLKAVEHIN KTIIVPALLEK KLSVVEQEKI DKVMLELDGT 101 ENKSKFGANA ILGVSLAVCK AGAAEKGVPL YRHIADLAGN SELVLPFAP 151 NVINGGSHAG NKLAMQEFMI LPVGASNFHE AMRIGAEVYH NLKAVIKAKY 201 GKDATNVGDE GGFAPNILEN NEALELLKAA IEKAGYDPKI VIGMDVAASE 251 FYRKGKYDLD FKSPDDPNRY ISGEKLGDL KSFISYYPV SIEDPFDQDD 301 WDTWKSFLST VDIQIVGDDL TVTNPKRIQK GVEQKACNCL LLKVNQIGSV 351 TESIQACKLA QSSGWGVMVS HRSGETEDTF IADLVVGLCT GQIKTGAPCR 401 SERLAKYNQL MRIEELGDK AKFAGNRFRN PRGK 1 MPHAYPFLTP EQKKELSDIA QRIVAPGKGI LAADESTGSV AKRFQSINAE 51 NTEENRRLYR QLLFTADDRV KPCIGGVILF HETLYQKTDG GKVFSYDLKE 101 RGMVVGKVD KGVVPLAGTN GETTTQGLDG LYERCAQYKK DGADFAKWRC 151 VLKITTPTPS RLAIENANV LARYASICQM HGIVPIVEPE ILPDGDHDLK 201 RCQYVTEKVL AAVYKALSDH HVYLEGTLLK PNMVMTAGHAC SQKNTPQEIA 251 MATVTALRRT VPPAVPGVTF LSGGQSEEEA TLNLNAMNQC PLHRPWALTF 301 SYGRALQASA LKAWGGKKEN GKACQEEFIK RALNNSQACV GKVVSSGDKG 351 AAAGESLFVA NHAY
7	gi 147904511	34%	9	
9	gi 41282154	43%	15	

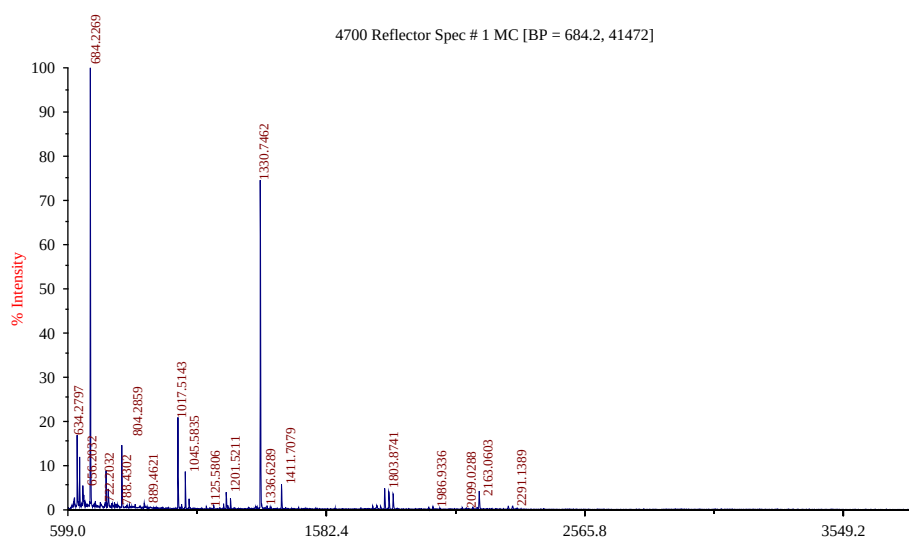


Figure 4. Mass spectra map of spots 11, 12 and 13 proteins.

Xenopus laevis. The protein sequences of these three allergens were shown in Table IV.

Discussion

The amino acid sequence of spot 5 was submitted to the allergen database, and showed 70.77% homology to Hevb 9, the main allergen of *Hevea latex* (Wagner et al. 2000).

The 37kDa allergen was identified as fructose-bisphosphate aldolase, which catalyses the reversible aldol condensation of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate to yield fructose bisphosphate. Class-I aldolase catalysis depends upon Schiff base formation with the substrate (Horecker et al. 1963). In class-II aldolases, found in fungi and prokaryotes, divalent cations are required for activity (Rutter 1964). A lot of allergens are cation dependent (Wopfner et al. 2007). For example, parvalbumin, the major marine fish allergen, requires calcium binding (Gajewski and Hsieh 2009).

Enolase (2-phospho-D-glycerate hydrolase) plays a very important role as a ubiquitous dimeric glycolytic enzyme that catalyses the dehydration of 2-phosphoglycerate to phosphoenolpyruvate (Lebioda et al. 1989). Its amino acids are highly conserved from bacteria to higher vertebrates (Vanderstraeten et al. 1991). Although there have not been reports of the enolase allergen residing in fish, enolase appears as a major antigenic protein in several organisms, including *Alternaria alternata* (Achatz et al. 1995), *Candida* (Ballantyne and Warmington 2000), *Rhodotorula mucilaginosa* (Chang et al. 2002), cockroach (Chuang et al. 2010), *Fusarium solani* (Verma et al. 2003), *H. latex* (Wagner et al. 2000) and more. Thus, the enolase-like protein may be an allergen in tilapia.

According to mass spectra shown in Figure 4, spots 11–13 actually represented one protein, with its most

intense signals at 1330.7 and 664.2 m/z . Because the full length is not match to any known protein and the score is not statistically significant. So there was not enough convincing data to confirm it as any of the known proteins in the databases. But we found that the peak with 1330.7 m/z identified as a T-cell receptor J-alpha wnix.2 in humans. (Accession No CAA50371), 644.2 m/z was identified as human T-cell receptor trans rearranged gamma delta-chain gene delta-1-D-delta-J-gamma region (Accession No AAA36682). This might be the reason why the protein reacted so strongly to the patients' sera. This may be a newly discovered allergen in fish that will be further studied.

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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