JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. XXXX, XXX, 000–000 A
DOI:10.1021/jf103942p

Characterization of Allergens Isolated from the Freshwater Fish Blunt Snout Bream (*Megalobrama amblycephala*)

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Fish are an important source of dietary protein for humans throughout the world. However, they are recognized as one of the most common food allergens and pose a serious health problem in countries where fish consumption is high. Many marine fish allergens have been extensively studied, but relatively little is known about freshwater fish allergens. This study identified two main allergens from blunt snout bream (*Megalobrama amblycephala*), a freshwater fish widely consumed in China. Sera from 11 patients with convincing clinical history of blunt snout bream allergy were utilized in IgE immunoblot analysis to identify prominent allergens. Several blunt snout bream proteins revealed specific binding to serum IgE, with the 47 and 41 kDa proteins being the most immunodominant among them. Two-dimensional gel electrophoresis (2D SDS-PAGE) enabled resolution of the 47 and 41 kDa proteins into several protein spots with distinct isoelectric points. 2D SDS-PAGE along with IgE immunoblot analysis further confirmed the strong reactivity of these protein spots with the pooled sera from blunt snout bream-sensitive patients. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of the peptides generated by trypsin digestion of the different spots corresponding to the 47 and 41 kDa proteins indicated that these spots were isoforms of enolase and muscle creatine kinase, respectively. The potential allergenicity of these proteins was further verified by an bioinformatics approach using the full-length and 80 amino acid sliding window FASTA searches, which revealed a significant amino acid sequence homology between blunt snout bream allergens and several known inhaled and crustacean allergens.

KEYWORDS: Fish allergen; blunt snout bream; IgE immunoblotting; enolase; creatine kinase

INTRODUCTION

Fish are an important source of dietary protein for humans. They also provide nutrients that are vital for health and maintenance of the human body. In addition to their nutritional value, some fish contain large amounts of the omega-3 fatty acids, which can reduce the risk of cardiovascular disease (*1*). Fish consumption has steadily grown worldwide. Proteins derived from fish, crustaceans, and mollusks account for 13.8−16.5% of the total animal protein intake of the human population (http://www.who.int/nutrition/topics/3_foodconsumption/en/index5.html). However, fish also ranks among the eight most significant food allergens that can trigger gastrointestinal symptoms, asthma, oral allergy syndrome, allergic dermatitis, and even life-threatening anaphylaxis (*2*). Due to this potential health risk, extensive research has been devoted to fish allergens. A variety of allergens have been identified from marine fish such as cod, salmon, pollack, mackerel, tuna, herring, wolfish, and halibut (*3*−*7*). However, few allergens have been identified in freshwater fish.

China has one of the highest rates of fish consumption (*8*). Freshwater fish are a major source of protein in the Chinese diet. The blunt snout bream (*Megalobrama amblycephala*), known for its high nutritive quality and flavor, is one of these fish. The blunt snout bream lives in freshwater at depths ranging from 5 to 20 m and is abundant in Wuchang county, Hubei province, China. Due to the prevalence of freshwater fish in the Chinese diet, an interest in the adverse effects of freshwater fish-induced allergic reactions has also increased among the Chinese population.

In the past, one-dimensional protein electrophoresis (1D SDS-PAGE) and IgE immunoblotting analysis have been widely used for the identification of food allergens (*6*, *7*). Recently, two-dimensional protein electrophoresis-based approaches (2D SDS-PAGE) have been used for more accurate identification of food allergens (*9*−*11*). In this study, we identified two immunodominant allergens from blunt snout bream using a combination of 1D SDS-PAGE, 2D SDS-PAGE, IgE immunoblotting, and MALDI-TOF MS. Our study revealed that the two dominant allergens of the blunt snout bream are enolase and muscle creatine kinase.

MATERIALS AND METHODS

Patients and Sera. Eleven patients who had been previously diagnosed as allergic to blunt snout bream were chosen from Peking University Health Science Center, Beijing, China, for this study. All patients had a convincing clinical history and showed positive reaction to double-blind

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placebo-controlled food challenge and skin prick tests. Initial screening for the presence of specific IgE to blunt snout bream was performed using individual serum, but for subsequent studies the sera were pooled. For the control samples, serum was collected from nonatopic laboratory volunteers. Informed consent was obtained from each volunteer. Serum from the patients was stored at −80 °C until used.

Preparation of Fish Extracts. Blunt snout bream (*M. amblycephala*) specimens were purchased in a local supermarket. The age of the fish at the time of harvest was approximately 1 year. Specimens were euthanized and stored at −80 °C until used. Fish muscles (100 g) were homogenized in 200 mL of 20 mM Tris-HCl buffer (pH 7.5) for 90 s. The homogenate was clarified by centrifugation at 12000 rpm for 20 min. The resulting supernatant was filtered through a 0.45 μm filter (Millipore, Bedford, MA) and protein quantified using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Enzyme-Linked Immunosorbent Assay (ELISA). Ninety-six-well plates were coated with 0.1 μg of crude protein in 100 μL of 20 mM phosphate-buffered saline (PBS) buffer (pH 9.6). Coating was performed overnight at 4 °C. This was followed by two washings with PBST buffer (20 mM PBS with 0.05% Tween-20, pH 7.4) for 2 min each. Residual binding sites were saturated by incubation with blocking buffer (2% glutin in 20 mM PBS/0.1% Tween-20) for 1 h at room temperature. Patients' sera (diluted 1:50 v/v in blocking buffer) were added and incubated for 1.5 h at 37 °C. Unbound antibody was removed by four washings with PBST. To each well was added 100 μL of HRP-labeled goat anti-human IgE antibody (Sigma Chemical Co., St. Louis, MO) diluted 1:8000 (v/v) in blocking buffer. The plates were incubated at 37 °C for 30 min and then washed four times with PBST. Peroxidase substrate (KPL, Inc., Gaithersburg, MD) was added. Reactions were stopped after 10 min by adding 100 μL/well 6 N H_2SO_4, and optical densities (OD) were read at 490 nm on a microplate reader. For controls, serum from nonatopic laboratory volunteers was used instead of patients' sera. All ELISAs were performed in triplicate, and the data obtained were expressed as the mean values. Individual fish sample readings were considered to be significant if the average allergic subject reading (minus the background) was greater than the average control serum reading (minus the background) plus two standard deviations for that fish extract.

1D SDS-PAGE. One-dimensional protein separation followed the method of Laemmli (*12*). Aliquots of fish protein sample (10 μL) were resolved on 12.5% SDS-PAGE under reducing conditions and visualized by Coomassie Blue G-250 staining.

Immunoblot Analysis. Proteins separated by 1D SDS-PAGE were blotted onto nitrocellulose membrane (Pharmacia Biotech, San Francisco, CA) using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) for 2 h at 100 mA/gel. To reduce nonspecific binding, blotted membranes were incubated in blocking buffer (TBS/Tween-20 0.05%, 2% BSA) for 1 h. The nitrocellulose membrane was cut into several strips, and each strip was probed overnight at 4 °C with patients' sera (1:10 dilution). Strips incubated with nonatopic volunteers' sera served as the control. Bound IgE antibodies were detected with HRP-labeled goat anti-human IgE antibody (1:4000 dilution, Sigma). Blots were developed by the addition of 4-chloro-1-naphthol buffer (Sigma).

2D SDS-PAGE and IgE Immunoblotting. To perform 2D electrophoresis experiments combined with IgE immunoblotting, 1.5 mg of fish protein was diluted in 120 μL of IPG rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Bio-Lyte 3/10, 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 performed for a total of 80 kV·h (250 V ramp for 30 min, held at 1000 V for 1 h, ramped to 10000 V in 5 h, and held at 10000 V for 60 kV·h). Following IEF, the IPG strips were incubated in equilibration buffer (6 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 incubation with 4.5% iodoacetamide in equilibration buffer for another 15 min at room temperature. Protein separation was carried out with 60 mA constant current for 6 h in SDS-PAGE running buffer (2.5 mM MOPS, 2.5 mM Tris base, 0.005% SDS, 0.05 mM EDTA, pH 7.7). Each experiment was performed in duplicate. Two-dimensional gel IgE immunoblotting was carried out using a Trans-Blot SD semidry transfer cell (Bio-Rad) with 18 V for 40 min. Ponceau S stain was used to check protein transfer efficiency.

Protein In-Gel Digestion. Spot picking was carried out with preparative gels that were stained with Coomassie Brilliant blue. Protein spots of interest were excised and destained with 25 mM ammonium bicarbonate and 50% acetonitrile. Gel pieces were then dried completely by centrifugal lyophilization. In-gel digestion was performed with 0.01 μg/μL trypsin (Promega Corp., Madison, WI) in 25 mM ammonium bicarbonate for 15 h at 37 °C. The supernatants were collected, and the tryptic peptides were extracted from the gel sequentially with 5% trifluoroacetate (TFA) at 40 °C for 1 h and with 2.5% TFA and 50% ACN at 30 °C for 1 h. The extracts were pooled and dried completely by centrifugal lyophilization.

Protein Identification. Peptide mixtures were dissolved in 0.5% TFA, and 1 μL of peptide solution was mixed with 1 μL of matrix (4-hydroxycyanocinnamic acid in 30% ACN and 0.1% TFA) before spotting on the target plate. MALDI-TOF MS and tandem TOF/TOF MS were carried out on a 4700 Proteomics Analyzer (Applied Biosystems, Carlsbad, CA). Peptide mass maps were acquired in positive reflector mode, averaging 1500 laser shots per MALDI-TOF spectrum and 3000 shots per TOF/TOF spectrum (the resolution was 20000). The 4700 calibration mixtures (Applied Biosystems) 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 minimum signal-to-noise ratio of 15 were picked for tandem TOF/TOF analysis. Mass database search was carried out using Profound (http://prowl.rockefeller.du/prowl-cgi/profound.exe), Mascot (http://www.matrixscience.com), and MS-FIT (http://prospector.ucsf.edu) for all peptide mass data comparison and protein identification. Peptide tolerance was set at 50 ppm. All of the automatic data analyses and database searches were carried out with GPS Explorer software (version 3.6, Applied Biosystems). Known contaminant ions (e.g., trypsin, keratin) were excluded. Protein scores that were statistically significant ($p \leq 0.05$) are reported. Redundancy of proteins that appeared in the database under different names and accession numbers were eliminated. If more than one protein was identified in one spot, the single protein member with the highest score (top rank) was singled out from the multiprotein family. The molecular weight and p*I* values of most proteins were consistent with the gel regions from which the spots were excised.

RESULTS AND DISCUSSION

Reactivity of IgE in Patients' Sera with Blunt Snout Bream Crude Extract. The ImmunoCAP system (CAP) has been widely used in the evaluation of allergens. Currently, an ImmunoCAP assay to measure blunt snout bream-specific IgE is not available. Consequently, we tested the reactivity of IgE in 11 patient's sera with blunt snout bream crude extract by ELISA. Sera from all 11 patients reacted in a dose-dependent manner to blunt snout bream crude extract (**Figure 1A**). The reactivity of the sera from patients 1, 2, 5, and 6 to blunt snout bream crude extract was less pronounced than that of sera from patients 3, 4, and 7−11. We also performed a competitive ELISA inhibition experiment using commercially purchased cod parvalbumin, a major fish allergen (*13*). Interestingly, parvalbumin did not inhibit the IgE reactivities of the blunt snout bream allergic patients (**Figure 1B**). Because the sera from patients 3, 4, and 7−11 revealed strong IgE reactivities to blunt snout bream crude extract, they were chosen for further characterization by immunoblotting.

Identification of the Major Allergen from Blunt Snout Bream. The protein component of the crude extract from blunt snout bream was analyzed by SDS-PAGE. Several protein bands ranging from 10 to 200 kDa were detected. The most prominent among them had molecular masses of 115, 47, 41, 36, 26, and 11 kDa (**Figure 2**). Individual sera from patients allergic to blunt snout bream reacted to most of these abundant proteins as well as some low abundant proteins. Even though differences in the molecular weight of proteins and the intensity of IgE reactivity were detected among individual sera, the 47 and 41 kDa proteins showed positive reaction with sera from all blunt snout bream allergic patients. Under identical experimental conditions, sera obtained from nonatopic laboratory volunteers showed no reaction against any of the fish proteins (**Figure 2**). Previous studies have shown paravalbumin, a 12 kDa calcium-binding sarcoplasmic protein, to be a major fish allergen protein (*13*). This protein

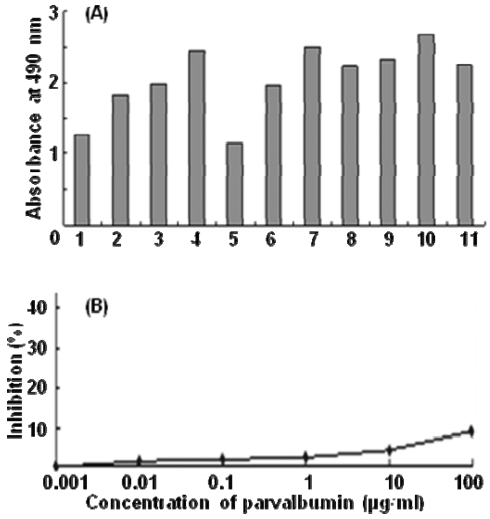

Figure 1. (**A**) Demonstration of IgE reactivity of patient sera against blunt snout bream protein extract by ELISA. Sera from 11 individuals were incubated with the blunt snout bream protein extract, and the specific binding was monitored with HRP-labeled goat anti-human IgE. (**B**) ELISA inhibition assay revealing the absence of IgE binding to cod paravalbumin. Inhibition assays were performed by incubating the pooled serum with increasing concentrations of cod paravalbumin.

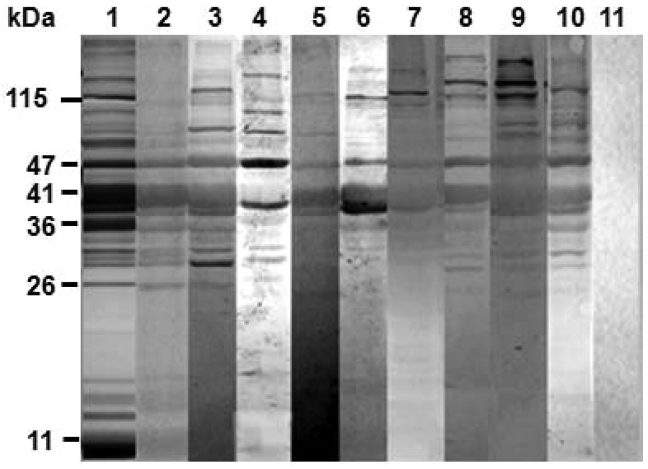

Figure 2. SDS-PAGE and IgE immunoblotting of blunt snout bream proteins. Crude blunt snout bream protein extracts fractionated by SDS-PAGE were transferred to nitrocellulose membranes, cut into strips, and incubated individually with sera from blunt snout bream-sensitive patients (lanes 4−10) or serum from an individual with no history of blunt snout bream allergy (lane 11). Lane 3 shows the reaction with pooled sera. Immunoreactive proteins were identified using anti-human IgE−horseradish peroxidase conjugate. Lane 1, blunt snout bream proteins visualized with Coomassie Blue R-250; lane 2, visualization of proteins transferred to nitrocellulose with Ponceau S. The sizes of the prominent blunt snout bream proteins in kDa are shown on the left of the figure.

is mainly responsible for food allergy in populations where fish consumption is high (*13*, *14*). A prominent 11 kDa protein is also present in the blunt snout bream protein extracts (**Figure 2**). To verify if the 11 kDa protein is related to paravalbumin, we performed immunoblot analysis using commercially available cod paravalbumin with pooled sera from patients allergic to blunt snout bream. No reactivity was detected against the purified paravalbumin, suggesting that this protein is not responsible for the blunt snout bream allergenicity.

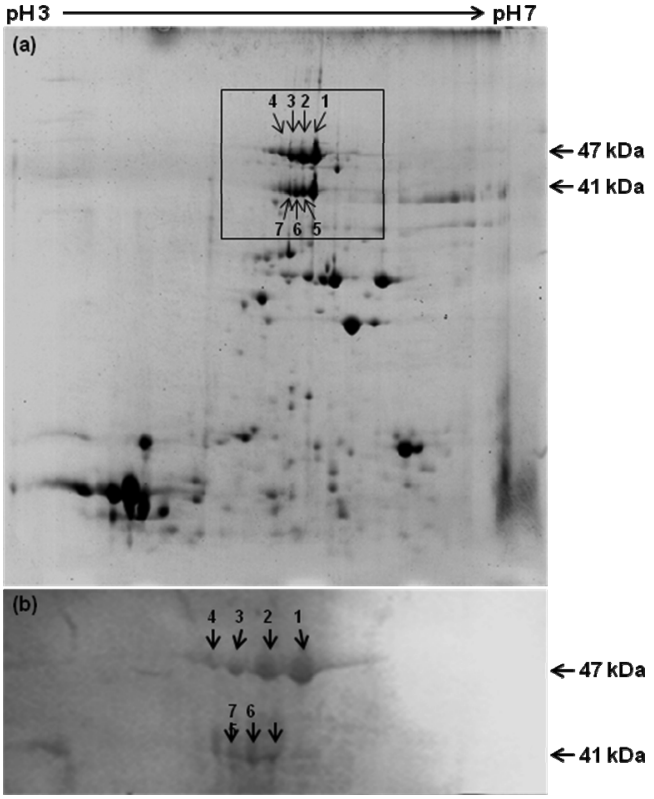

Figure 3. 2D SDS-PAGE and IgE immunoblot analysis of blunt snout bream allergens: (**A**) Coomassie blue-stained 2D gel (the prominent 47 and 41 kDa proteins separated into distinct spots are enclosed in a box); (**B**) IgE immunoblot showing the positive reaction with the protein spots identified in panel **A**. Blunt snout bream proteins separated by 2D SDS-PAGE were transferred to nitrocellulose membrane and probed with pooled sera from blunt snout bream-sensitive patients. Immunoreactive proteins were visualized using anti-human IgE−horseradish peroxidase conjugate antibody. The sizes of the prominent blunt snout bream proteins in kDa are shown on the right of the figure.

2D Immunoblotting. To further characterize the proteins that reacted with the sera from patients with blunt snout bream allergy, the protein extract was subject to 2D SDS-PAGE. To exclude artifacts caused by electrophoresis conditions, 2D gels were run in triplicate. A representative 2D gel picture of blunt snout bream protein extract stained with Coomassie blue shows the visualization of > 100 protein spots ranging in molecular mass from 10 to 100 kDa (**Figure 3A**). The majority of these proteins spots have p*I* values ranging from 3 to 7. The prominent 47 kDa protein was resolved into six distinct spots with isoelectric points ranging from 5.5 to 6.5, respectively (**Figure 3A**). Similarly, the 41 kDa protein was resolved into five distinct spots having isoelectric points ranging from 5.5 to 6.5, respectively (**Figure 3A**). To identify immunoreactive proteins, the blunt snout bream extract was separated by 2D gels, transferred to a nitrocellulose membrane, and incubated with pooled sera. Four spots comprising the 47 protein and three spots of the 41 kDa protein showed strong reaction with patients' sera (**Figure 3B**). Very weak reaction with other protein spots were also detected; however, these proteins were not further characterized.

MALDI-TOF MS Identification of Blunt Snout Bream Allergens. Because the 47 and 41 kDa proteins were recognized by sera from all 11 patients examined in this study, we focused on these two groups of proteins for further characterization. The immunoreactive protein spots were excised from the gel, subjected to in-gel digestion with trypsin, and analyzed with MALDI-TOF MS.

Table 1. MALDI-TOF MS Tryptic Peptide Ions Matched from Spots 1−4

start−end	observed	M_r(exptl)	M_r(calcd)	ppm	missed sequence	sequence
10−15	732.3848	731.3775	731.3813	−5	0	R.EILDSR.G
33−50	1764.8923	1763.8851	1763.9166	−18	0	R.AAVPSGASTGVHEALELR.D
90−103	1652.7822	1651.7750	1651.8127	−23	1	K.IDKFMLELDGTENK.S (M)
104−120	1734.9182	1733.9109	1733.9134	−1	0	K.SQFGANAILGVSLAVCK.A
104−126	2262.1128	2261.1055	2261.1838	−35	1	K.SQFGANAILGVSLAVCKAGAAEK.G
127−132	704.4067	703.3995	703.4017	−3	0	K.GVPLYR.H
133−141	938.4869	937.4797	937.4981	−20	0	R.HIADLAGNK.D
142−162	2119.0745	2118.0672	2118.1222	−26	0	K.DVILPVPAFNVINGGSHAGNK.L
184−193	1143.5908	1142.5835	1142.6084	−22	0	R.IGAEVYHNLK.N
203−228	2743.2903	2742.2830	2742.3348	−19	0	K.DATNVGDEGGFAPNILENNEALELLK.S
240−253	1540.7673	1539.7601	1539.8007	−26	0	K.IIIGMDVAASEFFK.S (M)
254−262	1072.5065	1071.4992	1071.5237	−23	1	K.SGKYDLDFK.S
257−262	800.3697	799.3624	799.3752	−16	0	K.YDLDFK.S
263−269	814.3975	813.3902	813.3981	−10	1	K.SPDDPKR.H
269−281	1529.7690	1528.7618	1528.7998	−25	1	K.RHITGEQLGDLYK.S
270−281	1373.6704	1372.6631	1372.6987	−26	0	R.HITGEQLGDLYK.S
286−306	2612.0781	2611.0708	2611.1139	−16	0	K.NYPVQSIEDPFDQDDWENWSK.F
307−326	2105.0259	2104.0186	2104.0689	−24	0	K.FTGSVDIQVVGDDLTVTNPK.R
327−334	1032.5156	1031.5083	1031.5182	−10	1	K.RIQQACEK.K
328−334	876.4147	875.4074	875.4171	−11	0	R.IQQACEK.K
335−343	1119.5704	1118.5632	1118.5940	−28	1	K.KACNCLLLK.V
336−343	991.4817	990.4744	990.4990	−25	0	K.ACNCLLLK.V
344−358	1633.7815	1632.7742	1632.8141	−24	0	K.VNQIGSVTESIQACK.L
373−394	2353.1135	2352.1062	2352.1519	−19	0	R.SGETEDTFIADLVVGLCTGQIK.T
395−400	661.3118	660.3046	660.3013	−5	0	K.TGAPCR.S
407−412	824.4033	823.3960	823.4010	−6	0	K.YNQLMR.I
407−412	840.3876	839.3804	839.3959	−19	0	K.YNQLMR.I

Table 2. MALDI-TOF MS Tryptic Peptide Ions Matched from Spots 5−7

start−end	observed	M_r(exptl)	M_r(calcd)	ppm	missed sequence	sequence
2−11	1175.5283	1174.5210	1174.5520	−26	0	M.PFGNTHNNFK.L
26−32	851.4186	850.4113	850.3868	29	0	K.HNNHMAK.V
26−32	867.4059	866.3987	866.3817	20	0	K.HNNHMAK.V oxidation (M)
108−116	1093.5510	1092.5437	1092.5451	−1	0	K.TDLNFENLK.G
117−130	1507.6678	1506.6606	1506.6951	−23	0	K.GGDDLDPNYVLSSR.V
139−148	1111.5472	1110.5400	1110.5570	−15	0	K.GYALPPHNSR.G
173−177	683.3577	682.3504	682.3690	−27	0	K.YYPLK.S
210−215	759.3266	758.3193	758.3347	−20	0	R.DWPDAR.G
216−223	997.4569	996.4496	996.4777	−28	0	R.GIWHNENK.T
224−236	1657.8074	1656.8001	1656.8260	−16	0	K.TFLVWVNEEDHLR.V
260−265	778.3979	777.3907	777.4272	−47	0	R.IEEIFK.K
267−292	2994.3701	2993.3628	2993.4178	−18	0	K.HNHGFMWNEHLGFVLTCPSNLGTGLR.G
308−314	907.4789	906.4717	906.4811	−10	0	K.FEEILTR.L
321−341	1994.9146	1993.9073	1993.9342	−13	0	R.GTGGVDTASVGGVFDISNADR.I
342−358	1832.8709	1831.8636	1831.8986	−19	0	R.IGSSEVEQVQCVVDGVK.L
359−365	879.4355	878.4282	878.4241	5	0	K.LMVEMEK.K

Using Mascot, the empirically determined mass-to-charge ratios of peptides were compared with peptides of known proteins listed in the National Center for Biotechnology Information nonredundant database (**Tables 1** and **2**). The peptide mass fingerprinting (PMF) spectrum of protein spots 1−4 were identical. Similarly, protein spots 5−7 also revealed similar PMF spectra. This observation indicates that spots 1−4 are the same protein. Likewise, protein spots 5−7 represent the same protein. The resolution of the 47 and 41 kDa proteins into several distinct protein spots may be the result of post-translational modification (e.g., glycosylation, acylation, or phosphorylation).

Mascot search results showed 31 peptides from spots 1−4 having significant sequence homology to enolase 1 from zebrafish (*Brachydanio rerio*) (**Table 3**). Using this sequence we conducted a FASTA search (*15*, *16*) against the Food Allergy Research and Resource Program (FARRP) Protein Allergen Online Database (http://www.allergenonline.org). Examination of the full-length FASTA search revealed significant homology with enolase from different sources. Enolases are homodimeric enzymes that catalyze the reversible dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate as part of the glycolytic and gluconeogenesis pathways. They are recognized as a class of highly conserved fungal allergens with conserved IgE binding epitopes (*17*). Enolase 1 from *B. rerio* showed > 64% amino acid sequence identity in a 431 amino acid overlap with enolase from *Candida albicans*, *Alternaria alternata*, *Rhodotorula mucilaginosa*, *Aspergillus fumigatus* and *Penicillium citrinum* (*17*−*20*). Interestingly, it also shared sequence homology with house dust mite allergen *Der f 2* (24% identity; 54% similarity in a 90 amino acid overlap) (*21*) and some known plant allergens including *Hevea brasiliensis* allergen *Hev b 9* (66% identity; 87% similarity in a 442 amino acid overlap) (*22*), isoflavone reductase related protein from *Pyrus communis* (27% identity; 51% similarity in a 160 amino acid overlap) and pathogenesis-related protein 10 from *Vigna*

Table 3. Protein Sequence of the Two Immunodominant Spots Identified with 2D SDS-PAGE and MALDI-TOF MS

spot	accession no.	sequence coverage (%)	no. of mass values matched	sequence
1−4	Q6TH14_BRARE	65	29	**1** MSISKIHAR**E ILDSR**GNPTV EVDLYTTKGR FR**AAVPSGASTGVHEALELR** **51** DGDKTRYLGK GTQKAVDHVN KDIAPKLIEKKFSVVEQEK**IDKFMLELDGT** **101 ENKSQFGANA ILGVSLAVCK AGAAEKGVPL YRHIADLAGN KDVILPVPAF** **151 NVINGGSHAG NK**LAMQEFMI LPVGAQNFHE AMR**IGAEVYH NLK**NVIKAKY **201** GK**DATNVGDE GGFAPNILEN NEALELLK**SA IEKAGYPDK**I IIGMDVAASE** **251 FFKSGKYDLD FKSPDDPKRH ITGEQLGDLY K**SFIK**NYPVQ SIEDPFDQDD** **301 WENWSKFTGS VDIQVVGDDL TVTNPKRIQQ ACEKKACNCL LLKVNQIGSV** **351 TESIQACK**LA QSNGWGVMVS HR**SGETEDTF IADLVVGLCT GQIKTGAPCR** **401** SERLAK**YNQL MR**IEEELGDK AKFAGKDFRH PKL
5−7	Q90X19_BRARE	43	16	**1** M**PFGNTHNNF K**LNYSVDEEY PDLSK**HNNHM AK**VLTKEMYG KLRDKQTPTG **51** FTVDDVIQTG VDNPGHPFIM TVGCVAGDEE SYEVFKDLFD PVISDRHGGY **101** KATDKHK**TDL NFENLKGGDD LDPNYVLSSR** VRTGRSIK**GY ALPPHNSR**GE **151** RRAVEKLSVE ALSSLDGEFK GK**YYPLK**SMT DAEQEQLIAD HFLFDKPVSP **201** LLLAAGMAR**D WPDARGIWHN ENKTFLVWVN EEDHLR**VISMQKGGNMKEVF **251** KRFCVGLQR**I EEIFK**K**HNHG FMWNEHLGFV LTCPSNLGTG LR**GGVHVKLP **301** KLSTHAK**FEE ILTR**LRLQKR **GTGGVDTASV GGVFDISNAD RIGSSEVEQV** **351QCVVDGVKLM VEMEK**KLEKG ESIDSMIPAQ K

radiata (27% identity; 63% similarity in a 114 amino acid overlap) (*23*).

The peptide mass data from protein spots 5−7 were also searched against the NCBI database using the Mascot search program. This analysis revealed 16 peptides from spots 5−7 having significant sequence homology to muscle-specific creatine kinase of zebrafish (**Table 3**). The amino acid sequence of zebrafish creatine kinase when subjected to full-length FASTA search revealed significant homology (47% identity; 72% similarity in a 347 amino acid overlap) with arginine kinase from *Litopenaeus vannamei* (Pacific white shrimp) (*24*), *Penaeus monodon* (giant tiger prawn) (*9*), *Bombyx mori* (domestic silkworm) (*25*), and *Plodia interpunctella* (Indianmeal moth) (*26*). The zebrafish creatine kinase also showed significant sequence homology with ABA-1 allergen from *Ascaris lumbricoides* (35% identity; 70% similarity in a 43 amino acid overlap) (*27*), *Pha a 5.4*, a major allergen of canary grass pollen (34% identity; 66% similarity in a 35 amino acid overlap) (*28*), and birch pollen allergen *Bet v 1* (44% identity; 59% similarity in a 34 amino acid overlap) (*29*).

In the present study, we have utilized 1D and 2D SDS-PAGE coupled with IgE immunoblot analysis to identify potential allergens from blunt snout bream. MALDI-TOF MS analysis identified the two immunodominant fish proteins as enolase and creatine kinase. To the best of our knowledge this is the first study that has identified these two prominent proteins as potential fish allergens. Even though enolase has been previously identified as a major fungal allergen (*17*−*20*), it has not been reported as a fish allergen. Serum from patients with mold allergy exhibit cross-reactivity with latex enolase *Hev b 9* and, consequently, it has been suggested that *Hev b 9* should be part of an allergen panel for diagnosis of mold allergy (*22*). The cross-reactivity could be explained on the basis of the high sequence homology seen among enolases from different organisms. It should be interesting to examine if IgE from serum from blunt snout bream allergic patients can also cross-react with latex enolase and fungal enolases. Interestingly, some of the blunt snout bream sensitive patients (3 of 11) also had histories of dust allergy, and thus the clinical relevance of IgE binding to fish proteins in these individuals needs further investigation.

The 41 kDa blunt snout bream creatine kinase identified in this study shows extensive sequence homology to arginine kinase. This enzyme catalyzes the transfer of a high-energy phosphoryl group from ATP to arginine, resulting in the generation of ADP and *N*-phosphoarginine (*30*). Some food allergens have been shown to exhibit regulatory and transport properties. For example, the major tropical fish allergen paravalbumin possesses calcium-binding properties (*31*), whereas shellfish tropomyosin is involved in actin binding and muscle contraction (*32*). By utilizing proteomics and immunological analysis Yu and his associates (*9*) first identified arginine kinase as a novel shrimp allergen. This shrimp allergen, Pen m 2, encodes a protein with a molecular mass of 40 kDa and a p*I* of 6.02, the same as observed for blunt snout bream creatine kinase. Creatine kinase catalyzes the reversible transfer of the γ-phosphoryl group of ATP to creatine, resulting in the formation of ADP and phosphocreatine. This enzyme plays a major role in energy homeostasis of cells with intermittently high energy requirements (*33*). Thus, the blunt snout bream creatine kinase, like Pen m 2, may also function as a novel fish allergen with regulatory and/or transport functions. Currently, little is known about the mechanism of allergic sensitization to enolase and creatine kinase. Even though we have shown that these two proteins bind IgE from the serum of patients with blunt snout bream allergy, the clinical relevance of immune response clearly needs further investigation.

ACKNOWLEDGMENT

We thank Zhang Tuo from Beijing Proteome Research Center for his dedicated assistance in this work.

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Received for review May 4, 2010. Accepted November 25, 2010.