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 a 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...
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 100 μL of fish crude extract in a concentration of 250 μg/mL, then washed with PBST (0.05% SDS, 0.05 mM EDTA, pH 7.7). Each experiment was performed in duplicate. Two-dimensional gel IgE immunoblotting was performed for a total of 80 kV h. Immunoblot analysis was performed 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.roche.com/; Prodigy.fun, Mascot (http://www.matrixscience.com), and MS-FIT (http://prospector.ucsf.edu) for all peptide mass data comparison and protein identification. Protein tolerance was set at 0.05. 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 < 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 pI 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 patients’ 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, and 5 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 (J3). 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 20 kDa. The reactivity of the 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 parvalbumin, a 12 kDa calcium-binding sarcoplasmic protein, to be a major fish allergen protein (J3). This protein of interest were excised and destained with 25 mM ammonium bicitarbate and 50% acetonitrile. Gel pieces were then dried completely by centrifugal lyophilization. In-gel digestion was performed with 0.01 μL trypsin (Promega Corp., Madison, WI) in 25 mM ammonium bicarbonate for 14 h at 37 °C. The supernatants were collected, and the tryptic peptides were extracted from the gel sequentially with 5% trifluoroaceticate (TFA) at 40 °C for 1 h 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-hydroxyxycinnamic 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). Protein scores that were statistically significant (p < 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 pI values of most proteins were consistent with the gel regions from which the spots were excised.
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.

2D Immunoblotting. To further characterize the proteins that reacted with the sera from patients with blunt snout bream allergy, the protein extract was subjected 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 pI 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.
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 non-redundant 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 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 phospho-enolpyruvate as part of the glycolytic and gluconeogenic 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.
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 A. lumbricoides (27% identity; 63% similarity in a 114 amino acid overlap) (27). 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), Bet v 1 (35% identity; 59% similarity in a 34 amino acid overlap) (28), and Porcellio scaber allergen. This shrimp allergen, Pen m 2, encodes a protein with a molecular mass of 40 kDa and a pI of 9.4, shown to exhibit regulatory and transport properties. For example, the major tropical fish allergen parvalbumin 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 pI of 9.4, 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
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LITERATURE CITED

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

<table>
<thead>
<tr>
<th>spot</th>
<th>accession no.</th>
<th>sequence coverage (%)</th>
<th>no. of mass values matched</th>
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<td>1–4</td>
<td>Q6TH14_BRARE</td>
<td>65</td>
<td>29</td>
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<tr>
<td>5–7</td>
<td>Q90X19_BRARE</td>
<td>43</td>
<td>16</td>
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</tbody>
</table>

1  MSISKIHARE ILDSRGNPTV EIVLDYLTGKR FRAAVPSGASTGVHEALEL 51  DGDKTTRYLQG GTOCAVHDNK IDKPMKIEEKR EIKFDMELE DGT 10  ENSKFSGANA ILGVSLAVCK AGAAEKGVPIL TRHADLAG KDVILPVPVF 51  NVINGSGHAG NKLAMQGEM FLPVQAGNHE AMRIGEAVYN LKNIK hiding 20  QKDATVNGDE GGFAPNILENIAELELLK IKEAGYDPKI IGMVDVEA 51  FFKSGQYDLD FKSQDKPRKR ITGEOQLDYL KSFKNVPQV SIEDPPQQDD 30  WENWSKFTGS VDIQVVDLTL TNTPKRIQG ACEKKACNCL LKVINIQGSSV 35  TESIQACKLA QSNWGVVMHS HRSGETEDTF IADLTVGCLT QIIGT 40  SLRALKYNQL MRREEELEGDK AFKAGKDIFHR PKL 40  1 MPFGNTHNHF KLNSVDEYEE PDLKSHNNHM AKVLTKEMYQ KLKDQKPTP 51  FTVDDVQTVG VDPGPQHPFIM TTVGQADGEE SYEVEFKDLFD PVISDHRGGY 101  KATDKHKTDL NFNELKGDG LDNPNYLSRA VARTGISBKGY ALPPHNSRGE 151  BRAVEKLSE ALSSLDEGPQ GKYPPKLSMT DAEGEQLIDQ HFLDQPKVGP 201  LLLAAMGDAR WPDARGWIM ENKTLFWVNN EEDELRVSOKGNNMKNEVF 251  KRFVCGLORIE EIEFKINHHFG FMWNEHLGFV LTCPSNLGTG LRGVHVKLP 301  KLTSHAKFEE ILTRLRLOKR GTGVDVTASG GGFVDSNAD RIGGSSEVEQV 351  QCXVGVGDQKL VEMEKLEKGE ESIDSMIPAO Q 351

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