SUPPLEMENTARY MATERIAL

Supplementary Methods

Subjects

Thirty patients with red meat allergy (19 men and 11 women; age range: 37−88 years; average age: 69.2 ± 8.4 years), enrolled in the study, were referred to the Department of Dermatology at the Shimane University Hospital (Shimane, Japan). The diagnosis of red meat allergy was made following an episode of allergic symptoms after ingestion of red meat and positive serum IgE tests for beef and α-Gal (ImmunoCAP®, Thermo Fischer Diagnostics, Tokyo, Japan). Some of these cases have previously been described as beef allergy [6], and the clinical features of these patients are summarized in Supplementary Table 1. After ingestion of red meat, urticaria was the predominant allergic symptom observed in 29 of the 30 patients, whereas 7 of the 30 patients developed anaphylactic shock. More than 60% of the documented 35 allergy episodes of these 30 patients appeared 3 h after ingestion of red meat or later, and 23% of the episodes developed in over 5 h. Twenty of the 30 patients showed allergic reactions after ingesting flounder with roe that had been fished from the Sea of Japan during winter season. All of these 20 patients had generalized urticaria, and seven of them developed anaphylactic shock a few hours after a meal as with red meat allergy. The episodes of flounder roe allergy followed the episodes with red meat allergy in most of the patients, and the patients had no allergic symptoms after ingesting flounder without roe. Five healthy subjects who had no allergy against either red meat or flounder roe were enrolled as negative controls.

Allergen-specific IgE tests and skin prick tests

Allergen-specific IgE antibodies were measured using CAP-fluorescent enzyme immunoassay (ImmunoCAP®) for beef (f27), flounder meat (f254), and α-Gal (o215).

Skin prick tests were performed on the volar surface of the arm. Histamine solution (10 mg/mL) was used as positive control, and saline as negative control. Fresh flounder fish *Hippoglossoides dubius* with roe were procured from a local supermarket on each day of testing, and prick-to-prick tests were performed with heated and non-

doi: 10.18176/jiaci.0754

heated samples. Reactions were read after 15 min, and responses were compared with positive histamine controls.

Immunoblot analysis

Water-soluble and water-insoluble fractions of beef, flounder meat, and flounder roe were prepared as previously described [6]. Briefly, samples were homogenized in ice-cold phosphate buffered saline (PBS) supplemented with a cocktail of protease inhibitors (Roche Diagnostics K. K., Japan) using a TissueLyser (QIAGEN, Valencia, WA, USA). The resulting lysates were centrifuged at 20,000 × g for 15 min at 4 °C, and the supernatants were used as water-soluble fractions. The pellets were solubilized in 2% sodium dodecyl sulfate (SDS) solution and used as water-insoluble fractions.

Immunoblotting was performed with water-soluble and water-insoluble fractions as previously described [6]. Briefly, samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% polyacrylamide gels according to the manufacturer's specifications (Atto Corp., Tokyo, Japan). Separated proteins were blotted under semi-dry conditions on the polyvinylidene difluoride membrane (PVDF) membranes (Millipore Corp., Billerica, MA, USA). For IgE immunodetection of allergens, the PVDF membranes were blocked with 0.6% polyvinylpyrolidone in the solution containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20, and incubated overnight with tenfold diluted serum samples. IgE antibodies were detected using a horseradish peroxidase-conjugated mouse monoclonal anti-human IgE Fc antibody (ab99806, Abcam plc, Cambridge, UK) and an ECL-plus kit (GE Health Care, Tokyo, Japan). Proteins possessing a sugar chain were evaluated by detecting fluorescence using a ProQ Emerald 300 Glycoprotein gel stain kit (Invitrogen, Thermo Fischer Scientific, Yokohama, Japan) according to the manufacturer's instruction after separation by SDS-PAGE. Fluorescence was evaluated by a LAS-4000 instrument (GE Healthcare) with excitation and emission wavelengths of 280 and 530 nm, respectively. For α-Gal detection, a mouse anti-α-Gal monoclonal antibody (M86 clone; Enzo Life Sciences, Inc., Farmingdale, NY, USA) and a horseradish peroxidase-conjugated anti-mouse IgM (μ) antibody (Kirkegaard & Perry Laboratories, Inc., KPL, Gaithersburg, MD, USA) were used.

doi: 10.18176/jiaci.0754

Identification of flounder roe allergen

Step 1. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Samples were purified using a Ready-Prep 2-D Cleanup kit (Bio-Rad) and dissolved in 60 mM Tris-HCl buffer (pH 8.8) containing 6 M urea, 1 M thiourea, 3% CHAPS, and 1% Triton X-100. Protein concentration was determined using a DC Protein assay kit, and 200 micrograms of the total protein underwent 2D-PAGE by a previously described method [6]. Briefly, first dimension protein separation was carried out in agarGEL (pH 3-8, φ 2.5 9 75 mm; ATTO corp., Tokyo, Japan) at 300 V for 3.5 h. The agarGel was subsequently used for second dimension SDS-PAGE on a 7.5% acrylamide gel under reducing conditions. For IgE immunoblot analysis, proteins separated by 2D-PAGE were electrophoretically transferred to PVDF membranes and further incubated with tenfold diluted patient serum samples.

Step 2. N-terminal amino acid sequence

Protein spots corresponding to the positive IgE immunoblotting were manually excised from the CBB-stained PVDF membranes, and the N-terminal amino acid sequence was determined with a model PPSQ-33A protein sequencer (Shimadzu Co., Kyoto, Japan) as previously described [6]. Homologies of the amino acid sequence and predicted signal peptide cleavage site were analyzed using DDBJ BLAST search and Signal IP server, respectively.

Step 3. cDNA cloning

Total RNA was extracted from *H. dubius* roe using a RNeasy Fibrous Tissue Mini kit (QIAGEN). Full length cDNA encoding flounder roe allergen was cloned using 3′-rapid amplification of cDNA ends (RACE) and 5′-RACE methods. 3′-RACE was carried out using a 3′-Full RACE Core Set (TAKARA Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Four micrograms of total RNA were reverse-transcribed with Oligo dT-3 sites Adaptor Primer, and the resulting cDNA was further amplified by polymerase chain reaction (PCR) in the presence of degenerative primer synthesized according to the amino acid sequence of flounder roe allergen and 3 sites Adaptor Primer. PCR conditions were as follows: 4 cycles at 98 ℃ for 10 s, at 55 ℃ for 15 s, and 68 ℃ for 3 min, then 30 cycles at 98 ℃ for 10 s, at 64 ℃ for 15 s, and at 68 ℃ for 3 min. These PCR products were purified with a Minielute Gel

doi: 10.18176/jiaci.0754

Extraction kit (QIAGEN) and subsequently cloned into the pMD20-T vector (TAKARA Bio) using a Mighty TA-cloning Reagent Set for PrimeSTAR kit (TAKARA Bio), and transformed into *Escherichia coli* JM109. Nucleotide sequence of the cloned plasmids was elucidated using a primer walking method. 5′-RACE was carried out using a 5′-Full RACE Core Set (TAKARA Bio) according to the manufacturer's instructions. According to the nucleotide sequence of the plasmids identified by the primer walking method, 5′-RACE-RT-PCR primer, 5′-RACE-A1 primer, 5′-RACE-A2 primer, 5′-RACE-S1 primer, and 5′-RACE-S2 primer were constructed, and 5′-RACE was performed. The obtained PCR products were purified after separation in 0.8% agarose electrophoresis, ligated into the pMD20-T vector using a Mighty TA-cloning Reagent Set for PrimeSTAR kit, and transformed into *E. coli* JM109. Cloned plasmids were analyzed for the presence of the DNA sequence obtained by 3′-RACE.

Ethics

This study was approved by the Ethics Committee of the Shimane University Faculty of Medicine (approval No. 469). The study was explained to all subjects and written informed consents were obtained.

doi: 10.18176/jiaci.0754