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Comparative study of GH-transgenic and non-transgenic amago salmon (*Oncorhynchus masou ishikawae*) allergenicity and proteomic analysis of amago salmon allergens

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ABSTRACT

Genetically modified (GM) foods are beneficial from the standpoint of ensuring a constant supply of foodstuffs, but they must be tested for safety before being released on the market, including by allergenicity tests to ensure that they do not contain new allergens or higher concentrations of known allergens than the same non-GM foods. In this study we used GM-amago salmon into which a growth hormone gene had been introduced and compared the allergens contained in the GM and the non-GM-amago salmons. We used a combination of Western blotting with allergen-specific antibodies and a proteomic analysis of their allergens with patients' sera, a so-called allergenome analysis, to analyze allergens. Western blotting with specific antibodies showed no increase in the content of the known allergens fish parvalbumin and fish type-I collagen in GM-amago salmon, in comparison with their content in non-GM-amago salmon. The allergenome analysis of two fish-allergic patients allowed us to identify several IgE-binding proteins in amago salmon, including parvalbumin, triose-phosphate isomerase, fructose-bisphosphate aldolase A, and serum albumin, and there were no qualitative differences in these proteins between GM and non-GM-amago salmons. These results indicate that amago salmon endogenous allergen expression does not seem to be altered by genetic modification.

1. Introduction

Since genetically modified (GM) foods are beneficial from the standpoint of ensuring a constant supply of foodstuffs, numerous transgenic plants and animals have been developed in order to make them resistant to insects or herbicides, to enhance their growth, and so on.

Even after the establishment of the 2003 Codex guidelines concerning the methods of evaluation that should be used to assess the safety of GM foods, controversy remains regarding the methods of evaluation that should be used to assess the safety of GM foods, especially concerning their potential allergenicity. Known allergens in foods are currently quantified by Western blotting or enzyme-linked immunosorbent assay (ELISA) with specific antibodies, but foods may contain unknown allergens. Proteomic analysis of allergens with patients' sera, so-called allergenome analysis, may be a useful method of comprehensively comparing the allergens contained in GM and non-GM foods of the same kind. Batista et al. reported the finding that proteomic analysis is a useful tool for assessing the safety of non-transgenic and transgenic soya food, and they used it to identify two new soya IgE-binding[1] proteins (Batista et al., 2007).

Mori et al. recently developed growth-hormone-1 (GH-1)-transgenic amago salmon (*Oncorhynchus masou ishikawae*) by transferring a construct containing sockeye salmon growth hormone GH-1 fused to the metallothionein-B promoter into wild amago salmon (Mori et al., 2007), and they found that 1-year-old transgenic amago salmon (GM, 1-year) weighed approximately fivefold more than non-transgenic amago salmon of the same age (non-GM, 1-year), and that the body weight of 2-year-old non-GM-amago salmon (non-GM, 2-year) was 13-fold greater than that of non-GM (1-year) amago salmon.

Fish allergies account for approximately 5% of food allergies in

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[1] *Abbreviations used:* IgE, immunoglobulin E; PBS, phosphate-buffered saline, pH 7.2; HRP, horseradish peroxidase; DTT, dithiothreitol; CBB, Coomassie brilliant blue; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; MW, molecular weight; LC/MS/MS, liquid chromatography/mass spectrometry/mass spectrometry; 2D-PAGE, two-dimensional polyacrylamide electrophoresis.

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Japan, and patients seldom become grown out. Parvalbumins are Ca^{2+}-binding proteins involved in muscle contraction and are well-known major allergens in cod, carp, salmon, and mackerel (Elsayed and Aas, 1971; Elsayed and Bennich, 1975; Lindstrom et al., 1996; Bugajska-Schretter et al., 1998; Shiomi et al., 1998). Parvalbumins are present in a wide variety of fish species, and they are considered as potent fish cross-reacting allergens (Van Do et al., 2005).

Hamada et al. showed that fish type-I collagen is another allergen present in many fish species (Hamada et al., 2001). Several other fish allergens have been reported, including a 41 kDa minor allergen in cod (Galland et al., 1998; Das Dores et al., 2002), 35–90 kDa allergens in snapper, which is consumed locally in Malaysia (Rosmilah et al., 2005), and 94–105 kDa allergens in tuna and marlin (Kondo et al., 2006), but not much is known about them.

We applied qualitative and quantitative methods of allergen analysis to non-transgenic amago salmon and the GH-1-transgenic amago salmon developed by Mori's group. In the quantitative analysis, 1D-Western blotting with allergen-specific antibody was used to compare the expression of parvalbumin and fish type-I collagen by non-GM and GH-transgenic amago salmons. In the qualitative analysis, 2D-Western blotting with fish-allergic patients' sera was used to comprehensively identify IgE-binding proteins in non-GM and GM-amago salmons. We adopted dual fluorescence-labeled 2D-Western blotting to accurately confirm the IgE-binding spots.

2. Materials and methods

2.1. Fish extracts

GH-transgenic (GM, 1-year) and non-transgenic amago salmons (non-GM, 1- or 2-year) were grown in the Fisheries Research Agency of the National Research Institute of Aquaculture (Mie, Japan). Introduction of the GH-1 gene was confirmed by real-time PCR as described in Mori's paper (Mori et al., 2007). At least three specimens of the edible part (muscle and skin) of each amago salmon were collected at random and immediately stored at −80 °C until used. A 15 mL volume of PBS containing protease inhibitor cocktail (SIGMA, St. Louis, MO, USA) was added to a 1.5 g sample of each frozen amago salmon, and the sample was homogenized with a Polytron (Kinematica). The homogenate was centrifuged at 9000*g* for 10 min, and the supernatant was collected. The protein concentration of the amago salmon extracts was determined with a BCA assay Kit (Pierce, IL, USA).

2.2. Sera

We used sera from 22 fish-allergic patients, eight from Japan and 14 from the United States, who were positive for fish-specific IgE (Immuno-CAP scores 1–6). Table 1 shows the background information regarding the fish-allergic patients. Sera from allergy-free persons were used as a negative control. Informed consent was obtained from all patients and volunteers. Our study was approved by the Ethical Review Committee of the National Institute of Health Sciences.

2.3. 1D-Western blot analysis of GM and non-GM-amago salmon with patients' sera and allergen-specific animal sera

(i) 1D-Western blot with patients' sera

The proteins in crude extracts of non-GM and GM-amago salmons were separated by SDS–PAGE on a 5–20% acrylamide gel (DRC, Tokyo, Japan), and the proteins were transferred onto 0.22-μm nitrocellulose membranes (S&S, Dassel, Germany). The membranes were cut into 3-mm-wide strips and blocked with 0.5% casein–PBS for 2 h at room temperature (RT). Membrane strips were then incubated with the fish-allergic patients' sera (diluted 1:10 with 0.1% casein–PBS) for 1 h at RT, and then overnight at 4 °C. After washing, the strips were incubated with HRP-linked anti-human IgE (1:500 dilution, Nordic Immunology, Tilburg, Netherlands) for 1 h at RT, and color was developed with Konica Immunostain (Konica Minolta, Tokyo, Japan) as described in a previous paper (Teshima et al., 1993).

Table 1
Laboratory features of the fish-allergic patients.

Serum #	Major diagnosis	Fish-specific Immuno-CAP score
1	FA	Salmon (4)
2	FA, AD	Mackerel (6), sardine (5), flatfish (5), salmon (3)
3	–	Codfish (2), horse mackerel (2), sardine (2), tuna (2), salmon (2)
4	–	Tuna (1), salmon (2)
5	–	Codfish (2), tuna (1), salmon (2)
6	–	Codfish (3),salmon (3), trout (3)
7	–	Codfish (3), salmon (3), trout (3)
8	–	Codfish (3), salmon (4)
9	–	Codfish (3), salmon (3), trout (3)
10	–	Codfish (3), trout (4)
11	–	Codfish (2), salmon (2)
12	–	Codfish (2)
13	–	Codfish (2), salmon (2)
14	–	Codfish (3), trout (4)
15	FA, A, IR	Codfish (3)
16	FA, AD	Mackerel (3)
17	FA, AD	Mackerel (2), Japanese horse mackerel (3)
18	FA, AD	Tuna (3)
19	FA, AD	Mackerel (2), Japanese horse mackerel (2)
20	–	Fish (3–4)
21	–	Sardine (3)
22	–	Cod (2)

FA, food allergy; AD, atopic dermatitis; A, asthma; IR, immediate reaction.
The major diagnosis and fish-specific CAP scores of the 22 individuals with fish allergy are shown.
Sera 1–9, 11, and 13 were from patients with salmon allergy, and the others were from patients with allergies to other species of fish.
Sera 1–3 and 15–19 were from Japan, and the others were from the United States.
The correlations between the Immuno-CAP scores and specific IgE concentrations were: 1, 0.35–0.69; 2, 0.70–3.5; 3, 3.5–17.4; 4, 17.5–49.9; 5, 50.0–99.9; 6, >100 IU/mL.

(ii) 1D-Western blot with allergen-specific animal sera

The proteins in the crude extract from each of three bodies of non-GM (1-year and 2-year) and GM amago (1-year) salmons were separated by SDS–PAGE on a 5–20% acrylamide gel, and the proteins were transferred onto 0.22 μm nitrocellulose membranes. After blocking with 0.5% casein–PBS, the membranes were incubated with mouse anti-frog parvalbumin (1:2000 dilution, SIGMA) or rabbit anti-fish type-I collagen (1:500 dilution, S20171, NOVOTEC, France) for 1 h at RT. After washing, the membranes were incubated with HRP-linked anti-mouse IgG (1:1000, GE Healthcare UK Ltd., Little Chalfont, UK) or HRP-linked anti-rabbit IgG (1:1000, GE Healthcare), respectively, and Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences Inc., Boston, MA, USA) was used as the luminescence substrate of HRP. The images were acquired with a Diana III image analyzer (Raytest, Straubenhardt, Germany).

2.4. 2D-PAGE and immunostaining with patients' sera or anti-parvalbumin specific animal serum

Proteins were purified from a crude sample of amago salmon (10 μg) by using a 2D Clean-Up Kit (GE Healthcare) according to the manufacturer's instructions, and the proteins were dissolved