

Original Article

Differential patterns of fish sensitization in Asian populations: Implication for precision diagnosis

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CRD, component resolved diagnosis;
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assay; G-6-P, glucose-6-phosphate
isomerase; GAPDH, glyceraldehyde-3-
phosphate dehydrogenase; HK, Hong Kong;
OD, optical density; oFAS, ordinal Food
Allergy Severity Score; PBS, phosphate-

ABSTRACT

Background: The current diagnostics of fish allergy lack sufficient accuracy such that more reliable tests such as component-resolved diagnosis (CRD) are urgently needed. This study aimed at identifying fish allergens of salmon and grass carp and evaluating the sensitization pattern in fish allergic subjects from two distinct populations in Asia.

Methods: One hundred and three fish allergic subjects were recruited from Hong Kong (67 subjects) and Japan (46 subjects). Western blot and mass spectrometry were used to identify allergens from salmon and grass carp. Fish allergens were purified and tested against 96 sera on ELISA to analyze patients' sensitization pattern. The protein profiles of salmon meat prepared under different cooking methods until core temperature reached 80 °C were evaluated by SDS-PAGE and mass spectrometry.

Results: Three common allergens between salmon and grass carp, namely enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and parvalbumin, and two salmon-specific allergens collagen and aldolase were identified. Parvalbumin was the major allergen for both fishes showing an overall sensitization rate of 74.7%, followed by collagen (38.9%), aldolase (38.5%) and enolase (17.8%). Japanese subjects showed more diverse allergen sensitization pattern and more frequent IgE-binding to heat-labile salmon allergens. Compared with steaming and boiling, cooking by baking and frying retained more fish proteins inclusive of heat-labile allergens.

Conclusions: Fish allergic patients from different Asian populations show varying fish allergen sensitization profiles. The relevant extracts and components for diagnosis are population-dependent but

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buffered saline; PBS-T, 0.05% Tween-20/PBS; SPT, skin prick test; TBS-T, Tween-20 in Tris-buffered saline; TPI, triosephosphate isomerase; WHO/IUIS, World Health Organization and International Union of Immunological Societies

parvalbumin and collagen are important biomarkers. Cooking methods modify allergen composition of salmon and appear to influence patients' allergic manifestations.

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Introduction

Fish is one of the most frequent causes of food-induced allergy alongside cow's milk, hen's egg, peanut, tree nut and shellfish, and has been included in the European mandatory labeling legislation together with 13 other allergens.¹ Fish is a valuable source of healthy nutrients such as omega-3 fatty acids with increasing global demand. Fish allergy affects 0.1–0.4% of the world's population,^{2,3} has an early onset and tends to persist throughout life.⁴ Allergic reactions are mainly provoked through ingestion but also by skin contact or by inhalation of fish steam during fish processing. The allergic reactions triggered by fish can be life-threatening and even fatal resulting in a heavy socioeconomic burden and negative impact on quality of life.^{5,6}

β-parvalbumin represents the major fish allergen accounting for up to 90% of sensitization and has been described and characterized in a wide range of fish species.^{7–10} Parvalbumins are 8–12 kDa calcium-binding muscle proteins with extreme thermal stability and shared 55–95% amino acid sequence homology among various fish species. This might account for the cross-reactivity among fish species but case reports have shown mono-sensitization and clinical allergy to single fish species.^{11,12} Alongside parvalbumin, collagen, aldolase, enolase, pyruvate kinase, glucose-6-phosphate isomerase (G-6-Pi), creatine kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triosephosphate isomerase (TPI) and tropomyosin are other registered fish allergens in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) database.¹³ Among these allergens, collagen, aldolase and enolase are more well-characterized. Collagen is another heat stable protein, with reports showing anaphylaxis provoked by collagen and illustrating IgE binding to collagen only but not parvalbumin.^{14,15} Enolase and aldolase, on the other hand, are identified as major heat-labile fish allergens in cod, salmon and tuna.¹⁶

The current diagnostic workup of fish allergy relies on skin prick test (SPT), measurement of specific IgE and when indicated, an oral food challenge.¹⁷ Although a number of fish extracts are available on the ImmunoCAP platform, only parvalbumins from cod and common carp are available for IgE testing and other identified fish allergens have not been included for diagnosis due to the insufficient understanding of their allergenic properties and clinical relevance especially in the Asian populations with high fish consumption. Yet a considerable number of studies have illustrated the low specificity of SPT and specific IgE measurement while safety has always been a concern in oral food challenges.¹⁸ Together with the reports of monosensitization to some fish species and parvalbumin-independent IgE sensitization,¹⁹ a testing panel inclusive of the relevant fish allergens for precision component-resolve diagnosis (CRD) will be of great value.

This study identified fish allergens of salmon (*Salmo salar*) and grass carp (*Ctenopharyngodon idella*), which are among the most commonly farmed and consumed seawater and freshwater fishes in Asia. We compared the sensitization pattern towards the four major allergens, β-parvalbumin, enolase, aldolase and collagen, from both salmon and grass carp in fish allergic subjects from Hong Kong and Japan. Based on our results, we further characterized the allergen profiles in salmon meat cooked at the same core temperature by different methods to dissect the link between dietary preferences and allergen sensitization profiles.

Methods

Recruitment of fish allergic subjects

This study recruited subjects aged 1–18 years with reported history of IgE-mediated fish allergy from six regional hospitals in Hong Kong (HK; Prince of Wales Hospital, Queen Mary Hospital, Queen Elizabeth Hospital, Yan Chai Hospital, Princess Margaret Hospital and United Christian Hospital) and Sagami-hara National Hospital in Kanagawa, Japan. The inclusion criteria include (1) subjects with convincing history of immediate fish allergy as defined by immediate allergic symptoms within 2 h following fish ingestion within two years before recruitment and clinically diagnosed by an allergy specialist; and (2) subjects with evidence of IgE sensitization to fish by blood sIgE measurement against cod (f3), salmon (f41), tuna (f40) and/or grass carp (experimental ImmunoCAP developed in collaboration with Thermo Fisher) and/or SPT with commercial fish mix extract (ALK-Abelló). Exclusion criteria are subjects receiving maintenance immunosuppressive treatments that cannot be stopped for four weeks prior to study and who receive intravenous immunoglobulin treatment within three months. Severity of allergic symptoms to fish was calculated based on the Ordinal Food Allergy Severity Score (oFASS).²⁰ Briefly, Grade 1 includes reactions restricted to oral cavity. Grade 2 to 5 may include oral symptoms but with other target organs affected, by which grades 2 and 3 reactions involve skin, eye/nose and/or digestive system. Involvement of larynx and/or bronchi reactions are graded 4 while grade 5 would involve cardiovascular and/or nervous systems. Participants and/or their legal guardians gave written informed consent. Ethical approvals were obtained from Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (2019.034 & 2019.612) and the Sagami-hara National Hospital Ethics Committee (2019-003).

Preparation of raw and cooked fish extracts

Frozen salmon (*S. salar*) and fresh grass carp (*C. idella*) were purchased from local supermarket. Two milligrams of raw fish meat were manually homogenized in 8 mL ice-cold phosphate-buffered saline (PBS) until a smooth paste was achieved. Protein was then extracted overnight at 4 °C with constant stirring. The protein extract was centrifuged and supernatant was filter-sterilized through a 0.22 μm polyethersulfone membrane. The fish protein extracts were stored at –20 °C until use.

Several cooking methods including steaming (at 100 °C), boiling (at 100 °C), baking (at 150 °C), lightwave cooking (at 180 °C), frying (at 180 °C) and air-frying (at 200 °C) were used to prepare cooked salmon for comparison of its protein profile. Raw salmon meat, weighed 20 g per pack, was cooked until the core temperature of salmon meat reached 80 °C as measured using digital thermometer. After cooking, all samples were weighed and 1/10 of the raw and cooked fish samples were extracted in 8 mL PBS as described above. All protein extracts were stored at –20 °C until use.

SDS-PAGE and western blotting

Four microliters of raw or cooked fish extracts were separated on a 13.5% SDS-polyacrylamide gel according to their molecular

weights using a Mini PROTEAN SDS-PAGE system (Bio-Rad). Protein bands were stained with SimplyBlue SafeStain (Thermo Fisher). Western blotting was performed using the surf-blot antibody system (Idea Scientific). One hundred fifty microliters of raw fish extracts (in 2 mg/mL), purified fish parvalbumin or collagen (in 0.2 mg/mL) were resolved on SDS-PAGE (13.5% for extracts and parvalbumin, 6% for collagen) and transferred to PVDF membranes (Bio-Rad) with the Trans-Blot Turbo system (Bio-Rad). Non-specific binding of the membranes was blocked with 5% non-fat dry milk in 0.05% Tween-20 in Tris-buffered saline (TBS-T, blocking solution) for 1 h at room temperature. Patient sera diluted at 1:10, 1:20 or 1:30 (adjusted for sIgE levels) and HRP-conjugated anti-human IgE (Southern Biotech) diluted at 1:2000 in blocking solution were used for probing IgE-binding proteins. IgE-binding fish proteins were visualized by incubating the membranes with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher), and the signal was acquired with the ChemiDoc MP Imaging System (Bio-Rad).

Inhibition western blotting

IgE immunoblot inhibition experiments were performed as described with slight modifications.²¹ One hundred fifty microliters of parvalbumin from grass carp or salmon (in 0.2 mg/mL) were separated on 13.5% SDS-PAGE. Sera from pooled fish allergic subjects ($n = 10$ from HK and $n = 5$ from Japan) were diluted 1:10 in blocking buffer (5% non-fat dry milk in 0.05% TBS-T), and pre-incubated with either grass carp or salmon parvalbumin (50 μ g per 1 mL of diluted serum) for 2 h at 37 °C. Preabsorbed sera were then incubated with the membrane overnight at 4 °C. Bound IgE were then detected with HRP-conjugated anti-human IgE and SuperSignal West Pico PLUS Chemiluminescent Substrate as described above.

Mass spectrometry

The IgE-reactive bands of raw fish extracts were excised from the SimplyBlue SafeStain-stained SDS-PAGE gel, destained and digested with trypsin following our laboratory's established protocol.²² The tryptic digests were analyzed on the UltrafleXtreme MALDI-ToF/ToF System (Bruker) for obtaining the peptide mass fingerprints. Searches against the entire protein sequence database (NCBI and UniProt) using the MASCOT search engine were then performed for protein identification.

Preparation of recombinant fish allergens

The protein sequences of enolase (Sal s 2; Uniprot ID: B5DGQ7) and aldolase (Sal s 3; Uniprot ID: B5DGM7) of *S. salar* were downloaded from the Uniprot database and reverse translated by MEGA 11.0. Enolase (Accession no: GBKA01005072.1) and aldolase (Accession no: GEUQ01036650.1) sequences of *C. idella* were retrieved from the transcriptome shotgun assembly deposited at GenBank (Accession no: PRJNA325430) with tBLASTn search using Sal s 2 and Sal s 3 as the search templates. The nucleotide sequences encoding the full-length allergens were commercially synthesized and cloned into the His-tag expression vector pET30(a)+. His-tagged recombinant allergens were then expressed in *Escherichia coli* [BL21 (DE3)] by culturing in MagicMedia (Invitrogen). Allergens were then purified using the HisPur cobalt spin columns (Thermo Scientific) as per the manufacturer's instructions. For parvalbumin purification, raw extracts extracted in Tris-HCl (pH 8.0) were heated at 95 °C–100 °C for 15 min, followed by centrifugation to remove precipitated heat labile proteins. Supernatant containing soluble proteins were loaded into a MonoQ anion exchange column

(Cytiva) connected to a NGC 10 FPLC system (Bio-Rad). Fractions containing pure parvalbumin were separated by a gradient elution of 0–1 M NaCl in Tris-HCl (pH 8.0) and buffer exchanged into PBS for downstream applications with an Amicon centrifugal filter unit. Acid-soluble collagen was extracted from the muscle of salmon and grass carp as described previously.²³ Briefly, diced fish meat was resuspended in 10 volumes of 0.1 mol NaOH overnight at 4 °C with gentle agitation to remove non-collagenous proteins. Upon centrifugation at 3000 rpm for 10 min and pH neutralization with ultrapure water, fish meat was soaked in 10% butanol and incubated for 24 h at 4 °C with gentle agitation. After centrifugation and washing in ultrapure water, samples were resuspended in 10 volumes of 0.5 M acetic acid for 4 days at 4 °C with gentle agitation. The supernatant were then salted-out by adding NaCl to a final concentration of 2 M. The resultant precipitate was dissolved in 0.5 M acetic acid and dialyzed against distilled water.

The concentration and purity of purified fish allergens were determined on the NanoDrop OneC spectrophotometer (Thermo Scientific) and SDS-PAGE, respectively. Protein identity of the allergens was also confirmed by mass spectrometry. All purified allergens were stored at –20 °C until use.

Enzyme-linked immunosorbent assay (ELISA)

Purified fish allergens diluted in coating buffer (100 mM Na₂CO₃, 100 mM NaHCO₃, pH 9.6) were coated onto MaxiSorp microtiter plates (Nunc) and incubated overnight at 4 °C. After washing the plates with 0.05% Tween-20/PBS (PBS-T) and blocking the plates with 5% fetal bovine serum (Gibco) diluted in PBS (blocking buffer) at room temperature for 2 h, serum samples diluted at 1:10 in blocking buffer were added for overnight incubation at 4 °C. Intensity of IgE binding was detected by incubating the plates with biotinylated anti-human IgE antibodies (1:1000 dilution, Vector Labs), HRP avidin D (1:1000 dilution, Vector Labs) and TMB substrate (BD Biosciences). Upon terminating the reaction with 0.1 M sulfuric acid, the optical density (OD) at 450 nm was measured using a microplate reader (BioTek). Results were considered positive only if the OD is 3-fold higher than the mean of non-atopic negative controls.

Statistical analysis

GraphPad Prism (version 8.0, GraphPad Software) was used for graphical presentation and statistical analyses. Quantitative variables were described by median, range and interquartile range whereas qualitative variables were described by absolute frequencies and percentages. Differences in quantitative variables were analyzed by Mann-Whitney test, and categorical data was compared by Fisher exact test. Pairwise comparison of sIgE levels was assessed using the Wilcoxon matched-pairs signed rank test. Correlation analysis was based on Pearson r based on Fisher r-to-z transformation. Comparison between sensitization rate was based on Chi-square test. $P < 0.05$ was considered as statistical significance.

Results

Demographics of fish allergic participants

Sixty-seven and forty-six subjects fulfilling the inclusion criteria were recruited from the pediatric allergy clinics in HK and the Sagami-hara National Hospital in Japan respectively (Table 1, Supplementary Table 1 and 2). The median (range) age of fish allergic subjects at evaluation was 6 (1–16) years and 10 (5–18) years for these two populations, respectively. The most common clinical features shared among the two populations were itchy

mouth/throat (61.1%, 69/113) and urticaria (51.3%, 58/113) while subjects from HK also commonly displayed angioedema (59.7%, 40/67) upon fish ingestion (Supplementary Table 1 and 2). The two populations did not differ in terms of the symptom severity as reflected from oFASS (Mann–Whitney test; $p = 0.5251$). SPT to fish mix extract was only available for subjects recruited in HK with median (range) SPT wheal size of 5 (0–11) mm, and 48 subjects (84.2%) had positive SPT (cutoff at 3 mm). The sIgE results for cod, tuna, salmon and grass carp are summarized in Table 1 and detailed in Supplementary Table 1 and 2. No difference was detected in sIgE levels to these fish species between the two populations, except for grass carp that HK subjects had significantly higher sIgE level comparing to Japanese subjects (Mann–Whitney test, $p = 0.0020$). Of note, 65/67 (97.0%) and 34/37 (91.8%) subjects from HK and Japan had positive sIgE to salmon (cutoff at 0.35 kUA/L), of whom 72.3% and 70.6% had high sIgE level and above class III–VI, respectively. As for grass carp, 63/67 (95.5%) and 25/30 (83.3%) subjects from HK and Japan had positive sIgE, of whom 87.3% and 56.0% had high sIgE level and above class III–VI, respectively. An overall positive correlation between sIgE levels to salmon and grass carp was observed for both populations, by which a stronger correlation was found among the Japanese subjects ($r = 0.69$, $p = 0.0002$) than HK subjects ($r = 0.33$, $p = 0.0061$) based on Fisher r -to- z transformation ($z = 2.01$, $p = 0.04$). Sera of Japanese subjects yielded high sIgE response to salmon with an average grass carp/salmon sIgE ratio of 0.70 (0.02–1.93) while sera of HK subjects yielded higher sIgE response to grass carp with average ratio of 3.39 (0.02–20.8) (Supplementary Fig. 1). Pairwise comparison between grass carp- and salmon-specific IgE levels also showed statistically higher grass carp-specific IgE level among HK subjects but statistically higher salmon-specific IgE level among Japanese subjects (Wilcoxon matched-pairs signed rank test, $p = 0.0008$ and 0.0315 respectively).

Identification of IgE-binding proteins in salmon and grass carp

The extracted salmon and grass carp proteins were analyzed on SDS-PAGE (Fig. 1A). Sera from 45 subjects (30 samples from HK and

15 samples from Japan; marked with asterisks* in Supplementary Table 1 and 2) and non-atopic controls ($n = 2$) were used to investigate IgE-binding to salmon and grass carp proteins by western blotting (Fig. 1B, C). No IgE binding was observed for control sera. Serum samples from Japanese fish allergic subjects showed more diverse IgE binding compared to those from HK, including to grass carp extract despite of the lower IgE level measured on ImmunoCAP. For both populations, the 11 kDa band in salmon and 10 kDa band in grass carp represented the major IgE-binding protein. In HK fish allergic subjects, 18/30 (60.0%) and 16/30 (53.3%) showed IgE binding to this 10 kDa protein in grass carp and salmon respectively, while 10/15 (66.7%) subjects in Japan had a positive reaction to the 10 kDa proteins in salmon and grass carp extracts. Six (40.0%) Japanese subjects reacted to proteins with molecular sizes 130 kDa and 58 kDa.

Protein bands that showed IgE reactivity to at least three serum samples were then excised from SDS-PAGE for allergen identification on mass spectrometry. These included the 130 kDa, 43 kDa, 34 kDa, 25 kDa and 11 kDa bands from salmon extract, as well as the 43 kDa, 34 kDa and 10 kDa bands from grass carp extract. For salmon extract, the 130 kDa protein was identified as α -collagen, 58 kDa band as β -enolase, 32 kDa band as aldolase A, 25 kDa band as GAPDH and the 11 kDa band as β -parvalbumin (Supplementary Table 3). Similarly, the 58 kDa, 36 kDa and 10 kDa bands from grass carp extract corresponded to β -enolase, GAPDH and β -parvalbumin, respectively.

Differential sensitization patterns to fish allergens between populations

To understand the IgE sensitization pattern, we purified the well-characterized fish allergens including collagen, β -enolase, aldolase and β -parvalbumin from both salmon and grass carp. All allergens had >90% purity as validated by SDS-PAGE (Fig. 2A, B). IgE binding to these allergens coated onto ELISA plates was evaluated using 46 serum samples from each of HK and Japan subjects. The respective sensitization rates are summarized in Table 2.

For both fishes, parvalbumin was the major allergen that showed an overall sensitization rate of 74.7% (71/95) in fish allergic subjects (Fig. 2C–F) while enolase is the least common allergen (17.8% sensitization). Interestingly, the sensitization rates to salmon enolase, the heat-labile allergen, was statistically higher among subjects from Japan (15.2%) than HK (4.3%) (Chi-square test; $p = 0.0396$). No remarkable difference in parvalbumin and collagen sensitization rate could be found between the two study cohorts (Chi-square test; $P > 0.05$). Mono-sensitization to salmon or grass carp parvalbumin was the most common in both populations (28.3%–37.0%). Only 7/96 subjects did not show IgE binding to parvalbumin but other fish allergen, and collagen represented the leading sensitizer in these patients (6/7 patients reacted to collagen). Mono-sensitization to collagen only but not parvalbumin was further confirmed by western blotting (Supplementary Fig. 2), by which sera from these patients only bound grass carp or salmon collagen but not parvalbumin of the same fish. IgE-binding to both parvalbumin and collagen was the most common form of co-sensitization (13.0%–23.9%).

For both populations, the IgE avidity (i.e. OD450nm) of grass carp parvalbumin was higher than salmon parvalbumin as noted in the heatmap (Fig. 2) (Wilcoxon matched-pairs signed rank test; $p < 0.0001$ for both populations), despite of the significantly higher salmon-sIgE level than grass carp-sIgE level among the Japanese. By means of inhibition Western blot, it is noted that grass carp parvalbumin completely inhibit IgE binding to salmon parvalbumin for both populations (Fig. 3), while salmon extract did not produce the same inhibition effect against grass carp parvalbumin. This further

Table 1
Subject demographics.

Demographic Features	Hong Kong	Japan	p-value
N	67	46	NA
Gender: male, % (no.)	76.1 (51)	67.4 (31)	<0.0001*
Age (y): median [range]	6 [1–16]	10 [5–18]	0.3913**
oFASS [†] : median [range]	2 [1–4]	2 [1–5]	0.5251**
SPT wheal size (mm): median [range]	5 [0–11] [‡]	ND	NA
sIgE to cod (kUA/L): median [range]	5.58 [0–66.7]	4.83 [0.10–74.2] [§]	0.9706**
sIgE to salmon (kUA/L): median [range]	6.61 [0.02–100]	8.50 [0.10–118] [¶]	0.9179**
sIgE to tuna (kUA/L): median [range]	2.30 [0.01–100]	4.37 [0.10–71.9]	0.3482**
sIgE to grass carp (kUA/L): median [range]	21.7 [0.03–100]	3.14 [0.01–100] [#]	0.0020**

NA, not applicable; ND, not done.

Bolded p-value indicates statistical significance.

* Statistical analysis based on Fisher-exact test.

** Statistical analysis based on Mann–Whitney test.

[†] oFASS, Ordinal Food Allergy Severity Score.

[‡] 57 subjects had SPT results.

[§] 35 subjects had sIgE to cod tested.

[¶] 37 subjects had sIgE to salmon tested.

^{||} 27 subjects had sIgE to tuna tested.

[#] 30 subjects had sIgE to grass carp tested.

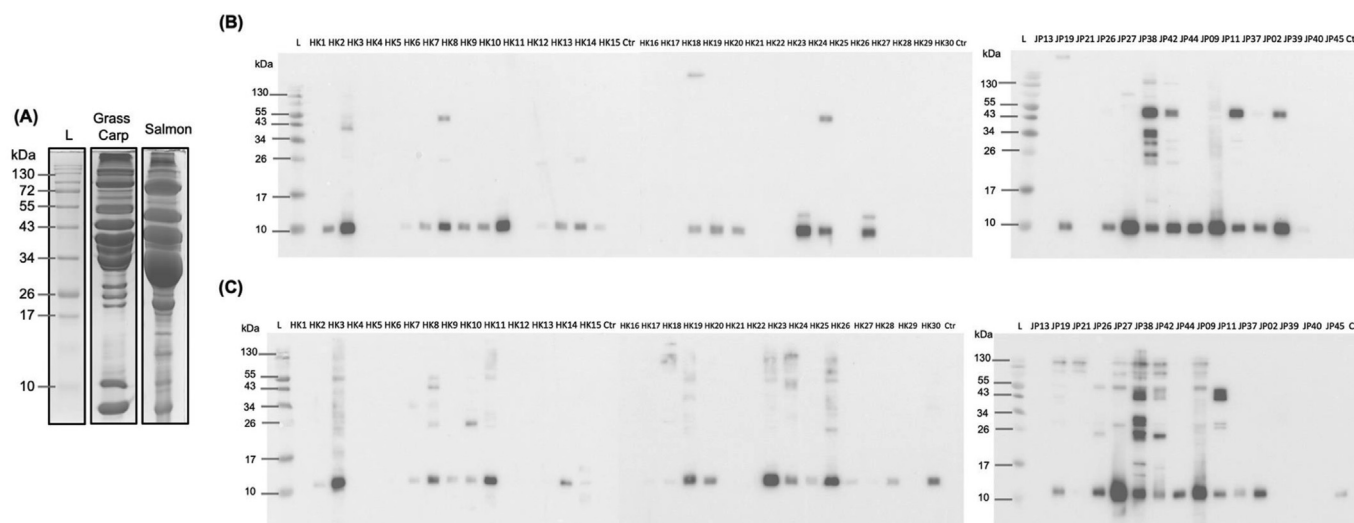


Fig. 1. Identification of IgE-binding fish proteins. **(A)** SDS-PAGE of the extracted raw salmon and grass carp proteins. **(B)** IgE-binding proteins of grass carp extract probed using sera from HK and Japan (JP) subjects. **(C)** IgE-binding proteins of salmon extract probed using sera from HK and JP subjects. A total of 30 HK samples and 15 JP samples were used for allergen identification as noted at each lane. Patient demographics and clinical features are detailed in [Supplementary Table 1 and 2](#), protein ladder. Ctr, non-atopic control.

illustrated the higher IgE avidity (and so allergenicity) of grass carp parvalbumin than salmon parvalbumin. Comparatively, inhibition by salmon parvalbumin was stronger with Japanese sera than by Hong Kong sera ([Fig. 3A](#)). However, no difference could be detected between the two populations in terms of the IgE binding intensity of grass carp parvalbumin (Mann–Whitney test; $p = 0.0562$) despite the significantly higher grass carp-sIgE level for the HK subjects than Japanese patients.

Relationship between dietary habit and sensitization patterns

Noting that Japanese subjects had higher salmon-sIgE level and a more diverse sensitization profile to salmon fish allergens, we sought to investigate if it might be related to different fish cooking preferences. Salmon meat was cooked using different methods until its core temperature reached 80 °C. Equal volumes of the extracted fish proteins were then resolved on SDS-PAGE. A significant portion of proteins were lost upon cooking ([Fig. 4](#)). Interestingly, retention of salmon proteins was the highest after baking followed by frying and airfry and the lowest after steaming, boiling and lightwave cooking. When salmon extracts were analyzed by mass spectrometry, protein bands at 130 kDa, 37 kDa and 8 kDa were retained following all cooking methods, and these bands were identified as α -collagen, GAPDH and parvalbumin, respectively ([Supplementary Table 4](#)). On the other hand, protein bands at 55 kDa, 43 kDa, 40 kDa, 38 kDa, and 34 kDa were visualized most abundantly in baked salmon extract, less after fry/airfry and absent in steamed or boiled extracts. By mass spectrometry analysis, these proteins corresponded to serum albumin, β -enolase, actin, aldolase A and tropomyosin, respectively.

Discussion

In this study, we delineated the allergen profiles of salmon and grass carp using sera from two Asian populations. With the purified allergens from both fish species, our study illustrates parvalbumin and collagen as important biomarkers for fish allergy diagnosis. Our results also indicate a wider allergen spectrum including sensitization to heat labile allergens, as well as stronger sensitization to salmon than grass carp among the Japanese fish allergic subjects where grass carp is unavailable. Such differences highlights that the relevant fish

species as diagnostic marker and allergen sensitization are distinct in different populations due to diverse dietary exposures and habits.

Ruethers *et al.* reported recently 10 allergens from catfish and 6 allergens from salmon.¹³ Specifically, tropomyosin and TPI identified as fish allergens in their study are known shrimp allergens.²⁴ Despite positive shrimp-sIgE by ImmunoCAP in six HK subjects, none showed reaction to fish tropomyosin or TPI in our Western blot. Our subjects also showed no detectable IgE binding to allergens including creatine kinase, pyruvate kinase, L-lactate DH and G-6-PI that were previously reported as catfish allergens. This might partly indicate that these are only catfish-specific allergens and/or the reactions are population-specific. Apart from the well-defined fish allergens (i.e. parvalbumin, enolase, aldolase and collagen), we consistently identified GAPDH as a putative fish allergen of both salmon and grass carp.¹² Such findings supported GAPDH to be an important fish allergen.

Our present report further focuses on studying the more well-defined fish allergens in terms of their sensitization rates. In agreement with most studies that parvalbumin accounted for 70–95% of allergic reactions,^{9,25} parvalbumin is the major allergen in grass carp and salmon with 74% sensitization rate determined by western blotting and ELISA. Collagen is the second commonest fish allergen with 37.5% of subjects being sensitized. The reported rates of IgE binding to collagen vary in different populations. For instance, Kalic *et al.* analyzed samples from 101 fish allergic patients in Australia and showed that 21% of subjects displayed IgE binding to collagen from tuna, barramundi and/or salmon.¹⁴ Kuehn *et al.* reported 19.3% sensitization rate to gelatin of cod, salmon and/or tuna in 62 fish allergic patients.¹⁶ With only 21 patients, Shimojo *et al.* reported a higher rate of 52.4% sensitization to collagen than to parvalbumin²⁶ while Kobayashi *et al.* reported also 50% of sensitization rate to mackerel collagen.²⁷ IgE binding to collagen was also more frequent in patients with more severe fish allergic symptoms than those with mild-moderate responses while independent reports have shown anaphylaxis provoked by gelatin/collagen.^{15,28} On the other hand, Kalic *et al.* reported that 8/21 of collagen-IgE positive patients showed IgE binding to collagen only but not parvalbumin.¹⁴ Comparatively, seven of our 37 collagen-IgE positive subjects demonstrated moderate IgE binding to collagen but absence of parvalbumin-specific IgE in both ELISA and Western blot. Taken together, collagen is a prevalent allergen in Asian

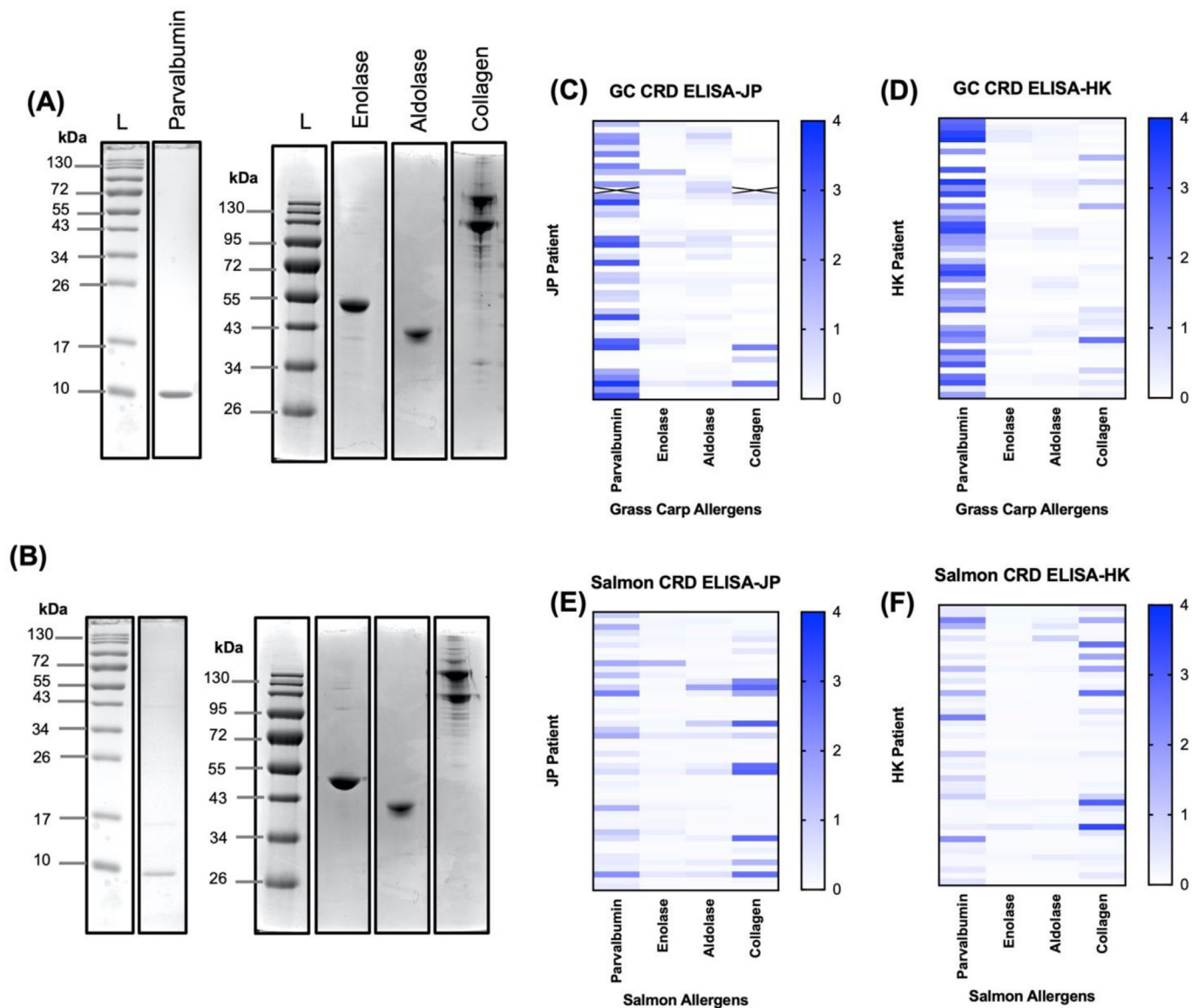


Fig. 2. Purified fish allergens and their sensitization pattern. Purified parvalbumin, enolase, aldolase and collagen from (A) grass carp and (B) salmon on SDS-PAGE. Their sensitization patterns were then evaluated on ELISA and shown on heat maps. IgE binding to purified grass carp allergens for (C) Japan and (D) HK fish allergic subjects, as well as IgE binding to purified salmon allergens for (E) Japan and (F) HK fish allergic subjects. L, protein ladder.

Table 2
Rate of sensitization to fish allergens[†]

	Salmon				Grass Carp			
	Parvalbumin	Enolase	Aldolase	Collagen	Parvalbumin	Enolase	Aldolase	Collagen
Japan	63% (29)	15.2% (7)	23.9% (11)	30.4% (14)	68.9% (31/45)	17.8% (8)	33.3% (15)	17.8% (8/45)
Hong Kong	56.5% (26)	4.3% (2)	15.2% (7)	43.5% (20)	78.3% (36)	15.2% (7)	28.3% (13)	30.4% (14)

[†] 46 subjects were included for analysis from each population, except only 45 Japanese subjects were tested for IgE reactivity against parvalbumin and collagen from grass carp due to insufficient serum.

populations. Fish allergic patients can have IgE binding to collagen only, supporting that testing for collagen should be included in the diagnostic workup for fish allergy.

To our knowledge, only one study with 62 fish allergic subjects reported the sensitization rates of 62.9% and 50.0% to enolase and aldolase, respectively.¹⁶ Another smaller study by Ruethers *et al.* showed that half of their 16 Australian patients showed weak to moderate IgE reactivity to enolase in commercial tuna and salmon

SPT extracts.²⁹ In our cohort, IgE binding to aldolase was detected in 38.5% of subjects while that to enolase was present in 17.8% of subjects. We did not anticipate a major difference in the reactivity of natural and recombinant aldolase and enolase as the reason for lower sensitization as the recombinant enzymes were confirmed to be reactive consistently by ELISA (data not shown) and western blots. Such discrepancy might, on the other hand, be explained by differences in the ethnicity and age of the studied populations: this

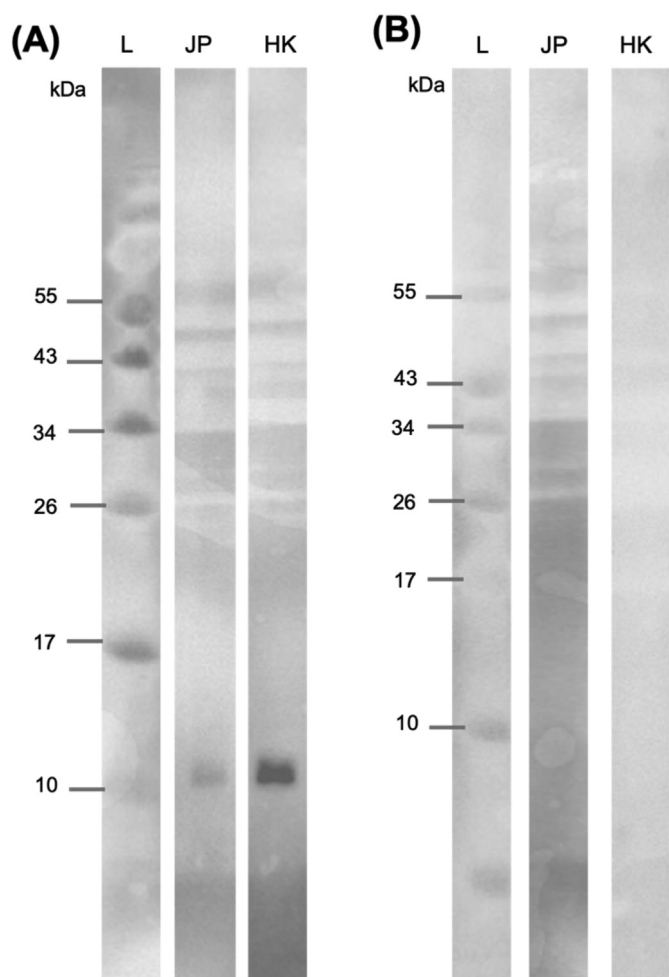


Fig. 3. Inhibition Western blot. Pooled sera from Japan (JP) and HK subjects were pre-adsorbed with (A) salmon parvalbumin and blotted against grass carp parvalbumin, or (B) with grass carp parvalbumin and blotted against salmon parvalbumin. Note that salmon parvalbumin partially inhibited IgE of Japanese subjects in binding to grass carp parvalbumin but exerted minimal effect to inhibit IgE of HK subjects. Grass carp parvalbumin completely inhibited IgE binding to salmon parvalbumin for both populations.

study only recruited children below 18 years old while many subjects studied by Kuehn *et al.* were young adults (mean age 28.7, range 13–62 years). Among seven subjects without parvalbumin-specific IgE in our cohort, only one Japanese subject showed IgE binding to salmon and grass carp aldolase. Testing of enolase and aldolase thus do not substantially improve the diagnostic yield for fish allergy in Asians while detecting IgE against parvalbumin and/or collagen might be sufficient to identify patients with fish allergy.

This study found minimal binding of sera from HK subjects to allergens other than parvalbumin by western blotting. A possible explanation was the use of different serum samples for ELISA and Western blot, different sensitivity of the assays, intrinsically low abundance of these allergens in fish extracts and the insoluble nature of collagen in aqueous solution.³⁰ Nevertheless, both ELISA and western blot assays robustly indicated a wider spectrum of allergen sensitization in Japanese than HK subjects. Japanese commonly consume raw fishes, but this is a less likely reason for this observation given the age-range of our recruited patients; the consumption of raw fish might not be a major attribute to the sensitization to a wider spectrum of allergen including the heat labile allergens. Our present study showed, for the first time, differences in fish protein and allergen profiles in relation to cooking methods at the same core

temperature. Despite higher cooking temperature with frying/baking compared with steaming/boiling, frying/baking retained more heat labile allergens in salmon meat. Steaming and boiling to make fish congee and soup are the preferred methods of fish cooking in Hong Kong while baking and frying of fish are more popular in Japan. For instance, 73.8% of the interviewed Japanese workers consumed fish by broiling (baking) comparing to only 14.1% for raw fish and 10% by stewing.³¹ Although we are aware that our present study is limited by the lack of direct investigation into the dietary habits of our studied populations, our results support the importance of dietary exposures, consumption and cooking preferences in determining fish allergen sensitization in particular when the two populations did not differ in terms of symptom severity. This factor should be considered during the selection of allergens for allergy testing and the interpretation of CRD results.

In the context of fish exposure, Japanese patients should not have eaten grass carp due to the unavailability of this freshwater fish. Thus, IgE binding to grass carp allergens in Japanese patients should be a result of cross-reactivity while HK subjects could be independently sensitized to the two fish species. This can be partly reflected by inhibition western blot that salmon parvalbumin partly inhibited IgE of Japanese patients in binding to grass carp parvalbumin that is in contrary to that by IgE of HK patients. However, we observed that more Japanese patients reacted to grass carp parvalbumin with higher OD values in ELISA and grass carp parvalbumin also completely inhibited IgE binding to salmon parvalbumin among the Japanese. This can be explained by the higher allergenicity of grass carp parvalbumin. Furthermore, Cten i 1 (grass carp parvalbumin) shares 82.41% protein sequence homology with salmon parvalbumin- β 2. While mackerel is a commonly consumed fish among Japanese, parvalbumin from Atlantic mackerel also shares 77.98% sequence homology with Cten i 1. Together with the consumption of other fishes, these might explain the higher IgE avidity to grass carp parvalbumin than salmon parvalbumin in Japanese subjects despite a lack of prior exposure. The higher salmon-sIgE level might thus be explained by IgE binding to other salmon allergens, particularly collagen, when considering the lower parvalbumin content in crude salmon extract, lower IgE affinity of salmon parvalbumin in ELISA, and the more diverse salmon allergen binding pattern in western blots. On the other hand, we also showed that one-fifth of subjects reacted only to grass carp parvalbumin, which indicated that grass carp parvalbumin has higher allergenicity than salmon parvalbumin. However, we are yet to know the clinical reactivity to grass carp among the Japanese subjects and so the question of improving assay sensitivity and specificity using fish allergen (parvalbumin) with higher allergenicity remains open. The varying allergenic potential of fish parvalbumins could be explained by the specific sensitization of patients from their clinical history.⁸ With the lack of grass carp in the local Japanese diet, our results suggested the presence of higher diversity, distinct and/or species-specific IgE-binding epitopes for grass carp parvalbumin.³² It is of note that IgE sensitization based on parvalbumin did not correlate well with patients' clinical allergy status,¹⁸ and we postulate that the identification of parvalbumin epitopes may help to resolve the inconsistency in cross-reactivity of different fish species.

We are aware that the sensitization pattern reported herein was based on ELISA that is not a standard platform. Each allergen might display different IgE binding kinetics in ELISA that leads to signal visualization bias. The cross-comparison of signal intensity among different allergen components might lack accuracy. Future studies replicating the experiments on different immunological platforms would be essential.

In summary, this study extends our understanding on the allergen profile of two important fish species that parvalbumin and

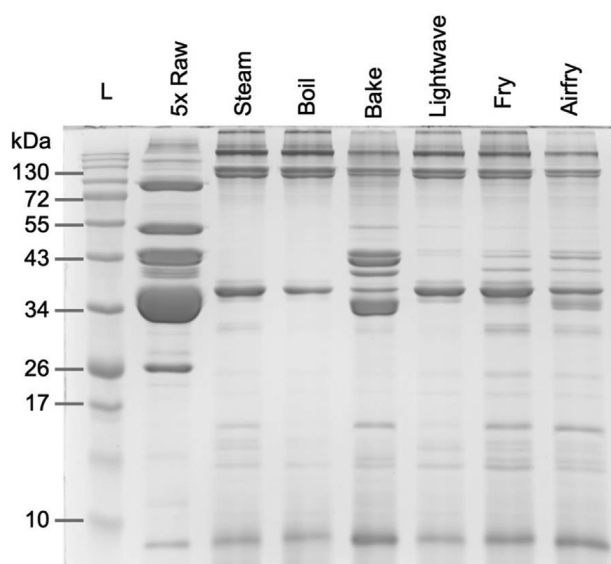


Fig. 4. SDS-PAGE of salmon meat prepared by different cooking methods. Raw protein was diluted by 5-fold for better band visualization. L, protein ladder.

collagen are important components for fish allergy diagnosis. We also illustrated that the fish species extracts relevant for diagnosis and allergen sensitization pattern differ among populations. Overall, this study provides additional information for the implementation of relevant extracts and components for precise fish allergy diagnosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2023.03.003>.

Conflict of interest

CYYW, NYHL, ASYL, TFL are inventors of a US Non-provisional patent on "Improved accuracy of fish allergy diagnosis using grass carp allergens" with publication no. US2020/0191797 A1 on June 18, 2020. ASYL received payment for FMSHK Certificate Course in Allergy 2022 & 2021. SS received honoraria for lectures from Viatrix, Torii Pharmaceutical. The rest of the authors have no conflict of interest.

Authors' contributions

Performing laboratory assays and contributing to data analysis: CYYW, NYHL, KJYX; Subject recruitment and sample collection: ASYL, NF, SS, YSY, JSRD, MYWK, JWCHC, NF, WHC, GTC, QUL, DCKL, PKH, JSCW, ICSL; Writing – original draft: CYYW, NYHL; Funding acquisition and work supervision: GWKW, ME, TFL. All the authors have read and approved the manuscript.

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