

Evaluation of Allergic Cross-Reactivity Among Fishes by Microfluidic Chips and MALDI-TOF MS

Xin Zhao and Hongyan Bi*

Cite This: *J. Agric. Food Chem.* 2022, 70, 7525–7534

Read Online

ACCESS |



Metrics & More



Article Recommendations



Supporting Information

ABSTRACT: Fish is one of the most common foods that cause allergic reactions. The study of cross-reactivity among fishes using mass spectrometry (MS) is still limited. We developed a strategy using microfluidic chips coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to evaluate cross-reactivity among fishes. The protocol employed commercial magnetic beads functionalized with anti-human IgE antibodies to carry out the IgEs immunomagnetic separation in blood samples, followed by the capture of allergens from seafood protein extracts in a single-straight microfluidic channel. After elution, the captured allergens were digested and identified by MALDI-TOF MS and high-performance liquid chromatography-tandem mass spectrometry and validated by enzyme-linked immunosorbent assay (ELISA). An investigation of the reproducibility revealed that the protocol can sense well the allergens in a food matrix. Seven fish species were analyzed to evaluate the allergic cross-reactivity among fishes. The commercial ELISA test gave consistent results with the presently developed strategy when the same allergenicity test was performed. Parvalbumins were detected from five of the seven analyzed fishes. The sequence alignment of parvalbumins revealed that the similarity of parvalbumins identified from the analyzed fishes is larger than 64%. Boiling may reduce the allergenicity of fish, as demonstrated by a marginal diminish in the parvalbumin content of crucian carp (*Carassius carassius*) muscle when boiling with water. The method can potentially be used to predict allergic cross-reactivity among fish species, provide advice and guidance to individuals with a history of seafood allergy, and ensure food safety in the food allergy community.

KEYWORDS: cross-reactivity, fish muscle, mass spectrometry, magnetic beads (MBs), microfluidic chips

INTRODUCTION

Food allergies are becoming a tremendous threat to people's lives and health with the changes in the structure of people's diets. It is particularly important to develop simple, rapid, and accurate methods to detect allergens in food matrices.

The U.S. Food and Drug Administration (FDA) enforces regulations requiring companies to list ingredients on packaged foods and beverages to protect those with food allergies and other food hypersensitivities. The ability to test allergens in ready-to-eat foods plays a very important role in food allergy control. In addition, it is usually desirable to test suspicious food samples in order to monitor the food supply chain for undeclared allergens and take action when they are found.

Unlike most other food allergies, seafood allergy persists for life in up to 90% of patients,¹ and it deserves more attention in seafood allergy studies. Bradford assay, enzyme-linked immunosorbent assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting have been used to evaluate the allergenicity of protein extracts of codfish, mackerel, common eel, flounder, cutlass, and catfish.²

Only a limited number of mass spectrometry (MS)-based methods have been reported for the seafood allergen test. Selected MS/MS ion monitoring MS was applied to analyze the major fish allergen, parvalbumin, in any foodstuff, where a methodology based on the purification of parvalbumins beta (β -PRVB) by treatment with heat, the use of accelerated in-solution trypsin digestion under an ultrasonic field provided by high-intensity focused ultrasound (HIFU), and the monitoring

of 19 β -PRVB peptide biomarkers was developed.³ We recently developed a strategy for the discovery and effective identification of unknown allergens in seafood matrices based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).⁴

The risk of clinical or allergic cross-reactivity needs to be understood to assist in food allergy optimal care, such as preventing over avoidance of safe foods and averting exposure to potentially allergenic foods.⁵ The cross-reactive allergens have been studied by enzyme-linked immunosorbent assay (ELISA) and inhibition ELISA.⁶ Mehrdana *et al.* evaluated IgE-cross reactivity of larval proteins from other zoonotic parasites present in freshwater and sea fish by *in vivo* (skin prick tests with parasites extracts) and *in vitro* methods (IgE-ELISA and IgE-immunoblot).⁷ Leung *et al.* investigated the cross-reactivity among parvalbumins from grass carp and other phylogenetically close (common carp) or commercially important (cod and salmon) species by competitive inhibition ELISA.⁸

Food processing in different regions may impact the prevalence of seafood allergies. Thermal processing methods

Received: April 8, 2022

Revised: May 25, 2022

Accepted: June 2, 2022

Published: June 13, 2022



such as baking, steaming, and a high hydrostatic pressure (HHP) were applied to improve digestibility, reduce allergenicity, and avoid protein oxidation in cod (*Gadus morhua*).⁹ Yu *et al.* investigated the effectiveness of some nonspecific proteases in reducing raw peanut allergenicity and found that Alcalase is relatively effective in reducing the overall allergenicity compared with those of bromelain and neutrase.¹⁰

Various sensors have been developed to detect allergens in seafood. A visible-light-driven photoelectrochemical aptasensor was developed for the detection of shrimp allergen tropomyosin using a graphitic carbon nitride–TiO₂ nanocomposite.¹¹ An electrochemical tropomyosin allergen immunosensor, based on a combination of amperometric transduction, magnetic particles, and disposable screen-printed electrodes, was developed for the detection of shrimp tropomyosin.¹² Gold microrods (AuMRs), Pd nanoparticles (PdNPs), and polyaniline (PANI) nanocomposite interface were fabricated on the screen-printed carbon-microelectrode (SPE) to detect tropomyosin in seafood samples.¹³ A surface plasmon resonance (SPR) sensor with gold-patterned biochips was built for detection and quantification of tropomyosin in shellfish.¹⁴

Microfluidic chips combined with various detection techniques have been widely applied for developing solutions for bioanalysis. Fan *et al.* summarized fluorescent analysis of bioactive molecules in single cells based on microfluidic chips.¹⁵ We previously proposed UV–vis spectroscopy-integrated microfluidic chips for the specific analysis of ingredients in sample matrices with complex backgrounds such as human blood.¹⁶ However, to the best of the authors' knowledge, there is no report on the study of allergic cross-reactivity of seafood based on microfluidic chips.

In the present report, a microfluidic platform combined with MALDI-TOF MS was developed for sorting and identifying the allergens from seafood matrices. The allergic cross-reactivity of seven fish species was analyzed and evaluated by using the developed strategy. The crude proteins from fish muscles, obtained by simplified protein extraction, were used for on-chip immunomagnetic separation (IMS) and MALDI-TOF MS analysis. The combination of microfluidics, IMS, and MALDI-TOF MS as a strategy for testing potential allergens holds the advantages of exempting purification of proteins of interest, a shortened detection time, and the use of a small volume of blood. The tryptic digests of eluted potential allergens were analyzed by MALDI-TOF MS and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) for the identification of proteins. The same allergenicity test was performed by commercial ELISA for the validation of the present strategy. The allergic cross-reactivity among fishes was evaluated by aligning the sequences of allergens that were identified. In addition, the raw seafood materials were processed or cooked and taken for the allergy test. Considering that fish muscles are often consumed as a soup by boiling in Asia, herein, the muscle sample of crucian carp (*Carassius arassius*, CC) was boiled for different periods of time to obtain a soup-like food, as a model of foods ready for consumption, and its allergenicity test was performed by using the developed method. It was found that boiling the fish muscle with water marginally diminishes the allergenic properties of parvalbumin from CC muscle. The method can be potentially used to predict the cross-activity among fish species to provide advice and guidance to individuals with a history of allergy.

MATERIAL AND METHODS

Chemicals, Seafood, and Sera. Acetic acid (AA, 36–38%), acetonitrile (ACN, 99.90%), ethylenediaminetetraacetic acid (EDTA) (≥99.0%), and 2-propanol (99.80%) were bought from Shanghai Titan Scientific Co., Ltd. (Shanghai, China). Ammonium sulfate (≥99.0%), sinapinic acid (SA, ≥99.0%), cytochrome c from the equine heart (≥95%), myoglobin from equine skeletal muscle (≥98%), trypsin from bovine pancreas (≥10,000 BAEE units/mg protein), and Tween 20 (analytical grade) were purchased from Sigma-Aldrich (St. Louis, USA). Bovine serum albumin (BSA, 96%) was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Hydrochloric acid (HCl, 36–38%) and phosphate-buffered saline solution (PBS, 10 mM, pH 7.4) were purchased from Collins Reagent Co., Ltd. (Shanghai, China) and Shanghai LabTop Bio-Technology Co., Ltd. (Shanghai, China), respectively. Sodium borate buffer (0.1 M, pH 9.5), trifluoroacetic acid (TFA, ≥99%), and tris(hydroxymethyl) aminomethane (Tris, 99.5–100.5%) were obtained from Shanghai Qincheng Biotechnology Co., Ltd. (Shanghai, China), Fisher Scientific (Loughborough, UK), and Adamas-Beta Co., Ltd. (Shanghai, China), respectively.

Tosyl-activated superparamagnetic polystyrene beads with a diameter of 1.5 μm were obtained from BioMag Scientific Inc. (Wuxi, China). Mouse monoclonal anti-human IgEs were obtained from AbD Serotec (Oxford, UK). A Milli-Q Simplicity system (Millipore, Bedford, MA, USA) was used to produce deionized (DI) water for the preparation of all the aqueous solutions.

Acetonitrile (ACN, ≥99.8%) from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), formic acid (99%) from J&K Scientific Ltd. (Beijing, China), and distilled water from Watsons Water Co. Ltd. (Shanghai, China) were used for HPLC-MS/MS analysis.

Large yellow croaker (*Larimichthys crocea*, shortened as LC in the present study), grass carp (*Ctenopharyngodon idella*, CI), Mozambique tilapia (*Oreochromis mossambicus*, OM), and crucian carp (*Carassius carassius*, CC) were purchased in April 2021 from Dingdong Maicai App (Shanghai, China). Japanese seabass (*Lateolabrax japonicus*, LJ) and silver carp (*Hypophthalmichthys molitrix*, HM) were purchased in May 2021 from Dingdong Maicai App (Shanghai, China). Slices of certified Patagonian toothfish (*Dissostichus eleginoides*, DE) were obtained in October 2020 from the Lingang Ftabest Free Trade Import Food Center (Pudong New Area, Shanghai, China). Information such as classification, size, and weight of the studied fish samples with their probable origins is listed in the [Supporting Information S1](#).

Sera of two subjects, age 3 and 8, respectively, with immunoglobulin E (IgE)-mediated food allergy were collected by the Shanghai Hospital (Shanghai, China). The allergy blood tests of these patients were performed by the corresponding hospital. The IgE titers in the sera of the patients are 186 and 143 IU/mL, respectively. In the present study, the 3 year old patient with an IgE titer of 186 IU/mL was referred to as patient 1; and the 8 year old patient with an IgE titer of 143 IU/mL was referred to as patient 2. [Table S3](#) lists the clinical history data tested by the hospital.

The blood samples were collected with the consent of the donors and were in agreement with the World Health Organization's rules for "the clinical use of blood." No research on genetic material was conducted.

Functionalization of MBs. The obtained MBs were functionalized according to the manufacturer's protocol. Briefly, 2.5 μL of 20 mg/mL MBs were washed 3 times with sodium borate buffer (0.1 M, pH 9.5), magnetically separated, and the pellets were collected and resuspended in 2.5 μL of 0.1 M sodium borate buffer (pH 9.5). 2.5 μL of anti-human IgEs (1 mg/mL), 6.3 μL of 0.1 M sodium borate buffer (pH 9.5), and 5.2 μL of 3 M ammonium sulfate were added to the MB suspension. The obtained solution was shaken at 850 rpm overnight at room temperature (RT) on an incubation machine (ThermoMixer C, Eppendorf, Hamburg, Germany) to avoid MB sedimentation. Then, the mixture was magnetically separated, and the pellets were collected and mixed with 20 μL of PBST (0.05% Tween 20 in 10 mM PBS, v/v, pH 7.4) containing 5% (m/m) of BSA and

then incubated at RT for 6 h to coat BSA onto the surface of the MBs for preventing the nonspecific binding of reactants, especially proteins in the analyzed biosamples. The obtained anti-human IgE-functionalized MBs were washed three times with PBST (0.05% Tween 20 in 10 mM PBS, v/v, pH 7.4) for further use.

The above-prepared anti-human IgE-functionalized MBs (ca. 1 μ L) was transferred into an Eppendorf tube, and then 13 μ L of the serum sample from a fish-allergic patient was added, and the mixture was incubated at RT for 5 min. Afterward, the mixture was placed on a magnetic separation rack (BMB-01, with 16 holes for 1.5–2 mL Eppendorf microcentrifuge tubes, BioMag Scientific Inc., Wuxi, Jiangsu, China) for the collection of MB pellets. The resultant IgE-coated MB pellets were collected to interact with seafood matrices after rinsing three times with PBST (10 mM, 0.05% Tween 20, pH 7.4) and once with DI water.

Extraction of Proteins from Fish Samples. 3 g of LC, OM, CC, CI, LJ, DE, and HM muscle samples were separately placed in a beaker with 20 mL of buffer solution (20 mM Tris–HCl containing 5 mM EDTA, pH 7.5) and homogenized for 10 min with an electric homogenizer (F6/10 handheld high-speed homogenizer, Shanghai Jingxin Technology Co., Ltd., Shanghai, China). Then, the suspension was centrifuged (S810 R, Eppendorf, Hamburg, Germany) at 6797 g and 4 °C for 30 min, and the supernatant was collected and interacted with IgE-coated MBs for the allergy test.

The crude fish sarcoplasmic proteins (FSPs), and eluted solutions of the captured allergens of seven fish samples were filtered through Whatman filter paper and then tested by a third party (Shanghai Jingkang Biology Engineering Co., Ltd., Shanghai, China) using a BCA kit (JC0001, 100T, Shanghai Jingkang Biology Engineering Co., Ltd., Shanghai, China) and a parvalbumin-specific ELISA kit (JLCA4402, 48T, Shanghai Jingkang Biology Engineering Co., Ltd., Shanghai, China), respectively for the determination of their protein contents.

Pretreatment of Microfluidic Channels. A 1 cm length of a single straight channel with a 200 μ m $\times$ 200 μ m cross-section was fabricated by polydimethylsiloxane (PDMS) soft lithography and bound to a glass substrate to form a sealed microfluidic chip. The BSA solution (5%, m/m, in PBST buffer (10 mM with 0.05% Tween 20, v/v, pH 7.4) was filled in the microchannels for 1 h to block the unspecific adsorption sites on the surface of the channels prior to the allergen capture. The PBST buffer (10 mM PBS buffer with 0.05% Tween 20, v/v, pH 7.4) was pumped through the channel twice to wash the loosely attached biomolecules away, and then the channel was washed once with DI water.

On-Chip Capture of Allergen. The fish sarcoplasmic protein solutions were mixed with the resultant IgE-coated MBs and filled in the microfluidic channels for 1 h. Then, the channels were magnetically fixed in the microfluidic channels by using a 60 $\times$ 40 $\times$ 5 mm³-sized NdFeB permanent magnet (FeiDiXing flagship store, Taobao, Jiangxi, China) and rinsed two times with the PBST buffer solution (10 mM PBS with 0.05% Tween 20, v/v, pH 7.4) and once with DI water. Afterward, 10 μ L of 5% (v/v) acetic acid solution was filled in the microfluidic channel to capture proteins on the surface of MBs, and 1 μ L of the eluted solution was deposited on the MALDI target plate (Bremen, Germany), and after air drying, 1 μ L of the matrix, sinapic acid (SA), was dropped onto the surface and dried for MALDI-TOF MS analysis.

Digestion of Allergens by Trypsin. 5 μ L of the above-eluted allergens were diluted 10 folds with PBS (10 mM, pH 7.4) and mixed with 1 μ L of 1 mg/mL trypsin for digestion at 37 °C for 14 h and terminated by controlling the final TFA concentration at 0.4% (v/v). The obtained digests were subjected to MALDI-TOF MS and HPLC-MS/MS, respectively, for further analysis.

MALDI-TOF MS Analysis. A Bruker microflex LRF mass spectrometer (Bremen, Germany) was used for performing all MALDI-TOF MS experiments. A protein aqueous solution containing cytochrome c (2 mg/mL) and myoglobin (4 mg/mL) as an external standard was used for the calibration of the MALDI-TOF mass spectrometer by utilizing the doubly and singly charged ions of cytochrome c (m/z = 6181 and 12,361, respectively) and myoglobin

(m/z = 8476 and 16,952, respectively). The matrix for the protein analysis contained 15 mg/mL of SA in a 70:30:0.1 (v/v) acetonitrile/water/TFA mixture. 1 μ L of the eluted samples with allergens were deposited on a MALDI target plate (MSP 96 target ground steel). After air-drying at RT, the sample spots were covered with 1 μ L of the SA solution (15 mg/mL) and dried prior to MALDI-TOF MS analysis.

The linear and positive ion modes were used to acquire mass spectra in the m/z range of 2000–20,000 or 2000–10,000. Sum spectra were made from 500 laser shots using the automated sample collection mode. Laser frequency, detector gain, and laser intensity were set as 20 Hz, 10.3 $\times$, and 65%, respectively. At least three mass spectra were acquired for each sample.

Identification of Allergens Captured by MBs via MALDI-TOF MS. Identification of allergens captured by magnetic beads (MBs) was achieved by using the search engine (<https://www.uniprot.org>) for searching against the UniProt database and assigning the measured ion peaks. Mass spectrometric data analysis of tryptic digests was performed with FlexAnalysis from Bruker (v. 3.4, Daltonik GmbH, Bremen, Germany) and the FindMod tool available on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) (<http://lexpasy.org/>). The masses and intensities of peaks were sent to FindMod for database searching. The peak identification was achieved by comparing the experimentally measured peptide masses generated from the unknown proteins with the masses of the theoretical peptides calculated from a specified Swiss-Prot/TrEMBL entry.

Identification of Allergens Captured by MBs via HPLC-MS/MS. The above-prepared tryptic digests of allergens were desalted with a desalting column (MonoSpin C18, GL Science Inc, Tokyo, Japan) on a centrifuge system (Hitachi centrifuge, Hitachi Koki Co., Ltd., Japan). The obtained solution was dried in a vacuum drying system (LNG-T98 vacuum dryer, Taicang Huamei Biochemical Instrument Factory, Jiangsu, China) at RT for about 40 min.

The column used for HPLC-MS/MS analysis was a ChromXP C18 column (120 Å, 0.3 $\times$ 150 mm, 3 μ m, Eksigent AB Sciex, Singapore). The mobile phase was composed of A (98% water with 2% ACN and 0.1% formic acid) and B (98% ACN with 2% water and 0.1% formic acid), with a gradient elution of 0–6 min (B, 0%), 6–6.5 min (B, 0–5%), 6.5–95 min (B, 5–28%), 95–100 min (B, 28–55%), 100–105 min (B, 55–80%), 105–112 min (B, 80%), 112–113 min (B, 80–5%), 113–120 min (B, 5%), and 120–124 min (B, 0%). The flow rate was set as 0.012 mL/min (0–6 min), 0.15 mL/min (6–120 min), and 0.02 mL/min (120–124 min), respectively. A column temperature of 40 °C was utilized. 27 μ L of the sample was injected.

An electrospray ionization (ESI) source was used to couple a quadrupole time-of-flight (QTOF) mass spectrometer (Triple TOF 4600, AB SCIEX, Foster City, CA, USA) with an HPLC system (Shimadzu 20A, Shimadzu, Tokyo, Japan). A positive ionization mode was utilized. ESI was set as 5500 V, and ion source parameters were optimized as follows: gas 1, 25 psi; gas 2, 15 psi; curtain gas, 30 psi; and source temperature, 350 °C. MS/MS data were acquired in the mode of information-dependent acquisition. The maximum number of candidate ions to monitor per cycle and the mass range were set as 40 and 350–1250 (m/z), respectively. MaxQuant (www.maxquant.org) was used to identify peptides from the HPLC-MS/MS data.

Deallergenization of Fish Muscle by Thermal Processing. 10 g of the CC muscle sample was added into 200 mL of DI water and boiled on a hot plate (RH digital, IKA, Staufen, Germany) for 5, 15, 25, 30, 45 min, and 1 h, respectively. The resultant aqueous extracts were collected and centrifuged at 4 °C and 11,000g for 20 min on a centrifuge system (S810 R, Eppendorf, Hamburg, Germany). The resultant supernatant was collected and filtered by using a 0.22 μ m membrane and then subjected to MALDI-TOF MS analysis.

Safety Consideration. TFA is considered hazardous. Use TFA only outdoors or in a well-ventilated area. Wear hand/eye/face protection, and wash face, hands, and any exposed skin thoroughly after handling TFA.

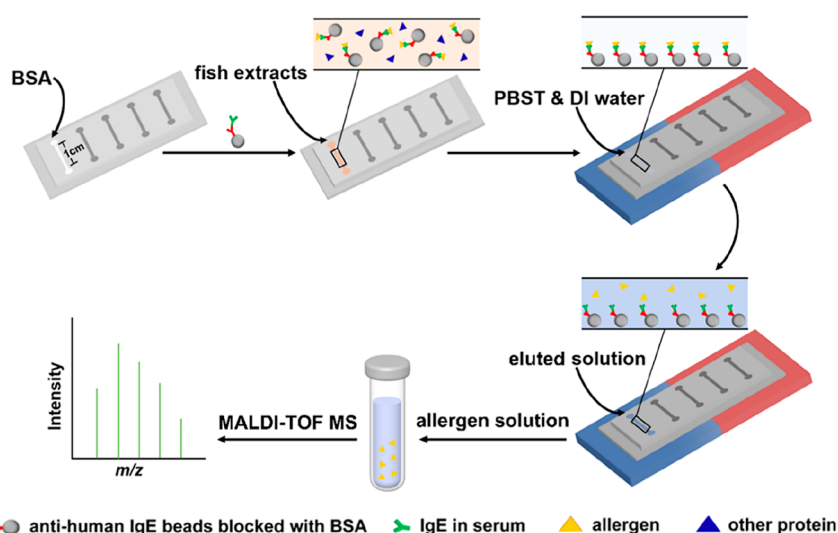


Figure 1. Procedure of the on-chip allergy test based on the MB-filled microfluidic channel and MALDI-TOF MS. MBs functionalized with anti-human IgE antibodies were used to carry out IgEs in the blood sample, followed by allergen capture from seafood protein extracts and rinsed with the PBST solution and DI water. After elution, the captured proteins were analyzed by MALDI-TOF MS.

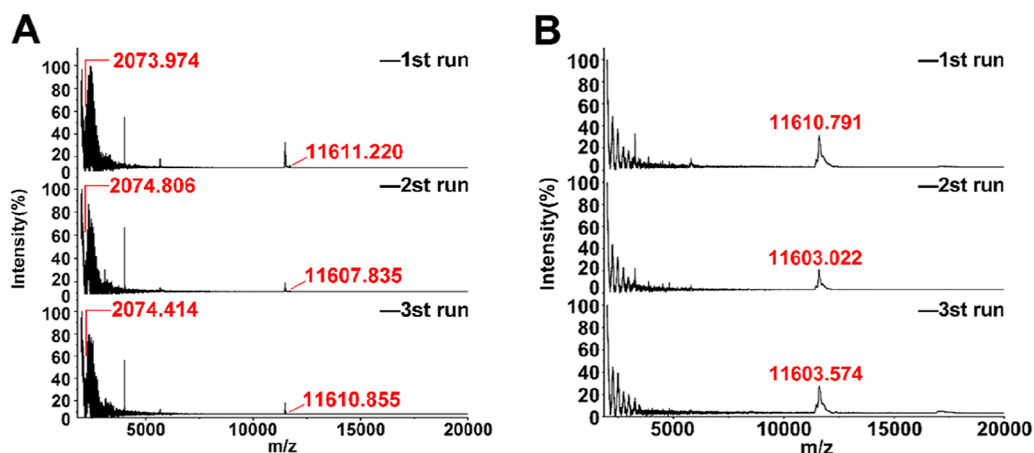


Figure 2. Representative MALDI-TOF mass spectra obtained from three independent experiments during the DE allergy test of the sera of patient 1 (A) and patient 2 (B). During the experiment, anti-human IgE-functionalized MBs interacted with human serum and then with DE sarcoplasmic proteins in a 1 cm length of a single straight channel with a $200\ \mu\text{m} \times 200\ \mu\text{m}$ cross-section. Functionalized MBs and the single straight channel were blocked with 5% (m/m) BSA. During the experiment, the serum samples were directly utilized and diluted 10 folds in (A) and (B), respectively. The freshly functionalized MBs and freshly extracted proteins from DE muscle samples were utilized in each experiment. Three independent microfluidic channels were used to perform the experiments. The peak m/z values that can be assigned to proteins are denoted in the diagram.

RESULTS AND DISCUSSION

Work Flow of Detection of Allergic Proteins by MBs on a Microfluidic Chip. In the present study, a strategy integrating microfluidic chips with MALDI-TOF MS was developed to evaluate allergic cross-reactivity among fishes. During the experiment, anti-human IgEs beads blocked with BSA were utilized to attach the IgE antibodies (IgEs) from the sera of allergic patients. As illustrated in Figure 1, the fish protein extracts containing substances that a patient is allergic/sensitized to were filled in the microfluidic channels for 1 h. Then, the microfluidic channels were magnetically fixed by a magnet for the separation of allergens and rinsed with PBST buffer solution and DI water. After elution of the captured proteins (allergens) from IgEs on the MB surface, the proteins were digested and analyzed by MALDI-TOF MS and HPLC-MS/MS to achieve the identification of captured allergens.

Reproducibility of Capture of Allergens on Chips. The reproducible performance must be considered when employing a sensor for a practical test. Herein, the FSP solutions mixed with the resultant IgE-coated MBs were injected into three independently prepared microfluidic channels that were blocked with BSA solution in advance. A 1 cm single-straight channel with a cross-section of $200\ \mu\text{m} \times 200\ \mu\text{m}$ was utilized to capture the allergens in the protein extracts of fish. The serum of allergy patient 1 was directly utilized to coat the surface of MBs for the allergy test. Both the surfaces of MBs and microfluidic channels were blocked by using BSA solution.

Three independent tests were performed to sense the allergens in the DE crude protein extracts by using the sera of two seafood allergic subjects. MBs were functionalized with anti-human IgE and blocked with BSA prior to the allergy test. The BSA solution [5% (m/m) in PBST buffer (10 mM with 0.05% Tween 20, v/v)] was filled in the microchannels for 1 h

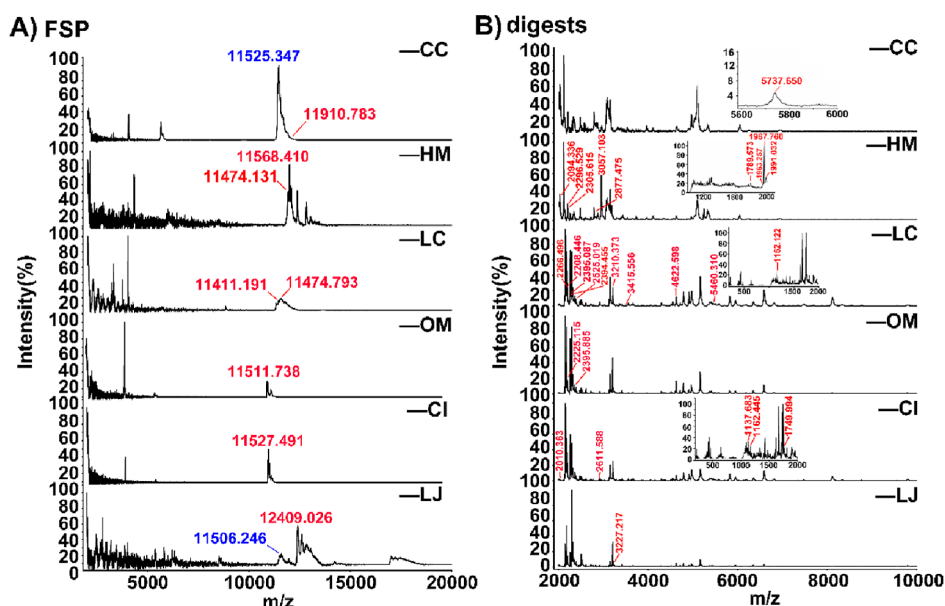


Figure 3. Representative MALDI-TOF mass spectra obtained during the fish allergy test (A) and spectra of tryptic digests (B) of the captured allergen in the FSP of CC (*Carassius auratus*), HM (*Hypophthalmichthys molitrix*), LC (*Larimichthys crocea*), OM (*Oreochromis mossambicus*), CI (*Ctenopharyngodon idella*), and LJ (*Lateolabrax japonicus*) by functionalized MBs, respectively. The serum sample from patient 2 was tested. During the experiment, anti-human IgE-functionalized MBs interacted with human serum and then with DE sarcoplasmic proteins in a 1 cm length of a single straight channel with a $200\ \mu\text{m} \times 200\ \mu\text{m}$ cross-section. Functionalized MBs and the single straight channel were blocked with 5% (m/m) BSA. The serum sample with a 10-fold dilution was used during the experiment. $5\ \mu\text{L}$ of the above-eluted allergens were diluted 10-fold with PBS (10 mM, pH 7.4) and mixed with $1\ \mu\text{L}$ of trypsin (1 mg/mL) at $37\ ^\circ\text{C}$ for 14 h for the digestion, which was terminated by controlling the TFA concentration at 0.4% (v/v). The peak m/z values that can be assigned to proteins are denoted in the diagram. The m/z of the peptides that match the theoretically predicted peptide masses are also denoted in the diagram. Red-color-labeled ions are the ones listed in Table 1. Blue-color-labeled ions are explained in the main text.

to block the unspecific adsorption sites on the surface of the channels prior to allergen capture.

Figure 2 shows the MALDI-TOF mass spectra obtained from three independent tests of applying MBs for the DE allergy test of the serum of seafood allergic patient 1 and patient 2. It can be found that in the three runs of measurements, the allergens can be captured, indicated by clear peaks in the obtained mass spectra. The results show that the developed protocol for the seafood allergy test is reproducible and has the potential to be applied in allergy test studies.

On-Chip Analysis of Allergens in Different Fishes. On-chip allergen analysis was conducted by using solutions of protein extracts of fishes, including LC, OM, CC, CI, LJ, DE, and HM, as listed in Table S1. Figure 3 shows the detected MALDI-TOF mass spectra of allergens in the protein extracts of the analyzed fishes where the serum used for preparing MBs was from patient 2. The specific proteins detected in the analyzed fish species were characterized by using the UniProtKB database. Parvalbumin was reported to be present in CC and LJ.^{17–19} Herein, the blue-color-labeled ions in Figure 3A with $m/z = 11,525.347$ and $11,506.246$ from CC and LJ, respectively, may be parvalbumin. Furthermore, the UniProt database search shows that the probable protein with an ion peak at $m/z = 11,474.793$ from LC may be parvalbumin beta. The ion peaks at $m/z = 11,527.491$ from CI and $m/z = 11,511.738$ from OM are parvalbumin. The probable protein with an ion peak at $m/z = 11,568.410$ and $m/z = 11,474.131$ from HM may be parvalbumin.

Parvalbumin is well-known as a major allergen present in fish.¹⁷ Parvalbumins are calcium-binding proteins with molecular weights (M_w) in the 10,000–12,000 Da range that

are relatively abundant in muscle tissues and well known as major allergy-eliciting proteins.²⁰ Parvalbumin is highly conserved in fish and leads to clinical and serological cross-reactivity among different fish species.⁸

In addition, the probable protein with an ion peak at $m/z = 11,411.191$ from LC may be cytochrome c. From a previous study, cytochrome c is an allergen from the pollens of the dicotyledons English plantain (*Plantago lanceolata*) and Paterson's curse (*Echium plantagineum*).²¹ The probable protein with an ion peak at $m/z = 11,910.783$ from CC may be peptidylprolyl cis–trans isomerase. Peptidylprolyl cis–trans isomerase is confirmed to be a potential allergen from *Periplaneta americana* (Linnaeus) by the Structural Database of Allergenic Proteins (SDPA).²² The probable protein with an ion peak at $m/z = 12,409.026$ from LJ may be the macrophage migration inhibitory factor (MIF). It has been found that the MIF is involved in several allergic disorders, including atopic dermatitis (AD), and can induce eosinophil accumulation in the skin.²³

Identification of Allergens in Fishes by Analyzing Their Tryptic Digests. To further identify the potential allergens, the on-chip captured allergens from the crude sarcoplasmic protein extracts of the analyzed fish species were digested with trypsin. The tryptic products of allergens were detected by MALDI-TOF MS and were identified by using the FlexAnalysis software from Bruker and the FindMod tool available on the ExPASy proteomics server of the SIB (<http://expasy.org/>). Figure 3B shows the representative MALDI-TOF mass spectra of tryptic digests of captured allergens on microchips from CC, HM, LC, OM, CI, and LJ. The protein identification was performed by using peptide mass fingerprinting (PMF) analysis.

Herein, the masses of peptides obtained from the tryptic digestion of the captured allergens were compared with the predicted masses of peptides from the theoretical digestion of proteins in the database. Table 1 lists the identified potential allergens and their theoretical masses of fragments for trypsin digestion. Table 1 also lists the identified peptides derived from the potential allergenic proteins captured by on-chip immunomagnetic separation (IMS) and MALDI-TOF MS. The MALDI-TOF mass spectra shown in Figure 3B display the digests of captured allergens and highlight the matched nine theoretically predicted peptide masses from parvalbumin originating from LC; one peptide from peptidylprolyl cis-trans isomerase originating from CC; eight peptides from parvalbumin beta and two peptides from cytochrome c originating from LC; two peptides from parvalbumin originating from OM; six peptides from parvalbumin originating from CI; one peptide from MIF originating from LJ and four peptides from parvalbumin originating from DE, respectively. The elucidation of these peptides allows the successful identification of parvalbumin, peptidylprolyl cis-trans isomerase, cytochrome c, and MIF as potential allergens. The detection of parvalbumins in five of the seven analyzed fishes further illustrates the occurrence of allergic cross-reactivity among fishes.

Validation of Parvalbumin in the Analyzed Fishes by ELISA and HPLC-MS/MS. To validate the results obtained by the present sensor, ELISA was performed to detect the presence of antigens in the protein extracts of fishes. In detail, the FSP of seven fish samples and the eluted solution of the MB-captured allergens on microchips in the FSP of the seven fish samples were tested by a third party using a parvalbumin-specific ELISA kit.

Table 2 lists the detected concentration of the crude protein and parvalbumin in the analyzed fish species. The results show that the concentrations of parvalbumin in the eluted solution of the captured allergens on the microchip by using the serum of patient 2 in crude protein extracts of HM, CC, LC, OM, CI, LJ, and DE are 19.11 ± 0.51 , 23.41 ± 0.27 , 22.75 ± 0.29 , 19.44 ± 0.19 , 19.68 ± 0.20 , 23.99 ± 0.16 , and 22.56 ± 0.49 $\mu\text{g/mL}$, respectively. The findings here further illustrate that the cross-reactivity of allergens between foods can be investigated based on MALDI-TOF MS and MBs in the microchip, keeping consistent with the results obtained by ELISA.

To further identify the protein, the tryptic digests of eluted potential allergens were analyzed by HPLC-MS/MS. The protein extracts of the fish muscle of DE were analyzed as a model. Figure S1 shows the high-performance liquid chromatography-tandem mass spectra of tryptic digests of captured allergens in the FSP of DE by anti-human IgE-functionalized MBs. The ion at 847.4287 is the mass of ALTAETK of parvalbumin from trypsin digestion. The result illustrates the existence of parvalbumin in the FSP of DE and keeps consistent with the result obtained by using microfluidic chips coupled with MALDI-TOF MS in the present study.

Analysis of Allergic Cross-Reactivity Among Fishes. The blue-color-labeled ions in Figure 3 with $m/z = 11,525.347$ and $11,506.246$ from CC and LJ, respectively, can be identified as parvalbumin. However, the entry of parvalbumin from CC at $m/z = 11,525.347$ and LJ at $11,506.246$ cannot be searched in the UniProt database. Herein the similarity of parvalbumin identified from CC and LJ was not analyzed. On the other hand, the UniProt database search shows that the proteins with

an ion peak at $m/z = 11,568.410$ and $m/z = 11,474.131$ in Figure 3 from HM can be identified as parvalbumin.

The sequences of parvalbumins identified from fishes HM, LC, OM, CI, and DE were aligned by using the tool on the UniProt website (<https://www.uniprot.org/align/>), and the similarity among them can be revealed. Figure S3 shows the results of sequence alignment of HM, LC, OM, CI, and DE. It can be found that the sequence similarity of parvalbumin identified from HM with $m/z = 11,568.410$ and the parvalbumins identified from LC, OM, CI, and DE is 64.22%; and the sequence similarity of parvalbumins with $m/z = 11,474.131$ is 65.14%. Allergic cross-reactivity among fishes can be evaluated by the presently developed microfluidic chips and MALDI-TOF MS strategy.

On-Chip Capture of Allergens for the Allergy Test.

The application of on-chip capture of allergens for the allergy test was performed by using sera of patients allergic to fish, with the certified DE as models. During the experiment, the interaction of the mouse monoclonal anti-human IgE-functionalized MBs and the serum of allergy patient 2 were performed prior to on-chip allergen capture. The serum of allergy patient 2 was directly utilized to coat the surface of MBs for the allergy test. A 1 cm single-straight channel with a cross-section of $200 \mu\text{m} \times 200 \mu\text{m}$ was utilized to capture the allergens in the protein extracts of fish. The surface of MBs and chips were blocked by using the BSA solution. The protein extract of DE was filled in the microfluidic chips. MALDI-TOF MS analysis of tryptic products of the captured allergens was performed. The UniProt database was used to further identify the detected proteins. The m/z values of peaks that can be assigned to proteins are labeled as shown in Figure 4. Peaks at $m/z = 11,614.173$ can be observed in the mass spectrum of the allergy test of the serum of a seafood allergic patient. The UniProt database search shows that the probable protein with an ion peak at $m/z = 11,614.173$ is parvalbumin, with the function of calcium ion binding, and the protein sequence is MALAGNLKEA DITAALAACK AAESFKHKEF FAKVGLSAKS ADDIKKAFLV IDQDQSGFIE EEELKLFLQN FSA-GARALTD AETKAFLKAG DIDGDGMIGI DEFATMVKA. Parvalbumin has been broadly reported as a major fish allergen.²⁴ By characterizing the tryptic digests of these proteins, using the database search engine at <https://www.uniprot.org> and matching in the UniProt databases, four peptides derived from parvalbumin can be matched. The results show that the on-chip captured allergens from protein extracts of DE can be identified as parvalbumins. This protein can be used as a marker allergen of DE protein extracts. The present protocol can be used to characterize allergens in seafood matrices.

Furthermore, the serum from another patient (patient 1) was also tested. A clear peak near $m/z = 11,600$ in the obtained MALDI-TOF mass spectra can be observed, as shown in Figure S2. Considering parvalbumin (M_w , 11.6 kDa) as a marker allergen contained in DE protein extracts, it can be predicted that the detected patient is also allergic to DE, showing consistent results with the hospital performed blood test. The findings here illustrate that a rapid allergy test can be performed by using the microfluidic platform, and the identified allergens in a food can be taken as a reference for fast characterization of potential allergens.

On-Chip Screening of the Effect of Thermal Processing on the Allergenicity of Crucian Carp Muscle. Food processing in different regions may impact the prevalence of

Table 1. Identification of Peptides Derived From the Potential Allergenic Proteins Captured by On-Chip IMS and MALDI-TOF MS, With Mass Spectra Shown in Figure 3.^a

fish species	ions with <i>m/z</i> at	potential allergen	identified peptides (calculated)	theoretical mass (Da)	detected mass (Da)	position	entry	function	length
<i>Hypophthalmichthys molitrix</i> (silver carp)	11,574	parvalbumin	SPDDIKKAFVIDQDK	1789.9330	1789.573	40–55	B6UV97	calcium ion binding	109
			AGDSGDGKIGVDEFAVLVK	1991.9919	1991.032	89–108			
	11,475	parvalbumin	DFFAKVGLSAKSPDDIKK	1966.0643	1967.760	29–46			
			KAFAIIDQDKSGFIEDELK	2296.1706	2296.529	46–65	B6UV98		
<i>Carassius carassius</i> (crucian carp)	11,915	peptidylprolyl <i>cis</i> -trans isomerase	AFAIIDQDKSGFIEDELKL FLQNFK	3058.5771	3057.103	47–72			110
			MAFAGILNDADIAALEACK AADSFNHHK	2878.3861	2877.475	1–28			
			VGLSAKSGDDVKKAFAIIDQ DK	2305.2397	2305.615	34–55			
			LFLQNFKAGARAL TDAETK	2094.1342	2094.336	66–84			
<i>Larimichthys crocea</i> (large yellow croaker)	11,471	parvalbumin	AGDSGDGKIGVDEFAALVK	1963.9606	1963.257	89–108			109
			HGGMGTLSMANAGPNTNGSQ	5735.7501	5737.650	57–110	A7M8X9	1) peptidyl-prolyl <i>cis</i> -trans isomerase activity; 2) protein folding	
			FFICTDDTAWLDDGKHVVEGK						
			VVEGIDVVQAMERK						
<i>Oreochromis mossambicus</i> (Mozambique tilapia)	11,415	cytochrome c	IGVDEFAALVK	1161.6513	1162.122	98–108	A0A0F8C4S0	calcium ion binding	104
			AFLIIDQDNSGFIEEELK	2210.0863	2208.446	47–65			
			TFLAAGSDGDGKIGVDEFA ALVK	2396.1979	2395.087	85–108			
			TFLAAGSDGDGKIGVDEFA ALVKQ	2524.2565	2525.019	85–109			
<i>Ctenopharyngodon Idella</i> (grass carp)	11,508	parvalbumin	ALTDGETKTFLAAGSDSDGDK	3211.6004	3210.373	77–108			109
			KGVDEFAALVK						
			AFLIIDQDNSGFIEEELKL FLQNFSAGAR	3414.7215	3415.556	47–76			
			MAFSVLSDADIQAALAGCA	4620.2094	4622.598	1–45			
<i>Lateolabrax japonicus</i> (Japanese seabass)	11,533	parvalbumin	AADSFNYKTHFKACGLSAKS EADV K	5464.6706	5460.310	47–97			109
			AFLIIDQDNSGFIEEELKL FLQNFSAGARAL TDGETKTF						
			LAAGSDSDGDK						
			SKGIVWNEDTLMYLENPK	2266.1060	2266.498	55–73	D5FQ06	1) electron transfer activity; 2) heme binding; 3) metal ion binding	
<i>Dissostichus eleginoides</i> (Patagonian toothfish)	11,606	macrophage migration inhibitory factor	SKGIVWNEDTLMYLENPKK	2394.2009	2394.455	55–74			109
			AFKIIDQDNSGFIEEELK	2225.0972	2225.115	47–65	Q0R3Z9	calcium ion binding	
			AFLSAGSDSDGKIGIDEFA ALVK	2396.1979	2395.885	85–108			
			AFFAKVGLSAK	1138.6619	1137.683	29–39	A0A4Y5R730	calcium ion binding	
<i>Lateolabrax japonicus</i> (Japanese seabass)	12,406	macrophage migration inhibitory factor	IGVDEFAALVK	1161.6513	1162.445	98–108			115
			SGDDVKKAFAIIDQDK	1749.9017	1749.994	40–55			
			AADSFNHHKAFKAVGLSAK	2009.0603	2010.363	21–39			
			AADSFNHHKAFKAVGLSAKS GDDVK	2610.3310	2611.588	21–45			
<i>Dissostichus eleginoides</i> (Patagonian toothfish)	11,606	parvalbumin	HLGISADRIYINFDMDAAN VAWNNTTFG	3225.5421	3227.217	87–115	A0ASCONQL8		109
			AGDIDGDMIGIDEFATMVK A	2125.9780	2125.358	89–109	C0LEK1	calcium ion binding	
			AFLVIDQDQSGFIEEELK	2210.0863	2209.290	47–65			
			AFLKAGDIDGDMIGIDEFA TMVK	2514.2254	2512.132	85–108			

Table 1. continued

fish species	ions with m/z at	potential allergen	identified peptides (calculated)	theoretical mass (Da)	detected mass (Da)	position	entry	function	length
			VGLSAKSDDDIKKAFLLVIDQ_DQSGFIEEEELK	3522.8213	3522.307	34–65			

^aPeptides of parvalbumin from HM, peptidylprolyl cis–trans isomerase from CC, parvalbumin beta and cytochrome c from LC, parvalbumin from OM, parvalbumin from CI, macrophage migration inhibitory factor from LJ, and parvalbumin from DE were identified. The serum of patient 2 was tested. The allergy test was performed using an optimized IMS procedure. Functionalized MBs were used to capture the allergens in a 1 cm length of a single straight channel with a 200 $\mu\text{m} \times 200 \mu\text{m}$ cross-section. The digestion was conducted following the protocol detailed in the experimental section “Digestion of Allergens by Trypsin.” The listed peptide masses are in the range of 2000 to 10,000. The theoretical peptides were calculated from a specified Swiss-Prot/TrEMBL entry.

Table 2. Results of the Concentration of the Crude Protein and Parvalbumin, Respectively, in Fish Species of *Hypophthalmichthys molitrix* (HM), *Carassius carassius* (CC), *Larimichthys crocea* (LC), *Oreochromis mossambicus* (OM), *Ctenopharyngodon Idella* (CI), *Lateolabrax japonicus* (LJ), and *Dissostichus eleginoides* (DE) Measured by the BCA Kit and ELISA Kit, Respectively^a

fish species	crude protein results (mg/mL) (n = 3)	parvalbumin results ($\mu\text{g/mL}$) (n = 3)
<i>Hypophthalmichthys molitrix</i> (HM)	4.58 $\pm$ 0.08	19.11 $\pm$ 0.51
<i>Carassius carassius</i> (CC)	4.61 $\pm$ 0.05	23.41 $\pm$ 0.27
<i>Larimichthys crocea</i> (LC)	4.85 $\pm$ 0.03	22.75 $\pm$ 0.29
<i>Oreochromis mossambicus</i> (OM)	4.43 $\pm$ 0.04	19.44 $\pm$ 0.19
<i>Ctenopharyngodon Idella</i> (CI)	4.82 $\pm$ 0.03	19.68 $\pm$ 0.20
<i>Lateolabrax japonicus</i> (LJ)	4.61 $\pm$ 0.04	23.99 $\pm$ 0.16
<i>Dissostichus eleginoides</i> (DE)	4.60 $\pm$ 0.05	22.56 $\pm$ 0.49

^aThe allergens in the FSP of seven fish samples were captured by on-chip IMS.

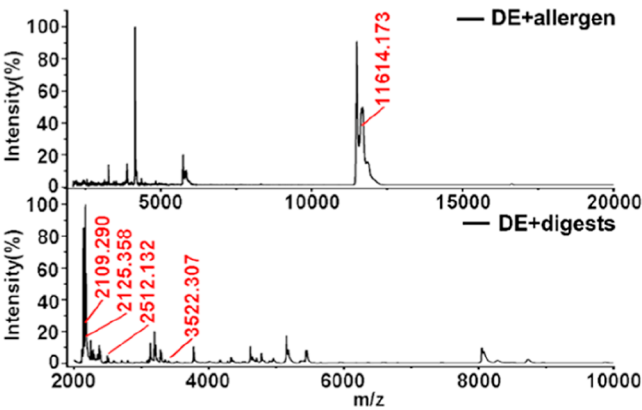


Figure 4. Representative MALDI-TOF mass spectrum obtained during the DE allergy test and spectrum of tryptic digests of the captured allergen in the FSP of DE by functionalized MBs. The serum sample of patient 2 was tested. During the experiment, anti-human IgE-functionalized MBs interacted with human serum and then with DE sarcoplasmic proteins in a 1 cm length of a single straight channel with a 200 $\mu\text{m} \times 200 \mu\text{m}$ cross-section. Functionalized MBs and the single straight channel were blocked with 5% (m/m) BSA. The serum sample with a 10-fold dilution was used during the experiment. The allergens captured by MBs were eluted by using 2 μL of 5% (v/v) acetic acid solution. 5 μL of the above-eluted allergens were diluted 10-fold with PBS (10 mM, pH 7.4) and mixed with 1 μL of trypsin (1 mg/mL) at 37 $^{\circ}\text{C}$ for 14 h for the digestion, which was terminated by controlling the TFA concentration at 0.4% (v/v). The peak m/z values that can be assigned to proteins are denoted in the diagram.

seafood allergy. The feasibility of the present protocol for the evaluation of allergenicity in ready-to-eat food was assessed. Fish muscles are often consumed after boiling as a soup in Asia. Herein, the CC muscle sample was boiled in water for different periods of time to obtain a soup-like food as a model ready-to-eat food. The results shown in Figure 5 illustrate that boiling the fish muscle using water may not completely alter the allergenic properties of the fish muscle but can reduce the level of allergenicity, indicating the decrease in the number of

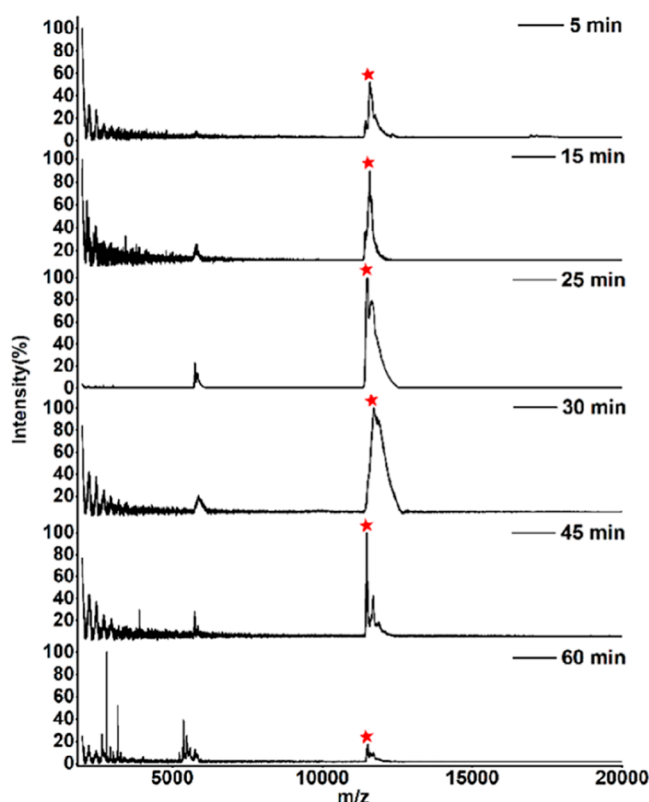


Figure 5. Representative MALDI-TOF mass spectra obtained during the CC allergy test by using the serum of patient 2. The protein extracts of CC were boiled for 5, 15, 25, 30, 45 min, and 1 h. MBs and the single straight channel were blocked with 5% (m/m) BSA.

allergens. These results keep consistent with a previous study where parvalbumin levels in boiled fish fillets were found to be up to 25% lower compared with the corresponding fresh fish sample.²⁵

Parvalbumins are water-soluble and relatively heat-stable.²⁵ A relatively mild food processing procedure can be boiling the raw food materials where the heat can be transferred from boiling water to food matrices. Boiling does not considerably change the extractable and detectable parvalbumin. Treatment of the fish muscle with heat by boiling water may purify the heat-stable proteins since the predominant precipitation of heat-labile muscle proteins causes a rather relative accumulation of proteins such as parvalbumin in the soluble fraction.²⁵ Overall, boiling the fish muscle in water marginally diminishes parvalbumin allergenic properties of the CC muscle.

In summary, the present study demonstrated the feasibility of MALDI-TOF MS coupled with microfluidic chips to evaluate cross-reactivity among fishes by using crude protein extracts of seven fishes. The developed protocol was found reproducible for the capture of allergens from seafood protein extracts. The results show that the allergens can be on-chip captured and are indicated by clear peaks in the obtained mass spectra in the three runs of measurements. Seven fish species were studied on the microfluidic channels to evaluate cross-reactivity among fishes and validate the present method. The allergens captured from crude DE sarcoplasmic proteins by using the serum of seafood allergic patient 1 were analyzed and identified by MALDI-TOF MS and HPLC-MS/MS. Meanwhile, the serum of patient 2 was used to capture the allergens from crude DE sarcoplasmic proteins. On-chip allergen capture

of fishes, including LC, OM, CC, CI, LJ, DE, and HM, was performed by using the serum of seafood allergic patient 2, and the results show cross-reactivity among the seven fish species. The cross-reactivity among the seven fish species was also confirmed by trypsin digestion and ELISA. The sequence alignment shows that the sequence similarity of parvalbumin identified from HM with $m/z = 11,568.410$ and the parvalbumins identified from LC, OM, CI, and DE is 64.22%; and the sequence similarity of parvalbumin identified from HM with $m/z = 11474.131$ is 65.14%. The muscle sample of CC was boiled for different periods of time to obtain a soup-like food and utilized for assessing the feasibility of the present protocol for the evaluation of allergenicity in foods ready for consumption. It was found that boiling the fish muscle with water marginally diminishes the allergenic properties of parvalbumin from the CC muscle. The present protocol can be successfully applied in the evaluation of allergenicity in ready-to-eat foods. The method can be potentially used to predict cross-reactivity among fish species to provide guidance and advice to allergic patients.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.2c02433>.

Information on the fishes analyzed in the present study; some information on the volunteers of the serums analyzed in the present study; identification of patient 1-specific IgE-reactive allergens by HPLC-MS/MS; and alignment of sequences of the identified allergens (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Hongyan Bi – College of Food Science and Engineering, Shanghai Ocean University, Shanghai 201306, China; orcid.org/0000-0003-2162-1829; Phone: +86-21-61900364; Email: hybi@shou.edu.cn; Fax: +86-21-61900365

Author

Xin Zhao – College of Food Science and Engineering, Shanghai Ocean University, Shanghai 201306, China

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.jafc.2c02433>

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful to Jia Yi and Winnie C. Soko for their assistance in instrument operation and proofreading, respectively.

■ ABBREVIATIONS

AA, acetic acid; ACN, acetonitrile; AD, atopic dermatitis; BSA, bovine serum albumin; CC, *Carassius carassius*; CI, *Ctenopharyngodon idella*; DE, *Dissostichus eleginoides*; DI, deionized; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; ExPASy, expert protein analysis system; FDA, Food and Drug Administration; FSP, fish sarcoplasmic proteins; HCl, hydrochloric acid; HHP, high hydrostatic pressure; HIFU, high

intensity focused ultrasound; HM, *Hypophthalmichthys molitrix*; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; IMS, immunomagnetic separation; LC, *Larimichthys crocea*; LJ, *Lateolabrax japonicus*; MALDI, matrix-assisted laser desorption/ionization; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MBs, magnetic beads; MIF, macrophage migration inhibitory factor; MS, mass spectrometry; OM, *Oreochromis mossambicus*; PANI, polyaniline; PBS, phosphate-buffered saline solution; PDMS, polydimethylsiloxane; PMF, peptide mass fingerprinting; QTOF, quadrupole time-of-flight; RT, room temperature; SA, sinapinic acid; SDPA, structural database of allergenic proteins; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SIB, Swiss Institute of Bioinformatics; SPE, screen-printed carbon-microelectrode; SPR, surface plasmon resonance

REFERENCES

- (1) Ruethers, T.; Taki, A. C.; Johnston, E. B.; Nugraha, R.; Le, T. T. K.; Kalic, T.; McLean, T. R.; Kamath, S. D.; Lopata, A. L. Seafood allergy: A comprehensive review of fish and shellfish allergens. *Mol. Immunol.* **2018**, *100*, 28–57.
- (2) Yuk, J. E.; Lee, J.; Jeong, K. Y.; Park, K. H.; Kim, J. D.; Kim, J.-T.; Lee, J.-H.; Park, J.-W. Allergenicity and Stability of 6 New Korean Bony Fish Extracts. *Allergy, Asthma Immunol. Res.* **2021**, *13*, 623–637.
- (3) Carrera, M.; Cañas, B.; Gallardo, J. M. Rapid direct detection of the major fish allergen, parvalbumin, by selected MS/MS ion monitoring mass spectrometry. *J. Proteomics* **2012**, *75*, 3211–3220.
- (4) Zhao, X.; Lu, J.; Long, S.; Soko, W. C.; Qin, Q.; Qiao, L.; Bi, H. MALDI-TOF MS and Magnetic Beads for Rapid Seafood Allergen Tests. *J. Agric. Food Chem.* **2021**, *69*, 12909–12918.
- (5) Cox, A. L.; Eigenmann, P. A.; Sicherer, S. H. Clinical Relevance of Cross-Reactivity in Food Allergy. *J. Allergy Clin. Immunol.* **2021**, *9*, 82–99.
- (6) Kobayashi, Y.; Hüge, J.; Imamura, S.; Hamada-Sato, N. Study of the cross-reactivity of fish allergens based on a questionnaire and blood testing. *Allergol Int* **2016**, *65*, 272–279.
- (7) Mehrdana, F.; Lavilla, M.; Kania, P. W.; Pardo, M. Á.; Audicana, M. T.; Longo, N.; Buchmann, K. Evidence of IgE-Mediated Cross-Reactions between *Anisakis simplex* and *Contracaecum osculatum* Proteins. *Pathogens* **2021**, *10*, 950.
- (8) Leung, N. Y. H.; Leung, A. S. Y.; Xu, K. J. Y.; Wai, C. Y. Y.; Lam, C. Y.; Wong, G. W. K.; Leung, T. F. Molecular and immunological characterization of grass carp (*Ctenopharyngodon idella*) parvalbumin Cten i 1: A major fish allergen in Hong Kong. *Pediatr. Allergy Immunol.* **2020**, *31*, 792–804 official publication of the European Society of Pediatric allergy and immunology/Pediatric Allergy and Immunology.
- (9) Zhang, Y.; Bi, Y.; Wang, Q.; Cheng, K.-W.; Chen, F. Application of high pressure processing to improve digestibility, reduce allergenicity, and avoid protein oxidation in cod (*Gadus morhua*). *Food Chem.* **2019**, *298*, 125087.
- (10) Yu, J.; Mikiashvili, N. Effectiveness of different proteases in reducing allergen content and IgE-binding of raw peanuts. *Food Chem.* **2020**, *307*, 125565.
- (11) Amouzadeh Tabrizi, M.; Shamsipur, M.; Saber, R.; Sarkar, S.; Ebrahimi, V. A high sensitive visible light-driven photoelectrochemical aptasensor for shrimp allergen tropomyosin detection using graphitic carbon nitride-TiO₂(2) nanocomposite. *Biosens. Bioelectron.* **2017**, *98*, 113–118.
- (12) Angulo-Ibáñez, A.; Elettigerra, U.; Lasheras, X.; Campuzano, S.; Merino, S. Electrochemical tropomyosin allergen immunosensor for complex food matrix analysis. *Anal. Chim. Acta* **2019**, *1079*, 94–102.
- (13) Mohamad, A.; Rizwan, M.; Keasberry, N. A.; Nguyen, A. S.; Lam, T. D.; Ahmed, M. U. Gold-microrods/Pd-nanoparticles/polyaniline-nanocomposite-interface as a peroxidase-mimic for sensitive detection of tropomyosin. *Biosens. Bioelectron.* **2020**, *155*, 112108.
- (14) Zhou, J.; Wang, Y.; Qian, Y.; Zhang, T.; Zheng, L.; Fu, L. Quantification of shellfish major allergen tropomyosin by SPR biosensor with gold patterned Biochips. *Food Control* **2020**, *107*, 106547.
- (15) Fan, Y.; Dong, D.; Li, Q.; Si, H.; Pei, H.; Li, L.; Tang, B. Fluorescent analysis of bioactive molecules in single cells based on microfluidic chips. *Lab Chip* **2018**, *18*, 1151–1173.
- (16) Bi, H.; Duarte, C. M.; Brito, M.; Vilas-Boas, V.; Cardoso, S.; Freitas, P. Performance enhanced UV/vis spectroscopic microfluidic sensor for ascorbic acid quantification in human blood. *Biosens. Bioelectron.* **2016**, *85*, 568–572.
- (17) Zhang, X.; Li, Y.; Tao, Y.; Wang, Y.; Xu, C.; Lu, Y. A novel method based on infrared spectroscopic inception-resnet networks for the detection of the major fish allergen parvalbumin. *Food Chem.* **2021**, *337*, 127986.
- (18) Vornanen, M.; Tiitu, V.; Käkälä, R.; Aho, E. Effects of thermal acclimation on the relaxation system of crucian carp white myotomal muscle. *J. Exp. Zool. Suppl.* **1999**, *284*, 241–251.
- (19) Gao, Q.; Zhenxing, L. I.; Nasha, M. L.; Lin, H.; Liangtao, L.; Zhang, Y.; Laboratory, F. S. J. F. S. Effect of Fermentation on IgE Binding Ability of Sea Bass (*Lateolabrax japonicus*) Parvalbumin. *J. Food Sci.* **2017**, *38*, 88–94.
- (20) Mazzeo, M. F.; Giulio, B. D.; Guerriero, G.; Ciarcia, G.; Malorni, A.; Russo, G. L.; Siciliano, R. A. Fish authentication by MALDI-TOF mass spectrometry. *J. Agric. Food Chem.* **2008**, *56*, 11071–11076.
- (21) Matthews, P. A.; Baldo, B. A.; Howden, M. E. H. Cytochrome c allergens isolated from the pollens of the dicotyledons English plantain (*Plantago lanceolata*) and Paterson's curse (*Echium plantagineum*). *Mol. Immunol.* **1988**, *25*, 63–68.
- (22) Ahmed, A.; Minhas, K.; Namood-E-Sahar; Aftab, O.; Khan, F. S. In Silico Identification of Potential American Cockroach (*Periplaneta americana*) Allergens. *Iran. J. Public Health* **2010**, *39*, 109–115.
- (23) Yoshihisa, Y.; Makino, T.; Matsunaga, K.; Honda, A.; Norisugi, O.; Abe, R.; Shimizu, H.; Shimizu, T. Macrophage Migration Inhibitory Factor Is Essential for Eosinophil Recruitment in Allergen-Induced Skin Inflammation. *J. Invest. Dermatol.* **2011**, *131*, 925–931.
- (24) Sharp, M. F.; Lopata, A. L. Fish allergy: in review. *Clin. Rev. Allergy Immunol.* **2014**, *46*, 258–271.
- (25) Kuehn, A.; Scheuermann, T.; Hilger, C.; Hentges, F. Important variations in parvalbumin content in common fish species: a factor possibly contributing to variable allergenicity. *Int. Arch. Allergy Immunol.* **2010**, *153*, 359–366.