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Determination of a major allergen in fish samples by simple and effective label-free capillary electrophoretic analysis after background suppression in ion-exchange chromatography

Linglin Fu[a,b], Jinru Zhou[a,b], Chong Wang[a], Yan Zhang[c], Aijin Ma[d], Yanbo Wang[a,b,*]

[a] Food Safety Key Laboratory of Zhejiang Province, School of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou 310018, China
[b] Zhejiang Engineering Institute of Food Quality and Safety, Zhejiang Gongshang University, Hangzhou 310018, China
[c] Hebei Institute of Food Quality Supervision Inspection and Research, Shijiazhuang 050091, China
[d] China National Institute of Standardization, Beijing 100191, China

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ABSTRACT

A simple analytical method using ion-exchange chromatography (IEC) coupled with capillary zone electrophoresis (CZE) was newly developed for detection and quantification of parvalbumin (PV), a major allergen, in fish. The procedure of IEC for simple enrichment of PV crude extract was optimized. By using 25 mmol/L borate-borax buffer with 15 kV separation voltage at pH 9.2 for CZE separation, the migration time, separation efficiency and electrophoretic resolution greatly improved. Under the optimal conditions, PV was determined in 2.8 min, with the limit of detection (LOD) at 0.71 μg/mL (S/N = 3) and the recoveries at 89.6%–104.7%. We also found that only when the concentration of PV was above LOD reported here was the protein capable of stimulating human mast cell degranulation, indicating the biological significance of the LOD. Finally, the use of this method to analyze fish samples with simple sample preparation highlights the applicability for detection of allergens in seafood matrices.

1. Introduction

Fish consumption has been increasing rapidly, and fish has been considered as a key part of a healthy balanced diet on account of rich micronutrients, macronutrients, and savory flavor (Ruxton, 2015). Unfortunately, fish represents one of the “big eight” categories of food allergies and is responsible for almost 10% of food allergies suggested by a multicenter study in emergency departments (Clark et al., 2004). According to current dates, almost 0.1–0.5% of individuals are allergic to fish in the US and Europe (Rona et al., 2007; Sicherer, Muñoz-Furlong, & Sampson, 2004). In Asia, seafood is a significant sensitizer that affects up to 40% of children and 33% of adults (Lopata & Lehrer, 2009). The prevalence data reported that 0.26% (Singapore) and 1.98% (Taiwan) of children, as well as 3.3% (Singapore) and 1.17% (Taiwan) of adults are allergic to fish (Connett et al., 2012; Thong, Cheng, Leong, Tang, & Chng, 2005; Wu et al., 2012).

Generally, with the development of aquatic products processing technology, comprehensive utilization ratio of aquatic resources is increasing, which leads to some difficulties either to distinguish seafood ingredients in food, or trace the allergens contamination in the process of food processing, and thus, the allergic individuals are more likely to contact allergenic foods.

Sensitive individuals mostly rely on allergen-avoidance to prevent allergic reactions, which depends on accurate detection and complete information of allergens obtained from labels (Taylor & Baumert, 2015). In accordance with Annex II of Regulation (EU) No 1169/2011, 14 allergenic ingredients, including fish allergens, are mandatory labelling and highlighting on food packages. Therefore, it is urgently required to develop reliable, highly sensitive and feasible analytical methods for detecting allergenic ingredients in seafood in order to provide powerful tools in easily determining seafood allergens. Moreover, evaluating the efficiency of allergen elimination approaches also requires effective detection methods.

Importantly, development of effective analytical methods is based on the identification of various allergens in given seafood raw materials, such as all kinds of fish. Until now, a number of allergenic components have been characterized in fish, among which, parvalbumin (PV) is considered the major allergen responsible for almost 95% fish-caused allergic reactions (Kuehn, Scheuermann, Hilger, & Hentges, 2010). PV was first identified as a IgE-reactive allergen of cod in 1968 and later named as Gad c 1 (Elsayed & Bennich, 1975; Lim et al., 2008). PV is an acidic, heat stable, water-soluble and EF-hand Ca^{2+}-binding

* Corresponding author at: 18 Xue Zheng Street, Hangzhou 310018, China.
E-mail addresses: wyb1225@163.com, wangyb@mail.zjgsu.edu.cn (Y. Wang).

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protein with a molecular weight (MW) of approximate 10–13 kDa, and presents in great amount in the muscle of a variety of vertebrates (Griesmeier et al., 2010). Due to the highly conserved structural of PVs in different fish species, the total serum IgE from almost 50% of the individuals who are allergic to PV in one fish species can cross-react with that in other fish species (Sharp & Lopata, 2014).

So far, there are two kinds of analytical methods for the detection of food allergenic ingredients: (i) protein-based methods, including enzyme-linked immunosorbent assays (ELISAs) and liquid chromatography (LC)-coupled mass spectrometry (MS), and (ii) DNA-based methods, mostly referring to polymerase chain reaction (PCR) methods (van Hengel, 2007). ELISA, a commercially available method, detects the presence of food allergens by utilizing specific mono- or polyclonal antibodies for target allergenic proteins. It has been reported that a sandwich ELISA method was established by using specific polyclonal antibody for the determination of PV in processed foods (Shibahara, Uesaka, Wang, Yamada, & Shiomi, 2013). Besides, two real-time PCR methods have been applied to analyze PV in a total of 25 species of finfish (Houhoula, Dimitriou, Mengjezi, Kyrana, & Lougovois, 2015), and fish allergens in fresh materials and processed products (Herrero, Vieites, & Espiñeira, 2014). However, ELISA is prone to nonspecific binding of employed antibodies with food matrix components and the results are highly affected by protein modifications upon food processing, which can lead to the strong possibility of false positive or negative results. DNA-based methods depend on the specific DNA sequences so that they do not detect the gene-unidentified target allergenic proteins in food or lack of specificity to bind double-stranded DNA. Although a LC-MS/MS approach has been developed to select 19 PV peptide biomarkers in 16 fish species, application of LC-MS in fish allergen detection still requires further study and validation (Carrera, Cañas, & Gallardo, 2012).

Alternatively, capillary electrophoresis (CE), which shows obvious advantages of high separation efficiency, simplicity of operation, extremely small amount of sample and rapid analysis, is considered a practical and powerful tool for detecting a large number of compounds in food and foodomics (Ibáñez et al., 2016). Moreover, some kinds of allergens have been detected using CE, such as birch pollen allergen (Bet v 1a) (Punzet, Ferreira, Briza, Ree, & Stutz, 2006), allergenic extracts from olive pollen (Zienkiewicz et al., 2014), camel milk proteins (β-lactoglobulin, α-lactalbumin, lactoferrin and serum albumin) (Omar, Harbourne, & Oruña-Concha, 2016), and cow milk allergens (Gasilova, Girault, & Chem, 2014). However, so far, there is no report regarding to the application of CE for detection of fish allergens, especially PV, because the variety of large background components from fish samples becomes a bottleneck in analyzing PV by CE.

In this work, for the first time, a simple and effective label-free CE method was newly developed to analyze the main allergen (PV) of fish. Furthermore, we evaluated this method in fresh fish samples by determination of precision, sensitivity and reproducibility, and the allergenicity of PV could be observed only when the concentration was above the limit of detection (LOD) of our approach, indicating the biological significance of the LOD.

2. Material and method

2.1. Chemicals

Hydroxymethyl aminomethane (Tris) and sodium chloride were purchased from Aladdin (Los Angeles, CA, USA). All chemicals used were of analytical grade. DEAE-Sepharose F.F. was purchased from General Electric Company, Fairfield, Connecticut. Positive blood sera of the patients allergic to fish were kindly offered from the second affiliated hospital of Zhejiang University (Hangzhou, Zhejiang, China). LAD2 cells were obtained from ATCC (Rockefeller, Maryland). Parvalbumin (PV) was obtained from GenScript, Piscataway, New Jersey. All solutions were prepared using ultrapure water purified using a Milli-Q system (Millipore, Billerica, Massachusetts).

2.2. Fish samples

Four species of different fish (*Ctenopharyngodon idellus*, *Hemiculter leucisculus*, *Carassius auratu* and *Mylopharyngodon piceus*) were purchased from a local supermarket (CenturyMart, Hangzhou, China).

2.2.1. Crude extract preparation of PV

Total protein extraction was performed using a commercial kit (KeyGEN, Nanjing, China). Fish muscle was grinded with liquid nitrogen into powder and the powder (0.1 g) was accurately weighed into a 1.5 mL centrifuge tube with the addition of 1 mL of mixture buffer (1 mL of Lysis Buffer, 1 μL of protease inhibitor, 10 μL of phosphodiesterases, 5 μL of 100 mM PMSF). After stationary incubation for 2 h at 4 °C, the extract was centrifuged in Microfuge 22 R Centrifuge (Beckman Coulter, Mississauga, ON) at 10,000*g*, 4 °C for 5 min. The supernatant was collected as crude extract and used for further extraction.

2.2.2. CE sample preparation by IEC

The crude extract (2 mL) was loaded into the DEAE-Sepharose F.F. column and retained for 30 min. Then, the column was washed with washing buffer (10 mM Tris-HCl, pH 7.5) and eluted by running a linear saline gradient buffer (0.1 M NaCl to 0.5 M NaCl). All effluents were collected and detected at 280 nm in Nanodrop 2000 (Thermo Scientific, Massachusetts, USA). Prior to injection into the CE device, samples were filtered with a 0.45 μm nylon membrane.

2.2.3. SDS-PAGE analysis

The concentration of total or column-collected proteins was determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, USA) with bovine serum albumin (BSA) as the standard. The purity of PV in crude extract and all effluents were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using AlphaView SA 3.4.0 software. The electrophoresis was carried out for 90 min at 120 V on 15% separation gels with a 5% stacking gel.

2.3. Capillary electrophoresis analysis

All experiments were performed on a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Mississauga, ON) with UV detector set at 214 nm. For data acquisition and analysis, a 32-Karat software was used. Fused silica capillaries with dimensions of 75 μm i.d., 375 μm o.d. (20 cm effective length, 30.2 cm total length) were obtained from Beckman (Beckman Coulter, Mississauga, ON).

In this work, the conditions including voltage, running buffer pH and concentration were investigated. The recovery test, peak height, migration time and relative standard deviation (RSD) were performed for verification of the accuracy of the method. In fish samples analysis, the external standard method was used for the quantification of PV. Standard curves were made with different concentrations (5–100 μg/mL) of PV and analyzed in triplicate.

2.4. Biological significance of the LOD

2.4.1. Cell culture and degranulation assay

LAD2 human mast cells were cultured in the presence of 9% (v/v) fetal bovine serum and 1% (v/v) penicillin–streptomycin in serum-free RPMI 1640 medium (GIBCO, Los Angeles, Southern California). Cells (1×10^6 cells/mL) were incubated with 10 μL of mixture of fish allergic-IgE antibody sera (2 ng/mL) in triplicate for 2 h at 37 °C (5% CO_2 and humidified atmosphere). After centrifuged at 900*g* for 5 min at 4 °C, cell pellets were washed and resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.38 mM $Na_2HPO_4{\cdot}7H_2O$, 5.6 mM glucose, 1.8 mM