



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

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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 Ca²⁺-binding

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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 auratus* 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 μ L of protease inhibitor, 10 μ L of phosphodiesterases, 5 μ L 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 10,000g, 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 μ m 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 μ m i.d., 375 μ m 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 μ g/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 μ L of mixture of fish allergic-IgE antibody sera (2 ng/mL) in triplicate for 2 h at 37 °C (5% CO₂ and humidified atmosphere). After centrifuged at 900g 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 Na₂HPO₄·7H₂O, 5.6 mM glucose, 1.8 mM

$\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 1.3 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4% BSA, pH 7.4). After that, the cell suspensions were stimulated with keyhole limpet hemocyanin (KLH), compound 48/80 (C48/80) and three concentration gradients (1, 0.1 and 0.01 $\mu\text{g}/\text{mL}$) of PV for 30 min at 37 °C, respectively. KLH was performed as negative control, and C48/80 was performed as positive control. The mixture was centrifuged at 900g for 5 min at 4 °C, and the supernatants were collected for ELISA analysis, while the cells were treated for transmission electron microscopy.

2.4.2. Transmission electron microscopy

LAD2 cells were incubated with glutaraldehyde fixative (2.5% glutaraldehyde in 0.05 M cacodylate buffer with 0.1 M NaCl, pH 7.5) at room temperature for 20 min. Then, cells were embedded in Spurr resin (Pelco, USA) and sections were cut with a knife on a LEICA EM UC7 ultratome (LEICA, Solms, Hessen) and observed using a Hitachi H-7650 transmission electron microscope (TEM) (Hitachi, Tokyo, Japan).

2.4.3. ELISA analysis

Histamine and tryptase as two indexes of mast cell degranulation was assayed using a double antibodies sandwich ELISA in the cell supernatants. According to the manufactures' instructions, a Human Histamine (His) ELISA Kit (BIM, San Francisco, CA) and a Human Tryptase (MCT) ELISA Kit (BIM, San Francisco, CA) were used.

2.5. Statistical analysis

All of the measurements were performed in triplicate. Statistical significance of the data was determined by one-way analysis of variance using SPSS.16.0 (IBM SPSS, Armonk, NY, USA) for windows. A *p* value of < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Optimization of sample treatment

As the aim was to develop a simple and quick method for analyzing PV in fish samples using the potential of the CZE separation and quantification method suitable for seafood matrices, we optimized the procedure of enrichment of PV crude extract.

First, the analysis of the composition of crude extract obtained from fish muscles indicated that twenty-four fractions were observed, including PV (Fig. 1A). Second, crude extract was subjected to IEC on DEAE-Sepharose F.F. to remove non-targeted proteins and reduce possible interfering components during CZE separation and quantification. During the flows, three major absorbent peaks were obtained and all fractions of the effluents were analyzed by 15% reduced SDS-PAGE (Fig. 1). Specifically, a major band with molecular mass of 10 kDa was detected, suggesting the substantial proportion of PV existed in the effluent of the peak 1 eluted by 0.1 M NaCl. In addition, as shown in Fig. 1B, the purity of PV in the crude extract and IEC-treated extract were approximately 5.6% and 13.2% of the total proteins, respectively.

The results indicated that the PV could be collected directly from eluting peak 1 and we ultimately employed 0.1 M NaCl as the elution buffer for IEC. Expectedly, our finding showed that the IEC-treated extract achieved a cleaner background with much fewer other protein bands compared to the crude extract (Fig. 1B). The two simple procedures of sample treatment provide the foundation for further characterization of PV by CZE.

3.2. Optimization of CZE separation conditions

In order to explore the separation conditions of CZE with UV detector, a standard of PV at concentration level of 10 $\mu\text{g}/\text{mL}$ was examined at 214 nm under the temperature of 25 °C.

3.2.1. Selection of running buffer

The properties of the running buffer significantly affect the electroosmotic flow (EOF) and reproducibility of migration time of analytes analyzed by CZE (Stoyanov & Righetti, 1997; Zhu, Changqin, & Liu, 2006). Borate-borax buffer, which can provide acceptable efficiency of separation, good peak shapes, and reduction of protein adsorption, was selected as the running buffer because PV was acidic protein.

3.2.2. Selection of separation voltage

Separation voltage is an important factor affecting the electrophoretic mobility of analyte and EOF of the separation system (Altria & Simpson, 1987). In theory, higher voltage can reduce migration time of analyte. However, the separation voltage is limited by Joule heat that leads to significant losses of resolution and peak efficiency (X. Xuan, Hu, & Li, 2006). Based on this, several voltages from 14 kV to 18 kV with 25 mmol/L borate-borax running buffer were investigated and the results were shown in Fig. 2. It was shown that the best separation performance was achieved when applying the separation voltage of 15 kV. Further increase of the separation voltage resulted in an unstable baseline, poor separation and low peak intensity.

3.2.3. Selection of buffer concentration

The concentration of separation buffer also influenced the EOF, the migration time and the separation resolution of the analyte (Cross & Cao, 1998). Normally, high concentration buffer can suppress wall interactions, but increase Joule heat that may cause peak broadening and decrease of separation efficiency (Xuan & Li, 2004). Various concentrations of borate-borax running buffer at a voltage of 15 kV were investigated in the present study (Fig. 3). We found that with the increase of borate-borax concentration, the degree of separation improved. Obviously, the best separation of PV was obtained at borate-borax buffer concentration of 25 mmol/L in terms of migration time, peak efficiency and resolution.

3.2.4. pH value of running buffer

Indeed, the pH of the running buffer can affect the mobility of the analyte, further affecting separation efficiency and electrophoretic resolution (Hayes, Kheterpal, & Ewing, 1993; Liu & Frank, 2015). The impact of the pH of the running buffer was investigated in the pH range of 9.0–9.4 (Fig. 4). As shown in Fig. 4, a sharp peak was observed at pH 9.2 for the borate-borax buffer, indicating the relatively high degree of separation of PV obtained under this condition. Collectively, we decided to employ a concentration of 25 mmol/L and a pH of 9.2 of borate-borax running buffer as well as a voltage of 15 kV as the optimal parameters for PV separation.

3.3. Method validation

On the basis of the above optimal conditions, this method was validated in terms of linear range, limit of detection (LOD), limit of quantification (LOQ), precision (relative standard deviation, RSD) and accuracy (recovery) using standard of PV.

3.3.1. Linearity, LOD and LOQ

Five concentration levels of standard solutions (5–100 $\mu\text{g}/\text{mL}$) were tested to determine the linear range of this method. The calibration curve was constructed by peak area against concentrations of PV. As shown in Fig. 5A (supplementary data), the correlation was: $y = 0.16x + 0.38$ with coefficient of determination (R^2) exceed 0.9938. We also determined that the LOD was 0.71 $\mu\text{g}/\text{mL}$ ($S/N = 3$) and the LOQ was 2.4 $\mu\text{g}/\text{mL}$ ($S/N = 10$), respectively.

Compared to previous studies, the LOQ (0.58 ng/ml) reported for ELISA (Shibahara et al., 2013), and the LOQ (2.5 copies) reported for real-time PCR (Tetzlaff & Mäde, 2016). Therefore, the obtained LOD in this method are higher than LODs obtained by using ELISA and real-

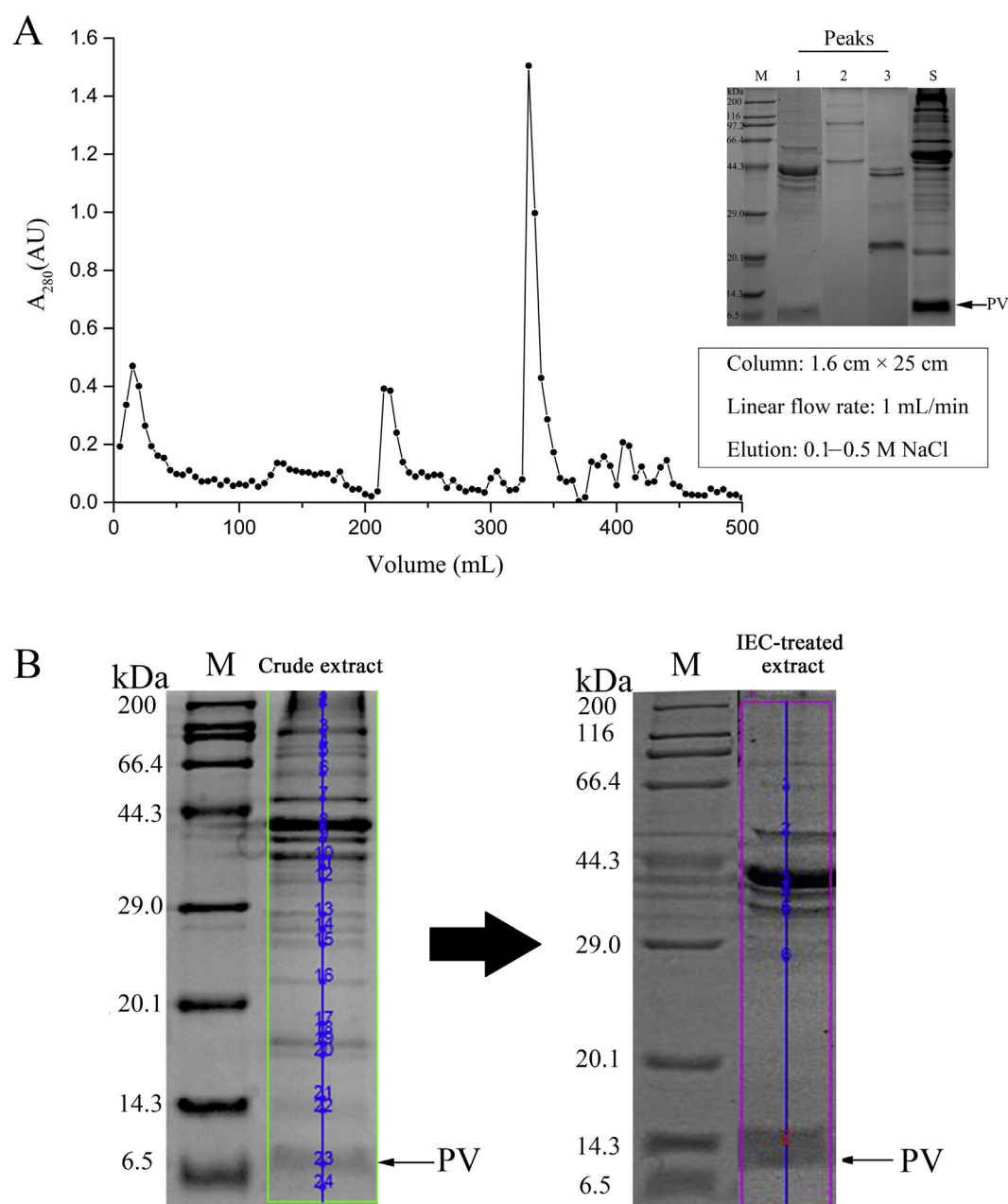


Fig. 1. SDS-PAGE analysis of sample preparation. (A) Elution characteristics and SDS-PAGE analysis of crude extract and IEC-treated extract; (B) The purity analysis of crude extract and IEC-treated extract. M, molecular weight markers.

time PCR. However, both the ELISA and real-time PCR methods have limitations in their application in quantifying allergens, as we indicated. Apparently, our newly developed method has key advantages, which are low cost, simplicity and accurate detection of PV within 2.8 min. Meanwhile, under the LOD of this method, it is more suitable for determination of PV in fresh fish samples, since PV concentration is rather high in fresh fish. Moreover, only when the concentration of PV is above the LOD we determined, the effective allergic response (Fig. 6) could be induced as we discuss in detail in the following section.

3.3.2. Precision and accuracy

The precision of the method was validated by performing six replicate separations of PV in fish samples obtained from a fresh fish under the optimal conditions. The RSD for peak area and migration time were shown in Fig. 5B. The RSD was 5.6% for the migration time and 6.3% for the peak area, respectively, which illustrated good

precision.

The recovery test was performed by selecting four fish samples mixed with PV standards at a concentration of 10 μ g/mL. The percentages of recovery are presented in Table 1 (supplementary data). The recovery of PV ranged from 89.6% to 104.7%, indicating a good accuracy.

3.4. Biological significance of the LOD

Theoretically, the LOD is considered best when lowest for analysis of hazardous substances polluted in food, such as pathogens, veterinary drugs or heavy metals. Nevertheless, as a native functional protein existed in aquatic product, PV actually would not induce anaphylactic reactions in sensitive individuals when under the threshold amount. Hence, we consider that it is necessary to have rapid, effective and feasible analytical methods that can also reflect the biological

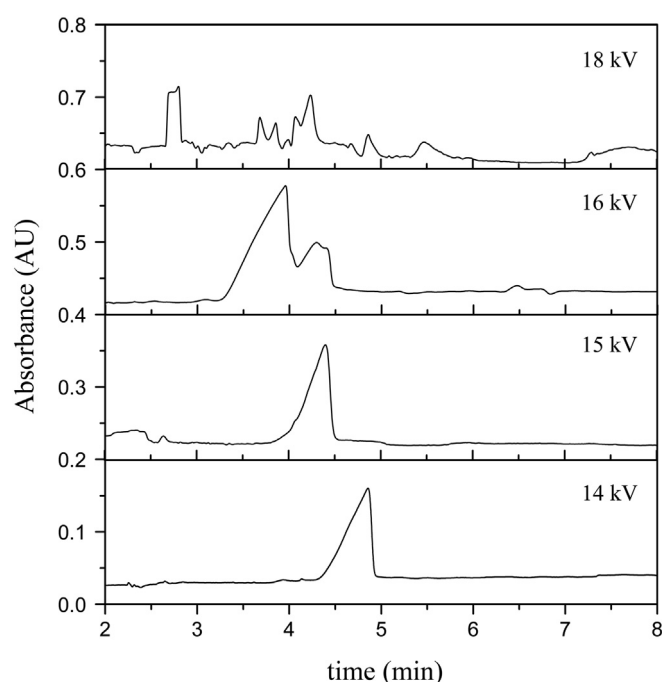


Fig. 2. Electropherograms showing the effects of voltages on the resolution of parvalbumin standards. Experimental conditions: 30 mmol/L borate-borax buffer with pH of running buffer at 9.1 under different separation voltages; injection sample 5 s under the pressure of 0.5 psi; capillary temperature set at 25 °C.

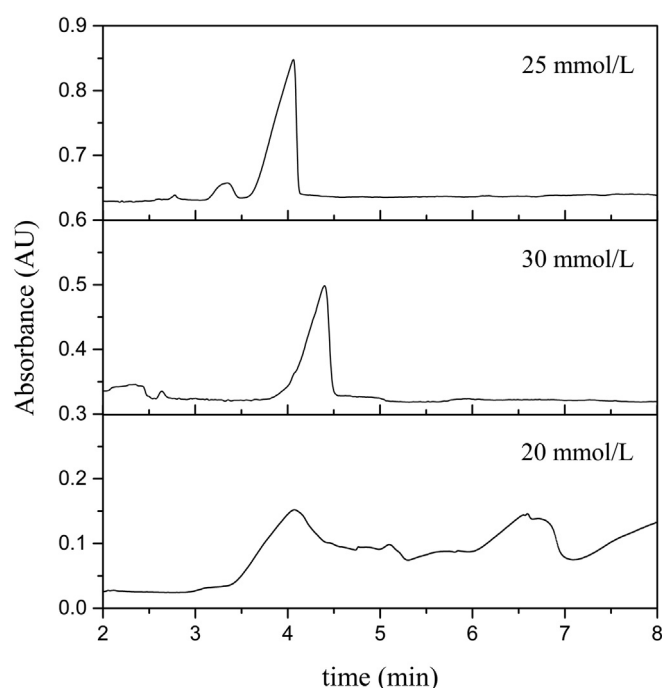


Fig. 3. Electropherograms showing the effects of concentrations of borate-borax buffer on the resolution of parvalbumin standards. Experimental conditions: 15 kV separation voltage and pH of running buffer at 9.1 under different concentrations of borate-borax buffer; injection sample 5 s under the pressure of 0.5 psi; capillary temperature set at 25 °C.

significance of the LOD in allergen detection.

Mast cells play a central role in IgE-mediated hypersensitivity (allergy) by releasing degranulation and inflammatory mediator, so the mast cell degranulation assay is usually employed to measure the

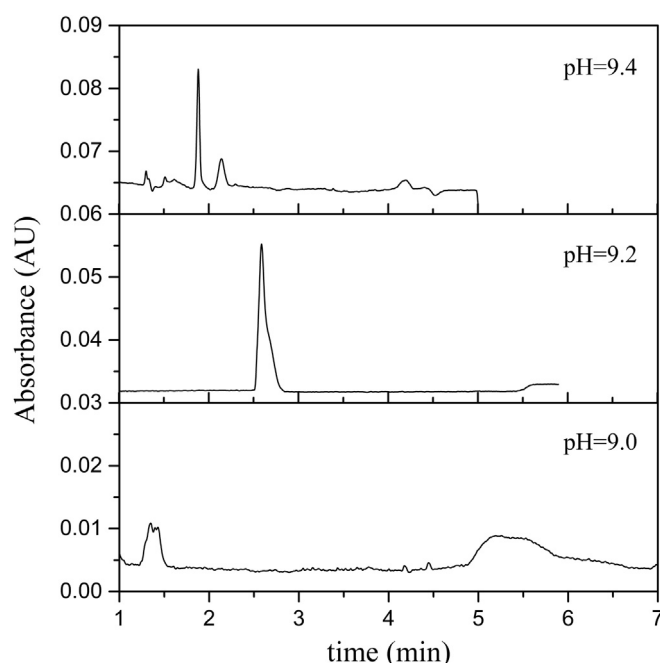


Fig. 4. Electropherograms showing the effects of pH values of borate-borax buffer on the resolution of parvalbumin standards. Experimental conditions: 25 mmol/L borate-borax buffer with 15 kV separation voltage at different pH values of running buffer; injection sample 5 s under the pressure of 0.5 psi; capillary temperature set at 25 °C.

allergenicity of potential allergens (Lo & Yokoyama, 1974). In order to determine whether a series of concentrations of PV above or below the LOD could induce human mast cell LAD2 degranulation, we investigated levels of two biomarkers (histamine and tryptase) and morphological changes of LAD2 cells. The C48/80-stimulated group was performed as positive control, because C48/80 is a potent activator to increase intracellular Ca^{2+} concentration in mast cells, which can cause the degranulation of proinflammatory mediators and further induce the release of preformed mediators, lipid mediators and cytokines (Pundir, Catalli, Leggiadro, Douglas, & Kulka, 2014).

3.4.1. Transmission electron microscopy (TEM)

As shown in Fig. 6A, representative KLH-stimulated LAD2 cells (negative control) displayed a non-segmented nucleus, a regular cytoplasm with numerous narrow surface folds and secretory granules distributed throughout the cytoplasm. Meanwhile, C48/80-stimulated LAD2 cells were characterized by an irregular nucleus and cytoplasm with secretory granules distributed outside the cytoplasm exhibiting a loss of membrane density. Remarkably, the ultrastructure of LAD2 cells treated with PV (1 $\mu\text{g}/\text{mL}$) is similar to that of the C48/80-stimulated. These cells showed disruption of the cell membrane, numerous secretory granules and some partly empty granules out of the membrane. However, similar to KLH-stimulated cells, LAD2 cells treated with PV at both 0.1 and 0.01 $\mu\text{g}/\text{mL}$ presented a normal ultrastructure, indicating that no degranulation induced when LAD2 cells were challenged at these two concentrations.

3.4.2. Release of histamine and tryptase

The effect of three concentrations of PV on the release of histamine (Fig. 6B) and tryptase (Fig. 6C) from LAD2 mast cells was investigated. The levels of histamine and tryptase were PV concentration-dependent, which showed the release of histamine and tryptase rising as the increase of PV concentration. Compared with the KLH group, incubation of mast cells with 1 $\mu\text{g}/\text{mL}$ PV or C48/80 significantly ($p < 0.001$) increased the levels of histamine and tryptase. In contrast, PV at concentrations of 0.1 and 0.01 $\mu\text{g}/\text{mL}$ did not activate LAD2 cell

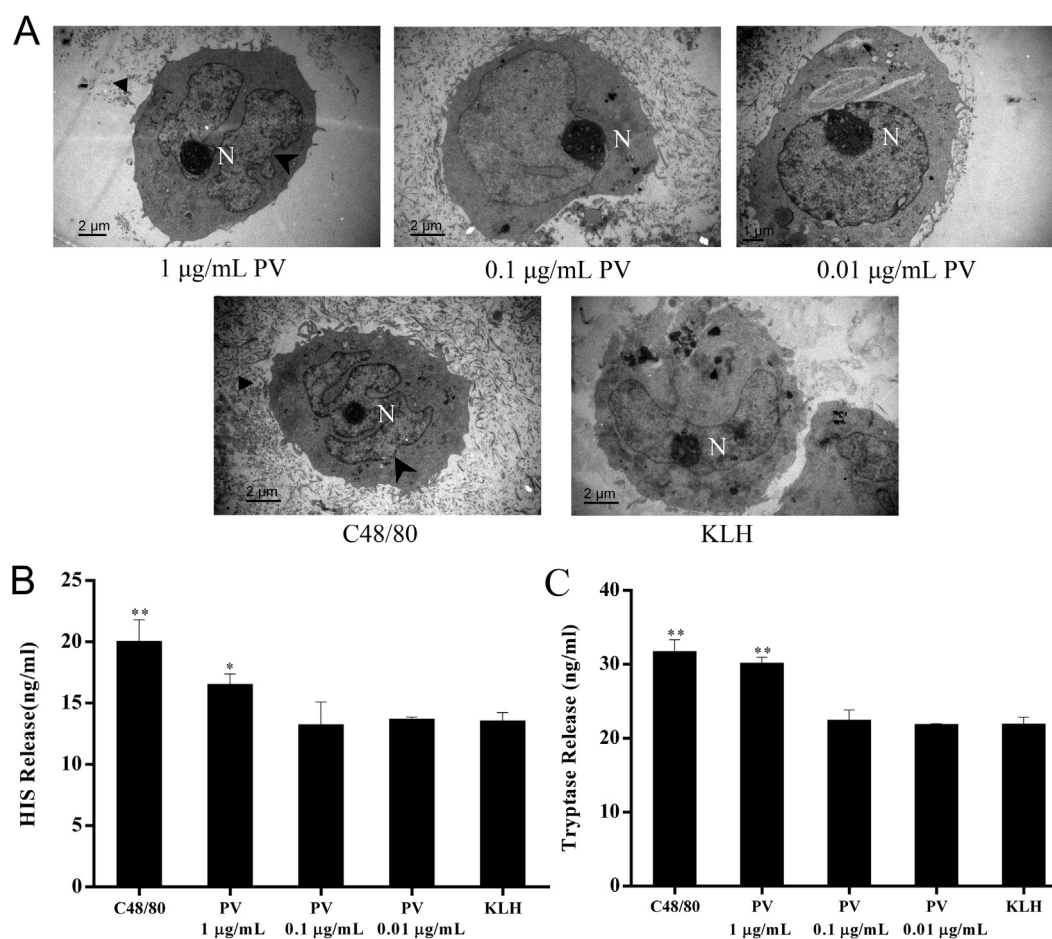


Fig. 6. The effect of different concentrations at 0.01, 0.1 and 1 µg/mL of parvalbumin on the degranulation of human mast cells. (A) TEM micrographs of LAD2 cells either unstimulated or stimulated using different concentrations of parvalbumin. Non-segmented nucleus (N), disrupted membranes (arrowheads), and degranulated granules (triangles) located outside the mast cell cytoplasm were indicated; (B) Levels of histamine and (C) levels of tryptase induced by different concentrations of parvalbumin ($n = 3$) were shown. * $p < 0.05$, ** $p < 0.001$ compared with negative control.

degranulation and production of preformed mediators, consistent with the phenomenon observed by TEM.

Altogether, the method established here was able to simply, effectively and quickly detect PV in fish at an allergenic concentration, suggesting the biological significance of the LOD.

3.5. Application of the method in fresh fish samples

To test the ability of this established method to function effectively and applicably in the presence of PV, we analyzed four species of raw fish samples. The concentrations of the major allergen in fish samples were back-calculated from the calibration curve, and the final amounts of PV existed in the raw fish samples were indicated as the calculated mass of allergens/total mass of fish powder. The results are summarized in Fig. 7 (supplementary data) and Table 2. These data showed that PV could be detected in *Ctenopharyngodon idellus*, *Carassius auratus* and

Mylopharyngodon piceus with abundances between 1.19 and 2.41 mg per g fish powder. However, PV was not detected in concentrations above the LOD in *Hemiculter leucisculus*. Our analysis of fresh samples provides information about the amounts of PV in four main fish species that are frequently consumed and highlights the possibility of the newly developed method for detecting the major allergen in fish, which may provide a practical way for the industrial and government to label allergens of fish and seafood products.

4. Conclusion

An effective improved CZE method with IEC as a simple background suppression treatment was first reported here for separation and determination of the major allergen in fish samples. To date, compared with the ELISA and real-time PCR methods reported previously, this system is the most effective and applicable method available for accurate quantification of the major allergen, PV, in fish samples containing diverse protein components. These results also highlight the applicability of CZE-based methods for the determination of allergens at the allergenic concentration range in complicated food matrices. Moreover, the method can overcome the problem of cross-reactivity and false positive results in immunoassays. Additionally, only when the concentration of PV is above the LOD reported here, the human mast cell LAD2 degranulation can be induced, indicating the biological significance of the LOD in the detection of natural functional proteins.

Table 2

Analytical results of parvalbumin in raw fish samples.

Fish species	Parvalbumin (mg/g) $\pm$ SD ^a
<i>Ctenopharyngodon idellus</i>	1.19 $\pm$ 0.058
<i>Hemiculter leucisculus</i>	ND ^b
<i>Carassius auratus</i>	2.41 $\pm$ 0.085
<i>Mylopharyngodon piceus</i>	1.97 $\pm$ 0.040

^a $n = 3$.

^b ND: not detected above the LOD.

5. Conflict of interest

All Authors declare that they have no conflict of interest.

6. Ethical approval

This article does not contain any studies with animals performed by any of the authors.

7. Informed consent

Not applicable.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version (Figure 5, Figure 7 and Table 1), at <https://doi.org/10.1016/j.foodchem.2018.04.037>.

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