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Development of a method for the quantification of fish major allergen parvalbumin in food matrix via liquid chromatography-tandem mass spectrometry with multiple reaction monitoring

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Dithiothreitol (PubChem CID:446094)
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Ammonium bicarbonate (PubChem CID:14013)
Formic acid (PubChem CID:284)
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ABSTRACT

The availability of analytical methods for quantification of allergens is crucial for the correct assessment and labeling of products in order to protect allergic consumers. For this purpose, a simple, sensitive and accurate technique was developed based on liquid chromatography-tandem mass spectrometry and multiple reaction monitoring mass spectrometry (LC-MRM-MS/MS). The proposed method uses a simple purification with heat and a completely optimized tryptic digestion. This method has been validated according to the requirements defined by ICH (Q2 [R1]), having a linear range from 0.10 to 1179.36 nM with $r > 0.999$. The parvalbumin beta in flounder (*Paralichthys olivaceus*) has been quantified at a low level down to 0.10 μg/g with satisfactory precision (RSD < 18.35%) and accuracy (< 13.3%). The new approach was successfully applied for the determination of parvalbumin beta in the other food matrices.

1. Introduction

Currently, food allergies have become one of the main concerns in the food safety sector. For sensitized individuals, a small amount of allergens could cause allergy symptoms ranging from minor (e.g. dermatitis, nausea, vomiting…) to severe (e.g. anaphylactic shock). However, there is no effective measure but to assure the total exclusion of allergens from the diet of sensitive individuals.

To guarantee the security of the allergenic customers, numerous regulations regarding food allergenic components have been enforced (Commission directive 2000/13/EC, 2000; Commission Directive 2007/68/EC, 2007; Regulation (EU) No 1169/2011, 2011; US Food and Drug Administration, Food Allergen Labeling and Consumer Protection Act, 2004). In the European Union (EU), the labeling legislation entails a mandatory declaration of the existence of 14 main allergenic foods including fish and its products in the labels (Regulation (EU) No 1169/2011, 2011). Usually, the warning applies in ambiguous and inconsistent manners (e.g. "may contain, be free of…"), and lack the sufficient interpretation of precautionary labels, thereby misleading the customers. Sometimes, the ignorance and negligence of allergenic peoples also lead to severe allergic reactions (Ballmer-Weber et al., 2015). The new terminology -"reference doses" of allergenic foods, a basis for the definition of impending threshold values, has been brought up in recent years (Ballmer-Weber et al., 2015; Heinonen, 2014; Taylor et al., 2014). The reference dose of fish protein was reported to be 27.3 mg of protein, likely provoking allergic reactions in 10% of the

Abbreviations: PV, Parvalbumin; HPLC-MS, High performance liquid chromatography-tandem mass spectrometry; MRM, Multiple reaction monitoring; VITAL, Voluntary Incidental Trace Allergen Labeling; ELISA, Enzyme-linked immunosorbent assay; RT-qPCR, Real-time quantitative polymerase chain reaction; RSD, Relative standard deviation; LOD, Limit of detection; LOQ, Limit of quantification; BCA, Bicinchoninic acid; CE, Collision energy; DP, Declustering potential

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sensitized individuals (Ballmer-Weber et al., 2015). As a consequence, considering the regulations and allergen threshold levels discussed above, it is necessary to establish accurate, sensitive and simple methods to detect traces of allergenic substances in the food and its products.

To date, enzyme-linked immunosorbent assay (ELISA) and real-time quantitative polymerase chain reaction (RT-qPCR) are widely used in routine laboratories to detect allergens in food (Jayasena et al., 2015; Khuda et al., 2012; Sathe, Teuber, & Roux, 2005; Verhoeckx et al., 2015). The ELISA method, which is based on the interaction of an allergen with its antibody, is the most commonly used method because of its sensitivity, operability and potential for standardization (Parker et al., 2015). But this method has significant drawbacks in terms of reproducibility and specificity. The qualification results from the same sample are not identical between commercial ELISA kit from different manufacturers, especially for the analysis of thermally processed foods (Poms et al., 2005). The interference of matrix components target allergen's epitopes which may be modified to be not detected during food processing, resulting in false-negative results (Fu & Maks, 2013; Jayasena et al., 2015; Khuda et al., 2012; Sathe et al., 2005; Verhoeckx et al., 2015). The RT-qPCR method is established using the specific DNA sequence of the allergenic protein as the biomarker (Eischeid, Kim, & Kasko, 2013). With regards to the characteristics of DNA (i.e., heat stable, specificity etc.), this method is less affected by heat processing and has a low chance of cross-reactions than protein-based method. Yet DNA will be separated and degraded during food production, as a result, might perform the false-negative results (Holzhauser & Röder, 2015).

Due to the limitations of the above mentioned two methods, high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) method has been developed for allergenic protein analysis in foodstuffs (Carrera, Canas, & Gallardo, 2012; Downs, Baumert, Taylor, & Mills, 2016; Koeberl, Clarke, & Lopata, 2014; Korte & Brockmeyer, 2016; Planque et al., 2016; Sayers et al., 2018). The combination of the effective separation techniques (e.g. nano-LC, HPLC, UHPLC) with stable monitoring techniques (e.g. Q-TOF, PRM, SRM, MRM, MS3) advances this method better in terms of sensitivity and specificity for the detection of multiple food allergens simultaneously both in raw and processed samples. The main steps involved in LC-MS/MS method includes signature peptide markers selection, minimization of interference from matrix components, optimization of mass spectrometric and chromatographic performances, and validation of method in terms of reproducibility, accuracy, sensitivity, linearity and quantification (Koeberl et al., 2014). The labeled or unlabeled peptides were frequently used as the chemical substitutes to quantify allergen peptide markers (Posada-Ayala et al., 2015; Ippoushi, Sasanuma, Oike, Kobori, & Maeda-Yamamoto, 2016). The mass spectrometry method based on multiple reaction monitoring mode (MRM) focused on proteotypic peptides, are more widely used (Korte et al., 2016; Lin, Chen, Zhang, Li, & Fu, 2018). Although several studies had been validated in terms of the limit of quantification (LOQ), limit of detection (LOD), signore-to-noise (S/N) and linearity curve, there are few methods systematically validated following the guidelines (Mindaye, Spiric, David, Rabin, & Slater, 2017; ICH.Q2 [R1], 2005; Commission Decision of 2002/657/EC, 2002).

Fish allergy is an important concern in the seafood processing environment. Numerous fish allergens including parvalbumin, enolase, aldolase and vitellogenin have been reported, and parvalbumins beta (β-PVs) are identified as the major allergens (Kuehn, Swoboda, Arumugam, Hilger, & Hentges, 2014). β-PVs are EF-hand calcium-binding globular protein having a molecular mass weight of 10–12 kDa, an acidic pI (3–5) and play a role of muscle relaxation. Due to this structure, especially the Ca^{2+} binding site, β-PVs are resistant to tryptic digestion and heat treatment (Swoboda et al., 2002). Both of the two characteristics increase the difficulty to quantify β-PVs using HPLC-MS/MS. Carrera et al. (2012) reported a rapid and direct HPLC-SMIM-MS/MS method for the qualitative detection of β-PVs in foodstuffs. This method allows the analysis of β-PVs in less than 2 h by ultrasound-assisted trypsin digestion, however, the selected peptides were not proved to be used in quantitative analysis and lacked the validation procedures.

The purpose of the present research was to establish a stable HPLC-MS/MS method for the quantitative analysis of β-PV in flounder (*Paralichthys olivaceus*). The method, using mass spectrometry with MRM, suits the routine laboratory equipment involved in food analysis. The established method was completely validated via a set of parameters in accordance with the guidelines of ICH Q2 (R1), including linearity, LOD, LOQ, precision and accuracy. Additionally, the validated method was employed for determining the presence of allergens in different matrices acquired from local supermarkets.

2. Materials and methods

2.1. Chemicals and reagents

Tris, glycine, dithiothreitol (DTT), ammonium bicarbonate (NH_4HCO_3), iodoacetamide (IAA) and BCA protein quantitation Kit were procured from Sigma-Aldrich (St. Louis, MO, USA). Formic acid and acetonitrile (ACN) of HPLC grade were obtained from Merck (Darmstadt, Germany). RapiGest SF acid labile surfactant (ALS) and sequencing grade trypsin were purchased from Waters Corporation (Manchester, UK) and Roche Diagnostics (Indianapolis, USA), respectively. Water was obtained from a Milli-Q Gradient A 10 system (Millipore, Bedford, MA, USA). DTT, IAA, RapiGest SF and trypsin were dissolved in 50 mM ammonium bicarbonate solution.

The reference peptides including ALTDAETK, SDFIEEDELK and LFLQNFSASAR (95% standard) were chemically synthesized by Sangon Biotech (Shanghai, China). Stock solutions (10.0 mM) were prepared in methanol/water (50/50, v/v) and diluted to 10.0 mL, then stored at −70 °C in dark for a maximum of 3 months. Working standard solutions at different levels were prepared for the calibration curves by diluting the stock solutions with the digests of turbot muscle matrix, and were kept at 4 °C in the dark and found to be stable for 48 h.

The recombinant β-PV (Gene Bank number: BAF98925.1) was expressed and purified from *Escherichia coli* in Sangon Biotech (Shanghai, China). Finally, 13.0 mg of recombinant β-PV was obtained with the purity of over 95%.

2.2. Preparation of fishes and matrices

Flounder (*Paralichthys olivaceus*), turbot (*Scophthalmus maximus*), brown-marbled grouper (*Epinephelus fuscoguttatus*), small yellow croaker (*Pseudosciaena polyactis*) and silver carp (*Hypophthalmichthys molitrix*) were obtained from local supermarkets. Food matrices including pork, shrimp, chicken muscle and beef were purchased from local supermarkets. To validate the method, incurred matrices were prepared by the addition of 1.0 g of the minced muscle of flounder to 9.0 g non-contaminated minced matrices with homogenization.

2.3. Sample extraction

The white muscle of flounder was homogenized using a homogenizer. 1.0 g of muscle was transferred into a 10.0 mL polypropylene-based centrifuge tube and extracted with 4.0 mL of 0.1 M Tris, 0.5 mM glycine and 1.0 mM DTT, then homogenized for 2.0 min using an Ultra Turrax T-18D (Ika, Staufen, Germany) at 8000 rpm/min. After centrifugation for 20.0 min at 4 °C and 12000 × *g*, the supernatant was pipetted into a new 10 mL polypropylene centrifuge tube. Then, the extracts were heated for purification in a water bath at 90 °C for 3.0 min. After centrifugation for 20 min at 4 °C and 12000 × *g*, the concentration of supernatants was measured by the bicinchoninic acid (BCA) kit (Sigma-Chemical Co., USA) (Smith et al., 1985). The other fish muscles