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Development of a liquid chromatography-tandem mass spectrometry method for simultaneous detection of the main milk allergens

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ABSTRACT

The goal of this study is development and validation of a method to confirm and quantify milk allergens in food products based on liquid chromatography-tandem multiple reactions-monitoring, mass spectrometry (LC-MRM/MS). Here, emphasis was placed on two whey proteins, α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), plus a third, α_{s1}-casein (α_{s1}-CN), known to be the main allergenic components of milk. Five marker peptides (one for α-La, two for β-Lg and two for α_{s1}-CN) and three quantitative marker peptides from the digestion of standard milk proteins were identified using matrix-assisted laser desorption/ionization with tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS). Optimization of enzymatic hydrolysis conditions were defined as 37 °C for 16 h. The linearity ranges for the three allergenic proteins (α-La, β-Lg and α_{s1}-CN) were 0.97–31.25 μg/mL, 0.48–31.25 μg/mL and 0.48–31.25 μg/mL, respectively. The assays were validated for absolute quantification of three milk proteins with satisfactory results, which indicates that the established mass method is suitable for the expression of levels in daily food.

1. Introduction

Food allergies have become a serious public health concern, especially within the past few years. According to a recent survey, up to 4%–5% of the total human population in industrialized countries suffers from a clinically-proven food allergy (Mehta, Ramesh, Feuille, Groetch, & Wang, 2014). Cow's milk allergy (CMA) has become a common disease in early childhood, its prevalence ranging from 1.6% to 2.8% among children younger than 2 years of age (Monaci, Tregoat, van Hengel, & Anklam, 2006). The most abundant milk proteins belong to the main classes of caseins and whey accounting for 80% and 20% of the total milk proteins, respectively (J. M. Wal, 1998). Casein (CN) represents the main fraction of milk proteins and is subdivided into a number of families (α, β, κ). They are characterized by a central hydrophobic part and a hydrophilic layer with phosphorylated sites and a hardly clear tridimensional structure suggesting the presence of preferentially linear epitopes (J. Wal, 2001). α_{s1}-Casein (α_{s1}-CN), a major cow's milk allergen, has nearly 70% unordered structure, with only a small amount of secondary structure, such as α-helix or β-sheets (Chatchatee, Järvinen, Bardina, Beyer, & Sampson, 2001; Cocco, Järvinen, Sampson, & Beyer, 2003). The whey proteinα-lactalbumin (α-La) is an important cow's milk allergen (J. M. Wal, 1998), comprising a water-soluble monomeric globular domain primarily α-helical in nature with some α-helix and β-sheet and a hydrophobic core well protected from the solvent in the interior by the rigid packing of side chains (Acharya, Stuart, Phillips, & Scheraga, 1990). β-Lactoglobulin (β-Lg) is the major whey protein of ruminant species and is also present in the milks (Kontopidis, Holt, & Sawyer, 2004), which consists of six different short fragments of the polypeptide chain, which are located especially in the β strands, covering a flat area on the allergen surface (Niemi et al., 2007).

Several analytical approaches for the detection of food allergens are available to-date. Mostly they target the allergenic proteins that indicate the presence of the allergenic food ingredient (Diaz-Amigo, 2010; Monaci, Brohée, Tregoat, & van Hengel, 2011). So immunoassays are currently commonly used for screening

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purposes because antibodies can recognize specific proteins in a complex mixture of compounds of different nature. Some target the DNA fragments of allergenic proteins to indirectly evaluate the allergenic food ingredient (Sun et al., 2015). The development of instruments and methods for accurate sequence-specific DNA analysis is essential for the efficient use of genomic information. Biosensors are applied in evaluation of allergenicity of food ingredient, through establishing the cell-based electrochemical sensor (Jiang et al., 2013) or cell-based fluorescence sensor (Jiang et al., 2015) et al. The enzymatic hydrolysis is incompetent in elimination or reduction of the allergenicity of food products (Panda, Tetteh, Pramod, & Goodman, 2015). In practice enzyme hydrolysis has often not reduced the allergenicity of complex foods and can result in an increase in allergenicity because of exposure of new antigenic epitopes due to protein breakdown (Lee et al., 2007). So the allergenic protein-based detection methods would not be suitable for evaluation of food allergens.

Methods based on mass spectrometry (MS) have advanced significantly in terms of sensitivity and specificity, having improved the identification, characterization, and determination of food allergens (Monaci, Pilolli, De Angelis, Godula, & Visconti, 2014; Shefcheck & Musser, 2004). LC–MS/MS has superior characteristics, improved reproducibility, recovery, sensitivity, dynamic range, and quantification (Koeberl, Clarke, & Lopata, 2014; Weber, Raymond, Ben-Rejeb, & Lau, 2006). In this sense, selected reaction monitoring (SRM) is a highly specific quantitative methodology based on the measurement of specific proteotypic peptide masses (peptide precursor and precursor fragments masses) corresponding to the protein of interest in a triple quadrupole (QQQ) mass spectrometer (Manes, Mann, & Nita-Lazar, 2015; Parker et al., 2015). A proteotypic peptide is defined as a peptide that identifies a protein uniquely; thus, this protein can be specifically quantified by peptide measurement.

This study explored a peptide-based LC-MRM/MS method for simultaneously sensitive determination of multi-allergens in milk samples and milk related food samples. The mainly allergenic protein, α-La, β-Lg and α_{s1}-CN, were treated with enzymolysis of trypsin, before conducted with MS analysis. With optimization of the enzymatic hydrolysis conditions and the design of peptides design, the mass detection method was of implementation with stability, precision and accuracy analysis. After the method verification, the real sample test was conducted with modified mass method.

2. Material and methods

2.1. Chemicals

Milk, cookie, cake and other samples were purchased from the Auchan supermarket (Wuxi, Jiangsu province, China). From Sigma-Aldrich Co. (Shanghai, China), α-lactalbumin (α-La, ≥ 90%), β-lactoglobulin (β-Lg, ≥ 85%) and α_{s1}-casein (α_{s1}-CN, ≥70%) were purchased. The peptide fragments used (purity ≥ 85%) were obtained from Sangon Biological Technology (Shanghai, China). Trypsase (for HPLC purification) was purchased from Promega (USA). Dithiothreitol (DTT, for HPLC purification) and iodoacetamide (IAA, also for HPLC purification) were purchased from Sigma-Aldrich Co. (Shanghai, China).

1.5 mg/mL standard protein stock solution, composed of 9 mg each α-La, β-Lg and α_{s1}-CN, were added to 6 mL of 25 mmol/L NH_4HCO_3 solution, then stored at −20 °C. Next, 0.5 mg/mL standard protein in-process solution was prepared, which was composed of 1 mL standard protein stock solution of α-La, β-Lg and α_{s1}-CN 1 mg/mL synthetic peptides stock solution, containing 1 mg peptides powder, was dissolved in water/acetonitrile (95/5, v/v) solution, sub-packaged and stored at −20 °C. Stock standard solutions of synthetic peptides (1 mg/mL) were prepared in water/acetonitrile (95/5, v/v) and stored at −20 °C. Working solutions were freshly prepared by appropriate dilution of the stock solutions. 20 mM Tris-HCl (pH = 8.2) and 25 mM ammonium bicarbonate (pH = 8.0) were prepared with ultrapure water. Trypsin solution of 0.01 μg/μL was prepared by 30 mM acetic acid and stored for approximately 1 month at 20 °C. Ultrafiltration was performed with Amicon ultra-15 Centrifugal Filter Devices with a volume of 15 mL and a 3 kDa cutoff (Merck Millipore Ltd. Ireland).

2.2. Sample preliminary treatment

For whole protein extraction from daily food samples (cookie, cake, etc.), 2 g of each sample was diluted with 30 mL of extraction buffer (20 mM Tris-HCl, pH = 8.2). Then the samples were vortexed and incubated at 60 °C in a water bath for 2 h with vigorous shaking. The mixture was centrifuged at 14,000 rcf for 25 min at 4 °C. The bottom layer was removed, and the supernatant was centrifuged at 14,000 rcf for another 25 min. The supernatant was ultra-filtered through a 3 kDa molecular weight cutoff filter (MWCO) centrifugal filter and washed 3 times with 8 mL of 25 mM Tris-HCl (pH = 8.2). The retained concentrate was collected and the filter washed with 20 mM Tris-HCl (pH = 8.2). The total protein concentrations of extracted fractions were determined by Enhanced BCA Protein Assay Kit. Proteins from milk were separated by gel electrophoresis. Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% separation gel, SDS running buffer, following the manufacturer's instructions (Wilson, Martinez-Villaluenga, & De Mejia, 2008).

2.3. Trypsin digestion

In gel digestion protocol: The gel bands (milk sample) were cut into spot, washed with water for twice. The gel spot were destained two times with 25 mM ammonium bicarbonate in 50% acetonitrile (ACN) followed by reduction with 1 mM Dithiothreitol (DTT) in 25 mM ammonium bicarbonate at 57 °C for 1 h. The sample was then allowed to room temperature, iodoacetamide (IAA) solution (25 mM NH_4HCO_3, 50 mM IAA) were added for alkylation at room temperature in the dark for 30 min. The gel spot then dehydrated with CAN, 20 μL digest solution (0.01 μg/μL, trypsin) were added at 37 °C for 1 h, the gels were extracted once with 50 μL extraction buffer (5% TFA, 67% ACN) at 37 °C for 30 min. The peptides mixture was stored at −20 °C for MALDI-TOF-TOF MS identification (Cutillas, Timms, Cutillas, & Timms, 2010).

In solution digestion was performed as follows: 50 μL of the final extract solution was transferred into a 2 mL tube and DTT (1 M in 25 mM ammonium bicarbonate) was added for protein reduction (final concentration 10 mM). The sample was incubated at 60 °C for 30 min. The sample was then cooled to room temperature, and IAA (1 M in 25 mM ammonium bicarbonate) were added into the mixture for protein alkylation, and the sample was placed at room temperature in the dark for 30 min. Trypsin digestion was done by adding prepared trypsin (0.01 μg/μL) to each sample (1:30, trypsin/protein ratio) and incubated for 16 h at 37 °C while mixing. Then 1% FA was added to the mixture to stop digestion. Finally the samples were centrifuged at 12,000 rcf for 20 min at 4 °C; the supernatant was transferred to another micro-centrifuge tube and stored at −20 °C before LC-MRM/MS analysis.

2.4. Identification of maker peptides by MALDI-TOF-TOF MS

Characterization of the peptides mixture was achieved using a matrix-assisted laser desorption ionization (MALDI) mass