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MINI REVIEW

Integrative Pediatrics

Application of Mass Spectrometry in the Identification of Gluten Proteins and Trouble Shootings

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Abstract

Gluten is a storage protein found in wheat and it is the main culprit that triggers celiac disease (CD). About 1% of the world population is affected by CD. Earlier CD was thought to be a disease of the pediatric population but now it is a well-known condition for adults as well. The only effective treatment for CD is to exclude gluten from the diet. However, complete removal of gluten from the diet is challenging. There are two main laboratory methods to identify gluten fractions in the food i.e. Enzyme-Linked Immunosorbent Assay (ELISA) and Mass Spectrometry (MS). ELISA method is a quicker and widely used method to identify gluten in the food. MS is a sophisticated analytical method that is highly sensitive to pick tiny fragments of gluten protein. There are multiple technical problems that require critical attention. Hence, MS cannot be established in every laboratory. In this article, we have given the specific method to perform the MS and also provided common technical difficulties and troubleshooting. This article could be very useful for researchers who remain puzzled while performing gluten identification. This article could also be helpful for beginners to established MS in the laboratory.

Keywords:*celiac disease; gluten; mass spectrometry; ELISA*

Introduction

Celiac disease is gluten mediated intestinal inflammation [1]. About 1-2% of the world population is affected by CD [2,3]. Earlier CD was thought to be a disease of the pediatric population but now it is a well-known condition for adults as well [4]. Gluten is a storage protein of wheat that contained epitopes that stimulated immunological reactions in genetically susceptible individuals. Gluten is found in wheat, rye, barley, and other related grains.[1] The only effective treatment for CD is to exclude gluten (wheat) from the diet and follow a life-long strict gluten-free diet (GFD). CD patients are reliant on naturally gluten-free food (rice, maize, etc) or commercially available gluten-free products [5].
However, gluten is ubiquitous in nature it is used almost in every food and non-food industry [6]. Therefore, the complete removal of gluten is challenging. CD patients are critically sensitive to traces of gluten, exposure of >10 mg/day gluten is enough to initiate an immunological trigger in most of the CD patients [7]. Based on gluten toxicity and amount of food intake, a safe amount of gluten exposure is defined which is <20ppm (mg/kg) of gluten [5]. This safe amount of gluten exposure is recognized by Codex Alimentarius and the US Food and Drug Administration (FDA) [8,9]. Gluten-free products with <20 ppm of gluten are considered gluten-free [5]. Nevertheless, in recent years, studies have shown that commercially available 'labeled gluten-free food' are not actually gluten-free. Up to 9-30% of gluten contamination is reported in commercially available labeled gluten-free products. The authenticity of such products are under great suspicion. [5,10,11]. Therefore, it is important to identify the gluten traces in gluten-free food products. There are two important methods to investigate gluten in food products. The first one is a widely used Enzyme-Linked Immunosorbent Assay (ELISA) method. R5 antibody-based ELISA method is an official method to quantify gluten in food products [5]. The second one is the Mass Spectrometry (MS) based method. MS is a highly sensitive method that can identify an even smaller fragment of gluten that may be missed from the conventional ELISA method [12]. MS is applied widely in sports doping, food authentication, and biomedical and pharmaceutical research [13-15]. However, MS is not a widely accepted method to identify gluten in food products because MS is an expensive and difficult analytical method to perform in the laboratory. It requires a trained and experienced researcher to perform and interpret the results. The protocol of performing MS is critical and requires several precautions and attention during the experiment. So, it is not standardized in every laboratory. As it is an expensive method, all laboratories do not have the MS facility [12,16]. MS processing required diverse reagents and solutions and during its performance, there could be several technical issues that need the full attention of the technician. In contrast, the ELISA method does not have such difficulties. That is why researchers give priority to the ELISA method. But, ELISA may provide false-positive results and it cannot distinguish the source of gluten

among cereals due to cross-reactivity [17]. MS provides an authentic result (Table 1). In this article, we have summarized the MS protocol and discussed the common difficulties during its performance and their troubleshooting.

S.No.	Factors	ELISA	Mass Spectrometry
1	Expense	Medium equipment cost	• Very high equipment cost • Low day-to-day reagent cost
2	Skilled and Trained staff	Less required	Highly required
3	Sample volume	High	less
4	Post translational modifications	Not identified	Identified
5	Automated	Semi-automatic	Almost completely automated
6	Range of analyte and antigen detected	limited	Wide range
7	Time	1-3 hours	5-10 hours
8	Sensitivity	High	High
9	Specificity	Poor (in terms of antibody batch)	High
11	Reproducible	Less	High
12	Cross reactivity	Less	No
13	Multiplexing	Limited multiplexing	High; multiple analyte can be screened
14	High throughput	High	Medium

Table 1. Major difference between gluten ELISA and MS**.**

Mass Spectrometry

Mass spectrometry is highly sensitive, accurate and a high throughput analytical tool that provides structural information of the analyte by measuring the mass-to-charge ratio (m/z) values [18]. In intact mass-analysis, the molecular weight of the undigested protein gets identified. A mass spectrometer consists of three major components (1) ionization source, (2) mass analyzer and (3) detector [15,18,19]. In the ionization source, the target protein sample is bombarded with high laser and the sample molecules or peptides to ionizes (charged). In mass analyzers, these ions are separated according to their mass-to-charge ratio and accelerated toward detector in the electric/magnetic field. The detector detects these ions and shows results in the form of the mass spectrum. Raw data generated from a typical MS analysis mass valued of all the peptides reached the detector. The target protein fraction is identified by matching experimentally observed peptide masses with the theoretical peptide masses present in the database [18-20].

Gluten isolation and result interpretation

A For the extraction of gluten is based on the protocol already described by Schalk et al. Briefly, for 100mg of flour, the floor is first treated with salt solution (2.0mL of 0.4M NaCl in 0.067M Na_2HPO_4/KH_2PO_4 buffer, pH7.6 for 10min at 22°C to extract albumin and globulin content. Then flour is treated with 1.5mL of ethanol/water (60/40,v/v) for 10min at 22°C to extract prolamins and in the third step flour is treated with (2.0mL of 2-propanol/water (50/50,v/v) in 0.1M Tris-HCl, pH7.5, containing 2 M (w/v) urea and 0.06 M (w/v) Dithiothreitol (DTT) for 30 min at 60°C under nitrogen to extract glutelins. The prolamins (gliadins) and glutelins constitute gluten thus both second and third solution should be mixed and lyophilized [21].

Gluten protein exhibits several proteomics challenges as gluten protein has high molecular weight, contains high amount of glutamine, proline and hydrophobic amino acids. Hence trypsin is not a suitable enzyme for gluten digestion because it is very precise and specific for lysine and arginine residues which are present in very few in numbers. The enzymes of choice for gluten digestion are chymotrypsin and thermolysin. After the digestion of gluten. The sample is shown as mass spectrum along with peak list generated as raw data. Raw data can be searched on instrument compatible software. Software ease our job and also tells us about neutral losses like H2O and CH3 etc. are common and post translational modification may occur which can be easily detected by the software. It gives all the information like

contribution, confidence of the peptide in the spectrum. It also tells gives the protein ID and number of peptides identifies hence tells the sequence coverage of the protein [22].

Precautions

IFor mass spectrometry experiments, high-quality reagents and solvents must be used, especially Mass Spectrometry grade chemicals. This will reduce the contaminants that could suppress the ionization. All the equipment (gel apparatus, scanner) should be rinsed with MiliQ and then with 70% ethanol to lessen contamination. Keratin contamination is the most notorious contamination in the protein sample that usually comes during numerous steps in protein digestion. To avoid keratin contamination, wear gloves during the entire process and change them often. All the buffers should be made and handled wearing gloves. Ammonium bicarbonate solution should be made daily and DTT and Iodoacetamide solution should be made fresh shortly before use. Gel casting and running should be done in keratin free environment. Gel bands should be cut on the clean glass plate with a clean razor. Gel band should be cut properly only up to stained corners and chopped into pieces of 1x1 mm so that buffer/protease could enter the gel pieces and also not so small to be picked and removed during washing with a pipette. Gel pieces should be transferred to fresh tubes and washing and rinsing should be done with MiliQ twice or thrice with vortexing are recommendable to remove any keratin present on the surface on the gel. Gel pieces should be washed properly between each step to remove extra contamination of salts, buffer, detergents, etc. Also, one should take extra precautions to avoid the use of Triton X-100 and tween-20 for sample solubility as they will suppress the signals. Buffers like tris, phosphate, and HEPES should not be used. Use of iodoacetamide (IAM) induced alkylation further improves the gluten digestion [12,19,23,24].

Troubleshooting

Dentsply Sirona's CAD/CAM technology has the longest track record of any of the existing digital impression systems in today's market place. The CEREC system has been in existence, in one form or another, for over 30 years. Dentsply Sirona's most recent system, the CEREC AC with Omnicam, was released in the summer of 2012. With the CEREC predecessors (RedCam, BlueCam), imaging was done via stitching together individual images creating a monochromatic yellow stone-like digital model. However, the Omnicam captures images, without the use of powder, via digitally streaming to create a full color digital model.

Keratin contamination

If after taking all the precautions still, keratin contamination is coming in the results. Create a general list for contaminants and add this in the exclusion list while searching for raw data so that the peaks coming from contaminants will be excluded during processing data.

Salt contamination

To clean salts/denaturants contamination which severely suppresses the signals. Ziptip can be used which binds the peptides and cleans the salts, buffers, and detergents. Thus sample ionizes well and a clear spectrum is attained.

Not many peaks are picked

The sample is not digested properly, 1% rapigest or 8 M urea can be introduced prior digestion but there conc. need to be adjusted in the sample prior adding Trypsin.

Conclusions

Though mass spectrometry being a very precise and accurate tool for gluten protein identification deals with some limitations – expensive, sophisticated tool, intolerance to contaminants and salts, low protein concentration, lack of complete protein databases and some technical limitation of proteome coverage. But if the suitable enzyme and digestion method is followed with proper precautions and a trained person operates the instrument and analyzes the data this can give more information about the sample origin along with better identification of gluten peptides. This technique has the possibility to be used as an established technique for gluten identification as a food contaminant.

Authors contributions

P.D.: wrote the first version of the article and drafted the manuscript. **P.D. and A.K.V.:** conceived the idea. **P.D.:** Collection and acquisition of data. **A.K.V.:** critically revised the manuscript. **P.D. and A.K.V.:** final approval of the version to be published. **P.D.:** overall guarantor of the manuscript. All authors approved the manuscript.

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