Advanced Proteomics and Systems Biology

Applied to Study Food Allergy

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SHORT TITLE: Proteomics and Systems Biology for Food Allergy

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HIGHLIGHTS

- Discovery proteomics allows the complete *de novo* sequencing of new allergens.
- Targeted proteomics allows the rapid detection of food allergens in foodstuffs.
- Proteomics-based systems biology allows the study of the mechanisms of food allergy.

1 **ABSTRACT**

2
3 Food allergy is considered to be the fourth most important public health problem by
4 the World Health Organization (WHO). As a consequence, accurate, sensitive and fast
5 detection methods for food allergy control and investigation are highly recommendable. In
6 this work, we present a short overview of the applications of the Advanced Proteomics and
7 Systems Biology approaches for the study and control of food allergy. For this purpose, two
8 consecutive proteomics strategies (Discovery and Targeted Proteomics) applied for the study
9 and control of food allergy are reviewed in detail. In addition, from an integrative point of
10 view, the emergence proteomics-based systems biology approaches applied for the study of
11 the mechanisms of food allergy are described. Future directions and new perspectives are also
12 provided.

13
14
15 **Keywords:** Discovery Proteomics, Targeted Proteomics, Proteomics-based Systems Biology,
16 Food, Allergy

1 INTRODUCTION

2
3 Food allergy is considered to be the fourth most important public health problem by
4 the World Health Organization (WHO). A total of 6-8% of children and 2-4% of adults are
5 affected [1]. Fourteen foods are declared by the European Food Safety Authority (EFSA)
6 as allergens: fish, shellfish, mollusks, eggs, milk, nuts, peanuts, soybean, celery, mustard,
7 sesame, sulphur dioxide/sulphites, lupin and wheat. The symptomatology emerges within
8 60 min of ingestion and include hives, abdominal pain, nausea, vomiting, diarrhea and
9 breathless. In the most grave cases, anaphylactic shock can occur [2,3]. To guarantee
10 consumer safety, several regulations regarding food allergy have been executed (Directive
11 2007/68/EC). Recently the EFSA recognized a world-wide affecting high risk and
12 accepted the necessity of developing new methodologies for its control. Consequently,
13 rapid, sensitive and accurate detection methodologies for food allergen control are highly
14 recommendable.

15 At present, the mechanisms by which the intracellular signaling of the immune
16 system triggers the susceptibility to food allergy are poorly understood. Regarding B and T
17 lymphocytes, it is well known that the B-cell receptor (BCR) and the T-cell receptor (TCR)
18 signaling pathway plays an essential role [4]. Understanding the intracellular mechanisms
19 by which the immune system signaling triggers the susceptibility to food allergy is a
20 relevant key to identify potential pathways and significant targets for its study and therapy.

21 In this sense, the powerful of the -omics methodologies, which are achieving a
22 great impact in the research community and in the research of modern food science, can
23 contribute to the study and control of food allergy. In fact, a new discipline, called
24 Foodomics has been reported [5,6]. This discipline studies food and nutrition areas through
25 the application of advanced -omics methodologies to improve consumer's well-being,

confidence and health. A number of high-throughput technological –omics approaches like genomics, transcriptomics, proteomics, lipidomics and metabolomics combined with bioinformatics and systems biology are involved in this new emerging field. Although the number of scientific publications found in the literature which incorporates proteomics and systems biology approaches in the area of food allergy is limited so far, we consider that the emergence advances in proteomics and systems biology tools are offering new strategies and perspectives for food allergen control and investigation that will have a promising significance [7,8].

Therefore, in this short review the main applications of the advanced proteomics and systems biology methodologies for the study and control of food allergy are summarized in three major areas: (i) Discovery proteomics, (ii) Targeted proteomics, and (iii) Proteomics-based systems biology (Figure 1).

DISCOVERY PROTEOMICS IN FOOD ALLERGY

Discovery proteomics involves the large-scale analysis of a particular proteome at a particular time. It is based by the unbiased discovery of the proteome, the determination of the protein abundance changes, the characterization of post-translational modifications and the identification of protein/peptide biomarkers [9,10]. In this approach, using a bottom-up proteomics workflow, the protein/s of interest are separated by two-dimensional gel electrophoresis (2-DE), or by capillary electrophoresis (CE) or liquid chromatography (LC), converted into peptides using enzymes such as trypsin and the resulting peptides are analyzed by mass spectrometry (MS) (Figure 2).

In the context of food allergy, using a bottom-up proteomics approach, several new food allergens have been characterized (Table 1) [11-48]. Thus, using a classical 2-DE protein separation, immunoblotting with sera of allergic patients and analysis by matrix-assisted laser

desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) or by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), were identified and characterized new food allergens from shrimp, sesame, hazelnut, pistachio, fruit and wheat [11-14]. Besides, the use of 2-DE together with the application of multi-enzymatic protein digestion followed by LC-MS/MS peptide identification and/or *de novo* MS sequencing were employed for the partial characterization of new food allergens as gluten and arginine kinase from shrimp [14,15] (Table 1).

It is important to emphasize that a novel and advanced discovery proteomics workflow for the complete *de novo* MS sequencing of new food allergens was developed [16]. This workflow was employed for the characterization of all parvalbumins beta (β -PRVBs) from all commercial fish species belonging to the Merlucciidae family. β -PRVBs are the major fish allergens [49]. This workflow is based on the combination of a conventional 2-DE bottom-up proteomics strategy with the accurate determination of the M_r of the intact proteins by Fourier-transform ion-cyclotron resonance (FTICR)-MS and selected tandem mass spectrometry (MS/MS) ion monitoring (SMIM) of the peptide mass voids. Thus, for each β -PRVB isoform, mass spectra acquired by LC-MS/MS from two peptide digests (Glu-C and trypsin) were analyzed by database searching using Mascot and Sequest and were *de novo* sequenced by hand with the assistance of two software DeNovoX and PEAKS. The peptide sequences were organized and the theoretical M_r for the resulting protein sequences was calculated. Then, for each β -PRVB isoform experimental M_r was estimated with high mass accuracy by FTICR-MS and the masses of certain missing peptide voids were estimated by comparing the experimental and theoretical M_r . MS/MS spectra corresponding to these ions were obtained by LC-MS/MS in the SMIM scanning mode. To finish, all peptide sequences were ordered to construct the final protein sequences.

1 This proteomics workflow reached the entire *de novo* MS-sequencing of 25 new β -
2 PRVB isoforms.

3 4 **TARGETED PROTEOMICS IN FOOD ALLERGY**

5 Targeted proteomics is a hypothesis-driven strategy that is focused on the selective
6 analysis of a subset of proteins representing candidate potential biomarkers [50]. The MS
7 analyzer is centered on analyzing only the compounds of interest by selective/multiple-
8 reaction monitoring (SRM/MRM) mainly on triple-quadrupole (QqQ) or on hybrid
9 quadrupole-ion trap (Q-Trap) mass spectrometers or by SMIM in ion trap mass spectrometers
10 [51]. In addition, targeted proteomics on hybrid quadrupole-Orbitrap (Q-Orbitrap) mass
11 spectrometers, known as parallel reaction monitoring (PRM), is improving the speed,
12 resolution and dynamic range of the targeted scanning mode [52]. Thus, monitoring specific
13 transitions, pairs of precursor and fragment ions, constitute a conventional assay to identify
14 protein biomarkers (Figure 2).

15 To provide a better management of food allergy, these targeted proteomics scanning
16 modes have been applied to detect and quantify in the foodstuffs the presence of several food
17 allergens (Table 1). Thus, using a LC-SRM/MRM assay on QqQ or Q-Trap instruments the
18 reliable specific detection and quantitation of the presence of gluten [14,20], eggs
19 [17,18,22,23,25], milk [17,18,21-24], soy [18,22,23], hazelnut [18,22,23], walnut [22,23],
20 almond [22,23] or peanuts [18,21,22,23,26,28] in complex food products was effectively
21 performed. Moreover, strategies for the multi-allergen detection were performed for five
22 allergens (eggs, milk, soy, hazelnut and peanut) [18] and for the detection of seven different
23 allergens (milk, egg, soy, peanut, hazelnut, walnut and almond) by LC-SRM/MRM [22,23].
24 Using a high-resolution mass spectrometer (HRMS) as the Q-Orbitrap instrument was

possible to detect and quantify the presence of peanuts [36], milk [39] or eggs [45] in trace amount in other food products.

An innovative workflow published by our laboratory permits the rapid detection of fish β -PRVBs in any food product [43] (Figure 2). The strategy is based on the use of a fast purification step of the β -PRVBs by treatment with heat (time: 45 min), the acceleration of in-solution protein digestion by high-intensity focused ultrasound (HIFU) (time: 2 min), and the monitoring of 19 β -PRVBs peptide biomarkers by LC-MS/MS SMIM in a linear ion trap mass spectrometer (time: 60 min). The procedure allows the rapid detection of occurrence of β -PRVBs in any foodstuff, including precooked and processed products, in less than 2 hours. To our knowledge, this is the quickest methodology to achieve the direct detection of this food allergen in foodstuffs. This strategy was validated using several commercial sea-foodstuffs including processed and even battered precooked products. This workflow was also successfully applied for the rapid authentication of fish species [42] and for the rapid detection of the thermostable and allergenic protein Ani s 9, characteristic of the Anisakids species, in any seafood products in less than 2 hours [44].

The use of data-independent acquisition (DIA) mode is currently emerging due to recent innovations in MS instruments, such as sequential window acquisition of all theoretical mass spectra (SWATH), where all peptide ions that are present at a time in the mass analyser are fragmented. Monaci et al. (2013), combining DIA with HRMS reported values of limits of detection ranged from 0.4 to 1.1 $\mu\text{g/mL}$ for traces of egg-white powders and caseinate in white wines [45]. In addition, mass spectrometry based analysis employing DIA coupled with ion mobility mass spectrometry (DIA-IM-MS) was used to investigate the allergen composition of raw peanuts and roasted peanut flour ingredients used in challenge meals [46].

Top-down proteomics can support the development of methods for the detection and quantification of food allergens at the intact protein level [47,48]. The absolute quantitation of

the whey protein beta-lactoglobulin using LC-MS in various milk products was performed using appropriate internal standards [47]. In addition, the sensitive detection of cow milk proteins (lactalbumin, lactoglobulins A and B) in mixed-fruit juice samples were achieved with a limit of detection of 1 µg/mL. The key objective in top-down analysis in HRMS instruments is an efficient fragmentation of intact protein to obtain sequence-specific information for database searching and protein identification. An easy and robust method for fish allergen detection has been developed utilizing the high speed, high resolution and fragmentation capabilities of the Orbitrap Fusion mass spectrometer implemented with an ultraviolet photodissociation (UVPD) source [53]. Using PRVB proteoforms as a signature for the allergen detection the method showed several benefits such as, minimal sample preparation, high sensitivity, throughput and practically a complete protein sequence coverage.

PROTEOMICS-BASED SYSTEMS BIOLOGY IN FOOD ALLERGY

One essential objective of proteomics is to understand how the proteins are integrated and participate in the biological systems using a new approach known as proteomics-based systems biology. This approach use the advanced proteomics information together with the capacity of bioinformatics and computational modeling to understand and to predict the properties of biological systems using a holistic point of view.

In the context of food allergy, the mechanisms by which the intracellular signaling of the immune system triggers the susceptibility to food allergy are poorly understood. In the case of B and T lymphocytes, it is well known that the BCR and TCR signaling pathway play an essential role [4]. In addition, their interplay with other signaling pathways may be important to identify new channels for food allergy prevention and treatment. Towards this goal, the building of a model that represents B and T-cell signaling might help researchers to

1 understand the mechanisms of food allergic reaction. Previously, proteomics-based systems
2 biology approaches have been applied to elucidate biological pathways and networks to
3 retrieve information about the functional role of a particular proteome [54]. In that article, a
4 preliminary map of the sarcoplasmic fish interactome was built up including functional gene
5 ontologies and term enrichment *in silico* studies. The whole network revealed the biological
6 role of the sarcoplasmic fish proteome from an unprecedented interactomics perspective.
7 Although the number of studies is limited so far, we consider that this topic will have a
8 promising significance.

9 In view of that, our group is currently working using proteomics-based systems
10 biology approaches to compile information about T-cell signaling in the case of fish allergy;
11 involved, mainly, in the study of protein abundance changes, protein-protein interactions and
12 the dynamics of post-translational modifications (PTMs), primarily phosphorylations at
13 different time points upon stimuli in T-cells. Phosphorylations play crucial roles in regulating
14 the biology of the cell since they can change a protein's physical or chemical property,
15 activity, localization and interactions. Several proteomic techniques have been developed to
16 identify and quantify the phosphoproteome [10]. These datasets will be integrated in to
17 functional and dynamical networks using computational systems biology approaches. The
18 integration of the data in a holistic T-cell model for food allergy will provide a valuable
19 signaling map to understand the mechanism of action of specific allergens.

CONCLUDING REMARKS AND NEW PERSPETIVES

As is presented in this short review the capability of the advanced proteomics and systems biology tools for the study and control of food allergy is noticeable. Two sequential stages of the proteomics pipeline (Discovery and Targeted proteomics) allows the complete *de novo* sequencing of new food allergens. Besides, the monitoring of several allergen peptide biomarkers by SMIM or PRM, allows the unambiguous and rapid detection of food allergens in any foodstuff in less than 2 hours. Therefore, the use of reliable and sensitive MS-based proteomics approaches, for both discovery and monitoring of food allergens, will enhance the safety to the consumers. The application of absolute quantitation by AQUA-LC-MRM [55], the use of CE coupled to a top-down proteomics approach to detect intact protein allergens in HRMS instruments [56], and the employ of new complementary top-down MS/MS fragmentation modes like high-collision dissociation (HCD), electron-transfer-high-collision dissociation (ETDhcD) and UVPD, for the characterization and *de novo* sequencing of whole allergens, are new directions that will provide new valuable insights. We also considered that incorporating these results into portable biosensor devices based on lab-on-a-chip, microfluidics or protein arrays will be very advantageous. These devices will make possible for the food industry companies and food control authorities to perform the food routine allergy control test in their own facilities without the need for expensive instrumentations and/or qualified staff.

Proteomics high-throughput analytical platforms, combined with computational data management using bioinformatics and mathematical models has opened up new views in our understanding of complex biological systems inside to the area of systems biology. Tools for data mining, interpretation and data integration, are useful to manage this huge amount of data. Thus, the study of the crosstalk mechanisms between PTMs (i.e. phosphorylations and glycosylations) at different time point upon stimuli in of immune cells with the antigen (i.e.

1 food allergen) is one of the current challenges of the scientific community [57]. The dynamics
2 of the interplay of PTMs and their integration using computational systems biology
3 approaches will provide a valuable signaling map for further discoveries in the context of
4 food allergy. This global perspective offer tremendous potential in the clarification of the
5 mechanisms of food allergy as well as defining crucial pathways, networks and potential
6 targets for therapeutic interventions. To carry out a comprehensive elucidation of the
7 mechanism of action of food allergens, in vitro assays using cell culture and/or animal models
8 are necessary. Thus, the methodologies and tools now disposable in proteomics-based systems
9 biology offer sensational opportunities to develop the emerging field of systems biology in
10 the area of food allergy.

FIGURE CAPTIONS

Figure 1: Main areas of advanced proteomics and systems biology for the study of food allergy.

Figure 2: Proteomics pipeline used for the characterization and detection of food allergens.

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CONFLICTS OF INTEREST

None. All the authors confirm that there are no conflicts of interest associated with this publication.

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Figure 1

Figure 2

1 **Table 1.** Overview of the research articles found in literature in the last years, which use proteomics approaches to study food allergy.

Approach	Food Allergen	Main Goal	Proteomics Tools	Reference
Discovery Proteomics	Fruit	In-gel identification	2-DE, immunoblot	Marzban et al., 2008 [11]
	Shrimp, sesame, hazelnut, pistachio	In-gel identification	2-DE, immunoblot	Grishina et al., 2017 [12]
	Wheat	In-gel identification	2-DE, immunoblot, MALDI-TOF MS	Akagawa et al., 2007 [13]
	Gluten	Partial peptide identification	LysC, trypsin, chymotrypsin, LC-MS/MS DDA	Martínez-Esteso et al., 2016 [14]
	Shrimp (Arginine kinase)	<i>De novo</i> MS-sequencing (partial)	Bottom-Up proteomics, trypsin, LC-MS/MS DDA	Ortea et al., 2009 [15]
	Fish (Parvalbumins, PRVBs)	<i>De novo</i> MS-sequencing (complete)	Bottom-Up proteomics, trypsin, Glu-C, LC-MS/MS DDA, FTICR-MS, SMIM	Carrera et al. 2010 [16]
Targeted Proteomics	Gluten	Detection	LC-SRM	Martínez-Esteso et al., 2016 [14]
	Eggs and milk	Detection and quantification	LC-SRM	De Angelis et al., 2017 [17]
	Eggs, milk, soy, hazelnut, peanut	Detection and quantification	LC-SRM	Pilolli et al., 2017 [18]
	Peanut	Detection and quantification	LC-MRM	Careri et al., 2007 [19]
	Gluten	Detection and quantification	LC-MRM	Sealey-Voyksner et al., 2010 [20]
	Milk	Detection and quantification	LC-MRM	Ansari et al., 2011 [21]
	Milk, egg, soy, peanut, hazelnut, walnut, almond	Detection and quantification	LC-MRM	Heick et al., 2011a [22]
	Milk, egg, soy, peanut, hazelnut, walnut, almond	Detection and quantification	LC-MRM	Heick et al., 2011b [23]
	Milk	Detection and quantification	LC-MRM	Lutter et al., 2011 [24]
	Eggs	Detection	LC-MRM	Cryar et al., 2012 [25]
	Peanuts	Detection and quantification	LC-MRM	Pedreschi et al., 2012 [26]
	Nuts	Detection and quantification	LC-MRM ³	Korte et al., 2016 [27]
	Peanut	Detection and quantification	ICP-MS, LC-MRM	Careri et al., 2008 [28]
	Gluten	Detection	LC-MS/MS DDA	Weber et al., 2009 [29]
	Eggs	Detection	LC-MS/MS DDA	Lee et al., 2010 [30]
	Milk	Detection	LC-MS/MS DDA	Hong et al., 2012 [31]
	Milk	Detection and quantification	LC-MS/MS DDA	Cereda et al., 2010 [32]
	Milk	Detection and quantification	LC-MS/MS DDA	D'Amato et al., 2010 [33]
	Milk	Detection and quantification	LC-MS/MS DDA (XIC)	Monaci et al., 2010 [34]
	Peanuts	Detection and quantification	LC-MS/MS DDA (XIC)	Hebling et al., 2013 [35]
	Peanuts	Detection and quantification	LC-HR-MS/MS DDA (XIC)	Monaci et al., 2015 [36]
	Peanut	Detection	LC-MS/MS DDA, LC-MS SIM	Chassaigne et al., 2007 [37]
	Lupin	Detection and quantification	LC-MS/MS DDA, LC-SRM	Mattarozzi et al., 2012 [38]

	Milk	Detection and quantification	LC-HR-MS/MS (XIC, SMIM)	Monaci et al., 2011 [39]
	Peanut	Detection and quantification	LC-MS/MS SMIM	Careri et al., 2008 [40]
	Shrimp	Detection	LC-MS/MS SMIM	Ortea et al., 2011 [41]
	Fish	Detection	HIFU-LC-MS/MS SMIM	Carrera et al., 2011 [42]
	Fish	Rapid detection of all PRVB in food	HIFU-LC-MS/MS SMIM	Carrera et al., 2012 [43]
	Anisakids (Anis s 9)	Rapid detection of Anisakids	HIFU-LC-MS/MS PRM	Carrera et al., 2016 [44]
	Eggs and milk	Detection and quantification	LC-HR-MS/MS DIA (XIC)	Monaci et al., 2013 [45]
	Peanut	Detection and quantification	LC-DIA-IM-MS	Johnson et al., 2016 [46]
	Milk	Detection and quantification	Top-Down proteomics, LC-MS	Czerwenka et al., 2007 [47]
	Milk	Detection and quantification	Top-Down proteomics, LC-MS SIM	Monaci et al., 2008 [48]

1 2-DE, two-dimensional gel electrophoresis; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; FTICR-MS, Fourier-transform ion-
2 cyclotron resonance; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; HR-MS/MS, high-resolution tandem mass spectrometry; SIM, selected ion
3 monitoring; SMIM, selected tandem mass spectrometry ion monitoring; SRM, selected reaction monitoring; MRM, multiple reaction monitoring; MRM³, multiple reaction
4 monitoring cubed; PRM, parallel reaction monitoring; DDA, data-dependent analysis; DIA, data-independent analysis; XIC, extracted ion chromatogram; HIFU, high-
5 intensity focused ultrasound; ICP-MS, inductively coupled plasma mass spectrometry; IM-MS, ion-mobility mass spectrometry.

1 **Graphical abstract**

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