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Food allergen profiling: A big challenge

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ABSTRACT

Food allergy is an important public health and safety concern. The description of the pattern of individual allergens associated to each food is of crucial importance both for the diagnostic process and the setting of a safe diet for each allergic subject. However, we are still far from this goal as can be seen from allergen data resources like Allergome. Although some advances have been made during the last few years leading to the identification of new allergens in many allergenic sources and to their characterization, this knowledge is still fragmentary and does not allow the definition of a comprehensive pattern. The exploitation of new technologies and the improvement of those already existing has provided significant contributions, although the widespread use of specific technologies and/or methodologies without cross-checks between them may have generated some biases leading to the preferential identification of selected subsets of allergenic molecules. It would be now desirable to adopt new strategies based on a dynamic combination of different methodologies, spanning from the classic biochemistry-based ones to the innovative microtechnologies and bioinformatics, in order to obtain the best results and give a forward thrust to knowledge in this field.

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1. Introduction

Food allergy is widely reported to be on the rise (Fishbein & Fuleihan, 2012; Krause et al., 2002; Linneberg et al., 2000; Maziak et al., 2003; Nicolaou, Siddique, & Custovic, 2005; Sicherer, Munoz-Furlong, Godbold, & Sampson, 2010), but it is actually very difficult to have exact epidemiological data on this topic. The causes of the increase and spread of allergic reactions are still unclear and may be due to a combination of different factors (Fishbein & Fuleihan, 2012; Kemp & Bjorksten, 2003; Mari, 2004; Rottem, 2003; Rottem, Gershwin, & Shoenfeld, 2002). Food allergy can cause one or more symptoms that can be more or less severe and include angioedema, oral allergic syndrome, urticaria, abdominal pain, diarrhea, nausea, vomiting, itching and even life-threatening reactions, such as anaphylactic shock. Therefore, due to the increasing prevalence of allergic reactions and to the effect they have on the quality of life of allergic subjects, the appropriate management of these patients represents a growing public health concern.

As immunotherapy for food allergy is still far from being routinely available (Zuidmeer-Jongejan et al., 2012), the best treatment for food allergic subjects is still based on the avoidance of the allergen source, that means the exclusion of specific foods from the diet. The implication is that a proper management of these patients depends

on a reliable diagnosis providing a list of specific allergenic molecules/sources that each subject has to avoid. A reliable diagnosis of food allergy is important not only for the avoidance of foods that may cause a reaction, but also to avoid unnecessary dietary restrictions that may negatively affect the quality of life. In addition, an unequivocal diagnosis is also essential before considering the possibility of future immunotherapeutic treatments (Sastre, Landivar, Ruiz-Garcia, Andregnette-Rosigno, & Mahillo, 2012; Zuidmeer-Jongejan et al., 2012). However, the diagnostic systems now available sometimes fail in their aims and do not provide reliable responses.

The allergy testing methods are based on the detection of specific IgE and/or on the IgE-mediated patient's reaction to the hypothesized allergy source that can be used as a reagent (i) just as it is, or (ii) in the form of a protein extract, or (iii) as individual purified allergens. In the first option, the raw food is used to challenge the allergic subject by *in vivo* tests (prick-by-prick, food challenge), but sometimes these cannot be applied due to different types of limitations. The traditional, and still most common, allergy testing methods are based on the use of commercially available raw protein extracts derived from allergy sources. Protein extract preparations can be used to perform *in vitro* serological tests (Radioallergosorbent test, RAST; ImmunoCAP) and *in vivo* tests (skin prick test, SPT). However, it is well known that the allergen composition of extracts is very variable and their standardization appears impossible (Curin et al., 2011; Focke, Marth, & Valenta, 2009; Hildebrandt, Steinhart, & Paschke, 2008; Kottapalli et al., 2008; Larsen & Dreborg, 2008; Matthes & Schmitz-Eiberger, 2009; van Kampen et al., 2009). Quite recently, the use of individual purified

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allergen preparations has been introduced into the clinical routine allowing the so-called allergenic molecule-based diagnosis by *in vivo* (SPT) or *in vitro* tests such as Immuno Solid-phase Allergen Chip (ISAC) system (Phadia Multiplexing Diagnostics, PMD, Vienna, Austria) (Bublin et al., 2011; Deinhofer et al., 2004; Harwanegg & Hiller, 2005, 2006; Harwanegg, Hutter, & Hiller, 2007) and Allergenic Molecule-based micro-bead Array system, ABA (Pomponi et al., 2012). Clearly, in the third case, the reagents for molecule-based diagnosis allow a better standardization of the tests than the protein extract-based reagents.

Usually, data from more than one test system are comparatively evaluated in the attempt to reach a diagnosis which is as accurate as possible. False responses can be ascribed to different factors. Probably, the factor that most significantly affects the diagnostic response is the set of allergenic components, as well as their concentration, present in the reagent used to make the test. A false negative result could be due to the lack of one or more allergens in the reagent used for testing, whereas a false positive response could be due to possible contaminations of a food by other foods or by molecules deriving from non-food materials (Anibarro, Seoane, & Mugica, 2007; Polimeno et al., 2010; van der Veen et al., 1996). Ideally, a reagent used for the diagnosis of an allergy to a specific food should exactly contain all the potential allergens (all together in the raw food and extracts, or separated into individual purified allergens) of that food, and nothing more. To achieve this aim, two conditions should be fulfilled: (i) the entire profile of allergenic molecules contained in the allergenic sources should be known, and (ii) reliable protocols and methodologies, useful to assess the pattern of allergenic components really contained in the reagents used by the allergy test systems, should be available.

2. Known food allergens

Many allergens have been identified so far in different allergenic sources, as detailed in the Allergome database (www.allergome.org) (Mari, Rasi, Palazzo, & Scala, 2009; Mari et al., 2006), where 2415 allergenic molecules were listed as of October 15, 2012. Among the reported allergenic sources and individual molecules, those for which a sufficient immunological characterization is available have been officially named by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee (www.allergen.org). Searching in the database of the named allergens that are officially recognized by the WHO/IUIS Sub-committee revealed that overall 740 allergens were listed on September 7, 2012. Among them, 260 molecules (35%) were labeled as food allergens, belonging to the Animalia (phyla: Arthropoda, Chordata, Mollusca, Nemata) and to the Plantae (phyla: Liliopsida and Magnoliopsida) kingdoms (Table 1). Twelve allergens (4.6% of known food allergens) are not real food molecules because they are all proteins from *Anisakis simplex* (Armentia et al., 2006; Audicana & Kennedy, 2008; Kobayashi et al., 2011; Lopez & Pardo, 2011; Vidacek et al., 2010), a parasitic nematode that can infest some fish species. About 40% of the known food allergens belong to the species reported

in Fig. 1, showing the organisms for which 5 or more food allergens have been described.

3. The available allergen profiles of food allergenic sources

The description of the allergen profile of a food implies the identification of all the potentially allergenic molecules contained in it. This statement takes for granted the concept that the potential allergenic molecules represent a finite number of proteins, and the remaining components of the proteome of the allergenic source lack the features that cause the activation of the immune system leading to the allergic reaction.

Since the allergenic source may contain more than one allergen, an in-depth characterization is required to describe the complete allergome by classifying as “allergenic” or “not allergenic” the proteome components. However, the available knowledge does not provide sufficient data to classify any food protein as “definitely not allergenic” (Fig. 2). In fact, some proteins appearing not allergenic when a few subjects were analyzed, were recognized as allergens when a larger population, or a population selected on the basis of different criteria, was investigated. In this regard, the kiwifruit allergen Act d 11 can be considered as an example. In fact, initially it appeared non allergenic when it was tested on a population of subjects showing severe reactions after kiwifruit ingestion. However, this protein was identified as an allergen when it was tested on a population of subjects reacting to the birch pollen allergen Bet v 1, thus revealing its immunological correlation with the Bet v 1-like allergens (D'Avino et al., 2011). Indeed, the need for data from a very large, and possibly random, population arises from the observation that single allergic subjects display individual patterns of sensitivity to allergenic molecules.

At present several allergenic sources have been more or less investigated, but none has received an in-depth and targeted characterization allowing the classification of at least most of the proteome components as “allergenic” or “not allergenic”. Ideally, the availability of a panel of individual components of an allergenic source proteome would allow the identification of the complete pattern of allergenic molecules by probing them with individual sera from a very large population of allergic subjects. Indeed, the knowledge and the technologies now available do not yet allow the achievement of this goal.

4. IgE-binding of allergenic and non-allergenic food components

The word “allergy” is generally used to indicate the type I hypersensitivity, that is an IgE-mediated immediate reaction. Non-IgE mediated adverse reactions to food molecules can also occur, but this topic is beyond the scope of this review.

The presence in a patient's serum of IgE antibodies specifically recognizing one, or more, protein molecules contained in an allergy source is exploited in the process of allergy diagnosis and in the detection of allergenic molecules. In fact, the detection in human serum of IgE specific for a given molecule is considered the first clue suggesting the allergenicity of that molecule. Unfortunately, this is not sufficient to classify a molecule as a “real allergen” because some IgE-binding proteins have no clinical relevance since they do not cause any allergic symptoms. For instance, plant proteins bearing N-linked oligosaccharides are recognized by IgE specific for glyco-epitopes, known as cross-reactive carbohydrate determinants (CCD) (van Ree, 2002). The structure of these oligosaccharides is at least partially conserved in a large variety of plant and insect glycoproteins and represents an important cause of co-recognition by CCD-specific IgE (Mari, 2002). Ana c 2, that is the pineapple protease bromelain, is a well-known example of a glycoprotein detecting IgE antibodies specifically recognizing CCD. Ana c 2 seems to be a very rare cause of allergic symptoms, but nevertheless it is frequently tested positive in serum-based IgE antibody assays. In fact, positive results on Ana c 2 are obtained every time a serum of a subject producing CCD-specific

Table 1
Number of allergens found in different phyla and officially named by the WHO/IUIS.

Kingdom	Phylum	Total allergens	Food allergens
Animalia	Arthropoda	204	28
	Chordata	66	34
	Cnidaria	1	0
	Mollusca	3	3
	Nemata	14	12
Fungi	Ascomycota	82	0
	Basidiomycota	23	0
Plantae	Coniferopsida	14	0
	Liliopsida	84	25
	Magnoliopsida	249	158

Data were collected on September 7th, 2012.

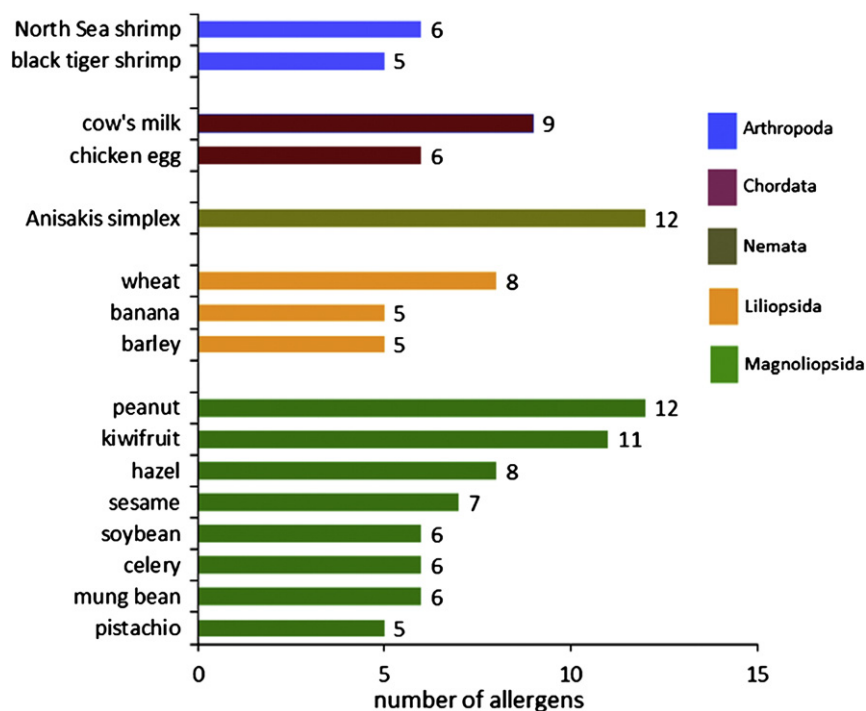


Fig. 1. Allergenic sources for which 5 or more allergens have been reported.

IgE is tested. However, this positive result is clinically irrelevant (Mari, 2002). In addition to Ana c 2, many other glyco-allergens have been described, and the list includes Api m 1 (Blank et al., 2011), Cup a 1 (Alisi et al., 2001), Hor v 17 (Mikami, Yoon, & Yoshigi, 1999), Ole e 1 (Castro et al., 2010) and Pla a 2 (Ibarrola, Arilla, Martinez, & Asturias, 2004). Unlike Ana c 2, some of these are reported to cause significant allergic symptoms and therefore they are considered clinically relevant. In the case of positive IgE binding on a glycoallergen, it is important to discriminate between glyco-epitopes, recognized by CCD-specific IgE, and the protein-epitopes associated to true allergic reactions. IgE-binding protein-epitopes also have a different relevance. In fact, some of them are associated to significant allergic reactions, whereas others are only involved in IgE co-recognition mediated by antibodies produced against a different sensitizing molecule, and sometimes they have no relevant clinical effects and cannot be considered real allergens.

This is an aspect to be considered in the process of allergy diagnosis, as well as of the validation of real allergens (Mari, 2008). The allergen profile of an allergenic source must include only validated allergens, that means IgE-binding proteins able to induce allergic symptoms. The IgE-binding proteins for which a clinical relevance has not been demonstrated cannot be considered as components of the allergen profile of an allergenic source.

5. Identification of new allergenic molecules

The classic route followed to identify a new allergenic molecule contained in a specific food begins with the observation that one or more subjects have allergic symptoms after the ingestion of that food. Next, the serum of each subject is used to probe a protein extract obtained from the same food. The detection and biochemical identification of one or more components recognized by the IgE contained in the subject's serum provide the preliminary information on new allergenic molecules. A combination of different methodologies can be exploited to achieve this aim, as reviewed in the literature (Gonzalez-Buitrago et al., 2007; Mari, 2008; Mari, Ciardiello, Tamburrini, Rasi, & Palazzo, 2010).

The above procedure has been widely used and has led to the identification of different allergenic protein families. Indeed, most of the allergens described so far belong to a few protein families and are characterized by a limited number of biochemical functions (Radauer, Bublin, Wagner, Mari, & Breiteneder, 2008). A search in the database of allergen families, AllFam, at www.meduniwien.ac.at/allergens/allfam/, shows that the Prolamin superfamily is the one including the highest number of allergens. Next, we found families with a decreasing number of components, such as the EF hand domain, Profilin, Tropomyosin, Cupin superfamily, Bet v 1-related proteins, and so on.

5.1. Possible selection of molecule sets in the identification of allergenic proteins

Several clinical observations and literature reports suggest that the allergenic molecules identified so far are much fewer than those actually contained in the allergenic sources (Dolle et al., 2011; Maeda et al., 2009; Sanz, de, Fernandez, & Gamboa, 2010; Trcka et al., 2012).

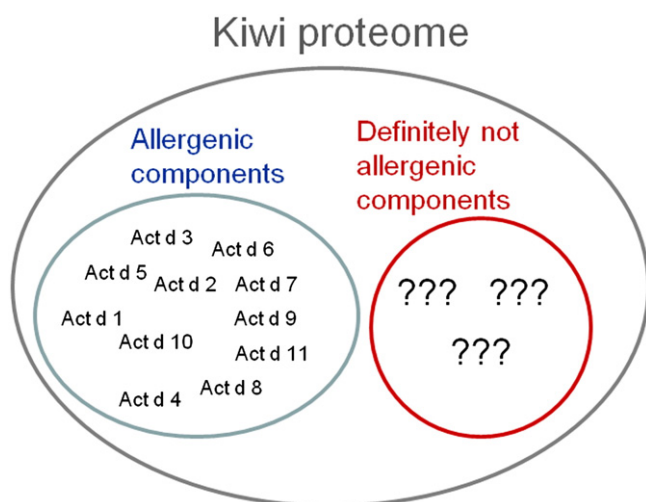


Fig. 2. Schematic representation showing that 11 components of the kiwifruit proteome have been classified as allergens and no one of the proteome components has been characterized as definitely not allergenic.

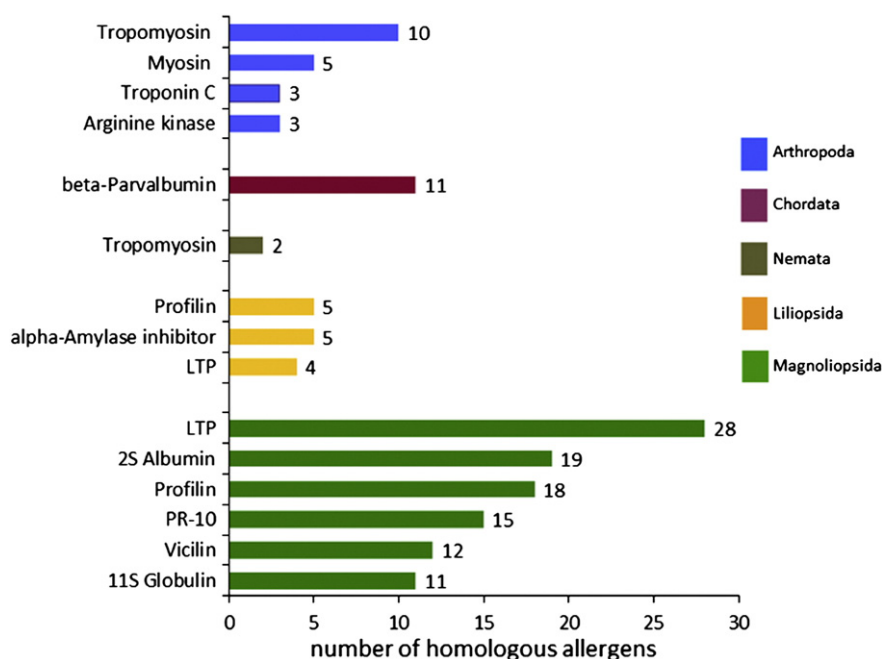


Fig. 3. Allergens of different phyla for which the highest number of homologs was registered by the WHO/IUIS.

A quick glance at the list of allergens currently known raises the doubt that some factors could have affected the process of allergen identification driving researchers towards selected molecules. In fact, apart from some exceptions, a high concentration in the natural source, and/or a high structural stability to proteolysis and high temperature, are two features frequently found in the best characterized food allergens. These two features can be considered as important causes of the sensitization of allergic subjects (Harrer et al., 2010; Mills & Mackie, 2008), but they also make the process of their identification, isolation and characterization easier. For instance, the first allergens identified in the chicken egg white (Gal d 1–4) are also the most abundant proteins in this food (Alessandri et al., 2012; Hoffman, 1983). The major protein components of egg yolk are the vitellogenin-derived proteins, and recently a fragment of the vitellogenin-1 precursor has been identified as an allergen (Gal d 6) (Amo et al., 2010). The first allergen identified in green kiwifruit is actinidin, Act d 1, that is also the most abundant protein component of the soluble fruit fraction (Grozdanovic et al., 2012; Palacin et al., 2008; Pastorello et al., 1996). Later, two additional major protein components of kiwifruit, the thaumatin-like protein (Act d 2) (Gavrovic-Jankulovic et al., 2002, 2008; Palacin et al., 2012) and kiwellin (Act d 5) (Bernardi et al., 2010; Bublin et al., 2011; Tamburrini et al., 2005; Tuppo et al., 2008) were isolated and their allergenicity was investigated. The kiwifruit allergen designated as Act d 3 was reported as a major allergen of kiwifruit in a study describing a significant link between the levels of IgE recognizing Act d 1 and Act d 3 and anaphylaxis in kiwi-sensitized subjects (Palacin et al., 2008). However, Act d 3 is still a poorly characterized allergen. We could speculate that the variable, and low, amount of this allergen in kiwifruit (Ciardiello et al., 2009), has impaired the characterization of Act d 3, as well as its inclusion in the allergy diagnostic systems. Pru p 3 is an abundant component of peach peel and displays a high thermal and protease resistance. It is also well known as an important peach allergen that has been associated to severe allergic reactions to fruit ingestion and has received an in-depth characterization, including structural and immunological comparison with homologs from different allergenic sources (Asero et al., 2006; Bernardi et al., 2011; Brenna, Pastorello, Farioli, Pravettoni, & Pompei, 2004; Cavatorta et al., 2009; Ciardiello et al., 2010; Leonart, Cistero, Carreira, Batista, & Moscoso del, 1992; Schulten et al., 2011; Tordesillas et al., 2009).

During the last few decades, the increasing use of molecular biology has allowed the characterization of many allergens, also those present in low amounts in the natural source. The exploitation of recombinant DNA technology has supported the characterization of many homologs of already known allergens, and after a sharp increase in the identification of new allergenic molecules during the nineties, not so many new allergen groups have been identified in the last decade. Fig. 3 shows the allergens of different phyla for which the highest number of homologs has been registered by the WHO/IUIS. The lipid transfer protein (LTP) and profilin are the allergens showing the highest values. In fact, LTP and profilin from Magnoliopsida and Liliopsida represent 12% and 8% of the total of officially named food allergens.

We cannot exclude the possibility that the preferential use of methodologies based on DNA technology could have generated a molecule selection promoting the study of homologous allergens from different sources (Mari, Ciardiello, Tamburrini, Rasi, & Palazzo, 2010). As an example, the allergy to kiwifruit has sometimes been associated to the allergy to the major birch pollen allergen, Bet v 1 (Gall, Kalveram, Forck, & Sterry, 1994; Voitenko et al., 1997). This observation suggested the presence of a Bet v 1-like molecule in this fruit. The Bet v 1-homolog was searched using two different approaches, one based on DNA technologies and the other based on proteome analysis. The first strategy led to the identification of a cDNA coding for Act d 8, a Bet v 1 homolog showing a high identity with the birch pollen allergen. This cDNA was used to obtain the translational product that was characterized (Oberhuber et al., 2008), although the natural molecule has never been isolated from the fruit proteome. Conversely, the analysis of the proteome, carried out using the classic biochemical methodologies, led to the detection in kiwifruit of significant amounts of Act d 11, which is immunologically correlated to Bet v 1, but belongs to a different protein family, MLP/RRP (D'Avino et al., 2011). The identification of Act d 8 and Act d 11 is a clear example of the generation of selected sets of molecules the detection of which depends on the strategies and methodologies that have been chosen to perform the study.

5.2. Methodological limitations in the detection and identification of allergenic molecules

During the last few years significant efforts have been made to improve some technologies exploited for proteome/allergome analysis.

These range from the development of new molecular biology-based technologies, such as the phage display systems (Lopez & Pardo, 2011; Manavski et al., 2012), to the improvement of traditional technologies for protein analysis, such as the mass spectrometry equipment and the analysis of the data obtained (Li, Zhang, Zaia, & Linhardt, 2012; Zaubert & Schulze, 2012). For instance, the sensitivity of mass spectrometers has been brought to such a high level that they can detect vanishingly small amounts of molecules. A well-known side effect of this improvement is that even tiny amounts of contaminants can impair a perfectly performed experiment. All the proteomics laboratories are aware of this and the adoption of “good laboratory practice” is a rule strictly applied to avoid contaminations. For instance, a frequent and well known contaminant is keratin, that is a protein that is normally present in the skin cells and spreads everywhere. Without appropriate precautions, keratin present on a researcher's hands, or in his/her exhaled breath (Hoffmann, Tabaksblat, Engchild, & Dahl, 2008), could be detected as a contaminant of the samples analyzed by modern mass spectrometers and in some cases it could result as being more abundant than the sample molecules. This underlines the very high sensitivity of these instruments, that can be exploited in many fields and for several aims. In fact, a significant part of the current knowledge about the food components relies on the availability of this technology.

As already reviewed elsewhere, a significant number of papers highlight the use of proteomics and peptidomics as a support for allergen identification and monitoring (Mamone, Picariello, Addeo, & Ferranti, 2011; Monaci & Visconti, 2009; Picariello, Mamone, Addeo, & Ferranti, 2011). The identification and characterization of two putative new allergens, MRJP8 and MRJP9, found in royal jelly, is an example of the most recent contribution of mass spectrometry-based proteomic analyses to this field (Blank, Bantleon, McIntyre, Ollert, & Spillner, 2012). A combination of specific protocols and technologies, including mass spectrometry, allowed the identification of twenty-seven potential wheat allergens, seven of which were newly reported as food allergens (Sotkovsky et al., 2011). Recently, a method based on isotopic dilution tandem mass spectrometry has been developed and successfully used for the simultaneous detection of two major snow crab aeroallergens, tropomyosin and arginine kinase, in their aerosolized forms in processing plants (Abdel Rahman, Gagne, & Helleur, 2012).

Some efforts have also been made to the improvement of technologies to be used to support mass spectrometers in the detection of trace amounts of already known proteome components (Geiser, Dayon, Vaezzadeh, & Hochstrasser, 2011; Geiser, Vaezzadeh, Deshusses, & Hochstrasser, 2011), including allergens contained in pollen (Shahali et al., 2012) and in food products (Picariello et al., 2012). Very recently a strategy has been reported, including the use of off-gel electrophoresis or strong cation exchange chromatography combined with LC-SRM (Liquid Chromatography-Selected Reaction Monitoring) mass spectrometry, that has allowed the detection of amounts even lower than 1 femtomole of isotope labeled peptides in a complex mixture of proteins used as a model (Schafer et al., 2012). However, a prerequisite of this strategy, as well as of other strategies recently described for the identification of low abundant proteome components, is the pre-selection of already known targets. This means that these strategies can be very helpful to detect traces of what is already known, such as known allergens in raw or industry-derived foods (Carrera, Canas, & Gallardo, 2012; Lutter, Parisod, & Weymuth, 2011; Mamone et al., 2011; Monaci, Losito, Palmisano, & Visconti, 2011; Pedreschi, Norgaard, & Maquet, 2012; Rebe, Liu, Norde, & Bremer, 2010). Conversely, the above strategies appear not to be so useful for the identification of new low-abundant allergens, and therefore for the elucidation of the allergen profile of an allergenic source.

An additional limitation affecting the methodologies based on mass spectrometry and used for the identification of protein molecules is the availability of the corresponding amino acid sequences in the databases. In fact, a common approach, known as “bottom-up proteomics”, is often

used for protein identification (Monaci & Visconti, 2009). It is based on the generation and analysis of a “peptide mass fingerprinting” deriving from the digestion of the molecule with a specific protease and the determination of the masses of the separated fragments by the appropriate mass spectrometer. The mass pattern is unique for each protein and can be exploited to search in specific protein-sequence databases using appropriate software. The peptide profile of the unknown protein is compared to the theoretical pattern of each protein present in the database and the results are statistically analyzed to find the best match. The success of this procedure depends on the protein sequences contained in the database. In fact, at least one homologous sequence of the unknown protein has to be already present in the database, otherwise it will not be found/identified. This is a limitation which is not strictly intrinsic in mass spectrometry technology, but nevertheless it can nullify the final result. Although the widespread use of DNA technology has prompted a fast increase in protein sequences contained in the databases, nevertheless too many sequences are still lacking and this impairs the detection/identification of proteome components by mass spectrometry.

When the protein sequences contained in the database do not allow an unequivocal identification of a protein, its peptides can be analyzed by tandem mass spectrometry for *de novo* sequencing (Chong & Leong, 2012; Pevtsov, Fedulova, Mirzaei, Buck, & Zhang, 2006). This methodology is used to predict full or partial sequences. However, the reliability of the prediction of peptide sequences is strongly dependent on the quality of the data obtained from MS/MS spectra. Therefore, although it is sometimes applied, *de novo* sequencing can still be considered an emerging technology needing further improvement, including the implementation of the computational methods used for the analysis of data generated by mass spectrometry (Chong & Leong, 2012). Among the limitations of sequencing by mass spectrometry is the inability to discriminate between amino acids with identical masses, such as leucine and isoleucine, lysine and glutamine. Significant efforts aimed at overcoming such limitations by the derivatization and fragmentation of amino acid residues are in progress (Cook, Collin, & Jackson, 2009). Conversely, the use of automated direct sequencing based on Edman degradation chemistry, provides unequivocal sequence data. In fact, it allows the identification of amino acids and their order in the protein molecule starting from the N-terminal residue (Niall, 1973). Although *de novo* sequencing has provided some contributions to the detection and monitoring of allergenic molecules (Carrera et al., 2010; Abdel Rahman et al., 2010) almost all of the identifications of new allergenic proteins achieved so far have been supported by Edman degradation chemistry. For instance, in green kiwifruit eleven allergens have been described; Edman degradation chemistry applied to the natural molecules was involved in the identification of nine of them, namely Act d 1 (Carne and Moore, 1978), Act d 2 (Gavrovic-Jankulovic et al., 2002), Act d 3 (Palacin et al., 2008), Act d 4 (Popovic et al., 2010), Act d 5 (Tamburrini et al., 2005), Act d 6 (Camardella et al., 2000), Act d 7 (Ciardiello et al., 2008), Act d 10 (Bernardi et al., 2011) and Act d 11 (D'Avino et al., 2011); the other two allergens, Act d 8 (Oberhuber et al., 2008) and Act d 9 (Bublin et al., 2010), were detected in the natural source by DNA technology.

Nevertheless, mass spectrometry offers the advantage of higher sensitivity since it can generate sequence data for peptides in low femtomole quantities, whereas the Edman degradation methodology needs at least a few picomoles to provide reliable data. However, it is worth noting that during the last few decades significant efforts to improve mass spectrometry performance have been made, whereas the Edman degradation technology has been disregarded. In the future, a desirable increase in the sensitivity of the Edman degradation technology could provide a useful contribution to this field. Even better, since both mass spectrometry and Edman degradation have advantages and disadvantages, the best strategy for the identification of a protein could be based on a dynamic combination of both these methodologies.

6. Desirable improvements

The isolation and identification of a new protein/allergen molecule need a concerted application of several technologies and often the mass spectrometry analysis represents only the final step. Therefore, it would seem to be important to improve the technologies supporting the sample preparation and data analysis, such as those exploited in the classic procedure used to identify a new allergen, which includes (i) the extraction of the protein molecules from the allergenic source, (ii) their separation by electrophoretic or chromatographic methods, (iii) the detection of the IgE-binding components and then (iv) the identification of the IgE-binding molecules. If a protein has been missed or damaged during the sample preparation at one of the above steps, then that protein will not be identified, even if a very sensitive technique is used at the final step of the procedure.

It appears that the process of identification of new allergens may be influenced by several factors, that sometimes represent methodological/technological pitfalls. As a final result, some allergens have been identified and others have been missed, producing fragmentary knowledge in this field. In fact, for no allergenic source has the complete allergen profile been described so far.

A systematic study of specific proteomes could be useful to provide the current knowledge with additional information. This would imply the biochemical and immunological analysis of a huge number of individual molecules, many of them only available in very low amounts. The development of new strategies, that may involve new or improved technologies, could be necessary to shift the investigation from the already quite well characterized set of abundant molecules to those contained in much lower amounts in the allergenic source.

7. Sensitivity of biochemical and immunological methodologies

Literature reports describe some examples of the higher sensitivity of immunological methods that can reveal the presence of allergenic molecules missed by some biochemical methods. For instance, the kiwi-fruit allergen Act d 10 was not detected in the protein extract when it was subjected to chromatographic separation by HPLC, but its presence in the extract was observed by means of an immunological test based on the IgE-binding inhibition of the same molecule spotted on the ISAC microarray (Bernardi et al., 2011). The lower sensitivity of some biochemical methods can sometimes be the cause of the incorrect response of some allergological tests (Aalberse, 2011; van der Veen et al., 1996). In fact, if the reagents contain hidden allergenic molecules, the presence of which was not detected when the purity of the preparation was checked by biochemistry-based methods, a positive response of *in vivo* or *in vitro* immunological tests could wrongly be attributed to the main component of the reagent rather than to the contaminant molecule, as recently reported for the peach allergen Peamaclein (Pru p 7) (Tuppo et al., 2012). The identification of this new allergen was actually driven by some discrepant results from immunological tests performed using different preparations of the peach allergen Pru p 3. Some of these reagents contained Pru p 7 as a hidden contaminant of Pru p 3. They produced a positive reaction also on some subjects negative to Pru p 3, but allergic to Pru p 7, although this allergen was present in very low amounts. The false positive results registered on the subjects not allergic to Pru p 3 could have important implications especially if, for instance, molecule-based immunotherapy is planned for them (Zuidmeer-Jonghejan et al., 2012). In fact, those subjects would risk following a desensitizing immunotherapy against the wrong molecule so failing to obtain any clinical effect.

The sensitivity of the immunological methods should be associated with similarly highly sensitive biochemical methods allowing the separation and identification of the target molecules. Ideally, we should be able to obtain all the high- and low-abundant components of a specific proteome, each one purified and available in individual test tubes in

order to analyze them separately by probing with subjects' sera and exploiting the current, or improved, microtechnologies.

In this context, the already available multiplex test systems based on microarray technology are very promising and their improvement could lead to the achievement of this objective (Mari et al., 2010). This technology allows the investigation of the IgE binding of many protein molecules with a single test. For instance, the ISAC system is already routinely used for allergy diagnosis in many allergological centers allowing the analysis of the sensitization up to the current 112 allergens (immobilized on the microarray) with a single run and using only 25 µl of the subject's serum (Bublin et al., 2011; Cabrera-Freitag et al., 2011; Ebo et al., 2010; Fall & Niessner, 2009; Harwanegg & Hiller, 2005; Mari, Alessandri, et al., 2010). In addition, this system has already been used successfully for research purposes (Alessandri et al., 2012; Melioli, Compalati, Bonini, & Canonica, 2012; Scala et al., 2010, 2011) and has contributed to the detection, identification and characterization of new allergenic molecules, either purified or present in complex mixtures, such as raw protein extracts (Bernardi et al., 2011; Pasquariello et al., 2012; Tuppo et al., 2012). The reported outcomes indicate that this technology is a powerful tool for the identification of new allergens. Two critical points could be the following: (i) the availability of all (or as many as possible of) the proteome components of the allergenic source to be spotted on the microarray and probed with the sera of allergic subjects and (ii) the availability of advanced technology allowing the management of a huge number of different samples and the data generated. To deal with these requirements, the improvement of the separation methods and the increase of the sensitivity of the biochemical methods used for the detection of the proteome components, combined with suitable bioinformatic support, would be very helpful (Mari et al., 2009).

8. An example of allergen profile investigation using the microarray technology

The multiplex biochip-based immunoassay on the ISAC system has been exploited to investigate the allergen profile of different apple cultivars (Pasquariello et al., 2012). The protein extracts from the fruit were used to perform inhibition tests by preincubating the sera of allergic subjects with the protein extract. IgE antibodies of the sera bound to the allergenic proteins contained in the extracts, thus becoming unavailable for the interaction with the microarrayed molecules for which the reactivity with the sera IgE had already been established. The IgE binding proteins present in the individual apple extracts were detected with a single run and using a very low amount of serum and of protein sample (Pasquariello et al., 2012). In line with some literature reports (Bolhaar et al., 2005; Carnes, Ferrer, & Fernandez-Caldas, 2006; Matthes & Schmitz-Eiberger, 2009; Sancho et al., 2008; Vlieg-Boerstra et al., 2011; Zuidmeer et al., 2006) this study has shown that the different apple cultivars have individual allergen profiles. However, in addition to the available literature, the use of this microtechnology has revealed the presence in the apple samples of not yet reported potential allergens. Additionally, the detection of IgE reactivity on homologs from other sources immobilized on the microarray has also provided information on the identity of still unknown apple allergens, which anyhow deserve further biochemical and immunochemical characterization to be fully recognized as allergens (Mari, 2008).

9. Conclusions

At present only fragmentary information on allergenic molecules contained in allergenic sources is available. The elucidation of comprehensive allergen profiles would represent crucial information that could be exploited to obtain a more reliable diagnosis, that is the reference point to formulate a safe diet for each allergic subject and, possibly, to apply the correct immunotherapy. To reach this goal, the adoption of new strategies allowing the analysis of low abundant proteome

components is necessary. The use of strategies based on a dynamic combination of different technologies, especially the emerging microtechnologies based on the use of microarrays, could be very helpful to meet this challenge, that is the biochemical and immunological analysis of the protein components present in much lower amounts in the proteome of the allergenic source.

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