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Review

Bioanalytical characterization of proteins

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Abstract

Allergens from the view of a protein chemist are quite normal proteins, not to distinguish from non allergenic proteins. The first task is therefore to recognize and identify the proteins responsible for the allergenic reaction. This is usually only possible if the allergenic structure is conserved during the purification procedures. For a detailed analysis of the allergenic protein modern protein chemical methods for characterization, identification, determination of posttranslational modifications and epitope characterization have to be applied. Such techniques are briefly described in this article. © 2001 Elsevier Science B.V. All rights reserved.

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Contents

1. Introduction

Foods contain an enormous variety of proteins but only a small fraction of all these polypeptides are allergens. Most food allergenic proteins have molecular masses ranging between 10 and 70 kDa [1] even

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though several low molecular mass proteins, below 10 kDa, e.g. in hydrolysed milk formulae and peanut extract have been separated [2,3]. Many of the allergens exist as isoallergens i.e. proteins with at least 67% identical amino acid sequences [4] and with differences in degree of glycosylation or protein amidation. Food allergens are often glycoproteins with an acidic isoelectric point [5]. These properties are however also shared by many nonallergenic proteins. Nevertheless, glycosylation of a protein may be of significance for its antigenic properties and contribute to immunogenicity [6–9]. Many allergens share characteristics displayed by viral and bacterial proteins to which the human body has developed non-adaptive defence mechanisms [6]. Furthermore, they are to a large extent soluble in aqueous solvents [5]. Many of the plant-food allergens are often storage proteins [1,10]. Food allergens are, with the exception of most fruit and vegetable allergens, to a high degree stable molecules resistant to processing, cooking and enzymatic digestion [1,10–19]. For instance Ara h 1, a major peanut allergen, is resistant to digestion by artificial gastric juice [12]. This stability may allow allergenic proteins to persist in various environments for comparatively longer times, e.g. in the acid pH of the stomach. However, due to this stability it might be difficult to enzymatically cleave such proteins for characterization purposes. For those proteins where the tertiary structure are modified by storage, heating, texturing and enzymatic degradation it is possible that new epitopes might be exposed [20,21]. It has been shown that heat generated during the storage of soybeans due to microbial and mold contamination derive new allergenic determinants [22]. Hidden epitopes could even be uncovered by the digestion process in the gut. Moreover, various fruit pesticides might change the structure of the allergen and thereby also its allergenicity. Chlorprophame have been shown to alter the structure of the apple allergen Mal d 1 [23]. Clearly, the biochemical properties of allergenic proteins are of importance for their processing at mucosal surfaces and how the immune system responds to them.

Common allergenic foods tend to be commonly consumed foods. Cows' milk, fish, egg, shellfish, nuts and various fruits and vegetables are among the most important sources for food allergens [17,24–26]. Many food allergens are also highly abundant proteins in foods. However, it is important to keep in mind that a minor ingredient in a prepared dish may be the offending allergen. This was the case as described recently in a case report where the spice dill caused an allergic reaction in a man who had been eating salmon garnished with dill [27]. Furthermore, there are proteins which are major allergens in one species but show no allergenic reactivity in another. The muscle protein tropomysin for example, is the major allergen in shrimps whereas in beef, pork and chicken the same protein is considered to be non-allergenic [5]. This distinction could be due to structural differences between the species. Agronomic conditions as well as the stage of development of plants or animals influence the type, amount and nature of the individual proteins in foods which in turn could have an effect on the allergenicity. For instance, it has been shown that the allergenic potency increased during ripening of apple fruits [28]. Not only ingestion of a food allergen elicit allergic symptoms. Also inhalation of aerosols e.g. generated during the cooking of fish or visiting an open-air fish marked can cause an allergic reaction [25,29]. Egg yolk and egg white proteins have also been reported to act as aeroallergens [30]. However, aeroallergens derivied from foods are not regarded as food allergens but as occupational allergens.

Allergic reactions are complex, multifactorial processes. Knowledge about the biochemical and biological properties of allergenic proteins are of significance for the understanding of allergic diseases as well as the underlaying causes of their progressive increase. Protein profile characterization or proteome analyses are of special importance since they reveal valuable information in this field. Certain features might predispose proteins to become allergenic. There is however no generally accepted, established, definitive procedure to define or predict a protein's allergenicity. One approach would be structure comparison of a certain protein antigen with known allergens and their allergenic epitopes to look for conserved structures or motifs and amino acid sequence resemblances for the purpose of detecting common epitopes shared by the allergens. In computer modeling studies potential antigenic sites could be predicted on the basis of hydrophilicity and surface probability in combination with secondary-

structure and backbone-flexibility [31,32]. Recently, a new structure alignment algorithm has been developed where the biologically alignment aspect is considered [33].

2. Structural analysis of isolated allergens

Studies to identify molecular features responsible for the allergenic nature of proteins are important. It is also of importance that allergen extracts used for allergy diagnosis are well characterized and well standardized. Western blotting is often the method of choice to analyze the allergenic potential of protein antigens present in complex mixtures using IgE antibodies in sera from allergic patients. Blotting provides important information on the occurrence, distribution and intensity of IgE antibody binding to different allergens as well as a good general view of the strongly varying individual IgE-binding patterns in sera from allergic patients. The immunoblotting technique is fully covered in the review by Becker and Reese in this issue. Various in vitro and in vivo techniques can be used to determine the biological activity of potent allergens as reviewed by Poulsen in this issue.

When a protein is recognized to be allergenic, one major task is to elucidate its molecular structure. Commonly these proteins are isolated by chromatographic or electrophoretic techniques as described in the review by Pastorello and Trambaioli in this issue. But even after such separation the protein of interest is still often present in a mixture and only visible as a stained band or spot in a polyacrylamide gel. In recent years, mainly due to the progress in proteomics, the analysis of such proteins became rather routine. Amino acid compositional and sequence analyses, chemical and enzymatic cleavage as well as mass spectrometric determinations (to be described in more detail below) are commonly used methods for identification and characterization of both allergenic and non-allergenic proteins. Protein identification techniques are fundamental in the field of proteomics. In the literature there are several reviews covering this subject from different aspects [34,35]. Furthermore, recombinant DNA techniques and gene-cloning have been used for molecular characterization of allergens as reviewed by Lorenz, Scheurer, Haustein, and Vieths in this issue.

2.1. Molecular mass determination

For the determination of the molecular mass of the intact protein two mass spectrometric techniques developed in the last decade can be used. Matrix assisted laser desorption/ionization (MALDI) mass spectrometry (MS) for surface immobilized samples or electrospray (ESI)-MS for liquid samples (for a recent review see Ref. [36]). In the former technique short intense pulses of laser light are used to induce formation of intact gaseous ions and in the latter ions are directly formed from small charged liquid droplets. Apple and peach allergens are examples of plant-food proteins which have been characterized by MALDI analysis [37]. Electrospray mass ionization has among other things been used to analyze *N*-glycans isolated from Ara h 1, a major peanut allergen [38]. The molecular masses of: the IgE-binding protein patatin in potato, the major allergen in cherry (Pru a 2), a major IgE-reactive isoform of carp parvalbumin and egg white allergens have been determined by MALDI-time of flight (TOF) [39–42]. It should be noted that the accuracy of these mass spectrometric methods is not good enough to identify a protein by its molecular mass alone. Furthermore, the concentration necessary for a mass determination (pmol/μl) cannot easily be reached. Recent developments try to determine the protein mass from electroblotted proteins [43] which may in the future lead to a rapid diagnostic tool of complex protein patterns [44]. The molecular mass information becomes especially valuable when the proteins identity is already elucidated by other means, i.e. partial sequencing and immuno assays. The molecular mass predicted from the database information helps to clarify if a posttranslational modification is present and thus to decide if more detailed work is required.

2.2. Amino acid analysis and N-terminal amino acid sequenceing

Amino acid analysis is used for quantification and determination of the composition of proteins, peptides or free amino acids. It is a two step process

involving complete hydrolysis of proteins and peptides, followed by quantification of the released amino acids. The cleavage of the peptide bonds is done either enzymatically or chemically. Derivatization of the amino acids are required to improve their chromatographic separation and/or detection. Two types of derivatization procedures exist. In the post-column derivatization technique free amino acids are separated by ion-exchange chromatography. The derivatizing reagent is added to the eluent and the mixture passes through a reaction coil and thereafter through the detector. In the pre-column derivatization technique a number of derivatizing reagents are used to modify the amino acids' chromatographic properties prior to separation by reversed-phase HPLC. Amino acid analysis is nowadays performed in the low picomole range which makes accurate analysis a challenge because of the presence of unwanted contaminants in chromatographic buffers, on glass surfaces and in hydrolysis acids.

Edman degradation is the classical sequencing method of proteins. Usually it is performed after cleavage of the polypeptide chain into small fragments by either enzymatic digestion using different endoproteases or by chemical splitting with for example cyanogen bromide. Subsequently the resulting peptides are separated by reversed-phase high-performance liquid chromatography (RP-HPLC) followed by sequencing. The order of the amino acids are determined by stepwise removal and identification of successive amino acids from the polypeptide amino terminal. The identity of a protein can also be explored by direct N-terminal amino acid sequence analysis after blotting of the protein to a chemically inert PVDF-membrane [45]. If the protein has a free alpha amino group a sequence of 15 to 40 amino acid residues in length can be expected. This is usually sufficient to identify the protein in a protein data base, or if the protein is unknown, to get an idea about the nature of the protein via homology studies. Numerous allergens, as for example from celery, potato, carrot, avocado, cherry, apple, peach and kiwi have been characterized by N-terminal sequencing [37,39,40,46–50]. However, only a small portion of the amino acid sequence of the protein may be covered and in many cases the protein can be N-terminally blocked, and therefore not amenable to Edman sequencing. About 50% of the naturally occurring proteins are N-terminally modified. Acetyl, formyl or pyroglutamic acid are common blocking groups. Sometimes blockage is introduced during sample purification and preparation because of increased pH during a purification step or due to impure chemicals and detergents which react with the N-terminal amino acid. With the aid of chemical or enzymatic digestion followed by chromatographic or electrophoretic separation of the blocked proteins internal peptide fragments can be sequenced. For example, the blocked amino terminal end of peanut allergen Ara h 1 was treated with trypsin in order to obtain protein sequencing data [51]. How to remove blocking groups with special techniques is described in [52]. Nevertheless, it may be difficult to differentiate between isoforms of a protein or to recognize posttranslational modifications essential for the protein function.

2.3. Molecular mass fingerprinting

In general the most efficient way to identify and characterize a protein is to enzymatically cleave the protein and analyze the generated peptide mixture in detail. The advantage is a much higher sequence coverage, that means information from all parts of the protein rather than from the N-terminal end only. To identify such proteins usually mass spectrometric techniques like MALDI-MS and ESI-MS are applied together with data base searches. The peptides resulting from chemical or enzymatic digests can be detected in MALDI analysis using different matrices of matrix mixtures. Each enzymatically cleaved protein will be compared with a computer generated mass pattern derived from a theoretical enzymatic cleavage of any protein in the protein data base. If the sequence of the protein investigated is stored in the protein data base almost always the protein can be identified rather unambiguously. However, if the protein is derived from a protein family (i.e. similar isoforms exists), if only a few peptide fragments can be observed (e.g. with membrane proteins) or if the proteins are heavily modified, sometimes the identification is not unequivocally. Thus, this mass fingerprinting approach will only lead to a successful protein identification if the protein sequence is stored in the data base.

2.4. Internal sequencing

When the protein is unknown or if the database search gives unclear results further time consuming efforts have to be made to characterize the protein in detail. In general, methods capable of generating sequence information have to be used. Here again, since a few years mass spectrometric techniques are quite common. With the two mass spectrometric techniques, MALDI–MS and ESI-MS, structural information can be obtained by different fragmentation mechanisms. In postsource decay (PSD)-MALDI–MS peptide fragments are generated spontaneously during the flight time within the flight tube of the MALDI-TOF instrument [53]. The PSD-fragments carrying the sequence information exhibit the same velocity but the energies differ from the parent ion. Therefore, they can be separated by an energy filter, the so called reflector, and focused on a separate detector. ESI-MS instruments are usually equipped with several tandem quadrupole mass analyzers. In a typical triple quadrupole instrument a certain ion can be selected in the first mass spectrometer and thereby isolated and guided to a second mass spectrometer, where the selected ions are forced to collide with gas atoms (collision cell). Due to the collisions peptide molecules are fragmented mainly at the peptide bond sites and the masses of the generated peptide fragment ions can be determined in the third quadrupole. The most modern type of instruments are Q-TOF or hybride type MS instruments [54]. An electrospray ion source is coupled with a reflector time of flight mass analyzer. These instruments have a high mass accuracy combined with a high sensitivity. It is also possible to analyze chemically modified peptides both with PSD-MALDI–MS and ESI-MS.

Manual interpretation of the complex fragment spectra is complicated and very time consuming. Therefore, computer programs were developed which could accomplish ‘‘on line’’ data evaluation and — similarly to peptide mass fingerprinting — compare experimental MSMS pattern with theoretical — in silico — generated MSMS spectra of data base derived peptides [55]. These results are usually very discriminating and highly significant in identifying a protein present in a data base. Here, not only proteins with a fully determined amino acid sequence stored in the data base can be identified, but also partial protein sequences stored in EST (expressed sequence tags) data bases can be searched. However, if one is dealing with an unknown protein whose sequence is not stored in a data base more laborious methods have to be applied. In these cases still amino acid sequence analysis by Edman degradation is the way of choice. It gives unambiguous results down to the femtomol level. Mass spectrometric de novo sequencing is still difficult and error prone, but new software and better instruments are rapidly improving the situation. A flow scheme for the chemical characterization of proteins is given in Fig. 1.

2.5. Posttranslational modification

Various posttranslational modifications can occur like deamidation, oxidation, glycosylation, phosphorylation, sulfation, methylation and blocked N- and C-terminal ends [56,57]. If an indication exists that a posttranslational modification has functional consequences then the whole analytical arsenal has to be applied to clarify the position, nature and degree of modification. Also here mass spectrometry, together with sequencing, amino acid analysis, modification reactions etc., usually give sufficient tools to manage this task. However, the amount of protein needed is significantly higher (pmol to nmol) and is often the limiting factor.

Phosphorylation of proteins is a common posttranslational modification. Such proteins can be characterized by mass spectrometry since each phosphate group increases the mass with 80 Da. In-gel digested phosphorylated proteins are analyzed by MALDI–MS followed by alkaline phosphatase treatment of the peptides. Subsequently, the phosphopeptides are identified by differential peptide mapping, i.e. before and after treatment with alkaline phosphatase. Phosphorylation sites in proteins can also be determined by parent ion scanning. Here, loss of specific diagnostic fragment ions from the peptide is recorded. Another commonly employed technique to detect different forms of phosphorylation is immunoblotting where antibodies against specific types of phosphorylation are used [58].

A special problem is the analysis of complex and heterogeneous glycostructures, where not too many successful reports have been published. Some of the

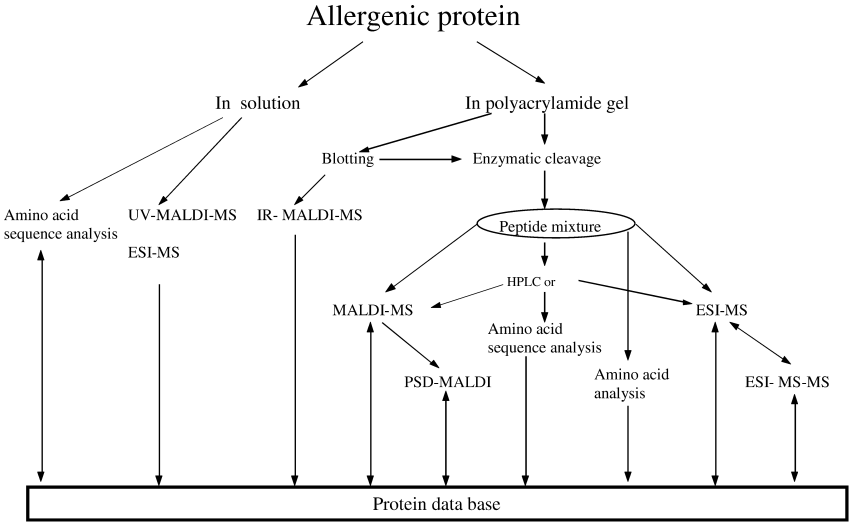

Fig. 1. Strategy for chemical characterization of proteins.

mass spectrometric approaches used to analyze glycoproteins are summarized in [59]. Carbohydrate-containing peptides can be sequenced by Edman degradation, despite their strong hydrophilicity, if they are covalently attached to a modified PVDF-membrane which contains arylamine groups allowing the coupling via the side chains and the C-terminal carboxy group and where liquid trifluoroacetic acid is used as a transfer solvent [60]. To determine the carbohydrate content of a glycoprotein glycosidases, enzymes which chemically cleave the carbohydrates, can be used followed by measurement of the molecular mass with the aid of MALDI–MS. For example has MALDI-TOF been used to determine the molecular masses of native and deglycosylated ovomucoid, a major allergen in hen's egg-white [61]. Glycoproteins can also be studied with other methods [62]: Periodic acid/Schiff (PAS) staining is a useful technique to detect glycoproteins on gels and blotting membranes. This method has for instance been used to investigate glycosylation of proteins in kiwi fruit [46]. The sensitivity of the PAS method can be improved by extending it with an enzymatic detection method using digoxigenin (DIG)/anti DIG alkaline phosphatase (AP) labeling. This procedure requires periodate treatment of the glycoprotein to enable the attachment of digoxigenin. The carbohydrate structure is destroyed by this oxidation procedure. Glycoprotein detection with periodate treatment has been used in the study of various plant food allergens from for example plum, tomato, kiwi, apple and celery as well as for fish allergens [63–67]. Furthermore, lectins can be used to probe for specific structures in the glycoconjugates. Several plant glycoallergens have been investigated by this method [38,64,65,67]. The monosaccharide composition of a glycoprotein can be analyzed by high-pressure anion-exchange chromatography with pulsed amperometric detection as shown in [38]. Here, the primary structures of the *N*-glycans of Ara h 1, a major peanut allergen, were determined.

3. Epitope identification

The identification of discrete sites on antigens that are recognized and bound by particular antibodies or T-cell receptors, so called epitopes, are important for the characterization of allergens. Epitopes may be composed of sequential contiguous residues along the polypeptide chain or nonsequential residues from segments of the chain brought together by folded conformation of the protein. Determination of al-

lergen-specific, IgE-binding epitopes is important for better understanding of the complex allergic reaction. Synthetic peptides have been used to determine such epitopes in several food allergens [68,69]. In this technique (reviewed by Becker and Reese in this issue) short overlapping peptides, corresponding to the allergenic protein sequence, are synthesized on derivatised polyethylene rods arranged in a microtiter plate format [70–72]. The set of synthesized peptides is subsequently scanned for sequential epitopes in an ELISA system using sera from allergic subjects. Overlapping peptides representing the entire length of a protein can also be synthesized on an activated cellulose membrane and subsequently probed with serum IgE from allergic individuals in order to determine IgE-binding regions throughout the putative allergen [73]. Synthetic peptides could be an effective tool in epitope-mapping studies. However, this technique is less efficient for those B cell epitopes which are highly dependent on their conformation. Discontinous B cell epitopes are quite often destroyed by reduction and alkylation. In order to resist the denaturation in the gut to the utmost possible extent many allergenic epitopes are linear [21]. Dominant antigenic regions within bovine BLG (beta lactoglobulin), one of the major allergens in cow's milk, have been studied both with the PEPSCAN technique and by the phage display technique [69]. In the phage display technique libraries of randomized short peptides fused to the coat proteins of filamentous phage are utilized as a source of epitopes for analysis. The results show that the majority of epitopes recognised are present on the surface of the antigen and particularly on loops and turns in the structure. BLG is a member of the lipocalin family. The proteins in this family have a similar three-dimensional structure where the polypeptide chain forms a β-barrel [74]. In return the sequence homology is low, only about 20% [75].

Essential residues for the epitope function and thereby for the IgE-binding can be pointed out by replacing each amino acid residue, one by one, in the IgE-binding epitope [73,76]. Furthermore, it is possible to mutate epitopes to non-IgE-binding peptides by single amino acid substitutions or amino acids deletions. This could be used for development of hypoallergenic forms of allergens which in turn could be useful for immunotherapy. Substitution of single amino acids for alanine in the peanut allergen was shown to prevent or reduce IgE binding [73,77].

4. Cross-reactivity due to sequential, structural or functional homologies

Although antigen–antibody reactions are highly specific, it happens that a particular antibody or T-cell receptor reacts with an unrelated antigen due to a shared epitope. In general, cross-reactivity is quite common between food allergens suggesting homogeneous molecular regions or conserved structures, i.e. due to resemblance in epitopes because of phylogenetical relationships. Cross-reactivity is for example observed among birch pollen, fruits, vegetables and nuts [37,47,48,67,78–84]. This could be due to cross-reactive carbohydrate determinants (CCD) which are widely distributed in the plant kingdom [82]. Panallergens, proteins which are ubiquitously spread in tissues of many species and have high degree of sequence homology, could also be responsible for the observed cross-reactivity. There are also speculations that functional aspects may contribute to the allergenic potential of proteins and that functional homologies might be another reason for the cross-reactivity. There is for instance a surprisingly high degree of food allergies in latex-allergic patients [85–87]. This is probably due to conserved regions of common plant defence proteins shared by these plant-food species and latex from the rubber tree *Hevea brasiliensis*. Latex gloves should therefore not be used when handling foods to avoid contamination with latex proteins [85].

Cross-reactivity among a variety of fish species has been reported [66,88,89]. Parvalbumin, the major cod allergen (Gad c 1), has been shown to be a cross-reactive fish allergen [66]. This allergen has a three-dimensional structure which consists of three domains where two of them are able to bind calcium. It is a very stable allergen in respect to extreme pH, temperatures and exposure to dissociating agents [15,16]. However, removal of the calcium ions leads to a reduction of its IgE-binding capacity [66]. There are at least five IgE binding sites distributed along the polypeptide chain of Gad c 1 [90]. These regions contain repeated determinants. Cross-reactivity of

various fish species could therefore be due to a conserved homology in these domains. Gad c 1 is not a predominant protein in codfish.

5. Peptide libraries

In recent years large libraries of peptides have been constructed which are valuable in search for antigenic determinants or epitopes (see the article by Reese, Ayuso, Leong-Kee, Plante and Lehrer in this issue and Refs. [91–93]). With limited knowledge about the natural antigen or even for unknown antigens it has been possible to identify disease-specific epitopes from phage-displayed random peptide libraries using human sera [94]. The expressed peptides might not necessarily present the sequence of a natural allergen-derived peptide. This phage-display technology has great potential for application in the areas of drug discovery, vaccine developments and diagnostics.

6. Conclusion

Allergenicity appears to be a consequence of a complex series of interactions. Up to now the identification of the full spectrum of allergens is far from complete. There are considerable differences in the patterns of individual reactivities to various allergens. Phylogenetically conserved structures and determinants seem to exist within certain food groups. Once isolated, the protein chemical characterization of an antigenic protein is rather routine and limited mainly by the amount of material available. One of the main goals is to identify the allergenic epitopes. A careful determination of all properties of the molecular structure of an allergenic protein, such as amino acid exchanges, N- or C-terminal truncations and posttranslational modifications (especially glycosylation), is extremely important, since they may be connected with the allergenic phenotype. Thereby would it be possible to explain a protein's allergenicity on the basis of key physicochemical characteristics.

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