

REVIEW

## Overview of the most commonly used methods in allergen characterization

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*Abstract:* The characterization of an allergen is a troublesome and difficult process, as it requires both the precise biochemical characterization of a (glyco)protein molecule and the establishment of its susceptibility to IgE antibodies, as they are the main link to histamine release in some hypersensitivity states (type I allergies). As the characterization of an allergen includes molecular weight determination of the allergenic molecule, its structure determination, physicochemical properties, IgE binding properties of the allergen molecule, and its allergenicity, an overall review of which biochemical and immunochemical methods are used in achieving this goal are presented in this paper. The information on the molecular level on the structures of allergens indicates that allergens are considerably heterogeneous protein structures, and that there is no particular amino acid sequence which is responsible for the allergenicity. Therefore, information gained from detailed structural, functional and immunochemical studies of these intriguing molecules, which nowadays modulate a variety of pathophysiological conditions, would greatly improve our understanding of the underlying disease mechanisms, and the way to handle them.

*Keywords:* allergen, characterization, allergy, immunoglobulin E, recombinant allergen.

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### 1. INTRODUCTION

When a patient becomes allergic to one of the well recognized sources of allergens, an IgE antibody response has been produced to one or more of the proteins that are produced by mites, trees, grass, cats or fungi.

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The term "allergen" came to be used selectively for proteins that cause supersensitivity, *i.e.*, an allergen is an antigen which gives rise to hypersensitivity. A great majority of allergens are proteins the sizes of which range in molecular weight from 10 to 70 kDa. They are all freely soluble in aqueous solutions and have diverse biological functions. Cloning has revealed sequence homology between allergens and diverse proteins, including enzymes, carrier proteins, calyces and pollen recognition proteins. Any allergen can be described either by its source or by the nature of specific proteins. Some of the functions tied to the corresponding allergens are given in Table I.

TABLE I. Potential biochemical properties of some of the previously described major allergens. The appropriate literature references are mentioned in the text

| Allergen  | Function                     |
|---|------------------------------|
| Grass pollen  |                              |
| Group 1   | Endoglucanase (expansin)     |
| Tree pollen Jun a 3                                     | Endo $\beta$ -1,3-glucanases |
| Tree pollen Bet v 2 and allergens homologous to Bet v 2 | Profilin                     |
| Dust mites  |                              |
| Group 2   | Cholesterol binding          |
| Group 1   | Cysteine proteasae           |
| Group 14  | Lipid binding                |
| Mammals and insects                                     |                              |
| Lipocalins  | Lipid binding                |
| Peach fruit   |                              |
| Pru p 3   | Lipid transfer protein       |
| Kiwi fruit  |                              |
| Act c 2   | Antifungal activity          |

There is no doubt that the methods used in allergen characterization are complex, as allergen characterization is a difficult and troublesome task. The aims of the characterization of any allergen should include:

- physicochemical characterization and structure determination of the molecule
- insight into the potential biological function of a given molecule
- immunological characterization and allergenicity proven by *in vitro* and *in vivo* methods (IgE binding from the serum of an allergenic patient, ability to release histamine, ability to stimulate specific T-cell proliferation).

## 2. PHYSICOCHEMICAL CHARACTERIZATION

Probably one of the most popular and the most common techniques in the characterization of an allergen, isolated from natural sources, is Laemmli SDS PAGE followed by Western blotting.<sup>1</sup> The powerful resolution of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) usually makes it the first choice method for the preliminary screening of potential allergens of a certain source. By using an appropriate set of protein markers, this method enables a calibration curve to be constructed and the apparent molecular weight of a protein (*i.e.*, its mobility in different polyacrylamide gels) to be determined.

However, this method is not sufficiently accurate and can result in a large overestimation of a molecular weight ( $M_w$ ) or an underestimation of a  $M_w$ , especially in cases of highly basic proteins, such as Art v 1,<sup>2</sup> or highly acidic proteins,<sup>3</sup> where abnormal SDS binding occurs, leading to "false" results. Sometimes, especially when dealing with a monomeric protein containing a large number of disulfide bridges (such as the major kiwi allergen Act c 2), its molecular weight can also be misjudged and can vary depending on the presence of a reducing agent, due to the more compact fold in its unreduced form and, therefore, higher mobility in a polyacrylamide gel.<sup>4</sup> However, although for such anomalous cases, Tris-tricine systems<sup>5</sup> can be more appropriate, they are not in general use in allergen characterization.

Native PAGEs are not particularly applicable for the purpose of allergen characterization, due to the low resolution of the separation. However, they (both analytical and preparative) can also find application in the characterization of native and recombinant counterparts, such as in the case of Phl p 2.<sup>6</sup>

Capillary electrophoresis is also capable of separating proteins and peptides and includes the possibility of online-coupling to mass spectrometers.

As one-dimensional electrophoretic analysis of an allergen is usually not sufficient to characterize the individual specificity of the IgE response to certain allergens, two-dimensional electrophoreses are the methods of choice. They can both separate and define the physico-chemical characteristics of allergens, as they give isoelectric points and molecular mass of the analyzed samples. By means of 2D PAGE it is possible to characterize different isoforms of a particular allergen the so-called isoallergenic forms, as described for Fes p 4, a basic allergen of *Festuca pratensis* pollen.<sup>7</sup> For example, latex-glove proteins, which are allergens, can be absent from the sap extracts and the sensitization of these allergens could be underestimated. Individual 2-D analysis of the sensitization to latex allergens is useful to define the best allergen mixture required for diagnosis and needed for individual therapy monitoring.<sup>8</sup> With the introduction of immobilized pH gradient, high resolution and reproducible 2D electrophoresis could be obtained. It is possible to distinguish an enormous number of IgE binding proteins by means of 2D PAGE, especially when the immobilized ampholytes are used in the first dimension. This technique was also applied to separate the allergens of *Dactylis glomerata* pollen.<sup>9</sup>

However, the troublesome and time-consuming 2D PAGEs are not suitable for the rapid screening of potential allergens, therefore, the one-dimensional, usually reducing, SDS PAGE conditions are frequently the first choice of analytical biochemists when searching for a new allergen.

Once the protein of interest has been identified, its properties, such as pI value, could be determined by IEF using ampholytes or immobilized variants, as already mentioned. Perhaps, the final point of a preliminary characterization of an allergen would be to accurately determine its  $M_w$ . In such cases, the usually employed size exclusion chromatography, calibrated with a set of calibration proteins, could also be prone to error, especially regarding highly glycosylated proteins, the glyco component of which is usually voluminous.

However, size exclusion chromatography finds its application in comparisons of natural and recombinant counterparts. It is a great task to make a recombinant allergen. Very often, such a protein is produced in the form of inclusion bodies, hence, it is necessary to refold it correctly, as the correct 3D structure of an allergen is necessary for its biological activity (*i.e.*, IgE binding and subsequent histamine release from basophiles). In such cases, a comparison of the SEC profiles of recombinant proteins with those of natural ones would be a useful method to obtain an indication of the correct folding. As SEC mobility is highly dependent on the correct shape of the protein globule, the SEC mobility is not the same for properly folded (globular) and unfolded (random coil) proteins.

### 3. THE PRIMARY STRUCTURE AND DETERMINATION OF POST-TRANSLATIONAL MODIFICATIONS

During the last decade, since the introduction of electrospray ionization (ESI)<sup>10</sup> and MALDI<sup>11</sup> for large biomolecules, these techniques have become the most powerful tools for protein identification and characterization.

The method of choice for an accurate  $M_w$  determination is a mass spectrometry technique suitable for proteins, such as MALDI MS. As this is basically the mass spectrometry of (digested) protein samples, it could also be applicable for the purpose of identification of a known allergen, if the total sequence of the protein is available from a database.

Before the development of mass spectrometry for the analysis of biomolecules, protein sequences were determined by chemical or enzymatic methods, such as Edman degradation<sup>12</sup> or amino acid analysis.<sup>13</sup> The limitation of the Edman degradation is its inability to analyze peptides lacking a free *N*-termini. Furthermore, samples containing more than one peptide having a free *N*-terminus are problematic due to overlapping of the results. Nowadays, *de novo* sequencing<sup>14</sup> presents a new alternative to these methods and is capable of assigning peptide sequences to fragment mass spectra without the need for any database.

Peptide mass fingerprints are the fastest method for identifying proteins recovered from 2-D PAGE. After gel electrophoresis, the protein spots are excised,

washed and digested with a specific protease<sup>15</sup> (Table II). The generated peptides are subjected to mass spectrometry (MALDI-MS). The basic principle of this technique is the comparison of the measured peptide masses with calculated peptide masses from database entries. Every protein results in a unique set of peptide masses after cleavage with a specific protease. In the best case, only a few peptide masses are required for reliable protein identification. The same approach, the generation of peptide fingerprints, is used when assessing the primary amino acid sequence of an unknown protein by Edman degradation. The proteases listed in Table II are the most frequently used for this purpose. The enzymatic digestion usually takes advantage over chemical methods, due to the mild conditions and higher specificity.

TABLE II. Overview of the most frequently used proteases in protein analysis

| Endopeptidase | Type     | Specificity | pH range  | Inhibitors                 |
|---------------|----------|-------------|-----------|----------------------------|
| Chymotrypsin  | Serine   | Y, F, W     | 1.5 – 8.5 | Aprotinin, DFP, PMSF       |
| Trypsin       | Serine   | R, K        | 7.5 – 9.0 | TLCK, DFP, PMSF            |
| Glu C         | Serine   | D, E        | 7.5 – 8.5 | DFP                        |
| Lys C         | Serine   | K           | 7.5 – 8.5 | DEFG, Aprotinin, Leupeptin |
| Arg C         | Cysteine | R           | 7.5 – 8.5 | EDTA, Citrate              |

Concerning the glycocomponent of an allergen, controversy exists among allergologists in regards to how much the glycocomponent contributes to the clinical significance of an allergen.

The covalent addition of sugar moieties to the polypeptide backbone of proteins is one of the most widely occurring co- and post-translational modifications. It is commonly found in a broad range of organisms and in a variety of different forms. Glycoproteins are present inside cells, *e.g.*, the cytoplasm and the inner subcellular compartments, as well as outside cells, *e.g.*, in extracellular matrices.

Glycosylation is a major secondary modification of proteins and adds another dimension to the structural diversity, beyond that determined by genetic variation. Two principal kinds of proteoglycans exist, N-linked and O-linked, which are formed by different enzymatic pathways and comprise different carbohydrate structures. N-glycans have attracted more attention than O-glycans during the past decade, and several pollen allergens have been found to carry N-linked glycans. The functional consequences of glycosylation and the diversity it confers are, however, still poorly understood.

Differences in allergenic glycoproteins include the amino acid residue of the polypeptide backbone to which they are connected, the composition of the monosaccharide, the reducing sugar unit, the position and anomeric form of linkages between the individual sugar residues and the sequence of the monomeric units, including the occurrence of branched 3-D structures. Furthermore, a number of addi-

tional variable modifications, such as phosphorylation, sulfation, methylation and acetylation, may be found on glycan residues. In addition to the complexity of the glycan structure itself, glycoproteins typically exist as populations of diverse glycoforms consisting of a number of different glycan chains attached to individual glycosylation sites in variable molar amounts (microheterogeneity).

N-glycosylation is a complex biosynthetic process which occurs in the secretory pathway. A precursor oligosaccharide is initially attached to an asparagine residue of an acceptor site and the carbohydrate structure is then formed by iterative and consecutive steps of trimming and assembly. N-glycosylation requires the tripeptide Asn-X-Ser/Thr in the polypeptide chain, where Asn is the attachment point and X may be any amino acid except proline or aspartic acid. N-glycans are commonly divided into three classes based on their structure and complexity: high mannose type, hybrid type and complex type, although division into a fourth class, paucimannosidic type, has been suggested.<sup>16</sup>

While most parts of the N-glycosylation process are common to all eukaryotes, plants and invertebrates possess additional activities which generate structures which are not present in mammalian glycans. N-linked carbohydrates containing these structures,  $\beta$  (1,2)-xylose and (1,3)-fucose, are immunogenic and IgE antibody binding to plant and insect N-glycans was first reported by Aalberse *et al.*,<sup>17</sup> more than two decades ago. Sialic acid, which occurs as a terminal structure in mammalian N-glycans, has not been found in plants or invertebrates.

Due to the limited number of glyco structures in the proteins, they often contribute to the IgE binding in different *in vitro* assays, where there is no necessity of bridging two IgE molecules in order to promote the release of histamine from basophiles, a feature designated as allergic reaction type I (IgE-mediated hypersensitivity) with its typical clinical manifestations. However, the ability of a molecule to bridge two IgE molecules could be investigated in a wide range of *in vitro* and *in vivo* assays, such as histamine release or the typical skin prick assay.

Although the need for elucidation of a glyco component emerges, there is still no clear data on this, even from those assays made on neoglycoproteins (non-allergic proteins coupled to a glycan originating from a glycoallergen).<sup>18</sup>

Therefore, a complete analysis of a single glycoprotein would have to provide information regarding microheterogeneity at each glycosylation site and a structural characterization of the bound carbohydrate moieties. Very often the characterization of a single glycoprotein isoform, *e.g.*, separation by 2-DE, is sufficient to provide an overview of the overall complexity of the sample. The relative ionization efficiency of glycans during MALDI-MS has been demonstrated to be very similar.<sup>19</sup> Using MS techniques, it is possible to detect both the glycosylation sites and glycan structures, as well as the individual glycan distribution at each glycosylation site (microheterogeneity). The method is based on gel-separated glycoproteins and is extremely sensitive.

## 4. TERTIARY STRUCTURE AND FUNCTION

One of the very important aims regarding allergens is to reveal the structure and function of these molecules. The introduction of molecular biology to the study of allergen structure and function was initiated just over a decade ago. Since then, the knowledge gained from these studies has made a major contribution to the study of allergic disease. Most of the clinically significant allergens have now been described at the molecular level and their endogenous biochemical activities determined.

The ultimate task in a thorough biochemical characterization of an allergen is to determine its real 3D structure. The availability of recombinant proteins has greatly facilitated the ability of crystallographers to determine the three-dimensional structures of a variety of allergens. The 3D structure of numerous allergens has been determined by X-ray crystallography.

One of the first published 3D structures of an allergen is the 3D structure of Bet v 1, the major allergen of birch pollen. The three-dimensional structure of the major birch pollen allergen, the 17,500 M(r) acidic protein Bet v 1 (from the birch), was determined both in the crystalline state by X-ray diffraction and in solution by nuclear magnetic resonance (NMR) spectroscopy. This was the first experimentally determined structure of a clinically important major inhalant allergen. The structure shows three regions on the molecular surface predicted to harbor cross-reactive epitopes. This provides a structural basis for the allergic symptoms shown by birch pollen allergic patients when they encounter pollens from related trees, such as hazel, alder and hornbeam.<sup>20</sup>

The structure of Fel d 1 (the major allergen of cat dander), revealed by X-ray crystallography, presents a striking similarity to that of uteroglobin, a steroid-inducible cytokine-like molecule with anti-inflammatory and immunomodulatory properties.<sup>21</sup> The structure of Fel d 1 displays the localization of three Fel d 1 IgE binding epitopes on the surface of the protein. On the basis of the data obtained from structural studies, there will be structural foundations for experiments to verify the antigenicity of the proposed epitopes, as well as to design novel hypoallergenic forms of the protein suitable for diagnosis and treatment of allergies.

The other method applied in solving the structures of allergens is NMR. Very recently, the three-dimensional structure of Ole e 6 (a pollen protein from the olive tree) was determined in solution by NMR methods.<sup>22</sup> The global fold of Ole e 6 consists of two nearly antiparallel alpha-helices, spanning residues 3–19 and 23–33, which are connected by a short loop and followed by a long, unstructured C-terminal tail.

When there is not enough protein available to crystallize it or when crystallization is very difficult (such as in the case of native, glycosylated proteins), it is possible to perform some structural studies on a protein in solution, using methods such as small angle X-ray scattering (SAXS)<sup>23</sup> or by combining chemical cross-linking, enzyme digestion and MS to obtain data on the low resolution surface topology of a protein.<sup>24</sup> However, these methods have so far not found wide application in the characterization of allergens.

Although it is reasonable to expect that the allergen molecule itself might influence modulation of the immune system, hitherto, there has been no definite evidence for this. It is well established that Der p 1 is an active cystein protease. *In vitro*, this enzyme cleaves CD25 and CD24 off the surface of lymphocytes and can also disrupt tight junctions in epithelial cell layers.<sup>25,26</sup> One of the panallergens, profilin, is an actin-binding protein and is described from a variety of sources.<sup>27-29</sup> Profilins constitute a ubiquitous family of proteins that control actin polymerization in eukaryotic cells; in particular, profilin participates in the acrosomal reaction of animal sperm cells. Plant profilins might have similar functions in pollens during plant fertilization and, therefore, represent allergenic components in almost all pollens. Another ubiquitous family of proteins, lipid transfer proteins, also represents allergens found in wide range of plant sources, such as pollens and fruits.<sup>30,31</sup>

#### 5. CROSS REACTIVITY OF POLLEN ALLERGENS AND CLASSIFICATION

Among the most important airborne allergens worldwide and in our country<sup>32-35</sup> are proteins originating from pollen grains. The allergens of this origin are well characterized, usually highly cross-reactive among members of the same group and are classified into more than 10 different groups of allergens.

Group 1 allergens show 60 – 70 % sequence identity to expansins,<sup>36</sup> a family of proteins involved in cell wall loosening and extension in plants through their activity of unlocking wall microfibril and matrix polysaccharide structures.<sup>37</sup> In a functional assay, group 1 pollen allergens from *Phleum pratense*, *Lolium perenne* and *Zea mays* have indeed been found to possess expansin-like activity, and a functional role of group 1 allergens in facilitating pollen tube growth through the female flower tissue upon germination has been suggested.<sup>38</sup> The family of expansins has been divided into  $\alpha$ - and  $\beta$ -expansins; the latter including group 1 grass pollen allergens, as well as members expressed in vegetative tissues.

A number of tree pollen and fruit allergens belong to the group of plant proteins known as pathogenesis-related proteins (PRs). These proteins are either constitutively expressed or produced in response to development signals, physical stress and infection. There are currently 14 groups, with most of the denominated allergens belonging to PR-2, -3, -4, -5-, -10 and -14.<sup>39</sup>

Enzymatic activity, particularly carbohydrases (PR-2, -3, -4) and ribonucleases (PR-10), as well as thaumatin-like proteins (PR-5) characterize many of the groups. The PR-5 thaumatin-like proteins demonstrate some homology with the sweet-tasting protein thaumatin from the fruit of the plant *Thaumatococcus danielli*, and at least four allergens have been shown to belong to this group, including the cherry allergen Pru av 2,<sup>40</sup> the apple allergen Mal d 2<sup>41</sup> and the kiwi allergen Act c 2.<sup>4</sup> However, the PR-5 proteins *per se* do not possess the property of sweetness. Their true function in plants is unclear, although they have been shown to possess antifungal properties.



## 6. RECOMBINANT ALLERGENS

Commercial allergen extracts are routinely used for the diagnosis of allergic diseases, both *in vitro* and *in vivo*. The evaluation of the quality of an allergen extract for testing means the demonstration of consistent protein and IgE-reactive patterns, the quantification of the individual allergens, as well as a measurement of total allergen-specific IgE-reactivity of the extract. The production of allergen extracts from natural sources of consistent quality places considerable demands on manufactures.<sup>42</sup>

The outcome of *in vitro* IgE determinations and skin tests largely depends on the quality of the extracts used.<sup>43</sup> Complex allergenic sources, such as mites, moulds and foodstuffs, represent heterogeneous mixtures containing hundreds of proteins, carbohydrates, proteolytic enzymes and lower molecular weight components, including histamine. For example, the quality of extracts from fruits, vegetables and other plant foods can vary due to the inherent presence of proteolytic enzymes of these allergen sources.<sup>44</sup> This instability also leads to consistency problems with different lots of commercial extracts, or to large variations between extracts from different manufactures.

The driving force for moving into recombinant allergen technology was the recognition of the possibility to formulate optimal preparations of diagnostic applications, as well as allergen-specific immunotherapy using recombinant allergens and derivatives thereof.

The introduction of recombinant technology into the field of allergen characterization has considerably improved knowledge on allergenic structures. The first report on a genetically engineered allergen was reported in 1988. Since then an impressive number of allergens from different allergenic sources have been expressed and characterized.

Starting from a cloned cDNA, the encoded gene product can be biotechnologically produced as a highly pure protein of consistent quality in almost unlimited amounts. Therefore, a great number of allergenic molecules have been cloned, characterized and tested clinically during the past decade.<sup>45</sup> The results obtained showed that recombinant allergens can, in some aspects, be superior to commercially available extracts in terms of specificity and sensitivity for diagnostic purposes.<sup>46</sup> Nevertheless, before their application in diagnosis, structural/functional features and immunological properties of recombinant allergens have to be evaluated with their natural counterparts.

Although different expression systems have been employed for allergen expression, most of the existing recombinant allergens have been expressed in *E. coli*. For example, almost all timothy grass pollen allergens, except Phl p 4, have, so far, been expressed in *E. coli*.

In spite of the many advantages, prokaryotic recombinant allergens, on the other hand, have some limitations: their tertiary structure may be incorrect and

post-translational modifications, such as glycosylations (see above), which can be important for correct folding and, if not correct, can lead to a lack of allergenicity. Upon production, recombinant proteins are usually stored in inclusion bodies, so their purification demands buffers with denaturing agents, such as 6 M guanidine hydrochloride or 8 M urea. Correct refolding of allergens with a high content of Cys residues (more than 6) can sometimes be a problem.

Apart from expression in bacteria, yeast systems are also used for allergen expression. Different allergens from grass pollen (Phl p 1, Dac g 1), cat dander (Fel d 1), and mite allergens (Der p 1, Der f 1) have been expressed in *Pichia pastoris*. However, it has been shown that allergens expressed in *P. pastoris* are often overglycosylated compared to their natural counterparts.

Easy transformation and cultivation make plants also suitable for the production of many recombinant proteins. Plants are capable of carrying out not only post-translational protein modifications required for the biological activity of many eukaryotic proteins, such as glycosylation, but also acetylation, phosphorylation, and other modifications. Numerous heterologous (recombinant) proteins have been produced in plant leaves, fruits, roots, tubers and seeds, and targeted to different subcellular compartments, such as cytoplasm, endoplasmic reticulum, or apoplastic space.

However, the extraction and purification of proteins from biochemically complex plant tissues is a laborious and expensive process which is a major obstacle to large-scale protein manufacture in plants.<sup>47</sup>

Foreign genes can be produced in plants by permanent insertion or by transient expression using virus-based vectors. Several allergens have been successfully produced *via* a tobacco mosaic virus (TMV) expression system, *i.e.*, Bet v 1, the major birch pollen allergen,<sup>48</sup> Hev b 1 and Hev b 3, two *Spina bifida* associated latex allergens,<sup>49</sup> as well as the apple thaumatin-like protein Mal d 2.<sup>50</sup>

First studies investigating the IgE antibody reactivity of recombinant allergens have shown that recombinant allergens resemble the IgE epitopes of natural allergens and can be used for allergy diagnosis to determine the IgE reactivity profile of a patient at the molecular level.<sup>51, 52</sup>

Recently published results from a multicenter clinical trial on the development and evaluation of allergen-specific immunotherapy based on genetically engineered allergens suggest that immunotherapy with genetically engineered allergen derivatives has the potential to induce an allergen-specific, mixed Th2/Th1-like immune response. This immune response was characterized by an initial induction of IgE antibodies, followed by strong IgG1, IgG2, and IgG4 responses recognizing new epitopes as well as epitopes defined by the disease-eliciting IgE antibodies.<sup>53</sup> Therefore, in addition to application in component-resolved allergy diagnosis, genetically engineered allergens may also be used for the treatment of most common forms of allergy and even prophylactic vaccination.

## 7. IMMUNOCHEMICAL CHARACTERIZATION OF ALLERGENS AND THEIR MODE OF ACTION

Scientists involved in the research of allergic diseases and the understanding of Type I hypersensitivity have been searching for the answer to one question: what makes an antigen an allergen? In other words, which structural features make some antigens provoke an IgE instead of an IgG immune response? Almost all allergens which have been hitherto identified are proteins or glycoproteins by nature. Moreover, the surface structure (topology) is the most relevant for antibody binding. Therefore, a lot of research has been focused on determining allergenic structures and epitope mapping of IgE-binding epitopes.<sup>54,55</sup>

The term allergen is used to describe three distinct molecular features: to bind IgE antibodies; to sensitize, which means to provoke the immune system to produce high-affinity IgE antibodies; and to elicit allergic reactions *i.e.*, to trigger allergic symptoms in a sensitized person. In terms of these criteria, allergens can be divided into: complete allergens which possess all these features, and incomplete allergens which can elicit allergic symptoms but do not usually sensitize. This division mostly refers to food allergens, since it is believed that proteins which survive the environment of proteolytic enzymes present in the gastrointestinal fluids, will retain sufficient structural integrity to provoke the immune system, having passed the mucosal barrier of the gastrointestinal tract. All inhalant allergens (tree, grass, and weed pollen allergens, house dust mite allergens) are considered as complete allergens, since they encounter the immune system *via* the upper respiratory airways.

Several experiments can be performed to elucidate these features. Qualitative assays, such as dot blot or Western blot, are usually performed to validate whether the protein of interest has the potential to bind IgE from the sera of an allergic patient.

Cross-linking of IgE antibodies on FcεR1 by an allergen is the primary event by which mediators are released from basophils and mast cells. Hence, an allergen must possess at least two IgE binding epitopes in order to bridge two IgE molecules anchored on FcεR1 receptors and thus trigger allergic symptoms.

Another definition which has been introduced is the distinction between major and minor allergens, as a criterion, which addresses the issue of allergenicity. The definition of a major allergen is based on the prevalence of IgE or skin reactivity in persons that are sensitized to the allergen. An allergen is considered as a major if more than 50 % of the tested patients have allergen-specific IgE.

Molecular-level information on the structure of allergen indicates that allergens are considerably heterogeneous protein structures and that there is no particular amino acid sequence which is responsible for the allergenicity.<sup>56</sup>

It is estimated that over 20 % of the world's population suffer from IgE-mediated allergic diseases, such as asthma, rhinoconjunctivitis, eczema, and anaphylaxis. Therefore, the information gained from detailed structural, functional and immunochemical studies of these intriguing molecules that nowadays modulate a

variety of pathophysiological conditions, would greatly improve our understanding of the underlying disease mechanisms, and the way to handle them. All the efforts put into this would certainly improve our ability to handle the disease, both in a diagnostic and a therapeutical sense. The further development of (bio)chemical methods which could find application in any of the above mentioned aspects of allergen characterization would help in reaching the goal – knowing better, controlling better and, maybe, one day... treating better allergies.

## ИЗВОД

## ПРЕГЛЕД НАЈЧЕШЋЕ КОРИШЋЕНИХ МЕТОДА У КАРАКТЕРИЗАЦИЈИ АЛЕРГЕНА

ТАЊА ЋИРКОВИЋ ВЕЛИЧКОВИЋ, МАРИЈА ГАВРОВИЋ-ЈАНКУЛОВИЋ и РАТКО М. ЈАНКОВ

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Окарактерисати алерген је тежак и мукотрпан задатак, јер захтева прецизну биохемијску карактеризацију (глико)протеинског молекула, као и установљивање његове способности да везује IgE, јер је то главна спона ка ослобађању хистамина у неким стањима преосетљивости (алергије типа I). Карактеризација алергена подразумева одређивање молекулске масе, одређивање структуре, физикохемијских својстава, IgE везујућих особина и његову алергеност. Овај рад даје преглед које се биохемијске и имунохемијске методе најчешће користе ради постизања тог циља. Информације које су до сада добијене о структури алергена показују да су ови молекули изузетно хетерогене структуре и да не постоји одређена аминокиселинска секвенција која би могла да предвиди алергеност датог молекула. Међутим, информације добијене из детаљних структурних студија ових молекула ће допринети нашем разумевању патофизиолошких процеса који су у основи алергијских обољења и унапредиће начин на који их третирамо.

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