

Review

Immunological identification and characterization of individual food allergens

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Abstract

Food allergies of type-I-allergy are immunoglobulin E (IgE) mediated and caused by certain proteins or glycoproteins, which are called food allergens. An analytical marker of allergens is the IgE-reactivity to these substances. Normally food allergens are minor components in allergenic source material, which consist of a huge number of chemical different substances. Thus allergen extraction, separation and immunological detection methods are described which identify and characterize individual food allergens by a minimum of manipulation. Favoured separation methods of allergenic extracts are electrophoretic ones allowing the combination of highly resolved protein separations with immunological detection methods subsumed by the term immunoblotting. These techniques are a useful basis to characterize allergens by chemical methods. Once the primary protein structure of a food allergen is established, the way is cleared for the identification of epitopes. Epitopes are immunological detectable parts of a protein or glycoprotein generating the interface between chemical structure and immune-system. The nature of epitopes may differ, for instance, can be conformational, continuous, or built up by glycoconjugates, which determine the stability of food allergens, especially in the case of food processing. Progress in identification and characterization of food allergens will improve diagnostics and therapy of food allergy. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Food intolerances are a group of adverse reactions to foods and are caused by different pathophysiological mechanisms; they are therefore difficult to diagnose since the different forms may show similar symptoms. IgE antibody-mediated food allergies may express the full spectrum in severity of symptoms and clinical reactions. They range from mild forms such as Oral Allergy Syndrome (OAS) with itching and tingling sensation in the mouth to severe life-threatening anaphylactic reactions. In general, improvement of allergy prevention and therapy depends mainly on the reliability of the diagnosis and, thus, on the quality of the allergens used. To improve the quality of allergen extracts, it is important to identify allergenic molecules, to determine their primary, secondary and tertiary structure and investigate the interaction between allergen and IgE antibodies on the molecular level.

Food allergy is like other allergies a dysfunction of the immune system and is primarily IgE antibody mediated and thus, classified as an allergy of the immediate type or Type-I-allergy [1]. IgE is one of the five major immunoglobulin isotypes (IgG, IgM, IgA, IgD, IgE) representing with 17–450 ng/ml the lowest concentration in comparison to IgG antibodies with 8–16 mg/ml in blood which has the highest concentration [2]. One problem of measuring small quantities of IgE in the presence of large amounts of structurally very similar antibodies such as IgG antibodies is solved by using highly specific secondary antibodies that react exclusively with IgE. Another problem is the competition of specific IgE and IgG for the same binding sites on the allergen, which can be circumvented by using an excess of allergen in the test system. Up to now IgE-binding tests of the RAST (radio allergen sorbent test) prototype are used to quantify patients' allergen-specific IgE antibodies and identify the offending allergens in the allergenic source material. In the past, progress was seen in the development of

biologically pure allergenic source material, whereas the content and nature of allergenic components were of less importance. Up to now, allergists associated allergens with the allergenic source, rather than with single allergenic molecule. This gives rise to some confusion.

The growing demand for standardized and reliable allergenic source material moved the identification and characterization of allergenic components into the centre of interest. Even though some food allergens and their structures have been determined, much is left to do and the characterization of food allergens has not been completed yet.

The clinical significance of allergenic components depends strongly on the quality of clinical evaluation of the patients whose IgE is used. Since children are an important group suffering from food allergy they are in focus of research which finds its limitation by the small quantities of available serum. This problem may be softened by the alleviated use of micro methods (G. Reese in this issue). Once an allergen has been identified, allergen-specific monoclonal antibodies can be produced which in turn may serve as surrogates for human IgE. Furthermore, monoclonal antibodies are very useful for the establishment of secondary standards [3]. A secondary standard is a substance (e.g. a certain allergen in an extract), whose mass is determined indirectly by a quantifying assay such as an ELISA using monoclonal antibodies in contrast to a primary standard (e.g. a pure recombinant allergen or natural one) whose mass is simply determined by an analytical balance. Since antibodies do not recognize the whole molecule but only determinants (epitopes) of the allergen or antigen, the specificity of an antibody depends on the uniqueness of the epitope; if an epitope is present on different allergens, even epitope-specific monoclonal antibodies would bind to different proteins.

Epitopes are the interfaces between the chemical structure and the immune system [4]. Therefore, the identification and characterization of epitopes is the

next step after characterizing the structure of an allergenic component.

However, the first challenge is the separation and purification of allergenic components from the source material since allergens are primarily defined by their ability to induce allergen-specific IgE antibodies and not by their biochemical properties. Nevertheless, the antigenicity of substances depends on some physicochemical and immunological features. Proteins or glycoproteins should have a molecular mass greater than 10 kDa, polysaccharides greater than 50 kDa and the substances should be “not self” for the immune-system in question. Additionally, allergens must be able to induce an IgE response and induce a mast cell or basophilic cell degranulation via bridging of cytotoxic IgE. Thus, it is very important to keep in mind that IgE reactivity is only an operational marker to define allergens, since only the reproduction of an allergic reaction by a purified allergen would prove its allergenic potential. By this standard, only few antigens have been identified as allergens.

To separate allergens from other non-allergenic molecules, two physicochemical features of allergens — molecular mass or isoelectric point (pI) — can be utilized. Furthermore, allergen separation should be linked to immunological detection systems that allow reliable detection of IgE-reactive components. These requirements are met by electrophoretic separation techniques and immunoblotting. The identification and characterization of individual food allergens should follow a general protocol, however, each step must be adapted and optimized to the particular allergen that is being studied. The following steps need to be addressed and optimized to obtain reproducible and reliable information about allergens: the proper choice of the allergenic source material, the optimal extraction method to enrich allergens, electrophoretic separation and blotting methods that preserve the allergenicity of proteins, and sensitive and specific detection methods.

2. Allergenic source material and extraction methods

The aims of *in vitro* diagnostics of food allergy are to identify the offending allergen and quantify the

amount of allergen-specific IgE antibodies. But what is the right choice of the allergenic source material and its proper preparation? The accepted guideline is to mimic the conditions under which allergic subjects are confronted by the allergens. In order to identify allergens it is wise to use pure and well defined source material; for example, chocolate is not a good source material, it may contain allergens of different origins such as milk, nuts and peanuts. In the case of food allergy, different fruit and vegetable varieties may complicate the right choice; for example, different apple varieties may contain different amounts of allergens. Granny Smith contains more allergens than Hammerstein and is the better choice for the preparation of a more potent extract. This is supported by the clinical history of apple-allergic subjects and verified by immunoblot analysis [5].

Another question is whether processed food might be the better allergen source. This question is not inept since the possible generation of neoallergens by food processing can not be dismissed. However, the clinical experience shows that most patients, but not all, react to naive, unprocessed foods. Therefore, the risk of generating neoallergens through processing seems to be of minor relevance. A problem of more importance is the relationship between allergen and matrix and how it may influence the extract composition in regard to allergen content (representative extract). As a first step, foods are minced to make the extraction process more effective. To avoid denaturation, we freeze food at -80°C and mince it under addition of dry ice in a coffee grinder. In our hands, this method is a gentle, non-denaturing procedure and even useful as a first step of a mRNA preparation. Even fatty foods like peanuts become brittle and are easily pulverized. In other cases, grinding foods under liquid nitrogen N_2 with mortar and pestle is a good alternative [6]. The question whether the pulverized material should be defatted before extraction is a subject of controversy in literature. Some authors describe defatting with petroleum ether as necessary step prior to extraction [7,8], whereas others suggest conditions which could denature proteins like the usage of organic solvents should be avoided [9]. In a small study, we investigated the influence of the defatting step on hazelnut extracts by petroleum ether (F. Schocker, unpublished data); no differences in regard to the allergens content and composition was observed. As a conse-

quence, we omit the defatting step and only discard the lipid layer after extraction.

Another question is the avoidance and prevention of denaturation during the extraction procedure; here the methods that are utilized may vary (this is described by Pastorello and Trambaioli in detail in this issue). However, a moderate protease activity might be tolerated without losing much of the extract activity. Stabilization of extracts with antioxidants and protease inhibitors [10] depends on its future use. Thus skin prick test preparations need preservative substances, normally used in food stuffs. Coca's solution (0.25% NaHCO_3 and 0.5% NaCl) is widely used and a good extraction buffer to obtain active extracts. As an alternative, 125 mM ammonium carbonate buffer (pH 8.0) is a good extraction buffer; it has the advantage that it can be completely removed from the extract by lyophilization. Extraction of peanuts with ammonium carbonate buffer resulted in an extract that was comparable with the supernatant of chewed peanuts [11].

Once the allergens are identified, optimization of extraction procedure and conditions are strongly recommended. Niemeijer et al. [8] could show a dramatic influence of pH of the extraction buffer on the extract composition and antigen yield. Lehrer and Reese [12] showed that in addition to water-soluble allergens, corn contained isopropanol-soluble (extraction with 55% isopropanol in water) allergens. These results emphasize the need to optimize the extraction procedure individually depending on the source material and the purpose.

3. Separation methods

Allergenic source materials and especially foods are — from the chemical point of view — mixtures of different substance classes and concentrations. Therefore, the extraction procedure is an important step to enrich allergens which in most cases are proteins or glycoproteins and, in select cases, polysaccharides. In general, the allergenic components have a molecular mass in the range of 10 to 70 kDa. Thus, electrophoresis is the method of choice to separate allergens and characterize them in regard to their molecular mass and isoelectric point [13].

3.1. Isoelectric focusing (IEF)

In an electric field, proteins are separated according to their isoelectric points by isoelectric focusing (IEF). Basically, the proteins migrate in a gel through a pH gradient towards their isoelectric points (pI). When they reach their pI the net charge of the protein is zero and the migration stops. IEF is considered to be a non-denaturing separation technique. There are several ways to set up an IEF and to optimize the system. First, there is the choice how to create the pH gradient. The easiest way to run an IEF is performed by use of ampholytes containing agarose gels. This technique is simple and quick and in combination with immunoprinting has been successfully used to characterize allergens from a wide variety of source materials [14]. However, the method of choice for highly reproducible IEF is the immobililine technique. The pH gradient is polymerized (immobilized) into acrylamide gel [15]; in contrast to IEF-based on ampholytes there is no gradient shift during prolonged separation times. Furthermore, the pH range of an immobilized pH gradient (IPG) gel may be narrow for highest resolution, or broad for a first overview. For allergen screening by immunoblotting IEF is less useful, since most allergens consist of multiple isoallergens, which differ from their primary structure by some amino acid exchanges resulting in multiple bands in IEF. Indeed, many allergens were first identified by SDS–PAGE immunoblotting (see below). However, in order to investigate whether antibodies react to conformational or linear epitopes the comparison of antibody reactivity to the IEF immunoblot and to Western blot may give first hints since conformational epitopes may be destroyed under Western blot conditions.

3.2. Separation by molecular masses

The electrophoretic separation of proteins may also be performed by molecular mass by SDS–PAGE under reducing or non reducing conditions [16,17] or native electrophoresis [18,19]. SDS–PAGE is the most frequently used separation technique for proteins.

Proteins that are loaded with negative charges by SDS are separated in a polyacrylamide gel. Depend-

ing on the gel composition (acrylamide and cross-linker concentration) proteins in the molecular mass range between 10 to 200 kDa can be separated with the most widely used SDS–PAGE buffer system according Laemmli [16]. The separation capabilities can be increased to a molecular mass range of 3 to 30 kDa using spacer gels and tricine-containing electrophoresis buffers [20]. However, in our experience it is difficult to achieve reproducible results by using home-made gels for peptide electrophoresis. For occasional use commercially available gels (e.g. Novex™) (see F. Schocker in this issue) are recommended. Alternative gel systems have been developed; for example the cationic detergent cetyltrimethylammonium bromide (CTAB) was used instead of SDS [21]. An advantage of CTAB is its lower denaturing power as compared to SDS. In practice this method is rarely used. Only in special cases, native electrophoresis is applied because this technique has to be optimized for each single protein [22], since the proteins are separated according to molecular mass and net charge.

4. Immunoblotting

4.1. Transfer membranes

The perfect transfer membrane that binds all proteins to the same degree is not available because of the heterogeneous nature of proteins (glycoproteins, lipoprotein, hydrophilic, hydrophobic, small, large) [23]. Thus the choice depends on the nature of the protein under investigation. Different transfer membranes assorted with different pore sizes are available (0.45, 0.2 or 0.1 μm) whereby the 0.45 μm pore membranes are most frequently used. Nitrocellulose binds proteins via hydrophobic interaction independent of the charge of the proteins. Positively charged nylon membranes bind negatively charged proteins very strongly which may result in a high background [24]. Nowadays, polyvinylidene difluoride (PVDF) and polypropylene membranes are recommended because of their mechanical stability [25]; their chemical resistance allows gas phase sequencing of blotted proteins. PVDF membranes may have slightly different binding properties in comparison with nitrocellulose membranes. Activated glass fiber

membranes are also recommended due to their chemical stability [26], but are not widely used. CNBr activation of nitrocellulose membranes results in improved binding properties; proteins and peptides can covalently bind to the membrane. CNBr-activated nitrocellulose membrane is especially effective in binding small protein and peptides. The CNBr activation can be performed safely by the protocol of Demeulemester et al. [27].

4.2. Blotting methods

There are mainly three blotting procedures: diffusion or capillary blotting [28], electroblotting [29] and thermoblotting [24]. The easiest method of protein transfer is the print technique preferably used with agarose IEF. The application of this method for polyacrylamide gels is limited by the high molecular mass of the proteins because of the narrow pore structure of polyacrylamide gels. Thermoblotting (50°C) may help to overcome this limitation, but here the possibility of heat-denaturation has to be taken into account. Electroblotting, preferably in the semi-dry format [29], has very effective transfer properties, but the blotting time should be optimized for each individual problem. The major problem of blotting is not the elution of the proteins from the gel but the binding of the proteins to the membrane over the whole course of the procedure [30]. This can be controlled by inspecting the back of the membrane after staining or the use of a second membrane behind the first.

4.3. IEF/SDS–PAGE/2 D electrophoresis/immunoblotting

The benefit of the agarose IEF/immunoblotting is that up to between 40 and 70 samples such as sera, monoclonal antibodies or saccharide-specific lectins can be tested per run. Thus one and the same run offers the opportunity of examining various features of a given extract. With the aid of patients' IgE we have the opportunity of identifying minor and major allergens, and their individual reaction patterns. Once allergenic molecules are identified this method allows further characterization, e.g. such as the identification of glyco-conjugates with lectins, enzymatic activity or as targets of monoclonal antibodies. IEF/

immunoblotting is a method with the highest resolving power with regard to native 1-D separating procedures. Moreover, this method enables us to give semi-quantitative estimates of the binding reactivities. However, the intensity of signals is only comparable if the allergens are in excess and the binding affinity of the antibodies is identical. This does not exist under normal circumstances. Thus by IEF/immunoblotting the IgE reactivity to single allergenic components in an extract is tested simultaneously, whereas in diagnostics patients' IgE binding to allergenic source material is quantified by RAST-like procedures without differentiation between single allergenic components. Despite the obvious advantages of IEF/immunoblotting in allergy research this method has not gained general acceptance; this is due to the fact, that allergens normally appear in a number of isoallergens, which complicates the identification of allergens. Isoallergens may differ by up to 23% in their amino acid sequences from the IgE-reactive reference (counterpart) in the primary protein structure. Moreover, under the condition of isoelectric focusing proteins sometimes generate conformational isomers resulting in a characteristic pattern of bands. This can easily be shown when an isomer is isolated from the gel separated again by IEF resulting in the same isomer pattern [31].

This problem is overcome by SDS–PAGE/immunoblotting (Western blotting), which simplifies allergen patterns of an allergenic source. Western blotting separates the samples under denaturing conditions. Therefore, the benefit of simplified IgE-binding patterns is paid for by the loss of IgE-reactive conformational epitopes (with exceptions) and by the disappearance of distinguishable isoallergens. The highest resolution of protein separation is achieved by 2-D electrophoresis with subsequent blotting. In the first dimension the sample is separated by IEF with the immobiline technique (IPG) as the first choice. IEF is used in the first dimension, because running SDS–PAGE as the first dimension the detergent SDS would disturb the following IEF separation. A problem of 2-D electrophoresis is to realize reproducible runs. This is directly and indirectly achieved by IPG in that up to forty gel strips with identical quality can be gained from one gel [15]. Another feature of IPG is their

tolerance to high protein loads of up to 100 $\mu\text{g}/\text{cm}$ without loss of sharpness of the separated bands. After a 2-D electrophoresis run we are able to take four or more replica blots [32]. Thus, it is possible to identify a substance on a spot by patient's serum or monoclonal antibodies, by lectin binding, by enzymatic activity, and by N-terminal sequencing or mass spectrometry (MS). The establishment of the described electrophoresis/immunoblot method makes it possible to use the full impact of the hybridoma technique to generate monoclonal antibodies. The monoclonal antibodies give a new dimension to the electrophoresis/immunoblot technique in that they identify the allergens unambiguously.

5. Immunological tools to identify and quantify allergens

Antibodies are widely used as analytical tools to identify and quantify substances in various environments and matrixes with high sensitivity and specificity. If pure antigens are available for immunization, conventional production of polyclonal antibodies is not necessarily a drawback in comparison with monoclonal antibodies, because monospecificity is in such circumstances a solvable problem. However, in allergy research the allergenic source material consists of a multitude of substances. Here the hybridoma technique is the first choice to produce monospecific, monoclonal antibodies [3,33]. A prerequisite for generating monoclonal antibodies is a quick, efficient test system in order to select antibodies with the desired specificity out of several hundred hybridomas that are typically generated. After a preselection with a quick assay such as dot tests or ELISA for hybridomas that produce antibodies against the allergen extract, the electrophoresis-immunoblot technique is the most straight forward technique to characterize the specificity of monoclonal antibodies. The specificity of the monoclonal antibody can then be compared with the IgE reactivity patterns of allergic subjects. Once a monoclonal antibody had been selected it can be a tool for further applications such as allergen purification, detection or allergen standardization. Allergen-specific monoclonal antibodies can be used to develop

quantitative assays in allergen standardization or to measure “hidden” allergens in foods.

However, specificity alone is not enough for a successful usage of monoclonal antibodies in a quantitative assay or in affinity chromatography; the binding constant of an antibody must be suitable for the application. Quantitative assays need antibodies with high binding constants to be sensitive, whereas in affinity chromatography, an antibody with lower affinity works best since it allows the elution of the purified allergen under mild conditions and prevents damage to the antibody as well as the allergen. In some cases it is difficult to obtain the antibodies with the desired specificity. In this situation we were able to generate allergen-specific antibodies by using an allergen band that was cut out from an immunoblot for immunization or by using the cascade technique, where the immunodominant allergens are removed from the extract by the newly generated antibodies [34,35]. The remaining extract components are used for immunization and this process is repeated until an antibody with the desired specificity is obtained.

6. Characterization of allergens by chemical methods

To analyze and to characterize allergens in detail in regard to their structure partial sequence, information of the purified allergen are required. This will help to clone the allergen in question.

In the past, this was difficult since it required substantial amounts of purified allergen which were not easily obtained. Today, nanomolar amounts are sufficient to perform N-terminal sequencing based on Edman degradation. 2-D electrophoresis in combination with blotting provides these amounts of highly purified allergen. Furthermore, excised spots from 2-D blots can be pooled to perform digestion experiments by chemical cleavage (i.e. CNBr-cleavage) or enzymatic digestion with different proteases. The cleavage products are then analyzed by SDS–PAGE/ blotting followed by N-terminal sequencing. Following this method we were able to identify Ara h 1 [32] as ConA-reactive which is in contrast to findings by Burks et al. [36]. First, the suspected Ara h 1 was enriched by affinity chromatography using the immobilized lectin ConA. The bound components were

eluted by methylmannoside and further separated by 2-D electrophoresis. The Ara h 1 fraction was digested with the protease Lys-C and the digests were separated by SDS–PAGE followed by N-terminal sequencing. Via the N-terminal sequences of the starting material and two internal sequences it was shown that the ConA-reactive allergen of peanut is the truncated version of Ara a 1 since the sequences of the ConA-reactive allergen fit in the sequence of the recombinant Ara h 1.

In the near future, mass spectrometry (MS) in the form of matrix-assisted laser desorption/ionization (MALDI) in combination with time-of-flight (TOF) mass analysis may allow us to estimate the mass of such allergens and their peptides using blotted material directly. Another application of this technique (MALDI–MS) may be C-terminal sequencing [37]. Whether this method will achieve a broad application remains to be seen. Furthermore, electrospray tandem mass spectrometry may permit direct sequencing of natural allergen including the possibility of identifying post-translational modifications which is not possible with molecular biological cloning and sequencing since it only provides the amino acid sequence. This technique may also be helpful to compare recombinant and natural allergens and to provide a method of choosing the optimal expression for recombinant allergens when a product is required to be as close as possible to its natural counterpart.

7. Epitopes

As stated above, epitopes are those parts of an antigen or allergen that interact with the immune system, e.g. as binding sites of an antibody, MHC-molecule or T-cell-receptor. In proteins, two types of epitopes are described: conformational or discontinuous epitopes and continuous epitopes. Conformational epitopes are built by different sections of the primary structure and/or depend on the 3-D structure. It is evident that conformational epitopes may be easily destroyed by denaturation. This is one explanation why cooked apple or roasted hazelnut lose their allergenicity for some patients. Sometimes it is possible to find antibody reactivity to denatured conformational epitopes, since continuous parts of

the discontinuous epitope are large enough to serve as a binding site for antibodies. Another important question is whether the conditions of Western blotting destroy conformational epitopes. In practice these epitopes are mostly reactive and are explained with renaturation on the nitrocellulose membrane [38]. Experimentally, it is very difficult to identify conformational epitopes, since up to now X-ray crystallography of the allergen-antibody-complex is needed for the epitope identification. A well studied example in this regard is the egg lysozyme. These experiments were performed on egg lysozyme antibody (Fab) complexes for the first time. Three epitopes were identified (reviewed in [39]) by performing X-ray crystallography of complexes of egg lysozyme antibody and Fab antibody fragments. These discontinuous epitopes were located on the surface of the molecule and were approximately 750 Å² large. The amino acids that form the epitope recognized by the monoclonal antibody HyHEL-10 are residues 15–16, 20–21, 63, 73, 75, 89, 93, 96–98 and 100–102. In the future, NMR-techniques may facilitate the identification of conformational epitopes if the 3-D structure of the allergen is known. Nuclear magnetic resonance (NMR) spectroscopy makes it possible to observe signals from individual atoms even in complex biological macromolecules in solution [40]. The interaction of Fab-fragments with allergens are measured by signal-shifts of amino acids that are part of a particular epitope. However, it is possible to elucidate conformational epitopes indirectly. One way is to produce overlapping large 100 amino acid residues long recombinant fragments of the allergen and test these fragments for IgE antibody reactivity [41]. Reactive fragments are then further divided into smaller overlapping fragments and tested again for IgE antibody binding. Loss of antibody reactivity would indicate the presence of conformational epitopes. Sometimes site-directed mutagenesis is successful in localizing epitopes [42,43].

The identification of continuous epitopes is performed by testing overlapping synthetic peptides of identical length for IgE antibody reactivity. These peptides have a length of 6–16 amino acid with an offset of one to five residues. The offset is defined as the difference between the starting positions of two consecutive peptides (reviewed in [44]). An econ-

omical strategy to identify IgE-binding regions is to synthesize 15 residue-long peptides that span the entire allergen sequence with an offset by five residues. The peptides overlap nine residues which should be large enough to localize consecutive epitopes. In a second step, shorter peptides with smaller offsets may be tested to identify the shortest peptide that is able to bind significant amounts of IgE antibodies. Two different techniques are popular: (1) The pepscan method which uses activated plastic pins as the solid-phase for peptide synthesis [44]. This system is compatible with the 8×12 micro titer system. The pins are reusable after regeneration and can be used to identify epitopes of several allergic subjects or monoclonal antibodies. (2) The SPOTs technology which uses activated filter paper as the solid-phase. This technique is described in detail in the article of Reese et al. in this issue.

Besides epitopes that consist of amino acids carbohydrates may also contain epitopes. Cross-reactive carbohydrate determinants in food allergens may reduce the specificity and sensitivity of *in vitro* diagnostic assays and may be one cause for the resistance of glycosylated allergens to denaturation. It is relatively easy to determine whether an allergen is glycosylated. Most carbohydrate staining methods are based on oxidative cleavage of vicinal diols with meta-periodate, a reaction which is almost exclusive to saccharides. The generated aldehydes or ketones form a colored complex via the periodic acid Schiff (PAS) staining that indicates the presence of glycoproteins [13]. This method is preferably applied to stain glycoproteins in electrophoretic gels. Alternatively, glycoproteins can be labeled with (radioactive) Tritium by the reduction of aldehydes with NaBT₄. Another method is the formation of hydrazide derivatives via hydrazone formation of aldehydes or ketones. The most popular reagent of this type is the Dig-hydrazide which is very effective in staining glycoproteins on blots [45]. A different staining method of glycoconjugates on blots is performed by saccharide-specific lectins. It is recommended to evaluate the specificity of lectin binding by inhibiting the reaction with lectin-specific mono- or disaccharides. However, a negative lectin reaction does not exclude the presence of carbohydrate moieties since lectins are sugar-specific and no lectin recognizes all carbohydrate structures. An

example for the identification of carbohydrate-containing epitopes is described in the paper of Petersen and Mundt in this issue.

8. Conclusion

Why is it important to identify and characterize food allergens? Food allergy is caused by various foods and has a strong molecular basis. Since allergens are “pathogens” their identification and characterization has a clear relationship to the disease allergy. As mentioned earlier, the *in vitro* diagnosis is based on the identification and quantification of allergen-specific IgE reactivities to allergenic source material but not to single allergens. Since the same allergenic source material may induce different clinical symptoms in allergic individuals, IgE-reactivities to various allergenic substances may be the cause for these differences. For example, some celery-allergic subjects react only to fresh unprocessed material whereas others develop symptoms after exposure to heated material. Vieths and co-workers demonstrated that the celery analogue to birch profilin, Api g 1 and cross-reacting carbohydrate determinants (CCD) are the molecular basis for this clinical observation [46]. It was shown by Western blotting and enzyme allergosorbent test (EAST, an enzyme-based modification of RAST) that the heat sensitivity of these components were time dependent and that Api g 1 lost its IgE-binding ability upon heating, whereas CCDs were heat-stable for up to 30 min at 100°C.

In this context it is imperative to identify and characterize the most potent allergens and understand the basis of their potency since these allergens survive processing and may be the reason for severe anaphylactic reactions even in minute amounts (“hidden allergens”). A deeper understanding of the resistance of food allergens to enzymatic and heat degradation and of their epitopes is needed. Food allergens with these described features are found in crustaceans and peanuts and both are now under intense investigation [43,47,48]. Up to now, avoidance is the only rational therapy for food allergy. However, as our understanding of the molecular basis of allergenicity increases allergen-specific immunotherapy will come in focus as new therapeutical

concepts such as the usage of allergens with reduced anaphylactic potential [49] and DNA-based vaccines [50].

9. Nomenclature

IgE	immunoglobulin E
OAS	Oral Allergy Syndrome
pI	isoelectric point
IEF	isoelectric focusing
IPG	immobilized pH gradients
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
RAST	radioallergosorbent test
CTAB	cetyltrimethylammonium bromide
MS	mass spectrometry
MALDI	matrix-assisted laser desorption/ionization
TOF	time-of-flight

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