REVIEW

Characterization of plant food allergens: An overview on physicochemical and immunological techniques

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Allergy to plant-derived foods is a highly complex disorder with clinical manifestations ranging from mild oral, gastrointestinal, and cutaneous symptoms to life-threatening systemic conditions. This heterogeneity in clinical manifestations has been attributed to different properties of allergenic molecules. Based on this fact, symptom elicitors were grouped into class I and pollinosis-associated class II food allergens, but clear distinction is rather ambiguous. Moreover, mechanisms underlying food sensitization are not fully understood yet, and food allergy management most often relies on patient's compliance to avoid suspected foods. Therefore, recent efforts aim at the investigation of plant food allergies at the molecular level. This review provides an overview on currently available techniques for allergen characterization and discusses their application for investigation of plant food allergens. Data obtained by an array of physicochemical analyses, such as allergen structure, integrity, aggregation, and stability, need to be linked to results from immunological methods at the level of IgE and T-cell reactivity. Such knowledge allows the development of computational algorithms to predict allergenicity of novel foods being introduced by biotechnological industry. Furthermore, molecular characterization is an indispensable tool for molecule-based diagnosis and future development of safer patient-tailored specific immunotherapy in plant food allergy.

Keywords:
Class I food allergen / Class II food allergen / Immunological characterization / Plant food allergens / Physicochemical characterization

1 Introduction

Recent epidemiologic data suggest rising prevalence of food allergy in westernized nations, meanwhile affecting up to 4% of the population. Although the diversity of human diet is enormous, only a limited number of foods account for the majority of food allergies [1], and only a few food-derived molecules are capable of eliciting an allergic response in atopic individuals [2]. To date, the official list of the International Union of Immunological Societies Allergen Nomenclature Subcommittee comprises 156 allergens originating from 55 plant foods (http://www.allergen.org). In the last years, it became evident that most allergens display a narrow functional distribution and can only be found in a very restricted number of protein families [3]. In fact, based on common structural properties, the currently known plant food allergens can be grouped into only about 30 protein families [4]. These observations strongly suggest the existence of so far unknown properties that would render a protein allergenic [3].

Notably, food allergens might have to fulfill additional requirements. According to the underlying sensitization

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Abbreviations: AUC, analytical ultracentrifugation; **BAT,** basophil activation; **CAST,** cellular allergen stimulation; **CD,** circular dichroism; **DSC,** differential scanning calorimetry; **FTIR,** Fourier Transform-infrared spectroscopy; **LTP,** lipid transfer protein; **NOE,** nuclear Overhauser enhancement; **PR,** pathogenesis related; **SAXS,** small-angle X-ray scattering; **SEC,** size exclusion chromatography; **TCC,** T-cell clone; **TCL,** T-cell line

pattern, class I food allergies can be distinguished from class II food allergies. Class I food allergens share common properties, such as stability to food preparation and gastric digestion, which enable them to provoke allergic sensitization in the gastrointestinal tract. In contrast, class II food allergens are considered to be more sensitive to heat and digestive enzymes and, therefore, cannot cause sensitizations *via* the oral route. Instead, they provoke allergic reactions in already sensitized patients by means of IgE cross-reactivity between sensitizing aeroallergens and symptom-eliciting food allergens leading to pollinosis-associated food allergies [5]. Moreover the different properties of class I and II food allergens also determine the clinical manifestation of food allergy. For example, allergy to fresh fruits is generally associated with mild symptoms, whereas allergy to peanuts is considered a major cause of severe anaphylaxis [6]. On the molecular level, this is caused by the peanut major allergens Ara h 1, 2, and 3, which are regarded as potent class I food allergens and have been identified exclusively in food but not in pollen. In contrast, allergens originating from fruits often constitute pan-allergens such as profilins and lipid transfer proteins (LTPs) that can be found in a multitude of allergenic pollen [7, 8]. However, a clear distinction between class I and II food allergens is not always possible. For example, extreme thermostability and resistance to pepsin digestion identify LTPs as potent class I food allergens [9]. In contrast, pollen LTPs have been reported to behave as primary sensitizing allergens in patients with IgE to both mugwort and peach LTP [10], indicating an involvement of LTPs also in class II food allergies.

Geographical factors, regional dietary habits, and food preparation contribute to the complexity of food allergies. For example, LTPs are regarded as major allergens of *Rosaceae* fruits in the Mediterranean area [11], whereas in Central and Northern Europe allergy to *Rosaceae* fruits seems to be associated with birch pollinosis and sensitization to Bet v 1 but not with LTPs [12]. Concerning food preparation, high temperatures during roasting have been reported to increase allergenicity of peanut allergens, a phenomenon not observed upon boiling of peanuts. This explains why peanut allergies are frequent in the United States, but despite comparable *per capita* peanut consumption, they are virtually not observed in China [1, 13].

Taken together, plant food allergies seem to represent complex disorders that are influenced by a multitude of factors. Intrinsic properties of the plant food allergen itself seem to influence its allergenicity in general, its sensitization potential, and the severity of elicited clinical manifestations. So far, the nature of these physicochemical properties is widely unclear. As shown in Fig. 1, bioinformatics-based calculations revealed plant food allergens to be heterogeneous in size (the molecular weights range from 8.8 to 87.2 kDa), charge (p*I*: 4.04–10.54), hydrophobicity (grand average hydropathicity: −1.66–0.62), and stability (instability index: 17.49–139.45). Interestingly, we observed significant differences between plant food allergens with homologues in pollen (putative class II) and those without identified pollen homologue (putative class I). For example, class II food allergens appear rather small, stable (instability index < 40) [14], and more hydrophilic (higher grand average hydropathicity) [15] than class I food allergens. These results contradict experimentally obtained data demonstrating high stability of class I food allergens, such as the peanut 2S albumins Ara h 2 and 6 [16], and enhanced susceptibility to denaturation of certain class II food allergens, as reported for the members of pathogenesis-related (PR)-10 family of allergens [17]. This conflicting data might be explained by the fact that the algorithms used are solely based on protein primary structure and, therefore, cannot consider impacts of the 3-D fold. Hence, a detailed experimental investigation of plant food allergies at the molecular level is indispensable.

This review gives an overview on currently available techniques for physicochemical and immunological characterization of plant food allergens. Linking detailed physicochemical and immunological data is essential for a better understanding of common properties of allergens across protein families and might support computational algorithms for prediction of a molecule's allergenicity [18] as required for safety assessment of novel foods that are introduced by biotechnological industry. Moreover, such knowledge has direct implications for the development of molecule-based diagnosis and patient-tailored allergen-specific immunotherapy [19, 20].

2 Physicochemical characterization of plant food allergens

To date, a substantial number of well-established techniques for the physicochemical characterization of allergens are applicable and an overview on their information content is provided in Table 1. As the physicochemical properties influence or may even determine the immunological behavior of an allergenic molecule [21], such knowledge is a prerequisite for a well-founded immunological characterization, and provides the basis for the generation of modified molecules, *e.g.* hypoallergens. The physicochemical parameters discussed in Section 2.1 include molecular weight and p*I*, identity defined by amino acid sequence, 3-D structure and conformation, the presence of modifications, protein integrity, thermal and/or proteolytic stability, homogeneity, and aggregation behavior.

2.1 Molecular weight, p*I* and microheterogeneity

Classically, extracts from allergenic sources or purified proteins are analyzed by SDS-PAGE providing information on purity and estimated molecular weight by migration behavior. 2-D PAGE gives additional information on the p*I*

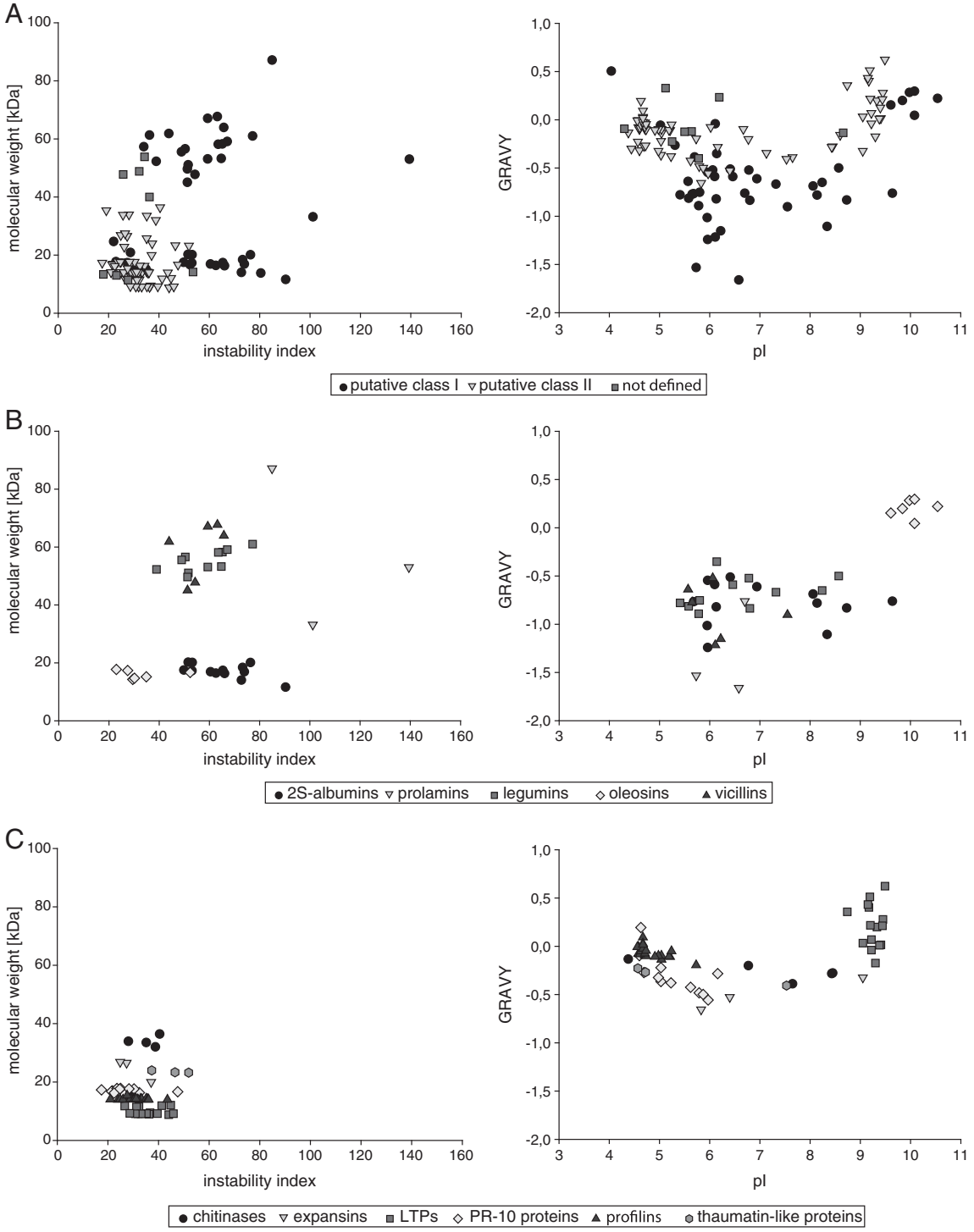

Figure 1. Bioinformatics-based calculations of physicochemical properties of plant food allergens. Theoretical molecular weight, p*I*, instability index, and grand average of hydropathicity were obtained using the ProtParam tool of the ExPASy proteomics server (http://www.expasy.org) of the Swiss Institute of Bioinformatics. (A) Physicochemical properties are shown for all plant food allergens registered in the official allergen list of the International Union of Immunological Societies Allergen Nomenclature Subcommittee (http://www.-allergen.org) with known protein sequence ($n = 119$). Allergens were distinguished in putative class I (without identified pollen homologues), putative class II (with identified pollen homologues), and a third not yet defined class of plant food allergens displaying no homologues in plant pollen but other allergen sources. (B) Class I and (C) class II plant food allergen families comprising more than three members are illustrated separately.

Table 1. Overview on information content of physicochemical characterization methods

	Molecular weight	Identity – primary structure	Integrity	Modifications	Quantity	Secondary structure	3-D structure	Thermal stability	Proteolytic/ acidic stability	Homogeneity	Aggregation behavior	Purity
SDS-PAGE, 2-D PAGE	✓		✓	✓	✓				✓	✓		✓
Edman sequencing		✓										
Amino acid analysis		✓			✓							✓
Intact MS (MALDI, ESI)	✓	✓	✓	✓								✓
NanoLC-MS/MS peptide mapping		✓		✓								✓
CD						✓		✓				
Fourier Transform infrared spectroscopy						✓		✓			✓	
X-ray crystallography		✓				✓	✓					
NMR		✓				✓	✓					
DSC								✓				
Atomic force microscopy											✓	
Fluorescence spectroscopy						✓			✓		✓	
SEC	✓		✓						✓	✓	✓	✓
Field flow fractionation	✓		✓							✓	✓	✓
AUC	✓									✓	✓	
Dynamic light scattering	✓							✓			✓	
Small-angle X-ray scattering	✓							✓			✓	

Unequivocal information content is indicated in black; methods giving partial information or requiring additional confirmation are indicated in gray.

of a molecule [22]. The issue of microheterogeneity due to the presence of various isoforms of natural allergen preparations [23] or post-translational modifications of recombinant products [24] can be monitored by isoelectric focusing with or without the second dimension of separation by size. Such microheterogeneities cannot be resolved by SDS-PAGE only, as they require much higher resolution for the determination of the molecular weight.

In contrast to electrophoretic methods, the accurate molecular weight of preferably purified allergens can be obtained by MS. Three issues being important for MS will be discussed, *i.e.* simple preparation, ionization, and resolution. Since ionization, as required for MS analysis, is very sensitive to the presence of salts or ionic detergents, it is advisable to pretreat purified proteins by solid-phase extraction (C_4 or C_{18} RP beads). For example, such a pretreatment has been applied for quality control of major apple allergen preparations, namely natural Mal d 2 and Mal d 3 and recombinant Mal d 1 and Mal d 4 [25]. For their quality assessment, the authors paid specific attention to potential modifications resulting from protein production. In principle, two modes of ionization are applicable for intact mass determination of proteins, *i.e.* MALDI and ESI. In MALDI, a gentle transition of sample from solid to gaseous phase is facilitated by co-crystallization with low-molecular-weight compounds such as gentisic acid, sinapic acid, or α-cyano-4-hydroxycinnamic acid, enabling energy transfer from light to primarily singly charged protein ions. Using this method, the α′- and β-subunits of soybean β-conglycinin have been characterized as potential new allergens [26]. In contrast to MALDI, a series of multiply charged protein ions with m/z of a few hundreds to several thousands for intact proteins are measured in the ESI mode. Within the past years, instrumental advances have been made toward better resolution in mass enabling atomic resolution of intact proteins. Recently, the isoform abundance of the natural peach LTP Pru p 3 was characterized by high-resolution MS [27]. Unequivocal isoform annotation has been achieved by top-down MS experiments. In top-down MS an oxidized intact allergen is fragmented directly (without prior proteolytic digestion) and sequence information obtained. Furthermore, the tissue distribution of Pru p 3 was studied by MALDI-MS imaging experiments, allocating the allergen mainly to the peel. Technological details on top-down MS methods are also regarding the investigation of protein modifications, which have been reviewed elsewhere [28].

2.2 Protein sequence, integrity and post-translational modifications

Since 1950, the N-terminal sequences of proteins have been determined by Edman degradation, a method in which the N-terminal amino acid residue is labeled and cleaved from the protein in a sequential manner [29]. Nowadays, mainly MS technology is applied for obtaining sequence information. With the exception of top-down MS [27], MS-based sequencing is conducted with peptides derived from proteolytic digestion usually performed with trypsin. In addition, V8 protease, endoproteinase Glu-C, proteinase K, thermolysin, pepsin, and CNBr are applicable to improve sequence coverage. Mass spectra are recorded after the separation of proteolytic fragments by RP-HPLC. For example, the sequences of Bet v 1-related allergens from celery and kiwifruit have been verified by this method [30, 31]. A proteomic workflow consisting of 2-D PAGE and MS-based protein sequencing is shown in Fig. 2, addressing the issue of microheterogeneity.

Using ESI-MS, online coupling to RP-HPLC is possible. In the usual miniaturized setup operating at flow rates smaller than 1 μL/min this procedure is referred to as nanoLC-MS/MS. At present, an array of high-resolution hybrid MS instruments is available, which combine either a linear/3-D ion trap or a quadrupole with a time-of-flight, a Fourier Transform-ion cyclotron resonance, or an orbitrap mass analyzer. Sequence information obtained by ESI-Fourier Transform-ion cyclotron resonance-MS revealed the existence of a novel isoform of Ara h 8, a Bet v 1 homologue in peanut which is an important food allergen for birch pollen-sensitized patients [23]. Applying tandem MS in the most commonly performed bottom-up direction, protein sequencing is performed on proteolytic peptides upon dissociation of peptide bonds induced by collision with helium or argon atoms. Resulting fragments (b/y-ion series) can be compared with databases for unequivocal identification of corresponding peptides. Furthermore, MS/MS technology enables *de novo* sequencing of new protein stretches or characterization of protein modifications. In addition to peptide mapping, MS-based methods have been used for disulfide bridge mapping. For example, conservation of the typical four disulfide bridges has been demonstrated for a novel 2S albumin from peanut [32]. Interestingly, this protein was not homologous to other 2S albumin family members (Ara h 2, 6, 7). It contains ten cysteine residues, of which the two supplement cysteines

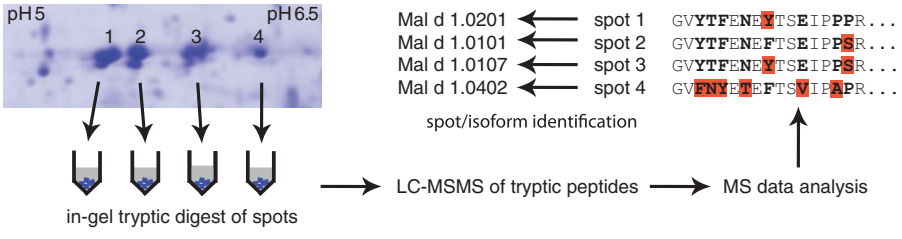

Figure 2. MS-based protein sequencing of different isoforms (microheterogeneity) of major apple allergen Mal d 1 resolved by 2-D PAGE.

were found to pair with each other. Thus, it was hypothesized that the overall five disulfide bonds would confer an even better stability against heat and proteolysis than observed for other family members.

2.3 Structural analysis

Secondary structure content and conformation of food allergens have been studied in many cases applying far-UV circular dichroism (CD) [16, 33–36]. Generally, five typical secondary structure elements, *i.e.* α-helix, β-sheet, β-turn, random coil, and poly-L-proline can be distinguished in a CD spectrum. The method depends on the presence of a chiral center in close proximity to UV-absorbing groups. In proteins, this is represented by the C_α atoms of the amino acids adjacent to peptide bonds. Wavelength scans are usually conducted from 190 to 260 nm. In this context, CD spectra being highly characteristic for seed storage globulins, which are rich in β-sheet structures, have been reported for the hazel allergens Cor a 9 and 11 [37]. In contrast, the CD spectrum for the LTP, Cor a 8, showed high α-helical secondary structure content.

The determination of secondary structure content of proteins relies on the availability of reference data sets with experimentally solved structures [38]. The ultimate method for 3-D structure determination represents X-ray crystallography, which operates by diffraction of monochromatic X-rays by well-ordered protein crystals. Based on the diffraction pattern, the electron density map of the macromolecule is converted by Fourier Transform algorithm and the protein sequence can be fitted into atomic coordinates. One of the most critical steps of X-ray crystallography is the generation of well-scattering crystals typically displaying a size of 50–100 μm. Crystallization is performed by dehydration of a saturated protein solution that usually takes several days to weeks [39]. For solving the structure, one may start from a structural model with significant similarity to the target protein. If such a structural homologue is not available, an isotopically labeled protein has to be expressed. Presently, more than 50 000 3-D structures derived from X-ray crystallography are available in the RCSB Protein Data Bank (http://www.pdb.org). For example, a high degree of structural conservation was found for peach LTP with cereal homologues by mainly focusing on the ligand-binding cavity and the protein surface. This might explain observed LTP cross-reactivity between peach and plant foods originating from botanical families other than *Rosaceae* [40]. More recently, the crystal structure of Ara h 3 has been published and important insights into its allergenic behavior have been gained [41]. Linear IgE-binding epitopes already known from the previous studies on Ara h 3 were mapped onto the structure and correlation of solvent exposure and IgE reaction frequency in patient sera was found.

Alternatively to X-ray crystallography, structural analyses of plant food allergens have been performed by NMR carried out in aqueous solution. Advantages include the possibility to examine structural flexibilities and conformational changes during protein–ligand interaction. However, high concentrations of allergen (5–10 mg/mL) are required, and structure determination is limited by size constraints, long data collection, and analysis times. Being based on nuclear spin of 1 H, ^{13}C, ^{15}N, and ^{31}P atoms, NMR requires isotopic protein labeling using enriched media for protein expression. Sequential assignment of all nuclei in the spectrum is crucial and can be achieved by multidimensional NMR experiments. The most important value for tertiary structure determination is nuclear Overhauser enhancement (NOE), which represents the cross-relaxation from one spin population to another and takes place through space. The NOEs between nuclei can be quantified and the corresponding inter-proton distances determined, which can be used for creating a 3-D model. The development of transverse relaxation-optimized spectroscopy based on $^{2}D/^{13}C/^{15}N$-triplelabeling and subsequent protonation of amide in H_2O has enabled efficient backbone assignments [42]. Thus, the former size limit of investigated proteins (< 25 kDa) could be overcome. At present, almost 8000 structures solved by NMR are deposited in the RCSB Protein Data Bank, and a whole list of NMR structures from 2S albumins has been determined including ricinus communis Ric c 3, sunflower seed SFA-8, rapeseed BnIa/BnIb, and peanut Ara h 6 [16, 43–45]. The highly protease-resistant core of Ara h 6 also served as a template for homology-based modeling the 3-D structure of the more allergenic Ara h 2. Although native Ara h 2 represented the more potent allergen than native Ara h 6, both cores retained similar and substantial IgE reactivity upon proteolysis. One-dimensional NMR spectroscopy has been used for folding assessment of three important hazelnut pan-allergens, Cor a 1.04, 2, and 8 from less concentrated protein solutions [46]. The authors estimated well-defined tertiary structures to be present in the protein preparations, as the spectra showed well-resolved NMR signals across the whole range; however, full assignment of the signals to individual protons could not be executed.

The structures of Api g 1 and Pru av 1, two PR-10 family members showing 42% sequence identity, are compared in Fig. 3. First, the crystal structure of the major celery allergen Api g 1 was determined at 2.9 Å resolution [47]. Second, the 3-D structure of the major cherry allergen, Pru av 1, has been calculated by 2438 restraints from five different NOESY experiments on ^{15}N, ^{13}C, and 1H nuclei [48]. Both molecules adopt similar structures consisting of a folded seven-stranded antiparallel β-sheet and two short α-helices arranged in a V-shaped manner wrapped around a long C-terminal α-helix forming a large hydrophobic cavity. However, major differences occur in the two loops 7-3 and 3-4. A backbone overlay of Pru av 1 with Bet v 1, its putative sensitizer in class II food-allergics, confirmed virtually identical secondary and tertiary structures leading to very similar molecular surface, rendering the existence of cross-

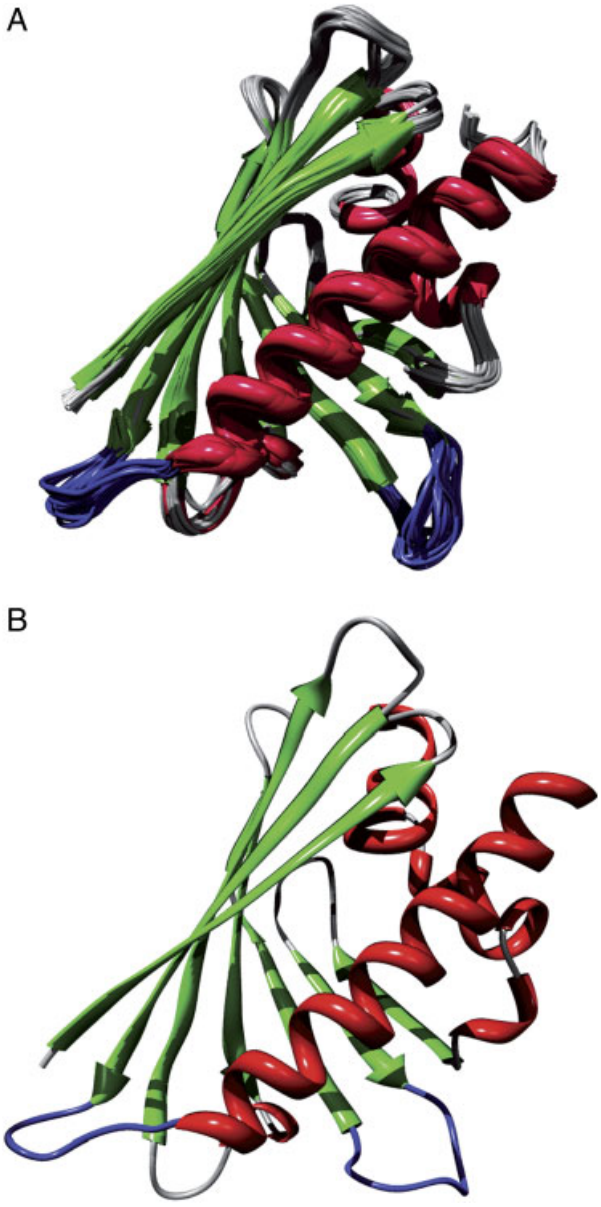

Figure 3. 3-D structures of PR10 proteins. (A) An overlay of 22 chains of Pru av 1 determined by NMR and B Api g 1 determined by X-ray crystallography. Both structures were retrieved from RCSB Protein DataBankTM (PDB ID: 1e09 for Pru av 1; PDB ID: 2bk0 for Api g 1). Highly flexible loops between β3-β4 and β7-α3 structures are shown in blue, β-sheets in green, and α-helices in red.

reactive IgE-binding epitopes most likely. Using IgE inhibition experiments with a serum pool of seven birch pollen-allergic patients, at least one IgE-binding epitope was found in Pru av 1 not present in Api g 1. As possible, candidate for such an epitope, the authors hypothesized the P-loop region displaying differences in charge and shape between these two homologues. A comparison of the solution structure (NMR) with the crystal structure (X-ray) may seem controversial; however, protein crystals contain 40–70% of solvent and usually solution and crystal structures are in good agreement. For example, no difference was found in the 3-D structures of the major birch pollen allergen, Bet v 1, which has been determined by both methods [49]. Notably, NMR spectroscopy can give information about dynamics of the molecule and the positions of hydrogen atoms can be assigned while remaining unresolved in X-ray crystallography [50].

2.4 Stability, homogeneity and aggregation

Food allergens often have to survive harsh conditions during food preparation and digestion. Therefore, stability to thermal denaturation, acidic environment, and gastrointestinal proteases are important parameters leading to the classification of class I and II food allergens. As *per* definition, epitopes of class I plant food allergens must be stable against degradation during digestive uptake to be able to function as sensitizers themselves, whereas class II may cross-react to IgE produced in response to inhalant allergens. Hence, the question of stability, homogeneity, and aggregation behavior of food allergens has been addressed extensively and a multitude of techniques has been applied.

2.4.1 Stability against proteolytic digestion

Stability to digestion and, therefore, prediction of allergenic potency have been investigated by simulated gastric or duodenal fluid assays [51]. Muskmelon profilin Cuc m 2, for example, was revealed to be stable in human saliva, whereas it was digested within seconds in simulated gastric fluid [52]. Increased stability of Cuc m 2 against proteolysis may be attributed to the presence of a number of saliva protease inhibitors. However, these results indicated that Cuc m 2 might be responsible for local oral syndromes in melon allergy. Moreover, as profilins are considered pan-allergens occurring in the majority of allergenic pollen, they may constitute important class II food allergens.

2.4.2 Stability against thermal denaturation

Effects influencing the heat-denaturation parameters of a protein can be investigated by CD. The denaturation temperature of an allergen equals the inflection point of the sigmoidal heating curve. Reversibility of protein denaturation can be assessed by comparing CD spectra at 25°C recorded before and after thermal treatment. Using CD, a detailed thermodynamic study was conducted on the major almond allergen amandin [53]. In general, 11S globulins have a high degree of thermostability, requiring temperatures of more than 70°C for denaturation, and a high resistance to proteolysis. It seems that such exceptional

conditions may play a role in determining the allergenic activity of seed storage proteins [54]. These proteins also have a propensity to form large aggregates upon heating [21] and still retain, to a large degree, their native secondary structure [55]. The hypothesis that class I food allergens display higher denaturation temperatures, better stability under acidic conditions, and a higher refolding capacity than class II food allergens was further substantiated by comparing the structural stabilities of the peach Bet v 1-homologue Pru p 1 and LTP Pru p 3 [17]. Latter proved to be more resistant to thermal treatment and acidic conditions (Fig. 4), a characteristic of many class I food allergens such as 2S albumins and α-amylase inhibitors [2]. The different susceptibilities to physicochemical treatment of the two peach allergens might reflect their variable allergenic potential. Effects of gastrointestinal digestion and heat-induced denaturation on the allergenicity of kiwi allergens have been investigated by gastrointestinal/duodenal *in vitro* digestion and CD. Both, serine-protease Act d 1 and thaumatin-like protein Act d 2 turned out to be quite resistant to digestion and heat-induced denaturation. Thus, the allergenic potency of kiwifruit has been attributed to the stability of these major allergens. Their roles as sensitizing agents through the gut have been proposed although homologues have been found in inhalant allergen sources [56]. Alternatively to CD, the thermodynamic behavior of food allergens during denaturation and aggregation can be determined by differential scanning calorimetry (DSC). In principle, DSC measures the heat required to increase the temperature of a sample compared with reference as a function of temperature. In this way endothermic phase transitions can be monitored and denaturation temperatures determined.

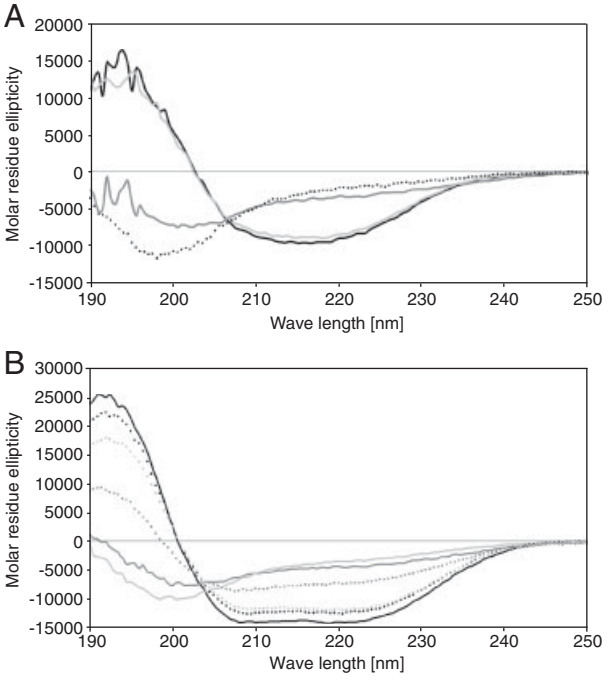

Figure 4. CD spectra showing the effects of pH and heating on the secondary structures of (A) recombinant Pru p 1 and (B) natural Pru p 3. Measurements were performed at pH 7.5 (solid line) and pH 3.0 (dotted line) and at 25°C (black), upon heating to 95°C (gray), and after heating and cooling to 25°C (light gray). Reproduced with permission from [17], copyright Wiley-VCH GmbH & Co. KGaA.

2.4.3 Monitoring protein aggregation

The aggregation behavior of plant food allergens upon cooking or various other heat treatments may play an important role for allergic sensitization upon uptake in the digestive tract, as aggregation may confer higher stability against proteolytic digest. Methodologically, atomic force microscopy allows visualization of structures at the molecular level. For example, atomic force microscopy has been used for detailed investigation of thermally induced aggregates of soybean 7S globulin in dependence of ionic strength. This storage protein, also known as Gly m 5 or β-conglycinin, has been shown to adopt fibers with diameter of 8–11 nm during heat-set gel formation. The transition was preceded by small changes in secondary structure at 75°C determined by CD and DSC [55]. Similar high denaturation temperatures were observed for Gly m 6, also known as glycinin, a highly abundant 11S storage protein of soybean [57]. Using NMR spectroscopy, heat-induced structural changes were allocated to a flexible α-helical region of the molecule. Aggregation by heat was detected by Fourier Transform-infrared spectroscopy (FTIR) to be due to β-sheet formation rendering glycinin even more stable to pepsin digestion. However, recently it was shown that IgE binding of glycinin does not correlate with protein stability [58]. IgE binding of hydrolyzates abolished after only 10 min. The authors concluded that these types of plant legumins do not withstand the digestive tract and are unlikely to represent sensitizers themselves. Therefore, they would represent less important allergens than originally suspected. In addition to using FTIR, the secondary structure content and heat-induced aggregation of the major peanut allergen Ara h 1 have been described [33]. However, its allergenicity was not effected by these changes, indicating that the recognition of conformational epitopes of Ara h 1 by IgE either is not a dominant mechanism or is restricted to parts of the protein that are not sensitive to heat denaturation. Methodologically, a frequency and an intensity shift of the longitudinal amide I vibration can be observed upon secondary structure changes in proteins. Furthermore, stabilizing or destabilizing effects of hydrogen bonds in β-sheets can be observed, as well as transitions from α-helices and intra- to inter-chain β-sheets as during protein aggregation. In another study, secondary structural changes of wheat gluten due to enzymatic deamidation were characterized by FTIR [59]. For quality control, FTIR was used to

show the absence of aggregation in a preparation of native Cor a 8, the hazelnut LTP, and during stability studies on Ses i 1 and Ber e 1, the 2S albumins from white sesame seeds and brazil nut, respectively [34, 37, 60]. Notably, FTIR can be performed on both liquid and solid samples. Thus, the influence of formulation (*e.g.* lyophilization and adsorption to aluminum hydroxide) on protein stability can be investigated physicochemically. This may be of importance for the development of therapeutical applications.

To get further insight at protein aggregation, fluorescence spectroscopy can be used. This technique monitors the local environment of tryptophan residues by a shift of the fluorescence maximum to lower wavelength when tryptophan becomes solvent buried, thus, indicating aggregate formation. Accordingly, quenching susceptibility using iodide has been used as indicator for compactness of potato Sola t 1 aggregates [61]. In this study, aggregation of patatin (Sola t 1) with other potato proteins accounted for a more pronounced decrease in IgE reactivity compared with self-aggregated patatin. It was concluded that the loss of IgE reactivity upon heating was due to irreversible aggregation with other potato proteins.

Information on quantity and size of plant food allergens or aggregates can be obtained by size exclusion chromatography (SEC) and analytical ultracentrifugation (AUC). In particular, SEC represents a simple and suitable method for quantification of aggregates. However, as aggregates have been shown to adopt irregular shapes, molecular weight cannot be deduced by simply comparing retention times of a sample with standards [55]. AUC is a widely used method for protein characterization with the sedimentation coefficient (*S*) firmly manifested in nomenclature of food allergens, *e.g.* the allergen families of 2S albumins and 7S or 11S globulins [4]. It can be operated either in equilibrium or in velocity mode. An advantage of equilibrium AUC is that it allows better estimates on aggregation or oligomerization state of protein unaffected of molecular shape. Equilibrium AUC was used for investigating the influence of heat on the structural properties of purified 7S globulin Ara h 1 [33]. AUC was run for 20 h at $180\,000 \times g$ in a 5–20% sucrose gradient until equilibrium was reached. Comparison to protein standards revealed an 8S complex suggesting a trimeric form. The presence of such Ara h 1 trimers was confirmed by a peak of approximately 180 kDa in SEC. The observed heat-induced changes in quaternary structure were not associated with the allergenicity of this peanut allergen.

Sophisticated methods being used for investigating the aggregation state of allergens include X-ray and various types of light-scattering techniques [20]. Multi-angle or right-angle light scattering detectors operating in flow can be coupled online to SEC and allow determination of molecular weight of eluting protein fractions and, thus, the aggregation state of the sample [62]. Aggregation behavior without prior separation can be investigated by dynamic light scattering and small-angle X-ray scattering (SAXS). Although dynamic light scattering determines the hydrodynamic radius of molecules from their diffusion speed, SAXS experiments give information on molecular dimensions from scattering intensity depending on particle size. High-end structural investigations on the behavior of soybean glycinin after drying and freezing or in environments as occurring in low-moisture food involved SAXS in combination with DSC, FTIR, and NMR [63].

3 Immunological characterization of plant food allergens

Detailed characterization of natural or recombinant allergens requires both physicochemical and immunological data. Immunological properties are not only restricted to binding of IgE, but also include knowledge of B- and T-cell epitopes. This knowledge might reveal new aspects on allergenicity, source of symptom elicitors, and factors contributing to cross-reactivity in class I and II food allergy. Furthermore, such knowledge might be particularly relevant for prediction of allergenicity and design of hypoallergenic molecules for diagnosis and therapy.

3.1 IgE reactivity and diagnosis of allergy

It is well established that IgE-binding epitopes of allergens are hallmark of allergic reactions. For clinicians, *in vivo* skin tests and *in vitro* serological assays are available to evaluate the presence of specific IgE to certain kinds of food in suspected allergic individuals, *i.e.* skin prick test, radio/enzyme-allergosorbent test, ELISA, and immunoblotting. However, skin and serological assays solely indicate sensitization but not clinically relevant to IgE reactivity resulting in food allergy [64]. Information on biological functionality of observed IgE reactivity can be provided by cell-based assays such as histamine release, basophil activation (BAT), or the cellular allergen stimulation (CAST) test, which have been used in an array of clinical studies on plant food allergy and component resolved diagnosis [65–68]. However, due to extreme variability in cellular responsiveness, these techniques have been suggested only as complementary methods in selected cases, and they are not broadly available [69]. Hence, double-blind placebo-controlled food challenges still represent the ''gold standard'' for food allergy diagnosis [70–72], revealing an urgent need in closing the gap between *in vitro* serological and cell-based assays frequently used in research to investigate IgE reactivity and biological potency of allergens.

3.2 B-cell epitope mapping

IgE epitopes can either induce antibodies themselves (class I food allergy) or cross-react with antibodies induced by other

proteins (class II food allergy) [47]. The difference between IgE immunogenicity and cross-reactivity appears to rely on molecular characteristics of allergens and route of sensitization resulting in two different forms of food allergy [70]. IgE immunogenicity of heat/acid-stable glycoproteins categorized as class I food allergens seems to result from sensitization *via* the gastrointestinal tract and can cause severe systemic reactions [73]. Prominent examples are the 2S albumin-type peanut allergens Ara h 2 and Ara h 6 with cores highly resistant to temperatures up to 100°C and proteolytic digestion [16]. In contrast, IgE cross-reactivity has been linked to respiratory sensitization by pollen allergens with generally milder symptoms limited to the oral cavity after ingestion of certain foods [74, 75]. Examples of common class II food allergies are birch pollen-allergic patients suffering pollinosis-associated food allergy to apple, carrot, and celery or the mugwort-birch-celery-syndrome [76]. With the exception of cross-reactive carbohydrate determinants, the clinical relevance of which is still under debate [77–80], IgE-binding epitopes are described to be either linear or conformational. As allergens usually lose conformational integrity during digestion, linear epitopes are considered to be more important in class I food allergy and conformation epitopes in pollen-related food allergy [81].

3.2.1 Sequential IgE-binding epitopes

Methods for mapping linear B-cell epitopes include enzymatic and chemical cleavage [82], generation of synthetic peptides in various formats [83], and the use of predictive algorithms [84, 85]. Enzymatic and chemical cleavage methods have been applied when sequence information was scarce. Peptide fragments obtained by protease digestion were screened with sera from allergic individuals and the resulting IgE-reactive fragments were subsequently sequenced. This procedure provided only rough information on the localization of IgE-binding epitopes and limitations included protease cleavage sites, peptide size, and quantity [81]. Further methodological refinements have been achieved by molecular techniques facilitating amplification and expression of small protein fragments allowing B-cell epitope mapping independently of proteolytic cleavage sites. Ultimately, advances in peptide chemistry led to the development of SPOTs technology, which has become a powerful tool for B-cell epitope mapping [86, 87]. Depending on the peptide library, the array of applications covers location of epitopes, identification of minimal peptide length required for antibody binding, and identification of key residues involved in antibody binding [83].

The methodological approach of identifying linear peptide epitopes by SPOTs technology is shown in Fig. 5. Briefly, short peptides covering the entire sequence of an

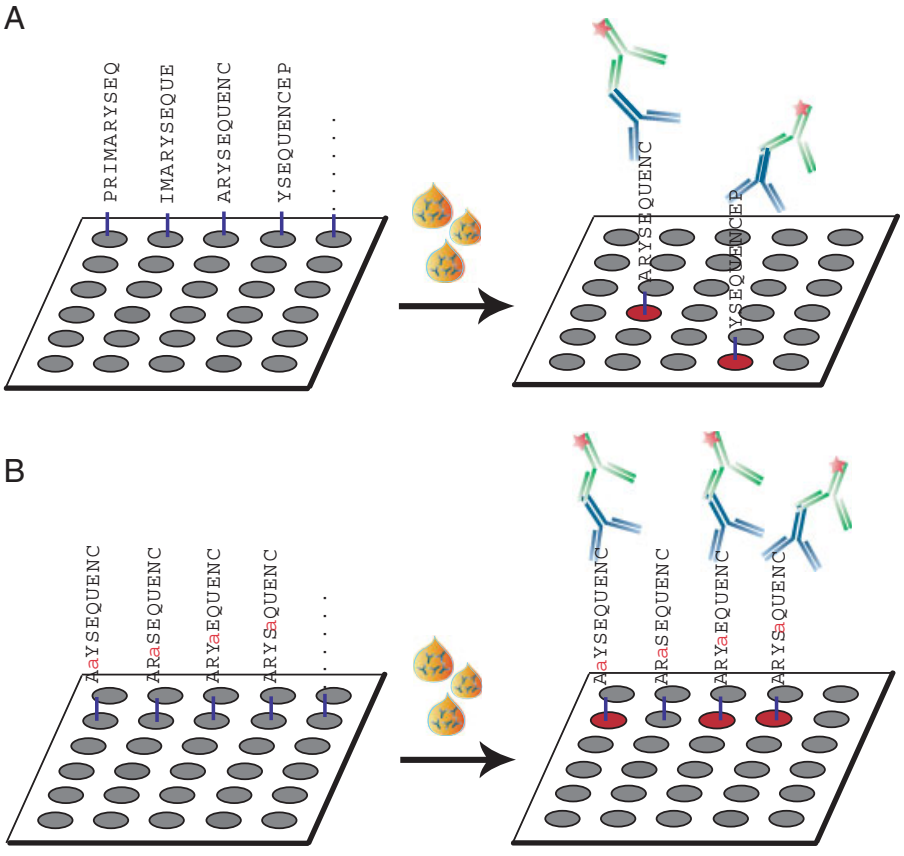

Figure 5. Mapping of linear B-cell epitopes and mutational analysis by SPOTs technology. (A) Overlapping peptides of entire allergen sequence synthesized on membranes are incubated with pooled human sera. IgE-binding peptides are identified by immunoenzymatic or radioactive detection. (B) Crucial IgE-binding amino acids are determined by sequential amino acid exchange followed by incubation with human serum.

allergen with a few amino acids offset are synthesized on derivatized membranes by repeated cycles of coupling, blocking, and deprotection reactions. These membranes are then incubated with pooled human sera and IgE-binding peptides are identified by immunoenzymatic or radioactive detection. Critical amino acids of an immunodominant epitope can be identified with the same technology by synthesizing multiple peptides with single amino acids being sequentially replaced by an alanine, glycine, or glutamine. Among the first plant food allergens to be characterized by this combinatorial approach of epitope mapping and mutational substitution analysis were the major peanut allergens Ara h 1, 2, and 3 [88–90]. However, major limitations are that large quantities of sera are required and the loss of information resulting from the frequent need to use pooled rather than individual serum samples. Adapting SPOTs technology to protein microarray format allowed large-scale IgE epitope mapping of individual patient sera. This refinement was introduced for parallel IgE-epitope analysis of Ara h 1, 2, and 3 revealing a remarkable heterogeneity in epitope recognition pattern between 77 patient sera [91]. This microarray-based immunoassay format has also been exploited to detect a correlation between peanut-specific IgE and IgG_4 epitope reactivity to Ara h 1, 2, and 3, with clinical severity [92, 93]. Besides peanuts, severe allergic reactions have also been described upon ingestion of tree nuts, especially cashew, walnut, and pecan [94, 95]. Linear immunodominant epitopes were mapped from various seed storage proteins including English walnut Jug r 1 (2S albumin) and cashew Ana o 1 (7S globulin), Ana o 2 (11S globulin), and Ana o 3 (2S albumin) [96–99]. Interestingly, all can be classified as complete food allergens, as homologues in pollen have not been found. The importance of the identified nut allergen epitopes is in good agreement with the theory suggesting that linear epitopes play a major role in class I food allergy.

Another important source of food allergens is soybean, a member of the legume family with more than 20% of its seed dry weight composed of glycinins. Several linear IgE-binding epitopes of P34/Gly m BD 30 K and two glycinin subunits G1 +2 have been identified by SPOTs technology [100, 101]. Interestingly, the two glycinins were found to share a conserved IgE epitope with peanut allergen Ara h 3, which might account for severe reactions to soybean in patients with known or latent peanut allergy [102, 103]. Finally, the consumption of wheat flour has been associated with severe allergic reactions such as wheat-dependent exercise-induced anaphylaxis. Due to the severity of the reaction, an increased allergen-uptake through the gastro-intestinal system was proposed. Accordingly, epitope mapping of wheat ω-5 gliadin revealed several immunodominant epitopes and determination of specific-IgE was reported to be a promising tool for diagnosis [104]. Summarizing, many sequential IgE-binding epitopes of various major food allergens have been mapped using SPOTs technology. However, as most B-cell epitopes are described to be conformational in nature, peptides identified by sequential approaches may render limited information and exhibit reduced antibody-binding affinity [105].

3.2.2 Conformational IgE-binding epitopes

Conformational epitopes are of major importance in pollen-related food allergy as they provide structural features for cross-reactive IgE-binding [81]. They are composed of non-adjacent amino acids in the allergen sequence brought into proximity by folding of the polypeptide. Therefore, systematic characterization of conformational epitopes is a much more difficult task compared with the identification of linear epitopes. In the past, the issue of conformational IgE epitopes has been addressed by investigating influence of heat denaturation and reduction of disulfide bonds as early demonstrated on the house dust mite-allergen Der p 2 [106, 107]. However, with these methods the exact localization of IgE epitopes remains obscure, as loss of allergenic activity simply relies on destruction of conformational epitopes. In a more sophisticated approach utilizing mutational analysis and IgE-inhibiting monoclonal antibodies to investigate IgE cross-reactivity within the PR-10 family, *i.e.* Pru av 1 and Api g 1, a conformational epitope located in the P-loop motif was identified [108]. Another recently published strategy to investigate cross-reactive B-cell epitopes involved in pollen-associated food allergy was the construction of a chimeric molecule by grafting selected short stretches of Mal d 1 onto Bet v 1 [109]. IgE reactivity to this construct was tested by ELISA and showed significant less reactivity of sera from birch-allergic donors compared with sera from birch-allergic patients with concomitant apple allergy.

Phage display methodology, which can be generally applied for the identification of both, linear and conformational epitopes, enabled precise localization of conformational IgE-binding epitopes of within the PR-10 family [110]. In this study, patient-specific IgE epitope patterns were identified and cross-reactive epitopes were compared between Bet v 1, Gly m 4, Ara h 8, and Pru av 1. Furthermore, a similar approach combining the use of a random phage display library with 3-D modeling identified conformational mimic epitopes, termed ''mimotopes'', of the peach LTP, Pru p 3 [111].

As characterization of conformational B-cell epitopes depends on knowledge of the tertiary structure, NMR-based methods, such as hydrogen exchange NMR, have been introduced for mapping antibody binding sites. Interacting amino acid positions between antibody and allergen can be measured, because antibody-binding results in a reduced rate of amide hydrogen exchange. This method was successfully used for the identification of IgE-binding epitopes of the house dust mite major allergen Der p 2 [112]. Although this technique has not been applied for plant food allergens yet, it represents a very promising tool.

The phenomenon of reduced hydrogen exchange rate has been employed in amide deuterium exchange mass spectrometry for mapping protein–protein interfaces and studying

structural dynamics [113]. Deuterium on the labeled allergen can be exchanged with hydrogen in H_2O, while this process is prevented in the IgE-binding epitope (Fig. 6). By adjusting the sample to a low pH and 4°C, a shift toward slow exchange conditions is achieved, and the amides within the interface remain deuterated. Subsequently, localization of deuterated amino acids is elucidated by progressive proteolysis with pepsin followed by nanoLC-MS/MS-based peptide mapping. One advantage over NMR is that MS-based proteomic technology is more readily accessible. So far, this method has not been used for the characterization of plant food allergens but was successfully applied to map subunit interactions of protein kinase A [114].

The ultimate method for elucidating B-cell epitopes, in specific conformational epitopes, is represented by crystal structure determination of complexes of allergens with specific Fab fragments, as it has been reported for Bet v 1 [115] and bee venom hyaluronidase [116] using a murine Fab fragment. It has to be emphasized that the identified B-cell epitopes do not necessarily correspond to B-cell epitopes recognized by serum IgE. A more advanced approach has been reported for the major grass allergen Phl p 2 [117] using specific human IgE Fab isolated from a combinatorial library constructed from lymphocytes of a pollen-allergic patient. However, no food allergen has been mapped so far using this high standard methodology.

3.3 *In silico* prediction and mapping of B-cell epitopes

Experimental data obtained by B-cell epitope mapping are nowadays collected in databases facilitating bioinformatics-based predictions of IgE-binding sites [18, 118]. Thus, pure theoretical antigenicity prediction schemes could be replaced by experimentally determined IgE-binding sequences and controversies between different algorithms would be solved [84, 85]. However, major limitations are that the list of IgE-binding epitopes is incomplete and that conformational epitopes are not included in these databases [81].

Nevertheless, available data on elucidated structures and determined sequences can be utilized for molecular modeling and *in silico* prediction of IgE-binding regions. In a recent approach combining experimentally obtained linear sequence data with 3-D models, potential IgE cross-reactivity between Ara h 2 and structurally related walnut Jug r 1, pecan nut Car i 1, and Brazil nut Ber e 1 was investigated. Mapping of the IgE-binding epitopes on the surface of Ara h 2 was followed by conformational analysis of the corresponding regions of the tree nut allergens. According to this study, 2S albumins are not predicted to be involved in cross-reactivity observed between walnut and Brazil nut with peanut, as no structurally homologous linear epitopes were found [119]. Another approach utilizing bioinformatics and molecular modeling of Ara h 3 combined with surface mapping of linear IgE-binding epitopes identified two conserved epitopes potentially accounting for cross-reactivity to other legumins. Interestingly, some of the epitopes were shown to comprise extended epitopic regions on the molecule surface with potential implication of enhanced binding capacity and variability of recognition sites [120].

Within the group of Rosaceae fruits, surface mapping of IgE-binding epitopes of LTPs from apple (Mal d 3), apricot (Pru ar 3), peach (Pru p 3), and plum (Prus d 3) identified by SPOTs technology revealed four consensus IgE-binding

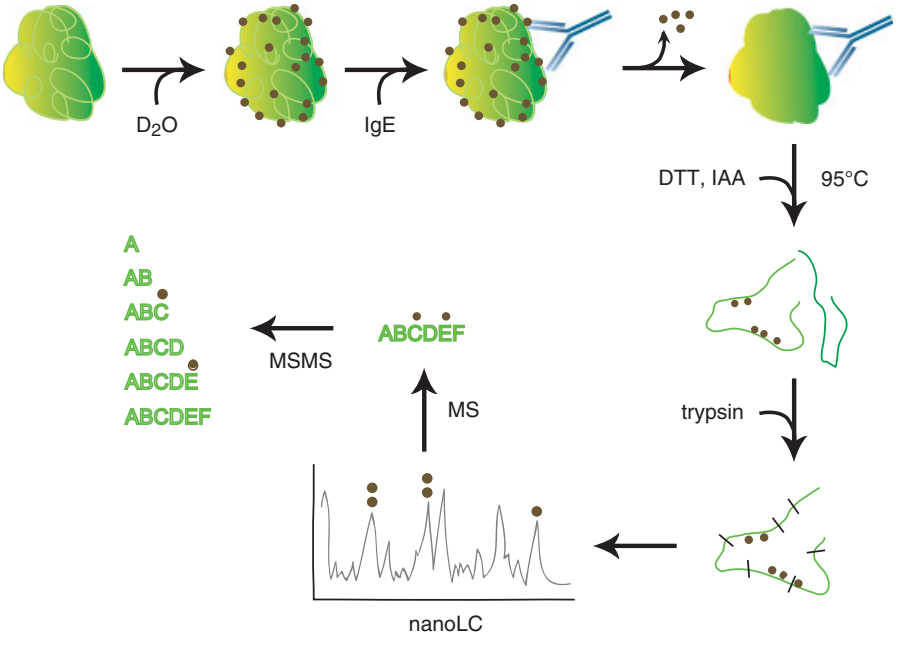

Figure 6. Mapping of conformational IgE-binding epitopes by deuterium exchange MS. Deuteration of allergen by incubation in D_2O followed by incubation with IgE. Deuterated amides involved in IgE-binding resist off-exchange. Deuterated amino acid residues forming conformational IgE-epitopes are identified by trypsin digestion, RP-LC, and MS/MS. DTT (reducing reagent); IAA, iodoacetamide (alkylation reagent).

epitopes covering more than 40% of the molecular surface [121]. Similar to Ara h 3, these epitopes also comprise extended conformational epitopes potentially contributing to strong allergenicity reported for Rosaceae LTPs [122].

Computational techniques are also increasingly utilized to investigate the molecular basis of sensitization and cross-reactivity in the profilin panallergen family. Based on the calculation of solvent-accessible surface areas and structural homologies, two highly cross-reactive IgE epitopes were predicted for celery Api g 4 and bell pepper Cap a 2 [123]. However, although good correlation with inhibition data was found, no strong connection to clinical cross-reactivity could be established. Similarly, strong and weak IgE-binding epitopes have been located on the melon profilin Cuc m 2 [124].

3.4 Allergenic potency of IgE-binding epitopes

Identification and characterization of immunodominant regions representing IgE-binding epitopes are prerequisite to understand the interactions of allergens with the immune system. Thus, multiple IgE epitopes found distributed throughout an allergen were in agreement with the fact that divalency is a minimum requirement for eliciting an allergic response in sensitized patients [125]. A major limitation of *in vitro* serological tests used to investigate IgE binding is that they provide no information on the biological potency of IgE reactivity. Latter is the result of efficient IgE receptor cross-linking triggering immediate release of potent vasoactive mediators (histamine and sulfidoleukotrienes) and depends, in part, on the affinity between specific IgE and the respective allergens [126] and the geometry of the epitope array [127]. Cross-linking capacity of allergens can be measured by an array of cell-based formats like β-hexosaminidase determination with rat basophil leukemia cells transfected with the α-chain of the human high-affinity IgE receptor [128], histamine release assays with autologous or passively sensitized heterologous human basophiles [68, 69, 129, 130], or sulfidoleukotriene release assays with peripheral blood mononuclear cells (CAST) [65, 131]. Furthermore, the human LAD2 mast cell line [132] has been reported as important tool for investigating allergic disease [131]. An alternative readout of allergenic activity is to analyze upregulation of basophil-specific activation markers CD63 [133, 134] or CD203c [135, 136] by flowcytometry (BAT). BAT and CAST have been mainly used to address clinically relevant research issues such as component-resolved diagnosis of pollen-associated food allergy [66, 133, 134]. Potentials and pitfalls of these methods are extensively discussed [69, 137–140].

These cell-based methods are important tools to differentiate biologically relevant cross-reactive IgE epitopes from cross-reactive IgE not capable of eliciting an effector cell response in pollen-related food allergy [141–144]. Furthermore, they have been used to show unchanged allergenicity after treatment with digestive enzymes of the major class I peanut allergens Ara h 2 and six raising speculations on their potential to sensitize atopic individuals [16]. In contrast, PR-10 family allergens of apple, hazelnut, and celery were shown to lose their allergenic potential upon digestion with pepsin and trypsin [145]. Intriguingly, this digestive treatment did not destroy the T-cell-activating capacity of these PR-10 family allergens, a topic leading over to methods to investigate T-cell-mediated reactivity.

3.5 T-cell epitope mapping

To date, many recombinant food allergens have been produced. The availability of cDNAs and recombinant allergens allowed investigations of T-cell responses to single allergens and mapping of T-cell epitopes. Generally, T-cell epitopes represent short, linear peptides in the primary sequence of a protein, which, in case of allergens, are presented on MHC class II molecules. To identify T-cell determinants, synthetic peptides covering the entire primary sequence of the allergen are routinely used to stimulate specific T-cell lines (TCLs) or T-cell clones (TCCs) generated from peripheral blood mononuclear cells [146] (Fig. 7). MHC class II molecules predominately bind peptides of 12–15 amino acids, and therefore, synthetic peptides for most studies were designed in a favorable length (10–20 residues). Peptides should furthermore contain broad overlaps of the primary sequence to ensure that epitopes are not excluded by experimental design.

Detailed investigations on the cross-reactivity of Mal d 1, Api g 1, and Cor a 1 at the T-cell level were conducted in comparison to Bet v 1 [147–149]. Bet v 1 and food-specific TCLs and TCCs were stimulated with 12-meric peptides covering the entire sequence of each allergen. T-cell epitopes identified in food allergens corresponded to T-cell-activating regions previously found in pollen Bet v 1. Interestingly, T-cell cultures induced with Mal d 1 and Api g 1 proliferated more pronouncedly in response to Bet v 1 than to food proteins, suggesting that Bet v 1-related proteins comprise class II food allergens [150]. Though members of the PR-10 family undergo rapid gastric digestion leading to total loss of IgE-binding activity, small peptides representing T-cell epitopes may survive gastrointestinal degradation. In fact, fragments of Mal d 1, Api g 1, and Cor a 1 obtained after treatment with pepsin and trypsin were still able to activate Bet v 1-specific TCLs and TCCs [145]. Such epitopes were proposed to be clinically relevant for pollen-allergic patients without oral symptoms, as they might stimulate pollen-specific T cells throughout the whole year [151]. From the LTP family T-cell epitope mappings were performed from Pru p 3 by two study groups with patients from Southern Europe [152, 153]. Although different epitopes were identified, a dominant TH2-type cytokine response was assayed from supernatants of peach-allergics by both study groups.

In regard to class I food allergens, Ara h 2 was investigated using peanut-specific TCLs, and proliferative

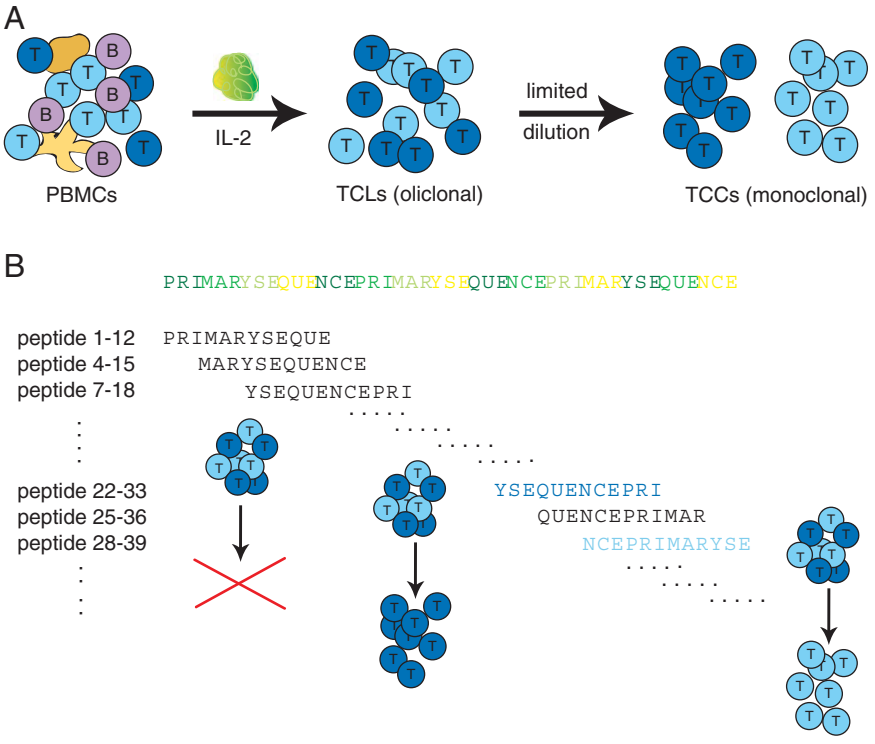

Figure 7. T-cell epitope mapping. (A) Oligoclonal TCLs are cultivated from peripheral blood mononuclear cells by stimulation with allergen and IL-2. TCCs specific for a single epitope are obtained by limited dilution. (B) Relevant T-cell epitopes are identified by proliferation of T cells upon stimulation with overlapping peptides of allergen sequence. T-cell proliferation is measured by ^{3}H-thymidine incorporation.

responses were assessed with overlapping 20-meric peptides covering the entire molecule. In total 8 out of 17 peptides showed significant proliferation, and T-cell reactivity was shown to be clustered in two regions (19–47 and 73–119). Interestingly, a major IgE-binding epitope was located in region 27–36, which overlapped with one prominent T-cell epitope. A potential use for T-cell peptides in treatment of peanut allergy was proposed, but further clarification of the precise localization regarding this epitope is needed. In principle, fragments with single amino acid exchanges can be exploited to identify critical residues necessary for T-cell receptor or MHC class II binding, such as shown in for the ryegrass pollen major allergen Lol p 5 [154]. However, synthetic overlapping peptides currently used in T-cell epitope mapping cannot reflect naturally processed MHC-bound peptides in regard to size, quality, and relative proportion. Therefore, *ex vivo* pulsing of antigen-presenting cells followed by MHC peptide elution and MS-assisted sequencing offers the possibility to identify naturally processed T-cell determinants [155]. Nevertheless, so far, no naturally processed food allergen-derived MHC ligands have been identified.

Finally, databases fed with sequence data obtained by experimental T-cell epitope mapping and the fact that T-cell epitopes as well as many B-cell epitopes represent short linear peptides made *in silico* prediction of these epitopes feasible. A summary of immunoinformatic resources including web-based interactive tools for T-cell epitope prediction and B-cell analysis is given elsewhere [118].

4 Concluding remarks

Allergy to plant-derived foods is caused by a still unknown interaction of molecular properties rendering proteins allergenic and the susceptibility of individuals to become sensitized. Deducing general aspects from the investigation of plant food allergies is additionally complicated by geographical factors, regional dietary habits, and food preparation. Thus, knowledge of molecular features of allergens, including their stability to heat, acidic conditions, and proteolytic digestion, is a prerequisite for understanding allergic sensitization to plant foods. In terms of elucidating the central question "what makes an allergen an allergen" these features have to be linked to the capacity of the molecule to cross-linking IgE receptors on effector cells.

Detailed characterization of plant food allergens using a broad array of physicochemical techniques in combination with immunological data and a cautious interpretation is necessary for revealing new aspects on allergenicity. Accordingly, heat-treatments leading either to chemical modification or to aggregation have been reported to influence IgE reactivity [13, 61]. Other interesting examples involve surface modeling of linear IgE-binding epitopes forming epitope clusters that were shown to account for enhanced IgE binding [119, 120]. Also on the T-cell level, interesting observations have been made indicating T-cell stimulation of Bet v 1-allergics to occur throughout the year, as peptides derived from plant food allergens of the PR-10 family obviously survived protease digestion [147–151].

Moreover, comprehensive physicochemical and immunological data of plant food allergens can be collected in databases created for putative prediction of cross-reactivity and allergenicity of novel plant foods introduced by biotechnological industry [18, 126]. As increasing numbers of purified natural and recombinant (hypo)allergens are becoming available for the substitution of allergenic extracts in diagnosis and therapy, their extensive characterization according to physicochemical and immunological parameters is mandatory [156].

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