

Molecular mechanisms in allergy and clinical immunology

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Molecular properties of food allergens

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Plant food allergens belong to a rather limited number of protein families and are also characterized by a number of biochemical and physicochemical properties, many of which are also shared by food allergens of animal origin. These include thermal stability and resistance to proteolysis, which are enhanced by an ability to bind ligands, such as metal ions, lipids, or steroids. Other types of lipid interaction, including membranes or other lipid structures, represent another feature that might promote the allergenic properties of certain food proteins. A structural feature clearly related to stability is intramolecular disulfide bonds alongside posttranslational modifications, such as N-glycosylation. Some plant food allergens, such as the cereal seed storage prolamins, are rheomorphic proteins with polypeptide chains that adopt an ensemble of secondary structures resembling unfolded or partially folded proteins. Other plant food allergens are characterized by the presence of repetitive structures, the ability to form oligomers, and the tendency to aggregate. A summary of our current knowledge regarding the molecular properties of food allergens is presented. Although we cannot as yet predict the allergenicity of a given food protein, understanding of the molecular properties that might predispose them to becoming allergens is an important first step and will undoubtedly contribute to the integrative allergenic risk assessment process being adopted by regulators. (*J Allergy Clin Immunol* 2005;115:14-23.)

Key words: Food allergens, ligand binding, membrane interaction, protein stability, protein mobility, glycosylation, glycation, repetitive sequences, aggregation, rheomorphism

Abbreviations used

FAO: Food and Agriculture Organization of the United Nations
βLg: β-Lactoglobulin
nsLTP: Nonspecific lipid-transfer protein
PR: Pathogenesis-related protein
SPT: Skin prick test
TLP: Thaumatin-like protein
WHO: World Health Organization

Recent epidemiologic data suggest that almost 4% of the American population have food allergy.¹ This prevalence has notably risen from the previously reported 2%.² Food allergies are most prevalent during infancy, affecting up to 6% of young children.³ In a recent update on food allergy, Sampson⁴ stated that although the diversity of the human diet is enormous, there are few foods that account for the majority of food allergies. In this context it is worth noting that plant food allergens also fall into rather few protein families.⁵ The scientific community and regulatory authorities are both highly interested in assessing the allergenic potential of a given protein, an essential facet of the safety assessment of novel foods, including those derived from biotechnology.

To this end, there has been considerable interest in using bioinformatic approaches to identifying potential allergens.⁶ A joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) expert consultation on foods derived from biotechnology held in 2001 on the evaluation of allergenicity of genetically modified foods (www.fao.org/es/ESN/food/pdf/allergygm.pdf) proposed that a query protein be regarded as potentially cross-reactive if it has an identity of at least 6 contiguous amino acids or more than 35% sequence similarity over a window of 80 amino acids when compared with known allergens. Subsequently, Hileman et al⁷ analyzed a total of 59 proteins using the FASTA algorithm⁸ and suggested that an 8-amino-acid window was more appropriate because it reduced the frequency of spurious matches. A new strategy to predict allergenic proteins on the basis of 52 sequence motifs identified from

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a comprehensive allergen database combined with a pairwise sequence alignment was proposed by Stadler and Stadler.⁹ This method identified potential allergens with greater sensitivity and improved precision compared with other methods. Recently, Soeria-Atmadja et al¹⁰ introduced new bioinformatic procedures to be used as part of an integrated assessment scheme for potential protein allergenicity. These *in silico* methods generally provide a quantitative measure of the global similarity of a given protein sequence with that of known allergens. Two other types of approach have been described that are aimed at identifying potential IgE epitopes, especially relevant to identifying potential cross-reactive allergens. One combines homology searching to identify short contiguous sequences between allergens and query proteins, followed by an analysis of their potential antigenicity.¹¹ Another combines sequence and structural information to identify whether query sequences match known IgE epitopes. This has been used to identify potential cross-reactive IgE epitopes for the thaumatin-like pathogenesis-related protein (PR) 5 protein allergen of cedar pollen (Jun a 3).¹²

However, as is emerging for inhalant allergens,¹³ the structure and function of a protein and the habits of food consumption coupled with any genetic predisposition of a given individual to become atopic, rather than sequence per se, are probably more important determinants of food protein allergenicity. This is reflected in the more recent deliberations of the third session of the FAO/WHO Codex Alimentarius Commission Ad Hoc Intergovernmental Task Force on Foods Derived from Biotechnology (FAO/WHO Report of the third session of the Codex Ad Hoc Intergovernmental Task Force on Foods derived from Biotechnology, ALINORM 03/34, March 4-8, 2002, [ftp://ftp.fao.org/codex/alinorm03/Al03_34e.pdf](http://ftp.fao.org/codex/alinorm03/Al03_34e.pdf)), during which it was decided not to elaborate the decision tree approach. Because no single criterion is sufficiently predictive of allergenicity, they recommended that the risk assessment process should adopt an integrated stepwise case-by-case approach that takes account of information of several types. Thus, Aalberse¹⁴ simply considered structural aspects of allergens using a proximity plot as a 2-dimensional representation of protein structure. He came to the conclusion that allergens have no characteristic structural features that set them aside from all other proteins. The author, however, did not address molecular properties that might be shared by food allergens. When studying the question of what establishes a protein as an allergen, Bredehorst and David¹⁵ concluded that functional aspects of some allergens might play a role in sensitization and the allergic response beside the structural aspects of allergens. In a recent review, Bannon¹⁶ discussed some biochemical characteristics associated with food allergens, such as the presence of multiple, linear IgE-binding epitopes and the resistance of the protein to digestion and processing.

Recent years have seen an extensive effort in the allergy community to identify and characterize many food allergens. Do they show common properties and functions, and how might they influence allergenic potential? It is the aim

of this review to summarize, with examples, a number of potentially important functional and physicochemical characteristics shared by many food allergens that might promote allergenicity.

LIGAND BINDING

A number of food allergens are able to bind ligands, ranging from metal ions to lipids. Certain ligands, such as metal ions, become integrated into the 3-dimensional structure of a protein, often buried deep within the molecule. The loss of a metal ion frequently disrupts protein folding, with an increase in polypeptide mobility and a transition in some instances into a partially folded form. Some proteins form a cavity into which a ligand fits: this might be a metal ion, steroids, or a variety of lipid molecules. Other proteins possess a tunnel into which ligands fit, whereas yet others bind ligands through superficial surface interactions. Ligand binding can have the overall effect of reducing mobility on the polypeptide backbone, increasing both thermal stability and resistance to proteolysis, with many proteases requiring flexibility in substrate proteins. Proteins such as the lipocalins and nonspecific lipid-transfer proteins (nsLTPs), which possess a lipid-binding pocket, show increased stability when the pocket is occupied. Thus the thermostability of β -lactoglobulin (β Lg) increases on lipid binding,¹⁷ as does that of the nsLTP of wheat.¹⁸ These properties are described in more detail below for several notable allergens, including the major fish allergen parvalbumin, the cow's milk allergens casein and β Lg, and the nsLTPs and Bet v 1 homologues found in a variety of plant foods.

Parvalbumins

A widely found calcium-binding domain is known as the EF-hand,¹⁹ a motif that consists of a 12-residue loop flanked on both sides by a 12-residue α -helical domain. Parvalbumins comprise 3 such EF-hand motifs,²⁰ 2 capable of binding calcium and the remaining silent domain forming a cap covering the hydrophobic surface of the 2 functional domains (Fig 1, A).²¹ Abundant (up to 5 mg/g fresh weight) proteins in the white muscle of many fish species, parvalbumins, are thought to be important for the relaxation of muscle fibers through the binding of free intracellular calcium.²² Loss of the protein-bound calcium triggers a large change in conformation and an associated loss of conformation-dependent IgE epitopes.²³⁻²⁵ Parvalbumins show a remarkable resistance to heat, denaturing chemicals, and proteolytic enzymes.²⁶ However, although circular dichroism analysis of carp parvalbumin revealed a remarkable stability and refolding capacity of the calcium containing form after heating,²⁵ the IgE-binding activity of canned fish has been estimated to be 100 to 200 times lower than in boiled fish.²⁷ Nevertheless, sufficient IgE-reactive epitopes remain after cooking to trigger allergic reactions in individuals, as demonstrated by the double-blind, placebo-controlled food challenge performed by Bernhisel-Broadbent et al.²⁸

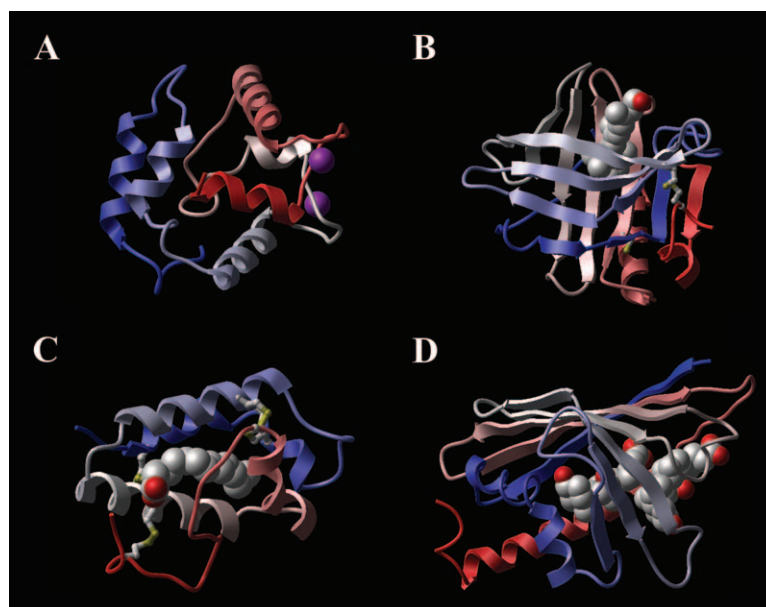


FIG 1. Ligand binding. Ribbon representations of allergens colored by a blue-red gradient from the N-terminus to the C-terminus, with ligands and disulfides shown. **A**, Carp parvalbumin (PDB code 4CPV), with the 2 calcium sites in purple. **B**, Bovine β Lg, Bos d 5, with bound retinol (1GX8). **C**, Corn nsLTP, Zea m 14, with a single bound palmitic acid (1MZM). **D**, Birch pollen allergen Bet v 1l with 2 molecules of deoxycholate (1FM4).

Caseins

Major cow's milk allergens, caseins, also bind calcium but in a very different way than parvalbumins. Clusters of phosphoserine residues, phosphothreonine residues, or both in α_{s1} -, α_{s2} -, and β -caseins chelate metal ions, including calcium. These form microstructures called nanoclusters²⁹ in which amorphous calcium phosphate is sequestered within a shell formed from casein polypeptides. These then assemble into much larger structures comprising around 1000 nanoclusters that correspond to the casein micelles found in milk.³⁰ Such structures allow calcium levels in milk to exceed the solubility limit of calcium phosphate. The significance of nanoclusters for determining the allergenic potential of caseins remains to be shown, although dephosphorylation reduces IgE binding to caseins.³¹

β Lg

β Lg belongs to the lipocalin superfamily of extracellular lipid-binding proteins and is one of the best characterized lipid-binding proteins known (Fig 1, B). It binds a diverse range of molecules, including retinol and its analogues, β -carotene, saturated and unsaturated fatty acids ranging in chain length from C12-C18, and aliphatic hydrocarbons.³² The affinities for these compounds range from $4.8 \times 10^5 \text{ M}^{-1}$ for heptane to $4.8 \times 10^7 \text{ M}^{-1}$ for retinol.³² These ligands bind in the central calyx of the characteristic lipocalin β -barrel,³³ and it has been proposed that ligand binding is determined by a pH-dependent opening-closing of the EF loop, akin to a lid opening on the ligand-binding site.³⁴

nsLTPs

Unlike β Lg, nsLTPs are generally able to bind lipids in a tunnel lined with hydrophobic residues running through the protein (Fig 1, C) that varies in size for the different nsLTPs structurally characterized thus far. Wheat nsLTP binds a range of fatty acids and phospholipids varying in chain length with high affinity, with dissociation constants in the order of 0.2 to 0.3 μM , depending on the lipid.^{18,35} The cavity is highly plastic and can bind a wide range of lipophilic molecules, including sphingolipids, prostaglandins, amphotericin B, and other hydrophobic drugs.^{36,37} It can actually accommodate 2 lipid molecules, which, on the basis of molecular modeling, appear to lay side by side in the tunnel.¹⁸ These properties seem to be a general feature of nsLTPs and might be related to their putative role in plants in transporting lipids involved in the synthesis of waxy cutin and suberin layers in outer plant tissues in seeds and pollen. The 3-dimensional scaffold found in nsLTPs is characteristic of the prolamin superfamily, and it is becoming evident that in some forms of the protein (eg, AceAMP1 from onion roots), the tunnel has been lost, along with the ability to bind lipids, the cavity being filled up with tryptophan and phenylalanine side chains.³⁸

Bet v 1-homologous food allergens

The first indication that Bet v 1-homologous proteins might be involved in the transport of steroid ligands came from the discovery of a high structural similarity between Bet v 1 and the steroid-binding domain of the human MLN64 protein, which possibly plays a role in promoting steroidogenesis in the placenta and brain.³⁹ This structural

homology led Neudecker et al⁴⁰ to use NMR spectroscopy to indicate that the Bet v 1 homologue from sweet cherry, Pru av 1, interacted with phytosteroids. Molecular modeling showed that the hydrophobic cavity of Pru av 1 is large enough to hold 2 such molecules.⁴⁰ Subsequently, Markovic-Housley et al⁴¹ provided evidence that suggested a plant steroid carrier function for Bet v 1 and other PR-10 proteins by showing the interaction of the isoform Bet v 11 with 2 molecules of the plant steroid brassinolide (Fig 1, D).⁴¹ It is generally accepted that oral allergy syndrome symptoms are associated with labile allergens, whereas anaphylactic reactions are attributed to stable food allergens. Generally, pollen-related allergens tend to be labile during heating and digestion.⁴² Surprisingly, the Bet v 1-homologous allergen Gly m 4 of soybean was found to be able to induce an oral allergy syndrome of extraordinary severity and severe systemic reactions in individuals with pollen allergy.⁴³ It is tempting to speculate that the difference in stability between Gly m 4 and other PR-10 allergens might be contributed to by a high-affinity binding of Gly m 4 of its ligands, a theory that will have to be tested in the future.

INTERACTION WITH MEMBRANES AND OTHER LIPID STRUCTURES

Many plant food allergens are also able to associate with cell membranes and other types of lipid structures formed in foods. One commonly observed mode of action whereby proteins can protect plants against microbial pathogens is through destabilization of bacterial or fungal membranes resulting in leakage. Proteins acting in this way include thionins, thaumatin-like proteins (TLPs), 2 types of prolamin superfamily members (2S albumins and nsLTPs), and some defensins.⁴⁴

TLPs

Many plant species that are used for human consumption produce proteins that share sequence and structure similarities with thaumatin, an intensely sweet-tasting protein from the fruit of the West African ketemfe (*Thaumatococcus daniellii*) plant. These proteins are called TLPs. TLPs, also known as family 5 of the PR proteins, have been isolated from a wide range of plant species.⁴⁴ Several allergenic TLPs from fruit and pollen have been described.⁴⁵ Many TLPs have been reported to inhibit the growth of fungi, including the apple allergen Mal d 2.⁴⁶ Although the precise mechanism of such action is not clear, it has been proposed to result from permeabilization of fungal membranes.⁴⁷ TLPs might also interact directly with the lipid bilayer of the fungal plasma membrane, which is composed of sphingolipids, phospholipids, and sterols, components that occur in membranes of all living organisms. The membrane-permeabilizing activity of linusitin, an antifungal TLP from flax seeds, was shown to depend on membrane composition and was amplified by an increased content of negatively charged phospholipids and the presence of sterols.⁴⁸

Prolamin superfamily members

Although a major function of the 2S albumins is undoubtedly as seed storage proteins, other functions have been ascribed to them, including antifungal activity resulting from membrane permeabilization in radish (*Raphanus sativus*) and rape (*Brassica napus*).^{49,50} An elegant study by Oñaderra et al⁵¹ has also shown that the allergenic 2S albumin Sin a 1 from mustard, another member of the *Brassica* genus, interacts with phospholipid vesicles. Around 20 lipid molecules were found to associate with each 2S albumin molecule, with the protein also causing vesicle aggregation, mixing of lipids between vesicles, and leakage. Although acidic bilayers are not generally found physiologically, the extracellular leaflet of intestinal brush boarder membranes contains greater amounts of such phospholipids compared with other plasma membranes.⁵² This led Oñaderra et al⁵¹ to propose that such interactions might affect the uptake and processing of such allergens in the gastrointestinal tract, offering the possibility that the biologic activity of these proteins plays a role in attenuating their allergenic potential.

Similarly, there is emerging evidence that nsLTPs are also able to interact with lipid structures because this is implicit in their ability to transfer lipids between donor and acceptor vesicles,⁵³ although this transfer activity is nonphysiologic. They have been found to interact with several types of lipid structures in model systems,^{54,55} with nsLTPs being able to form bridges between lipid structures. However, the physiologic importance of such interactions in relation to the allergenic potential of nsLTPs remains to be demonstrated.

In addition to interactions with membrane lipids, many food proteins can interact with lipids to form emulsified and other structures. Such interactions are deliberately introduced during the preparation of foods (eg, whey protein or egg proteins interacting with oil to form emulsified sauces, such as mayonnaises). Many allergenic food proteins, including the whey proteins (α -lactalbumin and β Lg), caseins, and various seed proteins, such as 2S albumins⁵⁶ and the 11S and 7S seeds storage globulins of, for example, soy,^{57,58} are widely consumed in foods in an emulsified form. When proteins adsorb to a lipid layer in an emulsion, they unfold, revealing hydrophobic regions of the molecules that favor interactions with lipids. The proteins also aggregate to form a 2-dimensional gel-like layer that has the elastic properties necessary to stabilize oil droplets effectively in an emulsion. Although emulsions and other lipid structures are widely used as adjuvants in the raising of antibodies, nothing is known of their effect on the allergenic potential of foods, but it is evident that any protein presented in this form to the immune systems will be at least partially denatured.

Membrane-bound receptors

One remaining type of interaction between proteins and membranes is through membrane-bound receptors. There are known receptors on the surface of hybridoma lines, with clear evidence of internalization of the protein within

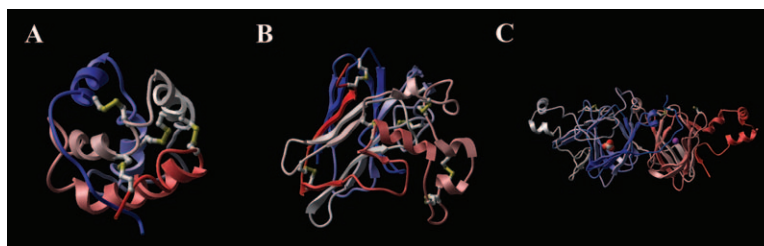


FIG 2. Stability. Ribbon representations of allergens or their homologues colored by a blue-red gradient from the N-terminus to the C-terminus, with cysteines and disulfides shown. **A**, The single-chain 2S albumin from sunflower, SFA-8 (1S6D). **B**, The sweet-tasting protein thaumatin from *Thaumatooccus daniellii* (1RQW) showing the disulfides and the β -barrel. **C**, Soybean glycinin: a single A3A4 subunit of a homohexamer is shown (1OD5). A carbonate ion binds to the N-terminal cupin barrel, and a magnesium ion binds to the C-terminal cupin barrel.

the cell.⁵⁹ It has been proposed that plasma membrane receptors exist in enteroendocrine cells for casein (in particular α -casein), which are responsible for casein-induced, L-type, calcium channel-mediated increases in intracellular calcium observed in an enteroendocrine cell line, STC-1.⁶⁰ Recently, nsLTPs have been demonstrated to bind to ubiquitous plasma membrane receptors in plant cells, and the authors speculated that there might be the possibility of similar interactions taking place with animal membranes that might be relevant to allergenic potential.⁶¹

PROTEIN STABILITY AND MOBILITY

The term *stability* is used to describe the ability of a protein to retain its original, native, 3-dimensional structure after treatments (chemical, such as urea, or physical, such as temperature), as well as resistance to degradation by proteases. No single structural motif is associated with such stability properties, nature having used a breadth of strategies to develop stable proteins. There are some indications that thermostable proteins might have a greater propensity to adopt β -structures and are on average smaller, possibly as a result of their lower heat capacity⁶²; that the shortened loops in the smaller proteins lead to a smaller difference in entropy between the folded and unfolded states, thus stabilizing the former, or both.⁶³

Disulfide bonds

One of the structural features clearly related to stability is disulfide bonds, although the relationship can be complex. In general, both intrachain and interchain disulfide bonds constrain the 3-dimensional scaffold, such that perturbation of this structure by heat or chemical means is limited and frequently reversible. There are some notable food allergens that are highly disulfide bonded, including members of the prolamin superfamily (nsLTP, 2S albumin, and inhibitors of trypsin and α -amylase found in cereals, together with the TLPs).

2S albumins are compact molecules because of the presence of 4 conserved disulfide bonds (Fig 2, A), which exhibit high thermostability and resistance to extreme pH and proteolysis, as indicated by the allergenic 2S albumin from mustard seeds.⁶⁴ The Brazil nut 2S albumin Ber e 1

was also significantly more resistant to proteolytic digestion than any other Brazil nut protein.⁶⁵ Their structural homologues, the nsLTPs, also possess 4 disulfide bonds (Fig 1, C) and are resistant to proteolysis, harsh pH changes, or thermal treatments, refolding to their native structure on cooling.⁶⁶ Thus the allergenic lipid-transfer protein of corn maintained its IgE-binding capacity after cooking at 100°C.⁶⁷ nsLTPs have also been shown to survive the digestive tract as fully IgE-reactive structures that can trigger mast cells in the vicinity of the epithelial monolayer.⁶⁸

Similarly, the conformation of TLPs is stabilized by 8 disulfide bonds (Fig 2, B).⁶⁹⁻⁷² This extensive disulfide cross-linking renders zeamatin, a TLP from corn, highly resistant to proteolysis,⁷³ whereas the allergenic TLP of kiwi was degraded by the simulated gastric fluid within 1 minute,⁷⁴ although such digestion can be dependent on the *in vitro* digestion system used.

A large group of antifungal proteins known as chitinases degrade the polymer chitin, a structural component of insect exoskeletons and fungal cell walls. Chitinases secreted by plants are subdivided into 4 major classes, class I chitinases of fruits being of allergologic relevance.⁵ They contain an N-terminal, cysteine-rich, chitin-binding domain (also found in the latex allergen hevein) linked by a hinge to a highly conserved central region.⁷⁵ The chitin-binding domain is highly conserved, with 8 cysteine residues forming intramolecular disulfide bridges, where the main IgE-binding epitopes are located.⁷⁶ The avocado class I chitinase allergen Pers a 1, which is involved in the latex-fruit syndrome, was extensively degraded in simulated gastric fluid. However, the resulting peptides, particularly those originating from the chitin-binding domain, resulted in positive skin prick test (SPT) responses,⁷⁷ probably because the 4 disulfide bonds hold the digested protein together in an immunologically active structure.

Aspartyl proteases, such as pepsin, require a certain degree of flexibility in their substrates because they act on 6 to 8 residue stretches of polypeptide chain that must lie across its active site in an extended conformation. Thus the structural features, such as extensive disulfide bonding, that increase protein stability, such as compactness with few mobile loops, will also render them poor substrates for proteases, such as pepsin.

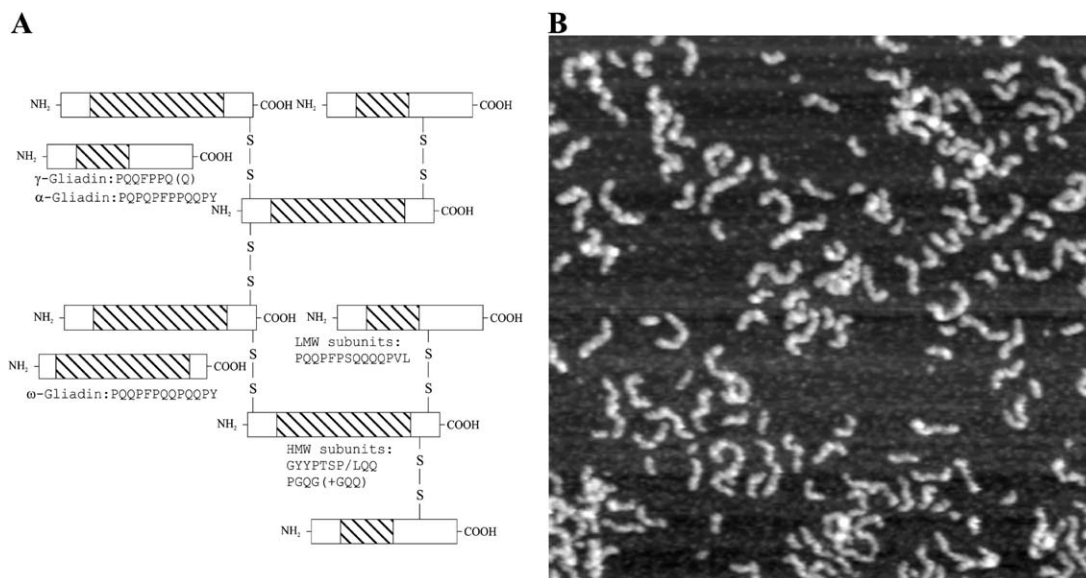


FIG 3. Repetitive structures and aggregates. **A**, Summary of the domain structures of typical wheat seed storage prolamins. The consensus amino acid sequences of the repeat motifs are shown. **B**, Atomic force micrograph of thermally induced aggregates of the 7S seed storage globulin of soy, β -conglycinin. Data analysis was performed on multiple images obtained for β -conglycinin heated to 100°C for 10 minutes.

This is indicated from studies of enzymes, such as thermolysins.⁷⁸ However, absence of disulfide bonds does not indicate a lack of stability because the cupin barrel, for example, is highly stable and possesses no disulfide bonds (Fig 2, C).

Rheomorphic proteins

Much of this review has focused on globular allergens for which we have well-defined 3-dimensional structures. However, a new class of proteins is emerging that contains large regions of disordered structures.⁷⁹ It might also be that certain domains or regions of conventional globular proteins also possess a disordered structure. Such proteins are dynamic, with polypeptide chains adopting an ensemble of secondary structures that are in equilibrium with each other and structurally resemble unfolded, denatured, or partially folded proteins. They can be considered to be rheomorphic, a term coined by Holt and Sawyer⁸⁰ in relation to caseins. As a consequence of their dynamic nature, unlike globular proteins, they do not undergo a sharp transition from one conformational state to another on heating and hence possess many potential thermostable epitopes.

In addition to caseins, the seed storage prolamins can also be considered rheomorphic. Although they possess, to various extents, remnants of the characteristic cysteine skeleton of the prolamins superfamily, this has been disrupted by the insertion of a central domain consisting of repeated sequences (see below) of varying lengths, depending on the type of prolamins.⁸¹ The repetitive domain adopts rapidly interconverting β -turn and β -sheet secondary structures that have a propensity to form aggregates linked by intermolecular β -sheets. It is these structures that are largely responsible for the insolubility of seed storage prolamins because they are actually

hydrophilic in nature and bind water avidly. Like the other rheomorphic proteins, the seed storage proteins do not possess a compact globular structure and therefore do not undergo a conventional transition from native to denatured states when subjected to thermal treatments.

GLYCOSYLATION

Many extracellular proteins (including many food allergens) undergo glycosylation during their passage through the endoplasmic reticulum. The immunologic activity of IgE antibodies directed against the carbohydrate portion of glycoallergens has been a matter of debate since the discovery of N-glycan-specific IgE.⁸² Carbohydrate-specific IgE antibodies of patients with pollen allergy cross-react with virtually all plant foods, without the patients having clinical symptoms. This poor immunologic activity has been confirmed by means of SPTs and *in vitro* basophil histamine release.⁸³ Mari⁸⁴ reported a high prevalence of anti-carbohydrate IgE in patients with multiple pollen sensitizations, yet SPT results and clinical histories confirmed poor immunologic activity of these IgE antibodies.⁸⁴ In contrast, carbohydrate-specific IgE was reported to induce *in vitro* basophil histamine release for Lyc e 2,⁸⁵ a glycoallergen of tomato, as well as for Api g 5, a glycoallergen from celery tuber.⁸⁶ However, in both cases the allergen concentrations needed to achieve mediator release were relatively high.

In addition to the immunologic implications, glycosylation also affects the biologic properties of allergens. N-glycosylation can have a significant stabilizing effect on protein structure,⁸⁷ and there is evidence that it increases the stability of, for example, the 7S globulin of pea and its resistance to chemical denaturation.⁸⁸ The 7S seed storage

globulins are frequently glycosylated, with between 1 and 2 N-linked glycosylation sites being located in the C-terminal domain. The 7S globulin of peanut, Ara h 1, is known to be glycosylated and has one asparagine-linked consensus carbohydrate addition site, the glycan moieties being a heterogeneous mixture of N-glycans, including Man₅₋₆GlcNAc₂ and Man₃₋₄XylGlcNAc₂.⁸⁹ Multiple forms of those 7S globulins possessing more than one glycosylation site have been characterized, notably for the 7S globulin of French bean, phaseolin. This protein has 2 glycosylation sites at Asn 228 and 317 with species that are singly glycosylated at Asn 228 and doubly glycosylated at both asparagine residues having been identified.⁹⁰

The 11S globulins are rarely glycosylated, an exception being that from lupin, in which the major acidic subunit becomes glycosylated.⁹¹ An N-glycosylation consensus sequence resulting from the introduction of serine residue is found in a conserved region toward the N-terminus of the acidic subunit as a result of a single point mutation. Around 1% (wt/wt) carbohydrate becomes attached to the lupin 11S at this site and appears to affect subsequent folding, assembly, and proteolytic processing of the globulin.⁹²

REPETITIVE STRUCTURES, AGGREGATES, AND GLYCATION

Both the presence of repetitive structures and a propensity to aggregate, either under physiologic conditions or as a result of food-processing conditions, might affect sensitization and aggregation, at least by enhancing immunogenicity.⁹³ Elicitation potential might also increase with increasing IgE epitope valency and hence the ability to trigger histamine release by mast cells. Indeed, it appears that aggregates are able to break tolerance to recombinant self-therapeutic proteins, such as IFN- α .⁹⁴ It is also intriguing to note that one major epitope site recognized by parasite-neutralizing antibodies in malaria corresponds to a serine-rich repeat sequence region.⁹⁵ Food allergens with repeating structures include the tropomyosin allergens of shellfish and seed storage prolamins, and many are able to form aggregates.

Repetitive structures

Tropomyosins are a family of closely related proteins present in muscle and nonmuscle cells,⁹⁶ and together with actin and myosin, they play a key regulatory role in muscle contraction. They contain a 7-residue (heptad) repeat of the form a-b-c-d-e-f-g, where a and d are generally apolar residues,⁹⁷ with most isoforms having an unbroken repeat series of 40 continuous heptads. These adopt an α -helical structure, with 2 molecules wound around each other to form a parallel dimeric α -helical coiled-coil structure. These then bond head to tail with a short overlap to form an unbroken coiled-coil cable that winds around the actin helix.⁹⁸ It is tempting to speculate that similar linear epitopes are to some degree unaffected by means of heat denaturation and partial proteolysis. Tropomyosins are heat-stable cross-reactive food aller-

gens. Extracts from boiled *Penaeus indicus* shrimp were found to contain Pen i 1 with unchanged allergenicity⁹⁹ and water-soluble shrimp allergens that had been released into the cooking water during boiling.¹⁰⁰

Repetitive structures are also a characteristic feature of many rheomorphic proteins, with the seed storage prolamins probably exhibiting the most degenerate repeat sequences. These are based on several different short motifs, ranging from 4 to 8 residues in length, which are rich in proline and glutamine (Fig 3, A). Studies with mAbs have shown that they can be highly specific for particular repeat motifs but bind to several seed prolamin polypeptides containing the repeat motifs.¹⁰¹

Aggregates and glycation

The globulin seed storage proteins also share a propensity to form large structures existing in a salt-dependent equilibrium of trimers, hexamers, and (for the 11S globulin of soy) dodecamers, which are held together by non-covalent interactions.¹⁰² A characteristic of seed storage globulins is their ability to form heat-set gel networks after wet-thermal processing, such as boiling. Both 11S and 7S globulins, as with other members of the cupin superfamily, are thermostable. Thus 7S globulins have their major thermal transition at around 70°C to 75°C, and 11S globulins unfold at temperatures of greater than 94°C, as determined by means of differential scanning calorimetry, with precise values varying between plant species, protein concentration, and ionic strength. It seems likely that the cupin barrel remains intact, but the unfolding of other regions of the polypeptides results in a loss of quaternary structure, with concomitant formation of large aggregates.

When these aggregates become sufficiently large, they will form either flocculant precipitates, or, at very high protein concentrations of 5% to 10% similar to those found in food systems, heat-set gel networks.¹⁰³⁻¹⁰⁵ It is likely that heating results in the formation of partially folded molten globule structures that are generally metastable and rapidly go on to form large aggregated structures, becoming kinetically trapped in the unfolded states in the process.

Atomic force microscopy is a technique that allows us to visualize structures at a molecular level and has been used to follow the formation of thermally induced aggregates of β -conglycinin, the 7S globulin of soya.¹⁰⁴ These aggregates have a fibrous and cylindrical appearance with a height (diameter) of 8 to 11 nm and begin to form at temperatures of greater than the main thermal transition for this protein at 75°C, when a small change in secondary structure takes place (Fig 3, B). At higher protein concentrations (1% wt/vol), just below gelling concentrations, the linear aggregates form very large macroaggregates, which might be the precursors of protein gel formation.

Unlike soybean and lentils, peanuts and other nuts are often subjected to thermal processing at low water levels, such as roasting. This will affect protein stability because protein denaturation requires the presence of water, with proteins becoming more thermostable in low-water systems.¹⁰⁶ Thus Ara h 1 purified from residual soluble protein only becomes unfolded on roasting peanuts to

140°C for 15 minutes,¹⁰⁷ although much of the peanut protein becomes insoluble. This is probably because of covalent modification of the proteins through Maillard reactions. This involves sugars reacting with free amino groups on proteins to form Amadori compounds, which might then rearrange to produce a range of adducts, known as advanced glycation-glycosylation end products. In addition to forming during thermal processing and particularly during the application of dry-heating procedures, such as roasting, they are slowly formed over days and months as a consequence of the aging process of foods.

Such products have been shown to destabilize the quaternary structure of proteins, such as reducing the stability at low pH of the triple helix of collagen.¹⁰⁸ Indications are that glycation reactions might be responsible for the apparent increase in allergenic activity of peanuts after processes such as curing and roasting.^{109,110} It is likely that because heating in either wet (boiling) or dry processes (frying) can denature the proteins, modify the proteins, or both, this might underlie the different effects such processes have on the allergenicity of foods. In addition, thermal treatments might result in disulfide exchange and modifications of other amino acids, further cross-linking the proteins.

It seems a general phenomenon that for food allergens thought to sensitize through the gastrointestinal tract, even unfolded proteins can retain their IgE reactivity. This implies they either contain many thermostable epitopes or that individuals have IgE responses primarily toward short continuous epitopes found in cooked material. The presence of large aggregated protein structures in food as it is consumed might potentiate their allergenic properties. Such structures will, by their very nature, contain repeating units and be sufficiently large to contain multiple IgE epitopes, which might trigger histamine release more effectively than soluble monomeric forms. Currently, studies investigating allergenic properties generally focus on soluble proteins, despite the fact that human subjects are generally exposed to insoluble aggregated particulate forms of many allergens that might have very different properties.

CONCLUSION

It is a fact that plant^{5,111} and animal food allergens belong to very few of the several thousand known protein families. The rate at which allergens that belong to additional protein families are identified appears to be decreasing, and it is likely that the bulk of protein families that include allergenic members have already been discovered. This certainly challenges the notion that the allergenic potential of all proteins is equivalent. However, membership in one of a limited number of protein families in itself is not sufficient to determine allergenic activity. It seems that 3 factors work together to result in the sensitization of an atopic individual with any given allergen: (1) the genetic make-up of the exposed person, (2) the structure of the allergen, and (3) the biochemical and physicochemical properties of the allergen. This review describes

the types of properties that might facilitate the presentation of food allergens to the immune system (primarily through the gut) and that include stability enhanced by binding various types of ligands and large numbers of disulfide bonds, by oligomerization or aggregation, by glycosylation, or by potential interaction with cell membranes or lipid structures. Now we are in a position to begin to investigate which of these factors (and others as yet to be identified) truly affect allergenic potential, and the coming years will undoubtedly see us begin to understand what makes an allergen at a molecular level.

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