



Comparative Structural Proteomics of Allergenic Proteins from Plant Pollen

Dinakar M. Salunke^{1,2*}, Jasmita Gill¹ and Alka Dwevedi¹

Abstract | Major sources of allergy from plants are seeds and pollens. A large number of protein antigens have been identified to be causative agents of allergy and possible biochemical and structural attributes of these molecules have been explored. The studies so far have not been able to provide clear mechanistic details that could be generalized. The reason for this could be that the studies have not been systematically explored. The structural proteomics approaches adopted in our laboratory is an attempt in this direction to establish the structural basis of allergenicity. Known crystallographic structures of proteins from plant pollens have been analyzed in this context.

1 Introduction

More than 20 percent of the world's population suffers from allergies. Allergy is a hypersensitivity reaction initiated by immunological mechanisms. Allergy therefore describes a symptomatic reaction to a normally innocuous environmental antigen that may result from the induction of an immune response. Such reactions are mainly categorized into two types: (i) antibody-mediated reactions occurring within minutes of contact with an allergen, predominantly involving antibody (IgE)-mediated mast cell activation and (ii) delayed hypersensitivity reactions involving cell-mediated responses played by sensitized lymphocytes. IgE mediated allergy is also known as type I hypersensitivity. Both genetic and environmental factors contribute to the development of IgE mediated allergy. Very few people show an exaggerated tendency to mount a strong IgE response against antigens that are known to be safe for vast majority of population. This state is called atopy.¹ Atopy has a strong familial basis and is influenced by several genetic loci. Cell-mediated immunological mechanisms seem to be important in allergic diseases such as contact dermatitis and celiac disease associated with gluten sensitivity.¹

Plant proteins are a major source of allergy and majority of plant allergenic reactions are associated with seed and pollen proteins. Evaluating the characteristics of a protein antigen that confers its allergenic properties when it comes in contact with

an atopic immune system is a formidable challenge. It is clear that, in some cases, biochemical properties of the protein itself are important, such as the stability and proteolytic activity of the inhalant allergen (e.g. Der p 1 from house dust mite).² However, huge gaps exist in our understanding of the determinants of allergenic potential, correlation of protein function with allergenicity as well as the distinction between protein elicitors and sensitizers in allergy. We have recently initiated a comparative structural proteomics approach in the determination of allergenic potential of proteins from plant sources. As part of this initiative, structures of a couple of seed proteins linked with allergy have been determined. Indeed, it would be relevant to analyze correlation of structural features with potential IgE binding epitopes, understanding the factors responsible for sensitization process as well as the mechanism of the allergen specific immune reactions. While a large number of protein structures associated with food and pollen have been determined, a systematic analysis providing such a correlation is lacking. Here we have attempted an analysis of a small family of existing structures of pollen allergens towards exploring such a correlation.

All plants produce microscopic pollen grains for reproduction. The plant uses pollen either from its own flowers to fertilize itself, or in some species, cross-pollination occurs where pollen must be transferred from the flower of one plant

¹Regional Centre for Biotechnology, Gurgaon, India.

²National Institute of Immunology, New Delhi, India.

*dinakar.salunke@gmail.com

to that of another belonging to the same species. Plants rely on either insects or wind transport for transferring the pollen. Most common seasonal allergic reactions, including pollinosis and hay fever, are produced by pollens from trees, grasses or weeds that are transported through winds. The birch pollen-related allergy has been studied most intensively among all pollen-associated allergies, is more than 93 percent of clinical data available is based on birch pollen.³

2 Structures of Allergenic Proteins from Pollen

2.1 Immunoglobulin-like family

The three-dimensional structures of immunoglobulin-like fold proteins constitute a poorly conserved and highly unstructured glycosylated N-terminal stretch and two β domains (Fig. 1). The structure adopts a fold comprising six-stranded β -barrel which is majorly stabilized by three disulfide bonds. Structures of pollen allergen protein Phl p 2 from major timothy grass have been

determined (PDB IDs: 1WHO, 1WHP). As was evident from these structures, Phl p 2 has a typical immunoglobulin fold wherein an anti-parallel β -barrel is formed by two four-stranded β -sheets that are sandwiched together (Fig. 1a). Further, a complex of Phl p 2 with antibody Fab has also been determined.⁴ In this structure (Fig. 1b), the interaction between the Phl p 2 epitope and the antibody Fab involves 21 allergen residues and 25 IgE Fab residues, together with eight water molecules creating an extensive interaction interface which is discontinuous and involves allergen residues located mostly within the β strands. Nine of these residues interact directly with CDRs L1, L3, H1, H2 and H3 of IgE Fab largely by hydrogen bonding and van der Waals interactions. It is interesting to note that there is no significant overall conformational change in Phl p 2 upon complex formation.⁴ Superposition of Phl p 2 with two other similar pollen allergens Phl p 1 and Phl p 3 have highlighted the characteristic fold of the immunoglobulin-like fold family (Fig. 1c).

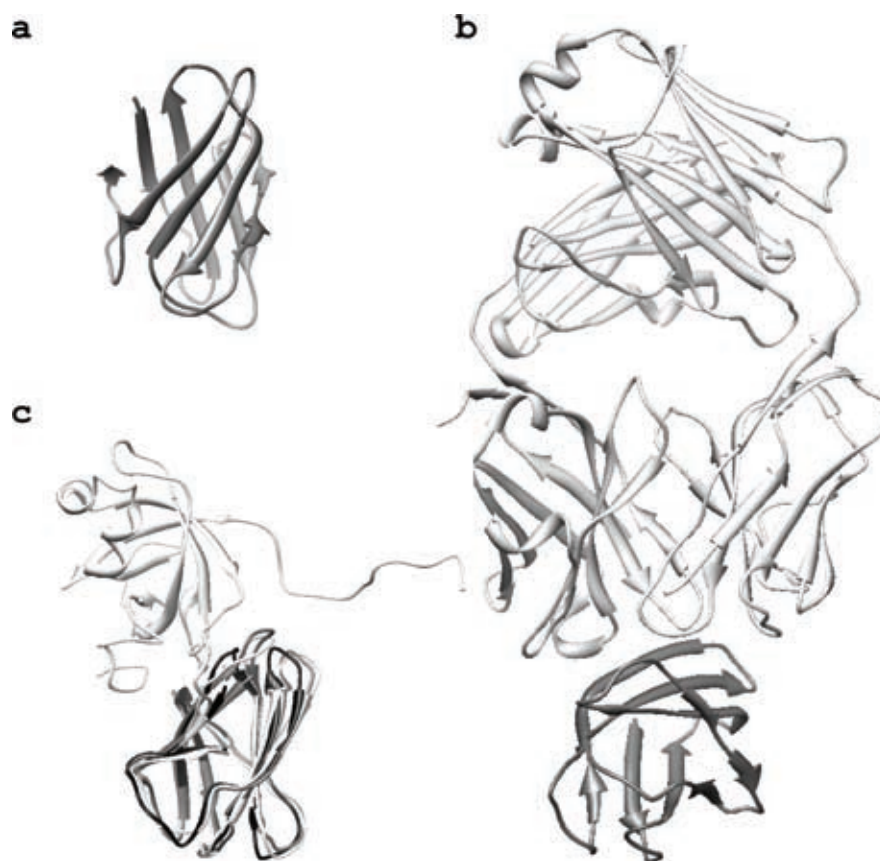


Figure 1: Immunoglobulin-like allergenic proteins. (a) Overall structure of pollen Phl p 2 (PDB ID: 2VXQ) (b) Structure of Phl p 2 in complex with an antibody Fab (PDB ID 2VXQ). Complex is shown as ribbons, Phl p 2 and Fab are colored dark and light grey respectively (c) Superposition of pollen proteins Phl p 2 (black), Phl p 1 (PDB ID: 1N10; light grey) and Phl p 3 (PDB ID: 3FT1; dark grey).

2.2 Bet v 1 related proteins

Majority of the pollen related allergies are associated with Bet v 1 family. The major birch pollen allergen, Bet v 1, is a member of the pathogenesis-related family of plant proteins. The Bet v 1 family is structurally well studied and various three-dimensional structures have been reported from different organisms. The most prominent attribute of the Bet v 1 family⁵ is the presence of a large hydrophobic cavity with an exterior opening, which has been defined as a potential ligand binding site as seen in Fig. 2a & b.

The crystal structure of Bet v 1 protein (Fig. 2c) was first reported from silver birch in complex

with an antibody Fab.⁶ The structure of a hypoallergenic isoform of Bet v 1 called Bet v 1l was reported in complex with two molecules of steroid deoxycholate.⁷ More recently, crystal structures of Bet v 1 variants in complex with an array of ligands and several point mutations have been reported.⁸ All these structures exhibit the classical Bet v 1 fold, consisting of a seven-stranded antiparallel β -sheet, two short α -helices and a long C-terminal α -helix (Fig. 2a–d). This structural arrangement leads to the formation of a large hydrophobic cavity, which is a distinguishing feature of this family. Further, only minor local rearrangements have been observed in Bet v 1l upon ligand binding.⁷

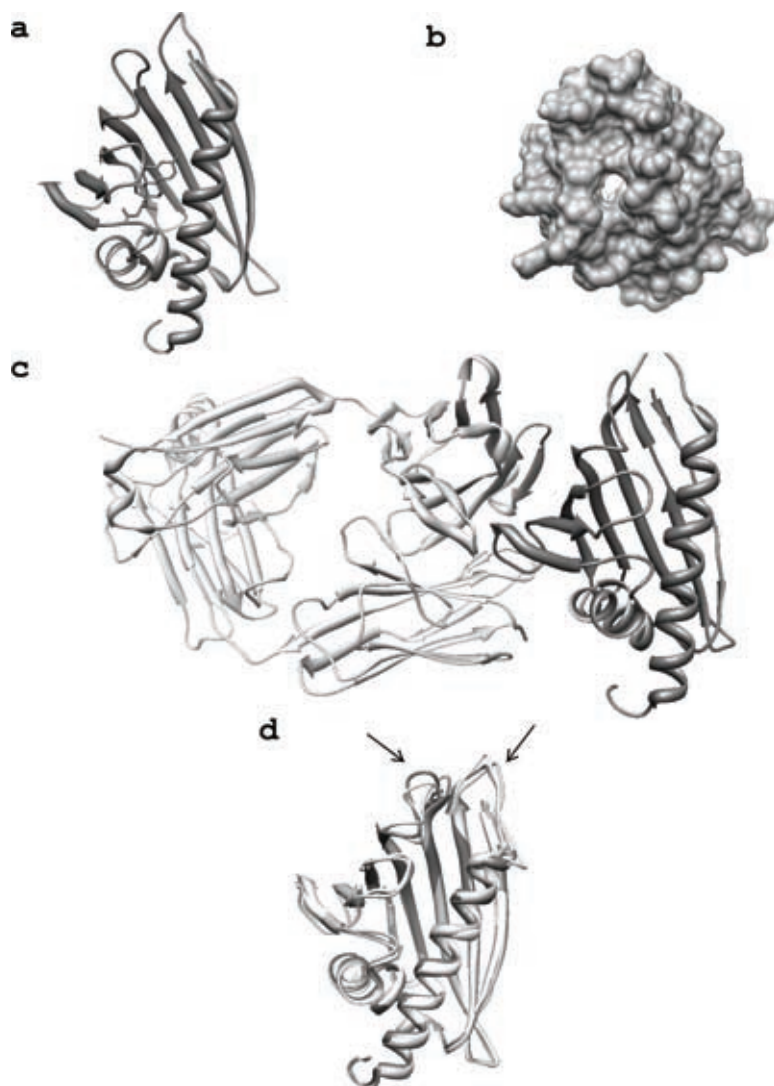


Figure 2: Bet v 1 related allergenic proteins. (a) Overall structure of Bet v 1 protein from silver birch in complex with ligand 8-anilinonaphthalene-1-sulfonate (PDB ID: 4A81). (b) Surface representation of Bet v 1 highlighting the ligand binding hydrophobic cavity. (c) Structure of Bet v 1 in complex with antibody Fab. Bet v 1 and Fab are shown as ribbons and colored dark and light grey respectively. (d) Superposition of Bet v 1 related structures from silver birch highlighting differences in the loop regions (PDB IDs: 1FM4, 1FSK and 4A81).

2.3 EF-hand domain family

The EF-hand domain family, consisting mainly of calcium binding proteins (polcalcin), comprises a conserved domain, which has a 12 residue calcium-binding loop flanked on both sides by α -helices of about 12 residues length. These proteins are majorly involved in either signaling or calcium buffering or transport. Signaling proteins usually undergo a conformational change upon calcium binding. Polcalcins are important calcium binding allergenic proteins from pollen of unknown function containing two EF domains.⁹

The first three-dimensional structure of a polcalcin belonging to this two EF-hand allergen family, Phl p 7, has been determined with bound calcium.¹⁰ Phl p 7 structure (Fig. 3a) has been shown to adopt an extended conformation as a novel dimer with unique features in contrast to previously well-known EF-hand proteins. In Phl p 7, two monomers assemble in a head-to-tail arrangement with domain-swapped EF-hand pairing forming an intertwined dimer.¹⁰ Each monomer comprises two calcium-binding helix-loop-helix domains (Fig. 3a). This unique assembly of Phl p 7 leads to the formation of an extended hydrophobic cavity providing a potential ligand-binding site. It has been observed that calcium binding acts as a potential conformational switch between its open

and closed dimeric forms.¹⁰ Also, the intermolecular pairing in Phl p 7 occurs between the C- and N-terminal domains of opposite chains of the dimer in contrast to classic EF-hands domain proteins. The structure of another polcalcin from silver birch, Che a 3 (Fig. 3b) has been reported¹¹ and a comparative analysis has highlighted the characteristic structural features of EF-hand domain family of allergens.

2.4 Profilin family

Apparently, almost all profilins assume a similar three-dimensional fold consisting of 7 β -sheets and 4 helices.¹² Crystal structure of profilin from birch pollen¹³ is shown in Fig. 4. In comparison to the other profilin structures, a significant change exists in the orientation of the N-terminal α helix in the birch pollen profilin which alters the topography of a surface hydrophobic patch thought to be involved in binding of proline-rich ligands, although it is not clear if it is directly relevant to its allergenic properties.¹³

2.5 Other families

The structure of the C-terminal key domain of Phl p 5b (Fig. 5a) from pollen is the first known allergen that is composed entirely of α -helices and forms a dimer stabilized by a disulfide bridge.¹⁴

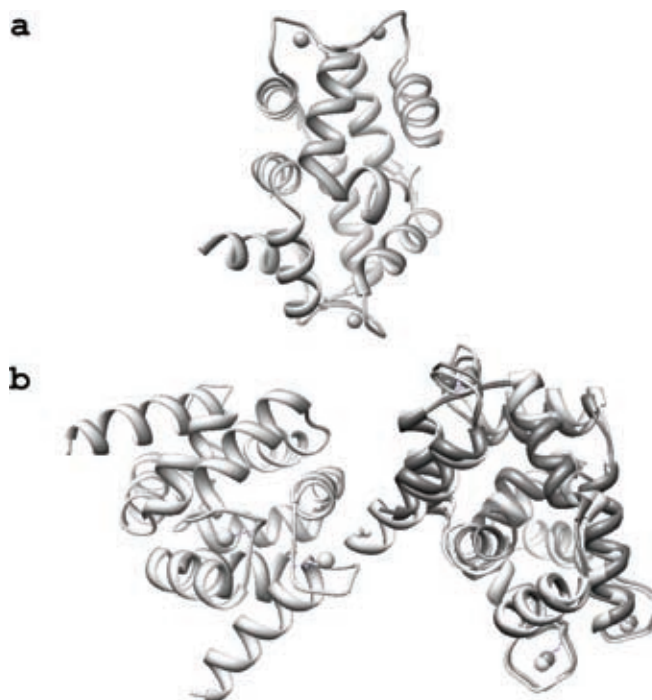


Figure 3: EF-hand domain allergenic proteins. (a) Overall structure of polcalcin Phl p 7 from timothy grass with calcium bound (PDB ID: 1K9U). (b) Superposition of polcalcins Phl p 7 from timothy grass and Che a 3 from silver birch (PDB ID: 2OPO). The proteins are shown as ribbons and colored white (Phl p 7) and dark grey (Che a 3).



Figure 4: Profilin allergenic proteins. Overall structure of profilin from birch pollen (PDB ID: 1CQA).



Figure 7: Pectate lyase Jun a 1. Overall structure of Jun a 1 from timothy grass shown in ribbon representation (PDB ID: 1PXZ).

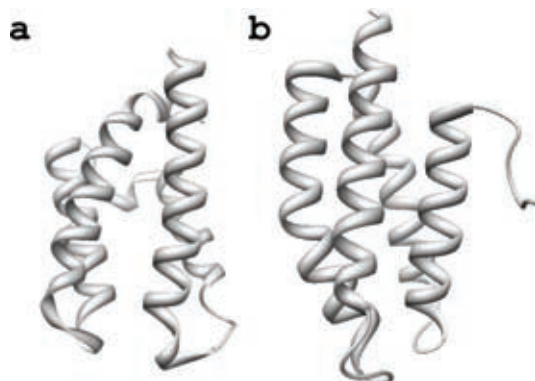


Figure 5: Four-helix-bundle allergens. (a) Overall structure of Phl p 5b protein from timothy grass (PDB ID: 1L3P). (b) Overall structure of Phl p 6 from timothy grass (PDB ID: 1NLX).



Figure 6: Berberine bridge enzyme Phl p 4. Overall structure of Phl p 4 from timothy grass shown in ribbon representation (PDB ID: 3TSJ).

Sequence homology experiments have suggested that all group V and VI grass-pollen allergens belong to this class of ‘four-helix-bundle allergens’ including Phl p 6 (Fig. 5b) from grass for which the structure has been reported in complex with Zn.¹⁴

Further, structures of proteins Phl p 4 (PDB IDs 3TSJ and 3TSH) and Jun a 1 have been reported from grass and mountain cedar respectively. Phl p 4 is a berberine bridge enzyme involved in glucose dehydrogenase activity (Fig. 6). Jun a 1 (Fig. 7) is a pectate lyase having the characteristic parallel β -helix commonly found in other pectin/pectate lyase.¹⁵

3 Relevance to Allergy

The structures of allergenic proteins from pollen described above, do provide interesting insights for exploring mechanistic basis of allergy. Similar protein folds have been observed in reported cases of pollen allergies with those associated with food but the generalization that proteins with a similar fold would result in similar allergenic effects is not necessarily true.¹⁶ Detailed analyses on several three dimensional structures of plant allergenic proteins have revealed highly conserved surface patches that may be relevant for allergy.¹⁷ Most interestingly, these patches are far from the functionally relevant ligand binding sites. Several features in allergenic proteins have been searched since it was confirmed that proteins are the factors responsible for causing type I hypersensitivity.¹⁸ Of course, analyses of the structures of cross-reactive

antigens for surface exposed and conserved residues remains the first approach in mapping conformational epitopes on disease-related antigens that are recognized by polyclonal patient antibodies. Effectively, systematically generated additional structural data on allergens may provide for understanding the allergenic determinants.

The experimentally studied IgE cross-reactivity between Bet v 1 and homologous allergens from plant foods has been shown to cause many birch pollen-allergic patients to show allergic reactions to various fruits and vegetables.¹⁹ The distinct hydrophobic cavity^{6–8} of Bet v 1 has been shown to be accessible to solvent and small molecules at three separate entry points. The binding of deoxycholate and modelling of other similar steroids into this pocket suggests the role of Bet v 1 as a more general steroid carrier. From the analysis of multiple structures of Bet v 1 in complex with myriad ligands, it has been suggested that the architecture of the Bet v 1 binding pocket is optimized for its promiscuous ligand binding to enable storage and/or transport of various lipidic mediators.^{7–8} Also, the Bet v 1 protein signature motif is believed to be the T-cell epitope.⁷

The IgE binding capacity of some EF hand-containing allergens has been shown to be calcium-dependent. It is well established that pollen germination and pollen tube growth critically depend on calcium.^{20–21} Furthermore, lipids are required for pollen tube guidance.²¹ The pollen-specific two EF-hand polcalcin proteins are thought to be involved in the control of calcium metabolism in pollen germination and pollen tube growth because of their ability to bind calcium.⁹ The description of the hydrophobic cavity in polcalcin Phl p 7 suggests a ligand-binding function rather than only calcium transport corroborating that Phl p 7 and its homologous proteins are expressed in low amounts in tissue in pollens.⁹ Further, Phl p 7 exists in two conformational states that can be achieved by binding or releasing calcium.¹⁰ It is well established that the IgE-binding capacity of such two EF-hand allergens from pollen is mainly modulated by the presence or absence of calcium.⁹ Based upon the structural data, it can be stated that surface-exposed amino acids may be potentially involved in epitope formation. The identical three-dimensional structures of another polcalcin from silver birch Che a 3 and Phl p 7 (Fig. 3b) explains the extensive cross-reactivity of allergic patients IgE Abs with two EF-hand allergens from unrelated plants. Further, these available structures of Che a 3 and Phl p 7 have been used to identify surface

exposed conserved amino acids to investigate potential surface patches as targets for the polyclonal IgE antibody response of allergic patients.¹⁰

Profilins from pollens have been identified as highly cross-reactive allergens that elicit IgE responses in 10–20 percent of pollen-allergic patients.²² It has been shown that profilin-specific IgE generally cross-reacts with homologs from every plant source, thus sensitivity to these allergens has been considered a risk factor for allergic reactions to multiple pollen sources and pollen-associated food allergy.^{22–23} The major IgE-reactive epitopes have been conformationally mapped onto the N- and C-terminal α helices and a portion of the protein containing two strands of the β sheet.^{12–13} The profilin epitope is located in regions with conserved sequence and secondary structure, and also overlap with the binding sites for natural profilin ligands. This indicates that native ligand-free profilin acts as the original cross-sensitizing agent.¹³

Three-dimensional structural studies have been done to identify T-cell and B-cell binding epitopes for the last decade but significantly very less information has been collected. Therefore, the question on features being responsible for allergenicity of proteins still needs to be answered. Probably in-depth analyses of IgE binding features on the proteins could provide new answers and may help identify common motifs discriminating allergenic from non-allergenic proteins.

Patients' sera have very low levels of IgE when sensitized with allergenic peptides from different proteins in most of the reported cases. Further, outcome of patients have revealed that intact allergenic proteins representing conformational epitopes have binding affinities several fold higher than that of linear peptides.²⁴ This implies that for the initiation of the allergenicity, conformation is important, whereas the immune response later broadens to smaller protein units. Moreover, digested peptides do not have the capacity to sensitize and may even harbor tolerogenic properties, whereas structural motifs are strongly associated with sensitization and IgE induction.

Pollen protein allergens reported so far have been shown to exhibit very good thermostability. Several factors have been proposed to explain this phenomenon: Pollen protein allergies have an ability to adopt β -structures, smaller in size (15 to 50 kDa) and have very short loops leading to very small difference in entropy while being treated with high temperatures. Structural studies on pollen allergenic proteins have found that

they have unique ligand binding sites; some plant allergenic proteins form a cavity while others possess a unique tunnel and have three or more disulphide linkages. Disulfide bonds are among the most important parameters responsible for their stability.²⁵ Both inter- and intra-chain disulphide bridges constrain the three-dimensional fold of allergenic proteins making them resistant to physical and chemical treatments. Further, presence of short loops helps compactness of allergenic proteins. These features render them extremely stable. However, there are various pollen allergenic proteins known to have extended loops, higher molecular mass and possess no disulfide linkages. While these contradictions exist, it is evident that the three-dimensional structure is critical for defining the determinants of protein allergenicity.

4 Future Perspectives

What makes a protein an allergen? Protein allergy is a complex process, which has yet not been understood properly. There is no intensive comparative characterization of allergenic and non-allergenic antigens. The identification of a large number of allergens from diverse sources has triggered the search for common properties of allergens. This would shed light on the mechanism of initiation of an allergic immune response. It appears that a single specific property may not make a protein allergenic. There ought to be various common structural features in plant protein allergens responsible for IgE sensitization. An essential requirement is to generate large enough structural data and analyse it under the biochemical and immunological constraints in order to arrive at more specific allergy determinants of the protein antigens.

The genetic makeup of an individual, specifically gene polymorphism is one of the important predisposing determinants for the development of allergic immune responses.²⁶ In addition, biogenic and anthropogenic environmental factors also play significant role in allergic sensitization and disease manifestation. Indeed, besides addressing the structural basis of allergenicity, it should be pertinent to analyze many additional factors from the environment, and a host that would determine the outcome of the response to allergens.

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References

1. B. Wüthrich, Clinical aspects, epidemiology, and prognosis of atopic dermatitis, *Annals of Allergy, Asthma & Immunology*, **83**, 464–470 (1999).
2. L. Gough, E. Campbell, D. Bayley, G. Van Heeke, and F. Shakib, Proteolytic activity of the house dust mite allergen Der p 1 enhances allergenicity in a mouse inhalation model, *Clin Exp Allergy*, **33**, 1159–1163 (2003).
3. N.E. Eriksson, H. Formgren, and E. Svenonius, Food hypersensitivity in patients with pollen allergy, *Allergy*, **37**, 437–443 (1982).
4. S. Padavattan, S. Flicker, T. Schirmer, C. Madritsch, S. Randow, G. Reese, S. Vieths, C. Lupinek, C. Ebner, R. Valenta, and Z. Marković-Housley, High-affinity IgE recognition of a conformational epitope of the major respiratory allergen Phl p 2 as revealed by x-ray crystallography *J Immunol.*, **182**, 2141–2151 (2009).
5. C. Radauer, P. Lacknerand, and H. Breiteneder, The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands, *BMC Evolutionary Biology*, **8**, 286 (2008).
6. O. Mirza, A. Henriksen, H. Ipsen, J.N. Larsen, M. Wissenbach, M.D. Spangfort, and M. Gajhede, Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1, *J Immunol*, **165**, 331–338 (2000).
7. Z. Marković-Housley, M. Degano, D. Lamba, E. Roepenack-Lahaye, S. Clemens, M. Susani, F. Ferreira, O. Scheiner, and H. Breiteneder, Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier, *J Mol Biol.*, **325**, 123–133 (2003).
8. S. Kofler, C. Asam, U. Eckhard, M. Wallner, F. Ferreira, and H. Brandstetter, Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen Bet v 1, *J Mol Biol.*, **422**, 109–123 (2012).
9. R. Valenta, B. Hayek, S. Seiberler, A. Bugajska-Schretter, V. Niederberger, A. Twardosz, S. Natter, L. Vangelista, A. Pastore, S. Spitzauer, and D. Kraft, Calcium-binding allergens: from plants to man, *Int. Arch. Allergy Immunol.*, **117**, 160–166 (1998).
10. P. Verdino, K. Westritschnig, R. Valenta, and W. Keller, The cross-reactive calcium-binding pollen allergen, Phl p 7, reveals a novel dimer assembly, *EMBO J.*, **21**, 5007–16 (2002).
11. P. Verdino, R. Barderas, M. Villalba, K. Westritschnig, R. Valenta, R. Rodriguez, and W. Keller, Three-dimensional structure of the cross-reactive pollen allergen Che a 3: visualizing cross-reactivity on the molecular surfaces of weed, grass, and tree pollen allergens, *J Immunol.*, **180**, 2313–21 (2008).
12. K. Kannan, and D.J. Pierre Moens, Structure and functions of profilins, *Biophysical Reviews*, **1**, 71–81 (2009).

13. A.A. Fedorov, T. Ball, N.M. Mahoney, R. Valenta, and S.C. Almo, The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profiling, *Structure*, **5**, 33–45 (1997).
14. K. Rajashankar, A. Bufe, W. Weber, S. Eschenburg, B. Lindner, and C. Betzel, Structure of the functional domain of the major grass-pollen allergen Phlp 5b, *Acta Crystallogr D*, **58**, 175–181 (2002).
15. E.W. Czerwinski, T. Midoro-Horiuti, M.A. White, E.G. Brooks, and R.M. Goldblum, Crystal structure of Jun a 1, the major cedar pollen allergen from *Juniperus ashei*, reveals a parallel β -helical core, *J Biol Chem.*, **280**, 3740–3746 (2005).
16. O. Ivanciuc, C.H. Schein, and W. Braun, SDAP: database and computational tools for allergenic proteins, *Nucleic Acids Research*, **31**, 359–362 (2003).
17. R.C. Aalberse, Structural biology of allergens, *J Allergy Clin Immunol.*, **106**, 228–238 (2000).
18. R. Bredehorst, and K. David, What establishes a protein as an allergen? *J Chromatogr B*, **756**, 33–40 (2001).
19. S. Vieths, S. Scheurer, and B. Ballmer-Weber, Current understanding of cross-reactivity of food allergens and pollen, *Ann N Y Acad Sci.*, **964**, 47–68 (2002).
20. J.L. Brewbaker, and B.H. Kwack, The essential role of calcium ion in pollen germination and pollen tube growth, *Am. J. Bot.*, **50**, 859–865 (1963).
21. V.E. Franklin-Tong, Signaling and the modulation of pollen tube growth, *Plant Cell*, **11**, 727–738 (1999).
22. R. Valenta, M. Duchene, C. Ebner, P. Valent, C. Sillaber, P. Deviller, F. Ferreira, M. Tejkl, H. Edelmann, and D. Kraft, Profilins constitute a novel family of functional plant pan-allergens, *J Exp Med.*, **175**, 377–385 (1992).
23. A. Mari, Multiple pollen sensitization: a molecular approach to the diagnosis, *Int Arch Allergy Immunol.*, **125**, 57–65 (2001).
24. E. Untersmayr, and E. Jensen-Jarolim, Mechanisms of type I food allergy, *Pharmacology & Therapeutics*, **112**, 787–798 (2006).
25. H. Breiteneder, and E.N. Clare Mills Molecular properties of food allergens, *J Allergy Clin Immunol.*, **115**, 14–23 (2005).
26. K.C. Barnes, Genetic epidemiology of health disparities in allergy and clinical immunology, *J Allergy Clin Immunol.*, **117**, 243–254 (2006).



Dr. Dinakar M. Salunke is currently the Executive Director of Regional Centre for Biotechnology, Gurgaon, India. The major focus of his research is to understand the physiological processes of self-nonsel self discrimination in terms of physicochemical principles of molecular interactions using structural biology, immunology and biochemistry. Dr. Salunke did his Ph.D. from the Indian Institute of Science, Bangalore, India in 1983, post-doctoral research at Brandeis University, Waltham (1984–1988) and led a vibrant structural immunology group at the National Institute of Immunology, New Delhi since 1988. He has authored more than 100 publications.



Dr. Jasmita Gill is pursuing post-doctoral research at the Regional Centre for Biotechnology, Gurgaon, India. Her area of interest is structural bioinformatics of immune complexes. She did her Ph.D. from the International Centre for Genetic Engineering and Biotechnology, New Delhi, India in structural biology and bioinformatics of disease-related malarial proteins.



Dr. Alka Dwevedi is pursuing post-doctoral research at the Regional Centre for Biotechnology, Gurgaon, India. Her area of interest is biochemistry of plant allergenic proteins. She did her Ph.D. from Banaras Hindu University, India in Biotechnology. She previously did post-doctoral research at Indian Agricultural Research Institute, New Delhi and University of West Ontario, Canada.