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Application of phage peptide display echnology for the study of food allergen epitopes

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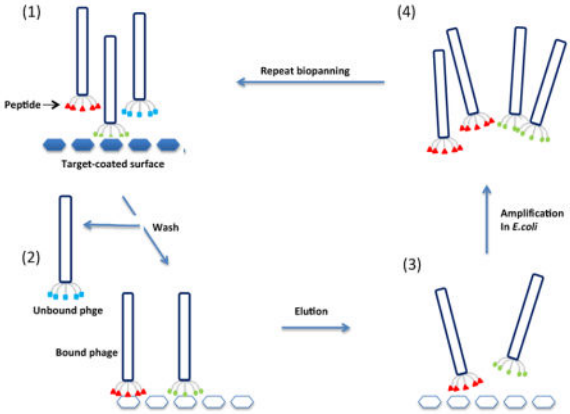
Abstract

Phage peptide display technology has been used to identify IgE-binding mimotopes (mimics of natural epitopes) that mimic conformational epitopes. This approach is effective in the characterization of those epitopes that are important for eliciting IgE-mediated allergic responses by food allergens and those that are responsible for cross-reactivity among allergenic food proteins. Application of this technology will increase our understanding of the mechanisms whereby food allergens elicit allergic reactions, will facilitate the discovery of diagnostic reagents and may lead to mimotope-based immunotherapy.

Graphical Abstract

Phage peptide display is a valuable technology that can be used to characterize the interactions between protein and protein at the molecular level. Only recently, an increasing number of researchers have applied this technology to the study of food allergens. Successful application of phage peptide display technology in conjunction with computer-based algorithms can lead to the identification of IgE conformational epitopes of important food allergens. Overall, this approach offers a promising alternative to crystallography for identification of allergenic epitopes, which contribute to allergen-antibody complexes.



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Keywords

Food allergy; conformational epitope; IgE epitope; mimotopes; phage peptide display

1. Introduction

Food allergy is a hypersensitivity reaction that affects approximately 4–10% of the population, and may be increasing in prevalence [1, 2].

The initiation of allergic reactions is due to allergen-induced cross-linking of allergen-specific IgE bound to high affinity receptors on mast cells and basophils, leading to cell degranulation and release of inflammatory mediators. Thus, binding of specific regions of an allergen to IgE that is part of IgE/FcεR1 complexes is a prerequisite for triggering allergic symptoms.

Binding sites recognized by IgE antibodies are called IgE epitopes. IgE epitopes are frequently categorized as either linear or conformational. Linear epitopes can be identified by analysis of IgE-binding to overlapping peptides derived from the primary sequence [3, 4]. Identification of conformational epitopes requires more elaborate methods. A variety of methods have been used to identify conformational epitopes including X-ray crystallography, nuclear magnetic resonance, hydrogen-deuterium exchange coupled to mass spectrometry, site-directed mutagenesis and shotgun mutagenesis [5, 6]. These approaches can, in varying degrees, provide high-resolution maps of antibody-antigen interactions and thus, a high-resolution structure of a mAb in complex with its target. However, these approaches are not always feasible because of the difficulty of obtaining sufficient quantities of correctly folded, properly processed allergens and can be laborious, time consuming and expensive. Phage display in combination with computational approaches is a cost-effective method to identify important conformational epitopes of clinically relevant allergens [7]. Strengths of this approach include the use of polyclonal antibodies from patients, the speed with which mimotopes can be identified, the relatively low amounts of allergen needed, the lack of need for crystals and, relative to some other techniques, technical simplicity. Drawbacks include the possibility of identifying off-target mimotopes, the need for sophisticated computer modeling to analyze the data and difficulties in validating the veracity of epitopes that are identified.

1. Phage display

Phage display, an advanced technology based on the expression of foreign peptides or proteins as fusions with coat proteins on the phage surface, was first described in 1985 by George P. Smith [8]. Phage display, due to its simplicity and efficacy, has proven to be a powerful versatile tool for studying specific interactions (protein with protein and protein with other molecules). Specific applications include, drug discovery [9–12], gene therapy, vaccine development [13–17], dissection of receptor interactions with agonists and antagonists [18, 19], epitope mapping [7, 20, 21] and identification of antagonists and inhibitors of enzymes [22–25].

Two types of phage are often employed for phage display. M13 filamentous phages are not only used for peptide display, but also for display of a variety of recombinant proteins. Most commonly, DNA encoding for a large library of random peptides is inserted into gene 3 or gene 8 of the filamentous phages. Filamentous phages infect Escherichia *coli* via the F pilus and fusion proteins are expressed on the surface of the bacteriophage. The infection caused by filamentous phage does not cause cell lysis, only "constant production", albeit with slower bacterial growth [26, 27]. A similar approach can be taken with the lytic phage, T7, in which peptides or proteins are displayed as fusions with capsid proteins. In this approach, the lytic cycle results in the destruction of the infected bacteria cells and the mature virions can infect other cells [28, 29]. In each approach, the investigator must devise a process to screen the expressed peptides that will lead to identification of peptides that mimic the interaction to be studied.

2. Filamentous phage display

Filamentous phages have been most commonly used as a phage peptide display platform [27]. Phage peptide libraries used in allergen research usually consist of small peptides, 7 to 12 amino acids in length (Table 1). Even though B cell epitopes are reported to consist at least 8 amino acids, energy calculations imply that epitopes of 5–6 amino acids are the key contributors to the binding between an antibody and its epitope. Heptameric peptides can be used to select the epitopes with the highest affinity to the specific IgE antibodies, while longer peptides enhance the affinity of interaction and increase the ability to detect important conformational epitopes that may be of lower affinity [30–32].

The most straightforward strategy for screening a phage library is the surface biopanning procedure, which consists of three main steps (Figure 1): (i) coating a plastic surface with the target, (ii) adding the phage library onto the immobilized target followed by removal of unbound phages by washing and (iii) eluting the bound phages usually with an acidic solution or by competition with the original antigen. Eluted phages are titrated and amplified in host bacteria and subjected to a second round of selection. Ideally, three rounds of selection are necessary to isolate target-specific phages [33, 34]. Because direct coating may cause some binding sites to be inaccessible due to steric blocking or target denaturation, an alternative method is phage panning in solution: the target is incubated with the phage in solution followed by affinity capture of the phage-target complexes onto an affinity matrix (usually beads) specific for the target protein [35]. For example, if the target protein has a polyhistidine affinity tag, the target-phage complexes can be captured on chelated nickel beads. Methods can be further optimized by using the avidin-biotin system which will increase sensitivity and enhance detection of relevant mimotopes [31]. A negative selection step can be introduced in which the amplified phage is pre-incubated with the beads in the absence of target to further minimize the retention off-target phages [36, 37]. Following the last selection round, the bound phages are eluted and individual clones from the final eluate are isolated and characterized for target specificity in a phage ELISA. The primary structure of the identified phages is then determined by sequencing the corresponding insert.

Validation of these peptides as true mimotopes is a challenge. The most direct way to address this is with inhibition assays characterizing the mimotope's ability to inhibit the

binding of antibody to the allergen. This is most successful with monoclonal and not polyclonal antibodies. Inhibition assays using polyclonal IgE have not been successful because of the polyclonal nature of the interaction (clearly one peptide cannot be enough). Unfortunately, patient-derived monoclonal IgE antibodies are not available to fully validate mimotopes. For additional validation, one can test the binding of peptides expressed on phage to specific IgE isolated from allergic sera as well as by their ability to elicit blocking IgG following immunization[37].

Phage libraries can be screened with monoclonal or polyclonal antibodies to identify multiple mimics of antigenic epitopes. In this application, the selected phage peptides mimic the native antibody-binding epitopes of the antigen by virtue of their physico-chemical properties [7], making this an ideal method for determination of individual epitope recognition patterns [38, 39]. Screening phage-displayed peptides with polyclonal IgE from allergic patients in combination with computer-based mapping of the peptide mimics onto the surface of the three-dimensional structure of the allergen is a promising novel tool to investigate IgE epitope specificity in individual patients [32, 40, 41]. Phage peptide display technology has been used to investigate allergen-antibody interactions for both respiratory and food allergens [7, 38, 42–44]. A summary of published reports in which phage peptide display technology has been applied for the identification of food allergen epitopes and of those food allergen epitopes that contribute to cross-reactivity among allergens is shown in Table 1.

3. Identification of conformational IgE epitopes

Extensive research has been performed to identify sequential epitopes on food allergens [45–47]. In contrast, few conformational epitopes have been identified, a situation resulting from the technical challenges associated with their characterization. Recently, increasing evidence indicates that conformational IgE epitopes may be particularly important in food allergen-induced allergic reactivity [48–50]. The identification of conformational IgE epitopes has also enhanced our understanding of the structural requirements for allergen-induced crosslinking of IgE/FcεR1 complexes that is necessary to activate basophils and mast cells. Specifically, the phage display technique has been used to define peptides (mimotopes) that mimic conformational IgE epitopes of food allergens. Alignment and mapping of identified mimotopes onto the allergen structure surface provide important information about epitope localization and the three-dimensional structure of the antibody binding sites [7].

Parvalbumin, a Ca^{2+}/Mg^{+}-binding protein, is a major fish allergen. Untersmayr et al. [51] screened a cysteine-flanked peptide phage library with Cyp c 1-specific IgE purified from fish-allergic patients and identified five mimotopes that mimic conformational epitopes. All showed similar binding patterns with both parvalbumin specific IgE and IgG in an ELISA, indicating that these mimotopes bind to both isotypes with similar specificity. Epitope mapping identified three matching regions. One of the matching regions was located in the EF domain, indicating that the Ca^{2+} binding loop of the EF domain is an immunogenic area. It was previously reported that Ca^{2+} depletion reduced the IgE binding capacity of parvalbumin because of dramatic conformational changes in the Ca^{2+} binding site [52].

Thus, it is likely that these conformational changes in the Ca^{2+} binding loop of the EF domain result in inaccessibility of critical residues for the binding of antibody.

Peach is frequently involved in allergic reactions in the adult population from the Mediterranean area [41]. In order to define peptides that mimicking conformational epitopes on Pru p 3, a major peach allergen, and to analyze the location of such epitopes on its molecular surface, Pacios et al. screened a random peptide phage display with Pru p 3 – specific IgE and performed three-dimensional modeling analysis. Pru p 3-specific IgE was purified from two pools of sera grouped by symptoms, OAS (Oral Allergy Syndrome) and SYS (Systemic Symptoms). A total of 21 individual phage colonies were identified that were confirmed by ELISA with individual patient sera from each pool. Peptide alignments revealed two consensus sequences, one for the OAS pool and the other for the SYS pool. The two consensus sequences mapped to the same surface area of Pru p 3, corresponding to the helix 2-loop-helix 3 region and to part of the non-structured C-terminal coil. These two relevant conformational IgE-binding regions of Pru p 3 may be useful for design of hypoallergenic variants of the allergen.

The peanut protein, Ara h 1, is an important allergen that is recognized by serum IgE from more that 80% of peanut allergic patients [53, 54]. In its native form Ara h 1 is a 63.5 kDa protein that forms stable homotrimers maintained by hydrophobic interactions between amino acids at the monomer-monomer contact points [55, 56]. Following digestion, Ara h 1 retains both its ability to sensitize and to elicit an allergic reaction, but not if subjected to chromatography. This suggests that, following digestion, small peptide fragments aggregate to form larger complexes [32, 57].

A phage peptide display library was screened with IgE from peanut allergic patients as well as IgE from Brown Norway rats immunized with either purified intact Ara h 1 or gastro-duodenal digested Ara h 1 products [32]. The bound phages were eluted with either intact or digested Ara h 1. Sequence analysis of the identified mimotopes with patient serum IgE revealed five epitope motifs that showed patterns of a minimum of five amino acids. All five motifs were identified on both intact and digested Ara h 1. However, such motifs were not identified by IgG from the immunized rats which showed a much more heterogeneous pattern. Despite the finding that the occurrence of motifs recognized by immunoglobulins was different between peanut allergic patients (IgE) and Ara h 1 immunized rats (IgG), the epitope mapping profile of IgE mimotopes from humans and IgG mimotopes from rats were very similar in terms of the immunoglobulin-binding fingerprints, amino acid distribution and the localization and clustering of epitopes on the Ara h 1 molecule. This indicates that humans and rats direct their antibody-response against the same areas of the allergen. Similarly, the IgE epitope profiles of intact and digested Ara h 1 were very similar when elution was made with intact or digested Ara h 1, respectively. These results suggest that the digested Ara h 1 is kept in a conformation resembling the native structure of Ara h 1, probably by non-covalently interactions, such as hydrophobic interactions.

Increased allergen-specific IgG4 concentrations are associated with development of tolerance to food allergens. This has been demonstrated in the natural development of tolerance to several food allergens and in desensitization by allergen-specific

immunotherapy [58–62]. Recently, several studies compared the specific IgE and IgG4 antibody-epitope recognition pattern in food allergic patients [45, 58, 60, 63] and suggested that similarities between IgE and IgG4 binding epitopes could be critical for the development of tolerance. However, these studies focused only on IgE and IgG4 linear epitopes. Bogh et al. [43] used the phage peptide display technique to study Ara h 1 specific IgE and IgG4 epitope recognition patterns, taking conformational epitopes into account. Phage library screening with IgE from three peanut allergic sera identified 146 Ara h 1 mimotopes. All mimotopes represented Ara h 1 conformational epitopes as none of the mimotope sequences corresponded to a full linear stretch of the Ara h 1 primary sequence and all mimotopes could be mapped to the surface of the Ara h 1 molecule by a computer-based algorithm. Three consensus sequences were recognized by IgE for all three patients. However, the IgG4 epitope pattern was more heterogeneous than the IgE pattern and no consensus sequence was detected. Furthermore, IgG4 epitopes did not coincide with IgE epitopes and were thought to have lower affinity than the IgE epitopes. Each patient had a distinct IgE and IgG4 epitope recognition profile, although some important IgE epitopes were common to all patients. These results demonstrate that the phage peptide display technique can be successful in distinguishing between the epitope patterns of IgE and IgG4, giving detailed information on fine specificity and relative affinity.

Ara h 2 and Ara h 6 are the two most potent peanut allergens [64–67]. Recently, Chen at al screened a phage peptide display library with IgE from four peanut allergic sera [31]. In order to increase the ability to detect important conformational epitopes, the authors optimized the method by using avidin-biotin system to enhance signal intensity. A total of 41 mimotopes that mimic conformational epitopes were identified that specifically bound to Ara h 2/Ara h 6-specific IgE. A computer-based algorithm [7, 68] showed that all mimotopes mapped to surface-exposed patches on the 3D model structures of Ara h 2 and Ara h 6. In the case of Ara h2, the highest scoring patches were located in the vicinity of a patch centered on Y63 and in the case of Ara h 6, the four top scoring patches centered on M80, T68, C73 and H29. Three of these patches, those centered on M80, T68, and C73, share several overlapping residues. Further structural alignment between Ara h 2 and Ara h 6 showed that the patch centered on H29 in Ara h 6 partially overlaps with the high scoring patch centered on Y63 in Ara h 2. These mimotopes were also recognized by IgG from rabbits immunized with either Ara h 2 or Ara h 6. Furthermore, the binding intensity of the mimotopes to rabbit anti-Ara h 2 and Ara h 6 specific IgG was highly correlated, which was consistent with the findings from the structural analysis. The mimotopes were further tested for their binding to IgE from 29 peanut-allergic sera. Eight of the 41 mimotopes were recognized by more than 90% of the tested peanut-allergic sera, suggesting that these 8 mimotopes mimic immunodominant epitopes. Each serum had distinct IgE recognition patterns but the patterns were not correlated to the concentration of peanut specific IgE or the clinical histories, indicating a broad variation in IgE conformational epitopes among patients suffering from peanut allergy.

4. Identification of cross-reactive IgE epitopes in primary food allergies and in pollen-associated food allergies

Cross-reactivity among foods or between food allergens and aeroallergens is a common clinical issue in patients with food allergy. Mimotope peptides are useful for studying potential cross-reactivity between homologous allergens.

Birch pollen-allergic patients often have birch pollen-associated food allergy which is mediated by cross-reactive IgE that reacts with Bet v 1-homologous allergens from foods [69]. Bet v 1-homologous food allergens are found in fresh fruits, vegetables, tree nuts, and legumes including peanut [69, 70]. Mittag et al. investigated cross-reactive IgE epitopes and epitope recognition patterns in individual patients [35]. A phage peptide-display library was screened with polyclonal IgE from five patients with birch pollen allergy in combination with several food allergies. Competitive ELISA confirmed the cross-reactivity between Bet v 1 (birch pollen), Ara h 8 (peanut), Gly m 4 (soy) and Pru av 1 (cherry) in the patient sera. The IgE bound phages specific for Bet v 1 or the homologous food proteins (Gly m 4, Ara h 8, and Pru av 1) were eluted competitively with the corresponding allergen. Thirty seven mimotopes with strong binding to specific IgE were identified and three potential IgE binding areas were identified on all four cross-reactive food allergens. One area was recognized by IgE from all patients, whereas, the other two areas were recognized by only 3 of the 5 patients. The results were consistent with the findings from cross-competitive ELISA and demonstrated that phage peptide screening in combination with computer-based mapping of the mimotopes on the surface of the three dimensional structure of the allergen is a promising tool to investigate IgE epitope specificity and to predict cross-reactivity in individual patients.

Melon allergy, an important food allergy in Spain [71, 72], is frequently associated with the sensitization to several pollens (*e.g.* birch, *Parieteria*, and grass) [73–75]. This cross-reactivity is due to homology among plant profilins. Cuc m 2, a melon profilin, is a major melon allergen [76]. By screening a phage peptide library with IgE from melon allergic patients, Tordesillas et al. identified and sequenced 12 individual Cuc m 2 specific mimotopes [77]. The mimotopes were mapped onto the 3D structure of the Cuc m 2 model and a consensus sequence $S_2W_3A_5Y_6D_9H_{10}T_{111}P_{112}G_{113}Q_{114}N_{116}M1_{17}R_{121}L_{122}$ was identified. This sequence was identical to homologous residues in Phl p 12 (timothy grass) and Bet v 2 (birch) but not to the homologous sequence in human profilin. The identified mimotopes most likely identify surface regions in Cuc m 2 that are involved in cross-reactions among food and pollen profilins and appear to explain the cross-reactivity observed in patients.

Peach Pru p 3, a major food allergen discussed above, is a lipid transfer protein [78, 79]. The homologous protein in wheat, Tri a 14, is thought to be important in occupational baker's asthma. Although Tri a 14 and peach Pru p 3 share 45% sequence identity, competitive ELISA results showed highly variable cross-reactivity between the two allergens among patients with baker's asthma, indicating different sensitization patterns to these allergens [80]. Tordesillas et al. used three approaches to characterize the IgE-binding epitopes of Tri a 14 and Pru p3: i) identifying linear IgE epitopes of Tri a 14 and Pru p 3 by IgE

immunodetection of synthetic decapeptides with IgE from patient with baker's asthma, ii) identifying Tri a 14 and Pru p 3 specific conformational epitopes by screening phage peptide display library with the same IgE, and iii) analysis of the surface electrostatic potential of both allergens [40]. Four linear epitopes were identified by IgE immunodetection, two of which were found to be shared by both allergens. However, one of the remaining epitopes was found only in Tri a 14 and the other, only in Pru p 3. By phage peptide library screening, a mimotope that mimics an important conformational epitope on both allergens was identified. Both Tri a 14 and Pru p 3 share the conformational regions involved in IgE-binding, but with different electrostatic features [40]. Thus, differences in the two linear epitopes and in the electrostatic potentials of the conformational epitope may explain the different sensitization patterns to the two allergens.

6. The potential for mimotope-based vaccines in food allergy

Filamentous phage are highly immunogenic and are known to induce humoral and cellular immune responses directed to their coat proteins resulting in a strong activation response [81–83]. Filamentous phage carriers have also been shown to elicit a more focused anti-peptide response compared to traditional carrier proteins [84]. Although still in early stages of development, phage-based vaccines have been used to induce protection against infectious diseases and cancers in preclinical studies [14, 85–89] and also have been tested in phase I/II human immunization studies [90].

Allergy to plant-derived foods is often associated with sensitization to pollen allergens. In the celery-mugwort-birch-spice syndrome, IgE cross-reactivity is associated with several classes of allergens [91, 92]. The celery allergen, Api g 5, a glycoprotein carrying fucosylated and xylosylated complex N-glycans, has been recognized as an important allergen. Lukschal et al. screened a phage peptide library with BIP3, a monoclonal antibody originally raised against birch pollen high molecular weight allergens, which also reacts to Api g 5 [93]. After phage screening and evaluation by an inhibitory ELISA, three mimotopes were identified, which specifically inhibited the binding of patient serum IgE to birch pollen allergens. These mimotopes were then used for immunization of BALB/c mice. The mimotopes were highly immunogenic and induced a strong IgG response against Api g 5 and birch pollen high molecular weight allergens, indicating that Api g 5 and its cross-reactive homologues are the major targets of BIP3. In order to determine whether the mimotopes are mimics of carbohydrate motifs of the epitopes, the authors used non-glycosylated grass pollen allergen, Phl p 5, and a model glycoprotein representing multiple carbohydrate species as controls. The results showed weak binding of the specific IgG to carbohydrates, suggesting that the BIP3-specific mimotopes predominantly mimic protein epitopes. These results demonstrate that the specific IgG was directed towards allergens relevant for human IgE binding in the celery-birch-mugwort-spice syndrome.

7. Conclusion

Phage peptide display is a valuable technology that can be used to characterize the interactions between proteins at the molecular level. It has been used to investigate protein interactions for drug discovery including anticancer peptides and proteins, receptor agonists

and enzyme inhibitors. Only recently, an increasing number of researchers have applied this technology to the study of food allergens. Successful application of phage peptide display technology in conjunction with computer-based algorithms can lead to the identification of IgE conformational epitopes of important food allergens. However, IgE mimotopes, when analyzed only through computational surface mapping, do not provide reliable evidence of true epitopes and should be further validated. Overall, this approach offers a promising alternative to crystallography for identification of allergenic epitopes that contribute to allergen-antibody complexes. When the interaction between serum IgE and specific allergens has been fully characterized, it will be possible to develop novel therapeutic agents based on the development of high-affinity competing antibodies and/or peptides.

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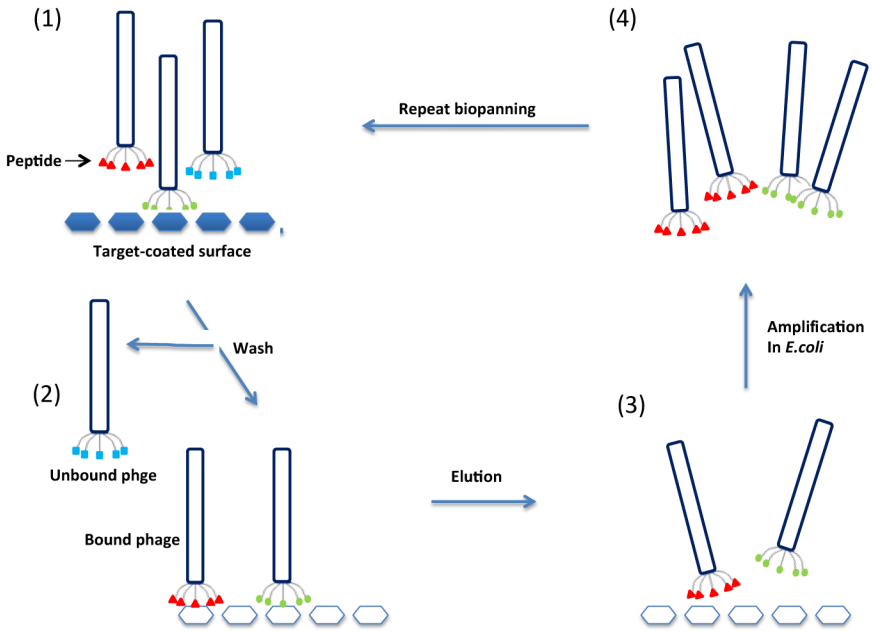

Fig. 1.

Schematic representation of biopanning. (1) Target proteins are immobilized on a solid surface and incubated with the phage peptide display library, (2) Unbound phage is washed away, (3) Bound phage is eluted through acidic solution or affinity eluted with specific ligands and (4) Eluted phage is amplified and used for repeated cycles of selection.

Table 1

Summary of studies using phage peptide display technology for identification of food allergen epitopes.

Allergy	Allergen	Allergic sera (*)or antibodies	Libraries**	Biopanning (***)	References
Conformational IgE Epitopes					
Fish	Cy p 1	Fish (16)	Cyc-10 on pIII	Solid surface (4)	51
Peach	Pru p 3	Peach (23)	Ph.D. – 12	Solid surface (3)	41
Peanut	Ara h 1	Peanut (3)	Ph.D. – 7	Solution (4)	43
	Ara h 1	Peanut (5) Peanut, rat IgG (5)	Ph.D. – 7	Solution (3)	32
Peanut	Ara h 2 & 6	Peanut (4)	Ph.D. – 12	Solution (3)	31
Cross-reactive IgE epitopes in primary food allergies or pollen-associated food allergies					
Birch pollen	Bet v 1	Birch pollen (5)	Ph.D. – 7	Solution (2)	35
	⮟				
Peanut	Ara h 8				
Soy	Gly m 4				
Cherry	Pru av 1				
Melon	Cuc m 2	Melon (17)	Ph.D. – 12	Solution (2)	77
	⮟				
Timothy grass	Phl p 12				
Birch pollen	Bet v 2				
Wheat	Tri a 14	Baker's asthma (8)	Ph.D. – 12	Solution (2)	40
	⮟				
Peach	Pru p 3				
The potential for mimotope-based vaccines in food allergy					
Celery-Mugwort-Birch-Spice Syndrome	Api g 5	mAb BIP3	Cyc-9 on pVIII	Solid surface (3)	93

*Number of individual sera or antibodies.

**Numbers refer to the number of amino acids in the peptides.

***Number of rounds of panning.