

Chapter 5

Cross-Reactivity

Wen Li and Zehong Zou

Abstract Cross-reactivity refers to an antigen–antibody reaction that occurs when a specific antibody reacts with antigen(s) other than just the primary sensitizing antigen. This phenomenon is common in allergic diseases. Cross-reactivity among allergens motivated us to reduce the number of allergens used in therapy and research to just the major representative allergens, which have subsequently been confirmed by the results of allergen component-resolved diagnosis and immunotherapy. This chapter addresses the history of the development of the theory of allergen cross-reactivity, the clinical manifestations, the biological basis of allergen cross-reactivity, and a bioinformatics analysis of cross-reactivity.

Keywords Cross-reactivity · Allergen · Co-sensitivity · AAFP · Pan-allergen · Epitopes

5.1 Formation and Development of the Theory of Allergen Cross-Reactivity

Allergens, as the core of the allergic reaction, have been a main focus of allergy research. Throughout a hundred years of history of allergy, allergen research can be divided into seven stages that also provide the foundation for the development of the theory of allergen cross-reactivity.

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5.1.1 Protein Extraction from Allergenic Sources

As early as 1819, John Bostock first reported a case of “hay fever”. In 1873, Charles Barkley further proved that “hay fever” is caused by pollen antigens, termed “allergens”, which led to the research of allergens (Garrelds et al. 1996). Allergen preparations are indispensable for the diagnosis and treatment of allergic diseases, so the first step into the research of allergens is the preparation of an allergen extract. Early studies of allergens included pollen, fungi, mites, food, etc. Allergen extracts can be used not only in clinical diagnosis (e.g., prick skin test) and in vitro testing but also for allergen-specific immunotherapy. However, batch-to-batch differences in allergen preparations can seriously affect the diagnostic accuracy, the efficacy, and safety of allergen immunotherapy. The document providing guidance on allergen-specific immunotherapy, released by WHO (World Health Organization) in 1998, stated that the quality of the allergen vaccine is critical for the diagnosis and treatment of allergic disease, and that standardized allergen vaccines should be encouraged for clinical use (Bousquet et al. 2008). Both the United States and Europe have developed standardized protocols for allergen preparations (Larsen and Dreborg 2008).

5.1.2 Identification of Novel Allergenic Sources

In recent years, with the development of modern life and genetically modified foods, novel allergens continue to be identified. Every hospital or clinic desires to be able to diagnose allergic disease caused by any and all allergens, thus, the greater number of allergens that are identified, the better allergy research and treatment becomes.

5.1.3 Identification, Characterization, and Cloning of Single Allergens

In the past two decades, with the development of PCR technology, IgE-binding detection technology and cloning and expression technology, allergens have been produced from in vitro expression systems and recombinant hypoallergenic proteins can be obtained through allergen site modification (Walgraffe et al. 2009). According to the nomenclature rules of the allergen nomenclature committee, allergens from the same species are considered to be homologous allergens (isoallergen) if their sequence identity is over 67 % (Radauer et al. 2014). In 1985, 12 novel and unique allergen sequences were cloned and there were no isoallergen genes. From 2000 to 2005, 1085 allergen genes were cloned. However, in this group, 900 allergen genes were found to be isoallergen genes and only 5 were novel allergen genes. Our survey, based on data between 2004 and 2012 from an allergen database (<http://www.allergen.org/>), showed the same

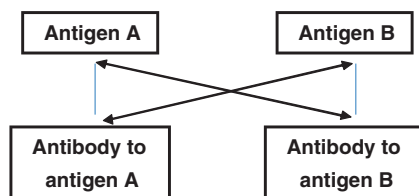
trend (Table 5.1). Most allergen research focused on mite allergen and the search for new allergens from related species was continued. A lot of similar work has been carried out on other allergens. Different research institutions study the same allergens either simultaneously or at different times resulting in similar allergen research. Just as Astwood pointed out, the golden age of new allergen discovery is over (Astwood et al. 1995).

5.1.4 Identification of Cross-Reactivity Among Different Allergens

Allergists have found that more than 90 % of *Dermatophagoides pteronyssinus* allergic patients are also allergic to *Dermatophagoides farina* and that *D. pteronyssinus* allergen preparations can be used to treat *D. farina* allergic patients (Fernandez-Caldas and Iraola Calvo 2005). When used in vitro, allergen proteins (mainly similar proteins) isolated from different sources can cross-react with each other. In addition, a comparative analysis of existing allergen sequences showed that the structures of many allergens are similar. Therefore, an interest in the exploration of allergen cross-reactivity began. Cross-reactivity refers to when an antibody to antigen A can react with antigen B and vice versa due to the presence of the same or similar sequences/epitopes/structures (Fig. 5.1).

Barnett defined the concept of allergen cross-reactivity as an allergic reaction caused by an allergen and a specific IgE antibody that was produced to another allergen resulting in a cross-reactivity between these two allergens (Barnett et al. 1987). However, cross-reaction needs to be distinguished from co-sensitization and multiple sensitizations. Co-sensitization or multiple sensitizations are generally related to mixed antigens or allergens, it is a cautious pre-judgment of “cross-reactivity” that a single serum positively reacts to different antigens/allergens. Co-sensitization or multiple sensitizations may be due to the presence of different antibodies reacting with different allergens, or may be caused by the presence of cross-reactive antigens. In general, when allergen cross-reactivity is suspected, co-sensitivity is the preferred term (Aalberse et al. 2001; Sidenius et al. 2001). However, in practice, true cross-reactivity of allergens and co-sensitivity may be difficult to differentiate based on the clinical presentation alone, especially in cases of cross-reactivity between nonhomologous allergens.

Fig. 5.1 Cross-reactivity of antigen A and antigen B



The cross-reactivity among different allergens occurs on several levels: First, different allergens from the pool of diverse species can have high similarities in their amino acid sequences, resulting in similar primary structures that induce the cross-reaction among different allergens. Analysis of the 280 major allergen entries retrieved from the public database resulted in them being able to be condensed into 21 major representative ones, thus allowing for the use of a smaller number of allergens when conducting comprehensive allergen testing and immunotherapy treatments (He et al. 2014). Second, pan-allergens such as profilin (Tao and He 2004), lipid transfer protein, tropomyosin, etc. are evolutionarily conserved in plants and humans and play an important role in cross-reactivity (Asero et al. 2001; Reese et al. 1999). Third, having highly similar 3D structures confer cross-reactions among different allergens, including those with remote homology. Last, but not least, allergen epitopes [antigenic determinants (AD)] play a key role in the cross-reactivity of allergens (Levin et al. 2014).

5.1.5 Major Representative Allergens

Major allergens are proteins that substantially bind to IgE from more than 50 percent of the patients with that specific allergy (Nordlee et al. 1996). Major allergens have usually been employed as internal standards in order to standardize allergen vaccines (Seppala et al. 2011). Detailed characterization of the important major allergens and their biological sources would allow for improved allergen standardization and, thus, help to obtain more effective and safer regimes for the diagnosis and therapy of many allergic diseases. The growing number of available allergen sequences and major allergens, in addition to the advancements of bioinformatics tools and methods, have made it possible for scientists to shed light on the evolutionary and structural relationships between allergens from different sources. In 2004, Tao and He analyzed the phylogenetic relationships between the newly obtained allergen profilin D106 (GenBank accession No AY268426) from short ragweed (Rg, *Ambrosia artemisiifolia* L.) and its cognates from other species and found that profilin D106 shared a similarity as highly as 54–89 % with different pollen allergens (from mugwort CAD12862 to apple Q9XF41) and as 79–89 % with different food allergens (from peanut Q9SQI9 to peach Q8GT39) (Tao and He 2004). This result made them contemplate the cross-reactivity between different allergens and the selectivity of allergen cloning. In 2005, Breiteneder discovered that most plant food allergens belong to only four structural families, including the cereal prolamin superfamily, the cupins, Bet v 1 (homologues of the major birch pollen allergen) and profilins, indicating that conserved structures and biological activities may play an important role in cross-reactivity (Jenkins et al. 2005). One year later, they found that pollen allergens belong to few protein families and show distinct patterns of species distribution (Radauer and Breiteneder 2006). In 2007,

Tao et al. gradually reduced the number of major allergens according to sequence similarity into fewer major representative ones (Tao et al. 2007), which facilitated recombinant allergen work.

5.1.6 Component-Resolved Diagnosis and Immunotherapy

Individual or few major allergens can be used not only to diagnose the genuine sensitization of patients to a given allergen or to cross-reactive molecules, but also for allergen-specific immunotherapy to yield the same effects as whole allergen extracts in allergic patients (Jahn-Schmid et al. 2003; Pittner et al. 2004). Intensive clinical evidence has proven that specific immunotherapy can prevent both the progression of allergies and the acquisition of new allergies. Immunotherapy with peptides containing T cell epitopes may provide an efficacious and safe alternative to conventional subcutaneous and/or sublingual immunotherapy using native full-length allergen preparations (Linhart et al. 2014).

Allergen is the term for the antigen that induces allergy and the accurate identification of allergens is vital for the treatment of allergic diseases. Allergen extracts are traditionally used in the diagnosis of allergic disease. But the allergen extracts include allergic and nonallergic substances, and natural extracts may contain pan-allergens, which can seriously affect diagnostic accuracy and safety. For example, house dust mite extract contains Der p 10, which belongs to the pan-allergen tropomyosin. If the patient is allergic to tropomyosin from one allergenic source but tolerant to house dust mite, testing using house dust mite extract could result in a misdiagnosis and affect subsequent desensitization therapy. A diagnosis of allergy using a single allergen or a few allergens can more accurately reflect true allergen sensitization or a cross-reactivity condition. It has been reported that treatment with a single allergen can produce the same curative effects as treatment with the allergen extract. Therefore, successful immunotherapy with one allergen can potentially prevent the development of allergy to other allergens.

The clinical use of allergen components can improve the diagnostic accuracy of allergic diseases and efficacy. However, some publications about allergen diagnosis have some general shortcomings: (1) the number of cases is small; (2) the geographical area of research is narrow (mostly concentrated in Europe); (3) the related race is limited; and (4) experiments using the same allergen were not repeated often (Incorvaia et al. 2014; Mohamad Yadzir et al. 2014). Nevertheless, it has been proven that treatment with a single allergen is safe and effective and that use of a single allergen or a few allergens not only identifies the true allergen sensitization or cross-reactive condition but also can be used for immunotherapy. Furthermore, successful immunotherapy with one allergen can prevent the development of allergy to other allergen sources (Ciprandi et al. 2011; Durham 2011; Malling 2003).

5.1.7 Progressive Clustering of Allergen Family Featured Peptides

Some protein families contain both allergens and nonallergens. Some nonallergens have high sequence identity with allergens, which suggests the presence of nonallergen components in allergens. In 2012, we developed the software SORTALLER that helped us reduce published allergens to 72 allergens with 534 characteristic peptides [Allergen Family Featured Peptides (AFFPs)] and can successfully predict more than 2290 nonredundant allergens (Zhang et al. 2012).

Cluster analysis was conducted on allergen sequences in each known allergen family. The allergen sequence was divided into peptides of 6–32 amino acids in length using a 1–10 amino acid sliding window, and then followed by a BLAST (Basic Local Alignment Search Tool, a local alignment search tool basic sequence) analysis with nonallergen sequences. Those peptides that were similar to nonallergen fragments were excluded. If the peptide did not match with nonallergen sequences and the E value of BLAST was lower than 10^{-5} , the peptide was retained and referred to as allergen featured peptide (AFP). AFFPs are each composed of 2–30 small adjacent peptides in the same allergen. IgE cross-reactivity may also result from a single conserved region despite a low overall level of sequence identity (Jenkins et al. 2005). In 2013, Breiteneder identified that only five consecutive amino acid residues are cross-reactive among Ara h 1, Ara h 2, and Ara h 3 (Bublin et al. 2013).

Previously, we retrieved online 478 allergen sequences and clustered them into eight groups by sequence similarity (Tao and He 2005). Subsequently, we focused on the major allergens, retrieved 280 entries, and retained 59 major allergens after initial reduction, which were initially classified into seven clusters. Two or more neighboring clusters were combined to form a new data source for further clustering. This procedure was iterated until the last clustering exhibited 21 allergens that were distantly related to each other (Fig. 5.2). Further alignment showed that several pairs of allergens could be, respectively, grouped together, however, less than 15 % share alignment of their amino acid sequences and most of them exhibited different tertiary structures (Fig. 5.3).

Continuing with the analysis of AFFPs using a progressive clustering procedure similar to the one mentioned above, groups with remote homology (<20–35 % in local region) were represented by single entries and 534 AFFPs were reduced into 20–22 allergens (Fig. 5.4) through five cycles of “cluster-selection-alignment.” All the core peptides contain 3–5 matching residues with adjacent mismatches.

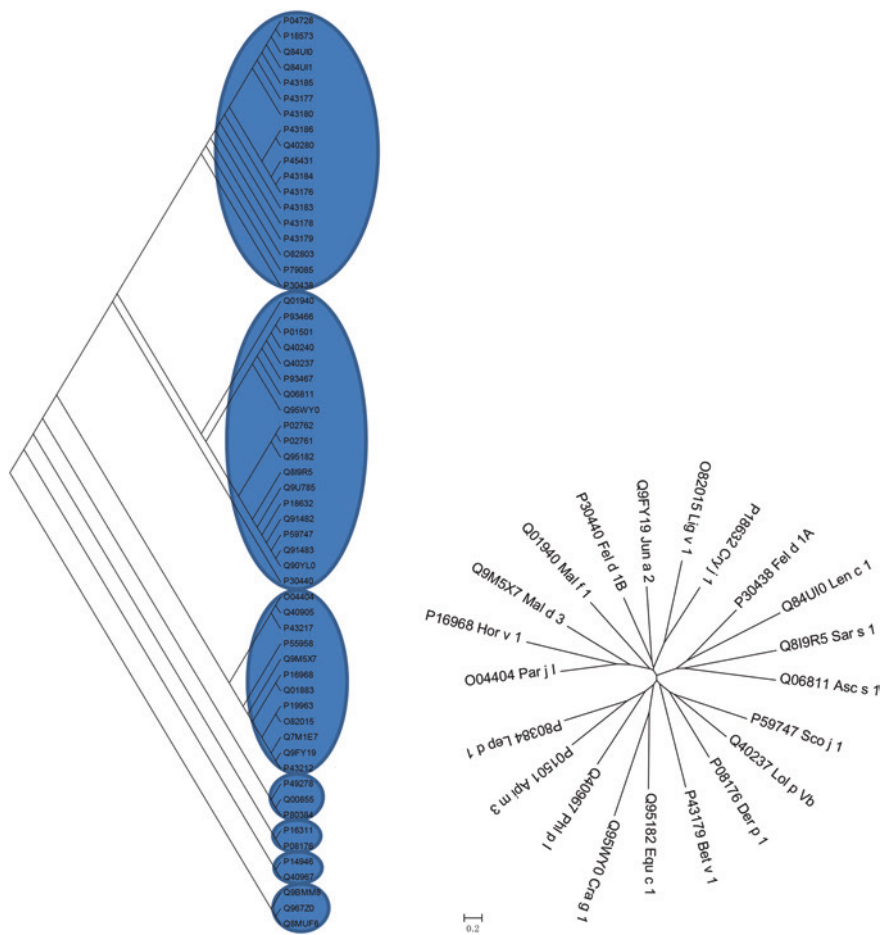


Fig. 5.2 Progressive clustering of the major allergens acquired in UniProtKB/Swiss-Prot database. **a** Phylogeny tree constructed and tested by Maximum Likelihood Estimation for 59 of 280 major allergens. **b** Bootstrap consensus tree of 21 major representative allergens reduced from 59 major allergens (He et al. 2014)

5.2 Cross-Reactivity Among Different Kinds of Allergens

Cross-reactions usually occur between the same species. For example, most dust mite-allergic patients are allergic to both *D. pteronyssinus* and *D. farina* (Fernandez-Caldas and Iraola Calvo 2005). But cross-reactions can exist between different species. For example, birch pollen-sensitive patients can have allergic symptoms after eating celery (Vieths et al. 2002). Cross-reactions between allergens are widespread in allergic disease. Thus, analyzing and understanding the clinical manifestations and molecular mechanisms of cross-reactivity among

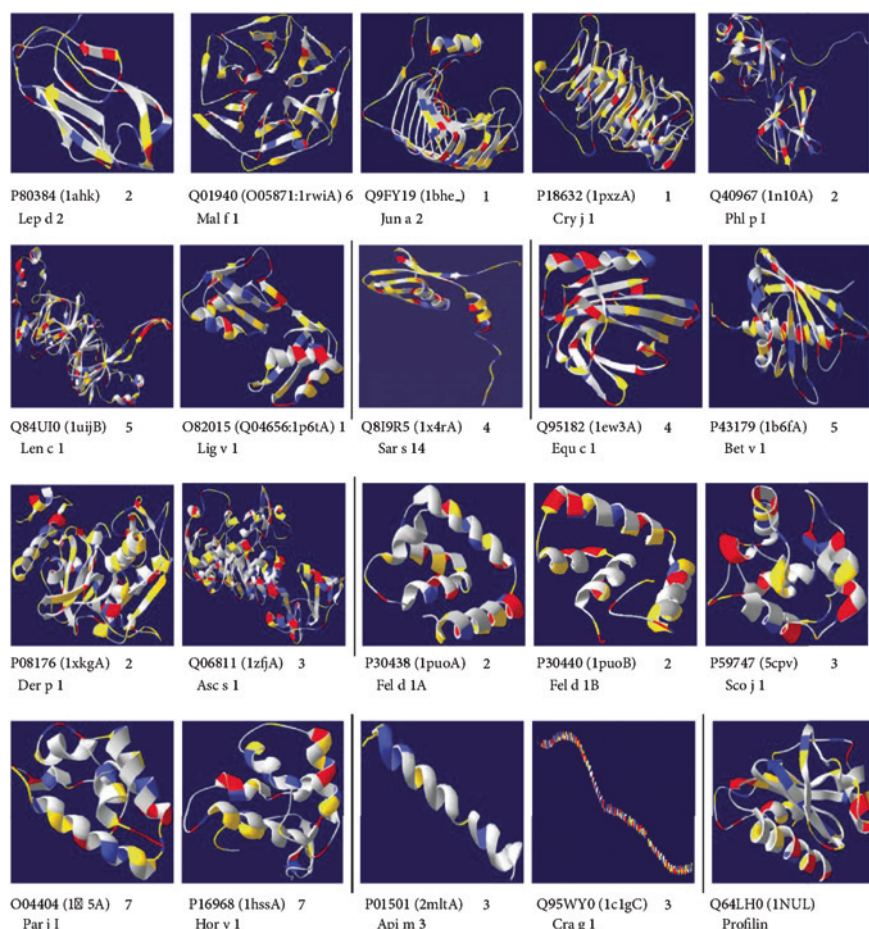


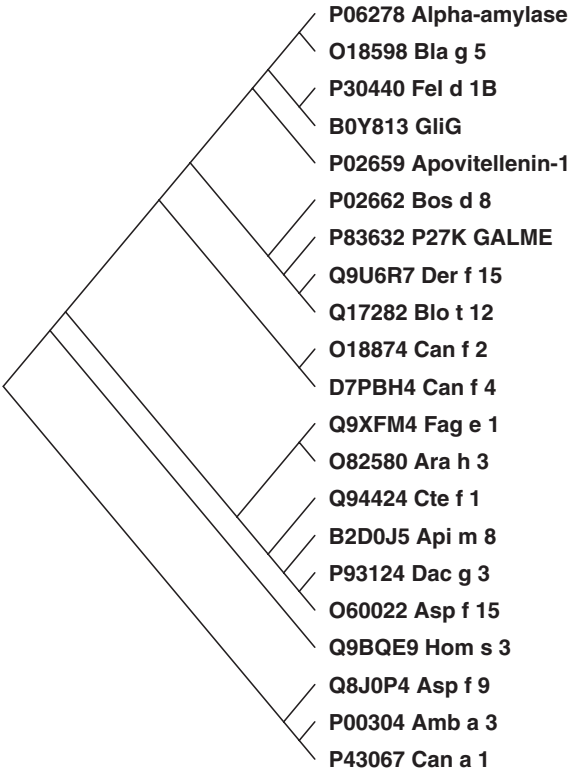
Fig. 5.3 Ribbon diagrams of profilin (Q64LH0) and 19 out of the 21 major representative allergens retained. The seven structural classes are separated by vertical lines. PDB codes are shown in brackets; the following number indicates the initial sequence clustering groups. Q64LH0 was not retrieved as a major allergen, thus is without an initial cluster number. Q9M5X7 and Q40237 were omitted for space consideration (He et al. 2014)

allergens has important significance in guiding the diagnosis and treatment of allergic disease and the development of allergen vaccines.

Allergens are the antigens that can induce the generation of specific IgE antibodies and react with them. They have a wide range of sources, including animals, plants, and microorganisms. Most allergens are proteins and a few of them are glycans, such as *Candida albicans* allergens (Savolainen et al. 1990).

Most allergens have potential biological functions. According to their biological activity or their similarity with proteins of known function, allergens can be

Fig. 5.4 Maximum Parsimony Tree deduced by MEGA5.10 for 21 representative AFFPs (Adapted from the reference He et al. 2014)



divided into several categories, such as enzymes, enzyme inhibitors, transport proteins, and regulatory proteins (Stewart and Thompson 1996). Common allergens include inhalant allergens and food allergens. Cross-reactivity between different inhaled allergens, food allergens, or the reaction between allergens of these two groups is discussed below.

5.2.1 Cross-Reactivity Among Inhalant Allergens

Inhalant allergens are the common causes of allergic rhinitis and asthma. They are usually divided into indoor allergens, outdoor allergens, and occupational allergens. Generally speaking, outdoor allergens are likely to cause seasonal rhinitis and indoor allergens are prone to cause principle perennial rhinitis and asthma (Braun-Fahrlander et al. 1997; Gergen and Turkeltaub 1992). Some of the more common inhalant allergens are dust mites, pollen, and cockroaches.

Most dust mite-sensitive patients are allergic to *D. farinae* and *D. pteronyssinus* (Fernandez-Caldas and Iraola Calvo 2005). In Sweden, *D. siboney* has strong

cross-reactivity with the other three kinds of dust mites (*D. farinae*, *D. pteronyssinus*, *D. microceras*) (Sidenius et al. 2001). Dust mites and *Euroglyphus maynei* have significant IgE cross-reactivity. Two hundred fifty suspicious allergic patients who had positive prick test results to *E. maynei* also reacted to dust mite. In these two studies, dust mite extract inhibition of reactivity to *E. maynei* was more obvious than the inhibitory effects of *E. maynei* extract on dust mite reactivity (Thomas et al. 2004). In addition, most studies found that storage mite allergy and dust mite allergy were positively correlated, with 60–88 % of the dust mite-sensitive patients also being allergic to storage mite. Cross-reactions between dust mites and *Acarus siro* or *Tyrophagus putrescentiae* are stronger than the cross-reactions between dust mites and *Lepidoglyphus destructor*. The inhibition of dust mite extract to IgE-binding reactivity of *A. siro* or *T. putrescentiae* is more remarkable than that of *A. siro* or *T. putrescentiae* extract to dust mite (Sidenius et al. 2001; Thomas et al. 2004).

Extensive cross-reactions exist among pollen allergens and can even sometimes occur between the pollens from different families. Wahl et al. reported that there was cross-reactivity between birch and ash tree pollen (Wahl et al. 1996). Castro et al. reported that plantain pollen allergens and olive pollen allergen cross-react (Castro et al. 2007). In India, it was reported that cross-reactivity occurs between proteins of the latex from *Hevea brasiliensis*, seeds and pollen of *Ricinus communis*, and pollen of *Mercurialis annua*, members of the *Euphorbiaceae* family (Palosuo et al. 2002). During the process of desensitization therapy, in order to control the disease by avoiding exposure, not only should real allergens be avoided, but also cross-reactive allergens. It is very important for the diagnosis and prevention of hay fever to understand cross-reactivity among pollen allergens.

Most cockroach-sensitive patients are allergic to both American cockroach and German cockroach (Bassirpour and Zoratti 2014). Moreover, cockroach allergy and dust mite allergy are positively correlated. In high-load areas of cockroach, the number of cockroach-allergic patients is higher than in the low-load regions. The positive correlation between cockroach allergy and dust mite allergy is partly because cockroach and dust mite allergens coexist in the same environment. Cross-inhibition between dust mite and cockroach occurs in most sera assays. Furthermore, inhibition of mite on cockroach reactivity is greater than that of cockroach on mite reactivity (Jeong et al. 2004).

5.2.2 Cross-Reactivity Among Food Allergens

It is very common to see cross-reactivity between cow and goat milk. 92 % of cow milk-sensitive patients are also allergic to goat milk (Bellioni-Businco et al. 1999). But only 4 % of the cow milk-sensitive children are allergic to mare milk (Businco et al. 2000). 10–20 % of the cow milk-sensitive children are allergic to beef, while 93 % of the beef-sensitive children are allergic to milk (Martelli et al. 2002; Werfel et al. 1997). In addition, cross-reactivity is

widespread among different types of poultry, mammalian meat, eggs (chicken, duck and goose), beans and nuts (Bernhisel-Broadbent and Sampson 1989; Ewan 1996; Langeland 1983; Restani et al. 2009). Mollusks cross-react with crustaceans and other invertebrates (arthropods, sponges, nematomorpha). Thus, avoidance of all crustaceans, mollusks, and other shellfish is generally recommended for people who are allergic to seafood (Leung et al. 1996; Reese et al. 1999).

5.2.3 Cross-Reactivity Between Inhalant Allergens and Food Allergens

Cross-reactions exist between pollen allergens and food allergens, especially vegetables and fruits. In 1942, it was reported that some fresh fruits and vegetables caused contact urticaria in patients with birch hay fever. This type of disease was termed the fruit-vegetable-pollen cross-reactive allergy syndrome or, more recently, pollen allergy syndrome (Caballero and Martin-Esteban 1998). Such cross-reactivity is caused by an actin-binding protein, which is widespread in eukaryotic cells, and is also known as profilin. Dust mite-snail allergy may be a very important issue in those countries that use snail as a food ingredient. Cross-reactions occur between dust mite allergens and other invertebrate allergens and can cause or aggravate allergies to foods such as snails, shrimp, etc. For example, we often find that dust mite-allergic patients who had never eaten snails had an allergic reaction the first time it was ingested and developed severe symptoms such as asthma, anaphylactic shock, generalized urticaria, and/or facial edema. Inhibition tests showed that specific IgE reactivity to snail can be inhibited by dust mite extract. However, snail extract did not significantly inhibit the binding between dust mite extract and dust mite-specific IgE. These results demonstrated the existence of cross-reactivity between dust mite and snail. It also showed that dust mites are a common allergen and that snail allergy was often caused by a cross-reaction with dust mites (Fernandez-Caldas and Iraola Calvo 2005; Sidenius et al. 2001).

5.3 The Biological Basis of Allergen Cross-Reactivity

Cross-reactivity is an immunological phenomenon that is influenced by at least three factors: (1) primary and tertiary structure of the allergen, (2) protein family (Pan-allergen), and (3) allergen epitopes. This section focuses on these three aspects.

5.3.1 Primary and Tertiary Structures of Allergens

The similarity between primary and tertiary structures determines the likelihood of cross-reactivity between allergens. The FAO/WHO predicts the chance of cross-reactivity based on the identity of six or more contiguous amino acids or a minimum 35 % sequence identity over a window of 80 amino acids (Umeda 2006). In particular, point mutations are able to inhibit IgE-binding capacity. The allergenicity of a mutant recombinant allergen was significantly reduced compared to wild type, but the immunogenicity was maintained, a phenomena that can be used to reduce immunotherapy adverse reactions (Vrtala et al. 2007).

Based on the original data released by the International Union of Immunological Societies, Clustal W 1.83, MEGA5.0 and other bioinformatics software that were used and then enhanced by manual selection, 59 major allergens from public databases were progressively analyzed for clustering. Seven clusters (Fig. 5.2a) were found that were then gradually reduced to 21 representative amino acid sequences without any relationship to each other (Fig. 5.2b). The 3D structures of the 21 major representative allergens are depicted in Fig. 5.3. When inspected from their spatial structural orientations and surface exposure of the allergens, all of the 21 allergens were shuffled against the initial clustering and, interestingly, fell into these seven structural classes (Fig. 5.3). However, this classification is complicated by the existence of similar structural scenarios in different structural classes (He et al. 2014).

- **Up-and-down β -barrel:** includes P80384, the major allergen Lep d 2 from Fodder mite (*L. destructor*), as well as other group 2 allergens from dust mite species, for example, Der f 2 (P49278) from *D. farinae*.
- **β -meander and/or ψ -loop constituted calyx:** shortened as (α) calyx, such as hexagon, cradle, and globose twins: includes allergens Q01940, Q9FY19, P18632, Q40967, Q84UI0, and O82015.
- **α - β structured crane:** includes Q8I9R5, an allergen from *Sarcoptes scabiei* type *hominis*.
- **α - β arranged banana string:** includes Q95182 and P43179.
- **(β) formed complex:** includes P08176 and Q06811.
- **α -helix built clips:** a large group including P30438, P30440, P59747, Q40237, O04404, Q9M5X7, and P16968. Interestingly, P30438 and P30440, chain 1 and chain 2 of cat allergen Fel d1, configure a pair of chiral molecules on the 3D level. O04404, Q9M5X7, and P16968, originating from different taxonomic species but classified into the same initial Cluster 7, here exhibited structures similar to each other.
- **α -helix spiral cord:** includes P01501 and Q95WY0, both of which came from the same initial cluster III. P01501 is an allergen Api m 3 from honeybee (*Apis mellifera*), also a main toxin of bee venom with strong hemolytic activity. Q95WY0 is the major oyster allergen and tropomyosin from the Pacific oyster *Crassostrea gigas*. These two allergens exhibited similar structures but displayed different lengths of their spiral cord.

Comparisons of 3D structures suggest that the properties conferred by protein folds in the allergens may potentiate protein immunogenicity, as well as allergenicity. Altogether, combining structural information with analysis of the conservation of primary structure indicates that the conservation of surface features (particularly main chain conformations that cannot be assessed by using simple amino acid similarities) would offer a sounder basis for assessing potential IgE cross-reactivity in novel proteins (Jenkins et al. 2005).

A comparison of sequence conservation within allergen family members, taxonomic distribution of identified allergens, and cross-reactivity data from the literature shows a high degree of correspondence. Omnipresent, highly cross-reactive allergens such as profilins and calcium-binding proteins show sequence similarities of more than 60 % between members of different plant families. In contrast, β -expansins from grasses share less than 50 % of their sequences with nonallergic homologues from other plant families, which do not cross-react with grass pollen expansins (Radauer and Breiteneder 2006).

A review of sequence similarities among allergenic and nonallergenic homologues of pollen allergens shows that the prerequisite for allergenic cross-reactivity is sequence identity of at least 50 %. The Bet v 1 family is the only known exception from this rule. Cross-reactive Bet v 1 homologues share only 37–67 % of their sequences with Bet v 1. This may be explained by the fact that surface exposed residues of Bet v 1 homologues are more conserved than buried ones, resulting in higher levels of similarity between surfaces compared with whole sequences (Radauer and Breiteneder 2006).

5.3.2 Protein Family (*Pan-Allergen*)

In general, for species, the closer they are in classification, the greater the likelihood of a cross-reaction. However, cross-reactivity between allergens from distantly related species has also been found. Allergen components are divided into different protein families according to their function and structure, and IgE cross-reactivity occurs in antibodies to the same protein family. Therefore, classifying allergen components helps to explain the problem of cross-reactivity. A quantitative comparison of plant allergens indicates that, in spite of the number and diversity of proteins encoded in plant genomes, the universe of plant allergens is rather small, with about 65 % of plant food allergens coming from only four protein families. Such information suggests that membership in one of these families is likely to result in a greater potential for a protein to become an allergen, providing that additional factors such as presence in plant foods are taken into account (Jenkins et al. 2005). Below are the four common families of proteins associated with cross-reactivity:

- **Pathogenesis-related protein family (PR)**

Represented by Bet v 1 (PR-10), the pathogenesis-related protein family is commonly found in defense proteins of higher plants. Its expression is induced by

pathogens or other external stimulating factors and plays an important role in plant defense against pathogens and external pressure and adaption to adverse environmental processes. Many food allergens have been reported to be members of protein families, such as hazelnut (Cor a 1), celery (Api g 1) and soybean (Gly m 4). Highly similar amino acid sequences in these families lead to cross-reactivity; however, heating or enzymatic hydrolysis easily destroys the binding ability of allergens to IgE. 50–90 % of birch pollen allergic patients develop allergic symptoms when ingesting apple, carrot, celery and hazelnut, which is the result of cross-reactivity with Bet v 1 (Vieths et al. 2002). Mal d 1 is the major apple allergen and has 64 % sequence similarity with Bet v 1, but there are still some differences in serum cross-reactivity and clinical symptoms. The IgE of the vast majority birch allergic patients is capable of binding to Mal d 1, but not all patients exhibit clinical symptoms. Further studies have shown that the cause of this phenomenon is that Bet v 1 has different B cell epitopes that bind IgE (Klinglmayr et al. 2009). The remarkable conservation of both surface residues and main chain conformations in the Bet v 1 family is of great importance in the conservation of IgE-binding epitopes and underlies the fruit-vegetable-pollen cross-reactive allergy syndrome (Jenkins et al. 2005).

- **Lipid transfer protein (LTP)**

Lipid transfer protein is the main allergen in *Rosaceae* fruit and *Amygdaloideae* fruit, especially in pericarp. Enzymatic digestion or heating does not destroy its antigenicity. Allergy symptoms, such as oral allergy syndrome and severe systemic allergic reactions, occur in the absence of pollen allergens. Therefore, LTP is often considered as a nonpollen-related allergen. However, evidence shows that allergy symptoms in some patients with hay fever are caused by LTP. In a study of 24 patients with *Artemisia* pollen allergy, more than 70 % of these patients had a positive LTP skin test. Mugwort LTP binding to IgE is not inhibited by pear LTP in inhibition ELISA experiments, while 50 % of patients tested for IgE binding to pear LTP showed inhibited IgE binding by mugwort LTP, indicating cross-reactivity with mugwort pollen (Lombardero et al. 2004).

- **Profilins**

As plant allergens, profilins are present in almost all eukaryotic cells. Initially reported as actin-binding proteins in 1977, profilin is the key factor in rapid actin reorganization (Ramachandran et al. 2000). Profilin-sensitized patients are allergic to a large number of pollens and foods. Patients with birch pollen allergy caused by Bet v 2 often develop oral allergy syndrome after ingesting pears, apples, carrots, and celery. In addition, grass allergic patients may react to carrots and celery due to cross-reactivity caused by profilins (Canis et al. 2011).

- **Tropomyosin**

Tropomyosin is a major allergen of invertebrates. This allergen exists in crustaceans (shrimp, lobster and crabs, etc.), mollusks (oyster, snail, abalone, scallop and turbo snail, etc.), and invertebrates (mite, cockroach, worms, etc.) (Table 5.2).

Table 5.2 Identification of the major invertebrate tropomyosin allergens

Species	Allergen name	UniProt number	Type of allergen
<i>Anisakis simplex</i>	Ani s 3	Q9NAS5	Food
<i>Blattella germanica</i>	Bla g 7	Q9NG56	Inhalant
<i>Charybdis feriatius</i>	Cha f 1	Q9N2R3	Food
<i>Chlamys nipponensis</i>	Chl n 1	O02389	Food
<i>Crassostrea gigas</i>	Cra g 1	Q95WY0	Food
<i>Dermatophagoides farinae</i>	Der f 10	Q23939	Inhalant
<i>Dermatophagoides pteronyssinus</i>	Der p 10	O18416	Inhalant
<i>Haliotis diversicolor</i>	Hal d 1	Q9GZ71	Food
<i>Helix aspersa</i>	Hel as 1	O97192	Food
<i>Homarus americanus</i>	Hom a 1	O44119	Food
<i>Metapenaeus ensis</i>	Met e 1	Q25456	Food
<i>Periplaneta americana</i>	Per a 7	Q9UB83	Inhalant
<i>Turbo cornutus</i>	Tur c 1	B7XC63	Food

According to the BLAST search results in UniPort, the oyster (*C. gigas*) allergen Cra g 1 shares sequence identity of 51 %, 50 %, 51 %, 52 % and 51 % with worm allergens (Ani s 3), German cockroach (Bla g 7), America cockroach (Per a 7), dust mite (Der f 10), and house dust mite (Der p 10), respectively. The homology of Cra g 1 is 47 % with crabs (Cha f 1), 54 % with *Metapenaeus* (Met e 1), 53 % with lobster (Hom a 1), and has similarity with other mollusks, such as scallops (Chl n 1) 58 %, abalone (Hal d 1) 66 %, snails (Hel as 1) 65 %, and *Turbo cornutus* (Tur c 1) 62 %. These similarities are near or exceed 50 %, reflecting the high similarity of tropomyosin between different species. Phylogenetic analysis of the main invertebrate allergen tropomyosin conducted by Mega 4.1 show that crustaceans and mollusks are in different clusters (Fig. 5.5). The similarity between mites, cockroaches, and crustaceans is higher than that of mollusks, which suggests that stronger cross-reactivity may exist between mites, cockroaches, worms, and other invertebrates. Mite-allergic patients may need to avoid eating aquatic foods such as crustaceans. But, in fact, cross-reactivity between crustaceans and mollusks is very common, and cross-reactivity between food allergens has been extensively studied. In contrast, cross-reactions between food allergens and inhalant allergens have been less studied, indicating a greater need for research and investigation in this area. In general, cross-reactive species have similar structures between allergens. The main allergic protein of the species above is tropomyosin, thus, the chance of cross-reactivity is very high. However, allergists have gradually reached a consensus that the main cause of cross-reactivity among different foods is due to the high similarity between allergen epitope regions, thus, common allergenic epitopes play an important role in cross-reactions between mites, cockroaches, other invertebrates, crustaceans, and mollusks (Reese et al. 1999).

The AllFam allergen database is allergen family database that elucidates the structure and biochemical properties of protein allergens, allowing for the

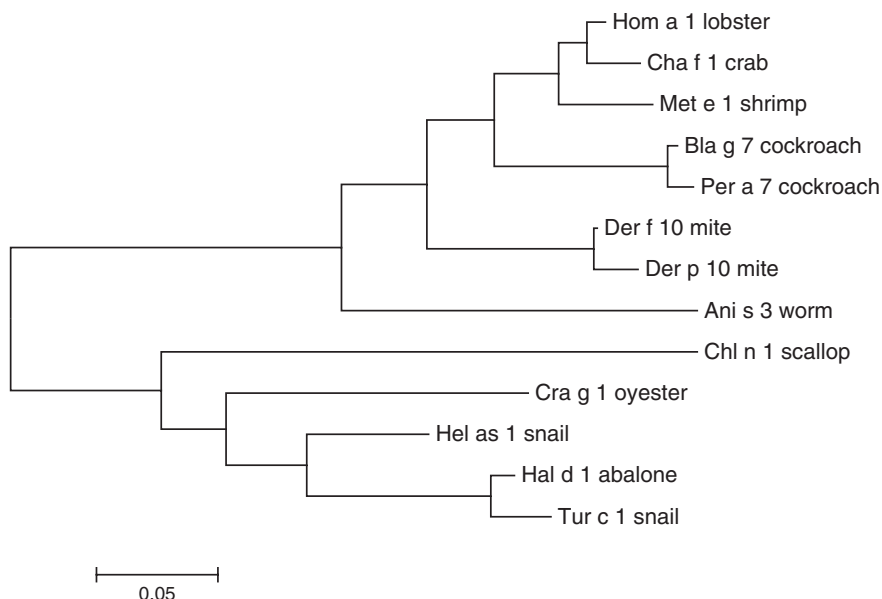


Fig. 5.5 Phylogenetic tree of the main invertebrate allergen tropomyosin (NJ method)

understanding of an allergen from a single allergen or allergen family as well as the systematic analysis of all allergens. This database includes 847 allergens, with 707 allergens classified into 134 AllFam families containing 184 different Pfam domains. Thus, allergen domains are only 2 % of the Pfam protein family database. Thirty-eight allergen families are grouped into 12 superfamilies or clans according to structure similarity or sequence motifs. The most important cluster is the triose phosphate isomerase (TIM) barrel glycosyl hydrolase superfamily containing seven allergens in the glycosyl family, and includes the major allergen families from mites, plants, fungi, and insect venom (Radauer et al. 2008).

As far as their biochemical function is concerned, allergens show a bias toward some activities such as hydrolysis of proteins, polysaccharides, and lipids, binding of metal ions and lipids, transport, storage, and cytoskeleton association. The cysteine protease Der p 1 allergen is a typical example of biochemical function correlated with sensitization (Radauer et al. 2008).

5.3.3 Allergen Epitopes

Allergic epitopes, known as AD, are specific chemical groups that determine antigenic specificity. Antigens bind to a corresponding lymphocyte surface antigen receptor at the AD, leading to activation of lymphocytes, which elicits an immune response. Antigens can also bind specifically to corresponding antibodies

or sensitized lymphocytes by the immune epitopes. Antigenic specificity is determined by the features, number, and spatial configuration of the epitopes. Allergens are the antigens that cause allergic reactions. Once an allergen enters the body, B cells are induced to produce IgE antibodies, which bind to surface FcεRI of target cells (mast cells or basophils). After two or more IgE molecules on the mast cell or basophil surface bind to the same bivalent or polyvalent allergen molecule, a cross-link is formed with FcεRI-IgE resulting in the activation of mast cells and basophils that then induce an allergic reactions (Gould and Sutton 2008). Therefore, cross-reactivity usually occurs between allergens that share the same IgE-binding epitope. There may also be the same or similar epitopes between the cross-reactive antigens. B cell epitopes (IgE-binding epitope) include both linear and 3D epitopes with most allergenic epitopes being three-dimensional. Five IgE-binding epitopes of a pan-allergen in invertebrates, tropomyosin, have been identified (Subba Rao et al. 1998). The amino acid sequence of tropomyosin in vertebrates and invertebrates is highly similar, but there are large differences in the amino acid sequence of the corresponding B cell epitope. This suggests that, for the cross-reactive allergens, the B cell epitope sequence is more important than the sequence of the protein (Klinglmayr et al. 2009).

Besides proteins, carbohydrates are another common cause of cross-reactivity, including cross-reactive carbohydrate determinants (CCDs), which generally do not cause clinical symptoms, and saccharides, which can cause other clinical symptoms. CCDs commonly include *N*-terminal glycoprotein oligosaccharides of plants and animals, wherein its two typical structures are α (1, 3) trehalose the *N*-acetyl glucosamine-glucosamine and β (1, 2) xylose connected to the man-nose core. These two epitopes, and other parts of sugars, synergistically induce CCD-IgE. Therefore, CCDs are the structural basis for a variety of cross-reactions (Altmann 2007; Vieths et al. 2002). Whether CCD-IgE can induce type I hypersensitivity allergy symptoms remains controversial. CCDs contain monovalent antigen epitopes and can only cause a positive result serological test in vitro. While the occurrence of type I allergy requires basophils or mast cells and other effector cell surface receptors to form dimeric IgE structures, an allergen must have at least two or more binding sites to cause IgE crosslinking. Pineapple enzyme protein only has one sugar chain, so the prick tests are negative. However, some glycoproteins may contain multiple *N*-glycosylation chains such as HRP but cannot cause basophil degranulation, which strongly argues that form-inactive CCD-IgE structures are due to monovalent antigen epitopes. There is also evidence showing that CCD-IgE can stimulate basophils, which can only occur if the IgE antibodies have biological activity, but it does not explain its clinical relevance (Eberlein et al. 2012; Mertens et al. 2010). Furthermore, o-glycans also exist in some plant proteins and have IgE-binding capacity and clinical relevance with the different saccharides mentioned above (Leonard et al. 2005).

Recent studies suggest that specific IgE against galactose- α -1, 3-galactose (α -gal) can cause serious reactions and even fatalities after exposure to cetuximab, and unusual delayed allergic reactions to beef, pork or lamb. This α -gal epitope is highly expressed in nonprimate mammalian tissue. It has been reported that a

tick bite causes not only the production of a tick-specific protein but also an IgE response to this carbohydrate (Hamsten et al. 2013).

Because the role of T cells in cross-reactivity is much more complex than that of B cells, it is more difficult to predict cross-reactive T cells, and relevant studies are very few. However, if T cell cross-reactivity exists, it can affect not only the outcome of the immune response, but also the results of the effector phase of allergic reactions (Aalberse 2005).

In-depth study of allergen cross-reactivity is very important for the development of component-resolved diagnosis and immunotherapy. Allergen cross-reactivity indicates that individual or few major allergens can be used not only to diagnose true sensitization of patients to a given allergen or to cross-reactive molecules, but also for allergen-specific immunotherapy to yield the same effects as whole allergen extracts in allergic patients (Blank et al. 2011; Bouaziz et al. 2015). Intensive clinical evidence has proven that specific immunotherapy can prevent both the progression of allergies and the acquisition of new allergic sensitizations. Immunotherapy with peptides containing T cell epitopes may provide an efficacious and safe alternative to conventional subcutaneous and/or sublingual immunotherapy using native full-length allergen preparations (Schein et al. 2007).

5.4 Allergen Family Featured Peptides (AFFP)

Cluster analysis was conducted for allergen sequences in each identified allergen family. The allergen sequence was divided into peptides of 6–32 amino acids in length according to a sliding window of 1–10 amino acids, followed by BLAST (Basic Local Alignment Search Tool, a local alignment search tool basic sequence) using the resulting peptide and nonallergen sequences. Those peptides similar to nonallergen fragments were excluded. If the peptide did not match with nonallergen sequences and the *E* value of the alignment was lower than 10^{-5} , it was characterized as an AFP. AFFPs are composed of 2–10 adjacent AFPs in the same allergen.

We developed an allergen prediction method, SORTALLER, which predicts allergens by using a novel algorithm on AFFPs that were substantially optimized on most of the SVM-based classifier parameters. SORTALLER outperformed other methods and achieved a perfect balance between high sensitivity and high specificity for discriminating allergenic proteins from diverse sources. This method could be used as the first step in the assessment of programmed allergenicity and a SORTALLER web server has been developed to allow for the simultaneous and rapid prediction of a set of amino acid sequences (Zhang et al. 2012).

A total of 2,359 allergenic protein sequences were first obtained from the Allergome database, the Swiss-Prot Allergen Index (<http://www.uniprot.org/docs/allergen.txt>), the Food Allergy Research and Resource Program allergen protein database (<http://www.allergenonline.org/>) and the Allergen Nomenclature database of the International Union of Immunological Societies (<http://www.allergen.org/>).

After filtering out negative IgE-binding results, 2,290 allergenic protein sequences were retained. The nonallergenic protein sequences were excluded from commonly consumed commodities by humans by searching in the UniProt/Swiss-Prot protein database. After a filtering process, 234,760 nonallergenic protein sequences were retained.

After analyzing the AFFP, we have selected 444–556 AFFPs from 211 known families and ungrouped allergenic proteins by adopting a specific screening procedure with different sliding window sizes. Of all the allergen families, more than half contained only one AFFP, and most AFFPs were shorter than 200 amino acids.

An independent dataset of 1000 sequences (including 500 allergens and 500 nonallergenic proteins) and 14 equivocal proteins (including at least four allergens referenced by IgE experiments) were compared using SORTALLER or with the following other methods: (i) the FAO/WHO evaluation scheme, based on the identity of six or more contiguous amino acids or a minimum 35 % sequence identity over a window of 80 amino acids; (ii) EVALLER; (iii) three different prediction methods of AlgPred; (iv) AllerHunter; (v) APPEL, and (vi) Allermatch. As shown in Table 5.3, the SORTALLER software significantly outperformed the other methods and achieved a perfect balance between specificity and sensitivity in discriminating allergens from diverse sources.

The following aspects may contribute to the superiority of SORTALLER: (1) using the Matthews correlation coefficient (MCC) as the performance measuring end point, which combines two parameters, specificity and sensitivity, to express an unbiased accuracy indicator; (2) having the appropriate number of AFFPs with sufficient information of the allergens and a synergistically optimized sigmoid function, and (3) BLAST for fast and reliable detection of the similarities of query sequences with AFFPs.

Table 5.3 Comparison of different prediction methods

Methods	SE (%)	SP (%)	ACC (%)	MCC
FAO/WHO ^a	99.2	9.6	54.4	0.198
EVALLER ^b	86.6	99.0	92.8	0.863
AlgPred (amino acid) ^c	92.4	80.2	86.3	0.731
AlgPred (dipeptide) ^c	88.8	88.2	88.5	0.770
AlgPred (ARPs BLAST) ^c	81.8	98.0	89.9	0.809
AllerHunter ^d	82.2	99.2	90.7	0.826
SORTALLER ^e	98.6	98.4	98.5	0.970

SE sensitivity, SP specificity, ACC accuracy, MCC Matthews correlation coefficient

^aAllergen sorting was conducted on a Perl script referenced in the FAO/WHO guidelines: cross-reactivity is predicted with an identity of at least six contiguous amino acids or a minimum of 35 % sequence identity with an allergen over a window of 80 amino acids

^b<http://www.slv.se/en-gb/Group1/Food-Safety/e-Testing-of-protein-allergenicity/e-Test-allergenicity/>

^c<http://www.imtech.res.in/raghava/algpred/>

^d<http://tiger.dbs.nus.edu.sg/AllerHunter/>

^e<http://sortaller.gzhmu.edu.cn/>

SORTALLER is unique because it is founded on the preferential panning of specific AFFPs and includes ideal information for sorting allergens based on a thoroughly optimized procedure. This novel classifier outperformed other methods and exhibits highly balanced sensitivity and specificity as well as higher accuracy. Moreover, the AFFP dataset incorporated in SORTALLER is useful for developing regimens for component-resolved diagnosis and peptide immunotherapy (Zhang et al. 2012).

5.5 Methods for Allergenicity Evaluation by Amino Acid Sequences

Cross-reactivity is an important phenomenon in allergic reactions. Information regarding known allergen cross-reactions can be searched in many allergen databases such as the Structural Database of Allergenic Proteins (SDAP, <http://fermi.utmb.edu/SDAP/>). However, a great number of allergen cross-reactions remain to be identified. Currently, the main serological methods used to study cross-reactions in vitro include allergosorbent test (RAST), enzyme-linked immunosorbent assay (ELISA), and protein immunoblotting (Houba et al. 1996; Kondo et al. 2002; Tee et al. 1987). In addition, bioinformatics technology has been widely used to predict unknown allergen cross-reactions before conducting allergenicity evaluating experiments. The potential for allergen cross-reactions is mainly analyzed by amino acid sequence alignment among different allergens. BLAST can be used to align two or more identical amino acid sequences of allergens (Altschul et al. 1990). The FAO/WHO evaluation scheme is based on the identity of six or more contiguous amino acids or a minimum 35 % sequence identity over a window of 80 amino acids (Umeda 2006). Expectation value (*E*-value) and *Z*-score are commonly used for evaluation of the alignment results of allergen amino acid sequences. The *E*-value measures how many matches with the same sequence similarity one would expect to occur by chance (randomly) in a database of a given size. Thus a low *E*-value (e.g., less than 10^{-5}) indicates a high significance of the sequence match. *Z* scores are indications of the quality of the match relative to the database random distribution. Higher *Z*-scores show better significance for the match (Schein et al. 2007).

We constructed and optimized the SVM-based classifier SORTALLER in concert with two major parameters, i.e. sliding windows of different peptide lengths for AFFP identification and the constant *C* value in sigmoid function for raw BLAST *E*-value scaling. It was shown that a length of 20 amino acids was the optimal peptide length for AFFP screening and a *C* value of 3 in the sigmoid function contributed to optimal allergen prediction. An MCC value as high as 0.970 showed the best prediction results acquired using this method (Zhang et al. 2012).

An integrative approach adopted by FAO/WHO is that any in silico analysis must include environmental factors, such as allergen exposure, dietary habits, and

the effect of food processing, as well as individual factors, including genetic background, which may predispose individuals to becoming allergic. It should be noted that available methods cannot, with 100 % accuracy, discriminate between similar proteins according to their allergenicity. Instead, they provide an indication that certain proteins may be cross-reactive (Schein et al. 2007).

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