Use of Recombinant Allergens for Component-Resolved Diagnostics (CRD) in IgE-Mediated Allergy

ÅSA MARKNELL DEWITT
Abstract

Immunoglobulin E (IgE)-mediated allergy occurs when our immune system causes a reaction to otherwise harmless substances (allergens). Allergens are predominantly proteins present in biological materials such as pollens, mites, animal epithelia, moulds and foods.

In vitro tests for specific IgE antibodies usually employ an allergen source extract as an antibody capturing reagent. The proportion of allergenic molecules in these biochemically complex extracts may vary.

Recombinant allergens may be obtained in large quantities with biotechnological techniques. These proteins can be characterized biochemically and immunologically, resulting in tests with minimal batch-to-batch variation. This thesis describes different uses of recombinant allergens in component-resolved diagnostics (CRD).

In CRD, single allergenic proteins are used to establish a sensitization profile of the patient. Two timothy grass (Phleum pratense) pollen allergens, Phl p 11 and Phl p 4, were cloned and expressed as recombinant proteins. They were subsequently characterized and can, for example, be used in a panel for grass pollen CRD.

Single allergens may be useful as diagnostic markers for allergic sensitization. This phenomenon was studied using tropomyosin, a major allergen from the shrimp Penaeus aztecus (Pen a 1). The characteristics of the recombinant and natural proteins were compared. The recombinant tropomyosin was then extensively tested using specific competition for IgE binding against extracts of other crustacean species, house dust mite and cockroach.

In cases when an important allergen is missing or underrepresented in a natural extract, the corresponding recombinant allergen may be added to the extract as a spiking reagent. Previous studies have shown that latex extracts for diagnostic testing may lack the allergen Hev b 5. Recombinant Hev b 5 was expressed from a synthetic gene construct, incorporating several adaptations to enable efficient large scale production of the recombinant protein, to be used as a spiking reagent.

Keywords: recombinant allergen, IgE, component-resolved diagnostics, CRD, tropomyosin, Phl p 11, Phl p 4, Pen a 1, Hev b 5

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"In theory, there is no difference between theory and practice; in practice, there is."

-Chuck Reid

To My Family
List of Publications

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals.


Reprints were made with the permission from the publishers (Blackwell Publishing and John Wiley & Sons, Inc.).
Front cover:
Scanning electron microscopy (SEM) image of Phleum pratense pollen.
Courtesy of the Palynological Laboratory,
The Swedish Museum of Natural History, Stockholm

Back cover:
Partial DNA sequence of the synthetic Hev b 5 gene wrapped around the ribbon structure of a tropomyosin segment.
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td><strong>Allergy</strong></td>
<td>12</td>
</tr>
<tr>
<td>Types of allergy</td>
<td>12</td>
</tr>
<tr>
<td>IgE-mediated allergy</td>
<td>12</td>
</tr>
<tr>
<td>Non-IgE-mediated allergy</td>
<td>13</td>
</tr>
<tr>
<td>Allergens</td>
<td>14</td>
</tr>
<tr>
<td>What makes an allergen an allergen?</td>
<td>14</td>
</tr>
<tr>
<td>Major and minor allergens</td>
<td>15</td>
</tr>
<tr>
<td>Allergen nomenclature</td>
<td>16</td>
</tr>
<tr>
<td>Protein families</td>
<td>16</td>
</tr>
<tr>
<td>Allergy diagnosis</td>
<td>22</td>
</tr>
<tr>
<td>Sensitization versus allergy</td>
<td>22</td>
</tr>
<tr>
<td>In vitro diagnostic methods</td>
<td>22</td>
</tr>
<tr>
<td>In vivo diagnostic methods</td>
<td>24</td>
</tr>
<tr>
<td>Food challenge</td>
<td>24</td>
</tr>
<tr>
<td>Cross-reactivity</td>
<td>25</td>
</tr>
<tr>
<td>Treatment</td>
<td>25</td>
</tr>
<tr>
<td>Immunotherapy with allergen extracts</td>
<td>25</td>
</tr>
<tr>
<td>Immunotherapy with purified allergenic proteins</td>
<td>26</td>
</tr>
<tr>
<td>Other treatments</td>
<td>26</td>
</tr>
<tr>
<td>Points of concern regarding immunotherapy</td>
<td>27</td>
</tr>
<tr>
<td><strong>Recombinant allergens</strong></td>
<td>28</td>
</tr>
<tr>
<td>Production of recombinant allergens</td>
<td>28</td>
</tr>
<tr>
<td>DNA templates</td>
<td>28</td>
</tr>
<tr>
<td>Expression systems</td>
<td>29</td>
</tr>
<tr>
<td>Purification</td>
<td>30</td>
</tr>
<tr>
<td>Component-resolved diagnostics</td>
<td>30</td>
</tr>
<tr>
<td>Single allergens</td>
<td>31</td>
</tr>
<tr>
<td>Panel of allergens</td>
<td>31</td>
</tr>
<tr>
<td>Multiple testing – arrays</td>
<td>32</td>
</tr>
<tr>
<td>Allergen mixes</td>
<td>32</td>
</tr>
<tr>
<td>Spiking of extracts</td>
<td>32</td>
</tr>
<tr>
<td>Present investigation</td>
<td>34</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>APT</td>
<td>atopy patch test</td>
</tr>
<tr>
<td>CCD</td>
<td>cross-reactive carbohydrate determinants</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CRD</td>
<td>component-resolved diagnostics</td>
</tr>
<tr>
<td>DBPCFC</td>
<td>double-blind placebo-controlled food challenge</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
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<td>HCW</td>
<td>health care workers</td>
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<tr>
<td>HMM-profiles</td>
<td>hidden Markov model based profiles</td>
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<td>immunoglobulin G</td>
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<td>I.U.I.S</td>
<td>International Union of Immunological Societies</td>
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<td>LTP</td>
<td>lipid transfer protein</td>
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<td>MBP</td>
<td>maltose binding protein</td>
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<td>nPhl p 4</td>
<td>natural Phl p 4</td>
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<td>polymerase chain reaction</td>
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<td><em>Phleum pratense</em></td>
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<tr>
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<td>point-of-care</td>
</tr>
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<td><em>Pichia pastoris</em></td>
</tr>
<tr>
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<td>prick-to-prick test</td>
</tr>
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<td>PR</td>
<td>pathogenesis-related</td>
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<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
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<tr>
<td>rHev b 5</td>
<td>recombinant Hev b 5</td>
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<td>rPen a 1</td>
<td>recombinant Pen a 1</td>
</tr>
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<td>recombinant Phl p 11</td>
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<td>RT-PCR</td>
<td>reverse transcriptase PCR</td>
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<td>SB</td>
<td>spina bifida</td>
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<td>SIT</td>
<td>specific immunotherapy</td>
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<td>SLIT</td>
<td>sublingual immunotherapy</td>
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<td>SPT</td>
<td>skin prick test</td>
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<tr>
<td>TLP</td>
<td>thaumatin-like protein</td>
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</table>
Introduction

"as civilisation and education advance, the disorder will become more common than it is at the present time" (Charles Blackley, 1873 [1])

Allergy has become a significant health problem in the western industrialized world, where the prevalence of allergy has increased dramatically over the past several decades to a current level of 20% or higher in the general population. From having been a rare condition 40 or 50 years ago, allergy is now a common disorder and affects many aspects of society and the lives of many individuals.

An accurate diagnosis is important with any disease. Together with a clinical assessment, an objective method to detect and quantify specific IgE antibodies can result in better management of allergic disease. Knowing which allergens that cause an allergic reaction can help the patient to avoid the offending allergen source and other sources that may contain cross-reactive components. This leads to a higher quality of life for the patient.

In vitro diagnostic methods in IgE-mediated allergy are typically based on a natural extract from an allergen source. The composition of the material, and thereby the presence and abundance of allergens, may vary dependent on, for example, the season and/or the geographical origin. Recombinant allergens, produced according to reproducible methods, can be used to enhance the properties of the natural extract, as stand-alone reagents or as a mixture replacing an extract, and to determine the allergen component profile of an individual for diagnostic or treatment purposes.

The only successful treatment for allergy today is specific immunotherapy. With a component-resolved allergen profile the eliciting allergens can be identified and the individual IgE binding reactivity can be monitored during immunotherapy. In the future it may be possible to tailor the specific immunotherapy to include only those allergens to which the individual patient is sensitized.
Allergy

Allergy has had an explosive development during the past decades [2-5], but the underlying causes of this “epidemic” are not fully understood. Most physicians and scientists agree that, beside the involvement of a hereditary factor [6-8], some aspect of our modern lifestyle must also influence the propensity to develop allergy [6, 9, 10]. The dramatic increase of allergy does not, however, appear to apply to all populations of the industrialized world. Epidemiological studies, investigating individuals of former West and East Germany and children from Sweden and Estonia, have shown that the prevalence of allergy is higher in the western countries as compared to the former eastern countries [11, 12]. It has also been demonstrated that adoption of a “western-like” life-style increases the prevalence of allergy [13]. Other factors that appear to influence the risk of sensitization include the diet at infant stage, being born during the pollen season and exposure to other children at an early age, where high interaction with other children appear to have a protective effect [14].

Allergy can give rise to a wide variety of symptoms, including rhinitis, conjunctivitis, urticaria, eczema, asthma, angioedema, oral allergy syndrome (OAS) and gastrointestinal reactions. In the majority of cases, symptoms are mild or moderately severe and can often be managed by medication or allergen avoidance. More rarely, allergic reactions can be severe or even life-threatening (anaphylactic shock) [15]. In such cases, major changes in lifestyle by the sufferer and his/her family may be required as a part of the disease management.

In addition to the discomfort, suffering and risks to individuals, allergic disease causes great expense to society [16] in terms of loss of productivity, health care, renovation of schools and other public places, dietary and sanitation requirements in food production facilities, and so on.

Types of allergy

IgE-mediated allergy

Allergy is defined as hypersensitivity to foreign substances caused by immunologic mechanisms [17]. There are several types of allergy, where immunoglobulin E (IgE)-mediated allergy is the common type of immediate
hypersensitivity, which underlies many of the symptoms mentioned above. As the term implies, this type of allergy is characterized by the formation of specific IgE antibodies against environmental substances (normally tolerated by non-atopic individuals), which elicit allergic reactions [17, 18].

Allergy requires allergen exposure and sensitization. Allergic sensitization can take place through different routes: the airways, the oral and gastrointestinal tract and the skin. A particular mode of sensitization occurs to venoms of bee, wasp and other stinging insects, which inject the sensitizing substance into the skin. Respiratory sensitization is the most common route in adolescent and adult individuals while dietary sensitization is predominant in infants and younger children [6].

Upon allergen exposure, specific IgE antibodies located on the surface of basophils and mast cells can bind and be cross-linked by allergens. Such cross-linking of cell-bound IgE triggers a cascade of cellular events leading to the release of inflammatory mediators, such as histamines and leukotrienes, which give rise to an allergic reaction [18] (Fig. 1).

![Image](image.png)

**Figure 1. Overview of the allergic reaction.** A) IgE bound to receptors on the surface of a mast cell or basophil. B) Cross-linking of IgE by an allergen results in mediator release.

**Non-IgE-mediated allergy**

Non-IgE-mediated allergy can, for example, be caused by immunoglobulin G (IgG) antibodies that form immune complexes with an antigen (as in serum sickness) or by allergen-specific lymphocytes (as in allergic contact dermatitis) [17]. Yet another type is the delayed-type hypersensitivity, which occurs when memory T-cells come in contact with the antigen it recognizes.
and thereby become activated and recruits phagocytes, which release mediators causing an allergic inflammation [18].

Some food-related disorders are sometimes erroneously referred to as food allergy. These include for example lactose intolerance, where the enzyme lactase is missing, and celiac disease, an autoimmune disorder [19]. These conditions should be referred to as nonallergic food hypersensitivity [17].

Allergens

What makes an allergen an allergen?

Allergy can be caused by a great variety of different biological materials such as pollens, mites, animal dander, moulds and foods [20-26]. Each such biological material contains distinct allergens, i.e. substances to which the allergic individual is sensitized and reacts. Allergens are predominantly proteins, usually with a size between 10 and 60 kDa, although both smaller and larger allergens exist [27, 28]. Some, but not all, allergens are glycosylated or carry other posttranslational modifications [26, 29]. All allergens do not belong to a particular group or family of proteins.

In general, there are certain criteria that have to be fulfilled for a protein to be an allergen. The decisive factor is of course the ability to trigger the immune response, which means that the molecule has to be exposed to the immune system. For this to happen the level of exposure to the allergen source is important, or in case of food allergy also the abundance of the allergen in the food source. For an allergen to elicit an allergic reaction it has to enter the body and be presented to IgE on mast cells and basophils. This can occur, for example, through the respiratory system, the gastrointestinal tract, the skin, or, in case of venoms, injection.

Airborne molecules can enter the body through the mucosa of the lungs. Allergens from food are usually digested before they are absorbed into the system and many are stable proteins which can withstand the extreme environment in the gastrointestinal tract. Examples of this are several storage proteins found in nuts and seeds [30-32] and a major allergen from shrimp [33] (see page 39). Some food allergens can elicit oral allergy syndrome (OAS), a condition where the symptoms mainly occur in the oral cavity. When these allergens encounter the gastric environment, or if they are denatured (e.g. heat-treated, as in cooking) before ingestion, symptoms may be absent. This is often the case for pathogenesis-related (PR)-10 proteins (see page 20), where the respiratory allergen from birch elicits upper-airway symptoms like asthma, rhinitis and conjunctivitis, while the homologous allergens from fruit and vegetables usually elicit mild to moderate OAS
symptoms, although more severe gastric and systematic reactions can occur [34].

The IgE antibody binds to specific epitopes present on the allergen. A minimum of two epitopes must be recognized on the allergen to cross-link the IgE. If more than two epitopes are present, the binding is more effective [35]. The epitopes can be linear or conformational. If a molecule that is susceptible to gastric digestion possesses both types of epitopes, the conformational epitopes may be abolished in the gastrointestinal tract while the linear epitopes will be retained and can still bind IgE [36, 37]. An additional theory concerning linear and conformational epitopes, is that allergy based on sensitization to linear epitopes will persist into adulthood, whereas the symptoms will disappear if the response is established to conformational epitopes [38].

In order to establish whether a novel protein is a potential allergen the amino acid sequence is determined and compared with sequences of known allergens. However, recent studies indicate that even though the amino acid sequence matters, structure and function of the protein is more important than the sequence itself [26]. Other important contributing factors are the level of exposure to the allergen and genetic susceptibility of the individual [36, 39].

Major and minor allergens

A simple way to classify allergens is into major and minor allergens. According to convention, an allergen is considered “major” when the allergen elicits a reaction in 50% or more of an allergic population and “minor” when less than 50% of the allergic population responds to the allergen.

Allergens may be classified as major or minor depending upon the allergen source or the demographics of the population being studied. It has been shown that some allergens are “major” when investigating for instance a group that have an excessive or unusual exposure to an allergen, as in certain occupations [40-43], or individuals from a restricted geographical area [28, 44, 45], but “minor” in other populations. An excellent example of the latter is fruit allergy caused by Rosaceae-fruits in Europe. In northern and central Europe a majority of the fruit-allergic patients are sensitized to the PR-10 proteins, which cross-react with the major allergen in birch, Bet v 1. A minority are sensitized to other allergens such as lipid transfer proteins (LTPs) and profilins. In southern Europe, the LTPs are more common sensitizers for reasons that are not fully understood [46].

Traditionally, major allergens are considered more important, but sometimes an allergen can be interesting for its ability to elicit severe clinical symptoms. As an example, the LTP in hazelnut is classified as a minor allergen in northern and middle Europe, while the PR-10 protein is acknowledged to be the major allergen in this region. LTP is suspected to have the
potential to cause more severe reactions than the PR-10 protein, which usually causes milder reactions [47-49].

Allergen nomenclature

Traditionally, allergens have been viewed in a species-specific way. When it became obvious that many allergenic proteins have homologues in closely or distantly related species, classification based on allergen sequence similarity or function has increased.

The nomenclature system for allergens derives from the allergen source and rules have been established by the International Union of Immunological Societies (I.U.I.S) Allergen Nomenclature Sub-committee to ensure that the identification is unique for each allergen [50]. The committee also maintains an “official list of allergens” which can be found at their web site (http://www.allergen.org). When a new allergen is identified that fulfills certain criteria regarding identity and IgE binding, the committee assigns a name to the allergen. The new allergen might be a novel or a known protein which has been discovered to have IgE binding properties. The name consists of the first three letters from the genus name followed by the first letter (or sometimes in case of conflict several letters) of the specific epithet. Finally an Arabic numeral is added. The first allergen to be discovered from a new allergen source will usually be assigned number one, the second number two and so on. The exception is when the protein has a known allergenic homologue that is already classified. The new allergen may in this case be assigned the same numeral as the known homologue [50]. Following these rules, the major allergen in birch (Betula verrucosa) is named Bet v 1, since it was the first characterized allergen from birch. Homologous proteins from other species include Mal d 1 from apple (Malus domestica) and Pru p 1 from peach (Prunus persica).

Protein families

By classifying allergens into protein families, common traits can be identified. These traits may provide diagnostic indicators to the physician to aid in treatment and/or dietary recommendations to patients. If an individual is allergic to birch pollen and it can be determined that Bet v 1 is the eliciting allergen, there is a risk that the individual will also react to homologous proteins in phylogenetically related species such as apple and hazelnut. Advice can then be given to the patient to be observant for allergic symptoms when ingesting such foods.
Pfam

One more recent way of grouping allergens is according to which Pfam family they belong. Pfam is a large collection of protein families and domains and the current version (21.0, November 2006) contains 8957 protein families [51]. The proteins are allocated to different families based on multiple sequence alignments and hidden Markov model based profiles (HMM-profiles) of complete protein domains. Pfam also models discontinuous domains, which results in domain definitions closer to structural databases [52].

For the plant food and pollen allergens a study was performed in silico to find out how the allergens from these sources would map to the families in the Pfam database [53]. Non-redundant sets of 136 plant food allergens, assembled from the PROTALL (www.ifr.ac.uk/protall) and FARRP (www.allergenonline.com) databases, and 152 pollen allergens assembled from FARRP, were mapped against the 3849 Pfam families that existed in the database at the time of the study (version 7.3, August 2004). The results showed that out of the 136 plant food allergens 129 were matched to only 20 different Pfam families (Table 1). The remaining 7 allergens could not be matched to a specific Pfam family. Sixty-five percent of the matches were grouped into only four families.

Table 1. Distribution of known plant food allergens among protein families defined by Pfam

<table>
<thead>
<tr>
<th>Family description</th>
<th>Plant food allergens (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolamin</td>
<td>39</td>
</tr>
<tr>
<td>Bet v 1 family</td>
<td>25</td>
</tr>
<tr>
<td>Cupin</td>
<td>18</td>
</tr>
<tr>
<td>Profilin</td>
<td>11</td>
</tr>
<tr>
<td>Lol p 1 family</td>
<td>9</td>
</tr>
<tr>
<td>Kunitz</td>
<td>7</td>
</tr>
<tr>
<td>Chitin binding</td>
<td>3</td>
</tr>
<tr>
<td>Chitinase I</td>
<td>3</td>
</tr>
<tr>
<td>Papain family</td>
<td>3</td>
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*a Adapted from Jenkins et al. J Allergy Clin Immunol, 2005.

A total of 136 plant food allergens were mapped to 3849 Pfam families. The table shows the protein families most frequently containing plant food allergens.

The largest group (39 allergens) belonged to the cereal prolamin superfamil, which is characterized by low-molecular-weight proteins containing 8 cysteine residues and with similar 3-dimensional structures. This superfamil include the prolamins; major storage proteins present in all cereals except rice and oat, the 2S storage albumins; storage proteins with high content of sulphur-containing amino acids, the non-specific LTPs (see page 21) and inhibitors of alpha-amylases and proteases.
The second largest group is the Bet v 1-homologues (25 allergens). Bet v 1 is the major birch pollen allergen, which belongs to the PR-10 family of proteins (see page 20). This 17 kDa protein was first described in 1983 [54] and almost all birch allergic individuals in northern Europe are sensitized to this molecule [55, 56]. Bet v 1 was first studied in the context of birch pollen allergy alone, but has now attracted additional interest after the discovery that the major allergen in many fruits and vegetables also belong to same protein family as Bet v 1 and that IgE produced in response to Bet v 1 often cross-reacts with the homologue protein from phylogenetically related species [57].

The last two major groups are the cupins, including 7S and 11S globulin storage proteins such as e.g. Ara h 1, 3 and 4 from peanut, and the profilins. Profilin is a 14 kDa actin-binding protein, present in all tissues and cells of all eukaryotic organisms [57, 58]. Homologous proteins with allergenic properties that are present in a wide variety of species are often referred to as pan-allergens [32, 33, 58]. Pan-allergens have the ability to cross-react serologically between phylogenetically distant species [33, 57] and may cause clinical symptoms.

When the 152 pollen allergens were investigated in the same way [53] they also mapped only to a limited number of Pfam families. 147 of the allergens were mapped to only 18 Pfam families and also in this case the majority (>70%) were mapped to four families (Table 2). The pollen allergens mapped with the highest frequency to the Bet v 1-family (49 allergens) followed by the Lol p 1-family (17 allergens) and the grass group 5 allergen family (17 allergens). The grass pollen group 5 allergens are major allergens that are exclusively present in pollen of a particular subfamily of grass species (Pooideae) and have so far not been found elsewhere [20]. The sequence and structure of these proteins are highly preserved and IgE antibodies cross-react with many of them to a high extent.

The forth most common family among the pollen allergens is the pectate lyase family (16 allergens). Pectate lyase is an enzyme that catalyses the cleavage of pectin, a major component of the cell wall of many plants [59]. This family includes the major pollen allergens from several cedar and cypress species.

When it comes to allergens from sources other than plants, the pattern is similar. All allergens on the official allergen list (published by the I.U.I.S Allergen Nomenclature Sub-committee in January 2007), from species belonging to the Animalia and Fungi kingdoms, were allocated into Pfam families using their UniProt designation or, if unavailable, their biochemical name. Isoforms, i.e. sequence variants, from the same allergen source were removed. Pfam version 21.0 (release November 2006), which contains 8957 families, was used (http://www.sanger.ac.uk/Software/Pfam/).
Table 2. Distribution of known pollen allergens among protein families defined by Pfam

<table>
<thead>
<tr>
<th>Family description</th>
<th>Pollen allergens (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bet v 1 family</td>
<td>49</td>
</tr>
<tr>
<td>Lol p 1 family</td>
<td>17</td>
</tr>
<tr>
<td>Grass group 5</td>
<td>17</td>
</tr>
<tr>
<td>Pectate lyase</td>
<td>16</td>
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<tr>
<td>Profilin</td>
<td>9</td>
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<tr>
<td>EF hand</td>
<td>8</td>
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<tr>
<td>Prolamin</td>
<td>4</td>
</tr>
<tr>
<td>Amb a 5</td>
<td>4</td>
</tr>
<tr>
<td>Pectinase</td>
<td>3</td>
</tr>
</tbody>
</table>

a Adapted from Jenkins et al. J Allergy Clin Immunol, 2005.
A total of 152 pollen allergens were mapped to 3849 Pfam families.
The table shows the protein families most frequently containing pollen allergens.

Of the 207 Animalia allergens in the database, 176 were matched to 50 Pfam families. Of these 51% (89/176) belonged to 6 families where the SCP-like extracellular protein family was the most common (21/176) (Table 3). Members of the SCP-like extracellular protein family have been proposed to be Ca\(^{2+}\)-chelating serine proteases and include venom allergens from both wasps and fire ants. The second most common family among Animalia allergens is the tropomyosin family (18/176). This protein is present in muscle and non-muscle tissue and is involved in the regulation of muscle contraction [33]. Tropomyosin is a major allergen in shrimp and other crustacea, but also a minor allergen in many other invertebrates [60, 61]. Third place is occupied by the lipocalins (16/176), which are common allergens in epithelia of cats, dogs and other domestic animals [62].

For the two different groups of Fungi, Ascomycota and Basidiomycota, 89 allergens were found in the official allergen list and 69 could be allocated to 28 different Pfam families. 65% of the allergens (45/69) belong to 8 families of which Peptidase S8, a family of serine proteases, was the most common (11/69) (Table 4). This family was previously known as the Subtilase family and includes several allergens from the Penicillin and Aspergillus species.
Table 3. Distribution of known Animalia allergens among protein families defined by Pfam

<table>
<thead>
<tr>
<th>Family description</th>
<th>Animalia(^a) allergens</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>SCP-like extracellular protein</td>
<td>21</td>
<td>11.9</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>18</td>
<td>10.2</td>
</tr>
<tr>
<td>Lipocalin</td>
<td>14</td>
<td>8.0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>12</td>
<td>6.8</td>
</tr>
<tr>
<td>Lipase</td>
<td>11</td>
<td>6.2</td>
</tr>
<tr>
<td>EF hand superfamily</td>
<td>9</td>
<td>5.1</td>
</tr>
<tr>
<td>Globin</td>
<td>8</td>
<td>4.6</td>
</tr>
<tr>
<td>Der p 2 / Der f 2 family</td>
<td>6</td>
<td>3.4</td>
</tr>
</tbody>
</table>

\(^a\)Animalia consists of Arthropoda, Chordata, Cnidaria, Mollusca and Nemata.
A total of 176 Animalia allergens were mapped to 8957 Pfam families.
The table shows the protein families most frequently containing Animalia allergens.

Table 4. Distribution of known Fungi allergens among protein families defined by Pfam

<table>
<thead>
<tr>
<th>Family description</th>
<th>Fungi(^a) allergens</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Peptidase 8</td>
<td>11</td>
<td>12.4</td>
</tr>
<tr>
<td>Ribosomal 60s</td>
<td>7</td>
<td>7.9</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>6</td>
<td>6.7</td>
</tr>
<tr>
<td>Redoxin</td>
<td>6</td>
<td>6.7</td>
</tr>
<tr>
<td>Enolase</td>
<td>5</td>
<td>5.6</td>
</tr>
<tr>
<td>Pro-isomerase</td>
<td>4</td>
<td>4.5</td>
</tr>
</tbody>
</table>

\(^a\)Fungi consists of Ascomycota and Basidiomycota.
A total of 89 Fungi allergens were mapped to 8957 Pfam families.
The table shows the protein families most frequently containing fungi allergens.

Pathogenesis-related protein families

Many plant allergens belong by homology to the plant defense system and another way of grouping allergens, based on function, are by pathogenesis-related (PR) protein families [63]. (Table 5). The major allergen in birch, Bet v 1, belongs to the PR-10 family as do the major allergens from the Rosaceae family. Even though the exact biological function of the PR-10 proteins is unknown, they are believed to be involved in plant pathogen defense response and their synthesis is regulated by environmental stress factors and varies through natural developmental stages [64, 65].
The non-specific LTPs are small, ~8 kDa proteins that are held together by four disulfide bonds. They are highly resistant to proteolysis, extreme pH and temperature [63]. The LTPs belong to the PR-14 family and are located in the outer layer of the peel of fruits such as peach, cherry and apple, but can also be found in nuts, seeds and vegetables. LTPs from different plant-derived foods are highly similar in structure and often cause IgE cross-reactivity. Sensitization to fruit LTPs is common in Mediterranean populations and is often associated with severe allergic reactions [63].

Another interesting allergen group is the chitinase class I proteins, also known as the PR-3 family. These allergens are present in exotic fruits and are involved in the “Latex-fruit-syndrome”. The causative allergen is probably the major allergen in latex, hevein, also known as Hev b 6.02. Hevein consists of only 43 amino acids and contains a domain (the hevein domain) which has high sequence homology to chitinase class I, present in several fruits such as avocado, mango, kiwi and chestnut, causing IgE cross-reactivity between latex and these foods [66, 67].

Because many allergens are defense-related proteins, a question arises as to whether the abundance of these proteins has increased over time? The manner in which we grow fruits and vegetables has changed over the past decades. Fertilizers and pesticides are used to achieve a greater harvest, plants are forced to grow faster and larger, and chemicals are used to preserve freshness [68]. All of these factors probably stress the plants and put them into a defensive state. It would be interesting to know if the amount of PR-allergens in the plant today is at the same level as it was several decades ago before our modern agricultural techniques were implemented.

For other allergen-containing PR-families, see Table 5.

Table 5. Allergens belonging to families of the plant defense system

<table>
<thead>
<tr>
<th>PR</th>
<th>Protein family</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR-2</td>
<td>Endo-β1,3-glucanases</td>
<td>Banana glucanase</td>
</tr>
<tr>
<td>PR-3</td>
<td>Class I chitinases</td>
<td>Pers a 1, Cas s 5</td>
</tr>
<tr>
<td>PR-4</td>
<td>Win-like proteins</td>
<td>Bra r 2</td>
</tr>
<tr>
<td>PR-5</td>
<td>Thaumatin-like proteins (TLPs)</td>
<td>Pru av 2, Mal d 2</td>
</tr>
<tr>
<td>PR-9</td>
<td>Peroxidases</td>
<td>Tri a Bd 36K</td>
</tr>
<tr>
<td>PR-10</td>
<td>Intracellular PR-proteins</td>
<td>Bet v 1, Mal d 1, Api g 1</td>
</tr>
<tr>
<td>PR-14</td>
<td>nsLTPs</td>
<td>Pru p 3, Cor a 8</td>
</tr>
</tbody>
</table>

*Adapted from Breiteneder et al. J Allergy Clin Immunol, 2004

Cross-reactive carbohydrate determinants

A particular type of IgE mediated cross-reaction is the one caused by specific carbohydrate structures present on many pollen and insect venom proteins [20, 69, 70]. These carbohydrate structures, also known as cross-reactive carbohydrate determinants (CCD) [71], are highly conserved and can therefore cause IgE cross-reactions between entirely different glycopro-
teins. CCD-mediated cross-reactivity is regarded mainly as a serological phenomenon, but certain studies indicate that CCD recognition may in some cases be clinically relevant [72, 73]. It has also been shown that the IgE binding to glycan epitopes usually are weaker than to peptide epitopes and it has therefore been proposed that a failure of CCD to cause in vivo reactions may be due to low affinity of the allergen/antibody interaction. Additionally, glycoproteins may be bound to CCD-reactive IgE antibodies in a monovalent manner without causing cross-linking [74, 75].

Allergy diagnosis

Several complementary methods are used to diagnose allergy. The first and most important step is to obtain clinical history from the patient, including symptoms, duration and other characteristics of the reaction and, if possible, indications of the offending agent. Secondly, the presence of sensitization to the suspected offending agent(s) is examined by one or several established in vivo or in vitro methods, of which the most commonly used are described below.

Sensitization versus allergy

Sensitization is defined as the formation of specific IgE antibodies after exposure of the immune system to an allergen. Sensitization is a prerequisite for IgE-mediated allergy, but sensitization may be present without clinical symptoms of allergy [76].

In vitro diagnostic methods

Specific IgE

The most common in vitro diagnostic method consist of measurement of specific IgE antibodies using an immunoassay such as an enzyme-linked immunosorbent assay (ELISA) [77, 78] (Fig. 2). A blood sample is taken from the patient and a small volume of serum or plasma is incubated with allergens that have been immobilized on a solid phase. After removal of unbound antibodies by washing, an anti-IgE antibody, conjugated with a reporter molecule, is allowed to attach to the allergen-bound IgE and, through the reporter molecule, the amount of specific IgE can be measured. A positive IgE results should always be evaluated in the context of the patient’s clinical case history.

The serum sample is usually sent to and analyzed in a laboratory, which requires a second visit to the physician for the patient. However, new tests
are being developed and specific IgE analysis can be achieved directly in the office of the physician in 20 minutes [79].

Advantages with *in vitro* diagnosis include patient comfort, no exposure to allergen, the possibility of quantitative measurements and the ability to perform multiple tests.

**Figure 2. Schematic description of an ELISA.** See text for details.

**Cellular based assays**

Other *in vitro* methods that can be used to diagnose allergic sensitization are different cellular based assays. In the histamine release assay, basophils in a blood sample are exposed to the suspected offending allergen. If allergen specific IgE is present on the basophils, mediator release will occur and histamine is measured in the supernatant. There are also assays that measures the release of leukotrienes, or basophil activation tests that measures the expression of basophil activation markers such as CD63 and CD203c using flow cytometry [35, 80, 81]. Some studies have shown a better clinical correlation for these biological assays compared to assays that measure specific IgE [81, 82].

A way to circumvent the need for fresh basophils from the patient is to use basophils from non-allergic donors that have been stripped from IgE followed by resensitization with the sera from the patient. There are also basophilic cell-lines from rat, which have been modified to bind human IgE, which can be used instead of stripped human basophils [35].
In vivo diagnostic methods

Skin Prick Test
The most common in vivo method is the skin prick test (SPT) where the allergen is applied to the skin, usually with a lancet [83]. If the individual is sensitized against the allergen, a local inflammatory reaction will occur and the size of the affected area can be measured. This method is fast and simple and can be completed while the patient visits the physician, but it includes exposure to the allergen, discomfort for the patient, subjectivity in evaluating the result and lack of standardized quantification. Another problem may be the lack of suitable skin surface to perform the desired number of tests, particularly if the patient is a child or has extensive eczema.

A variation to the traditional SPT is the “prick-to-prick” test (PPT) [83]. In PPT, a lancet is used to first prick the allergen source and immediately thereafter the skin of the patient [83]. This method can be used for fresh fruits and vegetables since commercial food extracts are often of inadequate quality.

As for the in vitro tests, a positive IgE results should always be evaluated in the context of the patient’s clinical case history.

Atopy Patch Test
The atopy patch test (APT) is sometimes used in children and infants with atopic dermatitis, especially when investigating late phase clinical reactions. To perform this test, a small chamber or pad containing the allergen of interest is attached to the patients back and left for a predetermined period of time. The test is considered positive if an erythema is seen after removal of the chamber or pad [84].

Food challenge

Open food challenge
When diagnosing food allergy, a challenge procedure with the suspected food can be performed. The simpler method consists of an open challenge, where the patient is given increasing doses of the food until a reaction occurs or, in case of tolerance, the top dose is reached. This is the easiest challenge to perform, but an impact of psychological factors on the outcome cannot be excluded, particularly if subjective reactions are recorded and considered indicative of a positive result [85].

Double-blind placebo-controlled food challenge
A more laborious procedure, which is considered more accurate, is the double-blind placebo-controlled food challenge (DBPCFC) [86]. When performing this test, two consecutive meals are served to the patient, one with and one without the suspected offending allergen. The two meals should be in-
distinguishable to the patient and the physician and neither of them should know the order in which they are served. For the challenge to be conclusive, the patient must have a positive reaction to the meal containing the active substance but no reaction to the placebo meal. The DBPCFC is considered the gold standard for diagnosis of food allergy today.

Disadvantages of challenge procedures include the extensive time required of the patient, medically trained personnel and a dietician (usually multiple visits over several days for DBPCFC), the equipment that is required, the risk of adverse reactions, such as anaphylactic shock, and the fact that the method has not been developed for all foods [86]. The consequence of these disadvantages is that challenge procedures are not performed on a routine basis and instead other, less costly, and potentially less reliable, methods are used.

Cross-reactivity
Allergens exhibit highly asymmetrical representation in different biological materials. Certain allergen types exist in a wide variety of species and tissues while others show a very restricted occurrence. Cross-reactions occur when specific IgE antibodies recognize a protein that has epitope structures similar to those of the protein they were formed against. Usually these epitopes are formed by the polypeptide chain of the allergen, but cross-reactivity can also occur through carbohydrate epitopes of glycoproteins [53, 57, 69, 71, 87].

Treatment
A cornerstone of allergy disease management is allergen avoidance. Correct diagnosis and accurate identification of the offending allergens is therefore of vital importance, especially in some types of food allergy, since symptoms may be severe and even life-threatening. Symptoms are usually treated with antihistamines, anticholinergic agents and corticosteroids or epinephrine in case of anaphylaxis [15].

Immunotherapy with allergen extracts
The only curative treatment for IgE-mediated allergy is specific immunotherapy (SIT) [88]. This involves desensitization to the offending allergen by administrating increased doses of the allergen over a period of time. Natural allergen extracts are used and this treatment is effective for many patients with allergies to pollen, cat dander and insect venoms [89-91]. The treatment always poses a risk of developing systematic reactions. Because of the risk of anaphylactic shock, SIT is usually not used for patients with food allergy.
Traditionally, SIT is performed by subcutaneous injection, but during the past decade the interest for sublingual immunotherapy (SLIT) has increased. One of the reasons for this is the inconvenience to both the patient and the health care professionals of frequent subcutaneous injections. The risk of severe adverse reactions has also contributed to the interest in alternative methods. Recently, a multicenter study investigating dose-dependent SLIT using grass pollen tablets was presented [92]. The treatment was well tolerated by the patients and was shown to reduce allergic symptoms to grass pollen as compared to a control group receiving placebo. Also, SLIT for hazelnut has been reported [93]. In the future SLIT might become available more widely and the treatment may consist of self-medication at home without risk for severe systemic reactions [92].

Immunotherapy with purified allergenic proteins

An alternative to allergen extracts for SIT is to use the allergens that elicit the allergic reaction in a pure form. This could be a single allergen [94], a set mixture of major allergens [95, 96] or possible a mix tailored to the individual reaction profile of the patient. Current research also focuses on developing hypo-allergenic SIT reagents (usually recombinant or denatured allergens with preserved T-cell epitopes, but destroyed B-cell epitopes) [97, 98] or to use T-cell targeting peptides to minimize the risk of anaphylaxis [99]. Studies with recombinant allergens as defined reagents for SIT, either unmodified [95, 96] or hypoallergens [94] have been reported in clinical trials.

Other treatments

Other therapeutic approaches against allergic disease, targeting the IgE antibody itself, have recently been introduced or are under evaluation. Already in clinical practice is omalizumab [100], a humanized monoclonal antibody designed to bind IgE and block its ability to bind the allergen. In clinical trials, asthma patients experienced reduced allergic symptoms and improved quality of life. The anti-IgE is delivered subcutaneously to the patient and regular injections are necessary. Another approach, circumventing this problem, has been tested in a rat model [101]. In this case a chimeric IgE, part rat and part from the phylogenetically distant species opossum, is injected to induce an autoimmune response against IgE. Animal models describing vaccination with DNA, coding for human T-cell epitopes, have also been reported. The aim of this model is to shift the Th2 allergic response to Th1. [102].
Points of concern regarding immunotherapy

One point with SIT is the standardization of allergen preparations. Since the constitution of extracts varies, depending on when or where the allergen source was collected or how it was cultivated, extracts from different manufacturers are rarely comparable and each manufacturer must spend great effort to maintain lot-to-lot consistency. Most manufacturers characterize their allergen preparations regarding major allergens, but since manufacturers define potency in different ways, comparisons are difficult to do. The CREATE project, with 29 partners in Europe, aim to develop reference materials (natural or recombinant allergens) and to validate ELISA assays for measurement of major allergens as aids in standardization of allergen vaccines [103].

Another point is that SIT can induce severe reactions, such as anaphylaxis [104]. Improved management of anaphylactic reactions and safer methods of administration, such as SLIT, is therefore desirable.

Component-resolved diagnostics (CRD) gives us a tool to monitor SIT in more detail compared to extract testing. The individual allergen profile of the patient can be determined and this may be of importance. There have been reports of sensitization to new allergens after SIT treatment [105] and cases where patients have developed new allergies [106]. Awareness and monitoring for cross-reactivity and new sensitizations may be important.
Recombinant allergens

“There is one part of the subject which would well repay careful investigation, viz., the chemical constitution of the pollen of various plants, and especially those of the order Graminaceae. Into this I have not been able to enter; but I cannot doubt that it would be well worth the time and trouble which would have to be expended on such an inquiry, if some able chemist would take up the matter and ascertain all that can be known of the constituents of pollen.” (Charles Blackley, 1873 [1])

In vitro tests that measure specific IgE antibodies are available as an aid in the diagnosis of allergy. Conventional tests usually employ an extract of the allergen source as antibody capturing reagent. Since these extracts are biochemically very complex and the proportion of allergenic molecules may vary, it is a difficult and elaborate task to ensure and verify a reproducible representation of all relevant allergens in different extract batches. During the past decade, recombinant DNA techniques have been used to obtain characterized recombinant allergens with immunological properties comparable to those of the natural molecules [107]. These allergens can be produced in large quantities [108] and the proteins can be characterized both biochemically and immunologically. Such documented characterization serves to facilitate the production of tests with minimal batch-to-batch variation [109]. Aspects that have to be considered when using recombinant allergens are that the primary structure is not always known, and that there maybe several important isoforms present. In addition, some allergens are not easy to obtain in a recombinant form, for example large or glycosylated proteins [110].

Production of recombinant allergens

DNA templates

To produce recombinant allergens a DNA template is needed. This template can be obtained in several ways. The traditional method was through screening of λ-phage libraries [111-113]. With this method it is possible to acquire DNA from many allergens at the same time. Difficulties with this method may include collection of the source material and knowledge of which tis-
sues actually express the gene. Screening of the library is labor intensive and a probe is needed. This probe may consist of a serum sample from a patient sensitized to the allergen.

Today most DNA templates are obtained through a polymerase chain reaction (PCR) using mRNA [114-116]. For this method, sufficient prior sequence information from the allergen is needed to be able to synthesize primers for the PCR. As with the first method, difficulties may be encountered in obtaining material that expresses the gene of interest. In general, a PCR-based approach is less labor intensive than a \( \lambda \)-phage library approach and a probe is not needed.

If there is no available source material a third method may be employed if the DNA or amino acid sequence for the allergen is known as it is possible to create a synthetic gene [117, 118]. For this method oligonucleotides covering the whole length of the gene, are ligated together and amplified by PCR. This method is particularly dependant on high quality oligonucleotides and correct original sequence information, since any sequencing mistakes will be transferred to the synthetic gene.

Expression systems

After the DNA template for the allergen of interest has been obtained it is inserted into an expression vector. The vector is chosen depending on the host it will be inserted into and may include sequences coding for different features that are desirable for the recombinant protein such as affinity tags, enzyme cleavage sites or additional initiator sequences.

The most common expression system uses the bacterium *Escherichia coli* (*E. coli*) as a host [119] and an advantage with bacterial systems is their short generation time. A consideration is that a high proportion of proteins produced in bacteria will not be properly folded, which may cause problems for purification and solubility. Proteins expressed in *E. coli* lack many major posttranslational modifications, such as glycosylations, which can be considered either an advantage or a disadvantage depending on the purpose of the produced protein [119, 120].

To obtain a higher proportion of soluble recombinant proteins with proper folding several eukaryotic expression systems can be used, and the yeast *Pichia pastoris* (*P. pastoris*) has recently become a popular eukaryotic alternative [119, 121]. As a eukaryote, *P. pastoris* possesses the ability to add posttranslational modifications and vectors exist that will direct the produced protein out of the cell, which results in a soluble protein that can be purified from the culture medium. Considerations when choosing *P. pastoris* may include their longer generation time and that extensive optimization to receive high yields may be necessary. Sometimes other eukaryotic hosts are used such as insect cells [122] or tobacco plants [123], but higher eukaryotes
have longer generation time and there might be problems to obtain larger quantities of protein.

Purification

If a recombinant protein has been successfully expressed it has to be purified from the host and media components. If the recombinant protein is accumulated intracellularly, the host cell must be ruptured through homogenization or sonication. Cell debris is separated from the recombinant protein through centrifugation and filtration.

To separate the recombinant protein from the host proteins, different separation techniques can be used. The proteins can be separated according e.g. its size, charge, affinity or isoelectrolytical point. Often an affinity purification step is used initially. This may require that the recombinant protein carries an affinity tag such as a histidine-tag or has a fusion partner such as the maltose binding protein (MBP). An advantage with affinity purification is that it is very specific to the protein of interest and most of the material that does not carry the tag or fusion partner can be discarded [124].

As a second step, size exclusion chromatography or ion exchange chromatography is often used. Size exclusion chromatography separates proteins with different sizes and aggregation forms. The method may be unpractical and difficult to handle when larger quantities of protein from large volumes are to be purified. Large volumes are more easily handled with ion exchange chromatography, but to achieve a pure product may require extensive optimization [124].

Purified recombinant allergens can for example be used as soluble reagents or to produce different diagnostic tests (Fig. 3).

Component-resolved diagnostics

In CRD, single allergenic proteins are used to establish a sensitization profile in the allergic patient, thereby adding clinically relevant information. In some cases, single allergens may also yield a higher diagnostic sensitivity than natural extracts. Further, selected recombinant allergens from a particular source can be combined in a mix to substitute a natural extract [109]. In cases when an important allergen is missing or underrepresented in a natural extract, the corresponding recombinant allergen may be added to the extract as a spiking reagent [125].
Figure 3. Recombinant allergens can e.g. be used as soluble reagents or to produce different diagnostic tests.

Single allergens

Some allergens can be indicative of more serious clinical reactions or a marker for primary sensitization. A test with these allergens can be used to determine the presence of specific IgE. An example of the latter is tropomyosin (see page 39), a major allergen in shellfish where IgE might cross-react with its homologues in mites, cockroaches and other invertebrates [33]. An example of the former may be LTP (see page 21), a protein that is believed to be able to elicit severe reactions. This type of molecular information may provide diagnostic indicators to the physician to enable treatment through dietary recommendations and/or choice of SIT reagents.

Panel of allergens

With CRD, an individual quantitative specific IgE-profile can be obtained. This is in contrast to an extract based test, which does not distinguish between different antibody specificities to components within that extract [109]. Depending on the sensitization pattern, cross-reactivity can also be predicted and aid the physician in e.g. dietary advice to the patient [126]. A
A panel of allergens from a biological source can be used as a tool in conjunction with SIT to characterize the antibody response of the patient. The sensitization profile may aid in the SIT extract selection and predict if SIT treatment will be successful [107]. The response to the SIT can also be monitored by measuring the levels of specific IgE or IgG antibodies against the allergens in the panel during treatment [127].

Multiple testing – arrays

*In vitro* testing using recombinant allergens have increased significantly during the past years. The possibility to perform CRD has given the opportunity to dissect the sensitization of the patient to different allergens, to study cross-reactivity and to monitor immunotherapy. As an increasing number of allergen components become available, the interest for multiplex testing and use of smaller blood sample volumes also increases [128].

Microarrays for nucleic acids have been common for more than a decade and several protein microarrays also exist. Many of these are intended for analysis of specific IgG antibodies, but since IgE antibodies are present in much lower concentrations in serum, it has been difficult to develop an allergen microarray test for specific IgE with sensitivity comparable to conventional assays. Today, most of the allergen arrays described require long incubation times and expensive instruments to measure and record the results [129, 130]. However, the advantage of using small sample volumes for multiplex testing is believed to inevitably lead to new arrays and multi-allergen tests on the market. One such test is a recently released rapid multi-allergen point-of-care (POC) test that can analyze specific IgE to a panel of ten allergens in 20 minutes [79].

Allergen mixes

Several allergens may be combined and replace a natural extract in a diagnostic test. One advantage of this controlled mixing is that all components are present in defined amounts and a mix using recombinant allergens will also circumvent CCD interference. This is difficult to accomplish with natural extracts since their allergen composition may be unbalanced and vary over time. In fruits and vegetables there are also proteases and other modifying or degrading activities, which affects the allergen content of the extract [131].

Spiking of extracts

Some natural allergen extracts are known to contain suboptimal amounts of one or several important allergens [132-134]. However, many extracts cannot be replaced by defined protein mixes since all of the important allergens
from these sources are yet to be identified and purified or produced with recombinant DNA techniques. An alternative solution is to spike the extract with the allergen which is underrepresented or missing. This approach has been practiced with latex extract in which a shortage of Hev b 5 had been noted [135] This allergen was therefore produced as a recombinant protein and added to the extract-based test to improve its diagnostic sensitivity [125].
Present investigation

Grass pollen allergens
Grass pollen is one of the most important airborne allergen sources and as many as 40% of the atopic population may be affected by grass pollen sensitization [4]. One of the most important and thoroughly studied species is timothy grass, *Phleum pratense* (*P. pratense*), and several of its allergens have been isolated and characterized.

The most important allergens in timothy grass pollen are Phl p 1 and Phl p 5 and over 80% of grass pollen sensitized individuals have specific IgE antibodies against each of these proteins. These allergens are grass pollen specific, which means that they are present in closely related grass pollen species, but cannot be found in more distantly related allergen sources [20]. Other allergens, such as profilin (Phl p 12) and the EF-hand protein (Phl p 7), are referred to as pan-allergens since homologous proteins, present in a wide variety of phylogenetically distant species, can cause IgE cross-reactivity [58, 136].

Fewer studies have been performed on high molecular weight grass pollen allergens and to date only two have been isolated and characterized. Of these, the most recently characterized is Phl p 13, an allergen of intermediate importance with homology to polygalacturonase, an enzyme involved in plant cell wall biogenesis and remodeling [137]. The other high molecular weight allergen, Phl p 4, is a basic glycoprotein that was first described in 1985 [138]. Since then, several studies regarding the biochemical and immunological properties of the protein have been reported [139-141]. Phl p 4 is a major allergen, but not considered as important as Phl p 1 or Phl p 5 since its IgE antibody binding intensity is generally lower [20]. The primary structure of this allergen was published in 2005 [142] and a distinctive homology to the berberine bridge enzyme family of proteins and to the celery allergen Api g 5 has been noted (II).

Other timothy grass pollen allergens identified include Phl p 2, Phl p 3, Phl p 6 [143-146] and Phl p 11 (I), which are all considered minor allergens.

Aim of the project
Many timothy grass pollen allergens have been thoroughly studied and most of them have also been cloned and recombinant allergens produced. The
study was part of a larger project which included identifying and producing, as recombinant proteins, all relevant allergens of timothy grass pollen.

The specific aims were as follows:

- To identify, clone and characterize a novel 20 kDa IgE binding protein from *P. pratense*
- To produce the novel 20 kDa allergen as a recombinant protein and study its IgE binding properties
- To obtain the primary sequence for Phl p 4, a major allergen from *P. pratense* previously only characterized in part biochemically
- To clone and produce recombinant Phl p 4, study its IgE binding properties and compare the findings to natural Phl p 4

Results and discussion (Paper I/II)

**Molecular and immunological characterization of a novel timothy grass (*Phleum pratense*) pollen allergen, Phl p 11 (Paper I)**

Sera from 150 individuals sensitized to timothy grass pollen were used to investigate the importance of different grass pollen allergens. All but one of these sera reacted to at least one of the known and characterized allergens available (recombinant Phl p 1, 2, 5, 6, 7, 12 and natural Phl p 4).

To investigate the IgE reactivity of this single discrepant serum, SDS-PAGE and immunoblotting with timothy grass pollen extract was performed. In a Coomassie-stained gel, a faint 20 kDa band was seen which aligned perfectly with a band in a corresponding immunoblot. The 20 kDa band was excised from the gel and subjected to N-terminal amino acid sequencing. A complete cDNA was cloned by PCR utilizing primers designed by back-translation of the obtained N-terminal amino acid sequence and 3’-RACE (rapid amplification of cDNA ends).

Sequence analysis revealed an open reading frame corresponding to a 143 amino acid polypeptide with a calculated isoelectric point of 4.8, a molecular mass of 15.8 kDa and one potential site for N-linked glycosylation. The polypeptide showed homology (32-95%) to pollen proteins from a variety of species and to soybean trypsin inhibitor. The highest homology, 95%, was found to Lop p 11, an allergen which had previously been purified from pollen of *Lolium perenne* [147], a species closely related to *P. pratense*. The recombinant protein, designated Phl p 11, was expressed in *E. coli* as a fusion to MBP and purified to homogeneity.

Serological analysis revealed that 32% of grass-pollen sensitized individuals (n=184) indicated specific IgE binding to recombinant Phl p 11 (rPhl p 11) and that about 20% of the IgE response to grass pollen proteins was directed against Phl p 11 (Paper I, Table 2).
In an immunoblot inhibition experiment, the ability of soluble rPhl p 11 to compete for IgE binding to natural Phl p 11 was examined. The autoradiography signal at 20 kDa was completely abolished when sera were pre-incubated with rPhl p 11 (Paper I, Fig. 5).

To investigate grass specific IgE directed to Phl p 11, dot blots were performed. Nine sera were pre-incubated with rPhl p 11 or the major allergen rPhl p 5 and then analyzed on nitrocellulose membranes where equal amounts of pollen protein extract had been spotted. The radiometric determination of membrane bound IgE showed that rPhl p 11 and rPhl p 5 inhibited on average 25% and 55% of the IgE activity, respectively (Paper I, Table 3).

Skin test and histamine release experiments were used to test the biological activity of rPhl p 11. In the skin test, two individuals with specific IgE reactivity to Phl p 11 showed distinct skin reactions after application of rPhl p 11, while no reaction was seen in four non-allergic individuals (Paper I, Fig. 6).

In the histamine release test, performed on basophil enriched cell preparations, a strong dose-dependent release of histamine was seen in one allergic individual (Paper I, Fig 7A), while only a weak reaction was seen in another allergic individual (Paper I, Fig 7B). No histamine release occurred when cells from a non-allergic individual were used (Paper I, Fig 7C).

In this study, a novel timothy grass pollen allergen, Phl p 11, was cloned and characterized. This was the first grass pollen group 11 allergen to be cloned and produced as a recombinant protein. It was shown that sera from approximately a third of grass pollen sensitized individuals contain specific IgE against Phl p 11 and that the recombinant allergen shares epitopes with the natural allergen.

Cloning, expression and immunological characterization of full-length recombinant Phl p 4, a berberine bridge enzyme-like protein with homology to celery allergen Api g 5 (Paper II)

The first group 4 allergen from grass pollen was initially characterized in 1983 [148] and Phl p 4 was first described in 1985 [138]. Since then, several reports have been published on IgE binding, cross-reactivity, cellular localization and the presence of glycan structures of group 4 allergens [139-141, 149-151]. Several attempts have been made to clone Phl p 4, but N-terminal blockage and resistance to trypsin has complicated the task.

To circumvent these difficulties, an approach was used that took advantage of known amino acid sequences from four peptides from the homologous group 4 allergen from Dactylis glomerata (cocksfoot grass), a species closely related to timothy grass [151]. These peptides were back-translated into DNA sequences, taking into account the codon preference of P. pratense, and used to design a set of forward and reverse primers for RT-PCR experiments. Since the internal order of the peptides in the D. glomerata was
not known, all combinations of primers had to be used in the initial experiments. Using first strand cDNA prepared from polyadenylated *P. pratense* RNA as template, six PCR reactions gave rise to a distinct amplification product and, based on the sizes resulting from different primer combinations, the order P20-P22-P15-P17 was deduced (Paper II, Fig. 1). The fragment spanning P20-P22 was used to design primers for 5'- and 3'-RACE, which generated terminal Phl p 4 fragments. The sequence from these fragments enabled the design of primers for amplification and cloning of full-length Phl p 4 cDNA.

Sequence analysis of full-length Phl p 4 revealed an open reading frame corresponding to 525 amino acids, including a 25-residue signal peptide. The calculated molecular weight of the deduced mature protein was 55.6 kDa and the isoelectric point 9.9. Upon searching for conserved motifs, two consensus N-glycosylation sites and a putative flavin binding domain were identified. Further, sequence similarity was found to recently reported genomic clones from several other Pooideae grass species and to Bermuda grass pollen allergen BG60. Similarity was also found to members of the berberine bridge enzyme family, including celery allergen Api g 5.

Recombinant Phl p 4 (rPhl p 4) was expressed in *E. coli* with a C-terminal hexahistidine-tag and purified using immobilized metal ion affinity chromatography. The purified protein bound specific IgE from 31 of 32 sera reacting to natural Phl p 4 (nPhl p 4; Paper II, Fig. 4). It was further shown that dose-dependent inhibition of IgE binding to rPhl p 4 could be achieved when sera were pre-incubated with soluble nPhl p 4 (Paper II, Fig. 5).

The presence of group 4-related epitopes among different extracts was investigated and compared to rPhl p 4, nPhl p 4 and nDac g 4 (group 4 allergen from *D. glomerata*) using anti-group 4 antibodies from rabbit and mouse IgG. The result showed that the polyclonal anti-Phl p 4 rabbit IgG antiserum bound all grass pollen allergen-derived tests used in the experiment, and that stronger binding occurred to species belonging to the Pooideae subfamily, than to species outside this taxon (Paper II, Table 3). Partly different results were obtained with mouse-anti-Dac g 4 monoclonal antibodies. These three monoclonal antibodies all bound stronger to rPhl p 4 than to nPhl p 4 and did not distinguish between Pooideae and non-Pooideae grass species (Paper II, Table 4).

In this study Phl p 4 was cloned and characterized. Phl p 4 share homology with other group 4 grass pollen proteins, with the berberine bridge enzyme family and the celery allergen Api g 5. It was shown that rPhl p 4 shares epitopes with nPhl p 4 and binds IgE from most patient sera reactive to nPhl p 4.

**Discussion (Paper I/II)**

In Paper I and II we have determined the primary structure for two allergens of *P. pratense*. In both cases, there was no prior knowledge of the nu-
cleotide sequence for the allergens or homologous proteins from closely related related species. Both allergens were expressed as recombinant proteins in *E. coli*, although they both appear to be glycosylated in their natural form.

In the case of Phl p 11 non-glycosylated recombinant protein was used to determine specific IgE binding to the protein core. In a defined mixture consisting of recombinant timothy grass pollen allergens rPhl p 1, rPhl p 2, rPhl p 5a, rPhl p 5b, rPhl p 6, rPhl p 7, rPhl p 11 and rPhl p 12 and the purified natural allergen nPhl p 4 it was found that the mixture was able to mimic the timothy grass pollen extract with respect to diagnostic sensitivity (sensitivity 100%, n=150) [20].

Grass pollen group 4 allergens are major allergens and have been studied biochemically and immunologically for over 20 years. However, their primary structures had not been determined. Phl p 4 shows homology to members of the berberine bridge enzyme family, which are proteins involved in the defense against plant pathogens. This is interesting since several other plant allergens also are pathogenesis related (see page 20). Phl p 4 also showed homology to fragments of celery allergen Api g 5. This glycosylated allergen is considered “CCD-specific”, i.e. without IgE binding epitopes on the protein core. It has been shown that both Phl p 4 and Api g 5 are expressed in similar tissues [140] and it would be interesting to determine if they share other epitopes than CCD.

Phl p 11 and Phl p 4 are part of a growing panel of timothy grass pollen allergens. In addition to being useful as reagents for CRD, to determine the allergen profile of the patient, they may also serve as suitable models to study the effect of glycosylation of allergens.

**Shellfish allergy**

Food hypersensitivity can be divided into IgE-mediated and non-IgE-mediated reactions. IgE-mediated food allergy is more common in children than in adults (8% versus <2%) and many children outgrow their food allergy [152]. Some foods are considered more allergenic than others, and the most serious reactions typically occur to peanut, tree nuts, fish and shellfish [152, 153].

Shellfish can be divided into two phyla: Arthropoda, which consists of Crustacea (shrimp, lobster, crab and crayfish species), and Mollusca, which are further divided into Gastropoda (abalone, limpet and snail species), Bivalvia (mussel, scallop, clam and oyster species) and Cephalopoda (squid and octopus species) [154].

Allergic reactions to shellfish are common in areas where they are consumed and it has been estimated that about 250 000 people in the U.S. may be allergic to shellfish. Shellfish allergy is particularly common among peo-
ple working with shrimps. This occupational form of shellfish allergy is often manifested with asthma-like symptoms [154].

Tropomyosin

Only a few shellfish allergens have been described in shellfish so far. Tropomyosin is the only major allergen characterized to date and has been regarded as dominant in crustaceans. Tropomyosin is a microfilament-associated glycoprotein, which predominantly exists in muscle tissue, but is also present in other tissues [33]. Shrimp tropomyosin is found both in the shrimp meat and in the boiling water used during food preparation. It has high homology to tropomyosins from other crustaceans (e.g., 98% homology with lobster tropomyosin) and high levels of cross-reactivity between tropomyosins have been demonstrated [33]. The protein exists in several isoforms in all animal species and the structure is highly preserved [33].

Extensive serological and clinical cross-reactivity exists among shellfish. In both crustaceans and mollusks, tropomyosin has been identified as the most important cross-reacting determinant. IgE directed to shellfish further cross-reacts with other invertebrates such as mites and cockroaches which is, at least in part, due to tropomyosin [87].

Aim of the project

The aim of this work was to biochemically and immunologically compare recombinant Pen a 1 (rPen a 1; tropomyosin from Penaeus aztecus), produced at large scale, with natural tropomyosin and to more systematically study serological cross-reactivity between rPen a 1 and extracts from crustaceans, mollusks and other invertebrates.

Results and Discussion (Paper III)

Recombinant tropomyosin from Penaeus aztecus (rPen a 1) for measurement of specific immunoglobulin E antibodies relevant in food allergy to crustaceans and other invertebrates

rPen a 1 was expressed in E. coli as a soluble protein with a C-terminal hexahistidine-tag. After purification to homogeneity, using immobilized metal ion affinity chromatography and size exclusion chromatography, biochemical analyses were performed to compare the folding properties of the recombinant protein and natural tropomyosin purified from shrimp tail muscle. In both analytical size exclusion chromatography and circular dichroism (CD) spectroscopy, recombinant and natural tropomyosin displayed practically identical properties.
To test for immunological similarity between the two proteins, an inhibition study was performed where a pool of six tropomyosin-reactive sera were pre-incubated with soluble rPen a 1 or the natural tropomyosin before analysis of IgE binding to immobilized shrimp extract. The two proteins out-competed a similar proportion of the IgE binding to the immobilized extract at the highest inhibitor concentration used (50 µg/mL) and an almost identical concentration was required to suppress 50% of the IgE binding (Paper III, Fig. 6). At the highest inhibitor concentration, a residual IgE binding of 10–20% was observed, indicating that a certain fraction of the IgE antibodies did not form a complex with tropomyosin in fluid phase.

The presence of tropomyosin in different species and cross-reactivity between these homologues was studied by comparing the magnitude of IgE antibody binding to pure tropomyosin and a variety of natural extracts. Sera from nine shrimp-sensitized individuals were used and IgE binding to recombinant and natural tropomyosin and extracts of *Pandalus borealis* (shrimp), *Homarus gammarus* (lobster), *Cancer pagurus* (crab), *Mytilus edulis* (blue mussel), *Octopus vulgaris* (octopus), *Dermatophagoides pteronyssinus* (house dust mite), and *Blatella germanica* (German cockroach) were analyzed (Paper III, Fig. 5). Seven sera showed a predominant reactivity towards extracts of crustaceans, while two had stronger IgE binding to house dust mite extract. All sera displayed comparable IgE binding to rPen a 1 and natural tropomyosin.

To evaluate cross-reactivity between rPen a 1 and tropomyosin from other species, a series of IgE inhibition experiments were performed. Dose-dependent inhibition was performed and analyzed using a pool of sera from six tropomyosin sensitized individuals pre-incubated with serial dilutions of rPen a 1, followed by measurements of IgE binding to extracts of shrimp, lobster, crab, blue mussel, octopus, house dust mite and German cockroach. rPen a 1 caused a dose-dependent inhibition of IgE binding to all extracts tested (Paper III, Fig. 7). The lowest level of inhibition occurred to house dust mite (21% reduction at the highest inhibitor concentration). An explanation for this could be that one or several of the sera used in the pool may come from individuals that are predominantly sensitized to mite allergens other than tropomyosin, to which rPen a 1 could not compete for IgE binding.

In another inhibition experiment, conducted with nine individual sera, rPen a 1 was used as inhibitor of IgE binding to extracts from the same species as in Fig. 5 (Paper III). Also in this experiment extensive inhibition of IgE binding was seen to extracts of all species tested, but with variations between the individual serum donors (Paper III, Fig. 8).

In this study we developed a method to produce high amounts of soluble rPen a 1 with biochemical and immunological properties similar to natural shrimp tropomyosin. It was shown that IgE to tropomyosin is highly cross-reactive among crustaceans and also to other non-edible invertebrates. This
protein may be useful as a defined and relevant diagnostic marker for allergic sensitization to invertebrate foods.

Latex allergy

Like IgE-mediated allergy in general, allergy to natural rubber latex (from here on only referred to as latex), has increased and the prevalence of sensitization to latex proteins has been estimated to ≈3.5% in the general population [155]. Certain groups, which are more intensely exposed to latex, are more likely to develop allergy to this allergen source. One such group is health care workers (HCW) where the prevalence of latex allergy is 5-10% [156]. However, due to implementation of preventive measures recommended by both research groups and task forces of several allergy associations, the trend indicates that, for HCW, the increase has leveled out [157]. A second group with increased contact with latex-containing products is individuals who undergo multiple surgical procedures, e.g. children with spina bifida (SB). In this group a prevalence of up to 37% has been reported [158]. Another group that recently attracted interest is individuals who are not only allergic to latex, but also to exotic fruits such as banana, avocado and kiwi [68]. This cross-reactive condition is usually referred to as the “Latex-fruit-syndrome” [67, 159].

Latex allergens

Latex comes from the rubber tree, *Hevea brasiliensis*, where it is produced in special cells called laticifers. The cytosol of these cells contains rubber particles and proteins, some of which can induce sensitization and allergy [160]. If the latex cytosol is fractionated by high speed centrifugation, three zones are obtained. The top layer consists of the rubber particles, the middle layer, also known as the C-serum, contain water soluble proteins and the bottom fraction, the B-serum, contain lutoids [161], a lysosomal microvacuolar compartment [162].

The 13 officially recognized latex allergens known to date (Hev b 1-13) can be found throughout these three zones. Hev b 1 and 3 are associated with the rubber particles and referred to as “spina bifida-specific”, since they often bind IgE in patients with multiple surgeries [158, 163, 164]. Hev b 2, Hev b 4 [165] and Hev b 13 [166] are found in the B-serum and the rest (Hev b 5 [160], Hev b 6 [167], Hev b 7 [156], Hev b 8 [168], Hev b 9 [167], Hev b 10 [167] and Hev b 11 [169]), except Hev b 12, are found in the C-serum. For Hev b 12 the localization is not known. Only recombinant Hev b 12, cloned from leaves and latex from *H. brasiliensis* by PCR using a 5’- and 3’-RACE method, has been studied and there is no conclusive evidence that the protein is in fact expressed in latex [170].
Aim of the project

The aim of the present work was to adapt the major allergen Hev b 5 for large scale production as an immunologically active protein, useful as a supplement to a latex extract based test in order to achieve a higher diagnostic sensitivity.

Results and Discussion (Paper IV)

Adaptation of the major latex allergen Hev b 5 for large scale production utilizing a synthetic gene approach

Hev b 5 is an important HCW-specific latex allergen [160, 171] present in latex products e.g. on the inside of latex gloves [172]. This major allergen has been reported to exist in suboptimal amounts in diagnostic tests based on the aqueous phase of the latex extract [125].

To be able to produce recombinant Hev b 5 (rHev b 5), a synthetic gene approach was used which allowed us to design a coding sequence based on codons favorable for expression in *E. coli*. Modifications to enhance expression and simplify purification of the recombinant protein were introduced. Several different constructs were evaluated which differed in the number and position of hexahistidine-tags, as well as the presence or absence of additional amino acids to enhance expression. Finally, as natural Hev b 5 lacks aromatic amino acid residues, a tryptophane residue was added to the recombinant allergen in order to render it traceable through chromatography.

The *E. coli* codon-optimized rHev b 5 that was chosen for large scale production carried hexahistidine-tags at both the N-and C-termini to enable efficient purification using affinity chromatography. At the N-terminal, there were four additional amino acids (Met-Arg-Gly-Ser) to enhance expression, and a tryptophane residue to monitor the absorbance at 280 nm. The identity of the recombinant protein was verified by mass determination using MALDI-TOF and N-terminal sequencing.

The *in vitro* IgE binding to purified rHev b 5 ImmunoCAP and latex extract was evaluated using 52 sera from an in-house collection. Specific IgE binding to rHev b 5 was detected in 21/52 (40%) of the sera. Of the 21 sera, two tested negative with the latex extract. Of the remaining 19 sera, the specific IgE reactivity to rHev b 5 was up to 45 times higher than the latex extract, with a median of 1.6 times higher to Hev b 5.

Subsequent to the work described in the manuscript, spiking of natural latex extract with rHev b 5 has been found to significantly reduce the proportion of false negative test results.
Concluding discussion

Even though most allergen extracts used in *in vitro* tests display adequate quality, it is a laborious and costly task to ensure batch-to-batch consistency and the amount of each of the different individual allergens present in the extract is difficult to control other than by indirect means. In the production of recombinant allergens, the absence of genetic and biological variation and the highly defined production process serve to minimize the batch-to-batch variation. The quantity of recombinant allergen can readily be measured and its physical quality analyzed by standard biochemical methods. However, whether the goal is to create a “synthetic extract”, i.e. a mixture of allergens with an allergenic complexity similar to the extract, or a panel of components for CRD, it is essential that all relevant allergens are included.

Biochemical and immunological characterization of allergens is important, but molecular characterization brings new information that cannot be obtained in any other way. With the knowledge of the primary structure, recombinant proteins can be produced and *in silico* studies can be performed, which allows predictions to be made regarding the structure and function of the protein. This in turn may give us another piece of the “what makes an allergen an allergen”-puzzle.
Allergi har under de senaste decennierna blivit en allt vanligare sjukdom, framför allt i de industrialiserade länderna i västvärlden. Varför prevalensen för sjukdomstillståndet har ökat så mycket under en relativt kort tid finns det ingen enkel förklaring på, men de flesta läkare och forskare är överens om att det hänger samman dels med vilken genuppsättning man har och dels hur och när kontakten skett med det ämne man är allergisk emot.

En allergisk reaktion sker när kroppen reagerar mot ett ämne som annars är helt ofarligt. Dessa ämnen, allergenerna, är vanligtvis proteiner som förekommer i en mängd olika allergenkällor såsom växter, djur, födoämnen, kvalster och mögel. Det första stadiet i sjukdomsprocessen är en immunologisk reaktion som kännetecknas av bildning av en viss klass antikroppar, immunoglobulin E (IgE), riktade mot allergenet.

Det första steget vid behandling av allergi är att man i möjligaste mån undviker det eller de ämnen man reagerar mot. Olika läkemedel riktade mot de allergiska symtomen används också. Den enda behandling som botar sjukdomen är immunterapi. Detta innebär att man behandlar patienten med en ökande dos av allergenet, i form av ett extrakt från allergenkällan, för att framkalla tolerans mot de allergener patienten reagerar mot.


Komponentupplöst diagnostik, CRD ("component-resolved-diagnostics"), kan användas för att fastställa en patients allergenprofil. Då används flera olika enskilda komponenter från t.ex. en allergenkälla och resultatet av analysen blir ett reaktionsmönster som skiljer sig åt mellan patienter. CRD kan användas i samband med immunterapi eller för att skaffa extra information.
om eventuella korsreaktiviteter. Om till exempel en patient med björkpollen-
allergi är allergisk mot huvuddallergenet i björk, Bet v 1, finns en risk att pa-
tienten även reagerar mot allergen som liknar Bet v 1 i närbesläktade frukter
och grönsaker, såsom hasselnöt, äpple och morot.

Vissa tester med enskilda allergener kan även användas som markörer för
att påvisa speciella IgE-antikroppar, t. ex där risk för allvarligare kliniska
reaktioner föreligger.

De enskilda allergenerna kan också sättas samman i bestämda koncentra-
tioner till en väldefinierad blandning som, om alla viktiga allergener ingår,
can ersätta ett extraktbaserat test.

Om det i ett extraktbaserat test fattas eller finns för lite av ett visst aller-
gen kan man producera det med hjälp av biotekniska metoder och sedan
sätta till det till extraktet, s.k. spetsning.

Avhandlingen beskriver exempel på ovan angivna användningsområden. I
delarbete I och II beskriver identifiering, karaktärisering och produktion av
två nya gräspollenallergener, Phl p 11 och Phl p 4. Dessa allergener kan ingå
i en panel med gräspollenallergener och kan användas för CRD.

I delarbete III undersöks ett muskelprotein från räka, tropomyosin. Detta
protein finns hos både ryggradsdjur och ryggradslösa djur och ser väldigt
lika ut också hos arter som inte är närbesläktade. Det rekombinanta proteinet
producerades med hjälp av bioteknik och jämfördes med det naturliga prote-
inet som framrenats från räka. IgE-antikroppars korsreaktivitet för tropomy-
osin från räka, andra skaldjur, mollusker samt andra ryggradslösa djur, så-
som kvalster och kackerlacka, studerades.

Delarbete IV beskriver en bioteknisk produktion av ett allergen från latex,
Hev b 5. Det hade tidigare visats att Hev b 5 saknades, eller endast fanns i
låg koncentration, i latexextrakt som används i diagnostiska tester, vilket
resulterade i att patienter med antikroppar mot Hev b 5 testade negativt eller
svarade med för låga IgE-antikroppsnivåer på extrakttestet. För att kunna
tillverka stora mängder av allergenet konstruerades en gen för produktion av
allergenet i speciella bakterier. Genen modifierades så att man på ett lätt sätt
kunde tillverka stora mängder av allergenet. Det producerade allergenet, re-
kombinant Hev b 5, är idag adderat till latexextraktet, vilket resulterat i ett
diagnostiskt test med förhöjd känslighet.
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