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Expression, purification and immunological characterisation of recombinant allergens from *Blomia tropicalis* and *Dermatophagoides pteronyssinus* in *Escherichia coli*

verfasst von / submitted by

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3.12.2019 Vienna Sarzsinszky Eszter

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Abbreviations

AIT – allergen-specific immunotherapy

APS – ammounium persulfate

CD – circular dichroism

CLR – C-type lectin receptor

CRD – component-resolved diagnosis

CTL – cytotoxic T lymphocyte

DAG – diacylglycerol

DAMP –danger associated molecular pattern

DC – dendritic cell

DMA - N,N-dimethylacrylamide

ECM – extracellular matrix

ELISA – enzyme-linked immunosorbent assay

EMTU – epithelial-mesenchymal trophic unit

FLG – filaggrin

HLA - human leukocyte antigen

HPLC – high-performance liquid chromatography

ICS – inhaled corticosteroids

ITAM – immunoreceptor tyrosine-based activation motifs

ILC – innate lymphoid cell

IPTG – isopropyl-β-D-1-thiogalactopyranoside

LB – Luria-Bertani

LPS – lipopolysaccharide

MAPS – [3-(methacryloyl-oxy)propyl]trimethoxysilyl

MD-2 – myeloid differentiation factor 2

NAS – N,N-acryloyloxysuccinimide

PAR – protease activated receptor

PIP2 – phosphatidylinositol 4,5-bisphosphate

PI3K – phosphatidylinositol-3-kinase

PKC – protein kinase C

PLC-y – phospholipase C-y

PMSF – phenylmethylsulfonyl fluoride

SEC – size-exclusion chromatography

SPT – skin-prick testing

TEMED - tetramethylethylenediamine

T_{FH} – follicular T helper cell

T_H – T helper cell

T_{reg} – regulatory T cell

TSH – thyroid-stimulating hormone

TSLP – thymic stromal lymphopoietin

1 Introduction

1.1 Hypersensitivity disorders and types of hypersensitivity

The adaptive immune system is, together with the innate immune system, responsible for protection against microbes, viruses, helminths and toxins as well as tumour cells. However, if the immune response is insufficiently controlled and targeted to self or environmental antigens that are otherwise harmless, the immune system by itself can become the source of disease.[1] These defective immune responses are called hypersensitivity reactions and, according to Coombs and Gell, are classified into four groups:

1) **Type I or immediate hypersensitivity** is characterised by the production of allergen-specific IgE-antibodies which is induced by the T_H2 cytokines IL-4, IL-5 and IL-13. IgE-associated allergy is the main topic of this thesis and will be explicated in greater detail in chapter 1.2.

2) **Type II or antibody-mediated hypersensitivity** is mediated by specific IgG antibodies that are targeted against antigens on the surface of cells or extracellular matrix. Fc receptors on leukocytes like NK cells or macrophages and molecules of the complement system are able to recognize these antibodies and activate effector mechanisms that cause tissue injury. We distinguish three different mechanisms where antibodies are targeted against tissue antigens:

   Cell surface antigens opsonised by antibodies can activate the complement system and the resulting complement products might lead to further opsonisation. This facilitates phagocytosis and destruction of affected cells through Fc receptors and C3b receptors.

   Antibodies directed against tissue antigens may be bound by neutrophils and macrophages through Fc receptors leading to the activation of these immune cells. Likewise, binding of complement molecules such as C3a or C5a to complement receptors can induce their activation. As a result of activation, neutrophils and macrophages release chemotactic molecules to recruit other leukocytes to the affected tissues. These cells release lysosomal enzymes and reactive oxygen species, triggering local inflammation and tissue damage.

Abnormal antibodies specific for receptors, hormones or neurotransmitters, i.e., autoantibodies, may disturb normal signalling and cell function leading to alterations in physiologic responses and diseases but not accompanied by inflammation (Figure 1). For instance, antibodies binding to thyroid-stimulating hormone (TSH) receptor cause hyperthyroidism (Graves' disease) without the presence of the natural ligand TSH. Another example are antibodies specific for acetylcholine receptors at motor endplates that inhibit binding of acetylcholine and, hence, block signal transmission in Myasthenia gravis.

The above mentioned reactions are typically caused by autoantibodies and are part of autoimmune reactions.[2]

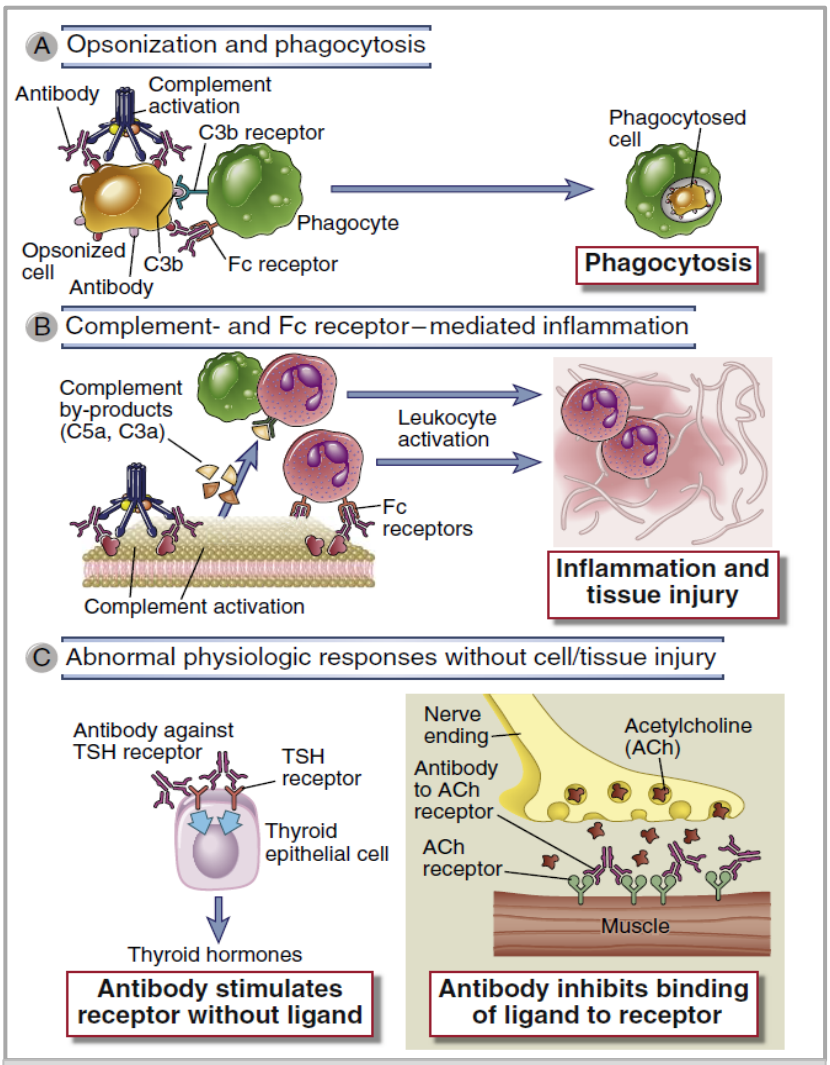

Figure 1. Mechanisms of antibody-mediated hypersensitivity[1]

3) Type III or immune complex-mediated hypersensitivity

The term was introduced by Clemens von Pirquet in the early 1900s who discovered this mechanism by treating patients with sera from horses immunised against diphtheria toxin. These patients reported joint inflammation, rash and fever at least one week after injection. His assumption that these symptoms were not antitoxin related was correct. Antibodies from the host against horse serum proteins formed immune complexes causing a disease today called serum sickness.

Acute serum sickness occurs if a large amount of foreign serum protein is introduced into the circulation and antibodies from the host form complexes with these protein antigens. Some of these complexes cannot be cleared by macrophages and sediment in the tissue (most prominently in joints, small arteries, renal glomeruli) causing a neutrophil-rich inflammation at the site of deposition. Chronic serum sickness can occur upon multiple injections, where small complexes are being formed and deposited in kidneys, lungs and arteries. A rare disease, Arthus reaction, typically occurs after vaccinations, at the site of antigen injection. Repeated inoculation of the antigen into the skin at the same site results in elevated levels of IgG antibodies and local formation of immune complexes, causing oedema and haemorrhage.[3]

4) Type IV or T cell-mediated hypersensitivity

This type of hypersensitivity is primarily elicited by T_H1 and T_H17 cells. Neutrophils, macrophages and many other types of leukocytes are recruited to the affected site by cytokines like IL-17 (produced by T_H17 cells), [4] interferon-y (IFN-y, produced by T_H1 cells), tumour necrosis factor and chemokines. Products like lysosomal enzymes, reactive oxygen species, nitric oxide, and proinflammatory cytokines from neutrophils and macrophages are responsible for tissue injury.

Type IV hypersensitivity mainly causes chronic diseases and manifests as delayed-type hypersensitivity reaction. This kind of disease starts with a primary sensitisation by an infection, vaccination or skin contact with certain chemical substances where the individual encounters the antigen. One to two weeks later a subsequent challenge with the same antigen elicits the so called delayed-type reaction taking shape as swelling, redness and induration approximately 48 hours later.

Chronic reactions are often organ-specific autoimmune diseases and are caused by T cells recognising self-antigens and releasing inflammatory cytokines (e.g., rheumatoid arthritis, multiple sclerosis, type 1 diabetes, psoriasis).

Strong T cell reactions associated with enhanced macrophage response against intracellular microbes like *Mycobacterium tuberculosis* can also induce tissue injury. The so-called tuberculin reaction occurs if the protein antigen of *M. tuberculosis* is injected intradermally into individuals that have already been exposed to this bacterium. This test is used in the clinics to prove previous or active tuberculosis infection.

Several diseases affecting the skin are caused by so-called neoantigens formed by certain chemicals bound to self-proteins and trigger contact sensitivity by activation of self-reacting T cells. These reactions may also become chronic, causing eczema.

Cytotoxic T lymphocyte (CTL) mediated diseases are elicited by $CD8^+$ T cells whose normal function is to protect against intracellular pathogens like viruses or certain microbes. However, if CTLs are not able to distinguish between harmless and cytopathic viruses they might destroy host cells. Likewise, CTLs can cause tissue injury in autoimmune diseases like type I diabetes, where the insulin-producing ß-cells are destroyed by autoreactive T cells.

1.2 Type I hypersensitivity

Among the four types of hypersensitivity reactions, type I which is commonly referred to by the term "allergy" is the most prevalent one. The term "allergy" was first devised by Clemens von Pirquet in 1906,[5] whose concept to describe systemic and local symptoms occurring after injection of antiserum was "a general change in reactivity to harmless or harmful environmental substances". He also emphasised that these reactions are not only dependent on the exogenous substance, but on the individual's predisposition as well.[6] Today, we use the term atopy to describe a genetic predisposition toward developing allergic sensitisation,[7] while allergic diseases are defined as hypersensitivity reactions associated with the production of allergen-specific IgE and expansion of allergen-specific T_H2 cell populations, directed against harmless environmental antigens, called allergens.[8] Allergic disorders have become extremely prevalent, affecting 20-30% of the population in industrialised countries, and thus became one of the most investigated immune disorders. Allergies can take shape of several clinical manifestations affecting the upper and lower respiratory tract, eyes, skin, the gastrointestinal tract and cardiovascular system,[9] including diseases like allergic rhinitis, conjunctivitis, atopic dermatitis, allergic asthma and food allergies.

Allergic inflammations resemble immune responses to helminth infestations or ectoparasite bites since they both involve T_H2 cells and antigen-specific IgE. However, under normal conditions, pathogen clearance turns on mechanisms to cease inflammation and tissue injury.[10] These immunosuppressive immune responses include activation of regulatory T cells (T_{reg})

that secrete interleukin 10 (IL-10) and TGF-β. It is hypothesised that in allergic disease these mechanisms do not fully develop.

Hypotheses about the pathogenesis of type I allergies stress both genetic, environmental and life style factors. According to the hygiene hypothesis, the infrequent exposure to parasites and microorganisms as a result of improved living conditions in terms of hygiene standards may be associated with an increased susceptibility to become sensitised. Exposure to micro-organisms at an early age "teaches" the immune system to develop a T_H1 dominated immune response and to suppress harmful immune reactions by T_{reg} cells.[11] Hence, lack of encounter of microbes can lead in genetically predisposed individuals to the tendency to develop a strong T_H2-response against various antigens. Regarding genetic factors, an association between certain HLA class II alleles and the allergic immune response to Amb a 5 was identified[12] already in 1982 and very strong influence of HLA-restriction on allergen-reactivity was shown at a population level. Similarly, a loss-of-function mutation in the gene encoding for filaggrin (FLG), has been shown to be associated with a higher risk for developing atopic dermatitis or asthma. This might be explained by an impaired epithelial barrier function, increasing the chance for allergen intrusion followed by sensitisation.[13]

1.2.1 The development of allergic inflammation

Primary sensitisation to allergens is very often associated with the disruption of epithelial barriers, however, there are many other routes to activate allergic immune responses (immune responses in house dust mite allergy are described in chapter 1.4.2). After having entered the tissue, allergens are taken up and processed by dendritic cells (DC, Figure 2). Subsequently DCs migrate to the local lymph nodes to present peptides derived from the allergens via MHC II to naïve helper T cells or to IL-4 producing follicular T helper cells (T_{FH}). In the presence of IL-4, T cells differentiate into mature T_H2 cells that activate B cells inducing the immunoglobulin class-switch. This means that the heavy chain locus of IgM expressing B cells is rearranged, removing the sequence encoding the μ-chain and putting the ε-chain gene segment in place allowing for IgE production.[1]

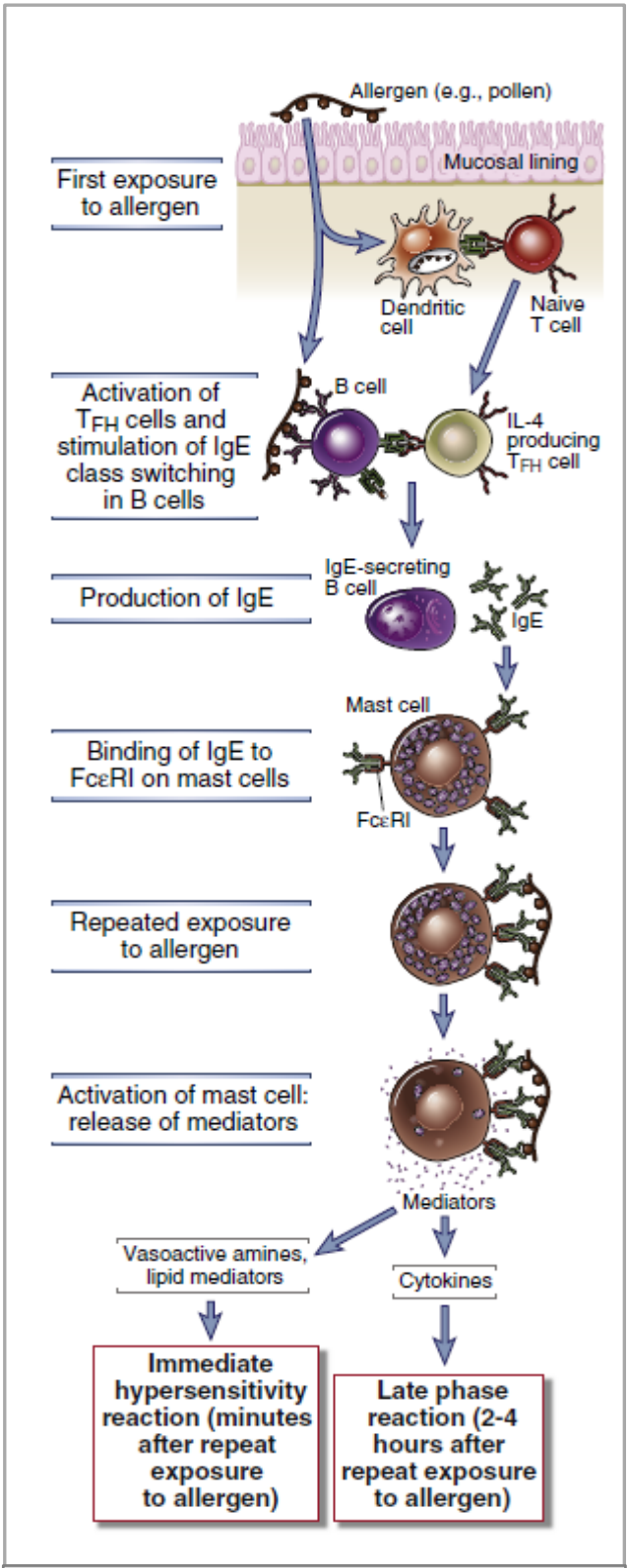

Figure 2. Immediate and late-phase reactions[1]

Early-phase reactions: Allergen-specific IgE binds to FcεRI, the high-affinity IgE-receptor, on mast cells and basophils. During a subsequent encounter of the allergen bound IgE antibodies specific to the same allergen are cross-linked, causing activation of intracellular signalling pathways. As a result of cross-linking, protein tyrosine kinases (LYN, FYN) are activated. LYN phosphorylates immunoreceptor tyrosine-based activation motifs (ITAM) on FcεRI and therefore activates SYK. FYN phosphorylates GAB2, activating the phosphatidylinositiol-3-kinase pathway (PI3K). LYN and SYK activate the RAS-MAPK and the phospholipase C-γ (PLC-

γ) pathways. PLC-γ cleaves PIP2 into DAG and IP3. IP3 activates the release of calcium from endoplasmic reticulum which, together with DAG activates PKC, leading to the release of granule contents and other preformed mediators.[8] Exocytosis of mediators stored in cytoplasmic granules and secretion of biogenic amines, enzymes and newly synthesised lipid-derived mediators lead to the manifestation of symptoms that are characterised as early-phase reactions. These include skin erythema, wheal formation caused by increased vascular permeability, contraction of bronchial smooth muscles and increased mucus secretion.

Late phase reactions are characterised as reactions developing 2-6 hours after allergen exposure and peaking after 6-9 hours. They are caused by a broad range of newly synthesised cytokines and mediators released from either resident or recruited mast or/and T cells. Some cytokines from mast cells play a role in attracting leukocytes (T_H2 cells, eosinophils, basophils, neutrophils) to the site of allergen contact, i.e., TNF-α, IL-8, LTB_8, CCL2, while others activate the cells of the innate immune system (TNF-α, IL-5). Elastase derived from neutrophils causes the activation of matrix metalloproteinases and type III collagen degradation, while eosinophil basic proteins can cause the disruption of epithelium, thus facilitating an easier penetration of new allergens. IL-13 produced by T_H2 cells is responsible for the increased mucus production, while Cys-LTs, TNF-α and IL-13 are involved in bronchoconstriction. Symptoms in the skin include oedema, redness, warmth and pain. These symptoms of late-phase reactions resolve normally after 24 hours without treatment (Figure 2).

1.2.2 Chronic allergic inflammation

Chronic allergic inflammation is elicited by continuous or repetitive allergen exposure. Several innate immune cells like eosinophils, basophils, neutrophils, macrophages and several adaptive immune cells like T_H2 cells, other T cells and B cells are involved in causing persistent changes in cells and extracellular matrix (ECM) of the affected tissues and organs. In chronic allergic asthma, for example, an increased number of mucus-producing goblet cells, thickening of lamina reticularis by the deposited ECM molecules and smooth muscle hyperplasia are observed in the airways. Chronic allergic inflammation causes repetitive epithelial injury and the repair response results in the formation of the so called epithelial-mesenchymal trophic unit (EMTU),[14] which sustains the inflammation by T_H2 cells. In patients with asthma, virus

infections (rhinoviruses, influenza viruses, respiratory syncytial viruses), tobacco consumption and air pollution might worsen the symptoms.[15]

1.3 Diagnosis and treatment of allergy

Allergy diagnosis nowadays is based on a detailed patient history, provocation testing (skin testing, bronchial, nasal, or oral provocation) and the detection of allergen-specific IgE antibodies in serum.

The most common provocation testing, skin-prick testing (SPT), has already been described in 1959 by Helmtraud Ebruster.[16] This technique was the primary diagnostic tool for the detection of type I hypersensitivity reactions back then and remained an essential diagnostic tool until now. SPT is traditionally performed on the forearm where a droplet of extract solution is applied, followed by pricking the epithelial layer of the skin with a metal lancet. After 15-20 minutes, the wheal responses triggered by degranulation of mast cells, resulting in histamine and mediator release, are quantified. According to European standards, the distance between two test solutions should be ≥ 2 cm to avoid false positive results. The largest diameter of the wheal is measured and considered positive if it exceeds 3 mm.[17] This technique is still widely used in the clinics, however, it has several limitations. Skin prick tests are typically performed using allergen extracts prepared from specimens obtained from the respective allergen source. These mixtures of allergens are very hard to standardise and often contain a variety of different proteins, glycoproteins, polysaccharides and nonallergenic materials. Therefore, the disease-eliciting allergens cannot be precisely determined.[18] As it was described in studies on timothy grass or birch pollen extracts, products from different manufacturers showed very high heterogeneity regarding the amount and presence of different allergen molecules, therefore leading to varying test results.[19,20] Furthermore, SPT is not applicable in patients with atopic dermatitis or urticaria and patients receiving antihistamine treatment might show false negative test results.

Immobilised extracts can also be used for the *in vitro* measurement of serum IgE, e.g., in the ImmunoCAP test system (Phadia AB, Uppsala, Sweden). However, as mentioned in the context of SPT, this approach only identifies the allergen source but not the molecules responsible for the symptoms and the same problem regarding lack of extract standardisation and

allergen content applies. The invention of recombinant allergen-based diagnosis and provocation tests has helped to overcome these problems. This type of diagnosis is termed component-resolved allergy diagnosis (CRD) or molecular allergy diagnosis.

1.3.1 Recombinant allergens in component-resolved diagnosis (CRD)

The term component-resolved diagnosis was introduced by Valenta *et al*. in two studies where tree and grass pollen allergic patients' sera were tested for IgE-reactivity using recombinant allergen molecules.[21,22] Many studies have demonstrated thereafter that using recombinant allergens for allergy diagnosis and immunotherapy have several advantages compared to the extract-based technology.[18]

The basic concept of this method is the generation of expression vectors accommodating a cDNA sequence encoding the allergen. These can be introduced into an appropriate expression system (bacteria, yeast, insect cells, etc.), expressed and purified with high purity. A major point of the quality control after protein expression is to analyse if IgE-binding to the recombinant protein is equivalent to the binding to its natural counterpart. Likewise, secondary structure of the protein ought to be comparable to the natural allergen's structure to retain its conformational epitopes. In some cases the structural similarity between certain recombinant and natural allergens is so high (like in the case of Der p 2 or Bet v 1) that the structural analysis can be performed using the recombinant protein.[23,24] Many bacterial strains, yeast or insect cells have been engineered into high-level expression systems of recombinant allergens. With a suitable expression system and appropriate conditions, a protein yield of several milligrams per litre of culture can be achieved. Well defined, standardised conditions and an adequate quality control allow for highly reproducible results in allergen production. In this respect, it is conceivable to assemble mixtures of different allergens with a defined concentration of each protein and without irrelevant nonallergenic molecules for testing and treating patients.[25]

Approximately 17 years ago, recombinant allergens were used for the construction of allergen chips which culminated in a novel tool for multiplexed allergy diagnosis. Allergen microarrays contain a great variety of allergens from the most common allergen sources which allows for the detection of serum IgE in a minute amount of serum and to screen for sensitisations to

numerous allergens in one step. The purified allergens are spotted on pre-activated glass slides with a surface that can either be coated by gels like agarose or polyacrylamide or with functional groups like aldehydes, epoxy- or amino groups.[26] The latest technology is using adsorption to a copolymer of N,N-acryloyloxysuccinimide (NAS), [3-(methacryloyl-oxy)propyl]trimethoxysilyl (MAPS) and N,N-dimethylacrylamide (DMA) where NAS is the reactive group able to bind proteins, DMA forms the polymer backbone and facilitates adsorption and MAPS stabilizes the coating.[27]

Using allergen chips for allergy diagnosis allows for the establishment of patients' individual IgE reactivity profiles. Detection of IgE to marker allergens specific for particular allergen sources and to cross-reactive allergens helps to identify the clinically relevant, disease-eliciting allergen components, i.e., to differentiate between co- and cross-sensitisation. With multiplex assays it is possible to resolve complex sensitisations in polysensitised patients and to predict symptoms and symptom severity already in childhood.[18,28,29,30,31] Based on these results, the clinician can precisely recommend avoidance of particular allergen sources and eventually allergen-specific immunotherapy (Figure 3).

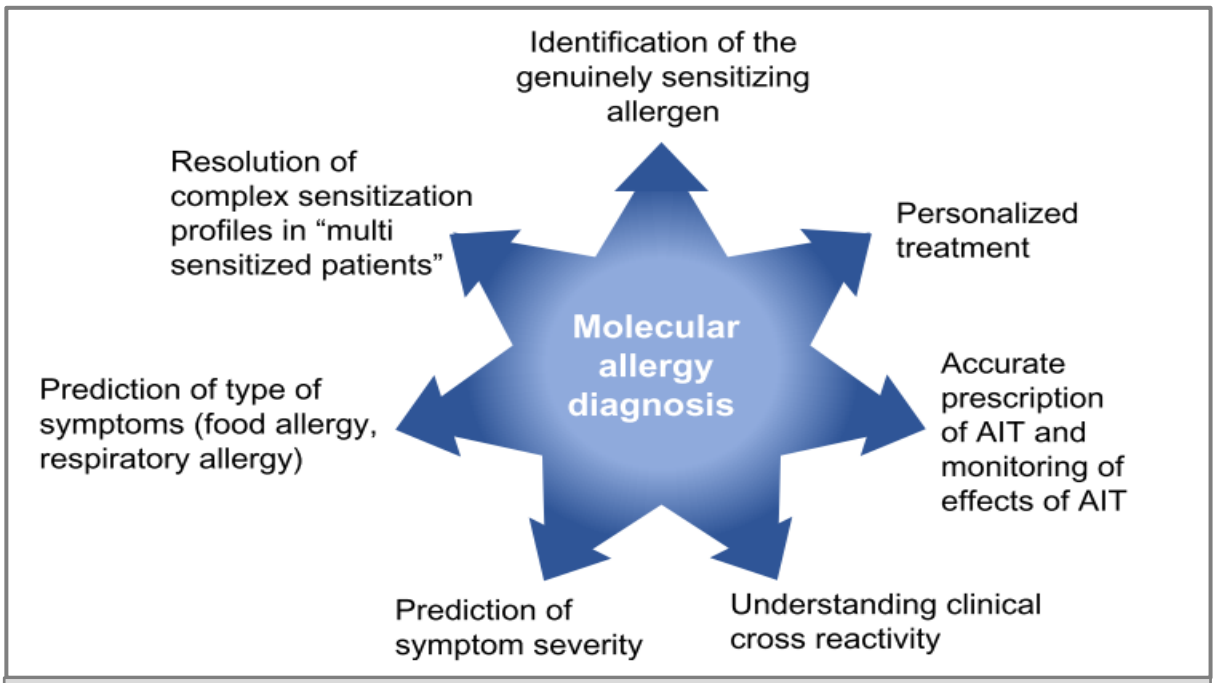

Figure 3. Advantages of molecular allergy diagnosis and allergen-specific immunotherapy[9]

Furthermore, allergen microarrays are a very helpful tool to monitor the immunological effects of immunotherapy. This is based on the fact that of each allergen only 50-200 fg are

spotted which is 10,000,000 times less than the amount of protein immobilised on the ImmunoCAP test system. Therefore, competition between antibodies of different isotypes specific for the same allergen will have an impact on the measured signal level. This unique feature allows for the detection of the induction of allergen-specific blocking IgG antibodies in the course of immunotherapy. Due to the low amount of allergen spotted onto the chip-surface, quality control and biochemical analysis of the purified allergens (folding, oligomerisation, presence of natural epitopes) is a very important point after protein expression.

Allergen microarrays can also be adopted for research purposes. In the European research programme MeDALL, a customised allergen chip was established. The MeDALL chip is a modified version of the ImmunoCAP ISAC (Phadia AB, Uppsala, Sweden) with an allergen panel that was increased to a total of 176 allergen molecules, with special attention on the extension of food and respiratory allergens.[32] This customised allergen-chip has been developed for the analysis of sera from European birth cohorts, i.e., to establish IgE and IgG reactivity profiles in several thousand serum samples in order to create a map of allergic sensitisations in Europe and to monitor the development of allergic diseases in childhood and adolescence.

1.3.2 Treatment of allergy

Allergic sensitisation is the first step in the development of allergic diseases which requires the encounter of the allergen. However, data on protective effects of allergen avoidance are controversial. Several studies have shown that avoidance of some allergens before or even after sensitisation has occurred can have a role in allergy prophylaxis.[33,34] On the other hand, evasion of peanut consumption during pregnancy can even increase the risk of peanut sensitisation, while high dose peanut exposure during infancy was shown to have a protective effect.[35] The opposite was observed for house dust mite allergens, where allergen exposure positively correlated with the degree of sensitisation which could be provoked by extremely low amounts of allergen.[33] In the case of already sensitised children, avoidance of food and aeroallergens was shown to be beneficial and resulted in improvement of asthma and rhinitis symptoms, however, similar data about adults yielded a less clear outcome.[36,37]

Avoidance of the allergens in some cases is very difficult or even impossible to maintain, especially in the case of aeroallergens. Therefore, allergy treatment by medication aiming at relieving symptoms is prescribed in many cases and helps to control or reduce the symptoms:

Corticosteroids suppress T_H2-mediated inflammation by interaction with glucocorticoid receptors, resulting in the inhibition of the expression of cytokine, chemokine and inflammatory genes regulated by the NF-κB and AP1 proinflammatory transcription factors. Inhaled corticosteroids are an effective treatment for airway inflammation in asthma.

β_2-adrenoceptor agonists activate G proteins through adrenoceptors resulting in increased production of cAMP and activation of protein kinase A. This enzyme phosphorylates and thus inactivates the myosin light chain kinase and mediates smooth muscle relaxation, opening of Ca^{2+}-dependent K^+ channels and bronchodilation. Short-acting β_2-adrenoceptor agonists like salbutamol and terbutaline are prescribed for the prompt relief of asthma symptoms. The other type, long-acting β_2-adenoceptor agonists like formoterol or salmeterol have a longer bronchodilatory effect for about 12 hours and are used for the treatment of asthma in combination with corticosteroids.[38]

Antihistamines act on histamine receptors as inverse agonists which means that they bind to the same receptor as the agonist but induce an opposed, i.e., inhibitory response.[39] They are used as short-term treatment for allergic symptoms and are characterised by the histamine receptors they act on. The relevant group by means of allergy treatment and clinical use is H_1-antihistamines that are inhibitors of the H_1 receptor activity in mast cells, smooth muscles and endothelium. Early products, the so called first generation antihistamines were the first drugs used for the treatment of allergy. However, despite relieve of the symptoms of allergy, the strong sedative side effects gave rise to the invention of second generation antihistamines like cetirizine, desloratadine, loratadine, fexofenadine, azelastine, etc., with a decreased ability to cross the blood-brain barrier, therefore reducing the adverse effects on the central nervous system.[40] Moreover, cardiac toxicity has successfully been overcome along with increased efficacy and selectivity. Antihistamines are currently the treatment for allergic rhinitis, allergic conjunctivitis and urticaria. Their direct effects are exerted mainly on small blood vessels and sensory neurons, including decreased vascular permeability, vasodilation and sensory nerve stimulation (itchiness). Effects on cellular signalling inhibit the NF-κB-induced expression of proinflammatory cytokines and regulate the function of calcium ion channels resulting in decreased mediator release.[41]

The pathophysiological role of leukotrienes in allergic diseases is wide-ranging, concerning the respiratory system as CysLTs are the most effective inducers of contraction of smooth

muscle cells[42] but they are also responsible for increased vascular permeability, bronchial hyperresponsiveness and leukocyte influx[43,44] in bronchial asthma, exercise-induced asthma and allergic rhinitis.[45] Moreover, their role in atopic dermatitis and allergic conjunctivitis has also been described.[46,47] Leukotriene receptor antagonists like pranlukast and montelukast are used as monotherapeutics in mild asthma but their anti-inflammatory properties are inferior compared to those of inhaled corticosteroids (ICS). Therefore they are currently mostly used as add-on therapeutics together with ICS for uncontrolled asthma symptoms.[48]

Another therapeutic target in allergy treatment is IgE, whose role in allergy has been discussed in the previous chapters. IgG antibodies targeted against the Cε3 domain of IgE can block the binding of IgE to high- and low affinity Fcε receptors (FcεRI, FcεRII/CD23) on mast cells and basophils. Omalizumab is a humanised IgG_1 specific for human IgE that binds circulating IgE and reduces IgE binding to effector cells and thus mediator release upon allergen encounter. Reduction of levels of free IgE furthermore leads to a down-regulation of high-affinity receptors which results in reduced allergic inflammation and asthma symptoms.[49] However, the level of free circulating IgE drops immediately after the first injection, clinical effects take up to 16 weeks to be seen.

Allergen-specific immunotherapy (AIT) is an immune-modifying treatment which has sustained effects on the disease. It is based on the induction of blocking IgG antibodies which can inhibit IgE-binding to the allergen and, therefore, prevent activation of effector cells of allergy. This type of treatment is described in chapter 1.3.3.

1.3.3 Allergen-specific immunotherapy

In 1911, Leonard Noon made the first attempt to cure hay fever symptoms by using an extract of the disease-causing allergen source to vaccinate patients. Even though the underlying hypothesis was misconceived, i.e., that the symptoms of allergy were caused by a pollen toxin, the vaccination successfully prevented the occurrence of symptoms even after discontinuation of allergen administration.[50] This attempt was the predecessor of the treatment now called allergen-specific immunotherapy (AIT). Today we know that the underlying mechanisms involve the induction of blocking IgG antibodies that compete with IgE for the binding sites on the allergens and thus prevent or mitigate the IgE-induced symptoms.[51] However, the outcome of extract-based AIT may be subject to considerable variation due to high variability

in terms of allergen composition and -concentration and to the presence of non-allergenic components, endotoxin or even contamination with allergens from other allergen sources. Next to this, AIT can cause severe side effects, in some cases even systemic anaphylaxis.

Recent experimental forms of AIT use well-defined allergen derivatives to controllably target distinct elements of the immune response. Administration of T cell peptides (targeting allergen-specific T cells) for example has the advantage that no IgE-mediated side effects are induced, but these peptides seemed to be too short for the induction of blocking IgG antibodies, therefore not able to reduce the allergic symptoms.[52] Genetically engineered recombinant hypoallergenic allergen derivatives have also reduced IgE reactivity and can induce the production of allergen-specific blocking IgG but at the same time induce delayed T cell-mediated side effects. Treatment with fusion proteins consisting of an unrelated carrier protein and non-allergenic peptides from IgE-binding sites of the allergen hold many advantages: no boost of allergen-specific IgE response, but induction of blocking IgG. By avoiding T cell epitopes for vaccine design, T cell-mediated side effects could be reduced (Figure 4). Finally, the vaccine can be produced at reproducible quality in expression systems like *E. coli* and, as higher dosages can be administered, a reduced number of injections is sufficient for the treatment.

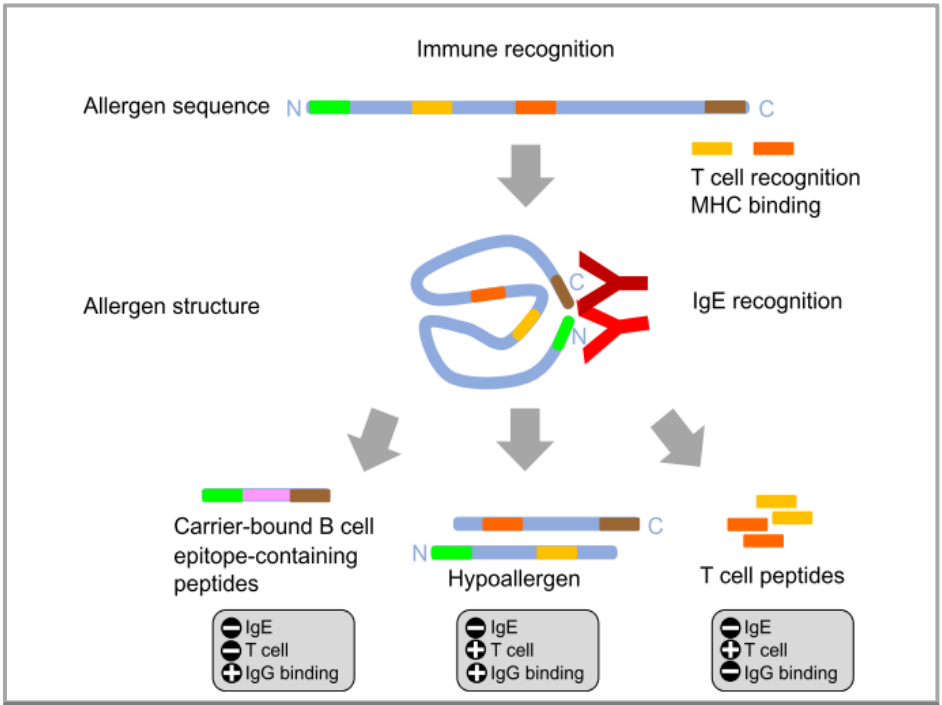

Figure 4. Novel approaches for allergen-specific immunotherapy[9]

1.4 House dust mite allergy

The identification of house dust as allergen source is dating back to the 1920s, however, identification of the component of house dust that elicits the allergic symptoms was first achieved in the 1960s. In 1967, Voorhorst *et al.* analysed several components (animal and human dander, moulds, yeast and several arthropods) of house dust and identified *Dermatophagoides pteronyssinus* as symptom eliciting allergen source by skin testing in patients allergic to house dust, using a *D. pteronyssinus* extract.[53]

Today we know that mites are indeed a significant cause of allergic asthma and allergic rhinitis but also affecting the skin and occasionally causing systemic symptoms. Sensitisation to mite allergens is the most common cause of allergy worldwide, affecting around 1-2% (65-130 million people) of the world's population.[54] The most important mite species in terms of allergic diseases are: *D. pteronyssinus, D. farinae, Blomia tropicalis* and *Euroglyphus maynei*. *Blomia tropicalis*, a storage mite, is mainly found in tropical and subtropical areas while the other species are house dust mites with almost worldwide appearance.

1.4.1 Classification, appearance and life cycle

Within the arthropod phylum, house dust mites are part of the arachnid class, therefore closer related to spiders or scorpions than to insects. The acarii order (order of mites and ticks) comprises ticks, chiggers, soil mites, parasitic mites and astigmata that contain the genera of all the above mentioned four important mite species (Figure 5). Adult mites have a body length of 250-350 µm and an off-white colour under light microscope. Their four pairs of legs end in small "suction cups" which facilitate adherence on different surfaces. Mites are photophobic organisms, however, instead of eyes they have a light receptor organ that helps them to avoid bright areas.[55] The

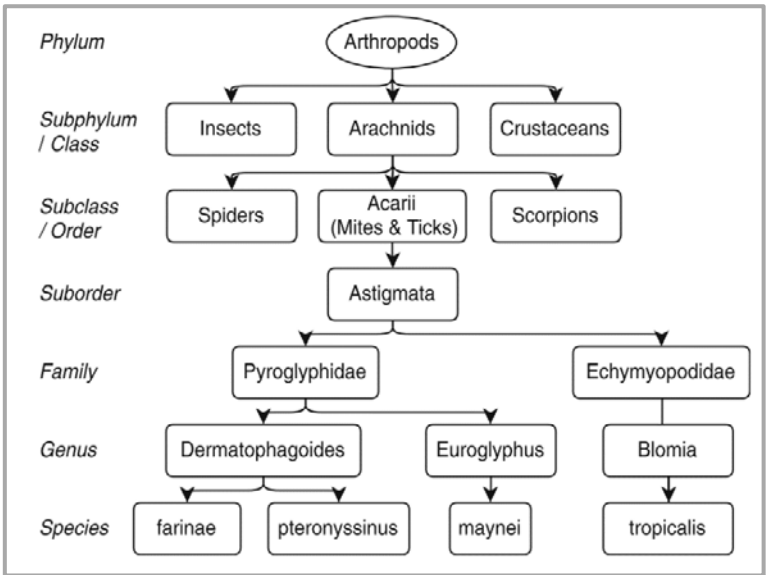

Figure 5. Simplified mite taxonomy[55]

development from the egg into an adult mite takes 3 to 4 weeks and contains a six-legged larval stage, a protonymph and a tritonymph stage. The total lifespan of the mature mites is 4 to 6 weeks, which allows the female to lay 50-80 eggs.[56] Components of the house dust such as keratin, cellulose and chitin serve as nutrients with keratin being the mites' primary food source. Digestive cells from the gut epithelium are excreted into the faecal pellets with digestive enzymes being a main cause of allergenicity.[57] Since they are highly dependent on air humidity, mites have special glands with a high content of sodium and potassium chloride, to be able to absorb water osmotically from air. The optimum humidity for mites is around 75%, with a temperature optimum of 23°C. Under these conditions, their reproduction is at the maximum, while if the humidity level drops below 50% a progressive decrease in life functions can be observed. The major determinants of their life function, i.e., humidity and temperature, are also reflected in their global and local distribution.[58] In areas with lower indoor humidity, significantly restricted numbers of mites can be observed. High altitudes for example do not favour their growth because of the decreased humidity and, therefore, lower sensitisation rates to HDM can be observed than at sea level.[59] Indoors, mites are most prominently found in shady areas, with high humidity, for instance upholstered furniture, carpets, mattresses, pillows or stuffed toys.

1.4.2 Allergic sensitisation to HDM and the role of mite-allergen specific IgE in the allergic response

In general, allergen contact leading to allergic sensitisation can occur at different sites, e.g., conjunctivae, upper and lower airways, skin or gut. Respiratory allergens are inhaled together with particles which they are attached to, and not as pure allergens,[60] with the size of the particles determining the likelihood of sensitisation. Smaller particles (< 4.7 µm) for example are less frequently inhaled than larger ones (> 4.7 µm) but reach deeper segments of the lung.[61] Faecal pellets from mites have a size of 20-25 µm, which allows them to easily become airborne, taking 20-30 minutes to settle again.

Allergic inflammation caused by HDM allergens is facilitated by the activation of T_H2 cells which, in turn, orchestrate an IgE-mediated immune response by the cytokines Il-4, IL-5 and IL-13. IL-4 is responsible for IgE class-switch, while IL-5 supports survival of eosinophils. IL-13

has many effects, including tissue remodelling and development of airway hyperresponsiveness (AHR). The recent identification of innate lymphoid cells (ILCs) including nuocytes and type 2 innate lymphoid cells (ILC2) might be the linkage between innate and adaptive immunity. These cell types are stimulated by IL-25 and IL-33 cytokines produced by epithelial cells, thus becoming producers of IL-5 and IL-13.

HDM allergens elicit allergic immune responses if the inhaled allergen particles pass through the airway mucosa and are taken up by dendritic cells (DC). DCs migrate to the draining lymph nodes to present the antigen-derived peptides to naïve helper T cells and promote T_H2 differentiation. Next to this conventional way, HDM allergens can activate the innate immune system via many pathways[62] and have also direct damaging effects on the airway epithelium by activating mast cells even in the absence of IgE.[63]

Due to their protease activity, group 1 HDM allergens are able to destroy epithelial tight junctions and activate protease activated receptors (PAR), whereas group 2 allergens mimic the myeloid differentiation factor 2 (MD-2) protein that forms a complex heterodimer with TLR4 and can recognise lipopolisaccharides (LPS), and activate the transcription of proinflammatory genes through TLR4 by presenting LPS. Tissue injury and disruption of epithelial cells mediated by proteases induce the release of danger associated molecular signals (DAMP) like ATP or uric acid. Uric acid, in turn, can induce necrosis by engaging the NLRP3 inflammasome and release of IL-1β, followed by an inflammatory response.

Glycan structures of Der p 1 and Der p 2 promote their uptake by DCs through C-type lectin receptors (CLR) playing a key role in T_H2 polarisation.[64] These signalling pathways promote the upregulation of the expression of many innate cytokines and chemokines like IL-6, IL-25, IL-33, thymic stromal lymphopoietin (TSLP), GM-CSF and CCL2. TSLP mediates IL-4 expression and thus T_H2 differentiation, while IL-25 and -33 activate ILC2, generating the main features of allergic airway inflammation (Figure 6).

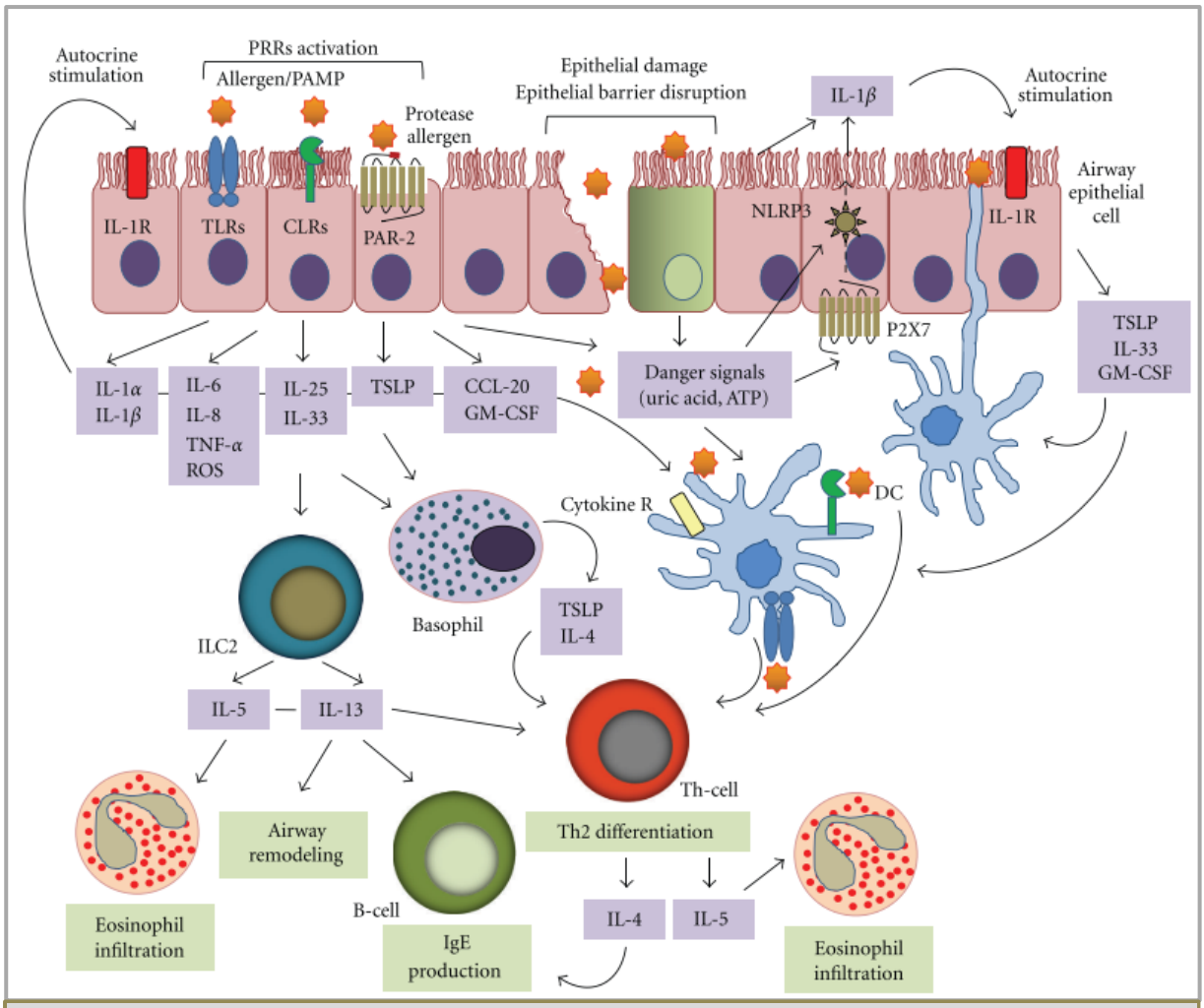

Figure 6. Simplified model of HDM-induced activation of the allergic immune response in allergic asthma[62]

1.4.3 House dust mite allergens

Currently, 30 allergens from *Dermatophagoides pteronyssinus* and 35 from *D. farinae* have been identified and officially classified by the International Union of Immunological Societies (IUIS, approved by the World Health Organisation). They are named according to the official allergen nomenclature, i.e., by the first three letters of the genus and the first letter of the species (Der p, Der f).[65] Both *D. pteronyssinus* and *D. farinae* show worldwide distribution and are the most characterised mite species. Their corresponding allergenic molecules display high sequence homology and similar biological activity. These allergens are categorised into four main families: proteases, allergens with affinity for lipids, non-proteolytic enzymes and non-enzymatic allergens.[66] The groups with protease activity are group 1 allergens that are papain-like cysteine proteinases and groups 3, 6 and 9 that are trypsin-like, chymotrypsin-like

and collagenolytic-like serine proteases. These allergens are most probably digestive enzymes found in the digestive tract and faeces of the mite. Group 2 allergens are MD-2-like lipid-binding proteins and, likewise, group 5, 7, 13, 14 and 21 show fatty acid/lipid binding activity. Group 5 and 21 are homologous proteins with 21% sequence identity in *D. pteronyssinus* and 40% in *B. tropicalis*, with their functions in mite being still unidentified. However, low degree cross-reactivity has been described between Blo t 5 and 21 and Der p 5 and 21, respectively, but Der p 21 and Blo t 21 were shown to be highly cross-reactive.[67,68] Group 7 contains bactericidal permeability inducing proteins but, unlike group 2 allergens, Der p 7 and Der f 7 cannot bind lipopolysaccharides.[69] Group 13 proteins are responsible for fatty-acid transport and found in mites in large quantity. However, their high abundance in mites does not correlate with their IgE-binding capacity. Group 14 allergens are large lipid transfer proteins that are homologous with those from other sources like vitellogenin of eggs or the retinoid transport proteins apoliphorin type I and II. Groups 4, 8 and 20 possess enzymatic activity but are non-proteolytic. Group 4 allergens are alpha-amylases showing high sequence homology between different mite species, causing cross-reactivity.[70] Sequence identity is very high, i.e., 86% between *D. farinae* and *D. pteronyssinus,* and 65% between *D. pteronyssinus* and storage mites. Even with mammalian amylases, *D. pteronyssinus* has 50% sequence identity. Group 8 represents gluthatione-S-transferase proteins that are scavengers of toxic compounds. Although the superfamily displays extremely diverse amino acid sequences, cross-reactivity between cockroach and mite group 8 allergens has been demonstrated.[71] Group 20 consists of arginine-kinases, a so far poorly characterised group of allergens in mite. However, they have been described in many invertebrates as cross-reactive allergens and in few organisms as symptom-eliciting major allergens.[72,73,74,75,76,77,78] Der p 20 shows 78% sequence identity to arginine kinase from *L. vannamei* (shrimp, Figure 7) and *S. paramamosain* (crab) and almost 80% to *P. pythagoricus* (spider) indicating that the structure of arginine kinases is highly conserved

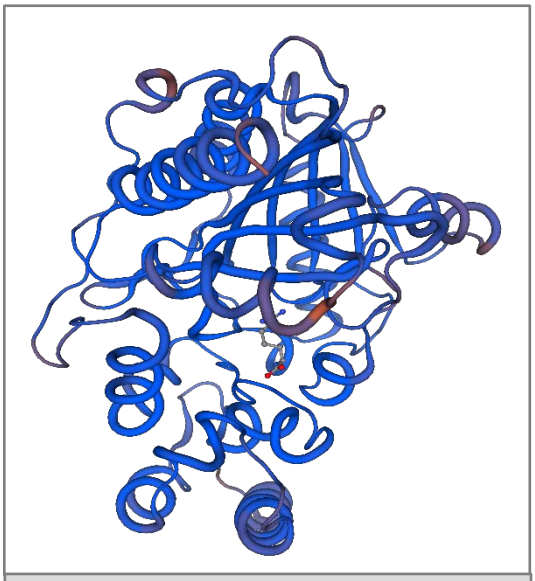

Figure 7. Structural model of Der p 20 based on the crystal structure of *L. vannamei* arginine kinase in a ternary analog complex with arginine, ADP-Mg and NO_3

among invertebrates. These enzymes are responsible for cellular energy homeostasis in the muscles of invertebrates catalyzing the reversible transfer of phosphate between phosphoarginine and ADP to provide ATP for the myofibrils in muscle contraction. The arginine kinase of *D. pteronyssinus*, Der p 20, will be discussed in detail in Chapters 5 and 6. Group 15 and 18 demonstrate homology with chitinases that are responsible for the catalysis of 1,4-ß linkage hydrolysis in chitin polymers,[79] while group 12, 23 and 37 allergens are peritrophin-like chitin-binding proteins. Der p 23, a recently identified major allergen in *D. pteronyssinus* is part of the chitinous peritrophic membrane, protecting the gut from degradation by digestive processes.[80] Groups 10 and 11, i.e., tropomyosin and paramyosin-like proteins, are both muscle-derived. Tropomyosins, similarly to arginine kinases have highly conserved amino acid sequences among invertebrates, causing cross-reactivity between many species, most relevant between HDM and shellfish. IgE-recognition frequencies and allergenicity of the allergen are relatively low, however, sensitisation to Der p 10 is a biomarker for broad HDM sensitisation indicating high total IgE levels.[81] Paramyosin is a large muscle protein in invertebrates, that shows less homology among the invertebrate phyla than tropomyosin, though Der p 11 demonstrates 95% identity to paramyosin from *B. tropicalis*.[82] Proteins of group 15 and 16 are gelsolin and EF-hand Ca^{2+}-binding protein-like allergens, respectively.[83,84]

1.4.4 Blomia tropicalis allergens

Blomia tropicalis is found in the tropics and subtropics. Albeit classified as storage mite, since it was initially described in storage facilities for grain,[85] it is frequently found in houses, therefore often mentioned on the same page with house dust mites. Compared to the extensively studied *Dermatophagoides* genus, *Blomia tropicalis* allergens are less studied in the literature and only twenty-five allergens have been identified so far and fourteen allergens have been officially acknowledged by IUIS. Available data on IgE recognition-frequencies and allergenicity show considerable discrepancy, presumably due to heterogeneity of study populations and varying quality of the recombinant protein preparations. Over the last decades, *B. tropicalis* allergens have been recognised as relevant cause of allergy, emphasising the importance of more comprehensive studies.

Unlike in *D. pteronyssinus*, the dominant allergens of *B. tropicalis* belong to groups 5 and 21 (Blo t 5 and Blo t 21). While in *D. pteronyssinus* groups 1, 2 and 23 represent the major allergens, Blo t 1 and 2 are of minor importance in respect of IgE-reactivity. Blo t 5 is by far the most studied and characterised allergen from *Blomia tropicalis*.[86,87,88,89] High-yield expression of the protein in many expression systems has been reported.[90,91] According to these studies, the recombinant protein was recognised by IgE in 10-98% of the patients. Despite high sequence homology between Der p 5 and Blo t 21, only low cross-reactivity was shown between these allergens. Similarly, low-degree cross-reactivity was shown for Blo t 5 and 21, but a high percentage of patients showed co-sensitisation to these allergens.[92,93]

Blo t 12 is a chitin-binding protein with IgE recognition frequencies of 22% to 70%.[94,95] Among the two identified isoforms, Blo t 12.0101 shows higher IgE-reactivity. Data on cross-reactivity with other Der p or Blo t allergens is not available for this allergen.

1.5 Aims of the thesis

The major aim of this thesis was to express and purify four mite allergens from *Blomia tropicalis* and *Dermatophagoides pteronyssinus*: Blo t 5, Blo t 12, Blo t 21 and Der p 20, to be used for the development of an allergen microarray that is based on the MeDALL allergen chip. The new research chip is planned to comprise even a higher number of relevant recombinant allergens to be able to analyse sera from other regions of the world like Asia, Africa or South America. We aimed to determine optimal conditions for expression, purification and refolding and to assess compatibility with conditions required for spotting the allergens onto the chip-surface. Furthermore, we intended to biochemically analyse the purified proteins by CD spectroscopy and size-exclusion chromatography.

In addition, we planned to study the relevance of Der p 20, a so far not well characterised allergen, by defining IgE-recognition frequencies in HDM allergic patients' sera and to investigate possible associations with sensitisation to other HDM allergens and with allergy symptoms. Reasoned by the high sequence homology between arginine kinases we planned to perform cross-reactivity studies between Der p 20 and AKs from different invertebrates by inhibition experiments.

2 Material and methods

2.1 Construction of vectors for expression of recombinant allergens

Blo t 5	
Size amino acids / base pairs	140 / 435
Amino acid sequence	MKFAIVLIACFAASVLAQEHKPKKDDFRNEFDHLLIEQANHAIEKGEHQLLYLQH QLDELNENKSKELQEKIIRELDVVCAMIEGAQGALERELKRTDLNILERFNYEEA QTLSKILLKDLKETEQKVKDIQTQHHHHHH
Molecular weight	14 kDa
Blo t 12	
Size amino acids / base pairs	131 / 408
Amino acid sequence	MADEQTTRGRHTEPDDHHEKPTTQCTHEETTSTQHHHEEVVTTQTPHHEEKT TTEETHHSDDLIVHEGGKTYHVVCHEEGPIHIQEMCNKYIICSKSGSLWYITVM PCSIGTKFDPISRNCVLDNHHHHHH
Molecular weight	Calculated by ExPASy: 15 kDa; SDS-PAGE:24 kDa
Blo t 21	
Size amino acids / base pairs	120 / 375
Amino acid sequence	MLPVSNDNFRHEFDHMIVNTATQRFHEIEKFLLHITHEVDDLEKTGNKDEKARL LRELTVSEAFIEGSRGYFQRELKRTDLDLLEKFFEAALATGDLLLKDLKALQKRVQD SEHHHHHH
Molecular weight	13 kDa
Der p 20	
Size amino acids / base pairs	362 / 1101
Amino acid sequence	MVDPATLSKLEAGFQKLQNAQDCHSLLKKYLTRDVFDQLKNKKTDMGATLLDVI QSGVENLDSGVGIYAPDAQSYKTFAALFDPIIDDYHKGFKPTDKHPKTDFGNIEN FVNVDPKNEYVLSTRVRCGRSLNGYPFNPMLTEAQYKEMETKVKGQLATFEGE LKGTYYPLLGMDKATQQQLIDDHFLFKEGDRFLQAANACRYWPVGRGIFHNDK KTFLMWVNEEDHLRIISMQKGGDLKEVYGRLVKAVKHIEQKIPFSRDDRLGFLTF CPTNLGTTIRASVHIKLPKLAADRKKLEEVAGRYNLQVRGTAGEHTESVGGIYDIS NKRRMGLTEYQAVKEMQDGILELIKMEKSMHHHHHH
Molecular weight	41 kDa

Table 1. Amino acid sequences and molecular weights of expressed proteins

2.1.1 Selection of amino acid sequences of mite allergens and design of expression vectors

Four house dust mite (HDM) allergens, Blo t 5 (O96870 Uniprot), Blo t 12 (Q17282 Uniprot), Blo t 21 (A7IZE9 Uniprot) and Der p 20 (B2ZSY4 Uniprot) were selected for protein expression

considering their IgE reactivity, allergenicity, cross-reactivity and ability to elicit allergic reactions. The amino acid sequences were obtained from the website of the International Union of Immunological Societies (IUIS) and NCBI GenBank (Blo t 5: U59102.1, Blo t 12: U27479.1, Blo t 21: AY800348.1, Der p 20: EU684970.1) (Table 1). Sequences were reverse translated into nucleic acid sequences and cloned into a pET-17b vector via an NdeI and EcoRI restriction site. The nucleic acid sequences for the pET-17b-allergen constructs were codon-optimised and synthesised by ATG:biosynthetics (Merzhausen, Germany). Their standard codon optimisation service improved the translational elongation efficiency in the given expression system (in our case *E. coli* BL21-Gold (DE3)) by adapting the sequence to the codon usage pattern of *E. coli*. The received DNA constructs (5 µg/tube), were dissolved in 50 µL DNase and RNase free water and stored at -20 °C.

2.1.2 Verification of cDNA sequences of the pET-17b constructs by DNA tube sequencing

To verify the cloned sequences, the constructs were sent for sequencing to Eurofins Genomics (Ebersberg, Germany), where automated Sanger sequencing was performed. According to instructions for the "Tube Sequencing Service", 15 µL of 100 ng/µL plasmid DNA were shipped. Since the cloned genes are under the control of a T7 promoter, T7 primers were chosen for sequencing.

2.1.3 Plasmid amplification in XL1 blue cells and plasmid preparation

The four pET-17b DNA plasmids (Blo t 5, Blo t 12, Blo t 21, Der p 20) were transformed into *E. coli* XL1 blue competent cells (Agilent Technologies, Santa Clara, USA) by adding 1 ng of DNA to 25 µL of cells. After adding the DNA, the competent cells were placed on ice for 30 minutes, then heat-shocked at 42°C for 40 seconds and placed on ice again for 2 minutes. For regeneration of *E.coli* cells, 150 µL SOC medium were added and the tubes were incubated at 37°C with shaking.

The cells were then plated on Luria-Bertani (LB) agar ampicillin plates and the positive clones were selected by ampicillin resistance. Single colonies were picked and inoculated into 50 mL

LB-medium cultures (from each plasmid type two single colonies were inoculated into separate cultures) with ampicillin (100 µg/mL final concentration) and incubated overnight at 37°C (Table 2).

SOC medium		LB agar-agar plates (500 mL)		LB medium (1000 mL)	
Yeast Extract	0.5 %	Pepton	5 g	Pepton	10 g
Tryptone	2 %	NaCl	5 g	NaCl	10 g
NaCl	10 mM	Yeast extract	2.5 g	Yeast-extract	5 g
KCl	2.5 mM	Agar-agar	7.5 g	pH 7.2 (adjusted with 5M NaOH)	
$MgCl_2$	10 mM	pH 7.2 (adjusted with 5M NaOH) autoclave			
$MgSO_4$	10 mM				
Glucose (added after autoclaving)	20 mM	500 uL ampicillin			

Table 2. Media and buffers used for plasmid amplification

The plasmid preparation was performed according to the PureYield™ Plasmid Midiprep System's protocol (Promega, Mannheim, Germany). According to the protocol, the cells were pelleted by centrifugation (5000 x g, 10 min) and the pellet was resuspended in Cell Lysis Solution (3 ml to 50 ml culture; 3 min, RT). After cell lysis, Neutralization Solution was added (5 mL to 50 mL culture; 3 min until white precipitate is formed) and the lysate was poured onto a PureYield™ Clearing column and incubated for 2 minutes. The column was centrifuged at 1500 x g for 5 minutes and the filtered lysate was poured onto a PureYield™ Binding Column and centrifuged again at 1500 x g for 3 minutes. The column was washed by addition of 20 mL of Column Wash Solution and DNA was eluted with 600 µl of Nuclease-Free-Water by centrifugation (1800 x g, 5 min). The concentration of the prepared plasmid DNA was measured by NanoDrop instrument (Nanodrop Technologies, Wilmington, USA).

2.1.4 Digestion of the plasmid DNA by restriction enzymes

In order to analyse the amplified and purified pET-17b plasmids harbouring the appropriate allergen sequences, a restriction digest using the enzymes NdeI and EcoRI (Roche, Vienna, Austria) was done. For each restriction reaction 900 ng of each plasmid DNA, 5 µL of Buffer H and 5 U of both endonucleases were added. The results of the restriction digest were analysed by agarose gels.

2.1.5 Agarose gel preparation

Since the DNA fragments of interest were in the range of 375 to 1101 base pairs, 1% and 1.2% gels were prepared to achieve a 0.4-6 kilo base resolution. The agarose was dissolved in 1x TBE and warmed up in the microwave. The hand-hot solution was supplemented with ethidium bromide to a final concentration of 0.5 µg/mL and cast into the gel tub followed by insertion of a comb. After solidification, 1x TBE (prepared from a 10x stock of TBE: 0.9 M Tris, 0.9 M boric acid and 0.02 M EDTA) was used as running buffer and GeneRuler DNA Ladder Mix as molecular weight marker (Thermo Fisher, Waltham, MA, USA). After loading the samples a voltage of 100 V was applied. The DNA on the agarose gel was visualised under UV light.

2.2 Protein expression in *E. coli* BL21 (DE3) and purification of allergens

2.2.1 Plasmid vector transformation and induction of protein expression

The four pET-17b constructs with the respective allergen sequences (Blo t 5, Blo t 12, Blo t 21 and Der p 20) were transformed into *E. coli* BL21 (DE3) competent cells (Agilent Technologies, Santa Clara, CA, USA). After adding 1 ng of DNA to 25 µL of cells, the competent cells were placed on ice for 30 minutes, then heat-shocked at 42°C for 40 seconds and placed on ice again for 2 minutes. Then SOC medium was added and the tubes were incubated for 1 hour at 37°C with shaking.

The bacterial cultures were plated on LB-agar ampicillin plates (100 µL and 10 µL, respectively) and positive clones were selected by ampicillin resistance conveyed by the expression vector.

2.2.2 Induction of protein expression and preparation of cleared *E. coli* lysates

The *E. coli* BL21 (DE3) strain contains a prophage (DE3) derived from a λ bacteriophage that carries a gene encoding the T7 RNA polymerase (T7 RNAP) under the control of a Lac promoter. Isopropyl-β-D-1-thiogalactopyranoside (IPTG), a compound mimicking allolactose, is able to induce the expression of the prophage T7 RNAP, which then binds to the T7 promoter on the pET-17b vector to initiate the transcription of the synthetic allergen genes cloned

downstream to the promoter. For each protein, optimal conditions for induction and expression were established, resulting in high protein yield. The localisation of the expressed protein in *E. coli* compartments was determined by SDS-PAGE and accordingly they were purified under denaturing or native conditions.

In the case of Blo t 5, single colonies were inoculated into 4 mL LB-Amp starter cultures and incubated at 37°C overnight. The entire volume of the starter culture was poured into 50 mL of LB-Amp media and protein expression was induced by IPTG (1 mM final concentration) at an OD_{600} of 0.5 for 4 hours. After induction, the cultures were centrifuged and the pellets were homogenised by stirring 3 hours in buffer B (the lysates were prepared under denaturing conditions, since the protein showed accumulation in inclusion bodies, see 2.3.2). The lysate was then centrifuged in an ultracentrifuge (18,000 rpm, 20 minutes, 4°C) to remove cell debris and intact cells. Induction and expression in larger culture volumes (250 mL, 500 mL) did not result in increased protein yield.

For Blo t 12-expression, a larger number of colonies was wiped off the agar plate and inoculated into 250 mL LB-Amp media. The cultures were induced by IPTG (1 mM final concentration) at OD_{600}=0.5 and incubated overnight at 37°C. Lysate preparation and protein solubilisation were performed under denaturing conditions as was done for Blo t 5.

Similarly, Blo t 21-expressing colonies were picked from the agar-plates and inoculated into 250 mL LB-Amp media and the cultures were induced at OD_{600}=0.5 for 4 hours.

Blo t 21 was expressed in the cytoplasm of *E. coli* BL21 and therefore the preparation of *E. coli* lysates occurred under native conditions using lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) and Ultra-turrax for opening the cells.

2.3 Purification of the recombinant allergens by Ni-NTA agarose chromatography

2.3.1 Purification under native conditions (Blo t 21 and Der p 20)

After centrifugation, the supernatants of *E. coli* lysates were incubated with 50% Ni-NTA suspension (1 mL Ni-NTA to 4 mL cleared lysate), at 4°C for one hour. Then, the mixture was loaded onto a column and, after collecting the flow through, washed with wash buffer, collecting flow-through in 1 mL-fractions (approx. 8 mL). Five mL of elution buffer were applied

and 0.5 mL aliquots of the eluate were captured in Eppendorf tubes separately (for composition of buffers, see Table 3). All fractions were analysed on an SDS polyacrylamide gel and those containing the pure protein were pooled for dialysis.

Lysis buffer		Wash buffer		Elution buffer	
NaH_2PO_4	50 mM	NaH_2PO_4	50 mM	NaH_2PO_4	50 mM
NaCl	300 mM	NaCl	300 mM	NaCl	300 mM
Imidazole	10 mM	Imidazole	20 mM	Imidazole	250 mM
pH 8		pH 8		pH 8	

Table 3. Buffers used for purification under native conditions

2.3.2 Purification under denaturing conditions (Blo t 5 and Blo t 12)

The lysates contained in buffer B were incubated with the Ni-NTA matrix at room temperature overnight. As described above, flow through and wash fractions using buffer C were collected. For protein elution buffers D and E were used (Table 4). Since the pI of Blo t 5 and Blo t 12 are 5.86 and 5.83, respectively, the pH of buffer D was adjusted to 5 instead of 5.9 as provided in the original protocol. Five and ten elution fractions of 2 mL each were collected during elution with buffers D (5x2mL) and E (10x2mL) and analysed subsequently by SDS-PAGE (Table 4).

Buffer B		Buffer C		Buffer D		Buffer E	
NaH_2PO_4	100 mM	NaH_2PO_4	100 mM	NaH_2PO_4	100 mM	NaH_2PO_4	100 mM
Tris	10 mM	Tris	10 mM	Tris	10 mM	Tris	10 mM
Urea	8 M	Urea	8 M	Urea	8 M	Urea	8 M
pH 8		pH 6.3		pH 5		pH 4.5	

Table 4. Buffers used for purification under denaturing conditions

2.3.3 Analysis of purified proteins by SDS PAGE

The 12.5% separating gels were prepared using 50% acrylamide (48% acrylamide, 1.3% bisacrylamide) and 4x Lower Tris (36.3% Tris, pH 8.85, 0.4% SDS) and, for the 5% stacking gel, 4x Upper Tris (1% Tris pH 6.8) was used. To facilitate polymerisation, ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added. As a protein marker we used PageRuler Unstained Protein Ladder (Thermo Fisher, Waltham, MA, USA) covering a range from 10 to 200 kDa. Two small gels were run at 170 V and the larger-sized gels at 180 mA in

1x electrophoresis buffer (10x stock solution: 60.6 g TRIS, 292 g glycine, 20 g SDS in 2 litres of MilliQ water, pH 8.3). For visualising the proteins, the gels were incubated with Coomassie Blue staining solution (0.25% Coomassie Brilliant Blue R 250, 50% methanol, 7.5% acetic acid) for 15 or 30 minutes (depending on the size of the gel) and destained for several hours in destaining solution (20% methanol, 10% acetic acid).

2.3.4 Dialysis under native conditions

Day 1	
NaCl	200 mM
Imidazole	100 mM
Na_2HPO_4/NaH_2PO_4	75 mM
pH 8.4 (7.5 for Der p 20)	
Day 2	
NaCl	100 mM
Imidazole	50 mM
Na_2HPO_4/NaH_2PO_4	75 mM
pH 8.4 (7.5 for Der p 20)	
Day 3	
NaCl	20 mM
Imidazole	10 mM
Na_2HPO_4/NaH_2PO_4	75 mM
pH 8.4 (7.5 for Der p 20)	
Days 4 and 5	
Na_2HPO_4/NaH_2PO_4	75 mM
pH 8.4 (7.5 for Der p 20)	

Table 5. Buffers used for dialysis under native conditions

After analysing the different fractions by SDS PAGE (flow through, washing steps, elution steps) all fractions containing the pure protein were pooled. Depending on the total volume of the pooled eluates, these were either loaded into Slide-A-Lyzer dialysis cassettes (Thermo Fisher, Waltham, MA, USA) with a molecular weight cut off (MWCO) of 3 kDa or into a SpectralPor dialysis membrane bag (VWR International, Vienna, Austria) with 6-8 kDa cut-off for Blo t 21 (Mw: 14 kDa) or 12-14 kDa MWCO for Der p 20 (Mw: 40 kDa). The cassettes and membranes containing the protein eluates were both dialysed against 2 litres of dialysis buffer overnight at 4°C, stepwise reducing Imidazole concentration and, in the last two dialysis steps, changing the buffer to 75 mM Na_2HPO_4 (Blo t 21: pH 8.4; Der p 20: pH 7.5; pH was adjusted with NaH_2PO_4; Table 5). The proteins were finally stored at -20°C.

2.3.5 Dialysis under denaturing conditions

Day 1	
Urea	4 M
Na_2HPO_4/NaH_2PO_4 pH 8.4	75 mM
Day 2	
Urea	2 M
Na_2HPO_4/NaH_2PO_4 pH 8.4	75 mM
Days 3 and 4	
Na_2HPO_4/NaH_2PO_4 pH 8.4	75 mM

Table 6. Buffers used for dialysis under denaturing conditions

Proteins that were expressed in inclusion bodies (Blo t 5, Blo t 12) required the stepwise reduction of urea concentration in the dialysis buffer (Table 6) in order to facilitate refolding of the denatured proteins. The rest of the dialysis process was identical to the one described in chapter 2.3.4.

2.3.6 Buffers used for the refolding of Blo t 12

All the buffers used for purification and dialysis of Blo t 12 under denaturing conditions were supplemented either with 20 mM ß-mercaptoethanol or gluthatione (GSH/GSSG, ratio 10:1, GSH concentration=4 mM - 10 mM). For the last two dialysis steps (days 4 and 5), the reducing agents were omitted and the protein was dialysed against a 75 mM phosphate buffer (see section 2.3.4).

2.4 Protein concentration measurement

2.4.1 MicroBCA

The concentration of the dialysed protein preparations was determined by using Micro BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, USA). A bovine serum albumin (BSA) dilution series was prepared as standard. From all samples, two dilutions were prepared for concentration measurement (1:20 and 1:100). Preparation of the solutions and samples was carried out following the instructions provided by the company using the “Microplate Procedure” protocol. The absorbance was measured by a Tecan Infinite F50 ELISA plate reader at 560 nm. If the concentration of the protein preparation was too low after dialysis, samples were con-

centrated by centrifugation (4°C, 4000 rpm) using Amicon Ultra 15 centrifugal filter units (Millipore, Billerica, MA, USA) with 3 kDa or 10 kDa cut-off, according to the respective protein size.

2.4.2 Protein concentration measurement of extract preparations by Bradford assay

Protein concentration of allergen extracts (see chapter 2.7) was measured by Coomassie Brilliant Blue G-250 based method, where binding of the protein to the dye results in a blue colorimetric reaction. A BSA-standard curve with different known concentrations of BSA (from 0.2 µg/mL to 200 µg/mL in a two-step serial dilution series) was established. The extract samples were diluted 1:10, 1:100, 1:1000 in PBS and 150 µL of each respective dilution were pipetted into a 96 well microplate (Nunc Maxisorp flat bottom 96-well plate, Thermo Fisher, Waltham, MA, USA). The Bradford solution (Bio-Rad, Hercules, California, USA) was diluted 1:5 in $_{dd}H_2O$ and 150 µL were added to each well and incubated for 5 minutes. The absorbance was measured at 595 nm.

2.5 Biochemical analysis of the recombinant proteins

2.5.1 Determination of the secondary structure by circular dichroism (CD) spectroscopy

The CD spectra of the four recombinant proteins were measured on a Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). The protein samples were loaded into a quartz cuvette with a concentration of 0.1 mg/mL in the same buffer they were stored in (75 mM phosphate buffer). The spectra were measured from 260 nm to 190 nm with a 0.5 nm resolution where the average of triplet scans was calculated and baseline measurement with the buffer was subtracted. The results were visualised as mean residue ellipticities recorded at a given wavelength and the percentages of the different types of secondary structure were estimated by SDSSTR software.

2.5.2 Oligomerisation study of the recombinant proteins by size-exclusion HPLC

Possible oligomerisation of the purified proteins was analysed by high performance liquid chromatography (HPLC). This method allows for the estimation of the relative size of a protein. The column contains a stationary phase (silica gel) with porous particles of different size. Larger proteins can only migrate around the particles without penetrating the pores, therefore they elute sooner. Small proteins can enter the silica gel and have more volume to transverse, thus they elute later.

For these experiments an UltiMate 3000™ (Dionex, Vienna, Austria) system was used and 500 µL of protein samples were injected into a Biosep SEC-s3000 column with a flow rate of 0.5 mL/min. The BioSep-SEC-S silica-based gel filtration column was equilibrated for 30 minutes at a flow rate of 0.5 mL/min using the dialysis buffer, i.e., 75 mM phosphate buffer either with pH 7.5 (Der p 20) or pH 8.4 (Blo t 5, 12, 21). Three protein standards with known molecular weight diluted in the same respective phosphate buffer were used: cytochrome c from horse heart (12.4 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa) and bovine serum albumin (66 kDa) and the retention times were analysed in separate runs. Of each purified recombinant protein, 100 µL with a minimum concentration of 100 µg/mL were loaded onto the column and run for 30 minutes at a flow rate of 1 mL/min. UV absorbance was detected at 225 nm and plotted against elution time. Molecular weights were calculated based on the elution times of the standard proteins.

To determine a possible reaction between the column material and the protein, the column was purged with 7 M urea in order to disrupt any secondary interactions and possible elution of protein was monitored for 1 hour.

2.6 Detection of allergen-specific IgE

2.6.1 IgE-measurement by Enzyme-linked Immunosorbent Assay (ELISA)

The recombinant proteins were diluted in bicarbonate buffer (0.393 g Na_2CO_3, 0.529 g $NaHCO_3$ in 100 mL $_{dd}H_2O$, pH 9.6) and coated on a Nunc Maxisorp flat bottom 96-well plate

(Thermo Fisher, Waltham, MA, USA) at a concentration of 3 µg/mL (100 µL/well). After overnight incubation at 4°C, the plates were washed 5 times with 200 µL TBST (10 mM Tris, 150 mM NaCl, 0.5% Tween 20, pH 8) and then saturated for 2.5 hours at 37°C with TBST/1% BSA. Patients' sera were diluted 1:10 in TBST/0.5% BSA and 100 µL were added to each coated well. After overnight incubation at 4°C and washing, the alkaline phosphatase-labelled mouse anti-human IgE detection antibody (BD Biosciences, San Jose, CA, USA) was added (1:1000 dilution in TBST/0.5% BSA) and the plates were incubated first for one hour at 37°C and then another hour at 4°C, followed by washing 5 times. Substrate was prepared by dissolving Sigma 104 phosphatase substrate tablets (Sigma-Aldrich, St. Louis, MO, USA) in ELISA substrate solution (97 mL diethylamine, 200 mg NaN_3 in 750 mL, pH 9.8) and 100 µl were added to each well. The absorbance was first measured after 10 minutes and measurements were continued until saturation was reached. Measurements were performed on a Tecan Infinite F50 ELISA plate reader at 405 nm and 550 nm reference wavelength.

2.6.2 Allergen-specific IgE detection by the MeDALL allergen-chip

IgE reactivity profiles in serum samples were established by the MeDALL-chip, a customised version of ImmunoCAP ISAC (Thermo Fisher/Phadia AB, Uppsala, Sweden), containing several allergens from *Dermatophagoides pteronyssinus* and *Blomia tropicalis*. The microarrays were warmed up to room temperature and then washed with washing buffer provided in the ImmunoCAP ISAC Assay Kit (Phadia AB, Uppsala, Sweden) for 5 minutes. The slides were dried in the centrifuge (1 min, 1000g, room temperature). Immediately after drying, the serum samples were added onto the arrays and incubated on a rocking platform at room temperature for 2 hours in a humid chamber. The slides were then rinsed with washing buffer using a spray bottle and washed and dried again as described above. Thirty microlitres of the detection antibody (anti-human IgE, ImmunoCAP ISAC Assay Kit) were added to each array. After 30 minutes incubation, the slides were washed, dried and scanned on the same day on a LuxScan-10 K microarray scanner (CapitalBio, Beijing, People's Republic of China).

2.6.3 Verification of the presence of arginine kinase in allergen extracts by Western blot

Of each extract, 300 µL (extracts from *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Blomia tropicalis, Lepidoglyphus destructor, Tyrophagus putrescentiae, Periplaneta americana, Blattella germanica)* or 200 µL (*Litopenaeus vannamei)*, respectively, were loaded onto a gel and subjected to electrophoresis. The gel was placed onto a nitrocellulose membrane, covered by filter paper and put into a blotting chamber that was filled with blot buffer (15.13 g TRIS, 72.06 g glycine, 1000 mL methanol, $_{dd}H_2O$ added to 5 litres). The proteins were transferred to the nitrocellulose membrane by applying 750 mAh. To check if proteins were successfully transferred, the nitrocellulose membrane was stained with Ponceau S staining solution containing 0.1% w/v Ponceau S (Sigma P 7767) dissolved in 5% v/v acetic acid. The blots were stained for 1 minute and then destained in $_{dd}H_2O$ for few seconds.

Additionally, for positive and negative controls, dot-blots were prepared using nitrocellulose stripes where 100 ng of protein (purified AK from moth, human serum albumin [HSA] and recombinant Der p 20) were dotted onto the same stripe and processed as described below for the blotted extracts.

Stripes with 4 mm width were prepared from the blots and blocked with gold buffer (37.5 g Na_2HPO_4, 5 g NaH_2PO_4, 25 ml Tween 20, 25 g BSA, 2.5 g NaN_3, $_{dd}H_2O$ added to 5 litres; incubation at room temperature for 2x10 min, then once for 30 min, replacing the buffer after each incubation step). Then sera (rabbit preimmune serum and anti-AK serum) were added in 1:1000 and 1:10,000 dilutions followed by overnight incubation at 4°C on a rocking platform. After washing three times (twice for 10 minutes, then for 30 minutes) the detection antibody, an anti-rabbit IgG, labelled with I^{125} was added for overnight incubation at room temperature. Next day, the blots were washed and then dried. Afterwards, an X-ray film was placed onto the blots and exposed at -80°C for several days, depending on the respective signal strength. Afterwards, molecular weight markers were indicated on the films based on the Ponceau S stained blots.

2.7 Extract preparation from different arthropod sources

For extract preparation, raw material from different mites and cockroach (*Dermatophagoides pteronyssinus, Dermatophagoides farinae, Blomia tropicalis, Lepidoglyphus destructor, Tyrophagus putrescentiae, Periplaneta americana, Blattella germanica)* were purchased (Allergon, Ängelholm, Sweden). An amount of 0.3 g of raw material was suspended in 5 mL PBS supplemented by a protease inhibitor (phenylmethylsulfonyl fluoride - PMSF, 1:1000 final dilution of a stock in EtOH with 10 mg/mL). This suspension was homogenised by Ultra-turrax IKA T18 basic (Staufen, Germany) for 1-2 minutes with speed 4 and then incubated overnight at 4°C on a rocking platform. Next day, preparations were centrifuged (4 min, 14,000 rpm, RT) and supernatants were stored at -20°C.

Extract from *Litopenaeus vannamei* was prepared from frozen, raw tail muscle, purchased in a local supermarket. Peeled and deveined shrimps were frozen in liquid nitrogen and homogenised in a mortar. Approximately 4 g of the resulting paste were added to 40 mL of PBS containing PMSF (10 µg/mL) and NaN_3 (0.05% w/v) to inhibit microbial growth. The homogenate was incubated overnight at 4°C on a rotating incubator and centrifuged on the next day at 3000 rpm, 10 min, 4°C and then at 15,000 rpm, 5 min, 4°C. The supernatant was stored at -20°C. Protein concentration was determined by Bradford assay as described above.

3 Results

3.1 Verification of cDNA sequences of the pET-17b constructs by DNA tube sequencing

Sequencing results confirmed that the translated cDNA sequence that had been cloned into pET-17b corresponded to the Der p 20 amino acid sequence found in the IUIS database.

3.2 Expression and purification of the recombinant allergens by Ni-NTA agarose chromatography

All four recombinant allergens were successfully expressed and purified from *E. coli* BL21 (DE3) cultures. During protein expression recombinant Blo t 5 and 12 were retained in inclusion bodies, therefore urea was applied to provide denaturing conditions for the preparation

and extraction of these insoluble proteins. Blo t 21 and Der p 20 were expressed in the soluble fraction of *E. coli* culture and purified under native conditions. In SDS-PAGE, Blo t 5, Blo t 21 and Der p 20 migrated at their expected molecular weight (14, 13 and 41 kDa, respectively), while Blo t 12 migrated at 25 kDa, i.e., higher than corresponding to the calculated molecular mass of 14 kDa (Figure 8). The highest yield was achieved in cultures expressing recombinant Blo t 21 (5 mg/litre of bacterial culture) and Blo t 12 (15 mg/litre of culture), while of Blo t 5 (1 mg/5x50 mL) and Der p 20 (2 mg/litre of bacterial culture) lower protein amounts were purified. Expression of Blo t 5 was limited to 50 mL LB-medium culture size, since attempts with larger cultures showed reduced yield.

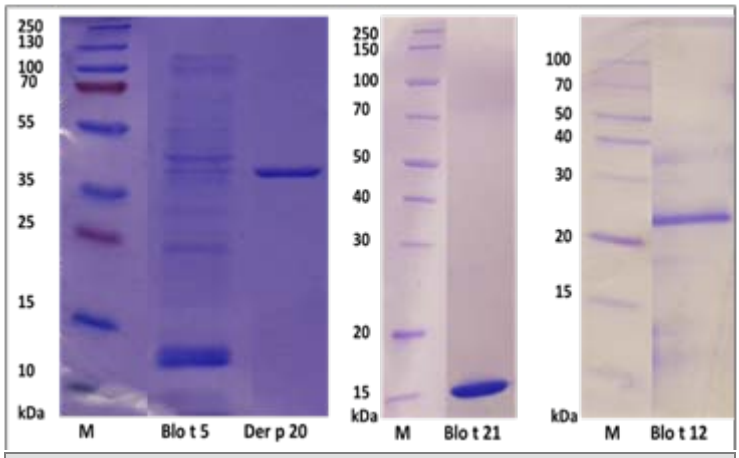

Figure 8. Reducing SDS-PAGE of purified recombinant proteins

3.3 Determination of the secondary structure by circular dichroism (CD) spectroscopy

The far UV spectra of recombinant proteins revealed for Blo t 5 (Figure 9A) two minima at 209 and 222 nm, for Blo t 21 (Figure 9C) at 211 and 225 nm and for Der p 20 (Figure 9D) at 209 and 223.5 nm. According to their CD spectra, these three recombinant proteins appeared to be properly folded in solution. Calculations using CDSSTR Software disclosed that Blo t 5 and Der p 20 are predominantly built up by α-helices and ß-strands while Blo t 21 contains primarily α-helices (Table 7). Blo t 12 (Figure 9B) showed a random coil CD spectrum which is in

	α-helix	ß-sheet	Turns	Unordered
Blo t 5	38.4 %	20.2 %	17.2 %	24.2 %
Blo t 12	29.0 %	28.0 %	21.0 %	21.0 %
Blo t 21	58.4 %	15.8 %	5.9 %	19.8 %
Der p 20	32.7 %	19.8 %	21.8 %	25.7 %

Table 7. Prediction of secondary structures based on the CD spectra of recombinant proteins

line with the data on protein structure available on Uniprot database (approximately 50% β-sheets and random coils). However, in a subsequent thermal denaturation assay, the recombinant protein showed no melting upon heating up to 95°C, indicating lack of β-sheet folding.

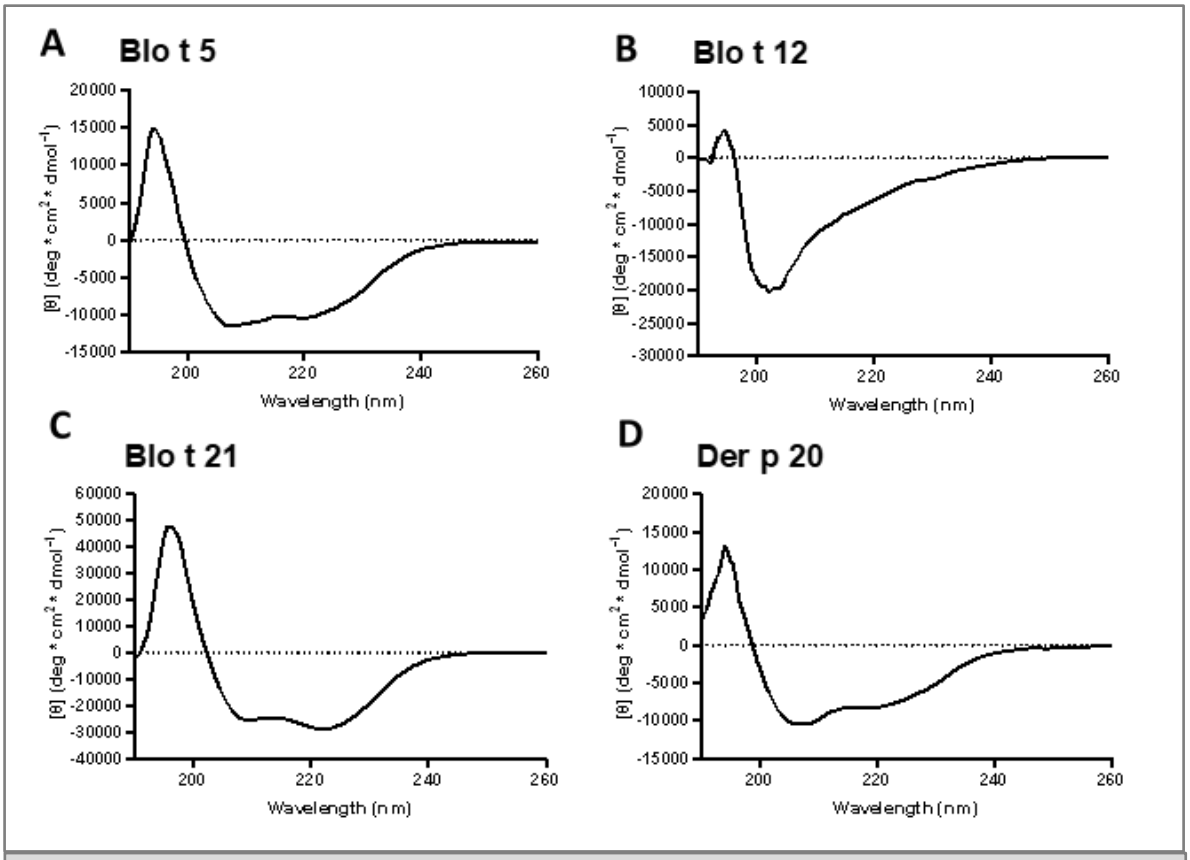

Figure 9. CD spectra of recombinant proteins

3.4 Oligomerisation study of the recombinant proteins by size-exclusion HPLC

Protein oligomerisation was analysed by size-exclusion chromatography (SEC). Measurements indicated that Blo t 5 (Figure 10A) and Blo t 21 (Figure 10B) are mainly found in monomeric form. For Der p 20 (Figure 10C), a lower molecular weight than calculated based on the amino acid sequence and revealed by SDS-PAGE results was measured by HPLC-SEC (see chapter 6.1.5). Blo t 12 (Figure 10D-F) showed strong aggregation after refolding, which might be the result of the considerable number of cysteines (n=6) in the amino acid sequence. To facilitate proper folding and formation of appropriate disulphide-bonds, two reducing agents, β-

mercaptoethanol and glutathione were applied during purification. Results showed that addition of glutathione helped to prevent oligomerisation to a certain degree (Figure 10F), but still obtaining only 23% of the monomeric form.

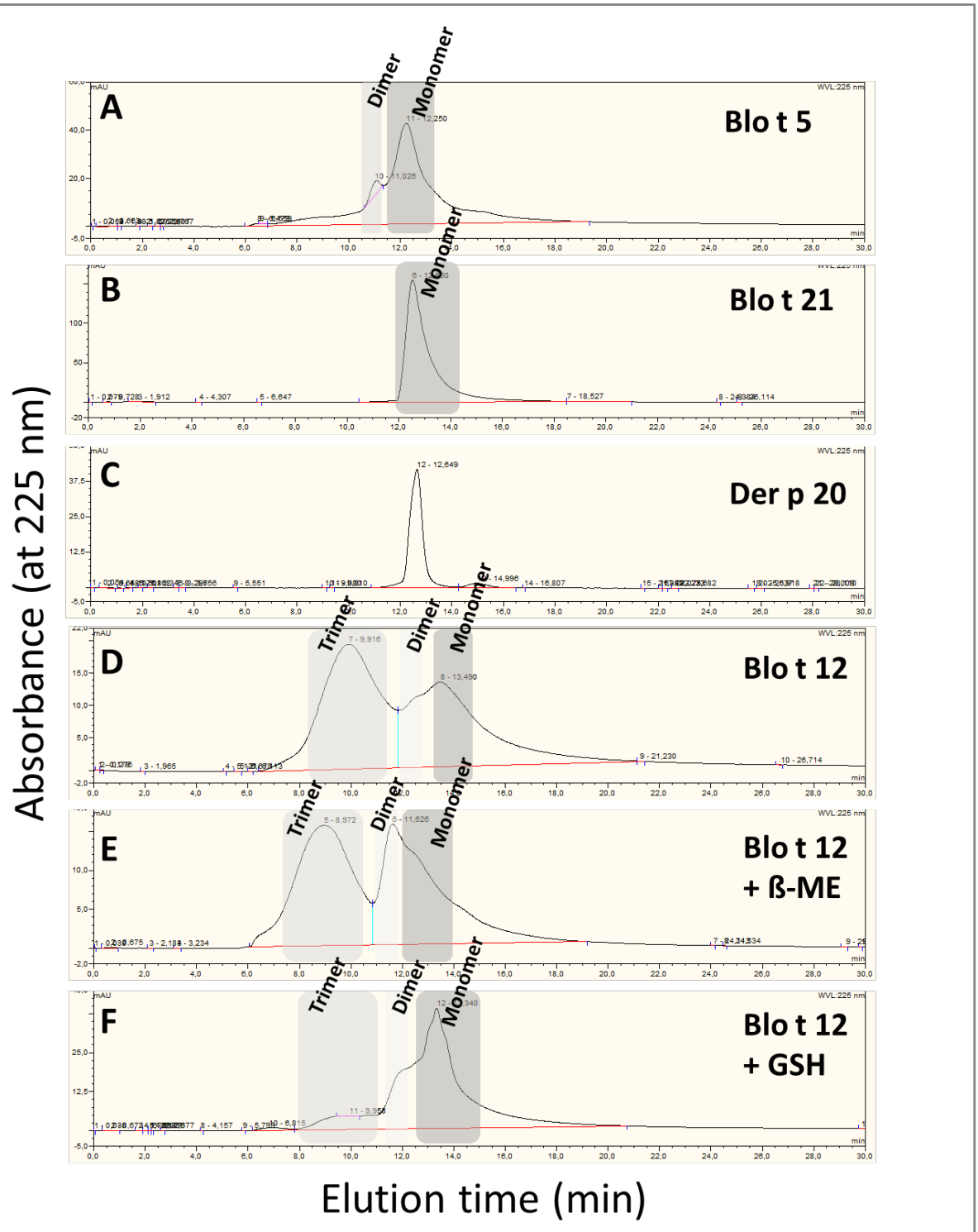

Figure 10. Analysis of oligomerisation of recombinant proteins by HPLC size-exclusion chromatography

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5 Manuscript

Expression in *Escherichia coli* and purification of folded rDer p 20, the arginine kinase from *Dermatophagoides pteronyssinus*: A possible biomarker for allergic asthma

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Abbreviations:

AK – arginine kinase

CD – circular dichroism

HDM – house dust mite

HSA – human serum albumin

IPTG – Isopropyl β-D-1-thiogalactopyranoside

MALS – multi angle light scattering

M_w – molecular weight

OD – optical density

rDer p 20 – recombinant Der p 20

SDS-PAGE – sodium-dodecyl sulfate-polyacrylamide gel electrophoresis

5.1 Abstract

Arginine kinase (AK) has been identified first as an allergen in the Indian-meal moth and subsequently was shown to occur as allergen in various invertebrates and shellfish. The cDNA coding for arginine kinase from the house dust mite (HDM) species *Dermatophagoides pteronyssinus*, Der p 20, has been isolated but no recombinant Der p 20 allergen has been produced and characterized so far. We report the expression of Der p 20 as recombinant protein in *Escherichia coli*. Recombinant Der p 20 was purified and shown to be a monomeric, folded protein by size exclusion chromatography and circular dichroism (CD) spectroscopy, respectively. Using AK-specific antibodies, Der p 20 was found to occur mainly in HDM bodies but not in fecal particles. Thirty percent of clinically well-characterized HDM allergic patients (n=98) whose IgE reactivity profile had been determined with an extensive panel of purified HDM allergens (Der f 1, 2; Der p 1, 2, 4, 5, 7, 10, 11, 14, 15, 18, 21, 23, 37) showed IgE reactivity to Der p 20. IgE reactivity to Der p 20 was more frequently associated with lung symptoms. AKs were detected in several invertebrates with specific antibodies and Der p 20 showed IgE cross-reactivity with AK from shrimp (*Litopenaeus vannamei*). Thus, Der p 20 is a cross-reactive HDM allergen and may serve as a diagnostic marker for HDM-induced lung symptoms such as asthma.

5.2 Introduction

Arginine kinase (AK) was first identified as an allergen in the Indian-meal moth (*Plodia interpunctella*) which is a frequent household pest in many countries.[1,2] Screening of a *Plodia interpunctella* expression cDNA library with serum IgE from an Indian-meal moth-sensitized allergic patient led to the isolation of a cDNA coding for AK. Recombinant AK from Indian-meal moth, Plo i 1, was expressed and shown to react specifically with IgE from sensitized patients and to induce IgE-dependent basophil activation and skin reactions.[1] Furthermore, IgE cross-reactivity with house dust mite (HDM), cockroach and shellfish was demonstrated. Arginine kinases are involved in cellular energy homeostasis in the muscles of invertebrates where they catalyze the transfer of phosphate between phosphoarginine and ADP to provide ATP for muscle contraction.[3] Like tropomyosin, AKs have been described later as possible cross-reactive allergens in many invertebrates including crustaceans, mollusks, insects and arachnids.[1,4,5,6,7,8,9,10,11] In many cases, HDM extracts strongly inhibited IgE-binding to AKs from the cross-reactive allergen sources but no significant inhibition was shown vice versa, suggestive of HDM as primary sensitizing agent.[12,13,14,15,16]

The HDM species *Dermatophagoides pteronyssinus* is one of the most important allergen sources world-wide containing several allergens in the body and fecal pellets[17] of which Der p 1, Der p 2, Der p 5, Der p 7, Der p 21 and Der p 23 have been identified as the clinically most relevant allergens.[18] The cDNA coding for Der p 20, the AK from *Dermatophagoides pteronyssinus*, has been isolated (GenBank: EU684970.1) but so far no recombinant Der p 20 has been expressed, purified and characterized regarding physiochemical and immunological properties and possible clinical relevance. In this study, we report expression and immunological characterization of Der p 20 and discuss its potential relevance as biomarker for allergic asthma.

5.3 Material and Methods (further details are provided in the supplement)

5.3.1 House dust mite allergic patients

Study participants (n=98), suffering from HDM-induced allergic rhinoconjunctivitis and mild asthma were enrolled and characterized as described[19] (52.9% female, mean age 24.9 ±4.7 years). Symptoms and IgE-sensitization to other HDM allergens were assessed by questionnaire and allergen microarray, respectively. Total nasal symptom scores were obtained during a controlled exposure to HDM-allergens in the Vienna Challenge Chamber. Approval of the study had been obtained from the Clinical Pharmacology Ethics Committee (Vienna, Austria).

5.3.2 Expression, purification and biochemical analysis of rDer p 20

The cDNA sequence of Der p 20 (GenBank: EU684970.1) with a DNA sequence coding for a C-terminal hexahistidine tag was ligated into pET17b. After transformation into *E.coli* BL21 (DE3), protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). Expression levels and localization of the target protein within the host cells were determined by SDS-PAGE. The protein was purified from the supernatant of *E.coli* lysate by nickel chelate affinity chromatography under native conditions. Secondary structure and oligomerization were determined by CD-spectroscopy and size-exclusion chromatography, respectively. Sequences of other arginine kinases with homology to Der p 20 were identified by comparing the amino acid sequence of Der p 20 with the sequences deposited in the UniProt data base (www.uniprot.org/blast/) using the BLASTP program.
(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=blasttab)

5.3.3 IgE-binding frequencies and association with clinical phenotypes

IgE-binding to Der p 20 was determined in patients' sera by ELISA. Differences concerning patterns of sensitization to other HDM-allergens, clinical symptoms, additional allergies and

sensitizations were statistically evaluated and compared between Der p 20-positive and negative patients.

5.3.4 Identification of AK in extracts from different invertebrates and inhibition assays

Extracts were prepared from specimens of different mite species (*Dermatophagoides pteronyssinus* and *farinae, Blomia tropicalis, Lepidoglyphus destructor, Tyrophagus putrescentiae*), cockroach (*Periplaneta americana, Blattella germanica*) and from Pacific white shrimp (*Litopenaeus vannamei*). Extracts were blotted onto nitrocellulose membrane and the presence of AK was investigated using rabbit-antibodies raised against AK from *Plodia interpunctella* (Indian-meal moth) (Davids Biotechnologie, Regensburg, Germany). Binding of IgG to AK was visualized by autoradiography.

IgE cross-reactivity between Der p 20 and AK from *Litopenaeus vannamei* was analyzed using serum from a Der p 20-positive patient. This serum was pre-incubated with rDer p 20 and IgE-binding to blotted extracts was visualized with ^{125}I-labelled anti-human IgE.

5.4 Results

Recombinant Der p 20 was purified from the soluble fraction of *E.coli* under non-denaturing conditions with a yield of approximately 2 mg of protein per liter of bacterial culture. Under non-reducing conditions the protein showed two bands on the SDS-PAGE that lay close to each other at a molecular weight of approximately 40 kDa whereas under reducing conditions only the larger band was visible (Figure 1A). Since the Der p 20 sequence (Genbank accession number: EU684970.1) contains 4 cysteine residues the smaller band observed under non-reducing conditions seems to correspond to a disulphide-bonded form whereas the larger band may represent the reduced, unbonded form. The analysis of the secondary structure of the protein by far-UV circular dichroism revealed two minima at 206 and 220 nm, respectively, and a maximum at 194 nm (Figure 1B). Calculations using CDSSTR Software indicated that Der p 20 consists of 32% α-helices, 20% ß-strands, 22% turns and 26% random coils which is in

line with data on secondary structure available at Uniprot-database. The thermal denaturation recorded by CD demonstrated a single melting point at 59°C without refolding upon cooling (data not shown).

Size-exclusion chromatography with Superdex 200 Increase 10/300 column material and multi angle light scattering (MALS) demonstrated that rDer p 20 occurs as a monomeric protein (Figure E1) with a molecular weight of 42 ± 4 kDa as measured by MALS which was in agreement with the molecular weight of 41.3 kDa calculated according to the sequence with the hexahistidine tag. Using rabbit antibodies specific for Plo i 1, the Der p 20-related arginine kinase from the Indian-meal moth, we could detect Der p 20 in extracts made from mite bodies whereas it was not detectable in the mite fecal pellets (Figure 1C).

The equivalence of rDer p 20 and natural Der p 20 regarding IgE reactivity was demonstrated by inhibition of serum IgE binding of a patient who was only sensitized to Der p 20 and Der p 2. Pre-incubation of the patient's serum with rDer p 20 completely inhibited IgE reactivity to natural Der p 20 (40 kDa) in house dust mite extract whereas IgE reactivity to Der p 2 (approximately 15 kDa) was not affected (Figure 2A).

Next, we determined the frequency of IgE reactivity to rDer p 20 in a cohort of clinically well characterized HDM allergic patients (n=98) by ELISA. We found that 30 out of 98 patients (i.e., 30.6%) showed specific IgE reactivity to rDer p 20 whereas 99% and 73.5% of the patients showed IgE reactivity to Der p 2 and to Der p 23, respectively. The median level of IgE specific for Der p 20 (OD=0.10) was lower than for Der p 23- (OD=0.24) and Der p 2 (OD=0.95); (Figure 2B).

Despite the fact that Der p 20-specific IgE levels were low we found that significantly higher percentages of Der p 20-sensitized patients reported symptoms of asthma (38.1% versus 17.9%), wheezing (42.9% versus 23.2%) and overall breathing problems (64.3% versus 39.3%) as compared to non-Der p 20-sensitized patients (Figure 2C). The observed differences regarding lower respiratory symptoms seem to be due to HDM sensitization because we found no significant differences regarding reported symptoms triggered by other common allergen sources such as birch, grass, mugwort and rye pollen, cat and dog dander between Der p 20-positive and negative patients (data not shown). This indicates that Der p 20 is a possible marker for HDM-triggered allergic asthma. No significant differences were found for reported symptoms of rhinitis, conjunctivitis or skin symptoms (Figure 2C) and there were no significant

differences regarding total nasal symptom scores measured during a controlled exposure to HDM-allergens in the Vienna Challenge Chamber (Figure E2A).

We also investigated if IgE reactivity to Der p 20 is associated with IgE reactivity to certain other HDM allergens or with IgE reactivity to a certain number of HDM allergens (Fig. E2B-D) but no such associations were found.

Figure E3 shows that AKs occur in various invertebrates, including mites, spiders, insects, crayfish, octopus, sea cucumbers and shrimps and that Der p 20 shares high sequence identities with these AKs. The highest sequence identities were found between Der p 20 and AKs from other mites (*D. farinae, E. maynei, S. scaibei, A. ovatus*) whereas the sequence identities with AK from *A. fangsiao*, *A. japonicas* and creatine kinases from vertebrates (*G. gallus, M. musculus, H. sapiens*) were lower.

Accordingly, we tried to detect AKs in extracts from different allergen sources (*Dermatophagoides pteronyssinus* (Dp), *Dermatophagoides farinae* (Df), *Blomia tropicalis* (Bt), *Tyrophagus putrescentiae* (Tp), *Lepidoglyphus destructor* (Ld), *Blattella germanica* (Bg), *Periplaneta americana* (Pa), *Litopenaeus vannamei* (Lv)) with rabbit antibodies specific for Plo i 1 (Figure E4). In Dp, Df, Pa, Tp and Lv extracts (Figure E4, lanes 1, 2, 5, 7, 8) bands at the respective molecular weights of species-specific AKs (40, 40, 43, 40, 39 kDa) were detectable whereas no bands were found in Bg, Ld and Bt extracts (Figure E4, lanes 3, 4, 6).

Some IgE cross-reactivity between Der p 20 and AK of the Pacific shrimp *Litopenaeus vannamei* was demonstrated by IgE inhibition experiments. Preincubaton of serum from a Der p 20 sensitized patient with rDer p 20 reduced IgE-binding to AK in shrimp extract (Figure E5, lane 1). However, this patient showed no allergic symptoms upon consumption of shrimps. Likewise, none of the 30 patients sensitized to Der p 20 among the 98 study participants reported symptoms of food allergy.

5.5 Discussion

Our study provides new data regarding prevalence of IgE-reactivity and the possible role of Der p 20, i.e., the arginine kinase from *Dermatophagoides pteronyssinus,* in HDM-allergy. Recombinant Der p 20 was expressed in *E.coli* and purified as folded, monomeric protein. IgE immunoblot inhibitions showed that rDer p 20 inhibits IgE binding to natural Der p 20 indicat-

ing that the recombinant allergens contains the IgE epitopes of the natural protein. Furthermore, we could show that Der p 20 is localized mainly in the body of the mite but seemed to be absent from fecal pellets. The IgE-recognition frequency of Der p 20 was measured in sera from 98 clinically well characterized HDM-allergic patients by ELISA. We found that 30% of the tested 98 HDM-allergic patients showed IgE-reactivity to rDer p 20. Although Der p 20-specific IgE levels were lower as compared to Der p 2- and Der p 23-specific IgE levels, IgE reactivity against Der p 20 was significantly associated with symptoms of asthma. In particular we found that an increased percentage of patients positive to Der p 20 reported breathing problems, in particular asthma and wheezing, whereas no significant associations were found with nasal, eye or skin symptoms. This result is in agreement with data found in the literature, where arginine kinases from spider, shellfish or snail were reported to be involved in asthma symptoms. Hence, IgE sensitization to Der p 20 could be a useful serological biomarker or at least indicator for lower respiratory symptoms.

Unlike for Der p 10 (tropomyosin),[20] no significant differences were observed for Der p 20 concerning sensitization-frequencies to other HDM allergens.

A possible role of AK as cross-reactive allergen in invertebrates and shellfish was supported by the demonstration of binding of AK-specific rabbit antibodies to AKs from HDM, cockroach and shrimps and by the demonstration that pre-incubation of serum from a Der p 20 sensitized patient with rDer p 20 inhibited IgE binding to shrimp AK.

Recombinant Der p 20 can now be used for diagnosis of HDM allergy and to study cross-reactivity among AKs from invertebrates and shellfish. Importantly, IgE reactivity to Der p 20 may be a serological marker for HDM-associated asthma.

5.6 Figure legends

Figure 1. (A) Coomassie Brilliant Blue-stained SDS-PAGE of purified recombinant Der p 20 under non-reducing (lane 1) and reducing conditions (lane 2). Lane M: Molecular weight marker. (B) Far UV circular dichroism analysis of rDer p 20. The graph displays molar ellipticities (y-axis) recorded at different wavelengths (x-axis). (C) Detection of Der p 20 in nitrocellulose-blotted *Dermatophagoides pteronyssinus* body (left panel) and feces (right panel). IS: rabbit anti-Plo i 1 immune serum; nrs: normal rabbit serum. Molecular weight in kDa is indicated on the left.

Figure 2. (A) rDer p 20 inhibits allergic patient's IgE-binding to nitrocellulose-blotted extracts from *Dermatophagoides pteronyssinus*. Serum from a Der p 20-sensitized patient was pre-incubated with rDer p 20 (lane 1), with human serum albumin (lane 2) or without inhibitor (lane 3). IgE-reactivity was detected with ^{125}I-labelled anti-human IgE and visualized by auto-radiography. Molecular weights in kDa are indicated on the left. (B) IgE levels determined for Der p 2, Der p 23 and Der p 20 (x-axis) for 98 HDM allergic patients by ELISA (y-axis): OD values corresponding to bound IgE, logarithmic scale). The statistical cut-off is indicated by the dashed horizontal line. Error bars indicate medians +/- interquartile ranges calculated using OD values of positive patients. (C) Percentages of the 98 patients (y-axis) reporting different types of symptoms (x-axis) are shown for Der p 20-positive (red bars) and -negative (blue bars) subjects. Statistically significant differences between the groups are indicated (*P<0.05).

Figure 1

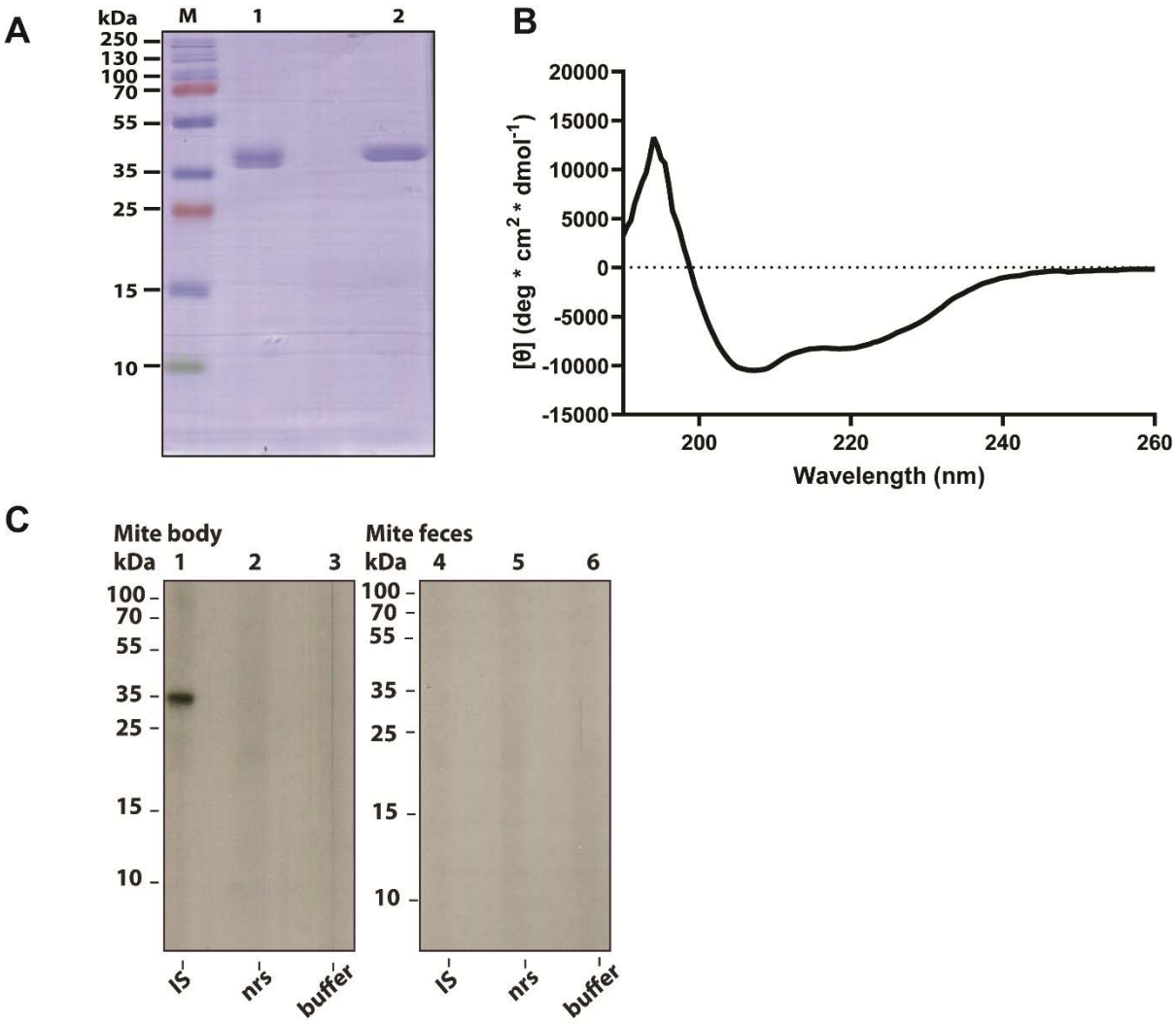
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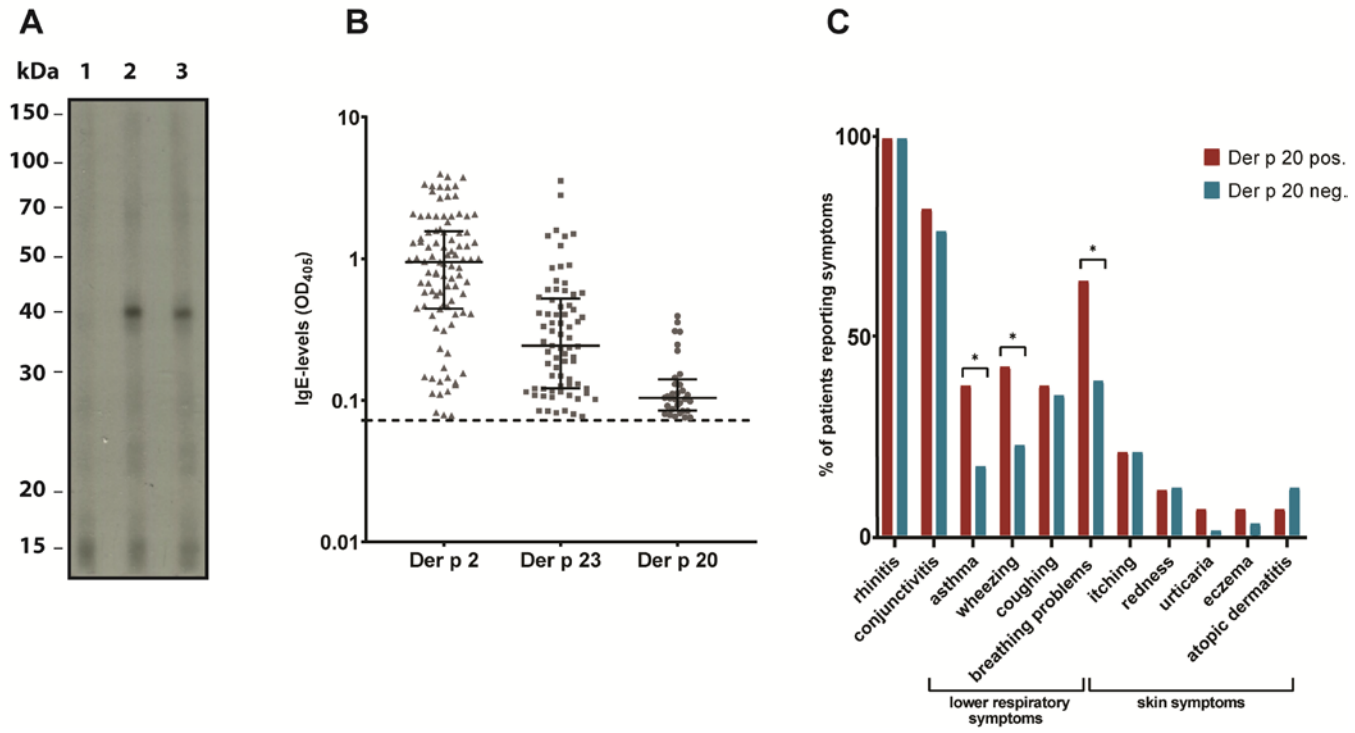
Figure 2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rhinitis
conjunctivitis
asthma
wheezing
coughing
breathing problems
itching
redness
urticaria
eczema
atopic dermatitis
lower respiratory symptoms
skin symptoms

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6 Online supplement

Expression in *Escherichia coli* and purification of folded rDer p 20, the arginine kinase from *Dermatophagoides pteronyssinus*: A possible biomarker for allergic asthma

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6.1 Material and Methods

6.1.1 House dust mite allergic patients

Ninety-eight patients, aged 18 to 65 years, suffering from HDM-induced allergic rhinoconjunctivitis and mild asthma were enrolled according to the following criteria: Positive skin prick test (SPT) and radioallergosorbent test result to *D. pteronyssinus*; forced expiratory volume (FEV_1) > 70%; total nasal symptom score (TNSS) ≥ 3 out of 9 in an HDM challenge test at baseline; regular contact with HDM at home. Severe asthma and severe atopic dermatitis were exclusion criteria. Further details regarding the study population are provided elsewhere.[1]
At the time when baseline serum samples were collected, data about allergic symptoms were obtained by questionnaire by asking patients if they suffered from one or more of the following symptoms: asthma, wheezing, coughing, breathing problems, itching, redness of the skin, urticaria, eczema or neurodermatitis. Sensitization to other allergens at baseline was assessed by ImmunoCAP ISAC (Thermo Fisher/Phadia AB, Uppsala, Sweden).[1]
Total nasal symptom score (TNSS) data was obtained in a controlled HDM exposure in the Vienna Challenge Chamber as described,[2] and are displayed as area under the curve (AUC) values.

6.1.2 Plasmid construction and amplification

The cDNA sequence of Der p 20 that is approved by the International Union of Immunological Societies (IUIS, www.allergen.org) was obtained from GenBank (accession number EU684970.1). A synthetic gene including a sequence encoding a C-terminal hexahistidine-tag was codon optimized for expression in *E.coli* and cloned into pET-17b vector via NdeI and EcoRI restriction sites (ATG:biosynthetics, Merzhausen, Germany). The lyophilized plasmid DNA was dissolved in DNase and RNase free water at a concentration of 100 ng/µL. To verify accuracy of the cloned sequences, the constructs were sequenced using T7 primers for amplification (Eurofins Genomics, Ebersberg, Germany).
The pET-17b/Der p 20 plasmid was transformed into *E.coli* XL1 Blue competent cells (Agilent Technologies, Santa Clara, CA, USA) for amplification (1 ng DNA was added to 25 µL of competent cells) and the cells were cultured on Luria-Bertani (LB) agar ampicillin plates and were

then inoculated into LB medium with ampicillin (100 µg/mL final concentration) and incubated overnight at 37°C in a shaker. Plasmid preparation was carried out using the PureYield™ Plasmid Midiprep Kit (Promega, Mannheim, Germany).
In order to confirm the length of the cloned inserts, the plasmid DNA was digested by NdeI and EcoRI restriction enzymes (Roche, Switzerland, Basel) and the fragments were analyzed on a 1.2% agarose gel. The concentration of the purified plasmid DNA was measured by NanoDrop instrument (Nanodrop Technologies, Wilmington, USA).

6.1.3 Expression of rDer p 20 in *E.coli*

The pET-17b/Der p 20 vector was transformed into *E.coli* BL21-Gold (DE3) competent cells (Agilent Technologies, Santa Clara, CA, USA). The plasmid DNA was added to 25 µl of cells at a final concentration of 0.04 ng/µL and incubated on ice for 30 minutes, followed by a 40 seconds heat shock at 42°C and 2 minutes incubation on ice. To 25 µL of bacterial cells, 80 µL of Super Optimal Broth (SOC) medium was added and incubated at 37°C with shaking for 1 hour for regeneration. After incubation, bacterial cultures were plated on Luria Bertani (LB) agar, containing ampicillin (0.1 mg/mL), and incubated overnight at 37°C. Several single colonies were inoculated into 250 mL LB-ampicillin media and incubated at 37°C. After reaching an OD_{600} of 0.5, protein expression was induced by addition of isopropyl-β-thiogalactopyranoside (IPTG, 1 mM final concentration) followed by incubation overnight at 37°C.

6.1.4 Purification of rDer p 20 by nickel chelate agarose affinity chromatography

After protein expression, bacterial cultures were centrifuged and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) with lysozyme (0.5 mg/mL) and homogenization was carried out using an Ultra-turrax IKA T18 basic (Staufen, Germany). The homogenate was centrifuged (10,000 g, 30 min, 4°C) and the supernatant was incubated with 50% Ni-NTA agarose (1 mL Ni agarose per 4 mL of cleared lysate; Qiagen, Hilden, Germany) for 1 hour at 4°C. After washing with 8 mL of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8), the protein was collected in 8 fractions of 1 mL of elution buffer each

(50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8). The elution fractions were analyzed by SDS-PAGE and fractions containing the protein were pooled and subjected to dialysis in a Slide-A-Lyzer dialysis cassette with a molecular weight cutoff of 3 kDa (Thermo Fisher, Waltham, MA, USA). NaCl and imidazole were removed by three consecutive dialysis-steps until the protein finally was dissolved in 75 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH 7.5, and stored at -20°C. Protein concentration was measured by Micro BCA Protein Assay Kit (Thermo Fisher, Waltham MA, USA).

6.1.5 Determination of the secondary structure of purified rDer p 20 by circular dichroism (CD) spectroscopy, thermal denaturation and size exclusion chromatography

Far UV CD spectra of rDer p 20 were measured on a Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). The protein (0.1 mg/mL in 75 mM Na_2HPO_4/NaH_2PO_4, pH 7.5) was measured in a quartz cuvette of 1 mm path length. The spectrum represents the average of triplet scans after subtraction of the blank, i.e., results for the buffer alone, collected in a wavelength range from 260 nm to 190 nm with 0.5 nm resolution, 50 nm/min scanning speed, 1 s time constant and a sensitivity of 100 mdeg. The results were visualized as mean residue ellipticity recorded over the wavelength range indicated above and the secondary structure composition was calculated using Dichroweb software, CDSSTR program.[3,4] Thermal denaturation of rDer p 20 was recorded at 222 nm, while heating from 10°C to 80°C and cooling down to 10°C again at a rate of 2°C/min with temperature measured in the sample.

Size exclusion chromatography with a Biosep SEC-s3000 column was not successful, presumably due to retention of the recombinant protein by the column material (data not shown). However, when using Superdex 200 Increase 10/300 column material and a multi angle light scattering (MALS) detector, a monomeric peak was obtained. Size exclusion chromatography was performed on Supradex 200 Increase 10/300 (GE Healthcare Chicago, Illinois, USA) at 0.5 mL/min in PBS with a miniDAWN TREOS multi angle light scattering detector (Wyatt, Santa Barbara, USA).

6.1.6 Measurement of IgE binding frequency to rDer p 20 in sera from HDM-allergic subjects by ELISA

Der p 20-specific IgE was measured in HDM allergic patients' sera (n = 98) by ELISA. Hundred microliter aliquots of rDer p 20, as well as of rDer p 2 and rDer p 23 as positive controls and HSA as negative control were coated overnight at 4°C on 96-well flat bottom plates (Nunc Maxi-Sorp, Rosklide, Denmark) at a concentration of 3 µg/mL in bicarbonate buffer, pH 9.6. Plates were washed 5 times with 200 µL TBST (10 mM Tris, 150 mM NaCl, 0.5% Tween 20, pH 8) and blocked for 2.5 hours at 37°C with TBST/1% BSA. Patients' sera were diluted 1:5 in TBST/0.5% BSA and 100 µL were added to the coated wells for overnight incubation (4°C). After washing, an alkaline phosphatase (AP) labelled mouse anti-human IgE antibody (BD Bi-osciences, San Jose, CA, USA) was added at a 1:1000 dilution and plates were incubated for 1 hour at 37°C and then for 1 h at 4°C. Following a final washing step, AP substrate solution (Sigma 104 phosphatase substrate tablets, Sigma-Aldrich, St. Louis, MO, USA) was added and absorbance was measured after 20 and 30 minutes, respectively, on a Tecan Infinite F50 ELISA plate reader at 405 nm and 550 nm. For normalization of OD values, reactivity of a standard serum positive to rDer p 2 was measured in 5 replicates per plate and individual normalization values were calculated for each plate. The statistical cutoff[5] was calculated for the results of all 98 sera to rDer p 20 to identify positive sera.

6.1.7 Preparation of allergen extracts

Raw material from *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Blomia tropicalis, Lepidoglyphus destructor, Tyrophagus putrescentiae, Periplaneta americana* and *Blattella germanica* was purchased (Allergon, Ängelholm, Sweden and *D. pteronyssinus* feces from LETIPharma, Barcelona, Spain). Of each allergen source, 0.3 g of specimens were suspended in 5 mL PBS supplemented with 10 µg/mL of a protease inhibitor (PMSF, stock prepared in ethanol at 10 mg/mL). The suspension was homogenized by Ultra-turrax IKA T18 basic (Staufen, Germany) for 1-2 minutes followed by incubation overnight at 4°C on a rocking platform. The homogenates were centrifuged (4 min, 23,500 g, RT) on the following day and the supernatants were stored at -20°C.

Extract from *Litopenaeus vannamei* was prepared from frozen, raw tail muscle. Peeled and deveined shrimps were homogenized in a mortar and approximately 4 g of the homogenate were added to 40 mL of PBS containing 10 µg/mL PMSF and, additionally, NaN_3 (stock: 20% wt/vol in ddH_2O) was added to a final dilution of 1:400 to inhibit microbial growth. The homogenate was incubated overnight at 4°C on a rotator and centrifuged on the next day at 1,700 g and 4°C for 10 minutes and a subsequent centrifugation step at 27,000 g and 4°C for 5 min. The supernatant was stored at -20°C. Protein concentration was determined by Bradford assay.

6.1.8 Identification of AK in different extracts and localization of Der p 20 by Western blot and inhibition assays

To verify the presence of AK in the different extracts, 300 uL of extracts from *D. pteronyssinus*, *D. farinae, B. tropicalis, L. destructor, T. putrescentiae, P. americana, B. germanica* and 200 uL from *L. vannamei* extract were loaded on a preparative polyacrylamide-gel, subjected to electrophoresis and blotted onto nitrocellulose membrane. The membranes were stained with Ponceau S staining solution (Sigma O 7767) to confirm transfer of samples and marker. Stripes of 4 mm width were prepared from each blotted extract and blocked by washing five times in gold buffer (40mM Na_2HPO_4, 6mM NaH_2PO_4, 0.5% V/V Tween 20, 70mM BSA, 70mM NaN_3). Antiserum from a rabbit that had been immunized with AK from *Plodia interpunctella* (Indian-meal moth) was diluted in gold buffer 1:1000 or 1:10,000, respectively, and added to the stripes. After overnight incubation at 4°C, the blots were washed in gold buffer and ^{125}I-labelled donkey anti-rabbit IgG detection antibody (Perkin Elmer, Boston, MA, USA), diluted 1:1000 in gold buffer, was added followed by overnight incubation. On the following day the blots were washed and dried and IgG-binding was visualized by autoradiography.

To analyze cross-reactivity between AK from mite and other arthropods, inhibition experiments were performed. Serum of a Der p 20 positive patient was diluted 1:10 in gold buffer and pre-incubated with rDer p 20 (20 µg/mL final concentration) for 1 hour at 4°C. Then, 1 mL of rDer p 20-pre-adsorbed serum or, for control purposes, serum that had been pre-incubated with HSA or buffer alone, was added to nitrocellulose-blotted *L. vannamei* extract overnight

at 4°C. After washing, ^{125}I-labelled anti-human IgE (BSM Diagnostica, Vienna, Austria) was added at a 1:10 dilution. IgE-binding was visualized by autoradiography.

To determine if Der p 20 is localized in the body or in the fecal pellets of the mites, blotted aqueous extracts of *D. pteronyssinus* body or feces were tested with the above mentioned rabbit AK antiserum at a 1:1000 dilution. Rabbit pre-immune serum and gold buffer were used as negative controls. As positive controls, purified AK (from *P. interpunctella* that had been used for rabbit immunization) and rDer p 20 and as negative control HSA were additionally dotted (100 ng/dot) onto nitrocellulose stripes. Binding of IgG to AK was visualized as mentioned above with ^{125}I-labelled donkey anti-rabbit IgG (Perkin Elmer).

6.1.9 Statistical analysis

Statistically significant differences between Der p 20 positive and negative groups were evaluated using the Mann–Whitney U test and the Chi-square test, respectively, by using GraphPad Prism 8.0.1 (San Diego, CA, USA). Differences were considered statistically significant if the p-value was equal to or lower than 0.05.

6.2 Figure legends

Figure E1. Size exclusion chromatography of rDer p 20. The graph shows the absorbance at 280 nm (y-axis) and the elution volumes in ml (x-axis). Molecular weights of standard proteins corresponding to the given elution volumes are indicated with black arrows. V0 – void volume. The standard based mass estimation of ~30 kDa indicates retardation due to interactions with the superdex medium (theoretical mass 41.3 kDa).

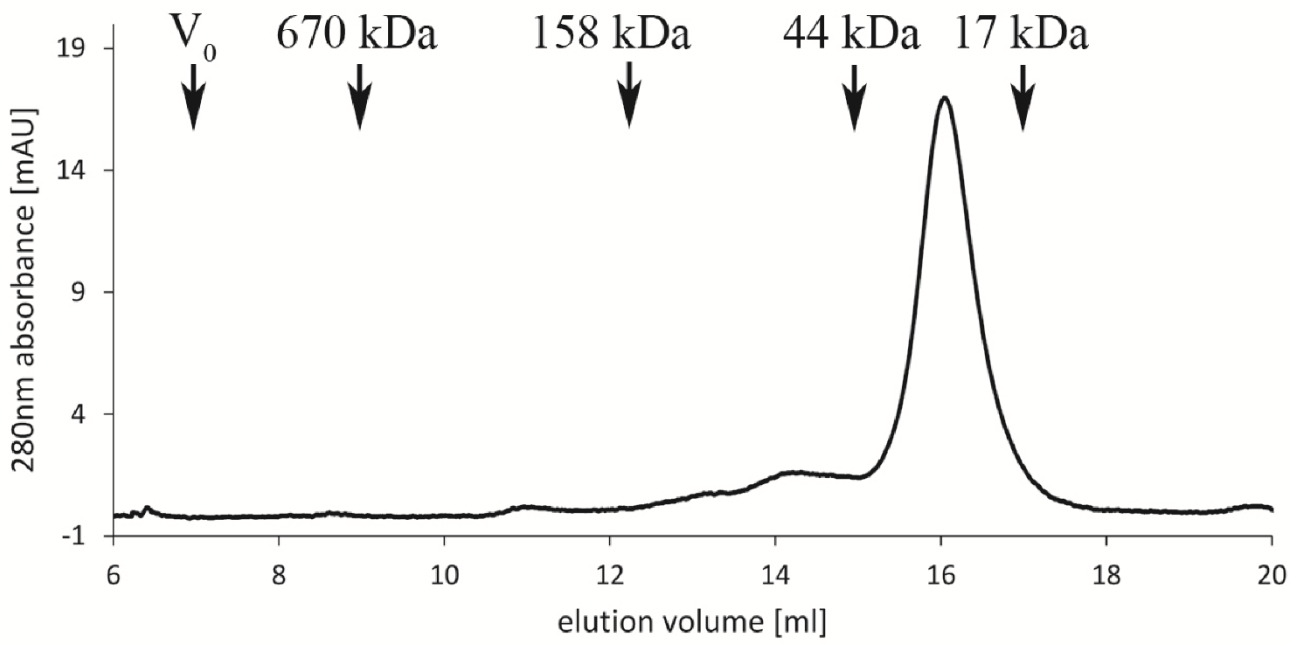
Figure E2. (A) Total nasal symptoms scores (TNSS) obtained during controlled HDM-allergen exposure in a challenge chamber are summarized as area under the curve (AUC) values (y-axis) for Der p 20-positive and -negative subjects (x-axis). (B) Percentages of Der p 20-sensitized (red bars) and -non-sensitized (blue bars) patients (y-axes) with specific IgE to other allergens from *D. farinae* and *D. pteronyssinus* (x-axis) and (C) with different numbers of sensitizations to other HDM allergens (x-axis). (D) Numbers of sensitization to other HDM-allergens (y-axis) are shown for individual patients for the Der p 20-positive and -negative group (x-axis). Error bars represent medians ± interquartile ranges. Ns - not statistically significant.

Figure E3. Percentages of amino acid sequence identity between Der p 20 and arginine kinases from other invertebrates. Identities of 60-70%, >70-80% and >80% (from light to dark orange) are highlighted by colored boxes.

Figure E4. Nitrocellulose-blotted extracts from (1) *Dermatophagoides pteronyssinus*, (2) *Dermatophagoides farinae*, (3) *Blattella germanica*, (4) *Lepidoglyphus destructor*, (5) *Periplaneta americana*, (6) *Blomia tropicalis*, (7) *Tyrophagus putrescentiae* and (8) *Litopenaeus vannamei* were probed with rabbit anti-Plo i 1-specific antibodies. Bound rabbit IgG was detected with ^{125}I-labelled anti-rabbit IgG and visualized by autoradiography. Molecular weights in kDa are indicated on the left margins.

Figure E5. Recombinant Der p 20 inhibits allergic patient's IgE-binding to AK in nitrocellulose blotted extract from *Litopenaeus vannamei*. Serum from a Der p 20-sensitized patient was pre-incubated with rDer p 20 (lane 1), with human serum albumin (lane 2) or without inhibitor (lane 3). IgE-reactivity was detected with ^{125}I-labelled anti-human IgE and visualized by autoradiography. Molecular weights in kDa are indicated on the left margins.

Figure E1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670 kDa
158 kDa
44 kDa
17 kDa
280nm absorbance [mAU]
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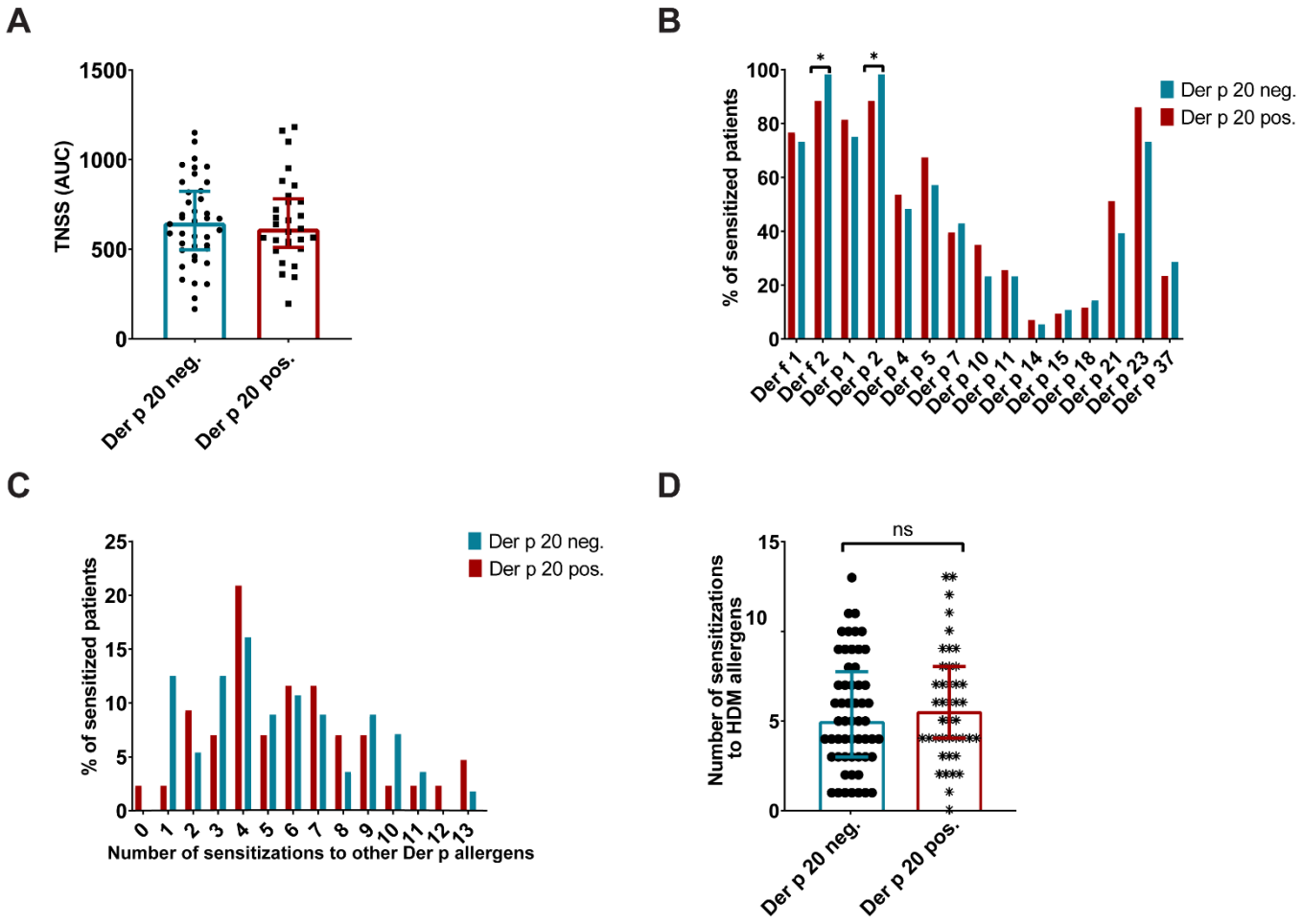
9
4
-1
6
8
10
12
14
16
18
20
elution volume [ml]

Figure E2


A
TNSS (AUC)
1500
1000
500
0
Der p 20 neg.
Der p 20 pos.
B
% of sensitized patients
100
80
60
40
20
0
*
*
Der p 20 neg.
Der p 20 pos.
Der f 1
Der f 2
Der p 1
Der p 2
Der p 4
Der p 5
Der p 7
Der p 10
Der p 11
Der p 14
Der p 15
Der p 18
Der p 21
Der p 23
Der p 37
C
% of sensitized patients
25
20
15
10
5
0
Der p 20 neg.
Der p 20 pos.
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Number of sensitizations to other Der p allergens
D
Number of sensitizations to HDM allergens
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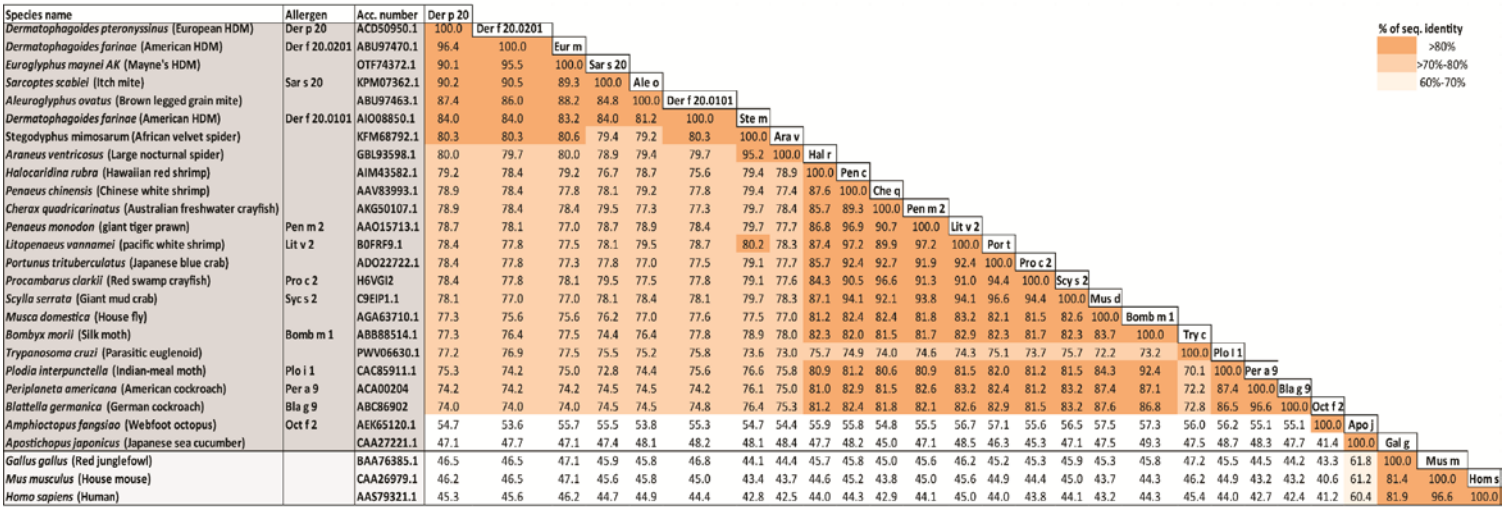
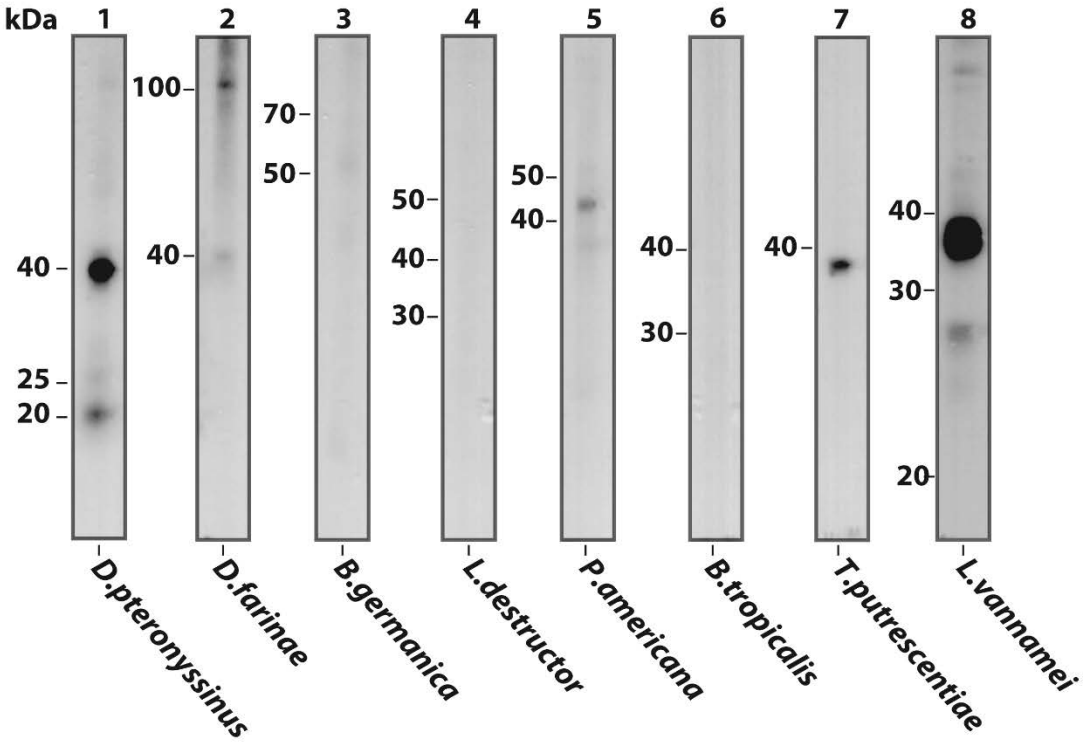
10
5
0
ns
Der p 20 neg.
Der p 20 pos.

Figure E3



Species name	Allergen	Acc. number	Der p 20	Der f 20.0201	Eur m	Sar s 20	Ale o	Der f 20.0101	Ste m	Ara v	Hal r	Pen c	Che q	Pen m 2	Lit v 2	Por t	Pro c 2	Scy s 2	Mus d	Bomb m 1	Try c	Plo i 1	Per a 9	Bla g 9	Oct f 2	Apo j	Gal g	Mus m	Hom s
Dermatophagoides pteronyssinus (European HDM)	Der p 20	ACD50950.1	100.0																									
Dermatophagoides farinae (American HDM)	Der f 20.0201	ABU97470.1	96.4	100.0																								
Euroglyphus maynei AK (Mayne's HDM)		OTF74372.1	90.1	95.5	100.0																							
Sarcoptes scabiei (Itch mite)	Sar s 20	KPM07362.1	90.2	90.5	89.3	100.0																						
Aleuroglyphus ovatus (Brown legged grain mite)		ABU97463.1	87.4	86.0	88.2	84.8	100.0																					
Dermatophagoides farinae (American HDM)	Der f 20.0101	AIO08850.1	84.0	84.0	83.2	84.0	81.2	100.0																				
Stegodyphus mimosarum (African velvet spider)		KFM68792.1	80.3	80.3	80.6	79.4	79.2	80.3	100.0																			
Araneus ventricosus (Large nocturnal spider)		GBL93598.1	80.0	79.7	80.0	78.9	79.4	79.7	95.2	100.0																		
Halocaridina rubra (Hawaiian red shrimp)		AIM43582.1	79.2	78.4	79.2	76.7	78.7	75.6	79.4	78.9	100.0																	
Penaeus chinensis (Chinese white shrimp)		AAV83993.1	78.9	78.4	77.8	78.1	79.2	77.8	79.4	77.4	87.6	100.0																
Cherax quadricarinatus (Australian freshwater crayfish)		AKG50107.1	78.9	78.4	78.4	79.5	77.3	77.3	79.7	78.4	85.7	89.3	100.0															
Penaeus monodon (giant tiger prawn)	Pen m 2	AAO15713.1	78.7	78.1	77.0	78.7	78.9	78.4	79.7	77.7	86.8	96.9	90.7	100.0														
Litopenaeus vannamei (pacific white shrimp)	Lit v 2	B0FRF9.1	78.4	77.8	77.5	78.1	79.5	78.7	80.2	78.3	87.4	97.2	89.9	97.2	100.0													
Portunus trituberculatus (Japanese blue crab)		ADO22722.1	78.4	77.8	77.3	77.8	77.0	77.5	79.1	77.7	85.7	92.4	92.7	91.9	92.4	100.0												
Procambarus clarkii (Red swamp crayfish)	Pro c 2	H6VGI2	78.4	77.8	78.1	79.5	77.5	77.8	79.1	77.6	84.3	90.5	96.6	91.3	91.0	94.4	100.0											
Scylla serrata (Giant mud crab)	Syc s 2	C9EIP1.1	78.1	77.0	77.0	78.1	78.4	78.1	79.7	78.3	87.1	94.1	92.1	93.8	94.1	96.6	94.4	100.0										
Musca domestica (House fly)		AGA63710.1	77.3	75.6	75.6	76.2	77.0	77.6	77.5	77.0	81.2	82.4	82.4	81.8	83.2	82.1	81.5	82.6	100.0									
Bombyx morii (Silk moth)	Bomb m 1	ABB88514.1	77.3	76.4	77.5	74.4	76.4	77.8	78.9	78.0	82.3	82.0	81.5	81.7	82.9	82.3	81.7	82.3	83.7	100.0								
Trypanosoma cruzi (Parasitic euglenoid)		PWV06630.1	77.2	76.9	77.5	75.5	75.2	75.8	73.6	73.0	75.7	74.9	74.0	74.6	74.3	75.1	73.7	75.7	72.2	73.2	100.0							
Plodia interpunctella (Indian-meal moth)	Plo i 1	CAC85911.1	75.3	74.2	75.0	72.8	74.4	75.6	76.6	75.8	80.9	81.2	80.6	80.9	81.5	82.0	81.2	81.5	84.3	92.4	70.1	100.0						
Periplaneta americana (American cockroach)	Per a 9	ACA00204	74.2	74.2	74.2	74.5	74.5	74.2	76.1	75.0	81.0	82.9	81.5	82.6	83.2	82.4	81.2	83.2	87.4	87.1	72.2	87.4	100.0					
Blattella germanica (German cockroach)	Bla g 9	ABC86902	74.0	74.0	74.0	74.5	74.5	74.8	76.4	75.3	81.2	82.4	81.8	82.1	82.6	82.9	81.5	83.2	87.6	86.8	72.8	86.5	96.6	100.0				
Amphioctopus fangsiao (Webfoot octopus)	Oct f 2	AEK65120.1	54.7	53.6	55.7	55.5	53.8	55.3	54.7	54.4	55.9	55.8	54.8	55.5	56.7	57.1	55.6	56.5	57.5	57.3	56.0	56.2	55.1	55.1	100.0			
Apostichopus japonicus (Japanese sea cucumber)		CAA27221.1	47.1	47.7	47.1	47.4	48.1	48.2	48.1	48.4	47.7	48.2	45.0	47.1	48.5	46.3	45.3	47.1	47.5	49.3	47.5	48.7	48.3	47.7	41.4	100.0		
Gallus gallus (Red junglefowl)		BAA76385.1	46.5	46.5	47.1	45.9	45.8	46.8	44.1	44.4	45.7	45.8	45.0	45.6	46.2	45.2	45.3	45.9	45.3	45.8	47.2	45.5	44.5	44.2	43.3	61.8	100.0	
Mus musculus (House mouse)		CAA26979.1	46.2	46.5	47.1	45.6	45.8	45.0	43.4	43.7	44.6	45.2	43.8	45.0	45.6	44.9	44.4	45.0	43.7	44.3	46.2	44.9	43.2	43.2	40.6	61.2	81.4	100.0
Homo sapiens (Human)		AAS79321.1	45.3	45.6	46.2	44.7	44.9	44.4	42.8	42.5	44.0	44.3	42.9	44.1	45.0	44.0	43.8	44.1	43.2	44.3	45.4	44.0	42.7	42.4	41.2	60.4	81.9	96.6

Figure E4


kDa
1
2
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4
5
6
7
8
100
70
50
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30
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40
30
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D.pteronyssinus
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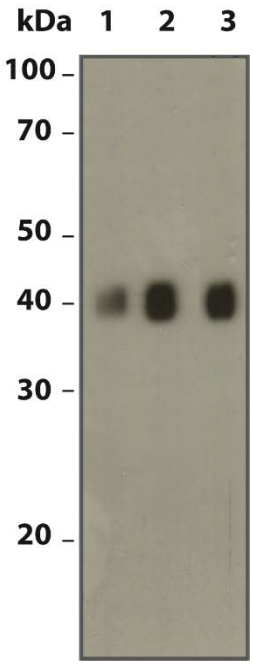
P.americana
B.tropicalis
T.putrescentiae
L.vannamei

Figure E5



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7 Summary

The first part of my work at the Medical University of Vienna was dedicated to the expression and purification of recombinant allergen molecules for the generation of a novel customised allergen microarray that also covers clinically relevant allergen molecules which are prevalent in areas outside Europe. After thorough literature search, three allergens from *Blomia tropicalis*, a storage mite that is an important allergen source in the tropics and subtropics, were selected for expression and purification. The three *Blomia* allergens (Blo t 5, Blo t 12 and Blo t 21) were selected according to clinical relevance, IgE-recognition frequencies, used expression system and protein yield. A fourth allergen, i.e., Der p 20 from *Dermatophagoides pteronyssinus* was the main topic of the manuscript in Chapters 5 and 6. Der p 20 is an arginine kinase (AK) and possibly cross-reactive with homologous proteins from other allergen sources that has not yet been characterised in detail.

cDNA sequences of Blo t 5, 12, 21 and of Der p 20 were obtained from Uniprot-database and ordered as synthetic genes in pET-17b expression vectors including a sequence encoding a C-terminal hexahistidine-tag to facilitate purification of the recombinant allergens. Expression-levels and localisation of the target proteins within the host cells were determined by SDS-PAGE. Proteins were purified either under denaturing or, if appropriate, under native conditions by affinity chromatography using nickel-agarose. For purification from inclusion bodies, proteins were solubilised by urea and refolded by stepwise dialysis, eventually after addition of glutathione to avoid oligomerisation. Purified allergens were analysed by CD-spectroscopy and size-exclusion chromatography to determine secondary structures and possible aggregation.

Der p 20 and Blo t 21 were purified as soluble proteins, while Blo t 5 and 12 accumulated in inclusion bodies. CD-spectra revealed proper folding of all proteins except Blo t 12 which showed a high percentage of random structures. To prevent formation of oligomers due to a considerable number of cysteines (n=6), purification and refolding of Blo t 12 were performed after addition of glutathione. Size-exclusion chromatography indicated that the presence of glutathione during purification actually reduced formation of oligomers, however, a thermal denaturation assay revealed that the recombinant protein was not folded.

IgE-recognition frequency of recombinant Der p 20 was determined by ELISA in sera from 98 patients suffering from HDM-induced allergic rhinoconjunctivitis and mild asthma. ELISA results showed that sera of twenty-seven HDM allergic patients contained allergen-specific IgE against recombinant Der p 20. Patients with IgE reactivity to Der p 20 more frequently reported breathing-problems compared to Der p 20 negative patients, indicating that Der p 20 might be a biomarker for more severe forms of HDM allergy. In addition, cross-reactivity between AKs from different organisms was studied by Western blot. An inhibition assay showed that rDer p 20 inhibited IgE-binding to AK in extracts from shrimp (*L. vannamei*), suggesting that Der p 20 might help resolve cases of cross-reactivity between shrimp and HDM that are negative to tropomyosin.

The purified mite allergens will be used to expand the spectrum of allergen molecules for the diagnosis of mite allergy and to study the importance of the *Blomia* allergens for allergy diagnosis and allergen-specific immunotherapy. Our results demonstrated the importance of Der p 20 as potential asthma biomarker and cross-reactive allergen. Further studies will be needed investigating the allergenic activity of Der p 20, its specific role in food and respiratory allergies as a potential cause of severe symptoms, as well as its use for the distinction between symptoms caused by tropomyosin or AK.

8 Zusammenfassung

Meine Masterarbeit an der Medizinischen Universität Wien widmete sich der Expression und Reinigung von rekombinanten Allergenen für die Entwicklung eines Allergen-Microarrays, welcher auch klinisch relevante Allergenmoleküle enthält, die in vielen Gebieten außerhalb Europas weit verbreitet sind. Nach gründlicher Literaturrecherche wurden drei Allergene aus *Blomia tropicalis*, einer Speichermilbe, die in den Tropen und Subtropen eine wichtige Allergenquelle darstellt, für die Expression und Reinigung ausgewählt. Die drei *Blomia*-Allergene (Blo t 5, Blo t 12 und Blo t 21) wurden nach klinischer Relevanz, IgE-Erkennungshäufigkeit, verwendetem Expressionssystem und Proteinertrag ausgewählt. Basierend auf den Daten aus der Expression und Charakterisierung eines vierten Allergens, Der p 20 aus *Dermatophagoi-*

des pteronyssinus, wurde ein Manuskript erstellt und zur Publikation in einem wissenschaftlichen Journal eingereicht (Kapitel 5 und 6). Der p 20 ist eine Argininkinase (AK), welche möglicherweise kreuzreaktiv mit homologen Proteinen aus anderen Allergenquellen ist. Dieses Allergen wurde jedoch bisher noch nicht im Detail charakterisiert.

cDNA-Sequenzen von Blo t 5, 12, 21 und Der p 20 wurden von der Uniprot-Datenbank bezogen und als synthetische Gene mit einer zusätzlichen Sequenz, welche einen C-terminalen Hexahistidin-tag codiert, in pET-17b Vektoren für die Expression in *E. coli* bestellt. Der C-terminale Hexahistidin-tag ermöglicht die Reinigung der rekombinanten Allergene mittels Nickel-Agarose. Expressionsmenge und Lokalisation der Zielproteine innerhalb der Expressionszellen wurden mittels SDS-PAGE bestimmt. Die Proteine wurden entweder unter denaturierenden oder gegebenenfalls unter nativen Bedingungen durch Affinitätschromatographie mit Nickel-Agarose gereinigt. Für die Reinigung aus *E. coli* Einschlusskörpern wurden Proteine durch Zugabe von Harnstoff gelöst und durch schrittweise Dialyse und gegebenenfalls Verwendung von Glutathion, um Oligomerisierung zu vermindern, wieder gefaltet. Gereinigte Allergene wurden mittels CD-Spektroskopie und Größenausschlusschromatographie analysiert, um Sekundärstrukturen und mögliche Aggregation zu bestimmen.

Der p 20 und Blo t 21 wurden als lösliche Proteine gereinigt, während Blo t 5 und 12 sich in Einschlusskörpern ansammelten. CD-Spektren zeigten eine korrekte Faltung aller Proteine mit Ausnahme von Blo t 12, das einen erhöhten Prozentsatz an Zufallsstrukturen zeigte. Um die Bildung von Oligomeren aufgrund einer erheblichen Anzahl von Cysteinen zu verhindern (n=6), wurde die Reinigung und Rückfaltung von Blo t 12 nach Zugabe von Glutathion durchgeführt. Die Größenausschlusschromatographie zeigte, dass dieser Schritt die Bildung von Oligomeren tatsächlich reduzierte. Ein thermischer Denaturierungstest ergab jedoch, dass das rekombinante Protein nicht gefaltet war.

Die IgE-Erkennungsfrequenz des rekombinanten Der p 20 wurde durch ELISA mit Seren von 98 Patienten mit allergischer Rhinokonjunktivitis und mildem Asthma, bedingt durch Hausstaubmilbenallergie, bestimmt. Die ELISA-Ergebnisse zeigten, dass Seren von 27 der 98 allergischen Patienten allergenspezifisches IgE gegen rekombinantes Der p 20 enthielten. Patienten mit IgE-Reaktivität gegen Der p 20 berichteten zusätzlich häufiger über Atembeschwerden im Vergleich zu Der p 20 negativen Patienten. Das deutet darauf hin, dass Der p 20 ein Biomarker für schwerere Formen von Haustaubmilben-Allergien sein könnte.

Darüber hinaus wurde die Kreuzreaktivität zwischen Der p 20 und AKs anderer Organismen durch Western Blot untersucht. Ein Inhibitionstest zeigte, dass rDer p 20 die IgE-Bindung an AK in Extrakten aus Garnelen (*L. vannamei*) hemmte. Der p 20 könnte somit helfen, Kreuzreaktivitäten zwischen Garnelen und HDM in Tropomyosin-negativen Patienten aufzuklären.
Die gereinigten Milbenallergene (Blo t 5, 12, 21 und Der p 20) werden verwendet, um das Spektrum der Allergenmoleküle für die Diagnose von Milbenallergien zu erweitern und die Bedeutung der *Blomia*-Allergene für die Allergiediagnostik und die allergenspezifische Immuntherapie zu untersuchen. Unsere Ergebnisse zeigten die Bedeutung von Der p 20 als potenzieller Asthmabiomarker und kreuzreaktives Allergen. Weitere Studien werden erforderlich sein, um die allergene Aktivität von Der p 20, seine spezifische Rolle im Rahmen von Atemwegsallergien als mögliche Ursache für schwere Symptome sowie seine Verwendung zur Unterscheidung zwischen Symptomen, die durch Tropomyosin oder AK verursacht werden, zu untersuchen.