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Doctoral Dissertation

Identification and characterization of novel allergens from fungal species commonly detected in the indoor environment

submitted by

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in partial fulfilment of the requirements for the academic degree

Doktorin der Bodenkultur (Dr.nat.techn.)

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Affidavit

I hereby declare that I have authored this dissertation independently, and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature are duly identified and cited, and the precise references included. Any contribution from colleagues is explicitly stated in the authorship statement of the published papers.

I further declare that this dissertation has not been submitted, in whole or in part, in the same or a similar form, to any other educational institution as part of the requirements for an academic degree.

I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

Vienna, 17.01.2022 Sandra PFEIFFER *(manu propria)*

This thesis is dedicated to Karl, Leopold, Elisabeth, and Barbara.

Scientific research is one of the most exciting and rewarding of occupations. It is like a voyage of discovery into unknown lands, seeking not for new territory but for new knowledge.

Frederick Sanger, Stockholm, 1981

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Preface

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Table of content

List of publications

Publications that comprise the main part of this cumulative dissertation

PAPER I (published):

Bibliography:

- Title: **Identification of *Ulocladium chartarum* as an important indoor allergen source**
- Authors: Pfeiffer S, Sandler P, Raith M, Pascal M, Munoz-Cano RM, San Bartolome C, Nöbauer K, Quirce S, Razzazi-Fazeli E, Focke-Tejkl M, Sterflinger K, Swoboda I
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S.P. and P.S. carried out the majority of the experiments described. M.R. performed the CD-spectroscopy. M.P., R.M.M.-C. and C.S.B. carried out the basophil activation assays and provided a part of the serum samples used in this study. S.Q. also provided serum samples. K.N. and E.R.-F. implemented the mass spectrometric analysis. M.F.-T. and K.S.-G. contributed to interpreting the results and planning the work by providing their scientific expertise. S.P. wrote the manuscript. I.S. supervised the project and contributed to the implementation of the research, to the analysis of the results and to the writing of the manuscript. All authors discussed the results and commented on the manuscript.

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- Title: **The emerging pathogen *Paecilomyces variotii* – a novel and important fungal allergen source**
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Authors contributions:

S.P. carried out the majority of the experiments described. M.R. performed the CD-spectroscopy. M.P., R.M.M.-C. and C.S.B. carried out the basophil activation assays and provided a part of the serum samples used in this study. S.Q. also provided serum samples. K.N. and E.R.-F. implemented the mass spectrometric analysis. M.F.-T. and K.S.-G. contributed to interpreting the results and planning the work by providing their scientific expertise. S.P. wrote the manuscript. I.S. supervised the project and contributed to the implementation of the research, to the analysis of the results and to the writing of the manuscript. All authors discussed the results and commented on the manuscript.

Additional contributions

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Bibliography:

- Title: **Analysis of the cross-reactive potential of the enolase pan-allergen**
- Authors: Pfeiffer S, Sperl CM, Nöbauer K, Razzazi-Fazeli E, Focke-Tejkl M, Sterflinger K and Swoboda I

Authors contributions:

S.P. carried out the majority of the experiments described. CM.S. carried out valuable preliminary experiments. K.N. and E.R.-F. implemented the mass spectrometric analysis. M.F.-T. and K.S.-G. contributed to interpreting the results and planning the work by providing their scientific expertise. S.P. wrote the manuscript. I.S. supervised the project and contributed to the implementation of the research, to the analysis of the results and to the writing of the manuscript.

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Abstract

Fungal allergy is a worldwide public health burden and problems associated with a reliable allergy diagnosis are far from being solved. The knowledge about allergenic fungal species is limited, especially the contribution of indoor fungal exposure to allergic diseases is not understood. One aim of this dissertation was to study the allergenic potential of two commonly occurring indoor molds *Ulocladium chartarum* and *Paecilomyces variotii*. The first allergens from *U. chartarum* and *P. variotii* were identified, recombinantly expressed in *Escherichia coli* and characterized, using different biochemical and immunological methods, as folded, IgE-reactive molecules with immunological features comparable to their natural counterparts. Among the allergens identified in both species were enolases, enzymes that have already been described as allergens. A second aim of the thesis was to analyze the cross-reactive potential of allergenic enolases. Investigations on the IgE-reactivity of fungal enolases and enolases present in plant pollen and animal-derived foods in a cohort of polysensitized patients by immunoblots and ELISAs, showed that enolases from different molds are highly cross-reactive, but also possess species-specific IgE epitopes. No cross-reactivity was found between fungal enolase and enolases of pollen or animal-derived foods. A third aim of the thesis was to investigate the impact of different environmental conditions on the expression of fungal allergens, which was studied by immunoblots using allergen-specific antibodies. The results showed that each species has different requirements for optimal allergen expression and the obtained information will help to improve fungal cultivation to produce highly potent diagnostic fungal extracts. Results presented in this dissertation expand the knowledge about fungal allergy and facilitate an improvement in the specificity of fungal allergy diagnosis. They will also form the basis for optimized treatment approaches.

Kurzfassung

Pilzallergie stellt eine weltweite Gesundheitsbelastung dar und eine zuverlässige Allergiediagnose ist nicht gewährleistet. Zudem ist das Wissen über allergene Pilze begrenzt, insbesondere der Beitrag von Pilzexposition in Innenräumen zu allergischen Erkrankungen ist unklar. Ein Ziel dieser Dissertation war es, das allergene Potenzial der Innenraumpilze *Ulocladium chartarum* und *Paecilomyces variotii* zu untersuchen. Die ersten Allergene vom *U. chartarum* und *P. variotii* wurden identifiziert, rekombinant in *Escherichia coli* exprimiert und mittels biochemischer und immunologischer Methoden als gefaltete, IgE-reaktive Moleküle mit vergleichbaren Eigenschaften wie die natürlichen Allergene charakterisiert. Unter den in beiden Pilzen identifizierten Allergenen befanden sich Enolasen, Enzyme, die bereits als Allergene bekannt sind. Ein weiteres Ziel war es das kreuzreaktive Potential von Enolasen zu untersuchen. Die Analyse der IgE-Reaktivität von Pilzenolasen und Enolasen in Pollen und tierischen Lebensmitteln mit einer Kohorte von polysensibilisierten Patienten zeigte, dass Pilzenolasen kreuzreaktiv sind, aber auch Spezies-spezifische IgE-Epitope besitzen. Es konnte keine Kreuzreaktivität zwischen Pilzenolasen und Enolasen aus verschiedenen tierischen Nahrungsmitteln und Pollen gezeigt werden. Das dritte Ziel der Dissertation was es den Einfluss von Umweltbedingungen auf die Pilzallergenexpression zu untersuchen, dies geschah mittels Immunblots und spezifischen Antikörpern. Die Ergebnisse zeigten, dass jeder Pilz unterschiedliche Kultivierungsbedingungen für eine optimale Allergenexpression benötigt. Die gewonnenen Informationen werden eine verbesserte Pilzkultivierung zur Herstellung von potenten Extrakten für die Allergiediagnose ermöglichen. Die in dieser Dissertation gezeigten Ergebnisse erweitern das Wissen über Pilzallergien und tragen zur Verbesserung der Spezifität der Pilzallergiediagnostik bei und bilden die Basis zur Entwicklung optimierter Behandlungsansätze.

1. Introductory overview

This section represents the framework paper of this cumulative dissertation and includes the theoretical background of this work, a detailed description of the methods used, as well as an overview of the relevant contributions and a summary of the thesis.

1.1. Theoretical Background

In the following chapters, relevant topics for this thesis such as characteristics of the fungal kingdom, allergenic mechanisms and the role of fungi as important respiratory allergen sources will be introduced.

1.1.1. The fungal kingdom

Fungi represent a separate entity within living organisms that comprises a taxonomic kingdom with millions of heterogenous species, ranging from the largest organism on earth, the plant pathogenic fungus *Armillaria ostoyae* that extends over more than 37 hectares of forest floor (1–3), to a myriad of microscopic species such as unicellular yeasts with a typical cell size of 3-4 µm (4–6). Furthermore, they form a significant portion of the Earth's biomass (25%), thus exposure to fungi and their products is ubiquitous (7,8). Even though the exact number of fungal species is unknown, high-throughput sequencing methods suggest that at least 5 to 6 million fungal species exist worldwide, of which approximately 100,000 species have been described so far (4,7,9,10). Thus, most fungal species remain uncharacterized, which gives the fungal kingdom the by-name "hidden kingdom" (4).

Growth and reproduction

In general, fungi are eukaryotic, non-chlorophyllus, non-motile, filamentous, and mostly spore-bearing organisms with a complex metabolism that differs from animals and plants (6). As they are heterotrophic organisms, they depend on external nutrients, which they externally digest by the secretion of enzymes followed by the absorption of the breakdown products through the cell membrane (6,8). Fungi, except for unicellular yeasts, typically have a thread-like or tube-like body composed of hyphae, which range from 2 to 10 µm in diameter

and then grow at their tips and frequently branch, resulting in an interconnected network called a mycelium (6).

A common feature of fungi is their quick and simple reproduction-cycle (11). They are principally dispersed as airborne sexual spores or asexual conidia, which vary in size and shape depending on the species and are common components of the atmospheric aerospora (12–14). In addition, unidentifiable viable hyphae fragments may also be aerosolized in large numbers and further disperse the organisms (12,14). This allows them to quickly occupy and inhabit suitable ecological niches, indoors as well as outdoors (11).

Fungal species manifest astounding diversity, resilience, and variability with regard to the environments they occupy depending on the optimal growth conditions of each species (4,15). Some are xerophilic, growing in relatively dry environments (e.g., *Penicillium* and *Aspergillus species*), whereas some are hydrophilic, requiring substrates with high water content (e.g., *Stachybotrys species*) (15). In addition, optimal growth temperatures vary among species, but are commonly between 18°C and 32°C (15). However, all fungi require oxygen for growth in addition to sources of carbohydrate and water (15).

The impact of fungi on the environment and society

The fungal kingdom is remarkable with respect to its profound impact on global health, biodiversity, ecology, agriculture, manufacturing, and biomedical research (4). It presents various opportunities as well as challenges to humanity through its high diversity, unique metabolic capacity, and rapid ability to change (4).

Fungal species interact with a broad range of other organisms, thus making them important members of diverse microbiota that promote ecosystem homeostasis (4,9). They live as saprophytes on non-living organic matter or as parasites or symbionts in living tissue of animals and plants (4,12). Their heterotrophic, absorptive nutrition allows them to play major roles as decomposers and mycorrhizal mutualists (9,16). Moreover, fungi are important factories of secondary metabolites that include important medical compounds such as antibiotics and immunosuppressants as well as products relevant for the food and cosmetic industry (e.g., enzymes) (4,11).

Besides the important positive impact fungi have on the environment and society, fungal organisms also represent an increasing public health problem worldwide as they can have serious determinantal effects on human health (15,17). Airborne fungi can impact human health in four different ways: (a) they can infect humans, (b) they can be toxigenic, (c) they may cause inflammatory reactions or (d) they may act as allergens (17).

Approximately 625 fungal species have been reported to infect vertebrates, 200 of which can be human-associated, either as commensals and members of our microbiome, or as pathogens that cause infectious diseases (4,15). There are diverse types of fungal infections that are determined by the fungal species and the immune status of the person infected (4,15). Fungal infections of humans range from superficial skin lesions to potentially fatal systemic mycoses (18). These eukaryotic pathogens, especially species of the genera *Aspergillus*, *Candida*, *Cryptococcus* and *Pneumocystis*, infect billions of people worldwide, killing more than 1.5 million per year (4).

In addition, some fungi produce toxic metabolites (= mycotoxins) that can be deleterious to human, animal, and plant health, causing acute, chronic, or both types of diseases (4,15,19). Toxigenic fungi include species of for examples *Alternaria, Paecilomyces* and *Trichoderma*, which occur commonly in soil, agricultural products, grain dust, and house dust (8).

Moreover, fungal cell walls are composed of layers of structures that are recognized by several receptors of the innate immune system, resulting in the production of proinflammatory cytokines (7). Such fungal exposure, for example to *Aspergillus species*, can, through a variety of mechanisms, result in inflammatory processes, leading to hypersensitivity pneumonitis, bronchopulmonary mycoses, fungal sinusitis, rhinitis, and asthma (4,7,15).

In addition to the impact on human health as infectious organisms, as producers of mycotoxins and as a cause of inflammatory reactions, fungi can also represent important allergen sources that affect millions of people worldwide (4,14).

1.1.2. Allergy

Allergic diseases constitute the most common cause of chronic illnesses in developed countries, affecting about 20-25% of the general population, and there is evidence of a steady

increase over time in the proportion of populations suffering from allergies (19–24). Allergy is defined as a hypersensitivity reaction initiated by specific immunologic mechanisms that are harmful rather than protective (25,26). In more detail, allergy describes a set of symptoms that are initiated by the exposure to a harmless stimulus at a dose tolerated by healthy individuals, which can be either antibody- or cell-mediated (25). If the reaction is mediated by immunoglobulins of class E (IgE), which is the most frequent underlying trigger, the allergic disease is defined as type I hypersensitivity (19,25).

Sensitization and allergic inflammation

Normally innocuous environmental substances, called allergens, will cause an allergic sensitization only in individuals with susceptibility, which is influenced by various factors including age, and heritable and acquired predisposition, and only if the allergen is encountered in sufficient quantities and via a relevant route of exposure (27).

For a protein to act as an allergen, several immunological events must occur during the first encounter that may then result in allergic sensitization (see **Figure 1**) (27). A B cell must differentiate into a plasma cell that then produces allergen-specific IgE antibodies (27). Therefore, the clonotypic B cell receptor, expressed on its surface, must bind to specific parts on the surface of the protein antigen, called epitopes (27). The IgE antibodies subsequently produced by the plasma cell, into which the B cell differentiates, have the same specificity as the B cell receptor, and are therefore able to bind specifically to the same B cell epitopes on the surface of the protein antigen (27). However, efficient secretion of antibodies normally requires that the B cells receive help from T-helper (Th) cells that specifically recognize separate epitopes on the same protein antigen (27). Such recognition is mediated by the T cell receptor, which recognizes the epitope on the protein antigen that was taken up, degraded, and presented by an antigen presenting cell (APC) (e.g., dendritic cells) via MHC (major histocompatibility complex) class II molecules (27). This interaction leads to the production of stimulatory intracellular signals to the T cell (27). These signals are able to activate resting, immature Th cells (designated Th0), causing their differentiation into mature, effector Th cells, which can be of either type 1 (Th1) or type 2 (Th2) (27). These mature T cells can help B cells to secrete immunoglobulins by releasing cytokines into the milieu that can bind to, and hence activate, specific cytokine receptors on the surface of the B cells (27). The

type of cytokines that stimulate the B cell can affect both the strength and the quality of the antibody response (27). Typically, Th2 cells produce a variety of cytokines including interleukin (IL)-4, IL-5, IL-6, IL-10, and IL-13 that, among other actions, encourage plasma cells to switch to the synthesis of IgE antibodies (27). In contrast, Th1 cells typically produce IL-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α/β, which together downregulate IgE synthesis (27).

At this asymptomatic or sensitization stage, the individual is sensitized to a given allergen source (28,29). Therefore, the term 'atopy' describes the personal and/or familial tendency to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens (25).

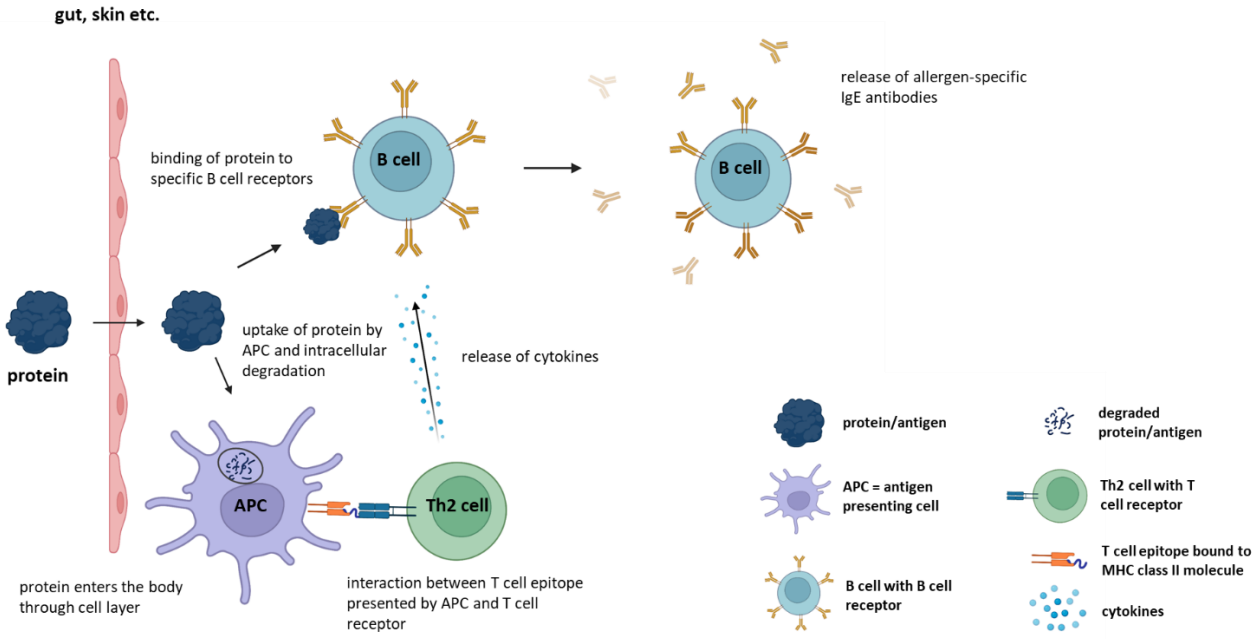

Figure 1. Immunologic events that occur during the first encounter to an allergen and lead to allergic sensitization. For IgE antibodies to be released from a differentiated B cell, two signals must be delivered: first, the B cell receptor must bind specifically to the B cell epitopes on the surface of the antigen. Second, help from T cells in the form of cytokines must be delivered by Th2 cells. Fir Th2 cells to be activated, their receptors must recognize fragments of the antigen (= T cell epitopes) that have been processed by antigen presenting cells (APCs) and displayed on their surface in association with MHC (major histocompatibility complex) class II molecules (adapted from Huby et al. and created with BioRender.com) (27).

In sensitized (atopic) individuals re-exposure to the offending allergen induces systemic distribution of the allergen-specific IgE antibodies, which, like all other types of antibodies, can bind specifically via its F(ab)-arms to the antigen that induced its production (27). Antibodies of the IgE isotype are also able to bind via their constant (Fc)-regions to specific

IgE receptors (FcεRI) found on the surface of (among other cells) mast cells and basophils (27). When two or more of these captive IgE molecules bind to their specific antigen, the FcεRI are cross-linked on the surface of the cell, initiating intracellular signaling events (27). These signals activate the cells, leading to degranulation with the release of histamine, and the production of prostaglandins, leukotrienes, and other inflammatory mediators that modulate cell adhesion, migrations, and signal transduction (see **Figure 2**) (27,30). Together, these agents mediate the symptoms of immediate-type hypersensitivity reactions such as allergic rhinitis, asthma, atopic dermatitis, and others (27,31).

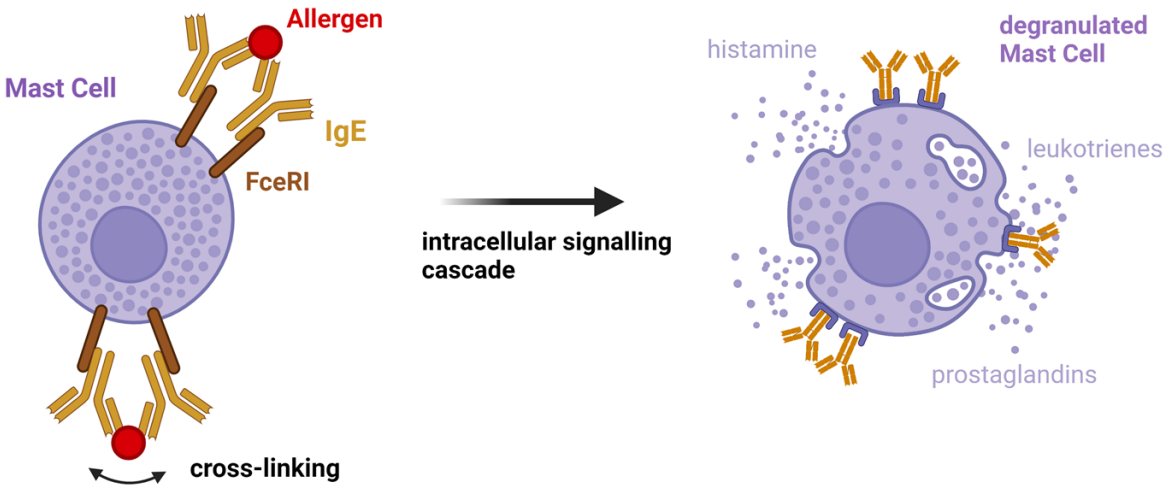

Figure 2. Hypersensitivity reactions are mediated by factors released by mast cells and basophils as a response to intracellular signals generated by the cross-linking of FcεRI on the surface of such cells. The cross-linking of FcεRI is initiated by receptor bound IgE antibodies that recognize and bind the allergen (adapted from Huby et al. and created with BioRender.com) (27).

Allergenic molecules

A wide range of different molecules, mainly proteins, from various sources, so-called allergens, can provoke severe allergic reactions (27,28,32). A molecule must possess the following distinct properties to be termed an allergen: (a) the property to sensitize and (b) the property to elicit an allergic reaction (33). Proteins that react with IgE antibodies induced by other proteins and elicit allergic symptoms are defined as cross-reactive allergens and are responsible for the well-known phenomenon of cross-reactivity leading to polysensitization,

which is frequently encountered in clinical practice (34,35). Furthermore, allergens can be classified as major or minor allergens, based on the prevalence of IgE- or skin reactivity in subjects that are sensitized to the corresponding allergen source (33).

There are different properties that can have an impact on the allergenicity of a molecule (capacity of an allergen to induce an allergic reaction) including the protein's solubility, stability, size, and the compactness of the overall fold (27,33,35). These aspects reflect the dependency of the allergenicity on the transport through mucosal barriers and susceptibility to proteases (33). Size and solubility of the intact protein are especially relevant factors for airborne allergens, whereas digestibility is an important factor to consider for food allergens (33). In addition, allergens have no characteristic structural features other than they need to be able to reach immune cells and mast cells (34). Many allergens are small, single-domain structures; however, complex multifold allergens do also exist, particularly among allergens that do not have to pass the airway mucosal barrier (e.g., venom phospholipase A) (33). Within this constraint, any antigen may be allergenic (34). Moreover, IgE immunogenicity is, to a very large degree, determined by factors other than the protein itself (34). In order of an allergen to induce sensitization in susceptible individuals, the allergen needs to be encountered in sufficient quantities and via a relevant route of exposure (as a food, as an airborne protein, or as an injected protein) (27,34). Depending on the route of exposure to the allergen (i.e., inhalation, ingestion, or skin contact), allergy can affect different organs like upper and lower respiratory tract, eyes, intestinal tract, and the skin, and may have systemic manifestations (19,28,36).

Respiratory allergen sources

Allergic respiratory diseases such as asthma or rhinitis affect approximately 20% of the population in industrialized countries and a dramatic increase in their prevalence has been observed during the last decades (18,37). It is suggested that this increase in prevalence is partially caused by the effects of air pollution and global warming (37,38). Damage to airway mucous membranes and impaired mucociliary clearance caused by air pollution may facilitate access of inhaled allergens to the cells of the immune system, thus promoting sensitization of the airway (37,38). Consequently, a more severe IgE-mediated response to aeroallergens and airway inflammation could account for the increasing prevalence of allergic respiratory

diseases in polluted urban areas (37,38). In addition, the effects of climate change and global warming on respiratory allergy are still unclear, but it is expected that it will affect the start, duration, and intensity of the pollen season on the one hand, and the rate of asthma exacerbations due to respiratory infections and/or cold air inhalation on the other hand (37,38).

Altogether, fungi are one of the three main causes of respiratory allergy along with mites and pollen, affecting millions of people worldwide (4). However, in contrast to various other respiratory allergen sources, fungal exposure occurs universally, outdoors, indoors, and in occupational settings, and is impossible to avoid completely (39,40). In addition, fungal exposure is very complex, which makes it difficult to systematically evaluate the relationship between airborne fungi and adverse health effects (39,41). Among allergenic fungi, species of the genera *Cladosporium* and *Alternaria* are the most prevalent outdoor molds, while *Aspergillus* and *Penicillium species* have been detected in high abundance in the indoor environment, followed by *Chaetomium, Ulocladium* and *Stachybotrys species* (17,23,41–43). Even though the indoor environment is an important source of health risk factors, particularly considering that most people spend more than 90% of their time indoors, the contribution of indoor fungal exposure to allergic diseases is still not completely understood (15,19).

Moreover, of all the various kinds of biologic particulate matter suspended in the atmosphere all over the world, fungal spores constitute the largest portion and frequently exceed pollen concentrations by 100-1,000-fold with a common concentration of 200-10^6 spores/m^3 (18,44). Furthermore, a substantial proportion of spores are small enough (<2-250 µm) to penetrate into the lower airways and may colonize the human body and further damage the airways by the production of toxins, proteases, enzymes, and volatile organic compounds (10,36). Thus, fungi can have a far greater impact on the patients' immune system than other respiratory allergen sources (10).

1.1.3. Fungi and their role as important allergen sources

Already at the beginning of the 18th century, exposure to a wide range of fungal spores and fragments was recognized as a potential cause of adverse respiratory symptoms (36). Nevertheless, the association between exposure to fungi and the occurrence of allergic

symptoms has been discussed controversially for a long time, and fungi are still a neglected allergen source (36,45). Nowadays, data from several epidemiological studies provide evidence for the important role of fungi in respiratory disease (36,45).

Prevalence

Fungal allergy is a common worldwide health problem as sensitization to fungi is playing a major role in the development, persistence, and severity of lower airway diseases (46,47). The prevalence of fungal allergy is estimated to range from 3% to 10% in the general population and up to 44% of atopics and 80% of asthmatics are sensitized to at least one fungal species (10,36). However, the precise prevalence of fungal sensitivity remains unclear, due to the high variability between different studies (36,45,46).

Clinical manifestations

Clinically, the IgE-mediated sensitization to respiratory fungal allergens can manifest as allergic rhinitis and rhinosinusitis, and allergic asthma (10,21,28). Allergic rhinitis results in nasal itching, congestion, sneezing and clear rhinorrhea and causes extra-nasal adverse effects including decreased quality of life, decreased sleep quality and obstructive sleep apnoea (28). Moreover, fungal allergy is clearly linked to a subset of chronic rhinosinusitis (28). Furthermore, the main allergic disease caused by fungal sensitization is asthma (28,48). The associations made between fungal exposure, sensitization, and asthma have suggested that fungal spores, alongside other airborne allergen sources, have a causal role in asthma development and severity (49). This is reinforced by the fact that fungal sensitization is found more often in patients with severe or life-threatening asthma (36). However, with some exceptions, the contribution of fungal sensitization to the severity of allergic reactions and asthma largely remains to be investigated (28).

Allergenic fungi

During the last decades, DNA sequencing has resolved 8 phyla of fungi of which the phyla Ascomycota, Basidiomycota and Zygomycota contain the most fungal genera associated with respiratory allergy (6,28,36). Among the 100,000 fungal species described so far, about 80

mold genera have been shown to induce type I allergic reactions in atopic individuals (10,15,28). However, epidemiological, environmental, and clinical research focused only on a limited number of genera and only a few species have been studied in depth (see **Figure 3**) (10,50).

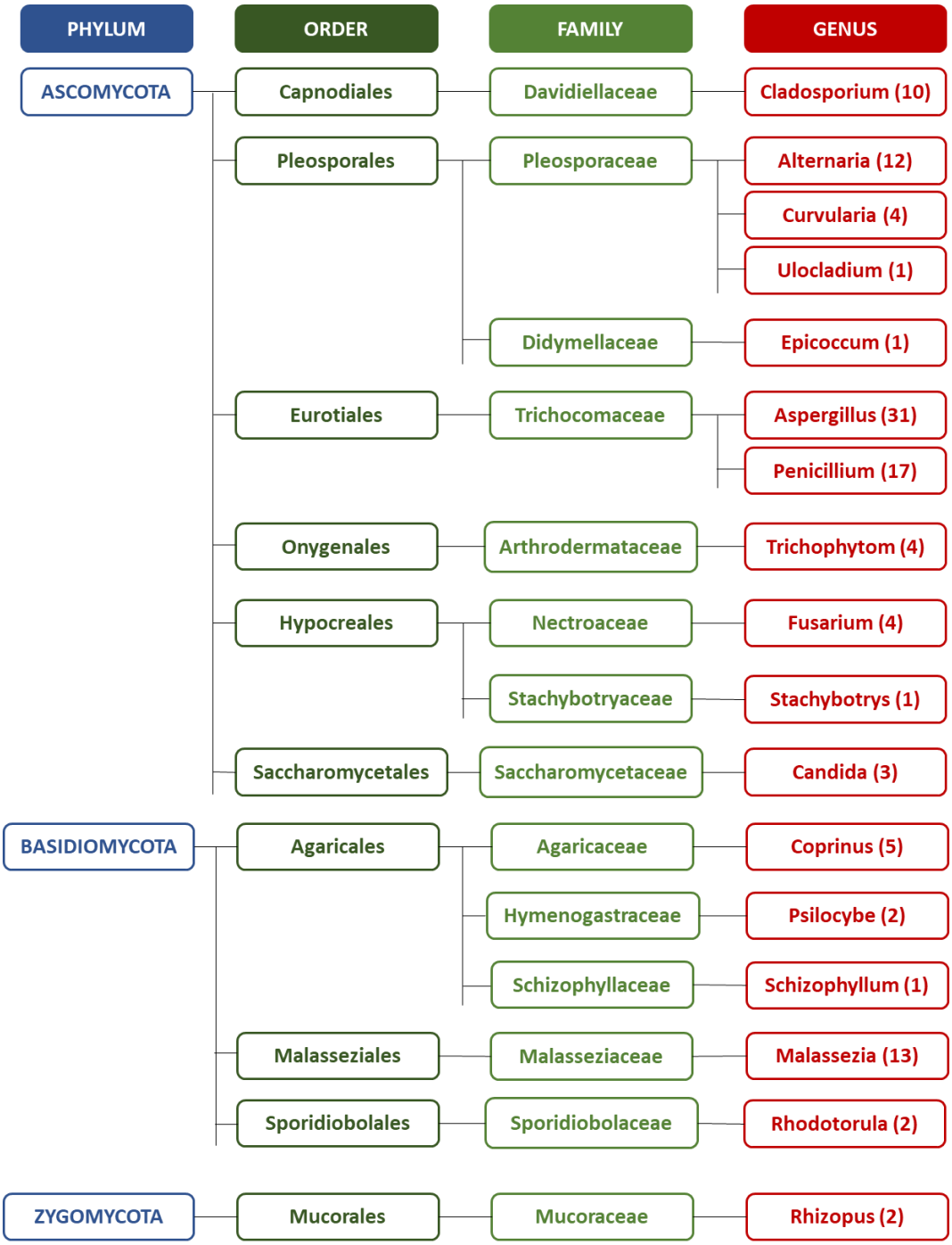

Figure 3. Fungal species that have been identified as a cause of type I allergy. The taxonomical tree includes all allergen producing fungal species with the corresponding number of allergens stated in brackets that have been listed by the Allergen Nomenclature Sub-committee of the International Union of Immunological Societies (IUIS) (51).

Among them, *Alternaria*, *Cladosporium*, *Aspergillus* and *Penicillium* are classically considered the most important fungal genera that cause allergic diseases (14,24,28,36,47,50).

Alternaria spp.

The genus *Alternaria* contains more than 270 species many of which are important plant pathogens and saprotrophs that occur worldwide (6). Furthermore, *Alternaria* spores are one of the most common spore types detected in the outdoor environment; however, these fungi are also commonly found indoors, especially growing on damp building materials (6,52). Among them, spores from *Alternaria alternata* are considered one of the most abundant and potent sources of airborne sensitizing allergens (10,52). A study showed that among a fungal-sensitized population, more than 60% reacted to *A. alternata* (50). In addition, a strong association between *A. alternata* sensitization and asthma severity was demonstrated (10).

To date, multiple allergens from *A. alternata* have been characterized including proteins that are restricted to a small number of fungal species that are taxonomically related to *A. alternata* as well as ubiquitous proteins that are conserved in different species (6,52). Alt a 1 represents the species' major allergen that is recognized by 80% of *A. alternata* allergic patients (5,53). Study results showed that Alt a 1 must be considered as a species- and family-specific allergen as it occurs in other species of the Pleosporaceae family and can thus be regarded as a marker for sensitization to this family (10). The enolase Alt a 6, in contrast, represents a minor allergen as approximately 15-22% of patients who are allergic to *A. alternata* show IgE-reactivity to Alt a 6 (53–55). However, this protein is of high diagnostic value as several authors demonstrated that Alt a 6-homologous allergens occur in distantly related species and might contribute to the phenomenon of cross-reactivity among several fungi and among species that belong to different phyla (54).

Cross-reactivity of *A. alternata* with other airborne fungal species has been extensively described and there is evidence that a significantly high percentage of patients sensitized to *A. alternata* are polysensitized to more than one other fungal species and might also be sensitized to other environmental aeroallergen sources such as pollens, mites, or even to food allergens (50,56).

Cladosporium spp.

Cladosporium contains more than 750 species that are frequently isolated from soil, food, paint, textiles, and other organic matters (6,57). The spores represent the most common fungal component isolated from outdoor air (6,57) and are well-adapted to be spread easily (10,55,57). *Cladosporium* species are known to produce several allergens that severely affect sensitized patients (6). Among them, *Cladosporium herbarum* represents one of the most important and prominent fungal allergen sources (10,55). The majority of the species' allergens identified so far are intracellular proteins with basic cellular functions with Cla h 1 and Cla h 2 being the most important allergenic molecules (6,55). Moreover, studies showed that the species' enolase, designated Cla h 6, represents another important allergen as 22% of *C. herbarum* sensitized patients reacted with the allergen (55).

Aspergillus spp.

The saprophytic genus *Aspergillus* includes more than 250 different species that are distributed ubiquitously in our environment (6). Furthermore, it includes a variety of important pathogens that are dominantly detected in the indoor environment (10). Of these, *Aspergillus fumigatus* is the most frequently encountered and virulent of all opportunistic fungal pathogens (6). Allergic sensitization to *A. fumigatus* is associated with severe persistent adult asthma allergic bronchopulmonary aspergillosis (6,58). The allergenic potential of this species has been thoroughly studied and 31 allergens from *A. fumigatus* have been identified and characterized (58). The species' major allergen Asp f 1 is considered, besides Alt a 1, to be the main cause of fungal sensitization (58). In addition, Asp f 1 also represents a species-specific allergen, which can be applied as a highly specific marker for IgE sensitization against the fungus (59).

Penicillium spp.

Members of *Penicillium* are among the most commonly occurring, economically important, and taxonomically difficult of all fungi (6). *Penicillium* includes more than 150 species that are frequent contaminants of foods and colonizers of damp building environments (6,10). Moreover, *Penicillium* is one of the most common airborne allergenic fungi that are often detected in the indoor environment (6). To date, 17 *Penicillium* allergens have been

characterized and are officially listed by the Allergen Nomenclature Sub-committee of the International Union of Immunological Societies (IUIS) (see **Figure 3**) (6).

Important cross-reactive fungal allergens

In general, patients are rarely sensitized to just one mold species, but in most cases to several ones (55,60). One reason for this might be the presence of conserved cross-reactive allergens and recently it was shown that IgE sensitization to fungal species reflected well their phylogenetic relationship since IgE-reactivity correlated better in closely related species compared to phylogenetically distant species (6,36,55).

Filamentous fungi contain an average of 10,000 genes and it is estimated that approximately 0.5-1.0% of proteins in each fungal proteome may be allergens (24). From a phylogenetical point of view, they can be subdivided into two classes: species-specific and cross-reactive allergens (60). Genome-wide studies of several fungal species revealed that fungi share a highly conserved set of allergen orthologues (proteins from genes in different species that evolved from a common ancestral gene; normally, orthologues retain the same function) (7,24). These appear to occur as functional groups such as proteins involved in proteolysis (vacuolar serine, acid, alkaline, aspartyl, and metalloproteases), proteins that potentially are involved in stress responses (peroxisomal membrane protein, thioredoxin, glutathione reductases, manganese superoxide dismutase etc.), proteins involved in protein synthesis and regulation (cyclophilins, heat shock proteins, acid ribosomal proteins P1 and P2), carbohydrate-metabolizing enzymes (enolase, alcohol and aldehyde dehydrogenases, glycosidases, and several multifunctional proteins of the Krebs and Emden-Meyerhof pathways), and a number of proteins with miscellaneous functions such as ribotoxins and nuclear factor 2 (7,24). These intracellular proteins seem to be highly homologous and widespread throughout the fungal kingdom (7). However, as previously mentioned, a few key allergens such as Asp f 1 (ribotoxin-like protein) and Alt a 1 (unknown function) appear to be highly specific to only a few species (7).

Allergens involved in proteolysis

Proteolytic enzymes play important roles in the fungal metabolism (7). Many of them are secreted in the surrounding environment, where they break down different substrates (7).

Examples of fungal proteases that are important allergens include vacuolar serine proteases (Asp f 18, Pen c 2, Pen c 18, Rho m 2), alkaline serine proteases (Asp f 13, Pen b 13, Tri r 2, Cur l 1, Epi p 1, Pen c 1, Tri r 4), metalloproteinase (Asp f 5), and aspartic protease (Asp f 10) (7).

Allergens potentially involved in stress responses

Peroxisomes are subcellular oxidative organelles, which are found in all eukaryotic cells and play key roles in lipid metabolism, antiviral defense, energy homeostasis and in the conversion of reactive oxygen species as response to oxidative stress (7). Peroxisomal membrane proteins identified as allergens include Asp f 3, Pen c 3, and Mal f 2 and Mal f 3 (7). Moreover, other fungal proteins that could be included in this group are manganese superoxide dismutase, thioredoxins, flavodoxins, catalase, and gluthathione S-reductase cascades, which could also all be involved in stress responses (7).

Allergens involved in protein synthesis and regulation

Heat shock proteins (HSPs) are highly conserved and very abundant proteins found in virtually all organisms (7). Different HSPs play important roles in the folding and unfolding of proteins, the assembly of multiprotein complexes, protein transport in the cell, cell-cycle control and signaling, and protection against stress and apoptosis (7). Moreover, HSPs interact with a wide variety of proteins including transcription factors and thus influence a wide variety of physiological processes (7). Examples of fungal HSPs identified as allergens include Alt a 3, Pen c 19, Mal s 10, Clad a 12 and Asp f 12 (7). Furthermore, acid ribosomal proteins P1 and P2 are unique phosphoproteins that are highly conserved in all eukaryotes and are involved in the regulation of transcription and DNA repair (7). Examples of acidic ribosomal proteins that are allergens from fungi include Alt a 12, Clad h 12, Pen b 26, Sac c P1, Alt a 5, Cla h 5, Asp f 8, Sac c P2 and Fus c 1 (7). In addition, cyclophilins are ubiquitous, multifunctional proteins that are present in all subcellular compartments and are important for protein trafficking and maturation, receptor complex stabilization, apoptosis, receptor signaling, and RNA processing (7). Examples of cyclophilins as allergens include Asp f 11, Asp f 27, Mal s 6, Psi c 2 (7).

Allergens involved in the carbohydrate metabolism

Several studies have demonstrated substantial IgE-reactivity to fungal glycosidase enzymes (7). A number of common nearly ubiquitous enzymes involved in carbohydrate metabolism have been identified as allergens from fungi (7). These proteins are abundantly expressed in many organisms and include enolase, alcohol dehydrogenase, mannitol dehydrogenase, aldehyde dehydrogenase, malate dehydrogenase, and aldolase (7). Alpha-enolase is a key enzyme in the metabolism of glucose and is therefore a constituent likely common to all fungi that further is recognized as an allergen in various species (e.g., Alt a 6, Cla h 6, Asp f 22, Can a E, Cur l 2, Pen c 22, Sac c E and Rho m 1) (7). It is most likely that other fungal enolases are also allergenic, making enolase a pan-allergen (7).

1.1.4. Fungal allergy diagnosis – an unsolved medical need

The first and most important step towards efficient allergen-specific forms of treatment is the proper diagnosis of allergy (36). Diagnosis of allergies, as routinely performed today, is a stepwise process, including anamnesis, determination of total and allergen-specific IgE antibodies, skin tests and, if necessary, other provocation tests (36). Any *in vivo* as well as any *in vitro* diagnosis of allergy depends on the quality of the material used for testing (28). In contrast to pollens or insect venoms, fungi as allergenic sources are extremely complex, and problems related to reliable diagnosis of fungal allergies are far from being solved (28).

Challenges in fungal allergy diagnosis

The discrepancy of diagnostic tests, commonly encountered in clinical practice, can partly be ascribed to the usage of fungal extracts of poor quality and high variability (36). Besides intrinsic factors, like strain variabilities and the tendency of fungi for spontaneous mutation, the fungal raw material (spores/mycelium) and manufacturing processes (culturing conditions, extracting procedures) affect the quality of fungal allergen extracts (36,61). As fungi are enzyme-rich organisms, degradation processes should also not be underestimated (36). All these factors may have an impact on the presence of certain allergens, protein/carbohydrate content, and allergenicity and antigenicity of fungal extracts (36).

Especially, the impact of the cultivation conditions on the fungal allergen expression has been shown (62–66).

Moreover, until now there are no generally accepted guidelines for the preparation of allergenic fungal extracts (36). It is therefore not astonishing that considerable differences in potency of fungal extracts between manufacturers and even batch-to-batch variations can be found (28,36). Due to the problems with standardization of fungal extracts, only a limited number of them are available for diagnosis (36). In addition, as already mentioned before, it is estimated that only a small proportion of allergenic fungal species have been identified so far, which has certainly contributed to the fact that fungal allergy has been underestimated for a long time (36).

Besides the inconsistency of allergen extracts, the presence of carbohydrates and other cross-reactive components hampers the precise identification of the disease eliciting fungal species (36). Cross-reactive allergens from related and non-related allergen sources distract from the primary sensitizing source (36). Especially in case of polysensitized patients, diagnosis based on extracts allows no discrimination between co- and cross-sensitization (36). The need for an improved component-resolved diagnosis is therefore evident, and it is clear that improvement can only come from the use of recombinant fungal allergens (36).

Component-resolved diagnosis

The rapid progress achieved in the fields of protein analysis and allergen cloning offers new opportunities for improved standardization of allergen preparations (67). Starting from the encoding cDNAs, the corresponding recombinant allergen can be biotechnologically expressed in heterologous hosts at laboratory scale up to industrial production and easily purified to homogeneity (67). If the recombinant allergens possess similar characteristics as their natural counterparts, they are suited for *in vitro* and *in vivo* diagnosis (36). Although cloning and characterization of single allergens may be time-consuming, the advantages are manifold (67). Problems related to contamination of the preparation with IgE-binding natural components, altered allergenicity due to extraction procedures and variability on allergen content can be circumvented (67). Moreover, the same allergen preparation can be used for both *in vitro* and *in vivo* tests allowing a direct comparison of the diagnostic results obtained

with different methods (67). In addition, the availability of the allergen repertoire in the form of single standardized recombinant components will allow to determine individual reactivity patterns for each single subject tested (67). In this way, sensitivity as well as specificity of diagnosis can be improved (36). Moreover, this component-resolved diagnosis opens new perspectives for the preparation of patient-tailored mixtures to be used in specific immunotherapy, thus also improving allergy treatment (67).

In conclusion, the future of the molecular diagnosis of fungal allergy is moving in two main directions: (a) the identification of new fungal allergens, their structure and biological activities, including markers of both primary sensitization and cross-reactivity; and (b) the establishment of the relationship between each allergen and the expression of the different clinical profiles that occurs in mold allergy (10,28,32,36).

1.2. Research questions

The list of fungal allergens officially approved by the Nomenclature Sub-committee of the IUIS includes 123 iso-allergens and variants from several fungal species (November 2021) (51). However, the number of fungal proteins able to elicit type I hypersensitivity reactions described in the literature is much longer, even though many of them are poorly characterized (10,28,36). Due to this still insufficient characterization of fungal allergens and the poor quality of test solutions used for allergy diagnosis, fungal allergies are often underdiagnosed (36).

It is known that fungi can occupy and inhabit nearly any suitable ecological niches, which makes them unique respiratory allergen sources that occur universally, outdoors, indoors, and in occupational settings (11). Thus fungal exposure is impossible to avoid completely, and even though the indoor environment is an important source of health risk factors, particularly considering that most people spend more than 90% of their time indoors, the contribution of indoor fungal exposure to allergic diseases is still not clear (15,19). Therefore, the focus of this thesis was on indoor fungi and the aim was to identify and characterize allergens from fungal species that are frequently detected in indoor environments. Expanding the knowledge about fungal species and fungal proteins able to elicit allergic diseases, helps to improve the sensitivity and specificity of allergy diagnosis and consequently will also improve allergy treatment strategies. To further improve allergy diagnosis, the cross-reactive potential of the pan-allergen enolase was analyzed. In addition, the impact of different environmental conditions on the expression of fungal allergens was studied, which helps to gain knowledge about fungal allergen expression to optimize fungal cultivation to produce highly potent fungal extracts. This also facilitates the analysis of the allergenic potential of fungal species.

<u>The project aimed to answer the following research questions:</u>

- Which of the fungal species, commonly detected in the indoor environment, are sources of allergens and are recognized by IgE-antibodies from mold-allergic patients?
- Which specific proteins, expressed by allergenic fungi, represent allergenic molecules?
- Do these allergens show relevant cross-reactivity to already known allergens?
- Which fungal cultivation conditions (e.g., temperature, time, medium composition) have an impact on the allergen production?

1.3. Methods

The following section provides a detailed description of the overall experimental design of the research.

1.3.1. Patient sera

Sera from polysensitized patients were obtained from the Department of Allergy, La Paz, University Hospital, Madrid, Spain, from the Hospital Clinic de Barcelona, Immunology Department, CDB, IDIBAPS, University of Barcelona, Spain and from the company AbBaltis (FDA-approved; Sittingbourne, United Kingdom). All patients agreed to participate in this study and gave informed consent. This study was approved by the ethics committee of the Hospital Clínic de Barcelone (approval number 2011/6605) and of the La Paz University Hospital in Madrid (EK565/2007). We confirm that all methods were performed in accordance with the relevance guidelines and regulations. Details concerning the demographic and serological characteristics of the patients are given in the respective manuscript.

1.3.2. Fungal cultivation

For the preparation of fungal protein extracts and RNA extracts, clones of *Alternaria alternata*, *Ulocladium chartarum*, *Paecilomyces variotii*, *Aspergillus fumigatus*, *Cladosporium herbarum* and *Penicillium chrysogenum* were cultured on malt extract agar-plates (= MEA, pH 5.0) containing 2% malt extract, 2% glucose and 1.5% agar-agar at 20-25°C for 7 to 14 days.

To determine the impact of carbon source, light exposure, cultivation temperature and cultivation time on the expression of the homologous major allergens Alt a 1 and Ulo c 1 as well as the homologous minor allergens Alt a 6 and Ulo c 6, clones of *A. alternata* and *U. chartarum* were cultured under varying conditions. In addition, the impact of the cultivation time on the expression of allergenic fungal enolases was evaluated for the species *A. fumigatus, C. herbarum* and *P. variotii*. An overview of the different cultivation conditions tested can be found in **Table 1**.

Table 1. Different growth conditions for fungal cultivation.

ANALYZED CONDITION	MEDIUM	LIGHT EXPOSURE	CULTIVATION TEMPERATURE	CULTIVATION TIME
Carbon Source	Glucose-based medium: 6.8% yeast nitrogen base, 2% glucose, 5% peptone from casein, 15% agar-agar, pH 6.0 Cellulose-based medium: 6.8% yeast nitrogen base, 2% cellulose, 5% peptone from casein, 15% agar-agar, pH 6.0 Methylcellulose-based medium: 6.8% yeast nitrogen base, 2% methylcellulose, 5% peptone from casein, 15% agar-agar, pH 6.0	Dark	20-25°C	10 days 15 days 20 days 25 days 30 days 35 days 40 days
Light Exposure	Malt Extract Agar	Dark or natural Light/Dark Rhythm	20-25°C	5 days 10 days 15 days 20 days
Temperature	Malt Extract Agar	Dark	20-25°C 30°C 37°C	5 days 10 days 15 days 20 days
Cultivation Time	Malt Extract Agar	Dark	20-25°C	5 days 10 days 20 days 30 days

1.3.3. Preparation of protein extracts

Protein extracts from different fungal species and from various fish and meat as well as from plant pollen were prepared and analyzed during this study.

Fungal protein extracts

Crude fungal protein extracts were prepared in two different ways from fungal mycelium and spores that were cultivated as described before. Fungal material was harvested from the agar-plates using a sterile scalpel and either ground in liquid nitrogen to a fine powder using a pre-cooled mortar and pestle or transferred into sterile MK28 2 mL Precellys tubes (Bertin Technologies SAS; Nontigny-le-Bretonneux, France). The ground powder was resuspended in fungal extraction buffer containing 50 mM $NaHCO_3$, 150 mM NaCl, 2 mM

phenylmethanesulforyl fluoride, 2 mM ethylene diamine tetra-acetic acid (EDTA) and complete EDTA-free protease inhibitor tablets (Roche Diagnostics, Mannheim, Germany; used according to the manufacturer's instructions). In contrast, phosphate buffered-saline (PBS) containing complete EDTA-free protease inhibitor tablets (Roche Diagnostics; used according to the manufacturer's instructions) was added to the samples in the MK28 2 mL Precellys tubes and the material was lysed, using the Precellys Evolution Tissue Homogenizer (Bertin Technologies SAS), by applying three times 4,500 rpm for 20 seconds with 30 seconds of pause between each round. After an overnight incubation of all samples at 4°C under continuous shaking, the samples were centrifuged at 4,000 g and 4°C for 10 minutes and the supernatants were stored at –20°C. The protein concentrations of the extracts were determined using the method of Bradford with bovine serum albumin (BSA) as a standard (68).

Food and pollen protein extracts

Protein extracts from various fish and meat including chicken leg, chicken breast, salmon, tuna, trout, and shrimp were prepared by cutting the flesh into small pieces, which were either boiled for 10 minutes in water or processed in raw form. The material was then ground to a fine powder under liquid nitrogen using a pre-cooled mortar and pestle and transferred into a sterile centrifugal tube, followed by the addition of 5 mL PBS per g meat. After an overnight incubation at 4°C under continuous shaking, the extracts were centrifuged at 4,000 g and 4°C for 30 minutes and the supernatants were filtered through a filter with a pore-size of 0.45 µm (Sarstedt AG & Co. KG, Nümbrecht, Germany). The filtrates were stored at -20°C and the protein concentrations of the extracts were determined as described before.

For the preparation of protein extracts from plant pollen, 10 mL of ultrapure water or PBS was added to 1 g of pollen and incubated overnight at 4°C under continuous shaking. Afterwards, the samples were centrifuged at 4,000 g and 4°C for 10 minutes and the supernatants were filtered using a 0.45 µm pore-size (Sarstedt AG & Co. KG). The filtrates were stored at -20°C and the protein concentrations of the extracts were determined as described before.

1.3.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, protein staining, immunoblot and inhibition immunoblot experiments

Two µg of recombinant proteins, or 1-10 µg of protein extracts were separated by 8%, 10%, 12% or 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGEs) as described by Laemmli (69). Proteins were then either stained with Coomassie-Brilliant Blue R250 (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) or using the Pierce Silver Stain Kit (Thermo Fisher Scientific, Waltham, MA) or transferred electrophoretically onto nitrocellulose membranes (0.2 µm; GE Healthcare, Chicago, IL) (70).

Detection with allergen-specific antibodies and with an anti-histidine tag antibody

To detect Alt a 1, Alt a 6 and related proteins, membranes were washed and blocked with PBS containing 0.5% Tween20 (PBS-T) and exposed to a rabbit antiserum either directed against a peptide of Alt a 1 (5'-KISEFYGRKPEGTYYNSLG-3') or against the recombinant enolase rAlt a 6 (Charles River Laboratories, Écully, France) (1:5,000 diluted in PBS-T). For inhibition immunoblots, the antisera were pre-incubated overnight with 20 µg of the respective recombinant allergen or, for control purposes, with PBS-T and then added to the proteins blotted onto nitrocellulose membranes. To detect the protein-bound antibodies, a goat anti-rabbit IgG antibody (Vector Laboratories Inc, Burlingame, CA; 1:10,000 diluted in PBS-T), conjugated to horseradish peroxidase (HRP), was used as detection antibody. The blots were developed using the SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific) according to the manufacturer's instructions and imaged using the UVP ChemStudio device (Analytik Jena, Jena, Germany).

For the detection of protein-bound histidine tags, nitrocellulose-blotted recombinant proteins were exposed to a monoclonal mouse anti-histidine tag antibody (Bio-Rad, Hercules, CA; 1:1,000 diluted in PBS-T) and a HRP-conjugated goat anti-mouse IgG secondary antibody (GE Healthcare; 1:10,000 diluted in PBS-T). The blots were then developed as described before.

IgE immunoblot and inhibition immunoblot experiments

For IgE-immunoblots, membranes were washed and blocked with PBS-T and exposed either to individual sera or to pools of sera from polysensitized patients. These pools contained either 2, 3, 5, 6, 7,9, 10 or 16 sera (1:10 diluted in PBS-T), resulting in dilutions of each serum of 1:20, 1:30, 1:50, 1:60, 1:70, 1:90, 1:100 or 1:130. For IgE inhibition immunoblots, individual sera or serum pools were pre-incubated with 20 µg of the respective recombinant protein or, for control purposes, with PBS-T and then added to the blotted proteins. For the detection of protein-bound human IgEs, a mouse anti-human IgE antibody, conjugated to HRP, was used (Southern Biotech, Birmingham, AL; 1:5,000 diluted in PBS-T). The blots were developed and imaged as described above.

An immunodetection control was included for all immunoblotting experiments, where the blotted proteins were incubated with the detection antibody only, followed by blot development and imaging as described before.

1.3.5. Liquid chromatography-mass spectrometric (LC-MS) analysis of proteins

Proteins recognized by human IgEs were excised manually from Coomassie Blue-stained SDS-PAGE gels and in-gel digestion was performed with 20 ng/µL trypsin (Promega, Madison, WI). Extracted peptides were then dried down in a vacuum concentrator and afterwards resuspended in 0.1% trifluoroacetic acid and injected into a nano-HPLC Ultimate 3000 RSLC system (Dionex from Thermo Fisher Scientific) and separated on a 25 cm Acclaim PepMap C18 column (Thermo Fisher Scientific).

For the mass spectrometric analyses, the nano-HPLC was directly coupled to a high-resolution quadrupole time of flight mass spectrometer (Triple TOF 5600 from Sciex, Framingham, MA) or to the high-resolution Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific). The database search was then performed using either the Software Protein Pilot (Sciex) or the Proteome Discoverer Software 2.2.0.388 or 2.4.0.305 (Thermo Fisher Scientific).

1.3.6. Fungal RNA extraction and cDNA cloning of fungal allergens into bacterial expression vector

For the recombinant expression of allergens, cDNAs coding for the proteins first had to be generated from RNA and then cloned into a bacterial expression vector.

RNA extraction

Total fungal RNA was extracted from fungal mycelium and spores as described for the preparation of protein extracts using either a mortar, pestle and liquid nitrogen or the Precellys Evolution Tissue Homogenizer (Bertin Technologies SAS). During this thesis the fungal RNA extraction procedure was optimized to receive high amounts of pure fungal RNA. Thus, the experimental set-up changed over the course of this thesis. First, RNA was purified following the method by Rio et al. (71) using TriReagent (Sigma-Aldrich, St. Louis, MO), followed by a second purification step. with the GeneJET RNA Cleanup and Concentration MicroKit (Thermo Fisher Scientific). As this procedure did not always result in sufficient amounts of extracted RNA of good quality, the innuSPEED Bacteria/Fungi RNA Kit form Analytik Jena was afterwards used for fungal RNA purification, following a second purification step using again the GeneJET RNA Cleanup and Concentration Micro Kit from Thermo Fisher Scientific. Lastly, the set-up was further optimized by purifying fungal RNA with the NucleoSpin RNA Plant and Fungi Kit (Macherey Nagel, Düren, Germany) and with the GeneJET RNA Cleanup and Concentration MicroKit.

cDNA synthesis

In general, cDNAs encoding fungal proteins were generated by reverse transcription polymerase chain reactions (RT-PCR) with the Access RT-PCR System from Promega. In case of *U. chartarum* proteins Ulo c 1 and Ulo c 2, information on the full-length sequences of the coding cDNAs first had to be obtained by 5'/3'-rapid amplification of cDNA ends (RACE) using the SMARTer RACE 5'/3' Kit (Takara Bio, Kyoto, Japan). Therefore, primers were designed based on the homologous *A. alternata* cDNA sequences of Alt a 1 (NCBI accession number: AY568627) and of the formate dehydrogenase (NCBI accession number: XM_018525579.1). To obtain the 5'- and 3'-end of the Ulo c 1 cDNA the following primers were used: 5'-AACGCATCCTGCCCTGTCACTACCGA-3' (for 3'-end amplification) and 5'-

GCTGTTCTCGCCGCAAGAGTACCAC-3’ (for 5’-end amplification). For the Ulo c 2 cDNA the following primers were used: 5’-GGAAAAGTTCTTCTCGTCCTCTACG-3’ (for 3’-end amplification) and 5’-CTTGCGCTGACCGTACGCCTTGG-3’ (for 5’-end amplification). Primers for full-length cDNA cloning of Ulo c 1 and Ulo c 2 by RT-PCR were then designed based on the sequences of the RACE-PCR-products. Since enolases are very conserved proteins, the cDNA coding for Ulo c 6 could be obtained by RT-PCR using forward and reverse primers designed based on the nucleotide sequence from the homologous *A. alternata* enolase Alt a 6 (NCBI accession number: U82437.2). Primers for the synthesis of cDNAs coding for *P. variotii* proteins were synthesized based on whole genome shotgun sequences that had been submitted to GenBank with the following accession numbers: GAD95401 (Pae v 1), GAD92095.1 (Pae v 2) and GAD96380 (Pae v 6). Primers for the different enolases’ cDNAs were designed based on the nucleotide sequence from the corresponding species’ enolase with the following NCBI accession numbers: AF284645.1 (Asp f 22), X78226.1 (Cla h 6) and AB091508.1 (Pen c 6). All primers used in this study to generate full-length cDNAs coding for the respective proteins are listed in **Table 2** (Microsynth, Balgach, Switzerland). Forward primers always introduced a recognition site for the restriction enzyme *Nde*I, whereas reverse primers added the coding sequence of a C-terminal hexa-histidine tag and the recognition site for the restriction enzyme *EcoR*I or *Xho*I.

Table 2. Sequences of the specific primers used for cDNA-cloning. Restriction sites of *Nde*I are shown in italics and sites of *EcoR*I and *Xho*I are underlined. In addition, the sequences encoding the C-terminal hexa-histidine tags are marked in bold.

Primer	Nucleotide sequence in 5'-3'-end orientation
Ulo c 1 Forward	GGTGGT*CATATG*GCTCCCCTCGAGTCCCGC
Ulo c 1 Reverse	GGTGGTGAATTCCGGTCA**GTCATGATGATGATGATG**CTCGTCATCGGGAAGGGT
Ulo c 2 Forward	GGTGGT*CATATG*GGAAAAGTTCTTCTCGTCCTCTACG
Ulo c 2 Reverse	GGTGGTGAATTCTTA**GTGATGATGATGATGATG**CTTGCGCTGACCGTACGCCTTGG TGGCGTAGTCTCCC
Ulo c 6 Forward	GGTGGT*CATATG*ACCATCACCAAGATCCACGCCCGC
Ulo c 6 Reverse	GGTGGTCTCGAGTTA**GTGATGATGATGATGATG**CAAGTTAACGGCAGTCCTGAA
Pae v 1 Forward	GGTGGTCATATGGCTTCCGCTCTCGACCAGCTC
Pae v 1 Reverse	GGTGGT*GAATTC*TTA**GTGATGATGATGATGATG**CGCGGTGATCTTCTGGCGCAG
Pae v 2 Forward	GGTGGTCATATGGTTAACGCTAAGATTGGTATC
Pae v 2 Reverse	GGTGGT*GAATTC*CTA**GTGATGATGATGATGATG**GGCGGAACCGGCGTCGACCTTG
Pae v 6 Forward	GGTGGT*CATATG*CCTATCACCAAGATCCACGCT
Pae v 6 Reverse	GGTGGTGAATTCCTA**GTGATGATGATGATGATG**CAGGTTAATGGAGTTGCGGAA
Asp f 22 Forward	GGTGGT*CATATG*CCTATCTCCAAGATCCACGCTCGTTCCG
Asp f 22 Reverse	GGTGGTGAATTCTTA**GTGATGATGATGATGAT**GCAGGTTGACGGCAGTGCGGAACTTGG
Cla h 6 Forward	GGTGGT*CATATG*CCTATCTCCAAGATCCACTCC
Cla h 6 Reverse	GGTGGTGAATTCTTA**GTGATGATGATGATGAT**GCAAGTTGATGGCAGTGCGGAA
Pen c 6 Forward	GGTGGT*CATATG*CCTATCTCTAAGATCCACGC
Pen c 6 Reverse	GGTGGTGAATTCTTA**GTGATGATGATGATGAT**GCAGGTTGACAGCAGTGCGG

The RT-PCR products were finally sub-cloned into the bacterial expression vector pET-17b (Novagen-Merck, Darmstadt, Germany) and nucleotide sequences were confirmed by Sanger Sequencing (Microsynth) using standard primers.

For control purposes, cDNA coding for Alt a 1 from *A. alternata* was also generated based on the sequence published by NCBI (accession number: AY568627) from *A. alternata* RNA by RT-PCR and was cloned with a C-terminal hexa-histidine tag into the bacterial expression vector pET-17b. For the enolase Alt a 6 from *A. alternata* (UniProt accession number: Q9HDT3), a plasmid containing the codon-optimized cDNA, for expression in *Escherichia coli*, in pET-17b was synthesized by GenScript Biotech (Piscataway Township, NJ) and used for recombinant protein expression.

In silico analysis of amino acid sequence homology

Deduced amino acid sequences of the generated cDNAs coding for the fungal proteins were obtained based on the *in silico* translation of the nucleotide sequences, using the open source Expasy translate software (72). To obtain information about sequence homologies the alignment software Clustal Omega (73) was used. For the detailed analysis of the homology between enolases from different sources, the deduced amino acid sequences of Pae v 6, Asp f 22, Cla h 6 and Pen c 6 were compared with the sequences of Alt a 6, of the enolases from *Oncorhynchus mykiss* (trout; NCBI GenBank accession number: OK349682[1]), from *Salmo salar* (salmon; UniProt accession number: B5DGQ7), from *Thunnus albacares* (tuna; UniProt accession number: I0J1J1), from *Gallus domesticus* (chicken; UniProt accession number: P07322), from *Phleum pratense* (timothy grass; UniProt accession number: K6Z748), from *Ambrosia artemisiifolia* (short ragweed; UniProt accession number: A0A1B2H9Q1) and of the human enolase (UniProt accession number: P06733). Resulting percentages of sequence identities were then used to create a sequence identity map.

1.3.7. Recombinant protein expression, extraction, and purification

Recombinant protein expression was carried out with the cDNAs coding for Ulo c 1, Ulo c 2, Ulo c 6, Alt a 1, Alt a 6, Pae v 1, Pae v 2, Pae v 6, Asp f 22, Cla h 6, and Pen c 6 that had been cloned into pET-17b, and for the enolase from rainbow trout (*Oncorhynchus mykiss*). The cDNA coding for the rainbow trout enolase had been generated based on the homologous sequence of the enolase from salmon, cloned into pET-17b and was already available in the laboratory. For the expression of the proteins, the generated cDNA encoding plasmids were transformed into *Escherichia coli* strain BL21-DE3 and recombinant protein expression was induced at 27°C (Ulo c 1, Ulo c 6, Alt a 1) or 37°C (Ulo c 2, Alt a 6, Pae v 1, Pae v 2, Pae v 6, Asp f 22, Cla h 6, Pen c 6, and recombinant trout enolase) by the addition of 5 mM isopropyl beta-D-1-thiogalactopyranoside.

[1] Nucleotide sequence has been submitted to the GenBank database.

Proteins were then extracted from the bacterial cells under native conditions, using the freeze-thaw lysis method described by Johnson et al. (74). Recombinant allergens were purified by immobilized metal ion affinity chromatography (IMAC) using Protino Nickel-Agarose (Macherey-Nagel). Proteins were eluted with a buffer containing 150-250 mM imidazole and were dialyzed against 10 mM sodium phosphate buffer (pH 7.0-8.0). The *U. chartarum* proteins rUlo c 1 and rUlo c 6 and the *P. variotii* protein rPae v 6 were further purified by IMAC using HisTrap HP columns (GE Healthcare) on the Äkta Start Protein Purification System (GE Healthcare). Proteins were eluted using a linear imidazole gradient (20-500 mM) and were again dialyzed against 10 mM sodium phosphate buffer (pH = 7.0-8.0).

The protein concentrations were then determined with the MicroBCA Protein Assay Kit (Thermo Fisher Scientific) using BSA as a standard and the purity of the proteins was assessed by SDS-PAGE followed by Coomassie-staining of the protein gels.

1.3.8. Secondary structure analysis using circular dichroism spectroscopy (CD-spectroscopy)

The secondary structure of purified rUlo c 1, rUlo c 2, rUlo c 6, rPae v 1, rPae v 2 and rPae v 6 was evaluated by circular dichroism spectroscopy on a Chirascan Plus spectrophotometer (Applied Photophysics, Leatherhead, United Kingdom) in 10 mM sodium phosphate buffer (pH 7.0-8.0) at 20°C and a protein concentration of 0.2 µg/µL using a quartz cuvette (Hellma Analytics, Müllheim, Germany) with a path-length of 1 mm. CD-spectra were recorded from 190 nm to 280 nm with a resolution of 0.5 nm and results were the average of three scans. The final spectra were corrected by subtracting the buffer baseline spectrum, obtained under identical conditions, and normalized to the number of peptide bonds by using the extinction coefficient of the measured protein at 205 nm (ε_{205}) (75). Results were expressed as mean residual ellipticity θ_{MRW} (deg cm² $dmol^{-1}$) at a given wavelength.

1.3.9. Enzyme-linked immunosorbent assay (ELISA) and inhibition ELISAs

The IgE-reactivity of recombinant allergens was analyzed by ELISAs performed with sera from mold-allergy and/or polysensitized patients. For this, 96-well nunc maxisorp ELISA plates (Thermo Fisher Scientific) were coated with 4 µg/mL of the recombinant proteins, diluted in

bicarbonate buffer (pH = 9.6). Following an overnight incubation at 4°C, the plates were washed and blocked with PBS-T for 2.5 hours at 37°C. Wells were then incubated overnight at 4°C with sera from polysensitized patients (1:5 diluted in PBS-T) or, for control purposes, with sera from non-allergic individuals (1:5 diluted in PBS-T) or, with PBS-T only. In case of IgE-inhibition ELISA experiments, patients' sera were pre-incubated overnight at 4°C with 20 µg of the respective recombinant protein before added to the wells. Bound IgE antibodies were detected with a HRP-labelled goat anti-human IgE antibody (SeraCare Life Sciences Inc., Milford, MA; 1:2,500 diluted in PBS-T) and using 1.8 mM of 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich) in 60 mM citric acid, 77 mM $Na_2HPO_4(H2O)_2$ and 3 mM H_2O_2 as a substrate. After 60 minutes of incubation in substrate solution, the optical density (OD) of the samples was measured at a wavelength of 405 nm using the Multiskan FC Photometer (Thermo Fisher Scientific). Experiments were always carried out in duplicates and the results are expressed as mean OD-values. The cut-off, used to differentiate positive and negative IgE-reactivity, was calculated from the mean OD-values plus two times the standard deviation of sera from three non-allergic patients.

To determine the levels of allergen-specific IgE antibodies in the sera, quantitative ELISAs were performed with human IgE-standards of different concentrations. For the generation of the standard curve, wells were coated with the major birch pollen rBet v 1, which was available in the laboratory, and incubated with defined concentrations (10, 5, 2.5, 1.25, 0.675, 0.338, 0.169 and 0 ng/mL) of an anti-Bet v 1 human IgE antibody (Absolute Antibody, Cleveland, United Kingdom). Protein-bound IgE antibodies were then detected with the HRP-labelled anti-human antibody. Based on the measurements obtained with the different anti-Bet v 1 IgE antibody levels, a standard curve was generated, which allowed calculating the allergen specific IgE antibody levels (in kU/L) in the sera.

1.3.10. Basophil activation tests (BATs)

To test the biological activity of recombinant molecules, basophil activation tests were performed using the Flow Cast Kit (Bühlmann, Schönenbuch, Switzerland) according to the manufacturers' instructions. Whole blood from mold-sensitized patients and, for control purposes, from two patients with no fungal sensitization, was incubated with increasing concentrations (1 to 1000 ng/mL) of the recombinant allergens. Basophil activation was

assessed by detecting the expression of CD63 by flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ). Activated basophils were gated as $SSC^{low}/CCR3^{+}/CD63^{+}$. At least 400 basophils were measured, and the percentage of CD63-expressing basophils, based on the total amount of basophils, was calculated.

1.3.11. Allergen Release Kinetics Experiments

Clones of *U. chartarum*, *A. alternata* and *P. variotii* were cultured on MEA-plates at 20-25°C for 7 days. Then, 10 mL of a buffer that mimics the natural nasal fluid (76) (160 mM NaCl, 30 mM KCl, 5 mM $MgCl_2(H_2O)_6$ and 2 mM $CaCl_2(H_2O)_2$) was added to the plates and spores were detached using a Drigalski-spatula. The spore solutions were transferred into centrifugation tubes and were incubated under continuous shaking at 20-25°C. Aliquots of 1 mL each were taken after 1, 5, 10, 20 and 60 minutes and were immediately centrifuged to separate the supernatants, containing the released proteins, from the fungal spores. The supernatants were stored at –20°C and the protein concentrations were determined using the method of Bradford with BSA as a standard (68). For the detection of released allergens, 0.1-0.5 µg of the supernatants were separated on 12% or 15% SDS-PAGEs, blotted onto nitrocellulose (as described before) or dotted onto nitrocellulose membranes and exposed to the antisera against Alt a 1 or rAlt a 6.

1.4. Contributions

Research that was carried out in course of this dissertation, was summarized in two published papers as well as in two unpublished manuscripts. An overview of these contributions is given in the following section.

1.4.1. Paper I

The main focus of this dissertation was to analyze the allergenic potential of fungal species that are commonly detected indoors. *Ulocladium chartarum* represents such a mold species, which is growing not only as a saprobe on plant material and in soil, but also on lumber, textiles, emulsion paint and paper (77–79). Furthermore, the species is frequently detected in the indoor environment of water-damaged buildings and is regarded as one of the indicators of water damage (77,78,80–85). Even though *U. chartarum* has been described as an opportunistic human pathogen (77,86,87), little is known about its allergenic potential and *U. chartarum* is not included in routine allergy diagnostic tests. The publication "Identification of *Ulocladium chartarum* as an important indoor allergen source" describes the verification of the allergenic potential of *U. chartarum* due to the identification and characterization of the species' first three allergens.

IgE immunoblots, performed with sera from patients sensitized to different molds, showed strong IgE-binding to several *U. chartarum* proteins, thus demonstrating the species' high allergenic potential. Three of these IgE-reactive proteins were identified by a proteomics-based approach and represent the first allergens described from *U. chartarum*. The proteins were identified as an Alt a 1-homologous protein, designated Ulo c 1, a formate dehydrogenase designated Ulo c 2 and an enolase designated Ulo c 6. cDNAs coding for the three newly identified allergens were then generated from *U. chartarum* RNA and cloned into a bacterial expression vector to produce recombinant proteins in *Escherichia coli*. Afterwards, the recombinant molecules' characteristics were analyzed by IgE immunoblots, ELISAs, CD-spectroscopy and basophil activation tests. ELISAs performed with sera from mold-allergic individuals proved that the three recombinant proteins represent IgE-reactive molecules. In addition, inhibition immunoblots showed that the recombinant allergens were able to specifically inhibit patients' IgE-binding to their natural counterparts, indicating that all the

molecules' IgE-binding epitopes are present in the recombinant proteins. Based on these results, it can be concluded that rUlo c 1, rUlo c 2 and rUlo c 6 could be used as tools for *in vitro* diagnosis of *U. chartarum* sensitization.

Previous studies have already suggested the expression of Alt a 1-homologous proteins in *Ulocladium* species (10,88–90). However, with the cDNA cloning and the production of an IgE-reactive molecule, this study provided evidence that an Alt a 1-homologous allergen indeed exists in *U. chartarum,* and inhibition immunoblots proved the IgE cross-reactivity between rUlo c 1 and rAlt a 1. Moreover, basophil activation tests showed comparable biological activity of rUlo c 1 and rAlt a 1.

In addition, analyzing the release of allergens from allergen sources can provide useful information about the potential exposure to these allergens. Thus, the release kinetics of the homologous allergens Ulo c 1 and Alt a 1 as well as Ulo c 6 and Alt a 6 from fungal spores with a buffer that mimicked the natural human nasal fluid was analyzed. The results indicated that the allergens are immediately released from the spores upon contact with nasal buffer. These findings suggest that rapid allergen elution might also occur when spores reach mucosal surfaces, where they can then elicit allergic reactions.

In conclusion, during this study the first allergens from *U. chartarum*, designated Ulo c 1, Ulo c 2 and Ulo c 6, have been identified, recombinantly produced and characterized. It was shown that all three molecules are IgE-reactive, with Ulo c 1 representing a major allergen and Ulo c 2 and Ulo c 6 being minor allergens of this species. The recombinant allergens produced in this study expand the repertoire of fungal allergens available for fungal allergy diagnosis, thus improving diagnosis' specificity and sensitivity.

1.4.2. Paper II

Besides the verification of the allergenic potential of the mold *U. chartarum*, the allergenic potential of the thermo-tolerant mold *Paecilomyces variotii* was studied as well. *P. variotii* represents a worldwide occurring fungus that has been isolated from soil, decaying plants, food products, and clinical samples (91–93). Moreover, the species is frequently detected in the indoor environment, where its colonization leads to the degradation of house construction materials (93–96). In addition, *P. variotii* is classified as an emerging pathogen,

causing serious, in rare cases life-threatening, infections in immunocompromised, but also in immunocompetent individuals (92,97–101). Even though *P. variotii* has been suggested as an aeroallergen source and occupational hazard, no allergens have yet been identified and characterized for this species (46,92,102). The publication "The emerging pathogen *Paecilomyces variotii* – a novel and important fungal allergen source" describes the verification of the allergenic potential of *P. variotii* due to the identification and characterization of the species' first three allergens.

IgE immunoblots, performed with sera from mold-sensitized patients showed the presence of several IgE-reactive proteins in *P. variotii* extract and mass spectrometry analysis of IgE-reactive protein bands led to the identification of the first three *P. variotii* allergens, which interestingly all represent enzymes: a transaldolase designated Pae v 1, a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) designated Pae v 2, and an enolase designated Pae v 6. Full-length cDNAs coding for the *P. variotii* proteins Pae v 1, Pae v 2, and Pae v 6 were then generated from *P. variotii* RNA, cloned into a bacterial expression vector and recombinant proteins were produced in *E. coli*. Afterwards, the recombinant proteins' characteristics were analyzed by SDS-PAGE, IgE immunoblots, ELISAs, CD-spectroscopy, and basophil activation tests. Results presented in this study showed that all recombinant proteins possess the capability to form disulfide bridges and to oligomerize under non-reducing conditions. In addition, circular dichroism spectroscopy indicated that the proteins represent properly folded molecules. Furthermore, ELISAs performed with sera from mold-allergic patients showed the IgE-binding capacity of the three recombinant proteins and led to the classification of Pae v 1 and Pae v 2 as minor allergens of *P. variotii*, whereas Pae v 6 represents a major mold allergen. Moreover, inhibition experiments showed that the recombinant allergens contain the IgE-binding epitopes of their natural counterparts and suggest that rPae v 1, rPae v 2, and rPae v 6 could be used as tools for *in vitro* diagnosis of *P. variotii* sensitization.

The availability of an anti-enolase antibody allowed for the investigation of the release kinetics of the enolase Pae v 6 from *P. variotii* spores. The experiments showed that the spores from *P. variotii* immediately released this major allergen upon exposure to a humid milieu. It can thus be expected that Pae v 6 would be rapidly secreted when the spores get in

direct contact with the human mucosa, leading to immediate allergic symptoms on the accessible mucosa surfaces.

In conclusion, during this study the first allergens from *P. variotii*, designated Pae v 1, Pae v 2 and Pae v 6, have been identified, recombinantly produced and characterized. It was shown that the three recombinant allergens represent IgE-reactive molecules, with Pae v 1 and Pae v 2 representing minor fungal allergens, whereas Pae v 6 can be classified as a major fungal allergen.

1.4.3. Unpublished manuscript I

In course of this dissertation, mold species *U. chartarum* and *P. variotii* have been identified as novel fungal allergen sources and the enzyme enolase was identified as an IgE-reactive molecule from both species. Besides the enzyme's vital functions within an organism, enolase has already been identified as an allergen in various sources. It has been recognized as an important respiratory allergen from several fungal species (103–105), as well as from plant (106) and pollen (107–110). Furthermore, it was identified as an allergen from animal-derived food sources including chicken (111), cod (112,113), salmon (112,114), and tuna (112). This gave rise to the question whether the novel allergens Ulo c 6 and Pae v 6, would show relevant cross-reactivity to already known allergenic enolases from other related and non-related species, as enolases have been suggested as a fungal pan-allergen family (7,60). However, the cross-reactivity between enolases from non-related organisms is largely unknown and has so far only been described between latex and the fungal species *Alternaria alternata* and *Cladosporium herbarum* (106). The existence of cross-reactive allergens often complicates the identification of the primary sensitizer species and results in defective management of the allergic disease (52,60). Thus, from both, diagnostic and treatment perspectives, it is important that polysensitization resulting from allergen cross-reactivity is distinguished from co-sensitization to multiple allergenic sources (52). Therefore, the unpublished manuscript "Analysis of the cross-reactive potential of the enolase pan-allergen" describes the investigation of the cross-reactive potential of enolases from different related and non-related allergen sources including molds, plant pollen and animal-derived foods, with the aim to gain more information about the enzymes' pan-allergenic character and consequently improve the specificity of allergy diagnosis.

Therefore, immunoblots were carried out to investigate whether an antiserum raised against the fungal enolase rAlt a 6, would also recognize other fungal enolases as well as enolases present in animal-derived foods and pollen. Moreover, it was analyzed, by immunoblotting and ELISA experiments, if polysensitized patients would recognize enolases from different species and whether these enolases share cross-reactive IgE epitopes.

In this study, the fungal enolases rAlt a 6[2], rCla h 6, rPae v 6, rAsp f 22 and rPen c 6 were recombinantly produced and sequence comparison revealed high amino acid sequence homologies of at least 85% among the different fungal enolases. Moreover, results showed that the anti-rAlt a 6 serum strongly reacted with fungal enolases from different species. IgE inhibition experiments performed with a cohort of polysensitized patients showed that rAlt a 6 inhibits IgE-binding to homologous enolases, suggesting the presence of cross-reactive epitopes. However, heterogeneity in IgE responses of individual patients also suggest the presence of species-specific IgE epitopes. These findings show that some of the IgE epitopes on the fungal enolase allergens are common to several fungal species while others are specific to one individual allergen.

So far, cross-reactivity between pollen, food, and mold enolases has not been investigated (115). Thus, protein extracts prepared from important food allergen sources such as salmon, tuna, chicken, trout, and shrimp as well as from the important pollen allergen sources ragweed, birch, and grass were prepared and analyzed in this study concerning the cross-reactive potential of the species' enolases. Sequence homology analysis revealed a high degree of sequence identity among enolases from different foods and among enolases from different pollen. Furthermore, a high sequence identity of >60% was also found among enolases from taxonomically distant organisms, which supports the high immunologic cross-reactive potential of enolases (115). Immunoblot experiments with an antiserum raised

[2] Previous studies have revealed that Ulo c 6 and Alt a 6 are 100% identical based on their amino acid sequences. Therefore, only Alt a 6 was included in this study to represent both, Ulo c 6 and Alt a 6.

against rAlt a 6 indeed showed that the antiserum raised against the fungal enolase recognizes also plant and animal enolases.

Furthermore, analyses of the IgE-reactivity of the animal-derived food and pollen extracts by IgE-immunoblots showed that polysensitized patients react with enolases from different allergen sources. Interestingly, a comparison of the IgE-reactivity detected for the recombinant fungal enolases by ELISA and for the protein extracts by immunoblots shows a strongly patient-dependent recognition of enolases. This heterogeneity in IgE-recognition was further verified by IgE inhibition immunoblots and suggest that, despite the high homology of enolases shown in this study, no cross-reactive IgE epitopes are shared between rAlt a 6 and the natural enolases from the allergen sources tested.

In conclusion, results presented in this study demonstrated the high homology of enolases from different organisms and showed the strong IgE cross-reactivity between enolases from various mold species, which reinforces the molecule's status as a fungal pan-allergen. Nevertheless, the data suggest that fungal enolases possess not only cross-reactive, but also species-specific IgE-epitopes. It will therefore be interesting to identify these cross-reactive and species-specific IgE-epitopes to enable the identification of the primary sensitizing species and therefore improve fungal allergy diagnosis and treatment. Furthermore, the IgE-reactivity of polysensitized patients to enolases from different food and pollen sources was shown. So far, no IgE cross-reactivity was detected between enolases from foods and pollen, and the fungal enolase rAlt a 6. However, further investigations could provide valuable information for allergy diagnosis and for developing patient-tailored prophylactic and therapeutic approaches.

1.4.4. Unpublished manuscript II

The first and most important step towards efficient allergen-specific forms of treatment is the proper diagnosis of allergy and problems related to reliable diagnosis of fungal allergies are far from being solved (28). Any *in vivo* as well as any *in vitro* diagnosis of allergy depends on the quality of the material used for testing and, although commercially available allergen solutions must pass through company-internal standardization procedures and quality controls, there are, until now, no generally accepted guidelines for the preparation of

allergenic fungal extracts (28). Thus, considerable differences in potency of fungal extracts between manufacturers and even batch-to-batch variations can be found (28,36,116). Due to problems with standardization of fungal extracts, as various factors such as the fungal cultivation or the extraction procedure, can affect the allergenicity and antigenicity of fungal allergen extracts differently, only a limited number of them are available for diagnosis (36,61).

The unpublished manuscript “Cultivation conditions and their impact on the expression of fungal allergens” describes the investigation of the impact of different environmental conditions, including carbon source, light exposure, and temperature, as well as the effect of cultivation time on the expression of fungal allergens. Information obtained in this study will facilitate the optimization of fungal cultivation to produce highly potent fungal extracts, which will help to analyze the allergenic potential of fungal species. In addition, if recombinant allergens are not available for allergy diagnosis, the use of highly potent fungal extracts as test solutions will also improve sensitivity and specificity of allergy diagnosis.

In this study, protein extracts from spores and mycelium of various fungal species that have been cultivated under specific conditions, were prepared. Afterwards immunoblots using specific antibodies directed against the major allergen Alt a 1 and the allergenic enolase rAlt a 6 from *Alternaria alternata* were carried out to investigate the expression of the homologous major fungal allergens Alt a 1, from *Alternaria alternata*, and Ulo c 1, from *Ulocladium chartarum*, as well as of allergenic enolases from *A. alternata, U. chartarum, Aspergillus fumigatus, Cladosporium herbarum* and *Paecilomyces variotii*.

Results of anti-Alt a 1 immunoblots showed that the growth on the different carbon sources glucose, cellulose, and methylcellulose did not lead to any differences in the expression of the major allergens Alt a 1 and Ulo c 1. Furthermore, results of analyzing the influence of light exposure on fungal allergen expression suggests an earlier expression of Alt a 1 when *A. alternata* is grown in the dark and a slightly increased production of Ulo c 1 by *U. chartarum* during light exposure. Nevertheless, this environmental condition does not seem to be of high importance for the expression of these allergens. Even though the type of carbon source and the exposure to light might not be crucial for the expression of Alt a 1 and Ulo c 1, a strong temperature-dependent allergen expression was detected as significant higher amounts of the allergens were expressed in the fungal species grown at 30°C as compared to fungi grown

at room temperature and 37°C. Moreover, differences in the expression of Alt a 1 and Ulo c 1 based on the cultivation time were detected as results of anti-Alt a 1 immunoblots showed that the amount of Alt a 1 and Ulo c 1 increases with continuing growth of the fungal species.

As already studied and described in the manuscript “Analysis of the cross-reactive potential of the enolase pan-allergen”, fungal enolases play an important role in the phenomenon of cross-reactivity among several fungi (54). Therefore, the impact of different cultivation conditions on the expression of the enzyme enolase by various fungal species was analyzed as well. Even though the allergenic enolases Alt a 6 and Ulo c 6 are completely identical based on their amino acid sequence (117), results revealed differences in the expression of the protein by the homologous fungal species *A. alternata* and *U. chartarum* as Alt a 6 could not be extracted from *A. alternata* mycelium and spores under any conditions. In contrast, results showed that the growth time had a major impact on the expression of Ulo c 6. The type of carbon source, the exposure to light, and the cultivation temperature, however, only influenced the time of expression of Ulo c 6. For example, Ulo c 6 was expressed after 30 days of growth when *U. chartarum* was grown at room temperature, whereas cultivation at 37°C led to an earlier onset of allergen expression already after 20 days of growth.

To analyze whether fungal enolases are differently expressed in related species, the growth-dependent production of the enolases Asp f 22 from *Aspergillus fumigatus,* Cla h 6 from *Cladosporium herbarum* and Pae v 6 from *Paecilomyces variotii* was also evaluated. Results of anti-rAlt a 6 immunoblots showed that the expression of each fungal enolase is not only dependent on the stage of growth, but also varies among different species.

In conclusion, results of this study showed that the cultivation conditions indeed have an impact on the allergenic content of fungal extracts and that the expression of fungal allergens is highly species-dependent, even in case of highly conserved, abundant enzymes such as enolases. This emphasizes the necessity of determining the optimal cultivation conditions for each fungal species separately, to obtain standardized extracts with a specific allergen content for fungal allergy diagnosis. Information obtained in this study will help to optimize fungal cultivation to produce highly potent diagnostic fungal extracts and therefore improve diagnosis’ specificity and sensitivity.

1.5. Summary and Conclusions

Fungal allergy is a worldwide public health problem affecting approximately 3% to 10% of the general population and can manifest as allergic rhinitis and rhinosinusitis, allergic asthma, and atopic dermatitis (10,28,36). Fungi are, in contrast to other allergen sources, extremely complex, and problems related to reliable diagnosis of fungal allergies are far from being solved (28). Even though data from several epidemiological studies provide evidence for the important role of fungi in respiratory diseases, fungi are still considered a neglected allergen source (36,45). Especially, the contribution of the exposure to the indoor mycobiota to allergic diseases is still not understood (7,8,19,39,40). Therefore, the main focus of this dissertation was to identify and characterize allergens from mold species that are frequently detected in indoor environments. Besides the fact that the limited knowledge about allergenic fungi is hampering an effective fungal allergy diagnosis, cross-reactive allergens further complicate the identification of the primary sensitizing species (52,60). Thus, the cross-reactive potential of the fungal pan-allergen enolase, derived from related and non-related allergen sources, was also analyzed to gain more information about the molecule's pan-allergenic character. To analyze the allergenic potential of fungal species, highly potent fungal extracts need to be produced first. To optimize fungal cultivation for the production of highly potent fungal extracts, the impact of different environmental conditions on the expression of fungal allergens was studied as well.

For the identification of novel allergens from the indoor mold species *Ulocladium chartarum* and *Paecilomyces variotii*, the IgE-reactivity of mold-allergic patients to protein extracts prepared from *U. chartarum* and *P. variotii* was tested in immunoblots. Following the identification of IgE-reactive proteins by mass spectrometric analysis, cDNAs coding for the identified proteins were generated from fungal RNA and cloned into a bacterial expression vector. Afterwards, the proteins were recombinantly expressed in *E. coli* and purified by immobilized metal affinity chromatography. Pure, recombinant molecules were then characterized concerning there biochemical and immunological characteristics by gel electrophoresis, CD-spectroscopy, immunoblot and inhibition immunoblot experiments, ELISAs, and basophil activation tests.

For the investigation of the cross-reactive potential of enolases from different allergen sources, protein extracts from various plant pollen and animal-derived foods were prepared. In addition, several fungal enolases as well as a fish enolase were recombinantly produced. The recombinant enolases and the enolases present in the protein extracts were analyzed concerning their amino acid sequence identity and immunoblots were carried out to investigate whether an antiserum raised against a fungal enolase would also recognize enolases from other organisms. Moreover, the IgE-reactivity of polysensitized patients to enolases from different species was analyzed by immunoblotting and ELISA experiments and IgE cross-reactivity was evaluated by inhibition experiments.

To evaluate the impact of different environmental conditions, including carbon source, light exposure, and temperature, as well as the effect of the cultivation time on the expression of fungal allergens, protein extracts from spores and mycelium of various fungal species that had been cultivated under specific conditions, were prepared. The protein extracts were then analyzed by gel electrophoresis and immunoblots using specific antibodies directed against the *A. alternata* allergens Alt a 1 and rAlt a 6 to investigate the expression of the homologous major fungal allergens Alt a 1 from *A. alternata* and Ulo c 1 from *U. chartarum*, as well as of enolases from different fungal species.

In this study, the high allergenic potential of the indoor mold species *U. chartarum* and *P. variotii* was verified as IgE immunoblots, performed with sera from mold-allergic patients, showed strong IgE-binding to several proteins in the extracts and led to the identification of the first allergens from these species, which were recombinantly produced. In case of *U. chartarum* three IgE-reactive proteins were identified: the Alt a 1-homologous protein Ulo c 1, a formate dehydrogenase designated Ulo c 2, and an enolase, designated Ulo c 6. Furthermore, the first three *P. variotii* allergens were identified: a transaldolase designated Pae v 1, a glyceraldehyde 3-phosphate dehydrogenase designated Pae v 2, and an enolase designated Pae v 6. Results showed that the recombinant proteins produced in this study represent IgE-reactive molecules, which contain the IgE-binding epitopes of their natural counterparts. Therefore, it can be concluded that rUlo c 1, rUlo c 2, rUlo c 6, rPae v 1, rPae v 2, and rPae v 6 could be used as tools for *in vitro* diagnosis of *U. chartarum* and *P. variotii* sensitization.

Results of investigating the cross-reactive potential of enolases from related and non-related allergen sources showed the high homology of enolases from different organisms as well as the strong IgE cross-reactivity between enolases from different mold species, which reinforces the molecule's status as a fungal pan-allergen. Nevertheless, the data suggest that some of the IgE epitopes on the fungal enolase allergens are common to several fungal species while other are specific to an individual allergen. Furthermore, the IgE-reactivity of enolases from different animal-derived food and pollen sources was shown, however, no IgE cross-reactivity between these and the fungal enolase rAlt a 6 was detected. In case of the well-known pan-allergens profilins and non-specific lipid transfer proteins, extensive cross-reactivity among related and unrelated allergen sources was shown (118). However, studies also revealed that these pan-allergens do not only lead to allergic diseases because of cross-reactivity, but also due to co-sensitization (118), which could be also the case for the enolase present in the allergen sources analyzed in this study. In addition, it was shown that fungal cultivation conditions indeed have an impact on the allergenic content of fungal extracts and that the expression of fungal allergens is species-dependent, even in case of highly conserved, abundant allergens such as enolases. Therefore, cultivation conditions have to be optimized for each fungal species individually to obtain potent allergenic fungal extracts.

In course of this dissertation, the knowledge about fungal species, able to elicit allergic diseases, was expanded as the allergenic potential of *U. chartarum* and *P. variotii* was verified and the obtained data suggest that these species should be considered as potential allergen sources and should therefore be included in routine fungal allergy diagnosis. Furthermore, the first allergens from *U. chartarum* and *P. variotii*, have been identified, recombinantly produced and characterized and thus expand the repertoire of fungal allergens available for fungal allergy diagnosis. By providing single allergens for allergy diagnosis, problems related to the production of natural allergen extracts, such as altered allergenicity due to extraction procedures and variability on allergen content, can be circumvented and will allow to determine individual reactivity patterns for each single subject tested (67). In this way, sensitivity as well as specificity of fungal allergy diagnosis can be improved, which also opens new perspectives for the preparation of patient-tailored mixtures to be used in specific immunotherapy, thus also improving allergy treatment (67). The role of enolases in allergic diseases has not yet been fully investigated and results presented in this study contribute to

a better understanding of the cross-reactive potential of enolases and could provide valuable information for allergy diagnosis and for developing patient-tailored prophylactic and therapeutic approaches. In addition, the results presented in this study showed the high variability of fungal allergen expression, and therefore it is important to mention that the analysis of the allergenic potential of a fungal species based on fungal extracts, is highly biased by the cultivation conditions under which the species was grown. Moreover, the findings of this research emphasize the necessity of determining the optimal cultivation conditions for each fungal species separately, to obtain standardized extracts with a specific allergen content when used for fungal allergy diagnosis. Thus, optimizing diagnosis' specificity and sensitivity, which is a prerequisite for improvement in safety and efficacy of extracts used for immunotherapy. However, the results also clearly reinforce the benefits of using single molecules instead of natural extracts for fungal allergy diagnosis, as a high variability in fungal allergen expression was detected.

In conclusion, results presented in this dissertation expand the knowledge about fungal allergy and facilitate an improvement in the specificity of fungal allergy diagnosis and thus, also enable optimized treatment approaches. Results further suggest the role of so far neglected indoor fungi as important allergen sources. It will therefore be interesting to study the allergenic potential of other indoor mold species, such as *Acremonium*, *Chaetomium*, *Wallemia*, and *Aureobasidium*.

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Authors contributions:

S.P. and P.S. carried out the majority of the experiments described. M.R. performed the CD-spectroscopy. M.P., R.M.M.-C. and C.S.B. carried out the basophil activation assays and provided a part of the serum samples used in this study. S.Q. also provided serum samples. K.N. and E.R.-F. implemented the mass spectrometric analysis. M.F.-T. and K.S.-G. contributed to interpreting the results and planning the work by providing their scientific expertise. S.P. wrote the manuscript. I.S. supervised the project and contributed to the implementation of the research, to the analysis of the results and to the writing of the manuscript. All authors discussed the results and commented on the manuscript.

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LETTER TO THE EDITOR

Identification of *Ulocladium chartarum* as an important indoor allergen source

To the Editor,

The constant exposure to fungal spores, which constitute the largest proportion of aerobiological particles, can cause severe health problems, including allergic diseases.[1,2] Appropriate management of fungal allergies is hampered by the fact that our knowledge about fungal allergy is still limited to a small number of thoroughly investigated fungi, whereas for the majority of species, it is not yet known whether they play a role in allergic diseases.[2] This lack of knowledge together with unreliable diagnostic results obtained with fungal allergen extracts of poor quality contributes to a general underdiagnosis of fungal allergy.[3,4] In the present study, we investigated the allergenic potential of *Ulocladium chartarum*, an opportunistic human pathogen[5] that can grow on various substrates.[6,7] Exposure to *Ulocladium* species often occurs in the indoor environment as they are commonly detected inside damp buildings and are regarded as an indicator of water damages.[6–9] The fact that *U. chartarum* belongs to the same family as the outdoor aeroallergen source *Alternaria alternata* suggests that *U. chartarum* might also represent an allergen source.[6,7]

IgE immunoblots, performed with sera from patients sensitized to different mold species (Table S1), demonstrated the high allergenic potential of *U. chartarum*, since patients' IgE antibodies recognized several *U. chartarum* proteins (Figure 1A), whereas exposure of the blotted proteins to sera from non-allergic individuals or to immunodetection reagent did not lead to any unspecific binding (data not shown). Interestingly, despite their phylogenetic relationship, the IgE-binding profile of *A. alternata* (Figure S1) differed significantly from the one of *U. chartarum*. Mold-allergic patients not only recognized more proteins in *U. chartarum* than in *A. alternata* extract, but also showed an overall stronger reactivity to them.

Peptide mass fingerprinting allowed to identify a 15 kDa *U. chartarum* protein (A in Figure 1A) as an Alt a 1-homologous protein, a 41 kDa protein (B) as formate dehydrogenase, a protein not yet known as an allergen, and a 48 kDa protein (C) as an Alt a 6-homologous enolase. The proteins were designated Ulo c 1[a] (A), Ulo c 2[b] (B), and Ulo c 6[c] (C). The IgE-reactive protein bands D, E, and F could not yet be identified by mass spectrometry. cDNAs coding for the identified allergens was generated and cloned into a bacterial expression vector, and recombinant proteins were produced in *Escherichia coli*. Circular dichroism spectroscopy revealed that the recombinant allergens contain considerable secondary structures. In case of rUlo c 1, mainly beta-sheet structures (minimum of far-UV spectrum at 213 nm) were found, whereas predominantly alpha-helical structures (minima at 207 nm and 220 nm) were detected for rUlo c 2 and rUlo c 6 (Figure S2).

ELISAs performed with sera from mold-allergic patients showed the IgE-binding capacity of the three recombinant allergens, with rUlo c 1 displaying the highest levels of IgE reactivity (Figure 1B). Furthermore, IgE inhibition immunoblots, where nitrocellulose-blotted protein extracts from *U. chartarum* were exposed to serum pools that had been pre-incubated with the recombinant proteins, showed that the recombinant allergens were able to completely inhibit patients' IgE binding to their natural counterparts (Figure 1C). This indicates that the recombinant allergens represent well-folded proteins which contain all the IgE-binding epitopes present in their natural counterparts and suggests that rUlo c 1, rUlo c 2, and rUlo c 6 could be used as tools for in vitro diagnosis of *U. chartarum* sensitization.

The prevalence of the molecules' IgE reactivity was analyzed by ELISA using sera from 85 individuals sensitized to different mold species (Figure S3). rUlo c 1 was recognized by 58% of the patients, indicating that this molecule represents a major mold allergen, whereas rUlo c 2 was recognized by 43% and rUlo c 6 by 40% of the patients, suggesting minor mold allergens.

Sequence comparison of the three *U. chartarum* allergens with homologous *A. alternata* proteins revealed protein sequence identities of 89% between Ulo c 1 and Alt a 1, 99% between Ulo c 2 and its homologous *A. alternata* protein, and 100% between Ulo c 6 and Alt a 6. The high sequence homology between Ulo c 1 and Alt a 1 and the suggested presence of Alt a 1-homologous proteins in other species of the Pleosporaceae family[10] prompted us to investigate the potential cross-reactivity between rUlo c 1 and

[a]NCBI Nucleotide Sequence Accession Number: MH716394.

[b]NCBI Nucleotide Sequence Accession Number: MN953052.

[c]NCBI Nucleotide Sequence Accession Number: MH910061.

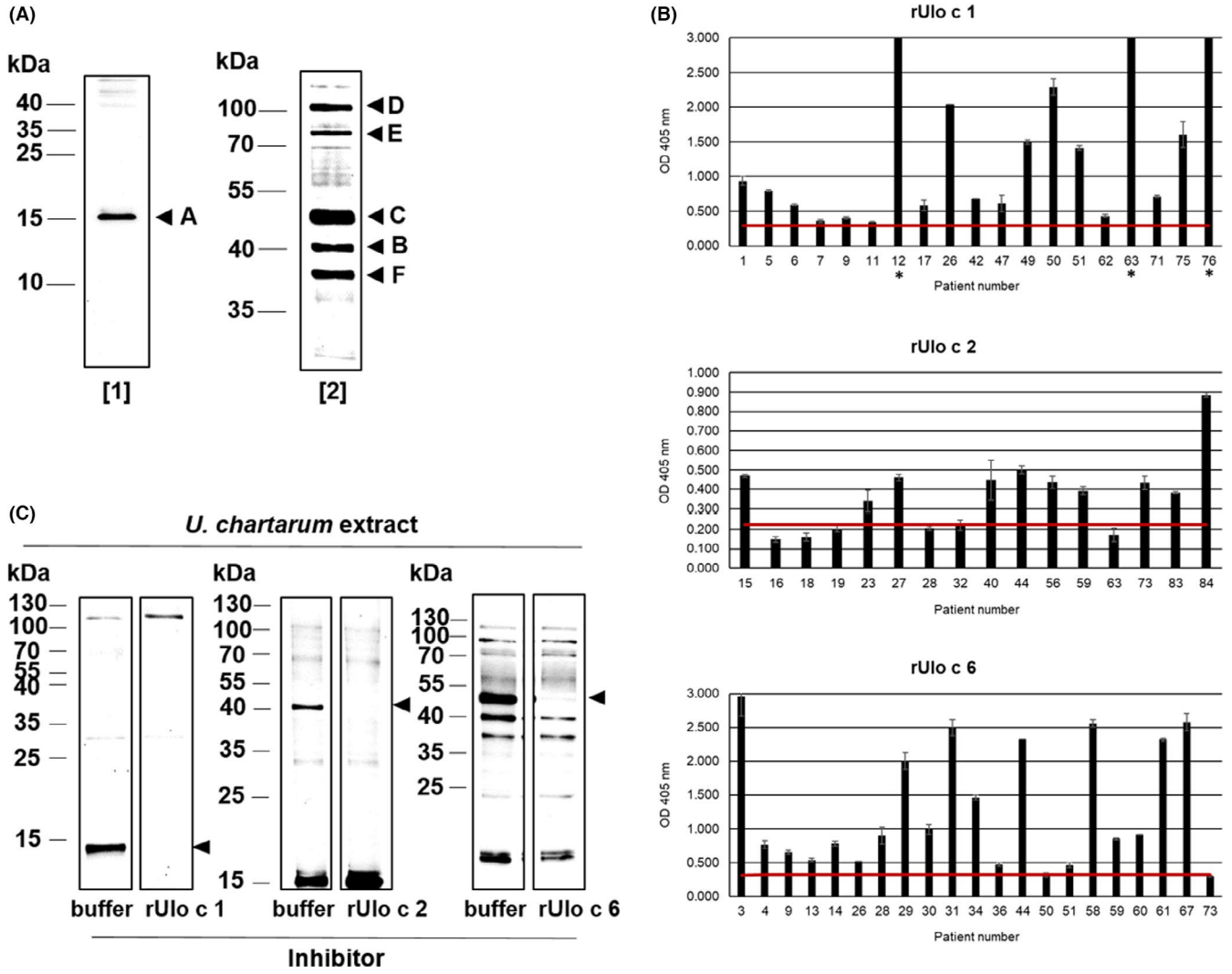

FIGURE 1 Identification and characterization of IgE-reactive proteins from *Ulocladium chartarum*. (A) Nitrocellulose-blotted proteins from *U. chartarum* were exposed to two pools of sera [pool 1: sera 1, 2, 45–47, and 74; pool 2: sera 3–4, 13–14, 28–31, 34–36, and 58–61] from mold-allergic patients. IgE-reactive proteins identified by mass spectrometry are marked with A, B, and C; strongly stained but unidentified bands are marked with D, E, and F. Molecular weight markers are indicated in the left margins. (B) Determination of the IgE-binding capacity of the recombinant allergens rUlo c 1, rUlo c 2, and rUlo c 6 by ELISA using sera from mold-allergic patients. Results are displayed as mean OD values, and standard deviations from two technical replicates are indicated as error bars in the bar charts. The red line represents the cutoff value, which was calculated from the mean OD values plus two times the standard deviation of sera from three non-allergic patients. Sera that gave OD values above 3.0 are marked with an asterisk (*). (C) Inhibition immunoblot: Blotted proteins of *U. chartarum* protein extracts were exposed to pools of sera (rUlo c 1: sera 25–27, 42, and 78; rUlo c 2: sera 15, 28–32, and 57; rUlo c 6: sera 3–4, 13–14, 28–31, 34–36, and 58–61) that had been pre-incubated with rUlo c 1, rUlo c 2, or rUlo c 6 or, for control purposes, with buffer only. Molecular weight markers are indicated in the left margins. Arrows point to reduction in IgE binding to natural Ulo c 1, Ulo c 2, and Ulo c 6 after pre-incubation with the recombinant proteins

rAlt a 1 in IgE inhibition immunoblots. As depicted in Figure 2A, pre-incubation of patients' sera with one allergen always significantly reduced or even abolished IgE binding to the other molecule, whereas IgE binding was not reduced by pre-incubation of the sera with the irrelevant respiratory allergen rBet v 1. This reduction in IgE binding obtained with rAlt a 1 and rUlo c 1 was comparable to the reduction obtained by self-inhibition and indicates that the two allergens share conserved IgE epitopes. Furthermore, both recombinant allergens, rUlo c 1 and rAlt a 1, induced a dose-dependent expression of CD63 in basophils from six of the eight analyzed mold sensitized individuals (Figure 2B), which provided evidence for the molecules' biological activity.

Analysis of the release of allergens from allergen sources can provide useful information about the potential exposure to these allergens. Interestingly, our investigations on the release kinetics of Ulo c 1 and Ulo c 6 from *U. chartarum* and Alt a 1 and Alt a 6 from *A. alternata* showed that the allergens were all immediately released from the spores (Figure S4). These findings suggest that rapid allergen elution might also occur when spores reach mucosal surfaces, where they can then elicit allergic reactions.

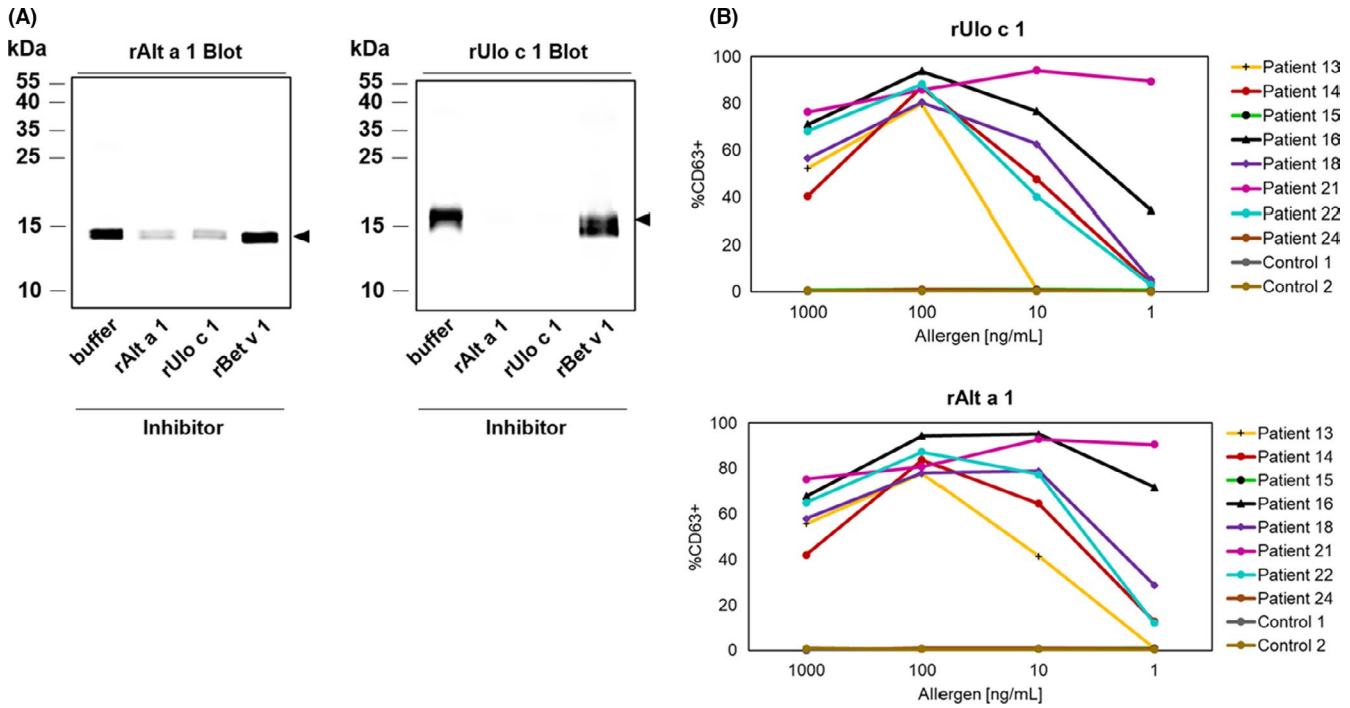

FIGURE 2 IgE cross-reactivity between the major fungal allergens rUlo c 1 and rAlt a 1. (A) Two micrograms of the recombinant allergens were separated by SDS-PAGE, blotted onto nitrocellulose, and exposed to a pool of sera (25–27, 42, and 78) from mold-allergic patients, which had been pre-incubated with rUlo c 1 or Alt a 1 or, for control purposes, with the non-relevant allergen rBet v 1 or with buffer only. Molecular weight markers are indicated in the left margins. (B) Basophil activation of rUlo c 1 and rAlt a 1 was determined by measuring the expression of CD63 by flow cytometry after incubation of the whole blood from eight *Alternaria*-sensitized patients (patients 13–16, 18, 21, 22, and 24) and from two non-atopic control individuals (control 1 and 2) with increasing allergen concentrations. The percentage of CD63-positive basophils (*y*-axis), based on the total amount of basophils, is displayed against the concentration of the applied allergen (*x*-axis)

In conclusion, in this study we provide evidence that *U. chartarum* represents an important, so far underestimated, allergen source that shows at least partial cross-reactivity to the evolutionarily related species *A. alternata*. Owing to the fact that in contrast to the outdoor mold *A. alternata*, *U. chartarum* is an important component of the indoor environment, it is especially important to raise the awareness that *U. chartarum* represents a potential cause of respiratory allergic diseases and to include the species into routine allergy diagnosis. We identified the species' first allergens, Ulo c 1, Ulo c 2, and Ulo c 6, and produced them as IgE-reactive recombinant molecules. These recombinant allergens will expand the repertoire of fungal allergens available for fungal allergy diagnosis, thus improving diagnosis' specificity and sensitivity.

The description of the used methods can be found in the supporting information.

KEYWORDS

allergy diagnosis, indoor allergens, mold allergy, recombinant allergens, ulocladium chartarum

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Methods

Patients' sera

Sera from mold-allergic patients were obtained from the Department of Allergy, La Paz, University Hospital, Madrid, Spain, from the Hospital Clinic de Barcelona, University of Barcelona, Spain and from the company AbBaltis (FDA-approved; Sittingbourne, United Kingdom). The demographic and serological characteristics of the patients are given in Table S1. Currently, *Ulocladium chartarum* is not included in the routine diagnosis of fungal allergy. Therefore, it was not possible to focus on sera of *U. chartarum* allergic individuals. Instead, patients with a sensitization to at least one of the fungal species used in routine diagnosis were included. All patients agreed to participate in this study and gave informed consent. This study was approved by the ethics committee of the Hospital Clínic de Barcelone (approval number 2011/6605) and of the La Paz University Hospital in Madrid (EK565/2007). We confirm that all methods were performed in accordance with the relevance guidelines and regulations.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblot and inhibition immunoblot experiments

Two µg of recombinant proteins or 5 µg of fungal protein extracts were separated on 10%, 12% or 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGEs), as described by Laemmli (S1) and were transferred electrophoretically onto nitrocellulose membranes (0.2 µm; GE Healthcare, Chicago, IL) (S2). For IgE-immunoblots, membranes were blocked with PBS containing 0.5% Tween20 (PBS-T) and were then exposed to pools of sera from mold-allergic patients, or for control purposes, to a pool of sera from three non-allergic individuals or to PBS-T only.

These pools contained either 3, 5, 6, 9 or 16 sera (1:10 diluted in PBS-T). For IgE inhibition immunoblots, pools of sera were pre-incubated overnight with 20 µg of rUlo c 1 or rAlt a 1 or, for control purposes, with the unrelated respiratory allergen rBet v 1 (available in the laboratory) or with PBS-T and then proteins, blotted onto nitrocellulose membranes, were incubated with these pre-incubated sera. For the detection of protein-bound human IgEs, a mouse anti-human IgE antibody, conjugated to horseradish peroxidase (HRP), was used as detection antibody (final dilution 1:5,000; Southern Biotech, Birmingham, AL). The blots were developed using the SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions and imaged using the UVP ChemStudio device (Analytik Jena, Germany).

To detect Alt a 1, Alt a 6 and related proteins, nitrocellulose-blotted proteins were incubated with rabbit antisera either directed against a peptide of Alt a 1 (5'-KISEFYGRKPEGTYYNSLG-3') or against the recombinant Alt a 6 (Charles River Laboratories, Écully, France). A goat anti-rabbit IgG HRP-labelled antibody (Vector Laboratories Inc, Burlingame, CA; diluted 1:10,000 in PBS-T) was used for detection and the blots were developed and imaged as described above for the IgE immunoblots.

cDNA cloning and recombinant expression of *U. chartarum* allergens

The full-length cDNAs coding for the *U. chartarum* proteins were generated from *U. chartarum* RNA by RT-PCR. For this, information on the full-length sequences of the Ulo c 1 and Ulo c 2 cDNAs first had to be obtained by 5'/3'-rapid amplification of cDNA ends (RACE) using the SMARTer RACE 5'/3' Kit (Takara Bio, Kyoto, Japan). Therefore, primers were designed based on the homologous *Alternaria alternata*

cDNA sequences of Alt a 1 (NCBI accession number: AY568627) and of the formate dehydrogenase (NCBI accession number: XM_018525579.1). To obtain the 5'- and 3'-end of the Ulo c 1 cDNA the following primers were used: 5'-AACGCATCCTGCCCTGTCACTACCGA-3' (for 3'-end amplification) and 5'-GCTGTTCTCGCCGCAAGAGTACCAC-3' (for 5'-end amplification). For the Ulo c 2 cDNA the following primers were used: 5'-GGAAAAGTTCTTCTCGTCCTCTACG-3' (for 3'-end amplification) and 5'-CTTGCGCTGACCGTACGCCTTGG-3' (for 5'-end amplification). Primers for full-length cDNA cloning of Ulo c 1 and Ulo c 2 by RT-PCR were then designed based on the sequences of the RACE-PCR-products.

Since enolases are very conserved proteins, the cDNA coding for Ulo c 6 could be obtained by RT-PCR using forward (5'-ACCATCACCAAGATCCACGCCCGC-3') and reverse (5'-CAAGTTAACGGCAGTCCTGAA-3') primers designed based on the nucleotide sequence from the homologous *A. alternata* enolase Alt a 6 (NCBI accession number: U82437.2).

The obtained sequences were submitted to GenBank and received the following accession numbers: MH716394 (Ulo c 1), MN953052 (Ulo c 2) and MH910061 (Ulo c 6).

cDNAs coding for Ulo c 1, Ulo c 2 and Ulo c 6 were cloned with C-terminal hexa-Histidine-tags into the bacterial expression vector pET-17b. Following the successful expression of the allergens as soluble proteins in *E. coli*, the molecules were purified under native conditions by affinity chromatography. The protein concentrations were determined with the MicroBCA Protein Assay Kit (Thermo Fisher Scientific) using BSA as a standard.

For control purposes, rAlt a 1 from *Alternaria alternata* was also produced in *E. coli.* For this, the Alt a 1 cDNA (NCBI accession number: AY568627) was generated from

A. alternata RNA by RT-PCR and was cloned with a C-terminal hexa-Histidine-tag into the bacterial expression vector pET-17b. rAlt a 1 expression and purification were carried out as described for the *U. chartarum* allergens.

Secondary structure analysis by circular dichroism spectroscopy (CD-spectroscopy)

The secondary structure of rUlo c 1, rUlo c 2 and rUlo c 6 was evaluated by circular dichroism spectroscopy on a Chirascan Plus Spectrometer (Applied Photophysics, Leatherhead, United Kingdom) in 10 mM sodium phosphate buffer (PH 7.0) at 20°C and a protein concentration of 0.2 µg/µL using a quartz cuvette (Hellma Analytics, Müllheim, Germany) with a path length of 1 mm. CD-spectra were recorded from 190 to 280 nm with a resolution of 0.5 nm and results were the average of three scans. The final spectra were corrected by subtracting the buffer baseline spectrum, obtained under identical conditions, and normalized to the number of peptide bonds by using the extinction coefficient of the measured protein at 205 nm (ε205) (S3). Results are expressed as mean residual ellipticity θ_{MRW} (deg cm² $dmol^{-1}$) at a given wavelength.

Enzyme-linked immunosorbent assay (ELISA) of recombinant fungal allergens

The IgE-reactivity of the recombinant *U. chartarum* allergens was analyzed in ELISAs performed with 85 sera from patients sensitized to different mold species. For this, 96-well Nunc MaxiSorp ELISA plates (Thermo Fisher Scientific) were coated with 4 µg/mL of the recombinant proteins, diluted in bicarbonate buffer (pH = 9.6). Wells were blocked with phosphate buffered saline containing 0.5% Tween20 (PBS-T) and were then incubated with sera from mold-allergic patients (1:5 diluted in PBS-T) or, for control purposes, with sera from three non-allergic individuals (1:5 diluted in PBS-

T) or with PBS-T only. Bound IgE antibodies were detected with a HRP-labelled goat anti-human IgE antibody (SeraCare Life Sciences Inc., Milford, MA; diluted 1:2,500 in PBS-T) using 1.8 mM of 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich) in 60 mM citric acid, 77 mM $Na_2HPO_4(H_2O)_2$ and 3 mM H_2O_2 as a substrate. After 60 minutes of incubation in substrate solution, the optical density of the samples was measured at a wavelength of 405 nm using the Multiskan FC Photometer (Thermo Fisher Scientific). Experiments were always carried out in duplicates and the results are expressed as mean OD-values. The cut-off, used to differentiate positive and negative IgE-reactivity, was calculated from the mean OD-values plus two times the standard deviation of sera from three non-allergic patients.

Basophil activation test (BAT)

To test the biological activity of the recombinant molecules, basophil activation tests were performed using the Flow Cast Kit (Bühlmann, Schönenbuch, Switzerland) according to the manufacturers' instructions. Whole blood from eight mold-sensitized patients and, for control purposes, from two patients with no fungal sensitization, was incubated with increasing concentrations (1 to 1000 ng/mL) of the recombinant allergens. Basophil activation was assessed by detecting the expression of CD63 by flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ). Activated basophils were gated as $SSC^{low}/CCR3^{+}/CD63^{+}$. At least 500 basophils were measured, and the percentage of CD63-expressing basophils, based on the total amount of basophils, was calculated.

Allergen Release Kinetics Experiments

Clones of *U. chartarum* and *A. alternata* were cultured on MEA-plates at 20-25°C for 7 days. Then, 10 mL of a buffer that mimics the natural nasal fluid (160 mM NaCl, 30 mM KCl, 5 mM $MgCl_2(H_2O)_6$ and 2 mM $CaCl_2(H_2O)_2$) (S4) was added to the plates and spores were detached using a Drigalski-spatula. The spore solutions were transferred into centrifugation tubes and were incubated under continuous shaking at 20-25°C. Aliquots of 1 mL each were taken after 0, 5, 10, 20 and 60 minutes and were immediately centrifuged to separate the supernatants, containing the released proteins, from the fungal spores. The supernatants were stored at –20°C and the protein concentrations were determined using the method of Bradford with BSA as a standard (S5). For the detection of released allergens, 0.1 µg of the supernatants were separated on 12% or 15% SDS-PAGEs, blotted onto nitrocellulose (as described before) and exposed to the antisera against Alt a 1 or rAlt a 6.

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FIGURE LEGENDS:

FIGURE S1. IgE-reactivity of *A. alternata* proteins. Nitrocellulose-blotted proteins from *A. alternata* were exposed to two pools of sera [pool 1: sera 1,2, 45-47 and 74; pool 2: sera 3-4, 13-14, 28-31, 34-36 and 58-61] from mold-allergic patients. IgE-reactive proteins are marked with G, H and I. Molecular weight markers are indicated in the left margins.

FIGURE S2. Structural analyses of recombinant *U. chartarum* allergens. Circular dichroism spectra were recorded for the recombinant allergens from 190 to 280 nm. Results shown are the average of three scans and are expressed as mean residual ellipticity θ_{MRW} (deg cm^2 dmol^{-1}) at a given wavelength.

FIGURE S3. IgE-reactivity profiles of mold-sensitized patients to different recombinant allergens. The binding of IgE antibodies from mold-sensitized patients to rUlo c 1, rUlo c 2 and rUlo c 6 was measured by ELISA and data are summarized in an IgE-reaction map. The optical density was measured in duplicates and the mean OD-value obtained with sera from three non-allergic individuals plus two times the standard deviation was set as a threshold for a positive reaction, indicated here in green. OD-values below the cut-off indicate negative results and are shown in red, whereas grey coloring indicates that the IgE-reactivity was not evaluated.

FIGURE S4. Ulo c 1, Alt a 1, Ulo c 6 and Alt a 6 are rapidly released from fungal spores. Proteins, released from *U. chartarum* and *A. alternata* spores after different times of hydration of the spores (0, 5, 10, 20, 60 min), were separated by SDS-PAGE and were blotted onto nitrocellulose membranes. Membranes were either exposed to

the anti-Alt a 1 or to the anti-rAlt a 6 antiserum. Molecular weight markers are indicated in the left margins.

Table S1. Summary of demographic and serological data of mold-allergic patients grouped based on their IgE-reactivity profiles (n = 85). Total IgE (in kU/L) and specific IgE (sIgE; in kUA/L) against different mold species were determined by ImmunoCAP analysis.

Patient numbers	Allergen source	Age (yr)	Total IgE [kU/L]	sIgE mold [kUA/L]
1-27	*Alternaria*	4-83	25-646	1.54-66.60
28-38	*Alternaria* *Cladosporium*	7-36	108-2229	*Alternaria*: 3.50 - >100 *Cladosporium*: 0.89-80.10
39; 44	*Alternaria* *Aspergillus*	75; N/A	862; N/A	*Alternaria*: 1.92-3.62 *Aspergillus:* 0.01-3.99
40-41	*Alternaria* *Aspergillus* *Penicillium*	35-48	121-1679	*Alternaria*: 01.13-6.00 *Aspergillus*: 1.06-1.94 *Penicillium*: 0.49-0.81
42	*Alternaria* *Epicoccum*	N/A	N/A	*Alternaria*: 3.37 *Epicoccum*: 2.01
43	*Alternaria* *Aspergillus* *Cladosporium*	N/A	762	*Alternaria*: 23.3 *Aspergillus*: 22.5 *Cladosporium*: 5.73
45-56	*Aspergillus*	12-74	185-1874	2.11-19.60
57-70	*Cladosporium*	4-42	220-3625	3.61-23.60
71-72	*Penicillium*	4-13	66-291	4.04-6.30
73	*Fusarium*	N/A	N/A	4.10
74-77	*Aspergillus* *Cladosporium*	10-11	924-984	*Aspergillus:* 4.54-29.00 *Cladosporium*: 7.53-24.70
78-80	*Penicillium* *Cladosporium*	N/A	N/A	*Penicillium*: 1.28-9.10 *Cladosporium*: 1.42-8.10
81-82	*Penicillium* *Cladosporium* *Aspergillus*	N/A	N/A	*Penicillium*: 6.93-7.72 *Cladosporium*: 8.67-8.99 *Aspergillus*: 10.80-11.30
83-85			N/A	

Abbreviation: N/A, information not available

Figure S1

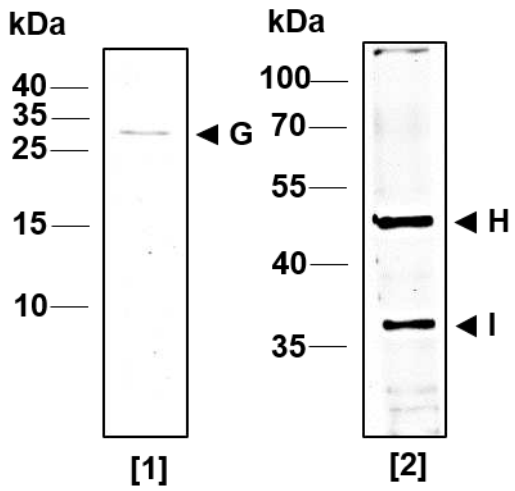

Figure S2

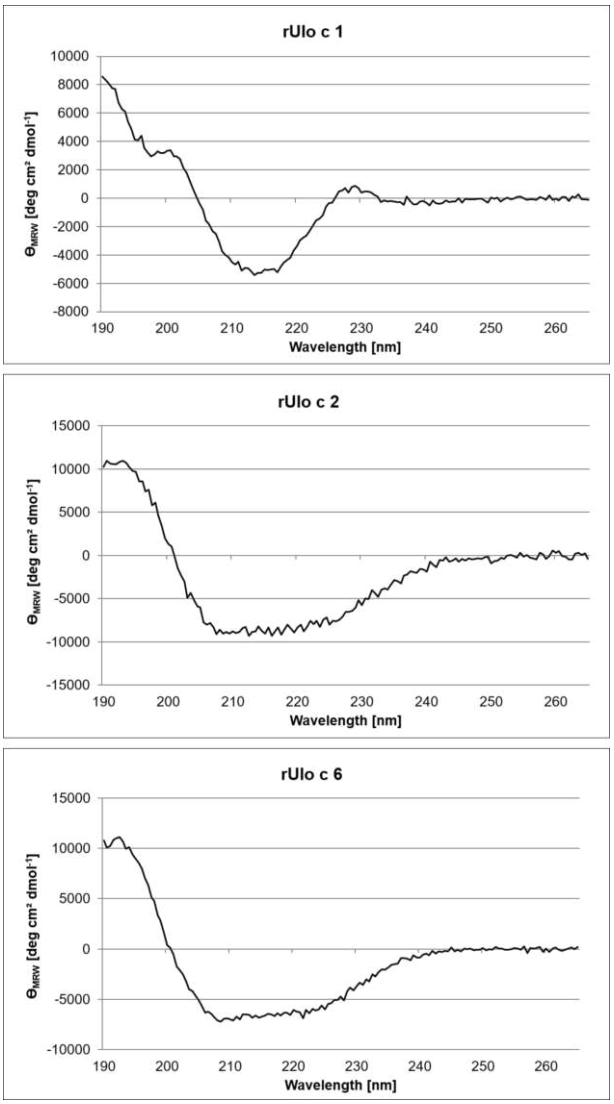
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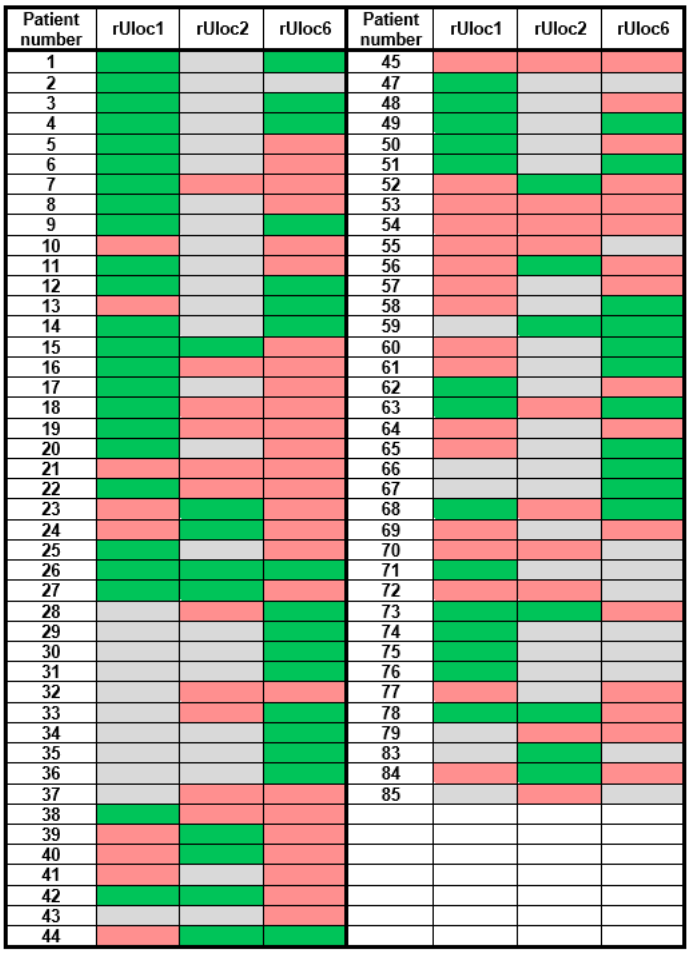
Figure S3



Patient number	rUloc1	rUloc2	rUloc6	Patient number	rUloc1	rUloc2	rUloc6
1				45			
2				47			
3				48			
4				49			
5				50			
6				51			
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11				56			
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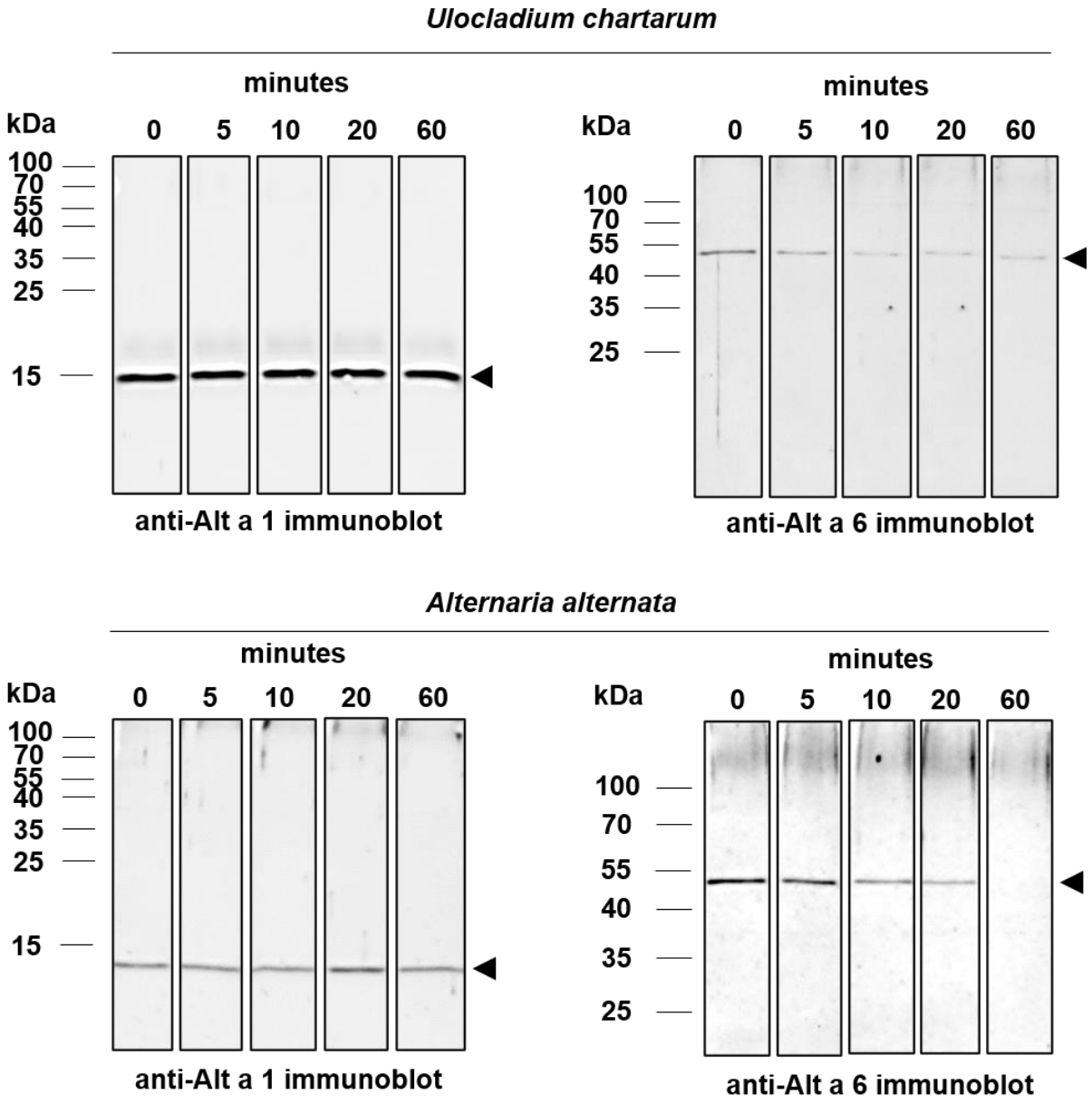
Figure S4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3. Paper II

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Authors contributions:

S.P. carried out the majority of the experiments described. M.R. performed the CD-spectroscopy. M.P., R.M.M.-C. and C.S.B. carried out the basophil activation assays and provided a part of the serum samples used in this study. S.Q. also provided serum samples. K.N. and E.R.-F. implemented the mass spectrometric analysis. M.F.-T. and K.S.-G. contributed to interpreting the results and planning the work by providing their scientific expertise. S.P. wrote the manuscript. I.S. supervised the project and contributed to the implementation of the research, to the analysis of the results and to the writing of the manuscript. All authors discussed the results and commented on the manuscript.

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LETTER

The emerging pathogen *Paecilomyces variotii* - a novel and important fungal allergen source

To the Editor,

Fungal allergy represents a worldwide public health burden.[1] Owing to their ability to colonize and germinate in the respiratory mucosa, fungi can have a far greater impact on the patients' immune system than other respiratory allergen sources.[1] However, the lack of knowledge about allergenic fungi is one of the factors that contribute to a general underdiagnosis of fungal allergy.[2] In the present study, we investigated the allergenic potential of *Paecilomyces variotii*, a heat-resistant mold ubiquitously found in soil, decomposing organic material, food products, and clinical samples, that is regarded as an emerging cause of serious, sometimes life-threatening, infections, and is often detected in the indoor environment.[3–6] Even though *P. variotii* has already been suggested as an aeroallergen source and occupational hazard, no allergens have yet been identified or characterized by this species.[7]

IgE immunoblots, performed with four different pools of sera from mold-sensitized patients (Table S1), showed the presence of several IgE-reactive proteins in *P. variotii* extract (Figure 1A), suggesting the species' high allergenic potential (no reaction was observed in immunoblots incubated with the detection antibody only—data not shown). Mass spectrometry analysis led to the identification of the first three *P. variotii* allergens, which interestingly all represent enzymes. Protein A (~36 kDa) was identified as a transaldolase, an enzyme that is already known as an allergen in different fungal species.[8] Protein B (~37 kDa) was identified as a glyceraldehyde 3-phosphate dehydrogenase, which represents an important allergen in various sources,[9] and Protein C (~48 kDa) was identified as an enolase. Enolases have also already been identified as highly conserved and cross-reactive allergens in various allergen sources including different fungi.[10,11] The proteins were submitted to the WHO/IUIS Allergen Nomenclature Subcommittee and were tentatively assigned the allergen names Pae v 1[a], Pae v 2[b], and Pae v 6[c]. The IgE-reactive protein bands marked D to H still have to be identified by peptide mass fingerprinting.

Full-length cDNAs coding for the *P. variotii* proteins Pae v 1, Pae v 2, and Pae v 6 were generated, cloned into a bacterial expression vector, and recombinant proteins were produced in *Escherichia coli* with a C-terminal hexa-histidine-tag. Reducing and non-reducing SDS-PAGEs (Figure S1) and immunoblots, performed with an anti-histidine-tag antibody, verified the purity of the recombinant proteins and showed that all proteins possess the capability to form disulfide bridges and to oligomerize under non-reducing conditions (Figure S1). Moreover, circular dichroism spectroscopy analysis of the recombinant allergens showed far-UV spectra with broad minima between 210 and 220 nm, which suggested the presence of both, alpha-helical structures, and beta-sheets, and indicated that the proteins represent properly folded molecules (Figure S2).

ELISAs performed with sera from mold-allergic patients showed the IgE-binding capacity of the three recombinant proteins. With OD values of more than 3.0 (Figure 1B), the enolase rPae v 6 showed the highest levels of IgE reactivity, whereas the IgE reactivities of rPae v 1 (maximum OD value of 0.8) and rPae v 2 (maximum OD value of 0.390) were considerably lower. Furthermore, IgE inhibition immunoblots, where nitrocellulose-blotted protein extracts from *P. variotii* were exposed to serum pools that had been pre-incubated with either one of the recombinant proteins or, for control purposes, with buffer only, showed that the recombinant allergens rPae v 1, rPae v 2, and rPae v 6 were able to significantly reduce patients' IgE-binding to their natural counterparts (Figure 1C). This indicates that the recombinant molecules contain the IgE-binding epitopes of their natural counterparts and suggests that rPae v 1, rPae v 2, and rPae v 6 could be used as tools for *in vitro* diagnosis of *P. variotii* sensitization. Pre-incubation with rPae v 2, a GAPDH, caused the reduction of IgE-binding to several proteins with molecular weights of 25–45 kDa. This suggests the presence of different Pae v 2 variants with different posttranslational modification and Pae v 2 degradation products in the natural fungal extract. The presence of GAPDH variants, which can also influence the molecule's different functions, has already been suggested in the literature.[12]

Analysis of the prevalence of IgE recognition in a cohort of patients, sensitized to different mold species, by ELISA revealed different frequencies of IgE reactivity. In Figure S3, the presence (in green) or the absence (in red) of IgE-binding to the recombinant allergens is displayed for each patient. rPae v 1 was recognized by 22% (11 out of 49) of the tested patients, rPae v 2 by 6% (3 out of 50), and 68% of the tested patients (39 out of 57) reacted with

[a]NCBI Nucleotide Sequence Accession Number: MN953053

[b]NCBI Nucleotide Sequence Accession Number: MN953054

[c]NCBI Nucleotide Sequence Accession Number: MN93795

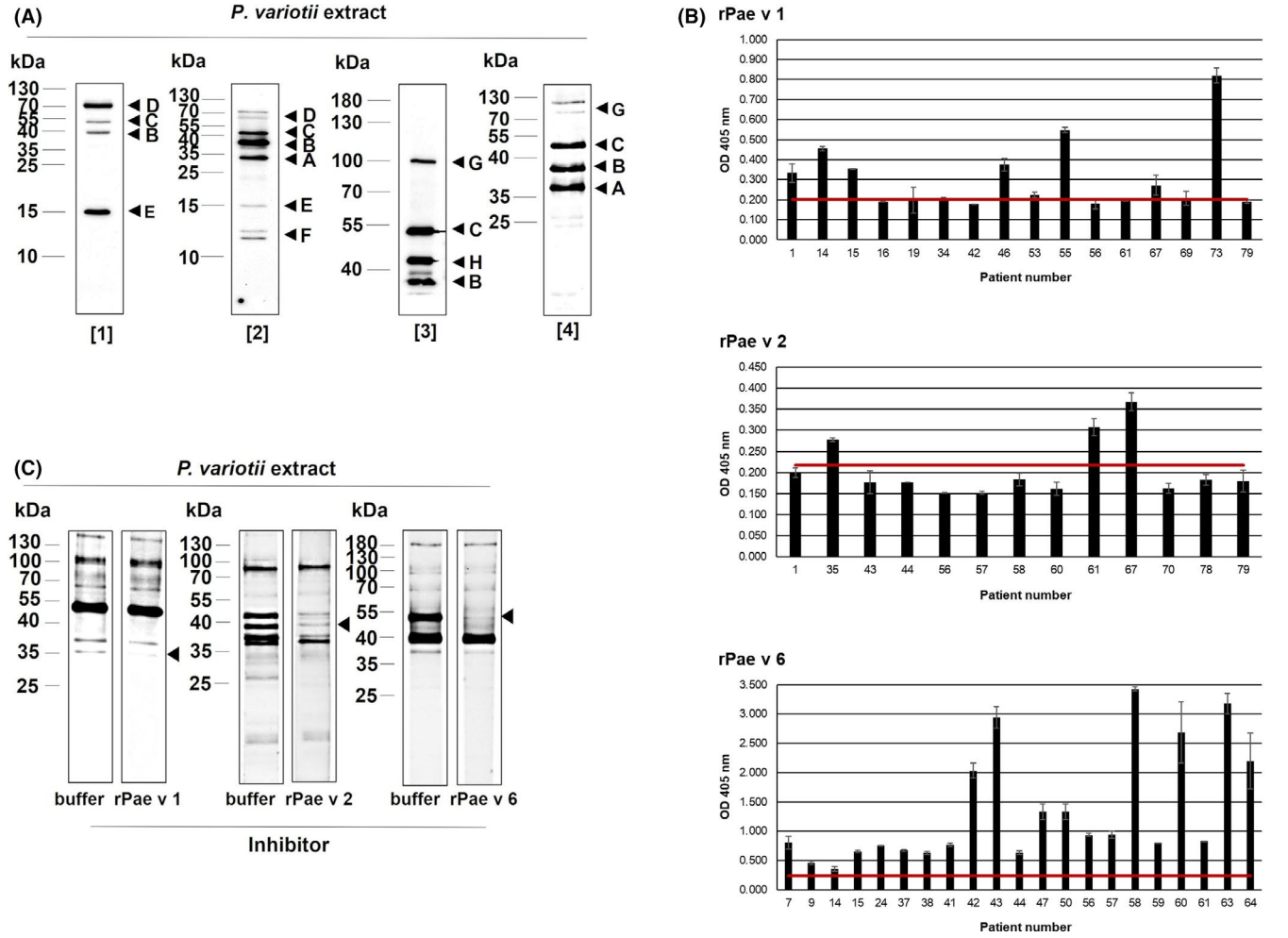

FIGURE 1 Identification and characterization of IgE-reactive proteins from *Paecilomyces variotii*. (A) Nitrocellulose-blotted proteins from *P. variotii* were exposed to four different pools of sera (pool 1: sera 1, 2, 26–28, and 65; pool 2: sera 3, 29–31, 40, 66, and 72; pool 3: sera 4–6, 48, 52, and 54; pool 4: sera 53, 56–61, 67, 77, and 78) from mold-allergic patients. IgE-reactive proteins identified by mass spectrometry are marked with A, B, and C; not yet identified bands are marked with D, E, F, G, and H. Molecular weight markers are indicated in the left margins. (B) Determination of IgE reactivity of rPae v 1, rPae v 2, and rPae v 6 by ELISA using sera from mold-sensitized patients. Results are displayed as mean OD values, and standard deviations from two technical replicates are indicated as error bars in the bar charts. The red line represents the assay's cutoff value, which was calculated from the mean OD values plus two times the standard deviation of sera from three non-allergic individuals. C, Inhibition immunoblots: blotted proteins in *P. variotii* extracts were exposed to different pools of sera (rPae v 1: sera 55 and 73; rPae v 2: sera 35, 39, and 61; and rPae v 6: sera 25, 57, 71, and 74–76), which had been pre-incubated with rPae v 1, rPae v 2, or rPae v 6, or, for control purposes, with buffer only. Molecular weight markers are indicated in the left margins. Arrows mark the molecular weights of the natural allergens Pae v 1, Pae v 2, and Pae v 6 in the fungal extract

rPae v 6. Among the patients tested with all three recombinant allergens, rPae v 1 was recognized by 22% (9 out of 41), rPae v 2 by 5% (3 out of 41), and rPae v 6 by 61% (25 out of 41) of the patients. Based on these results, rPae v 1 and rPae v 2 can be regarded as minor allergens of *P. variotii*, whereas rPae v 6 can be classified as a major mold allergen. Furthermore, evaluation of the allergenic activity of the recombinant major allergen rPae v 6 in basophil activation tests (BATs) showed a dose-dependent expression of CD63 in basophils from patient 4, suggesting the molecule's biological activity (Figure S4 in the supporting information).

The availability of an anti-enolase antibody allowed us to further investigate the release kinetics of the enolase Pae v 6 from *P. variotii* spores. The experiments showed that the spores from *P. variotii* immediately released this major allergen upon exposure to a humid milieu (Figure S5 in the supporting information). It can thus be expected that Pae v 6 would be rapidly secreted when the spores get in direct contact with the mucosa, leading to immediate allergic symptoms on the accessible mucosa surfaces such as the conjunctiva and the nose.

In conclusion, during this study the strong IgE reactivity of *Paecilomyces variotii* was demonstrated. Furthermore, the species first three allergens, tentatively designated Pae v 1, Pae v 2, and Pae v 6, were identified, recombinantly produced, and characterized regarding their biochemical and immunological features. It was shown that the three recombinant allergens represent IgE-reactive molecules, with Pae v 1 and Pae v 2 representing minor fungal

allergens, whereas Pae v 6 can be classified as a major fungal allergen. We are aware that the use of sera from patients allergic to known fungal allergen sources is a limitation of our study, because, in this way, it was much more likely to identify highly conserved, potentially cross-reactive allergens rather than species-specific allergenic molecules. Indeed, one of the three identified allergens is the highly conserved fungal pan-allergen enolase. However, since *P. variotii* has not yet been thoroughly investigated as an allergen source, it was not possible to overcome this limitation. Nevertheless, the obtained data together with the facts that exposure to *P. variotii* represents an important component of the indoor mycobiota[5] and is increasingly recognized as an important cause of infections[13] suggest that this species should be considered as a potential allergen source and should be included in routine fungal allergy diagnosis.

The description of the used methods can be found in the supporting information.

FUNDING INFORMATION

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CONFLICT OF INTEREST

All authors declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

METHODS

Patients' sera

Sera from patients allergic to fungi were obtained from the Department of Allergy, La Paz University Hospital, Madrid, Spain, from the Hospital Clinic de Barcelona, Immunology Department, CDB, IDIBAPS, University of Barcelona, Spain and from the company AbBaltis (FDA approved; Sittingbourne, United Kingdom). The demographic and serological characteristics of the patients are given in Table S1. Currently, *Paecilomyces variotii* is not included in routine diagnosis of fungal allergy. Therefore, it was not possible to focus on sera of *P. variotii* allergic individuals. Instead, patients with a sensitization to at least one fungal species used in routine diagnosis were included. All patients agreed to participate in this study and gave informed consent. This study was approved by the ethics committee of the La Paz University Hospital in Madrid (EK565/2007) and by the ethics committee of the Hospital Clínic de Barcelona (approval number 2011/6605). We confirm that all methods were performed in accordance with the relevance guidelines and regulations.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblot, inhibition immunoblot and dot blot experiments

Two µg of recombinant proteins and 5 µg of fungal protein extracts were separated on 8%, 12% or 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGEs) as described by Laemmli (S1) and were either stained with Coomassie-Brilliant Blue R250 (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) or transferred electrophoretically onto nitrocellulose membranes (0.2 µm; GE Healthcare, Chicago, IL) (S2). For IgE-immunoblots, membranes were washed and blocked with PBS containing 0.5%

Tween20 (PBS-T) and exposed to pools of sera from mold-allergic patients. These pools contained either 2, 3, 6, 7 or 10 sera (1:10 diluted in PBS-T). For IgE inhibition immunoblots, serum pools were pre-incubated with 20 µg of recombinant protein or, for control purposes, with PBS-T and then proteins, blotted onto nitrocellulose membranes, were incubated with these pre-incubated sera. For the detection of protein-bound human IgEs, a mouse anti-human IgE antibody, conjugated to horseradish peroxidase (HRP), was used (Southern Biotech, Birmingham, AL; final dilution 1:5,000). The blots were developed using the SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions and imaged using the UVP ChemStudio device (Analytik Jena, Germany).

For the detection of protein-bound histidine tags, nitrocellulose-blotted recombinant proteins were exposed to a monoclonal mouse anti-histidine tag antibody (Bio-Rad, Hercules, CA; diluted 1:1,000 in PBS-T) and a HRP-conjugated goat anti-mouse IgG secondary antibody (GE Healthcare; diluted 1:10,000 in PBS-T). To detect the enolase Pae v 6, 0.5 µg of protein extract were dotted onto a nitrocellulose membrane, which was incubated with a rabbit antiserum directed against the recombinant enolase from *Alternaria alternata* Alt a 6 (Charles River Laboratories, Écully, France). A goat anti-rabbit IgG HRP-labelled antibody (Vector Laboratories Inc, Burlingame, CA; diluted 1:10,000 in PBS-T) was used for detection and the blots were developed and imaged as described before. Furthermore, an immunodetection control was included for all immunoblotting experiments, where the blotted proteins were incubated with the detection antibodies only, followed by blot development and imaging as described before.

cDNA cloning and recombinant expression of *P. variotii* allergens

The full-length cDNAs encoding the *P. variotii* proteins were generated from *P. variotii* RNA by RT-PCR using the Access RT-PCR System from Promega and the primers listed in Table S2 (Microsynth, Balgach, Switzerland), which were synthesized based on whole genome shotgun sequences that had been submitted to GenBank with the following accession numbers: GAD95401 (Pae v 1), GAD92095.1 (Pae v 2) and GAD96380 (Pae v 6). The obtained sequences were submitted to GenBank and received the following accession numbers: MN953053 (Pae v 1), MN953054 (Pae v 2) and MN93795 (Pae v 6). cDNAs coding for Pae v 1, Pae v 2 and Pae v 6 were cloned with C-terminal hexa-histidine tags into the bacterial expression vector pET-17b. Following the successful expression of the allergens as soluble proteins in *E. coli*, the molecules were purified under native conditions by affinity chromatography.

Secondary structure analysis using circular dichroism spectroscopy (CD-spectroscopy)

Circular dichroism measurements of purified rPae v 1, rPae v 2 and rPae v 6 were carried out on a Chirascan Plus CD spectrophotometer from Applied Photophysics (Leatherhead, United Kingdom) in 10 mM sodium phosphate buffer (pH 8.0) at 20°C and a protein concentration of 0.2 µg/µL, using a quartz cuvette (Hellma Analytics, Müllheim, Germany) with a path-length of 1 mm. CD spectra were recorded from 190 to 280 nm with a resolution of 0.5 nm and results were the average of three scans. The final spectra were corrected by subtracting the buffer baseline spectrum, obtained under identical conditions, and normalized to the number of peptide bonds by using the extinction coefficient of the measured protein at 205 nm (ε_{205}) (S3). Results are expressed as mean residual ellipticity θ_{MRW} (deg cm^2 dmol^{-1}) at a given wavelength.

Enzyme-linked immunosorbent assay (ELISA) of recombinant fungal allergens

To analyze the IgE-reactivity of the recombinant proteins, ELISAs were carried out. Therefore, 96-well Nunc MaxiSorp ELISA plates (Thermo Fisher Scientific) were coated with 4 µg/mL of recombinant protein, diluted in bicarbonate buffer (pH = 9.6). Following an overnight incubation at 4°C, the plates were washed with PBS-T and afterwards blocked with PBS-T for 2.5 hours at 37°C. Wells were then incubated overnight at 4°C with individual patients' sera from mold-allergic patients (diluted 1:5 in PBS-T) or, for control purposes, with sera from three non-allergic individuals (1:5 diluted in PBS-T). Bound IgE antibodies were detected with a goat anti-human IgE HRP-labelled antibody from SeraCare Life Sciences Inc. (Milford, MA; diluted 1:2,500 in PBS-T) and using 1.8 mM of 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich), 60 mM citric acid, 77 mM $Na_2HPO_4(H_2O)_2$ and 3 mM H_2O_2 as a substrate. After 60 minutes of incubation in substrate solution, the optical density (OD) of the samples at a wavelength of 405 nm was measured, using the Multiskan FC Photometer from Thermo Fisher Scientific. Experiments were carried out in duplicates and the results are expressed as mean OD-values. The cut-off, used to distinguish IgE-reactive from non-reactive individuals, was calculated from the mean OD-values plus two times the standard deviation of sera from three non-allergic individuals.

Basophil activation test (BAT)

To test the biological activity of recombinant rPae v 6, basophil activation tests were performed using the Flow Cast Kit from Bühlmann (Schönenbuch, Switzerland) according to the manufacturer's instructions. Whole blood was exposed to increasing concentrations (1 to 1,000 ng/mL) of the recombinant allergen. Basophil activation was

assessed by detecting the expression of CD63 by flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ). Activated basophils were gated as $SSC^{low}/CCR3^{+}/CD63^{+}$. At least 400 basophils were measured, and the percentage of CD63-expressing basophils, based on the total amount of basophils, was calculated.

Allergen Release Kinetics Experiments

Clones of *P. variotii* were cultured on MEA-plates at 20-25°C for 7 days. Then 10 mL of a buffer that mimics the natural nasal fluid (160 mM NaCl, 30 mM KCl, 5 mM $MgCl_2(H_2O)_6$ and 2 mM $CaCl_2(H_2O)_2$) (S4) was added to the plates and spores were detached using a Drigalski-spatula. The spore solutions were transferred into centrifuge tubes and were incubated under continuous shaking at 20-25°C. Aliquots of 1 mL each were taken after 1, 5, 10, 20 and 60 minutes and were immediately centrifuged to separate the supernatants containing the released proteins from the fungal spores. The supernatants were stored at –20°C and the protein concentrations were determined using the method of Bradford with BSA as a standard (S5). For detection of released allergen, 0.5 µg of the supernatants were dotted onto nitrocellulose (as described before) and exposed to the anti-rAlt a 6 antiserum.

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FIGURE LEGENDS

FIGURE S1. Purity analysis and oligomerization potential of the recombinant *P. variotii* allergens. Two µg of the recombinant allergens rPae v 1, rPae v 2 and rPae v 6 were separated by 12% SDS-PAGE in the presence or in the absence of beta-mercaptoethanol. Proteins were either stained with Coomassie-blue or exposed to an antibody directed against the proteins' C-terminal hexa-histidine tag. Molecular weight markers are indicated in the left margins.

FIGURE S2. Circular dichroism spectra of purified, recombinant *P. variotii* allergens. CD-spectra were recorded for rPae v 1, rPae v 2 and rPae v 6 from 190 to 280 nm. Results shown are the average of three scans and are expressed as mean residual ellipticity θ_{MRW} (deg cm^2 dmol^{-1}) at a given wavelength (in nm).

FIGURE S3. IgE-reactivity profiles of mold-sensitized patients to different recombinant allergens. The binding of IgE antibodies from mold-sensitized patients to

rPae v 1, rPae v 2 and rPae v 6 was measured by ELISA and data are summarized in an IgE-reaction map. The optical density was measured in duplicates and the mean OD-value of the negative controls plus two times the standard deviation was set as a threshold for a positive reaction, indicated here in green. No significant reaction is shown in red, whereas grey coloring indicates that the IgE-reactivity was not evaluated.

FIGURE S4. Analysis of biological activity of the major *P.variotii* allergen Pae v 6. Basophil activation of rPae v 6 was determined by measuring the expression of CD63 by flow cytometry after incubation of the whole blood from an *Alternaria*-sensitized patient (4) and from two non-atopic control individuals (control 1, control 2) with increasing concentrations of rPae v 6. The percentage of CD63 positive basophils (y-axis), based on the total amount of basophils, is displayed against the concentration of the applied allergen (x-axis).

FIGURE S5. Analysis of release kinetics of the major *P.variotii* allergen Pae v 6. Proteins released from *P. variotii* spores after different times of hydration of the spores (1-60 minutes) were dotted onto a nitrocellulose membrane and exposed to an anti-rAlt a 6 antiserum.

Table S1. Summary of demographic and serological data of mold-allergic patients grouped based on their IgE-reactivity profiles (n = 84). Levels of total IgE (in kU/L) and specific IgE (sIgE; in kUA/L) against different mold species were determined by ImmunoCAP analysis.

Patient numbers	Allergen source	Age (yr)	Total IgE [kU/L]	sIgE mold [kUA/L]
1-25	*Alternaria*	2-83	25-646	1.5-66.6
26-39	*Aspergillus*	12-74	185-1874	2.1-26.7
40-51	*Cladosporium*	4-42	249-3625	3.6-23.6
52-53	*Penicillium*	4-13	66-291	4.0-21.7
55	*Alternaria* *Aspergillus*	75	862	*Alternaria*: 3.9 *Aspergillus:* 1.9
56-64	*Alternaria* *Cladosporium*	7-36	108-2229	*Alternaria:* 4.0- >100 *Cladosporium:* 0.9-80.1
65-69	*Aspergillus* *Cladosporium*	9-11	84-924	*Aspergillus:* 4.5-29.0 *Cladosporium:* 7.5-24.7
70-76	Various fungal species	6-48	121-1947	*Alternaria:* 1.1-67.6 *Aspergillus:* 0.01-21.1 *Cladosporium:* 0.01-29.1 *Penicillium:* 0.01-9.6
77-84*	N/A	N/A	N/A	N/A

*N/A, information not available; *sera were obtained from mold-allergic patients with unknown sensitization profiles*

Table S2. Sequences of the specific primers used for cDNA-cloning. Restriction sites of *Nde*I are underlined and sites of *EcoR*I are shown in italics. Sequences coding for the C-terminal hexa-histidine tag are marked in bold.

Primer	Nucleotide sequence in 5'-3'-end orientation
Pae v 1 Forward	GGTGGTCATATGGCTTCCGCTCTCGACCAGCTC
Pae v 1 Reverse	GGTGGT*GAATTC*TTA**GTGATGATGATGATGATG**CGCGGTGATCTTCTGGCGCAG
Pae v 2 Forward	GGTGGTCATATGGTTAACGCTAAGATTGGTATC
Pae v 2 Reverse	GGTGGT*GAATTC*CTA**GTGATGATGATGATGATG**GGCGGAACCGGCGTCGACCTTG
Pae v 6 Forward	GGTGGTCATATGCCTATCACCAAGATCCACGCT
Pae v 6 Reverse	GGTGGT*GAATTC*CTA**GTGATGATGATGATGATG**CAGGTTAATGGAGTTGCGGAA

Figure S1

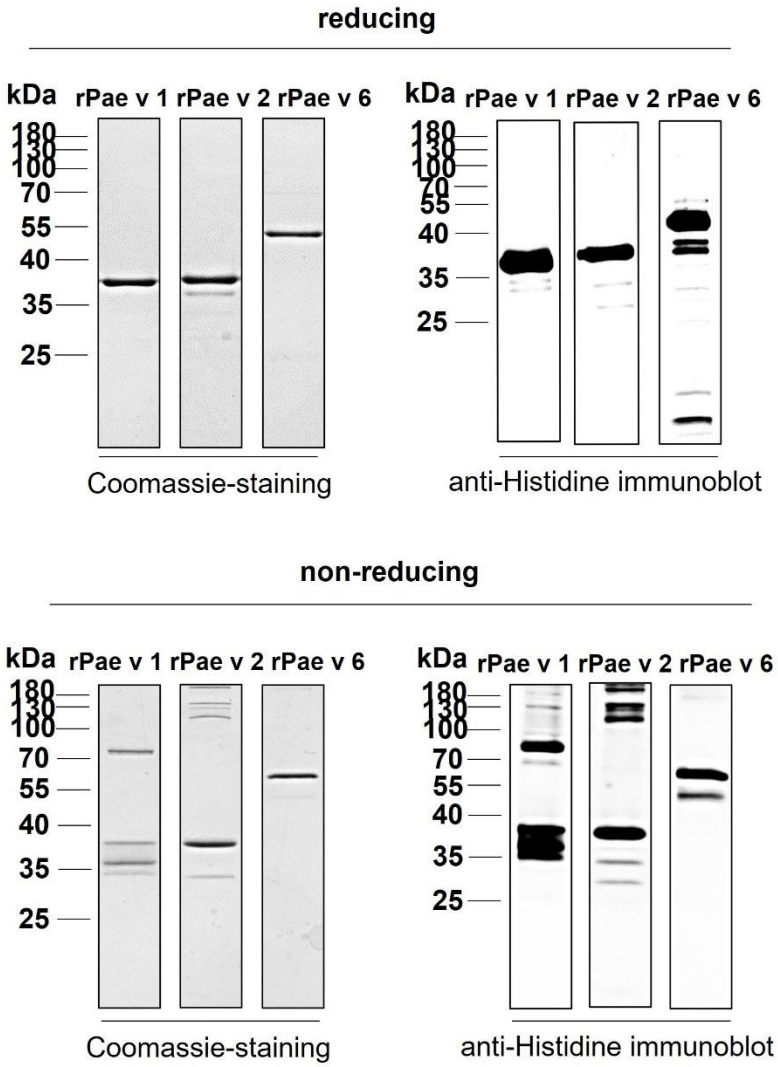
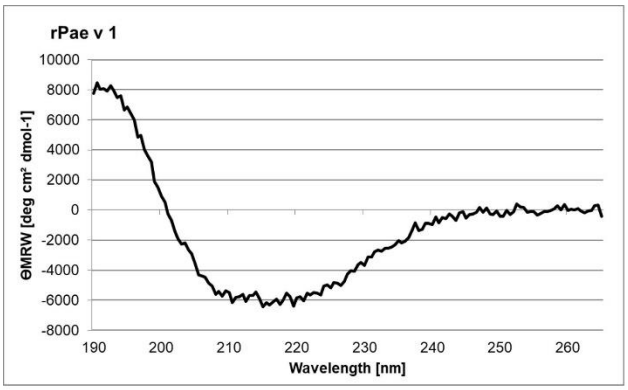
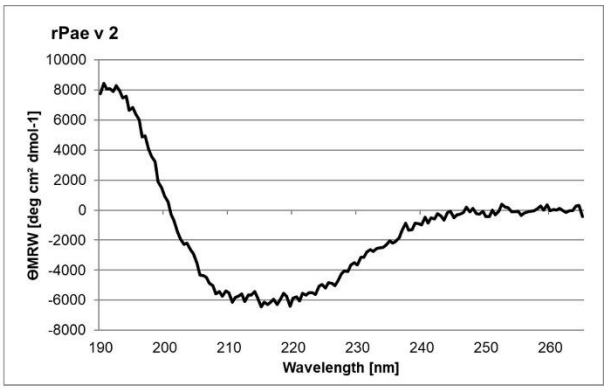

reducing
kDa
rPae v 1
rPae v 2
rPae v 6
180
130
100
70
55
40
35
25
Coomassie-staining
anti-Histidine immunoblot
non-reducing

Figure S2


rPae v 1
ΘMRW [deg cm² dmol-1]
10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
190
200
210
220
230
240
250
260
Wavelength [nm]


rPae v 2
ΘMRW [deg cm² dmol-1]
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8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
190
200
210
220
230
240
250
260
Wavelength [nm]


rPae v 6
ΘMRW [deg cm² dmol-1]
20000
15000
10000
5000
0
-5000
-10000
-15000
-20000
190
200
210
220
230
240
250
260
Wavelength [nm]

Figure S3

Patient number	rPae v 1	rPae v 2	rPae v 6	Patient number	rPae v 1	rPae v 2	rPae v 6
1				44			
4				45			
7				46			
8				47			
9				50			
10				51			
11				53			
12				55			
13				56			
14				57			
15				58			
16				59			
17				60			
18				61			
19				62			
20				63			
21				64			
22				67			
23				68			
24				69			
26				70			
30				71			
32				73			
33				74			
34				77			
35				78			
36				79			
37				80			
38				81			
41				82			
42				83			
43				84			

Figure S4

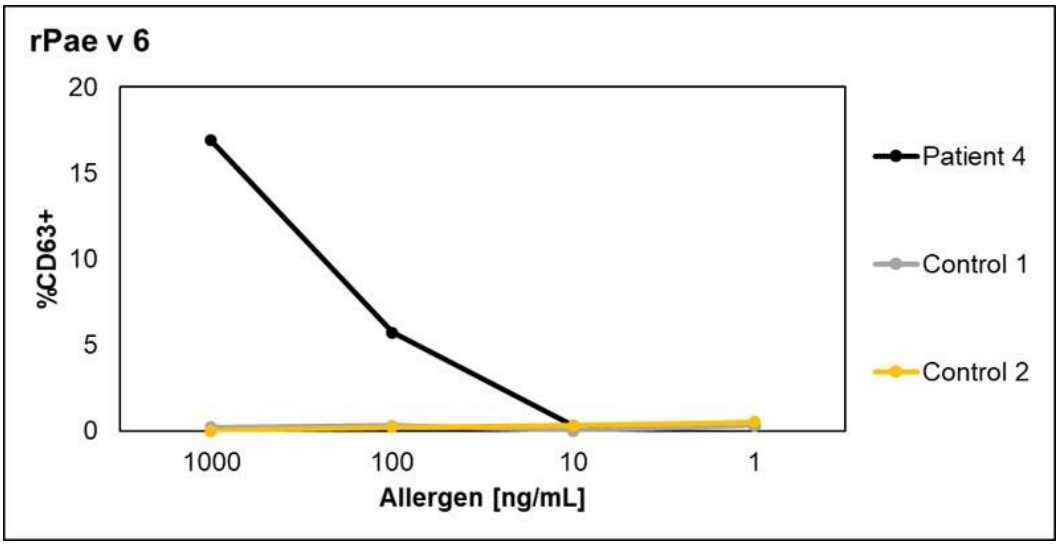
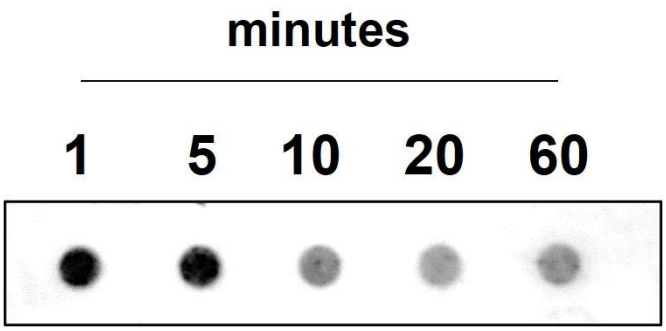

rPae v 6
20
15
10
5
0
%CD63+
1000
100
10
1
Allergen [ng/mL]
Patient 4
Control 1
Control 2

Figure S5



4. Unpublished Manuscript I

Bibliography:

Pfeiffer S, Sperl CM, Nöbauer K, Razzazi-Fazeli E, Focke-Tejkl M, Sterflinger K and Swoboda I.

“Analysis of the cross-reactive potential of the enolase pan-allergen”

Authors contributions:

S.P. carried out the majority of the experiments described. CM.S. carried out valuable preliminary experiments. K.N. and E.R.-F. implemented the mass spectrometric analysis. M.F.-T. and K.S.-G. contributed to interpreting the results and planning the work by providing their scientific expertise. S.P. wrote the manuscript. I.S. supervised the project and contributed to the implementation of the research, to the analysis of the results and to the writing of the manuscript.

Analysis of the cross-reactive potential of the enolase pan-allergen[1]

Introduction

Enolase, also known as phosphopyruvate hydratase (EC 4.2.1.11), was discovered in 1934 by Lohman and Mayerhof (1) and represents a glycolytic enzyme that catalyzes the interconversion of 2-phospho-D-glycerate and phosphoenolpyruvate in the last steps of the catabolic glycolytic pathway (2). Recent findings have shown that enolase performs several functions in addition to its innate glycolytic function and plays an important role in several biological and pathophysiological processes in organisms, such as the regulation of transcription, cell differentiation, thermal tolerance, and growth control (2–5).

Besides the enzyme's vital functions within an organism, enolase has been identified as an allergen in various sources with 16 allergenic enolases from different sources being listed by the Allergen Nomenclature Sub-committee of the International Union of Immunological Societies (IUIS) (6) (November 2021). Since its first identification as an allergen in *Saccharomyces cerevisiae* by Baldo and Baker (7), it has been recognized as an important respiratory allergen in several fungal species including the well-studied allergenic genera *Alternaria* (8,9), *Aspergillus* (10), *Cladosporium* (8) and *Penicillium* (10). Moreover, it is not only a recognized allergen from other respiratory sources including pollen of several plants (11–14), but was also identified as an allergen from animal-derived foods such as chicken (15), cod, salmon, and tuna (16). In general, approximately 20% of mold-allergic patients show a positive IgE-reaction to enolases, which classifies these proteins as minor allergens (10,17–19). However, fungal enolases seem to have a high diagnostic value as several authors have demonstrated that these allergens play an important role in the cross-reactivity phenomenon among several fungi (9,10,18,20).

Since enolases are phylogenetically highly conserved enzymes, it seems likely that sequence similarities could form the basis for their immunological cross-reactivity (2,3,21–23). Consequently, enolases have been suggested to represent pan-allergens (24,25). The Greek prefix "pan" meaning "all", emphasizes the ubiquitous occurrence of these molecules in living organisms (26). However, the cross-reactivity between enolases from distantly related

[1] Unpublished data

organisms is largely unknown and has so far only been described between latex and the fungal species *Alternaria alternata* and *Cladosporium herbarum*, suggesting a "latex-mold-syndrome" (27).

The recognition of cross-reactive molecules can lead to the well-known phenomenon of polysensitization, which is frequently encountered in clinical practice, as many patients display allergic reactions upon contact with multiple allergen sources (26,28). The existence of cross-reactivity may have implications for the diagnosis and treatment of allergies as it often complicates the identification of the primary sensitizer species and causes difficulties in the management of allergic diseases (24,29). Thus, from both, diagnostic and treatment perspectives, it is important that polysensitization resulting from allergen cross-reactivity is distinguished from co-sensitization to multiple allergen sources (29).

The purpose of this study was to investigate the cross-reactive potential of enolases from different allergen sources including molds, plant pollen and animal-derived foods with the aim to gain more information about the enzymes' pan-allergenic character. Therefore, IgE-reactivity to proteins from various animal-derived foods and plant pollen and to recombinant mold enolases was analyzed in a cohort of polysensitized patients by IgE immunoblots and ELISAs. In addition, IgE cross-reactivity between enolases was analyzed by inhibition experiments. Results obtained demonstrate the high sequence homology of enolases from different organisms and show that not only fungal enolases, but also enolases from non-related species are recognized by an antibody directed against the fungal enolase rAlt a 6. Furthermore, it was seen that fungal enolases possess cross-reactive, but also species-specific IgE-epitopes, whereas enolases from different animal-derived food and pollen sources lack IgE cross-reactivity with rAlt a 6. Information obtained in this study will contribute to a better understanding of the cross-reactive potential of enolases.

Methods

Fungal RNA extraction and cDNA cloning of fungal allergens into bacterial expression vector

Clones of *Paecilomyces variotii*, *Aspergillus fumigatus*, *Cladosporium herbarum* and *Penicillium chrysogenum* were cultured on malt extract agar-plates (= MEA, pH 5.0) containing 2% malt extract, 2% glucose and 1.5% agar-agar at 20-25°C for 7 to 14 days. In case of *P. variotii* and *C. herbarum*, fungal mycelium and spores were harvested from the agar-plates using a

sterile scalpel and ground in liquid nitrogen to a fine powder using a pre-cooled mortar and pestle. Total RNA was then extracted using the innuSPEED Bacteria/Fungi RNA Kit (Analytik Jena AG, Jena, Germany) according to the manufacturer's instructions, followed by RNA purification with the GeneJET RNA Cleanup and Concentration MicroKit (Thermo Fisher Scientific, Waltham, MA). In comparison, fungal mycelium and spores from *A. fumigatus* and *P. chrysogenum* were harvested with a sterile scalpel, transferred into MK28 2 mL Precellys tubes (Bertin Technologies SAS; Nontigny-le-Bretonneux, France), followed by cell lysis by applying three times 4,500 rpm for 20 seconds with 30 seconds of pause between each round using the Precellys Evolution Tissue Homogenizer (Bertin Technologies SAS) and RNA extraction was performed with the NucleoSpin RNA Plant and Fungi Kit (Macherey Nagel, Düren, Germany). The extracted RNA was then purified as described before with the GeneJET RNA Cleanup and Concentration MicroKit.

The cDNAs encoding the fungal proteins were generated by reverse transcription polymerase chain reactions (RT-PCR) using the Access RT-PCR System from Promega (Madison, WI) and the primers listed in **Table 1**. Forward primers always introduced a recognition site for the restriction enzyme *Nde*I, whereas reverse primers added the coding sequence of a C-terminal hexa-histidine tag and the recognition site for the restriction enzyme *EcoR*I.

Table 1. Sequences of the specific primers used for cDNA-cloning. Restriction sites of *Nde*I are shown in italics and sites of *EcoR*I are underlined. In addition, the sequences encoding the C-terminal hexa-histidine tag are marked in bold.

Primer	Nucleotide sequence in 5'-3'-end orientation
Pae v 6 Forward	GGTGGT*CATATG*CCTATCACCAAGATCCACGCT
Pae v 6 Reverse	GGTGGTGAATTCCTA**GTGATGATGATGATGATG**CAGGTTAATGGAGTTGCGGAA
Asp f 22 Forward	GGTGGT*CATATG*CCTATCTCCAAGATCCACGCTCGTTCCG
Asp f 22 Reverse	GGTGGTGAATTCTTA**GTGATGATGATGATGAT**GCAGGTTGACGGCAGTGCGGAACTTGG
Cla h 6 Forward	GGTGGT*CATATG*CCTATCTCCAAGATCCACTCC
Cla h 6 Reverse	GGTGGTGAATTCTTA**GTGATGATGATGATGAT**GCAAGTTGATGGCAGTGCGGAA
Pen c 6 Forward	GGTGGT*CATATG*CCTATCTCTAAGATCCACGC
Pen c 6 Reverse	GGTGGTGAATTCTTA**GTGATGATGATGATGAT**GCAGGTTGACAGCAGTGCGG

Primers for the cDNAs of the different enolases were designed based on the nucleotide sequence retrieved from the National Center for Biotechnology Information (NCBI). The accession numbers were the following: GAD96380 (Pae v 6), AF284645.1 (Asp f 22), X78226.1

(Cla h 6) and AB091508.1 (Pen c 6). The RT-PCR products were finally sub-cloned into the bacterial expression vector pET-17b (Novagen-Merck, Darmstadt, Germany) and nucleotide sequences were confirmed by Sanger Sequencing (Microsynth, Balgach, Switzerland) using standard primers.

For the enolase from *Alternaria alternata* (Alt a 6; UniProt accession number: Q9HDT3), a cDNA codon-optimized for expression in *Escherichia coli* was synthesized by GenScript Biotech (Piscataway Township, NJ) and cloned into the expression vector pET-17b.

In silico analysis of amino acid sequence homology

To obtain information about sequence homologies of enolases from different sources, the deduced amino acid sequences of Pae v 6, Asp f 22, Cla h 6 and Pen c 6 were compared with the amino acid sequences of Alt a 6, of the enolases from *Oncorhynchus mykiss* (rainbow trout; NCBI GenBank accession number: OK349682[2]), *Salmo salar* (salmon; UniProt accession number: B5DGQ7), *Thunnus albacares* (tuna; UniProt accession number: I0J1J1), *Gallus domesticus* (chicken; UniProt accession number: P07322), *Phleum pratense* (timothy grass; UniProt accession number: K6Z748), *Ambrosia artemisiifolia* (short ragweed; UniProt accession number: A0A1B2H9Q1) and of the human enolase (UniProt accession number: P06733) with the alignment software Clustal Omega (30). Resulting percentages of sequence identities were then used to create a sequence identity map.

Recombinant protein expression and purification of enolases

Recombinant protein expression was carried out for Alt a 6, Pae v 6, Asp f 22, Cla h 6, Pen c 6 and for the enolase from rainbow trout (*Oncorhynchus mykiss*). The cDNA coding for the rainbow trout enolase had been generated based on the homologous sequence of the enolase from salmon, cloned into pET-17b and was already available in the laboratory. For the expression of recombinant enolases, the cDNA encoding plasmids were transformed into the *E. coli* strain BL21-DE3 and recombinant protein expression was induced at 37°C by the addition of 5 mM isopropyl beta-D-1-thiogalactopyranoside. Proteins were extracted from the bacterial cells under native conditions, using the freeze-thaw lysis method described by Johnson et al. (31) and afterwards purified by immobilized metal ion affinity chromatography (IMAC) using Protino Nickel-Agarose (Macherey-Nagel). Proteins were eluted with a buffer

[2] Nucleotide sequence has been submitted to the GenBank database.

containing 150-250 mM imidazole and were dialyzed against 10 mM sodium phosphate buffer (pH 7.0-8.0).

The *P. variotii* protein Pae v 6 was further purified by IMAC using HisTrap HP columns (GE Healthcare, Chicago, IL) on the Äkta Start Protein Purification System (GE Healthcare). The protein was eluted using a linear imidazole gradient (20-500 mM) and again dialyzed against 10 mM sodium phosphate buffer (pH = 8.0). The protein concentrations were determined with the MicroBCA Protein Assay Kit (Thermo Fisher Scientific) using bovine serum albumin (BSA) as a standard and the purity of the proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie-staining of the protein gels.

Preparation of protein extracts

Fungal protein extracts were prepared from fungal mycelium and spores (cultivated as described for the preparation of RNA extracts), which were harvested from the agar-plates using a sterile scalpel and then transferred into sterile MK28 2 mL Precellys tubes (Bertin Technologies SAS). Afterwards phosphate buffered-saline (PBS) containing complete EDTA-free protease inhibitor tablets (Roche Diagnostics, Mannheim, Germany; used according to the manufacturer's instructions) was added and the material was lysed using the Precellys Evolution Tissue Homogenizer (Bertin Technologies SAS) by applying three times 4,500 rpm for 20 seconds with 30 seconds of pause between each round. Following an overnight incubation at 4°C under continuous shaking and a 10-minute centrifugation-step at 4,000 g, the supernatants were stored at -20°C and the protein concentrations of the extracts were determined using the method of Bradford with BSA as a standard (32).

Protein extracts from various fish and meat including chicken leg, chicken breast, salmon, tuna, trout, and shrimp were prepared by cutting the flesh into small pieces, which were either boiled for 10 minutes in water or processed in raw form and transferred into a pre-cooled mortar. The materials were then ground to a fine powder under liquid nitrogen using a pestle and transferred into a sterile centrifugal tube, followed by the addition of 5 mL PBS per g meat. After an overnight incubation at 4°C under continuous shaking, the extracts were centrifuged at 4,000 g and 4°C for 30 minutes and the supernatants were filtered through a sterile filter with a pore-size of 0.45 µm (Sarstedt AG & Co. KG, Nümbrecht, Germany). The filtrates were

stored at -20°C and the protein concentrations of the extracts were determined as described before.

For the preparation of protein extracts from plant pollen, 10 mL of ultrapure water or PBS was added to 1 g of pollen and incubated overnight at 4°C under continuous shaking. Afterwards, the samples were centrifuged at 4,000 g and 4°C for 10 minutes and the supernatants were filtered using a 0.45 µm pore-size sterile filter (Sarstedt AG & Co. KG, Germany). The filtrates were stored at -20°C and the protein concentrations of the extracts were determined as described before.

Patient sera

Polysensitized patients with IgE antibodies to several allergen sources were included in this study. Sera from allergic patients were obtained from the company AbBaltis (FDA-approved; Sittingbourne, United Kingdom) and the demographic and serological characteristics of the patients are given in **Table 2**.

Table 2. Summary of demographic and serological data of polysensitized patients (n = 12). Total IgE (IgE in kIU/L) and specific IgE (sIgE; in kUA/L) against different allergen sources were determined by ImmunoCAP analysis.

Patient number	Age (yr)/gender	Allergen source	sIgE [kUA/L]	IgE total [kIU/L]
1	53/M	Almond	11.7	N/A
		Peanut	57.8	
		Hazelnut	65.5	
		Brazil nut	21.2	
		Cashew nut	37.3	
		Hazelnut	76.4	
		Walnut	49.3	
		Pistachio	49.1	
		Soybean	56.0	
		Buckwheat	4.3	
		Anchovy	1.6	
		Watermelon	1.9	
		Pear	2.0	
		Cherry	1.2	
		Cacao	1.1	
		Maize flour	1.9	
		Cocksfoot	28.5	
		Chilly	4.0	
		Hazel	23.7	
		Redtop, bentgrass	55.2	
		Sweet Vernal Grass	55.6	
		Rye	42.3	
		Timothy Grass	49	
		Common Ragweed	28.4	
		American Beech	27.7	
		Tyrophagus putrescentiae	9.22	

		Aspergillus fumigatus	4.8	
		Cod	20.8	
		rCyp c 1 (carp)	21.4	
		Trout	26.1	
		Mackerel	2.3	
		Salmon	11.7	
2	49/M	Hazelnut	68.9	14485
		Peanut	77.5	
		Almond	10.3	
		Brazil nut	18.0	
		Cashew nut	35.4	
		Pistachio	34.3	
		Soybean	5.6	
		Avocado	5.0	
		Peach	5.8	
		Hamster epithelium	19.1	
		Rabbit	10.6	
		Cockroach	12.0	
		Mosquito	56.2	
		Horse dander	56.2	
		Cat dander	35.9	
		Latex	21.6	
		Redtop, bentgrass	48.7	
		Timothy Grass	24.6	
		Penicillium chrysogenum	6.9	
		Cladosporium herbarum	9.7	
		Aspergillus fumigatus	10.8	
		Rhizopus nigricans	4.2	
		Egg White	10.2	
		Egg Yolk	30.7	
		Clam	12.4	
		Blue Mussel	7.5	
		Shrimp	30.5	
3	47/m	Cockroach	35.3	N/A
		Alternaria alternata	12.7	
		Candida albicans	3.2	
		Fusarium proliferatum	4.1	
4	48/M	*Alternaria alternata*	6.6	356
		Dust mite	2.7	
5	56/M	Peach	1.6	N/A
		Celery	4.1	
		Cat dander	100	
		Horse dander	38.0	
		Tyrophagus putrescentiae	46.0	
		Dog dander	23.7	
		Storage mite	71.9	
		Guinea Pig	47.1	
		Rabbit	6.4	
		Dust mite	100	
		Common ragweed	48.9	
		Mugwort	26.5	
		Timothy Grass	17.1	
		Redtop, bentgrass	12.6	
		Common Silver Birch	10.0	
		Willow	12.6	
		Plantain Ribwort	13.1	
		Wheat	1.29	

		Cypress Pollen	2.9	
		Olive Tree	30.3	
		Grey Alder	9.9	
		Hazel	5.2	
		Oak	9.5	
		Maple Leaf Sycamore	10.5	
		European Ash Pollen	6.2	
		Penicillium chrysogenum	9.1	
		Cladosporium herbarum	8.1	
		Malassezia spp.	21.7	
		Candida albicans	27.6	
6	53/F	Peanut	1.1	N/A
		Coconut	1.2	
		Dust mite	23.8	
		Dog dander	47.7	
		Cat dander	11.2	
		Cow dander	4.5	
		Storage mite	6.8	
		Tyrophagus putrescentiae	10.2	
		Dermatophagoides pteronyssinus	35.2	
		Dermatophagoides farinae	22.5	
		Grey Alder	1.8	
		Timothy Grass	3.8	
		Cypress Pollen	1.1	
		Cladosporium herbarum	3.6	
		Pork	1.9	
7	31/M	*Cladosporium herbarum*	3.4	N/A
		Aspergillus fumigatus	4.3	
8	N/A	Cat dander	26.6	762
		Cladosporium herbarum	5.7	
		Aspergillus fumigatus	22.5	
		Alternaria alternata	23.3	
9	N/A	Apple	5.4	523
		Pear	5.0	
		Cherry	4.6	
		Common ragweed	1.6	
		Olive Tree	2.1	
		Bahia Grass	10.8	
		Timothy Grass	22.7	
10	N/A	Pistachio	10.4	N/A
		Cashew nut	2.1	
		Cockroach	14.1	
		Mugwort	2.2	
		Aspergillus fumigatus	5.0	
		Cladosporium herbarum	30.8	
		Egg White	13.5	
		Shrimp	72.7	
		Lobster	77.1	
		Cod	2.6	
		Mackerel	2.4	
		Mosquito	14.7	
11	35/F	Hazelnut	43.6	N/A
		Peanut	18.5	
		Anchovy	12.7	
		Avocado	9.9	
		White Ash	1.7	

		Cladosporium herbarum	1.3	
		Alternaria alternata	1.3	
		Cod	21.2	
		Crab	11.0	
		Shrimp	15.7	
		Lobster	12.6	
		Salmon	17.9	
		Mackerel	6.3	
12	21/F	*Alternaria alternata*	4.5	209

M, male; F, female; N/A, information not available

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Coomassie-staining, immunoblot and inhibition immunoblot experiments

Two µg of recombinant proteins or 10 µg of protein extracts were separated by 12% SDS-PAGEs, as described by Laemmli (33), and were either stained with Coomassie-Brilliant Blue R250 (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) or transferred electrophoretically onto nitrocellulose membranes (0.2 µm; GE Healthcare) (34).

For the detection of enolases in the different species, membranes were washed and blocked with PBS containing 0.5% Tween20 (PBS-T) and exposed to a rabbit antiserum directed against the recombinant *A. alternata* enolase rAlt a 6 (Charles River Laboratories, Écully, France) (1:5,000 diluted in PBS-T). For analysis of the specificity of the antibody binding to proteins present in the extracts, inhibition immunoblots were performed. For this, the antiserum was pre-incubated overnight with 20 µg of rAlt a 6 or, for control purposes, with PBS-T and then added to the blotted proteins. For the detection of protein-bound antibodies, a goat anti-rabbit IgG antibody (Vector Laboratories Inc, Burlingame, CA; diluted 1:10,000 in PBS-T), conjugated to horseradish peroxidase (HRP), was used as detection antibody. The blots were developed using the SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific) according to the manufacturer's instructions and imaged using the UVP ChemStudio device (Analytik Jena, Germany).

For IgE-immunoblots, nitrocellulose-blotted proteins were exposed to individual sera from polysensitized patients (1:10 diluted in PBS-T). For IgE inhibition immunoblots, sera were pre-incubated overnight with 20 µg of rAlt a 6 or, for control purposes, with PBS-T and then added to the blotted proteins. For the detection of protein-bound human IgEs, a mouse anti-human IgE antibody, conjugated to HRP, was used as detection antibody (diluted 1:5,000 in PBS-T;

Southern Biotech, Birmingham, AL) and the blots were developed and imaged as described above for the immunoblots performed with the anti-Alt a 6 serum.

Liquid chromatography-mass spectrometric (LC-MS) analysis of IgE-reactive proteins

Proteins recognized by human IgEs were excised manually from Coomassie Blue-stained SDS-PAGE gels and in-gel digestion was performed with 20 ng/µL trypsin (Promega). Extracted peptides were then dried down in a vacuum concentrator and afterwards resuspended in 0.1% trifluoroacetic acid and injected into a nano-HPLC Ultimate 3000 RSLC system (Dionex from Thermo Fisher Scientific) and separated on a 25 cm Acclaim PepMap C18 column (Thermo Fisher Scientific). The LC was directly coupled to a high-resolution Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific) and the database search was performed using the Proteome Discoverer Software 2.4.0.305 (Thermo Fisher Scientific).

Enzyme-linked immunosorbent assay (ELISA) and inhibition ELISAs of recombinant enolases

To evaluate the IgE-reactivity of the recombinant enolases rAlt a 6, rAsp f 22, rPen c 6, rPae v 6 and rCla h 6, ELISAs were carried out. For this, 96-well nunc maxisorp ELISA plates (Thermo Fisher Scientific) were coated with 4 µg/mL of the recombinant proteins, diluted in bicarbonate buffer (pH = 9.6). Following an overnight incubation at 4°C, the plates were washed and blocked with PBS-T for 2.5 hours at 37°C. Wells were then incubated overnight at 4°C with sera from polysensitized patients (1:5 diluted in PBS-T) or, for control purposes, with sera from three non-allergic individuals (1:5 diluted in PBS-T) or with PBS-T only. In case of IgE-inhibition ELISA experiments, patients' sera were pre-incubated overnight at 4°C with 20 µg of a recombinant enolase. Bound IgE antibodies were detected with a HRP-labelled goat anti-human IgE antibody (SeraCare Life Sciences Inc., Milford, MA; diluted 1:2,500 in PBS-T) and using 1.8 mM of 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich, St.Louis, MO) in 60 mM citric acid, 77 mM $Na_2HPO_4(H_2O)_2$ and 3 mM H_2O_2 as a substrate. After 60 minutes of incubation in substrate solution, the optical density (OD) of the samples was measured at a wavelength of 405 nm using the Multiskan FC Photometer (Thermo Fisher Scientific). Experiments were always carried out in duplicates and the results are expressed as mean OD-values. The cut-off, used to differentiate positive and negative IgE-reactivity, was calculated from the mean OD-values plus two times the standard deviation from results obtained with sera from three non-allergic patients.

To determine the levels of allergen-specific IgE antibodies in the sera, quantitative ELISAs were performed with human IgE-standards of different concentrations. For the generation of the standard curve, wells were coated with the major birch pollen allergen rBet v 1, which was available in the laboratory, and incubated with defined concentrations (10, 5, 2.5, 1.25, 0.675, 0.338, 0.169 and 0 ng/mL) of an anti-Bet v 1 human IgE antibody (Absolute Antibody, Cleveland, United Kingdom). Protein-bound IgE antibodies were then detected with the HRP-labelled anti-human antibody. Based on the measurements obtained with the different anti-Bet v 1 IgE antibody levels, a standard curve was generated, which allowed calculating the allergen specific IgE antibody levels (in kU/L) in the sera.

Results

Enolases from unrelated organisms show high sequence homology and are detected by an antibody raised against a fungal enolase

Functionally related molecules from unrelated organisms must share highly conserved sequence regions or three-dimensional structures to fulfill the requirements for potential IgE cross-recognition (26). To obtain information about the sequence homologies between enolases from different sources, the amino acid sequences of various enolases derived from fungi, plants, foods, and human were compared using the alignment software Clustal Omega. Resulting percentages of sequence identities were then used to create a sequence identity map, shown in **Figure 1**.

Enolase sequences of related organisms, for example of different fungal species, show a high identity of at least 85%. Moreover, enolase sequences of various animal-derived foods show a comparably high sequence identity of at least 79%, and sequences of plant enolases are at least 87% identical. Sequence identities between enolases from non-related species were variable, ranging from 60% homology between ragweed and fungal enolases to 72% homology between grass pollen and human enolases. Interestingly, high amino acid sequence identities of 88% were seen between tuna and human enolases.

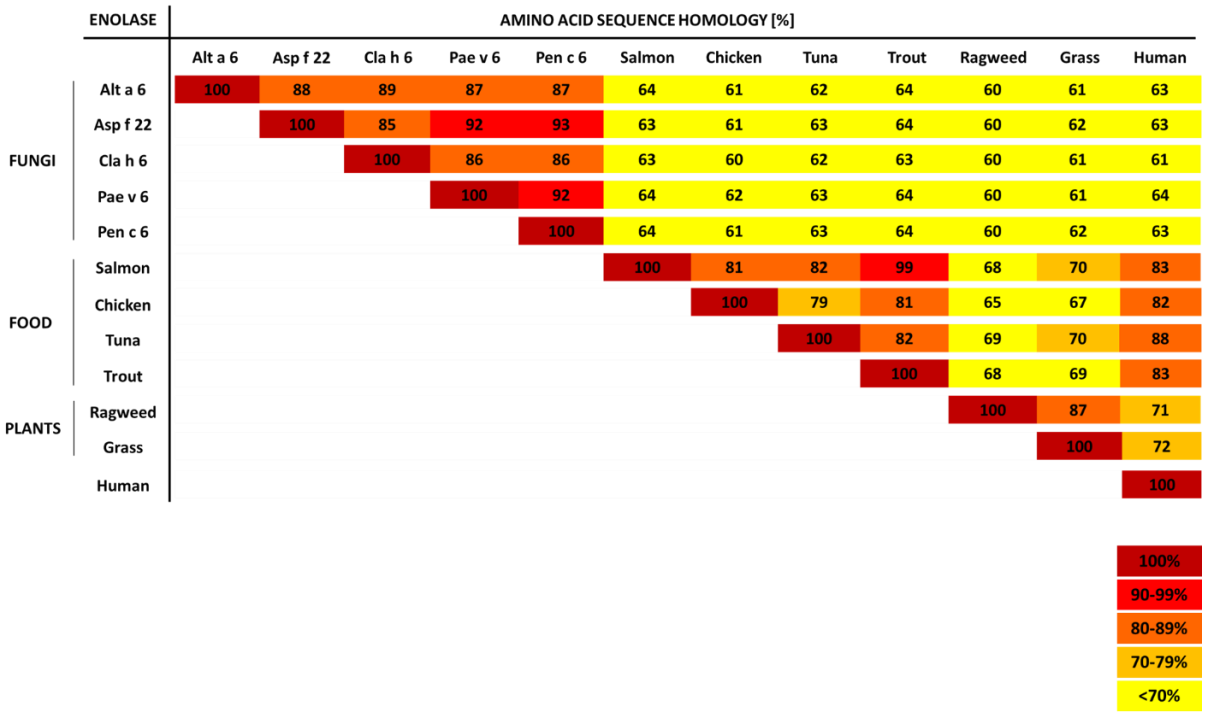


Figure 1. Amino acid sequence identity map of enolases from various related and unrelated species. Amino acid sequences of enolases derived from fungi, plants, animal-derived foods, and human were compared using the alignment software Clustal Omega and resulting percentages of sequence identities are shown.

It was then investigated whether an antiserum raised against rAlt a 6, the fungal enolase from *A. alternata*, would recognize other fungal enolases as well as enolases present in animal-derived foods and pollen. Therefore, two µg of recombinant proteins or 10 µg of protein extracts from various foods and pollen were separated by 12% SDS-PAGE and were either stained with Coomassie-Blue (**C**) or transferred electrophoretically onto nitrocellulose membranes. Proteins were then exposed to the rabbit antiserum directed against the recombinant enolase rAlt a 6 (**NI**). To analyze the specificity of the antibody binding, inhibition immunoblots were also performed, where the antiserum was pre-incubated with 20 µg of rAlt a 6 (**INH**).

Results, shown in **Figure 2A**, demonstrate that the antiserum raised against rAlt a 6 strongly reacted with fungal enolases (~47 kDa) from different species (**NI** in **Figure 2A**) and that the binding of the antiserum to all recombinant proteins was significantly reduced by the pre-incubation of the antiserum with rAlt a 6 (**INH** in **Figure 2A**).

Furthermore, the antiserum recognized also different animal-derived enolases of ~47-48 kDa. As shown in **Figure 2B**, the anti-rAlt a 6 serum strongly reacted with proteins of ~47-48 kDa present in the extracts prepared from raw or cooked salmon, tuna, and trout, as well as with the recombinant trout enolase (**NI**). Moreover, pre-incubation of the antiserum with rAlt a 6

always led to a significant and specific reduction of the antibody binding to these proteins (**INH**). In addition, Coomassie-staining of the fish protein extracts (**C**) revealed a relatively high abundance of the enolase in the extracts prepared from raw fish material, since protein bands of a molecular weight of ~47 kDa were clearly visible in the gels. However, significantly lower amounts of the proteins were seen in the cooked fish. Immunoblots also showed stronger antibody binding to the raw than the cooked extracts. This could be due to the degradation of the enolases during the heating process.

To analyze the reactivity of the chicken enolase to the antiserum, only protein extracts from cooked chicken breast and leg were prepared as chicken is usually not consumed in its raw form. Results of immunoblots (**Figure 2B**) showed that the antiserum recognized proteins of ~22 kDa and of ~47 kDa in the chicken breast as well as in the chicken leg extract, and that this antibody binding to both proteins was completely inhibited due to the pre-incubation with rAlt a 6.

Shrimp represents another important food allergen source, therefore, an extract prepared from cooked shrimp was also analyzed by immunoblot experiments. Results show (**Figure 2B**) the binding of the antiserum to proteins of ~38 kDa and ~47 kDa, which was reduced by the pre-incubation of the antiserum with rAlt a 6.

Besides the analysis of common food allergen sources, three respiratory pollen allergen sources were analyzed as well. Results in **Figure 2C** show that the anti-rAlt a 6 serum also recognized enolases, with a molecular weight of ~47-48 kDa, from ragweed, birch, and grass. Furthermore, inhibition immunoblots revealed that the pre-incubation of the antiserum with rAlt a 6 always reduced or completely inhibited the antibody binding to the enolases presents in the different pollen extracts.

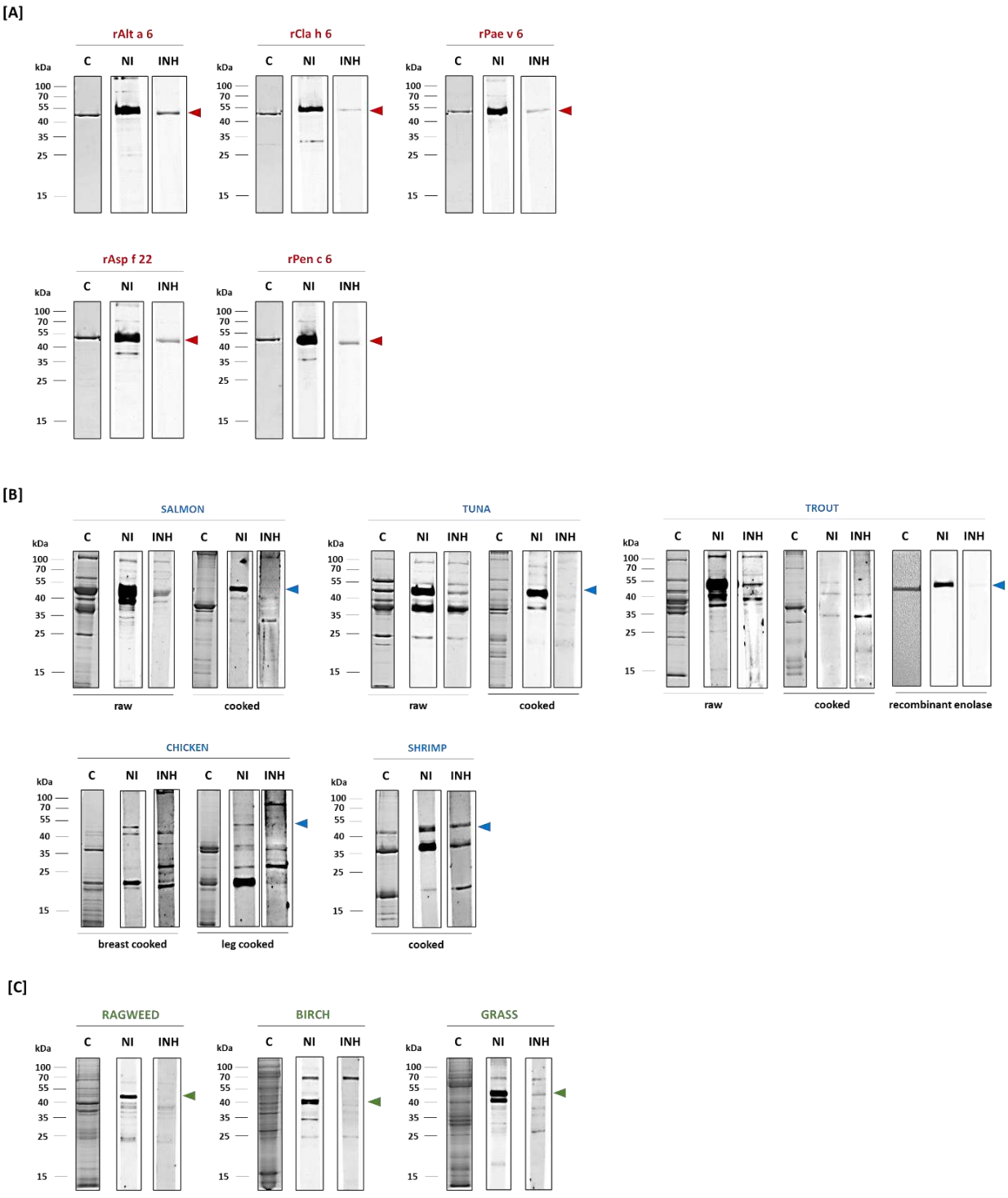


Figure 2. An antiserum raised against a fungal enolase recognizes enolases from distantly related species. [A] Two µg of recombinant fungal enolases rAlt a 6, rCla h 6, rPae v 6, rAsp f 22, and rPen c 6 were separated by 12% SDS-PAGE. **[B]** Ten µg of protein extracts prepared from salmon, chicken, tuna, trout, and shrimp and two µg of recombinant trout enolase were separated by 12% SDS-PAGE. **[C]** Ten µg of protein extracts prepared ragweed, birch, and grass were separated by 12% SDS-PAGE. Proteins were then either stained with Coomassie-Blue (**C**) or blotted onto nitrocellulose membranes. Blotted proteins were then exposed to a rabbit antiserum raised against rAlt a 6 that was either pre-incubated with 20 µg of rAlt a 6 (**INH**) or, for control purposes, with buffer only (**NI**). Molecular weight markers are indicated in the left margin. Protein bands of a molecular weight typical for enolases (~47 kDa) are indicated by a triangle.

Polysensitized patients react with enolases from different allergen sources

To evaluate whether polysensitized patients would recognize enolases from different fungal species, ELISAs were performed with the recombinant fungal enolases rAlt a 6, rAsp f 22, rPen c 6, rPae v 6, and rCla h 6 and with sera from twelve polysensitized patients. As can be seen in **Figure 3**, eight out of the twelve patients showed a reaction to more than one of the recombinant enolases. Highest IgE-reactivity was detected to rAlt a 6, rPen c 6 and rAsp f 22, whereas the reactivity to rCla h 6 and rPae v 6 was weaker. However, there were patient-dependent differences in the IgE recognition of all enolases. For example, patient 2 showed the strongest reactivity to rAlt a 6, whereas lower OD-values were obtained for rPen c 6 and rAsp f 22. In addition, patient 7 reacted strongly with rAlt a 6 and rAsp f 22 and showed a weaker reaction to rPen c 6. Furthermore, three of the patients showed a positive reaction to mainly one recombinant enolase. Patients 5 and 9 mostly reacted with the enolase from *P. chrysogenum*, whereas patient 8 showed a positive reaction only to rAsp f 22.

In addition, concentration of allergen-specific IgE levels against the recombinant fungal enolases were determined by quantitative ELISA and are summarized in **Table S1** in the supporting information. Results show that for six of the patients high IgE-levels of >30 kU/L against rAlt a 6, rPen c 6 and rAsp f 22 were detected in the sera. The highest amounts of IgE antibodies of >100 kU/L against these fungal enolases were detected in the sera from patients 2 and 7.

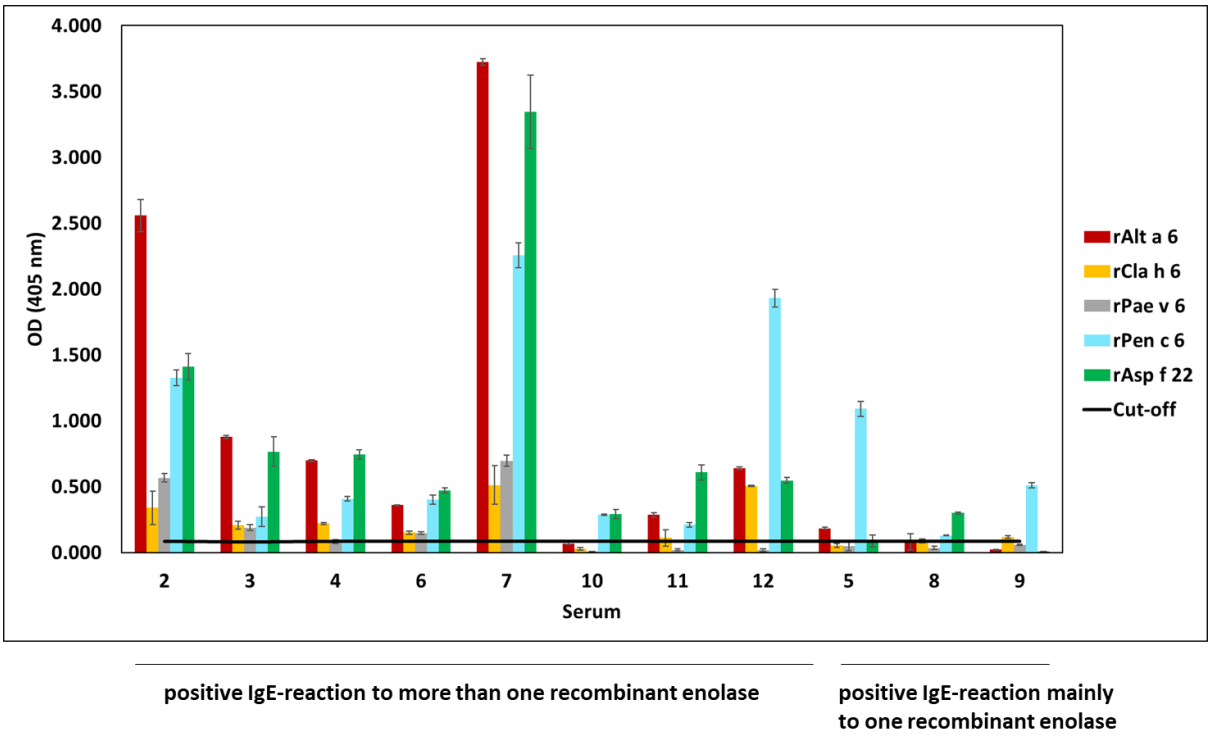


Figure 3. IgE-reactivity profiles of polysensitized patients to fungal enolases. IgE-reactivity to recombinant enolases rAlt a 6, rCla h 6, rPae v 6, rPen c 6, and rAsp f 22 was determined by ELISA. Results are displayed as mean OD-values and standard deviations from two technical replicates are indicated as error bars in the bar charts. Patients were grouped based on their reactivity profile. The black line represents the cut-off value, which was calculated from the mean OD-values plus two times the standard deviation from values obtained with sera from three non-allergic patients.

Furthermore, it was analyzed whether the polysensitized patients would also show IgE-reactivity to enolases from animal-derived foods and pollen. Therefore, again 10 µg of protein extracts were separated by 12% SDS-PAGE, transferred electrophoretically onto nitrocellulose membranes and exposed to individual patient sera. The number of extracts used in this analysis was reduced to one extract per species. This extract was either selected based on previous results (e.g., in the case of salmon and chicken higher amounts of the enolases were detected in the raw salmon extract and the cooked chicken leg extract) or based on the common form in which the food is consumed (e.g., tuna and trout are usually consumed in cooked form). Moreover, patients used for these experiments were selected based on previous immunoblots performed with pools of sera (data not shown) and only sera present in a pool that had shown a positive IgE-reaction to a protein of ~47-48 kDa were tested individually.

IgE-immunoblots (**Figure 4A**) showed that polysensitized patients react with various proteins from different animal-derived food sources. Patients 1, 2, 10 and 11 reacted with several proteins in salmon extract including a protein of ~47 kDa (**A**) that was identified by peptide

mass fingerprinting as an enolase (UniProt Accession Number: A0A152X522). In addition, three patients showed a reaction to at least one protein in the chicken extract with patient 3 reacting with a protein of ~47 kDa and patients 1, 2 and 3 reacting with a protein of ~22 kDa that was also recognized by the anti-rAlt a 6 serum (**Figure 2B**). These IgE-reactive proteins could not yet be identified by mass spectrometry. Out of the two allergic patients who showed a positive IgE-reaction to cooked tuna extract, patient 1 reacted with a protein of ~47 kDa (**B**), which was identified as an enolase (UniProt Accession Number: I0J1J1). Furthermore, IgE-antibodies of patient 10 bound to several trout proteins including a protein of ~47 kDa (**C**). Peptide mass fingerprinting analysis identified this IgE-reactive protein **C** as homologous enolase from *Salmo salar* (UniProt Accession Number: A0A152X522). Various patients showed a positive reaction to proteins present in shrimp extract including a protein of ~47 kDa. However, the strongest reaction was seen to a protein of ~38 kDa that was also recognized by the anti-rAlt a 6 serum (**Figure 2B**). Both IgE-reactive proteins could not yet be identified by mass spectrometry.

Moreover, patients' IgE-antibodies reacted with several pollen-derived proteins as shown in **Figure 4B**. Five patients showed a positive reaction to ragweed proteins with patients 9 and 10 reacting with a protein of ~47 kDa (**D**), which was identified as an enolase (UniProt Accession Number: A0A1B2H9Q1). In addition, out of the six patients that reacted with various proteins in birch pollen extract, patient 7 showed a reaction to a protein of ~47 kDa (**E**) that was also identified as the homologous enolase from *Ambrosia artemisiifolia* (UniProt Accession Number: A0A1B2H9Q1). Furthermore, the strong allergenic potential of grass pollen was demonstrated as nearly all patients showed a positive IgE-reaction to proteins in grass pollen extract. Three of those patients (patients 2, 3 and 7) reacted with a protein of ~48 kDa (**F**), which was identified by peptide mass fingerprinting as an enolase (UniProt Accession Number: K7AKY0).

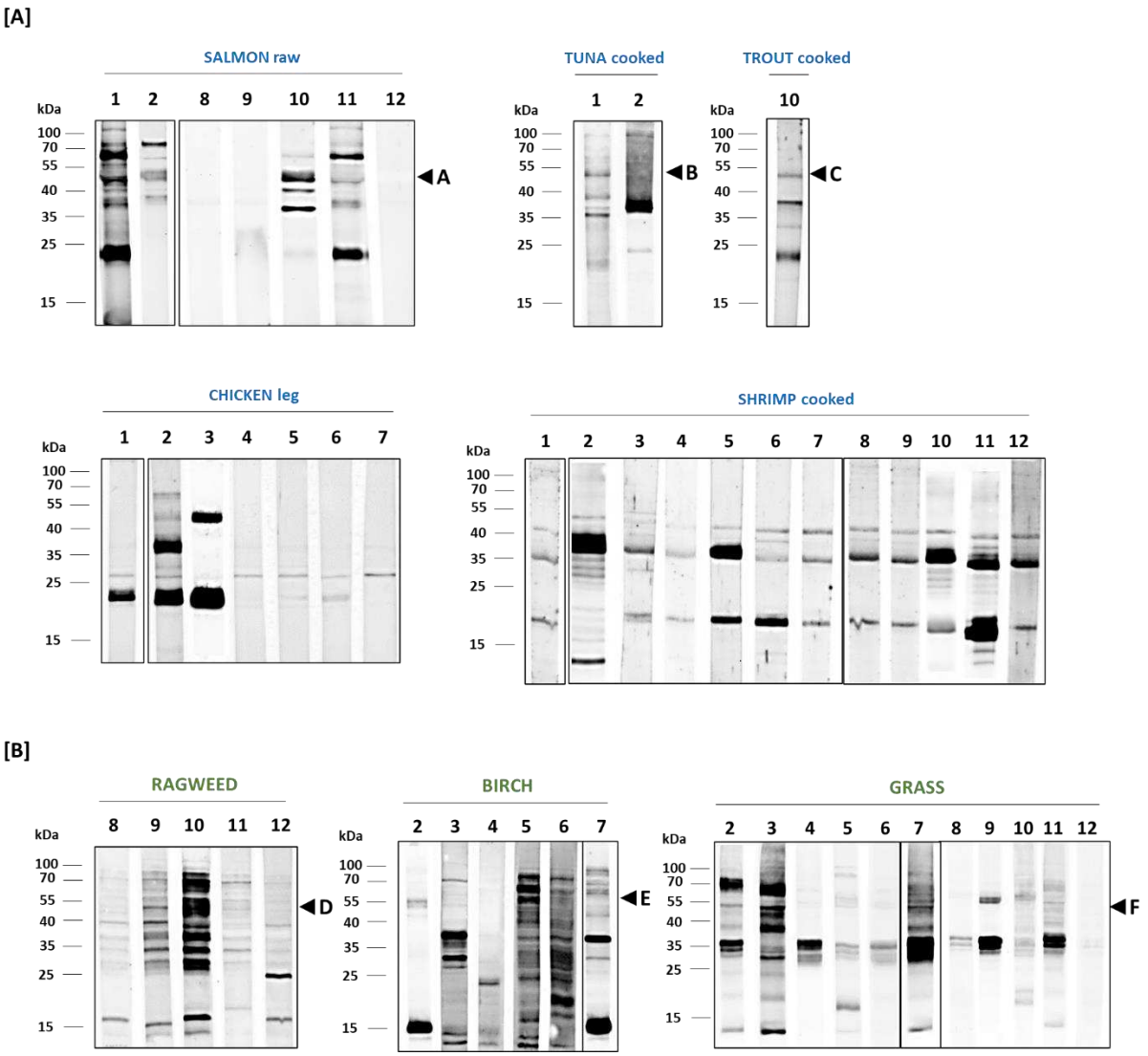


Figure 4. **IgE-reactivity profiles of polysensitized patients to animal-derived foods and to pollen. [A]** Ten µg of food extracts prepared from raw salmon, cooked chicken leg, cooked tuna, cooked trout, and cooked shrimp were separated by 12% SDS-PAGE. **[B]** Ten µg of pollen extracts prepared from ragweed, birch, and grass were separated by 12% SDS-PAGE. Proteins were then blotted onto nitrocellulose membranes and exposed to individual sera from polysensitized patients (diluted 1:10, indicated as 1-12). IgE-reactive proteins identified by mass spectrometry are marked with A, B, C, D, E, and F. Molecular weight markers are indicated in the left margin.

Homologous enolases possess cross-reactive and species-specific IgE-binding epitopes

To analyze the IgE cross-reactivity of the recombinant fungal enolases rAlt a 6, rAsp f 22, rPen c 6, rPae v 6, and rCla h 6, IgE-inhibition ELISA experiments were carried out. Therefore, plate-bound recombinant enolases were incubated with sera from allergic patients 7 and 12, which were pre-incubated with 20 µg of a recombinant enolase or, for control purposes, with buffer only.

IgE inhibition ELISAs, shown in **Figure 5A**, demonstrated that pre-incubation of the serum of patient 7 with rAlt a 6 led to a complete inhibition of IgE-binding to rAlt a 6, rCla h 6, and rPae v 6. However, this was not the case for rPen c 6 and rAsp f 22, where the IgE-binding was

significantly reduced, but not abolished. A similar effect was seen *vice versa*, as the pre-incubation of the serum with rPen c 6 led to a complete inhibition of IgE-binding to rCla h 6, rPae v 6, and rPen c 6, but only a reduction of IgE-binding to rAlt a 6 and rAsp f 22. In case of pre-incubation with rAsp f 22, complete abolishment of IgE-binding to all recombinant enolases except rAlt a 6 was observed.

The same experiment was carried out with the serum from patient 12 using only rPen c 6 as an inhibitor as this patient showed the strongest IgE reaction to rPen c 6 during previous experiments (**Figure 3**). A positive IgE-reaction, as can be seen in **Figure 5B**, was measured to rAlt a 6, rCla h 6, rPen c 6, and rAsp f 22 and pre-incubation of the serum with rPen c 6 led to a complete inhibition of IgE-binding to rCla h 6 and rPen c 6 and significantly reduced the binding to rAlt a 6. Interestingly, this pre-incubation led to almost no reduction in IgE-binding to rAsp f 22.

These results further showed that besides highly cross-reactive epitopes, fungal enolases also have species-specific IgE epitopes.

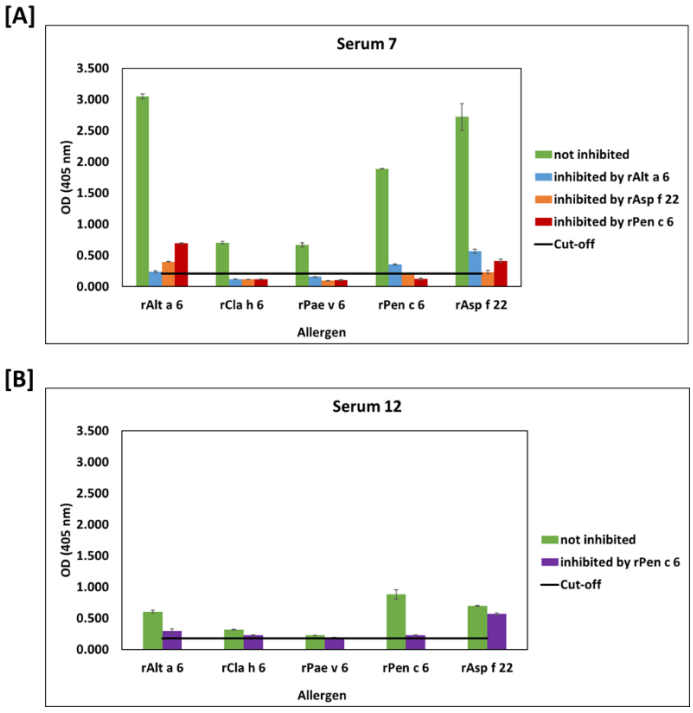


Figure 5. Determination of IgE cross-reactivity of the recombinant allergens rAlt a 6, rCla h 6, rPae v 6, rPen c 6, and rAsp f 22 by inhibition ELISAs using sera from two polysensitized patients. [A] Results for serum 7 are displayed as mean OD-values and standard deviations from two technical replicates are indicated as error bars in the bar charts. **[B]** Results for serum 12 are displayed as mean OD-values and standard deviations from two technical replicates are indicated as error bars in the bar charts. The patients' sera were either pre-incubated with 20 µg of a recombinant enolase or, for control purposes, with dilution buffer only as indicated in the legend of the figure. The black line represents the cut-off value, which was calculated from the mean OD-values plus two times the standard deviation from values obtained with sera from three non-allergic patients.

The IgE cross-reactivity of food and pollen enolases with the enolase from *A. alternata* was analyzed by IgE inhibition immunoblots as described before. Only a selected number of sera were analyzed based on the patients' IgE-reactivity profiles during previous experiments (**Figure 4**). Ten µg of protein extracts were separated by 12% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to individual sera from polysensitized patients, which were either pre-incubated with 20 µg of rAlt a 6 (**INH**) or, for control purposes, with buffer only (**NI**).

Results of IgE inhibition immunoblots are shown in **Figure 6A** for animal-derived food extracts and in **Figure 6B** for pollen extracts. No inhibition of IgE-binding to proteins present in the extracts was seen after pre-incubation of the sera with rAlt a 6, suggesting the lack of cross-reactivity between rAlt a 6 and enolases of these species.

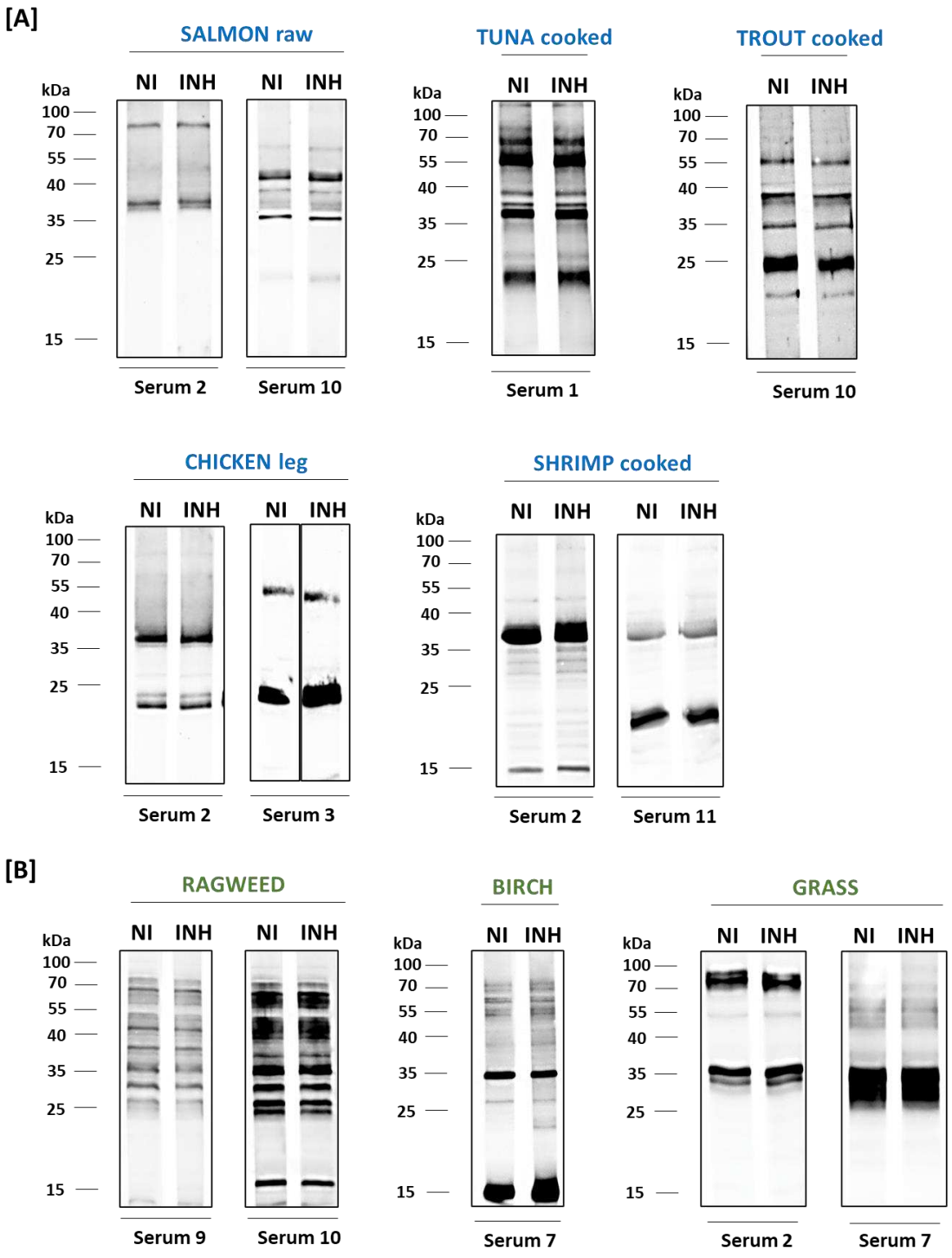


Figure 6. Analysis of IgE cross-reactivity of animal-derived food and pollen enolases with the fungal enolase rAlt a 6 by inhibition immunoblot experiments. [A] Ten µg of protein extracts prepared from salmon, chicken, tuna, trout, and shrimp were separated by 12% SDS-PAGE. **[B]** Ten µg of pollen extracts prepared from ragweed, birch, and grass were separated by 12% SDS-PAGE. Proteins were then blotted onto nitrocellulose membranes and were exposed to individual sera from allergic patients (diluted 1:10) that were either pre-incubated with 20 µg of rAlt a 6 (**INH**) or, for control purposes, with buffer only (**NI**). Molecular weight markers are indicated in the left margin.

Discussion

The glycolytic enzyme enolase plays an important role in the catabolic glycolytic pathway and is one of the most abundantly expressed cytosolic proteins in many organisms (2,35). Moreover, it has been identified as an important mediator of allergic responses in susceptible individuals (23). Since the first association with allergic diseases, allergenic enolases have been reported in a wide range of different allergen sources including animals, fungi, plants, and pollen (23). However, the role of enolases in allergic diseases has not yet been fully investigated and the cross-reactivity between pollen, food, and mold enolases has not been studied (23).

The purpose of this study was to investigate the cross-reactive potential of enolases from taxonomically related as well as unrelated allergen sources including molds, pollen, and animal-derived foods, with the aim to gain more information about the enzyme's pan-allergenic character. For this, immunoblots and ELISAs were first carried out using sera of polysensitized patients with protein extracts from different animal-derived foods and plant pollen and with recombinant mold and trout enolases. In addition, the cross-reactivity between enolases was analyzed by IgE inhibition experiments.

Enolases are one of the most prominent and conserved allergenic families in fungi and have been identified as minor allergens from many different fungal species (7,8,10,36–38). In this study, the fungal enolases rAlt a 6, rCla h 6, rPae v 6, rAsp f 22 and rPen c 6 were recombinantly produced and analyzed concerning their homology and cross-reactive potential. Sequence homology analysis revealed high sequence identities of at least 85% among different fungal species, which has already been described in the literature (23). These homologies tend to correlate with fungal phylogenetic relationship (e.g., enolases from the related species *Aspergillus fumigatus* and *Penicillium chrysogenum* show 93% amino acid sequence homology) (20,39). Besides analyzing sequence homologies, it was investigated whether an antiserum raised against the fungal enolase rAlt a 6 would also recognize other fungal enolases. Immunoblots showed that the anti-rAlt a 6 serum strongly reacted with enolases from different fungal species. The fact that the binding of the antiserum to all recombinant molecules was significantly reduced by pre-incubation with rAlt a 6, proved the specificity of the antibody binding.

Analysis of the IgE-reactivity profiles of patients to the recombinant fungal enolases by ELISA, showed that eight out of the twelve patients tested reacted with more than one of the recombinant enolases, whereas three of the patients reacted only with one of the recombinant allergens. This is also the first report of a positive IgE-reaction of mold-allergic patients to the enolase from *Penicillium chrysogenum*, designated Pen c 6. So far, only the enolase from the species *Penicillium citrinum* from the same genus was found to be IgE-reactive (10). The results further show that, besides the patients' strong reactions to homologous enolases, patient-dependent differences in IgE-reactivity occurred. For example, patients 2 and 7 strongly reacted with several enolases, whereas patients 5 and 9 only reacted with one fungal enolase. Moreover, for analysis of cross-reactivity, IgE-inhibition ELISA experiments were carried out with sera from two of the allergic patients. Results obtained lead to the assumption that homologous enolases possess not only cross-reactive, but also species-specific IgE-binding epitopes as pre-incubation with one fungal enolase often led to a reduction of IgE-binding to homologous enolases, but not to a complete abolishment. Already previous studies have observed heterogeneity in IgE responses of individual allergic patients to different fungal enolases (10,40). These findings along with the results obtained in the present study suggest that some of the IgE epitopes on the fungal enolase allergens are common to several fungal species while others are specific for an individual allergen. Thus, allergic patients may be sensitized primarily to an individual fungal enolase, but allergic symptoms may be induced by the exposure to enolases from other environmental fungi due to cross-reacting IgE antibodies. In addition, from a clinical point of view, it is important to note that patients can be exposed to enolases either in the outdoor environment, since *Alternaria* and *Cladosporium species* are important outdoor allergen sources, but also indoors, since *Penicillium* and *Aspergillus species* represent indoor allergen sources (23).

Fish and meat are common inducer of IgE-mediated food allergy, and enolases from different sources including nile perch (41), cod (16,41), salmon (16,42), catfish (42), tilapia (43), tuna (16) and chicken (15) have either been identified or suggested as minor allergens with the potential to cross-react (15,16,23). Thus, protein extracts prepared from important food allergen sources such as salmon, tuna, chicken, trout, and shrimp were also analyzed in this study. Furthermore, enolases have also been identified as allergens in other respiratory allergen sources besides fungi including Bermuda grass (13) and ragweed (11,12). In addition, several pollen-derived enolases have been suggested as potential allergens in the literature

(44–46). Therefore, the cross-reactive potential of enolases from important allergenic pollen, namely ragweed, birch, and grass, was also analyzed in the present study.

Sequence homology analysis showed a high degree of sequence identity among enolases from different animal-derived foods and pollen. In addition, a high sequence identity of >60% was also found among taxonomically distant organisms (e.g., between enolases from salmon and ragweed or tuna and grass). This degree of evolutionary conservation of enolases' protein sequences (3,21,47,48) supports the high immunologic cross-reactive potential of enolases (23). Moreover, immunoblot experiments with an antiserum raised against rAlt a 6 showed that the antiserum raised against the fungal enolase recognizes also plant and animal enolases. Furthermore, Coomassie-stained protein gels suggest the high abundance of enolases in the extracts prepared from raw fish, as protein bands of a molecular weight of ~47 kDa were clearly visible in the gels. In contrast, significantly lower amounts of this protein were seen in the cooked extracts, which corroborates previous studies which showed that enolases are sensitive to thermal treatment (23). The presence of enolases in highly consumed food sources as well as in highly abundant airborne pollen, suggests their importance as a group of allergens and hints at their potential as pan-allergens (23).

Analyses of the IgE-reactivity of animal-derived food and pollen extracts by IgE-immunoblots showed that polysensitized patients react with enolases from different allergen sources. For each allergen source, a positive IgE-reaction with a protein of ~47-48 kDa was detected with at least one of the patients. These reactive proteins could be identified by mass spectrometry as the enzyme enolase in salmon, tuna, trout, ragweed, birch, and grass. This is the first study that reports a positive IgE-reaction of allergic patients to enolases from trout, and birch pollen. Interestingly, comparing the IgE-reactivity detected for the recombinant fungal enolases by ELISA and for the protein extracts by immunoblots reveals a strongly patient-dependent recognition of enolases. For example, the majority of patients reacted with enolases from several sources such as patient 2, who showed a strong IgE-reactivity to several fungal enolases, but also recognized enolases in salmon, chicken, shrimp, and grass extract. In contrast, patients 4, 5, 6, and 12 reacted only with the recombinant fungal enolases and patient 1 recognized only fish enolases in salmon and tuna extract. This heterogeneity in IgE-recognition was further verified by IgE inhibition immunoblots, carried out with the different extracts and a selected number of allergic patients. These results showed that patients' IgE-

binding to enolases in the animal-derived food and pollen extracts was not inhibited by the fungal enolase rAlt a 6, suggesting that, despite the high homology of enolases, no cross-reactive IgE epitopes are shared between rAlt a 6 and the natural enolases from the allergen sources tested. However, it must be mentioned, that this is of course always a patient-dependent factor, therefore, cross-reactivity analyses will have to be performed in the future in a larger of cohort of patients.

In conclusion, results presented in this study demonstrate the high homology of enolases from related and unrelated organisms and showed the strong IgE cross-reactivity between enolases from various mold species, which reinforces the molecule's status as a fungal pan-allergen. Nevertheless, the data suggest that fungal enolases possess not only cross-reactive, but also species-specific IgE-epitopes. It will therefore be interesting to identify these cross-reactive and species-specific IgE-epitopes to enable the identification of the primary sensitizing species and therefore improve fungal allergy diagnosis and treatment. Furthermore, the IgE-reactivity of polysensitized patients to enolases from different animal-derived food and pollen sources was shown, and the sensitization and IgE-reactivity of the patients suggest that the highly conserved enolases might be one causative factor for the well-known phenomenon of polysensitization, which is frequently encountered in clinical practice (26). So far, no IgE cross-reactivity was detected between enolases from animal-derived foods and pollen, and the fungal enolase rAlt a 6. However, further investigations could provide valuable information for allergy diagnosis and for developing patient-tailored prophylactic and therapeutic approaches.

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SUPPORTING INFORMATION

Table S1. Allergen-specific IgE levels to recombinant mold enolases, measured by ELISA.

Allergen-specific IgE [kU/L]					
Serum	**rAlt a 6**	**rCla h 6**	**rPae v 6**	**rPen c 6**	**rAsp f 22**
1	**17**	**14**	**8**	**20**	**16**
2	**194**	**36**	**49**	**109**	**114**
3	**72**	**25**	**20**	**30**	**65**
4	**58**	**26**	**11**	**40**	**64**
5	**21**	**13**	**8**	**91**	**14**
6	**34**	**21**	**16**	**39**	**43**
7	**278**	**50**	**59**	**179**	**260**
8	**14**	**16**	**8**	**19**	**30**
9	**10**	**18**	**9**	**48**	**8**
10	**13**	**11**	**5**	**31**	**30**
11	**28**	**18**	**6**	**25**	**54**
12	**54**	**49**	**6**	**155**	**49**

5. Unpublished Manuscript II

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“Cultivation conditions and their impact on the expression of fungal allergens”

Authors contributions:

S.P. carried out the experiments described. M.F.-T. and K.S.-G. contributed to interpreting the results and planning the work by providing their scientific expertise. S.P. wrote the manuscript. I.S. supervised the project and contributed to the implementation of the research, to the analysis of the results and to the writing of the manuscript.

Cultivation conditions and their impact on the expression of fungal allergens[1]

Introduction

Fungal allergy is a worldwide public health problem and respiratory exposure to a wide range of allergenic fungal components is almost ubiquitous (1–3). The prevalence of fungal sensitization is estimated to range from 3% to 10% in the general population and up to 44% of atopic individuals are sensitized to at least one fungal species (4,5). However, the exact prevalence has not yet been reliably determined since the available reports of sensitization tests vary from 3% to 90% depending on the study population, geographical variations, and methodological limitations (6,7). However, a major factor that contributes to a general underdiagnosis of fungal allergy is the use of fungal extracts of poor quality as test solutions for allergy diagnosis (2,4,6).

Any *in vivo* diagnosis of allergy based on skin tests as well as any *in vitro* diagnosis of allergy based on the determination of allergen-specific IgE antibodies depends on the quality of the material used for testing (8). Although commercially available allergen solutions must pass through company-internal standardization procedures and quality controls, there are until now no generally accepted guidelines for the preparation of allergenic fungal extracts (4). It is therefore not astonishing that considerable differences in potency of fungal extracts between manufacturers and even batch-to-batch variations can be found (4,8,9).

Problems encountered in the standardization of fungal extracts derive from the complexity of the source material used, the culture conditions and medium applied to grow the fungus, the instability of the extracts due to protease content, and, of course, the extraction procedure (8–10). In contrast to pollens or insect venoms, fungi as allergenic sources are extremely complex due to strain variabilities and the tendency of fungi for spontaneous mutation (4,8). Moreover, the choice of fungal raw material used for protein extraction is of high importance as the allergen content in fungal spores or mycelium can be significantly different (9,11). It is therefore suggested that reference materials and commercial extracts contain both, spores and mycelial constituents (9). Moreover, the morphologic, biochemical, and antigenic characteristics of a given fungal strain are known to be dependent on the composition of the

[1] Unpublished data

culture medium (9,11). Physical conditions such as glucose supply, oxygen, light and dark cycles, temperature, pH, and growth period, might be important for sporulation and might affect allergen production (9,11,12). Furthermore, as fungi are enzyme-rich organisms, degradation processes should also not be underestimated as well as the impact of the extraction process (4,9). Moreover, fungal allergenic extracts contain a mixture of proteins, glycoproteins, polysaccharides, and other substances, both allergenic and non-allergenic, of usually unknown concentrations (11,12). This, along with the production of cell-bound, secreted, and intracellular allergens, makes it impossible to produce consistent extracts containing the three types of allergens in a single run without any non-allergenic molecules (8).

Due to the problems with standardization of fungal extracts, only a limited number of them are available for diagnosis (4). However, standardization of allergen extracts is essential for the improvement of diagnosis sensitivity and specificity, which is a prerequisite for improvement in safety and efficacy of extracts used for immunotherapy (12). The determination of the optimal growth conditions to produce highly allergenic fungal extracts is therefore the first step towards extract standardization (4,12).

The aim of our study was to evaluate the impact of different environmental conditions, including carbon source, light exposure, and temperature, as well as the effect of cultivation time on the expression of fungal allergens. Therefore, *Alternaria alternata, Ulocladium chartarum, Aspergillus fumigatus, Cladosporium herbarum* and *Paecilomyces variotii* were cultivated under different conditions, and protein extracts from spores and mycelium of the fungal species were prepared. The expression of the major fungal allergens Alt a 1 and Ulo c 1, as well as of fungal enolases was analyzed by immunoblots using specific antibodies directed against Alt a 1 and Alt a 6, the enolase from *A. alternata*. Results obtained in this study showed that the cultivation conditions indeed have an impact on the allergenic content of fungal extracts and that cultivation conditions are highly species-dependent. Information obtained in this study will help to optimize fungal cultivation to produce highly potent diagnostic fungal extracts and therefore improve diagnosis' specificity and sensitivity.

Methods

Fungal cultivation

Clones of *Alternaria alternata* and *Ulocladium chartarum* were cultured under different conditions to determine the impact of the carbon source, of light exposure, of the cultivation temperature, and of the cultivation time on the expression of the species' homologous major allergens Alt a 1 and Ulo c 1 as well as their minor allergens Alt a 6 and Ulo c 6. In addition, the impact of the cultivation time on the expression of fungal enolases was evaluated for the species *Aspergillus fumigatus, Cladosporium herbarum* and *Paecilomyces variotii*. An overview of the different cultivation conditions tested can be found in **Table 1**.

Table 1. Growth conditions for fungal cultivation.

ANALYZED CONDITION	MEDIUM	LIGHT EXPOSURE	CULTIVATION TEMPERATURE	CULTIVATION TIME
Carbon Source	Glucose-based medium: 6.8% yeast nitrogen base, 2% glucose, 5% peptone from casein, 15% agar-agar, pH 6.0 Cellulose-based medium: 6.8% yeast nitrogen base, 2% cellulose, 5% peptone from casein, 15% agar-agar, pH 6.0 Methylcellulose-based medium: 6.8% yeast nitrogen base, 2% methylcellulose, 5% peptone from casein, 15% agar-agar, pH 6.0	Dark	20-25°C	10 days 15 days 20 days 25 days 30 days 35 days 40 days
Light Exposure	Malt Extract Agar (MEA): 2% malt extract, 2% glucose, 15% agar-agar, pH ~5.0	Dark or natural Light/Dark Rhythm	20-25°C	5 days 10 days 15 days 20 days
Temperature	MEA	Dark	20-25°C 30°C 37°C	5 days 10 days 15 days 20 days
Cultivation Time	MEA	Dark	20-25°C	5 days 10 days 20 days 30 days

Fungal protein extracts preparation

Crude protein extracts were prepared from fungal mycelium and spores, which were harvested from the agar-plates using a sterile scalpel and ground in liquid nitrogen to a fine powder using a pre-cooled mortar and pestle. The powder was resuspended in fungal extraction buffer containing 50 mM $NaHCO_3$, 150 mM NaCl, 2 mM phenylmethanesulforyl fluoride, 2 mM ethylene diamine tetraacetic acid and complete EDTA-free protease inhibitor tablets (Roche Diagnostics, Mannheim, Germany; used according to the manufacturer's instructions). After an overnight incubation at 4°C under continuous shaking, the samples were centrifuged at 4,500 g and 4°C for 10 minutes and the supernatants were stored at –20°C. The protein concentrations of the extracts were determined using the method of Bradford with bovine serum albumin (BSA) as a standard (13).

For the cultivation experiment, in which the impact of the growth time on the expression of fungal allergens was analyzed, care was taken in the experimental set-up to minimize variability. Therefore, fungal mycelium and spores of three biological replicates were harvested as described before and then transferred into sterile MK28 2 mL Precellys tubes (Bertin Technologies SAS; Nontigny-le-Bretonneux, France). Afterwards phosphate buffered-saline (PBS) containing complete EDTA-free protease inhibitor tablets (Roche Diagnostics; used according to the manufacturer's instructions) was added and the material was lysed, using the Precellys Evolution Tissue Homogenizer (Bertin Technologies SAS), by applying three times 4,500 rpm for 20 seconds with 30 seconds of pause between each round. Following an overnight incubation at 4°C under continuous shaking and a 10-minute centrifugation-step at 4,500 g and 4°C, the supernatants were stored at –20°C and the protein concentrations of the extracts were determined as described before.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, protein staining and immunoblot experiments

Between one and three µg of fungal protein extracts, depending on the fungal species tested, were separated on 10%, 12% or 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGEs) as described by Laemmli (14). Proteins were then either stained using the Pierce Silver Stain Kit (Thermo Fisher Scientific, Waltham, MA) or transferred electrophoretically onto nitrocellulose membranes (0.2 µm; GE Healthcare, Chicago, IL) (15).

For immunoblots to detect Alt a 1, Alt a 6 and related proteins, membranes were washed and blocked with PBS containing 0.5% Tween20 (PBS-T) and exposed to a rabbit antiserum directed against either a peptide of Alt a 1 (5'-KISEFYGRKPEGTYYNSLG-3') or against the recombinant enolase rAlt a 6 (Charles River Laboratories, Écully, France) (1:5,000 diluted in PBS-T). For the detection of protein-bound antibody, a goat anti-rabbit IgG antibody (Vector Laboratories Inc, Burlingame, CA; diluted 1:10,000 in PBS-T), conjugated to horseradish peroxidase (HRP), was used as detection antibody. The blots were developed using the SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific) according to the manufacturer's instructions and imaged using the UVP ChemStudio device (Analytik Jena, Jena, Germany).

Results

Fungal growth on different carbon sources has no impact on the expression of the major fungal allergens Alt a 1 and Ulo c 1

To analyze whether the growth of *Alternaria alternata* and *Ulocladium chartarum* on media containing either glucose, cellulose, or methylcellulose as a carbon source, has an impact on the expression of the allergens Alt a 1, Alt a 6, Ulo c 1 and Ulo c 6, protein extracts from fungal spores and mycelium were prepared after different times of growth on these media. Proteins were then separated by 10% or 15% SDS-PAGE, and either stained with silver or transferred onto nitrocellulose membranes and exposed to antisera raised against Alt a 1 or against rAlt a 6.

Silver-staining of separated *A. alternata* proteins (**Figure 1A**) showed that no significant difference in the protein expression profile of the fungus due to the growth on different substrates can be seen. Furthermore, immunoblots performed with an anti-Alt a 1 antibody suggest that the species' major allergen Alt a 1, with a molecular weight of approximately 15 kDa, is expressed equally on media containing different carbon sources (**Figure 1B**). The allergen was strongly expressed between 10 and 35 days of growth, whereas lower amounts of Alt a 1 were expressed after a long cultivation time of 40 days. In contrast, the antibody directed against the species' enolase rAlt a 6 (~47 kDa), did not show any binding to any of the extracts, suggesting that Alt a 6 was not expressed in *A. alternata* growing on the different media (**Figure 1C**).

Immunoblots performed with extracts prepared from *U. chartarum*, grown on the different media showed that, comparable to *A. alternata*, neither the protein expression profile (**Figure 2A**) nor the expression of the Alt a 1-homologous major *U. chartarum* allergen Ulo c 1 (~15 kDa) was affected by the different media (**Figure 2B**). However, even though *A. alternata* and *U. chartarum* are highly homologous species and the amino acid sequences of the species' enolases are 100% identical (16), **Figure 2C** shows that Ulo c 6, in contrast to Alt a 6, could be detected by the rAlt a 6-specific antiserum after 10 days of growth on glucose- and cellulose-based medium. However, Ulo c 6 could not be detected in extracts prepared from *U. chartarum* grown on the media for longer periods of time. Interestingly, extracts prepared from fungal material growing on methylcellulose-based medium did not contain any detectable levels of Ulo c 6 at any cultivation time.

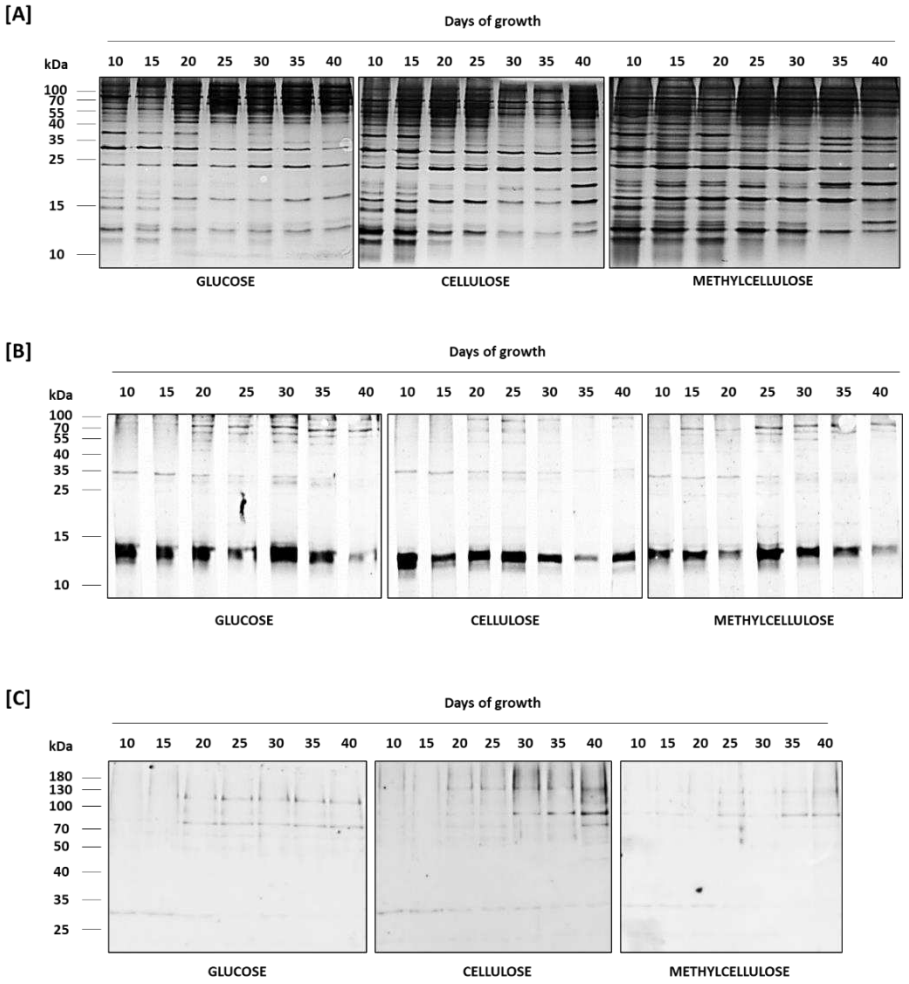

Figure 1: Analysis of the effect of glucose, cellulose or methylcellulose on the growth and allergen expression of *Alternaria alternata*. [A] Two µg of protein extracts were separated by 15% SDS-PAGE and proteins were stained with silver. **[B]** Two µg of protein extracts were separated by 15% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against Alt a 1. **[C]** Two µg of protein extracts were separated by 10% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against rAlt a 6. Molecular weight markers are indicated in the left margin.

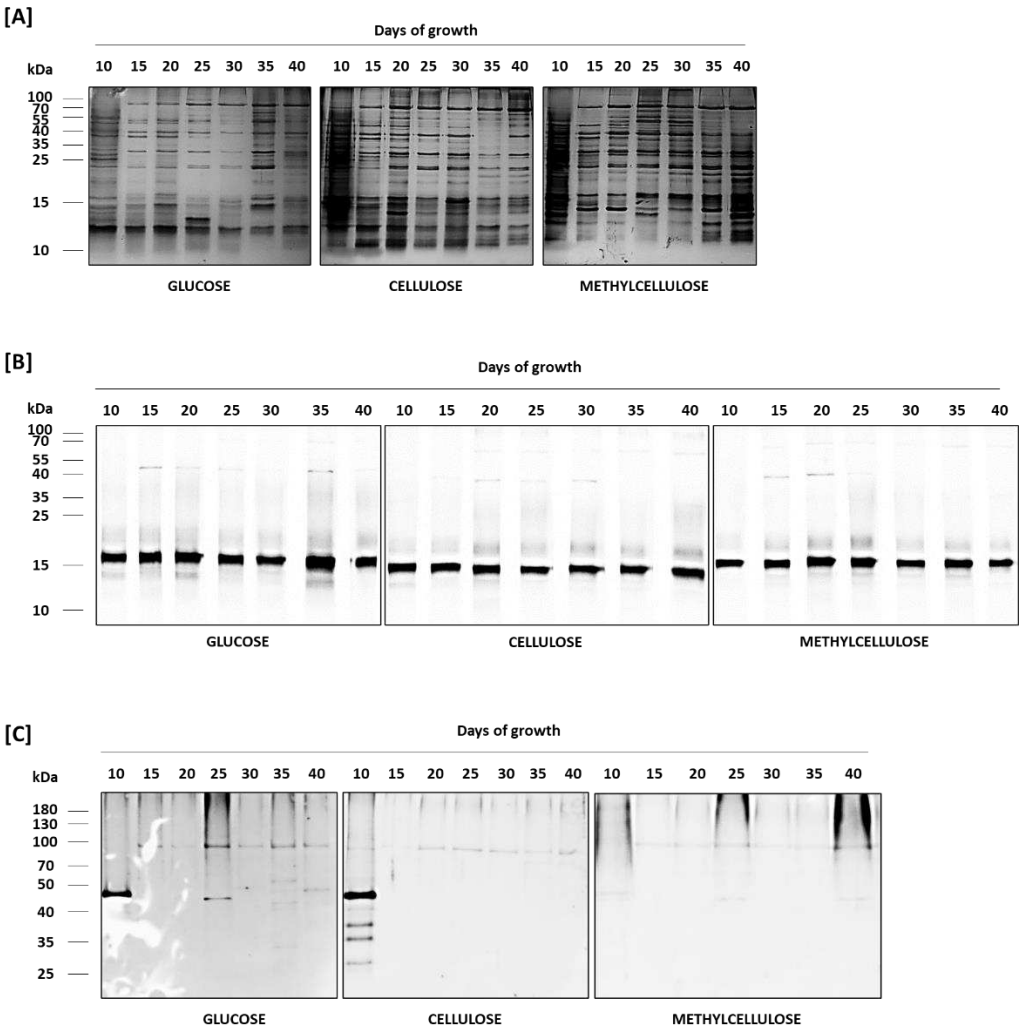

Figure 2: Analysis of the effect of glucose, cellulose, or methylcellulose on the growth and allergens expression of *Ulocladium chartarum*. [A] Two µg of protein extracts were separated by 15% SDS-PAGE and proteins were stained with silver. **[B]** Two µg of protein extracts were separated by 15% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against Alt a 1. **[C]** Two µg of protein extracts were separated by 10% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against Alt a 6. Molecular weight markers are indicated in the left margin.

<u>The cultivation temperature effects the expression of the fungal allergens Alt a 1 and Ulo c 1</u>

Next, it was analyzed whether different temperatures and different light conditions have an impact on the expression of Alt a 1, Alt a 6, Ulo c 1 and Ulo c 6. For this, protein extracts from fungal spores and mycelium were prepared after 5 to 20 days of growth on Malt Extract Agar, a standard medium for fungal growth. Proteins were then separated by 10% or 15% SDS-PAGE, transferred onto nitrocellulose membranes, and exposed to antisera raised against Alt a 1 or against rAlt a 6.

Immunoblots carried out with an anti-Alt a 1 serum (**Figure 3A**) suggest an expression of *A. alternata's* major allergen Alt a 1 already after 5 days of growth in the dark. In comparison,

expression of Alt a 1 was lower after 5 days if the fungus was also exposed to light. Independent of the exposure to light, the expression of Alt a 1 was highest after 10 and 15 days of growth and decreased after 20 days of growth on Malt Extract Agar. Analysis of the effect of the growth temperature on the expression of Alt a 1 showed that significantly higher amounts of Alt a 1 were expressed in the protein extracts prepared from fungi grown at 30°C compared to the samples from 20-25°C and 37°C. Interestingly, at growth temperatures of 20-25°C and 30°C the amount of Alt a 1 declined after 20 days of growth, whereas it increased at a growth temperature of 37°C. In immunoblots performed with the anti-rAlt a 6 serum (**Figure 3B**), again no Alt a 6 could be detected in any of the protein extracts, suggesting that Alt a 6 was not expressed under the tested conditions.

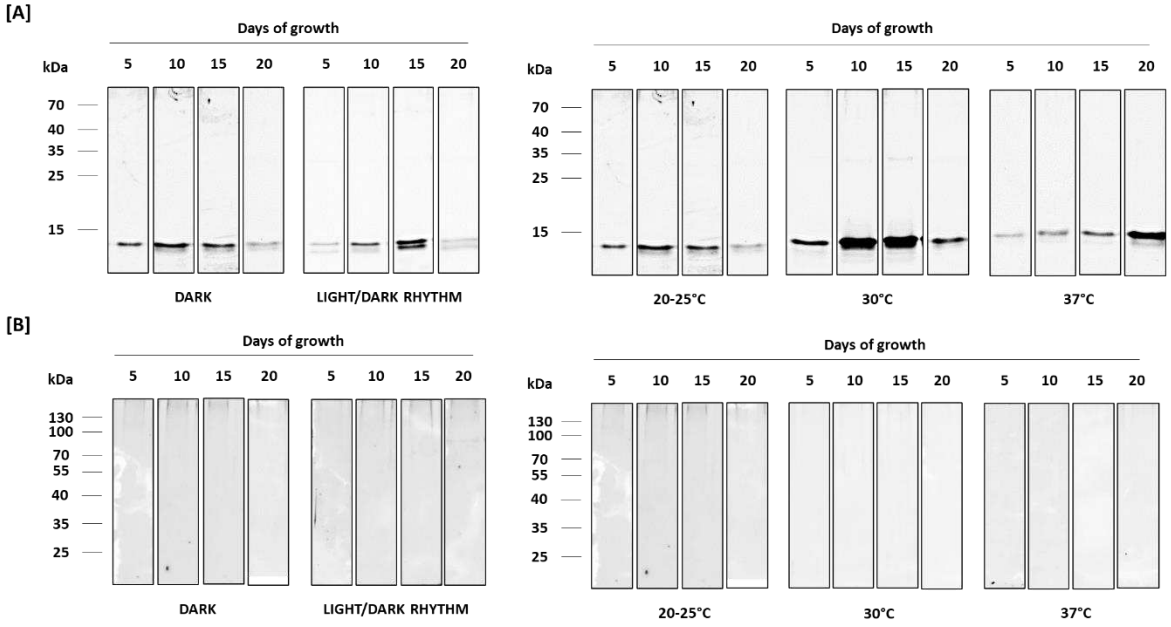

Figure 3: Analysis of the effect of different light conditions and temperatures on the expression of Alt a 1 and Alt a 6. [A] Two µg of *Alternaria alternata* protein extracts were separated by 15% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against Alt a 1. **[B]** Two µg of *Alternaria alternata* protein extracts were separated by 10% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against rAlt a 6. Molecular weight markers are indicated in the left margin.

Anti-Alt a 1 immunoblots performed with *U. chartarum* extracts showed that slightly higher amounts of Ulo c 1 were produced upon light exposure than in the dark (**Figure 4A**). Furthermore, also for Ulo c 1 a strong dependance of expression on the growth temperature could be seen (**Figure 4A**): cultivation of *U. chartarum* at 30°C led to the earliest and most constant production of Ulo c 1, whereas cultivation at 20-25°C and 37°C resulted in a weaker expression of this protein after shorter growth times (5, 10 and 15 days), whereas the highest amounts of Ulo c 1 were detected after 20 days of growth at 37°C. Immunoblots performed

with the anti-rAlt a 6 serum pointed again to differences in the expression of Ulo c 6 as compared to Alt a 6 since at least small amounts of Ulo c 6 could be detected in *U. chartarum* grown for 20 days either exposed to light or at 37°C (**Figure 4B**). However, only a weak expression of Ulo c 6 was seen.

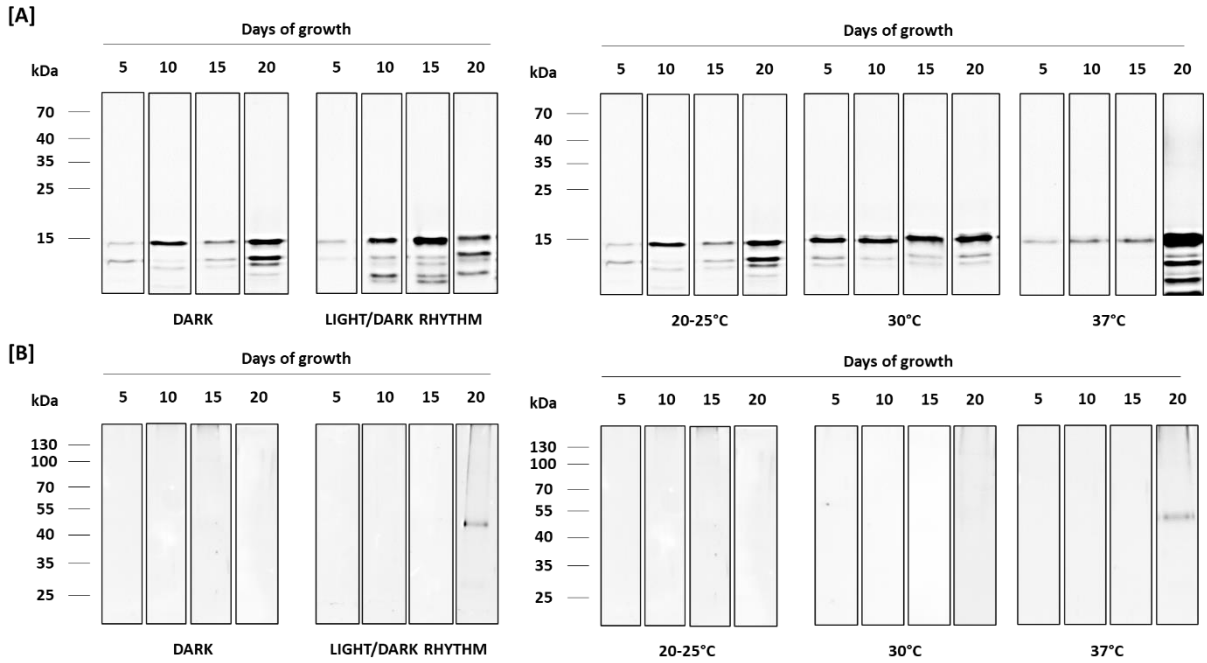

Figure 4: Analysis of the effect of different light conditions and temperatures on the expression of Ulo c 1 and Ulo c 6. [A] Two µg of *Ulocladium chartarum* protein extracts were separated by 15% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against Alt a 1. **[B]** Two µg of *Ulocladium chartarum* protein extracts were separated by 10% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against rAlt a 6. Molecular weight markers are indicated in the left margin.

<u>The expression of conserved, cross-reactive fungal enolases is time- and species-dependent</u>

To analyze whether the cultivation time has an impact on the expression of Alt a 1, Ulo c 1, Alt a 6, and Ulo c 6, protein extracts from *A. alternata* and *U. chartarum* were prepared after 5, 10, 20 and 30 days of growth on Malt Extract Agar and 20-25°C, using a different extraction procedure and biological triplicates to minimize variability. Proteins were then separated by 12% or 15% SDS-PAGE and transferred onto nitrocellulose membranes and exposed to antisera raised against Alt a 1 or against rAlt a 6.

Anti-Alt a 1 immunoblots suggest that the expression of the species' major allergen Alt a 1 increases with the growth time, whereas anti-rAlt a 6 immunoblots again indicated that Alt a 6 was not expressed under the conditions tested (**Figure 5A**). This correlates with the results previously obtained and suggests the difficulty in finding growth conditions ideal for the expression of this allergen. Immunoblots performed with *U. chartarum* extracts showed slight

increases in the expression of Ulo c 1 with the growth time (**Figure 5B**). However, the increase in expression was not as pronounced as in case of Alt a 1. Interestingly, in contrast to Alt a 6, Ulo c 6 could be detected after 30 days of fungal growth.

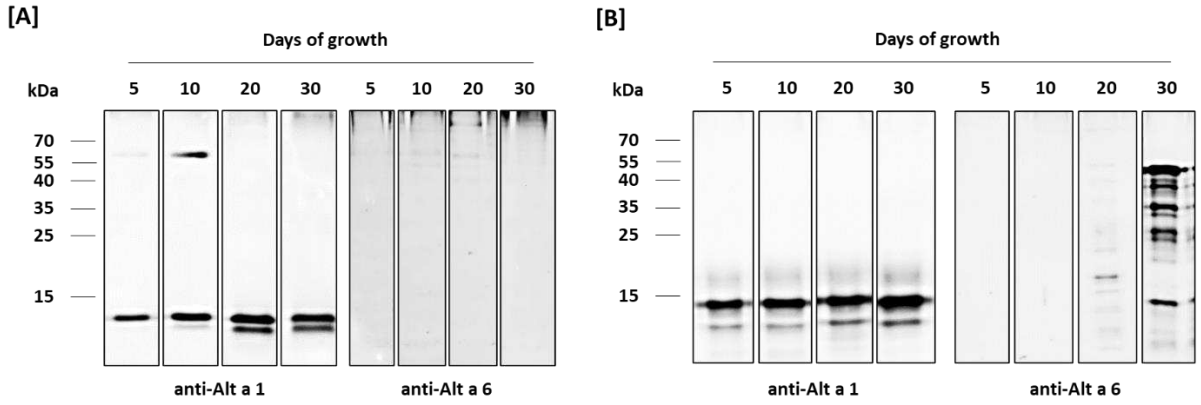

Figure 5. Analysis of the effect of the cultivation time on the expression of Alt a 1, Alt a 6, Ulo c 1, and Ulo c 6. **[A]** Two µg of *Alternaria alternata* protein extracts were separated by 12% or 15% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against Alt a 1 or against rAlt a 6. One representative replicate out of three biological replicates is shown for each time-point. **[B]** Two µg of *Ulocladium chartarum* protein extracts were separated by 12% or 15% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against Alt a 1 or against rAlt a 6. One representative replicate out of three biological replicates is shown for each time-point. Molecular weight markers are indicated in the left margin.

To analyze the expression pattern of fungal enolases also in other fungal species, clones of *Aspergillus fumigatus, Cladosporium herbarum* and *Paecilomyces variotii* were cultured under standard conditions for different times and protein extracts were prepared from harvested fungal material. **Figure 6** shows the results of immunoblots performed with the anti-rAlt a 6 serum for detection of the expression of the allergenic fungal enolases Asp f 22, Cla h 6 and Pae v 6.

The expression of all three enolases appeared to depend on the growth time. In case of Asp f 22, highest amounts of the protein could be detected after five days of growth, whereas lower amounts of Asp f 22 were detected in *A. fumigatus* grown for 10 and 20 days, and no Asp f 22 could be detected anymore after 30 days (**Figure 6A**). Comparable results were obtained for *P. variotii* extracts (**Figure 6C**), where highest amounts of the enolase Pae v 6 could also be detected after 5 days of cultivation. However, the expression of Pae v 6 decreased faster than the expression of Asp f 22, since no protein could be anymore detected after 10 days of growth. In contrast, analysis of *C. herbarum* extracts resulted in a different time-dependent expression pattern of the enolase. Cla h 6 expression increased during longer growth times and the protein was only detected after 30 days of growth (**Figure 6B**), which

correlates with the expression of Ulo c 6 (**Figure 5B**). These results clearly show that the expression of proteins is species-dependent, even in case of highly conserved, abundant enzymes such as enolases.

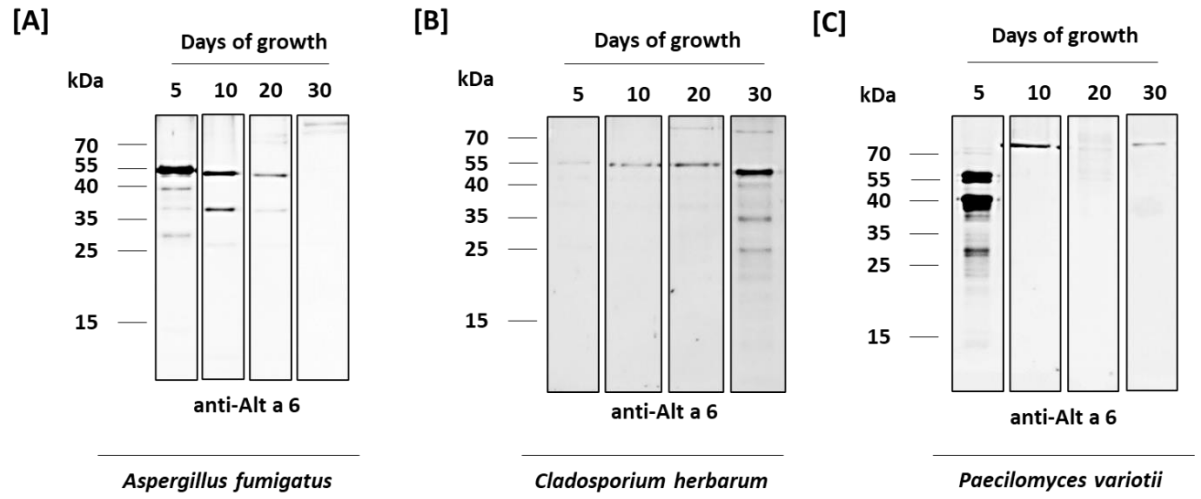

Figure 6. Analysis of the effect of the cultivation time on the expression of fungal enolases. [A] Three µg of *Aspergillus fumigatus* protein extracts were separated by 12% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against Alt a 6. One representative replicate out of three biological replicates is shown for each time-point. **[B]** One µg of *Cladosporium herbarum* protein extracts were separated by 12% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against Alt a 6. One representative replicate out of three biological replicates is shown for each time-point. **[C]** Two µg of *Paecilomyces variotii* protein extracts were separated by 12% SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to a rabbit antiserum raised against Alt a 6. One representative replicate out of three biological replicates is shown for each time-point. Molecular weight markers are indicated in the left margin

Discussion

Fungi represent extremely complex allergen sources, and problems related to the unreliable diagnosis of fungal allergies due to the poor quality of allergy test solutions are far from being solved (8). Moreover, until now there are no generally accepted guidelines for the preparation of allergenic fungal extracts, thus considerable differences in potency of fungal extracts between manufacturers and even batch-to-batch variations can be found (4,8). Besides intrinsic factors, the fungal raw material and manufacturing processes can affect the allergenicity of fungal allergen extracts (4,11).

It is known that fungi require different conditions for optimal growth and that the morphologic, biochemical, and antigenic characteristics of a given fungal strain change with variations in the nutritional composition of the culture medium (11,17). In addition, physical conditions such as the growth temperature or pH might also be important for fungal allergen expression (11,18–22). In this study, the impact of various environmental conditions as well

as the effect of cultivation time on the expression of different fungal allergens was determined by analysis of the expression of the major fungal allergens Alt a 1 and Ulo c 1, as well as of different fungal enolases by immunoblots.

Results of anti-Alt a 1 immunoblots showed that the growth on glucose, an easily accessible substrate, compared to the growth on cellulose or methylcellulose, which represent more complex carbon sources, did not lead to any differences in the expression of the major allergens Alt a 1 and Ulo c 1. Thus, the availability of only complex and unfavorable substrates will not necessarily prevent fungal growth and the production of the fungal allergens Alt a 1 and Ulo c 1. Previous studies have suggested that the exposure to light does not only have a profound effect on fungal mycelial growth, but also on fungal sporulation and on the germination of fungal spores (11,23). Furthermore, studies have shown that fungal spores tend to release more allergens when germinated, which is crucial for allergens that are predominantly found in spores, such as Alt a 1 (24–27). We therefore wondered whether the exposure of *A. alternata* and *U. chartarum* to a natural light-dark-rhythm would influence the expression of the species' major allergens in comparison to no light exposure. Results suggested an earlier expression of Alt a 1 when *A. alternata* is not exposed to light and a slightly increased production of Ulo c 1 by *U. chartarum* during light exposure. Nevertheless, this environmental condition does not seem to be of high importance for the expression of these allergens.

Even though, the type of carbon source and the exposure to light might not be crucial for the expression of Alt a 1 and Ulo c 1, a strong temperature-dependent allergen expression was detected. Temperature is the most important physical environmental factor for regulating the growth and reproduction of fungi (23). *A. alternata* usually shows the fastest growth at 25-30°C, whereas *U. chartarum* ideally grows at a temperature of 25°C, but can still grow well at 30°C and 35°C (23,28). It is therefore not astonishing that fungal growth at 30°C led, in both cases, to the earliest and highest production of Alt a 1 and Ulo c 1.

Analyzing the influence of the cultivation time, the experimental set-up was optimized to minimize natural and methodological variability and thus, improve reproducibility and comparability of fungal protein extracts. The improved set-up with biological triplicates and a standardized protein extraction procedure, made it possible to detect growth time-dependent differences in the expression of Alt a 1 and Ulo c 1 (**Figure 5**), which could not clearly be seen

during previous experiments (**Figures 1-4**). It has been shown that the amount of Alt a 1 and Ulo c 1 increases with continuing growth of the fungal species. These results correlate with the study implemented by Ibarrola et al., which describes a constant increase in Alt a 1 content up to one month of *A. alternata* growth (18).

Furthermore, the impact of different growth conditions on the expression of the highly cross-reactive fungal allergen enolase was also investigated. Results showed differences in the production of the identical, allergenic enolases Alt a 6 and Ulo c 6 from *A. alternata* and *U. chartarum*. In general, Alt a 6 could not be extracted from *A. alternata* mycelium and spores under any conditions. In comparison, protein extracts containing Ulo c 6 could be produced after 10 days of growth on glucose- or cellulose-based medium as well as after 20 days of growth either in the presence of light or when cultivated at 37°C. In addition, growth time-analyses showed that Ulo c 6 was only detectable after 30 days of growth under standard conditions. Despite the fact that enolase is a highly abundant enzyme (29), only small amounts of Ulo c 6 could be extracted from *U. chartarum* mycelium and spores. These results show the generally high variability of the allergenic content of fungal extracts and the difficulty in producing reliable allergen extracts for diagnosis.

Enolase represents a highly conserved fungal pan-allergen that has been identified as an allergen from various fungal sources besides *A. alternata* and *U. chartarum*, including *Aspergillus fumigatus, Cladosporium herbarum* and *Paecilomyces variotii* (1,10,16,30,31). To analyze whether fungal enolases have similar expression patterns, we analyzed the production of the enolases Asp f 22 from *Aspergillus fumigatus,* Cla h 6 from *Cladosporium herbarum* and Pae v 6 from *Paecilomyces variotii* after different growth times of the fungi. Anti-rAlt a 6 immunoblots (**Figure 6**) showed that the expression of the different fungal enolases varies in the different species. Asp f 22 and Pae v 6 could only be detected in early stages of growth with highest enolase concentrations observed already after 5 days, whereas Cla h 6 and Ulo c 6 were predominantly found in extracts prepared from older fungal material (30 days of growth). Due to the variability of the results obtained, it will be of interest to investigate the expression of the different fungal enolases mentioned also under varying physical conditions, such as cultivation temperature and the pH-value of the medium, to obtain more knowledge about the enzyme's expression profile.

In conclusion, results of this study confirm previous investigations by showing the impact of different cultivation conditions on fungal allergen production. Furthermore, our data clearly showed that the expression of fungal allergens is species-dependent, even in case of highly conserved, abundant allergens such as enolases. This emphasizes the necessity of determining the optimal cultivation conditions for each fungal species separately, to obtain standardized extracts with a specific allergen content for fungal allergy diagnosis. Information obtained in this study will help to optimize fungal cultivation to produce highly potent diagnostic fungal extracts and therefore improve diagnosis' specificity and sensitivity.

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Appendix A: Academic *Curriculum vitae*

SANDRA PFEIFFER, MSc

WORKING EXPERIENCE

Since 07/2017	**FH Campus Wien, Vienna, Austria** • Researcher / PhD student • Competence Center for Molecular Biotechnology • Working Group of Immunology – Ines Swoboda
07/2016-01/2017	**Danube University Krems, Krems, Austria** • Master student • Center for Integrated Sensor Systems • Thesis: *'Development of a reliable detection method for fungi and fungal spores in the air'* supervised by Karen Zuser
05/2015-06/2015	**Queens University Belfast, Portaferry, Northern Ireland** • Volunteer • Marine Biology Laboratory (EnAlge Project)
02/2015-05/2015	**Queens University Belfast, Belfast, Northern Ireland** • Bachelor student • Institute for Global Food Security • Thesis: *'Development of prototype immunoassays for the detection of a synthetic ciguatoxin'* supervised by Owen Kavanagh

ACADEMIC EDUCATION

Since 09/2017	**University of Natural Resources and Life Sciences, Vienna, Austria** • Study course: Doctoral Studies of Natural Resources and Life Sciences
09/2015-07/2017	**IMC FH Krems, Krems, Austria** • Study course: Medical and Pharmaceutical Biotechnology (Master, Full-time; English) • Concluded with distinction as Master of Science in Engineering (MSc)

09/2012-08/2015	**FH Wiener Neustadt Campus Tulln, Tulln, Austria** • Study course: Biotechnical Processes (Bachelor; Full-time; German) • Concluded with distinction as Bachelor of Science in Natural Sciences (BSc)
2008-2012	**AHS BG Rechte Kremszeile, Krems, Austria** • Concluded with school-leaving certificate ('Matura')

ACHIEVEMENTS

Scholarships Prizes	• Scholarship from the 'Hochschuljubiläumsstiftung' Vienna, Austria (2018) • Price for the best poster presentation during the EAACI Allergy School on Aerobiology and Pollution in Dubrovnik, Croatia (2018)
Public relations	• derStandard article (issue from 13.04.2021): **Wie die Promotion an einer FH abläuft**
Congress participations	• Poster presentation at the EAACI (European Academy of Allergy and Clinical Immunology) Allergy School on Aerobiology and Pollution in Dubrovnik, Croatia (2018) • Oral presentation at the 10th ÖGMBT (Österreichische Gesellschaft für Molekulare Biowissenschaften und Biotechnologie) Meeting in Vienna, Austria (2018) • Poster Flash at the 11th ÖGMBT Meeting in Salzburg, Austria (2019) • Poster presentation at the EAACI Annual Congress in Lisbon, Portugal (2019) • Poster presentation at the EAACI Annual Congress (digital event; 2020) • Oral presentation at the 32nd Mainzer Allergie Workshop (hybrid event; 2020) • Science Flash at the 13th ÖGMBT Meeting (digital event; 2021)