

Immunoglobulin E-binding and skin test reactivity to hydrophobin HCh-1 from *Cladosporium herbarum*, the first allergenic cell wall component of fungi

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Summary

Background For many years, fungal spores have been recognized as potential causes of respiratory allergies. All fungal allergens cloned so far represent either secreted or cytoplasmatic proteins, but nothing is known about the involvement of fungal surface proteins in allergic diseases.

Methods A phage surface displayed cDNA-library from the mould *Cladosporium herbarum* was constructed and phage displaying IgE-binding proteins were selectively enriched with immobilized serum IgE from *C. herbarum*-sensitized individuals. Inserts encoding putative allergens were sequenced, subcloned and used to produce recombinant proteins. Allergenicity of the proteins was evaluated by IgE binding in Western blots, enzyme-linked immunosorbent assay (ELISA) and skin prick test in a total of 84 patients sensitized to either *C. herbarum* or *Aspergillus fumigatus* and three healthy controls.

Results After four rounds of affinity selection, the cDNA-library was enriched for clones displaying IgE-binding molecules. Sequencing of inserts showed that one clone contained an open reading frame predicting a protein of 105 amino acids and a calculated molecular weight of 10.5 kDa showing the classical signature of members of the hydrophobin family. The recombinant protein, termed HCh-1, was able to bind IgE from six patients sensitized to fungi *in vitro*. Two of those patients were also included in a skin prick test survey and showed strong type I skin reactions to HCh-1, demonstrating the allergenic nature of *C. herbarum* hydrophobin and indicating a prevalence of sensitization in the range of 8–9%. In contrast, the hydrophobin HYP1 from *Aspergillus fumigatus* was not recognized by the sera of the same patients and controls investigated with HCh-1.

Conclusion *C. herbarum* hydrophobin represents the first component of the cell wall of fungi demonstrated to act as a rare but clinically relevant allergen *in vitro* and *in vivo*.

Keywords allergy, *Cladosporium herbarum*, fungi, hydrophobin, molecular cloning, recombinant allergens, skin test

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Introduction

Respiratory allergic symptoms have been correlated with indoor and outdoor exposures to fungal spores mainly from *Aspergillus*, *Alternaria*, *Cladosporium* and basidiomycetes [1–3]. Nevertheless, several problems in assessing exposure [4] and sensitization to fungi [5] remain. The main problem in routine diagnosis of allergy is due to the high variability in the content of allergenic components of commercial extracts from different sources and between batches of the same provider [6]. In recent years, molecular biology has considerably contributed to alleviating this problem. A great number of fungal allergens have been identified using different cloning techniques and produced in heterologous hosts [7]. The use of recombinant fungal

allergens contributes greatly to improve sensitivity and specificity of the diagnosis of fungal allergy both *in vitro* [8, 9] and *in vivo* [10, 11]. Thus, the importance of cloning methods can hardly be overestimated, especially in the field of mould allergy. All fungal allergens cloned so far represent either secreted or cytoplasmatic proteins [7, 12] spanning a wide variety of single structures and cross-reactive protein families [7, 13–15]. Interestingly, none of the cloned allergens was identified as a cell wall component in spite of the fact that the primary exposure results from inhalation of airborne spores [16, 17]. During the screening of a *Cladosporium herbarum* cDNA-library displayed on phage surface with IgE from *C. herbarum*-sensitized individuals, we discovered hydrophobin as an allergen representing a fungal cell wall protein.

Here we describe cloning, identification and characterization of the first hydrophobin from *C. herbarum*, HCh-1, which displays the characteristics of a class I hydrophobin, and the ability of the recombinant protein to bind serum IgE *in vitro* and to induce type I skin reactions *in vivo*.

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Materials and methods

Subjects and routine assessments

Sera from 24 individuals sensitized to *C. herbarum* and from 60 individuals sensitized to *Aspergillus fumigatus* were selected according to clinical history and immediate skin reactivity to commercial extracts. The titration of allergen-specific IgE was performed using Phadebas RAST (Pharmacia, Uppsala, Sweden) according to the package insert and converted into radioallergosorbent test (RAST) classes as described by the manufacturer. Commercially available *C. herbarum* and *A. fumigatus* extracts for skin prick test (SPT) solutions (ALK, Copenhagen, Denmark) were used to assess sensitization and considered positive if a weal diameter > 3 mm² was induced in absence of reaction to the saline control solution [18].

Construction and screening of a *C. herbarum* cDNA-library displayed on phage surface

Construction and screening of a *C. herbarum* cDNA-library displayed on phage surface was performed as described elsewhere [19]. Sera from 13 individuals sensitized to *C. herbarum* served as source of serum IgE for affinity enrichment.

Protein sequence analysis

A prediction of the signal sequence in HCh-1 was obtained using the neural network program SignalP V1.1 on the World Wide Web Prediction Server at the Center for Biological Sequence Analysis, Denmark (<http://www.cbs.dtu.dk/services/SignalP/>) [20]. The amino acid sequences were aligned using the program CLUSTAL W [21]. First the cysteines were aligned manually, and then the amino acids between the cysteines were aligned. Hydrophobicity plots were calculated according to Kyte and Doolittle [22] applying a six amino acid window.

Cloning, production and purification of HCh-1 and HYP1

For expression in *Escherichia coli*, the complete coding sequences of the HCh-1 was amplified by PCR using the following oligodeoxynucleotides: 5' primer: 5'-ATGGATC-CATGGCCTTCATCAAGTGTCTC-3' and 3' primer: 5'-TTAAGCTTAAAGAGGATGGCAAGG-3'. The gene encoding HYP1, a hydrophobin of *A. fumigatus*, was amplified using the primers 5'-ATAGATCTATGAAGTTCTCTTT-GAGCGC-3' and 5'-GTAAGCTTACAGGATAGAAC-CAAGG-3', based on the sequence from Parta et al. [23]. Both inserts were cloned into the high-level expression vector pQE30 (Qiagen, Hilden, Germany) and recombinant proteins produced cytoplasmatically after induction of exponentially growing cultures by addition of isopropyl-1-thio- β -D-galactoside (IPTG) to a final concentration of 1.0 mM. After 3 h of further shaking culture (37 °C, 220 rpm), cells were harvested by centrifugation at 4500 $\times$ g for 15 min at 4 °C. The supernatant was removed and cells resuspended in 25 mL 7 M guanidine hydrochloride, 100 mM NaH₂PO₄, 10 mM Tris pH 8.0 and lysed over night at 4 °C. After centrifugation (17500 g, 4 °C, 45 min) the cleared lysate was directly loaded on an equilibrated column containing 1.5 mL bedvolume Ni-NTA Superflow resin (Qiagen) and purified under denaturing conditions according to the supplier's recommendation. After dialysis against phosphate-buffered saline (PBS) protein concentrations were determined on the basis of the calculated ϵ_{280} absorption coefficients [24].

Titration of allergen-specific serum IgE and competition ELISA

For allergen-specific IgE titrations Maxisorp polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 5 μ g/mL of the hydrophobins in PBS, washed and blocked with 2% skimmed milk powder in PBS containing 1% Tween-20 (buffer A). After washing with PBS containing 0.1% Tween-20, sera were diluted 1:8 in buffer A, serially diluted twofold throughout the plate and incubated for 2 h at 37 °C. Subsequently TN-142 mouse anti-human IgE mAb (1 mg/mL) diluted 1:6500 in buffer A was added for 2 h at 37 °C. TN-142 was detected with alkaline-phosphatase-conjugated goat-anti-mouse IgG H + L chain antibody (0.6 mg/mL; Pierce, Rockford, IL, USA, 1:6500 dilution) in buffer A for 1 h at 37 °C. Enzyme-linked immunosorbent assay (ELISA) plates were developed with 1.5 mg/mL 4-nitrophenylphosphate disodium salt in diethanolamine buffer pH 9.8 for 45 min. Absorbance was measured at 405 nm with a Molecular Devices Reader (Menlo Park, CA, USA) and absorbancy values calculated by linear regression. Competition experiments were performed in ELISA plates as described [10, 15] and for graphic display, the measured absorbencies were plotted against the antigen concentration in fluid phase.

Western blotting

Recombinant proteins were separated on 4–20% Tris–glycine polyacrylamide gels (Invitrogen, Groningen, Netherlands) under reducing conditions, transferred to Immobilon-P[®] polyvinylidene-fluoride membranes (Millipore, Bedford, MA, USA) at 10 mA/cm² and processed as described [15]. After incubation with patients' sera [1:10 dilution in PBS containing 1% bovine serum albumin (BSA) and 0.1% Tween-20] binding of specific IgE was detected by incubation with TN-142 mouse anti-human IgE mAb (1 mg/mL) at 1:2000 dilution. Bound IgE was visualized after incubation with peroxidase-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark, 1 mg/mL, 1:5000) developing the blots with the ECL Plus substrate kit (Amersham Pharmacia Biotech, Rockford, IL, USA).

Skin test with recombinant proteins

SPT with recombinant proteins were performed according to established procedures [11, 18] in 24 patients (mean age 31.4 years; 16 female, nine male) who were SPT-positive to *C. herbarum* extract (ALK). Only 10 of those 24 patients also showed specific IgE against *C. herbarum* detectable by ImmunoCAP, indicating substantial differences between SPT extract used and the extract immobilized to the ImmunoCAP. Commercially available *C. herbarum* and *A. fumigatus* SPT solutions (ALK) were used to show sensitization to the moulds in the concentrations recommended by the supplier. Recombinant proteins were dissolved in 0.9% saline containing 0.4% (w/v) phenol (Evans solution) at a concentration of 100 μ g/mL. All the solutions were applied in duplicate in inverse order on the volar forearm of the patients with histamine (0.01%) and saline (0.9%) as positive and negative controls, respectively. Records of the skin reaction were outlined with a fine-tipped felt pen and transferred to paper with transparent tape 15 min after application of the allergen solution. A test was considered positive with a weal > 3 mm in the absence of reaction to the saline control

[18]. The study was approved by the ethical committee of the University of Zurich. A full oral and written explanation of the procedure was given to all the participants and written consent was obtained before testing.

Results

Identification of HCh-1 encoding cDNA

A cDNA-library from *C. herbarum* containing 8.9×10^8 independent clones was constructed in the pJuFoII phagemid and used to generate a library displayed on the surface of the filamentous phage M13 as described [25]. The phage surface display library was selectively enriched for phage displaying IgE-binding proteins by biopanning with serum IgE from *C. herbarum*-sensitized individuals. After four rounds of affinity selection and amplification, inserts of phage differing in length were sequenced using vector-derived primers. The deduced amino acid sequence of one of the selected clones (Fig. 1) shows a hydrophobicity plot characteristic for class I hydrophobins [26–28], which is that the cysteine doublets are followed by a set of hydrophilic amino acids. The clone encoding HCh-1 (EMBL accession number AJ496190) contains an insert of 663 bp consisting of a coding region of 315 bp (bases 33–350) predicting a protein of 105 aa with a molecular mass of 10 532 Da and a calculatory pI value of 4.03. The 3'-non-coding region has a polyadenylated tail of 22 bp. The correct open reading frame was identified by start and stop codons in the nearly correct consensus context [29]. The cysteine doublets at positions 50/51 and 83/84 are followed by some hydrophilic amino acids and the amino-terminus of the predicted pre-protein consists of a stretch of hydrophobic amino acids which could be identified as a signal sequence with a presumable cleavage site between Ala 18 and Ala 19 with a Y-score of 0.800 [20] (Fig. 2). Remarkably, position 18 coincides with a change from a hydrophobic to a hydrophilic stretch the polypeptide chain typical for signal peptides [30].

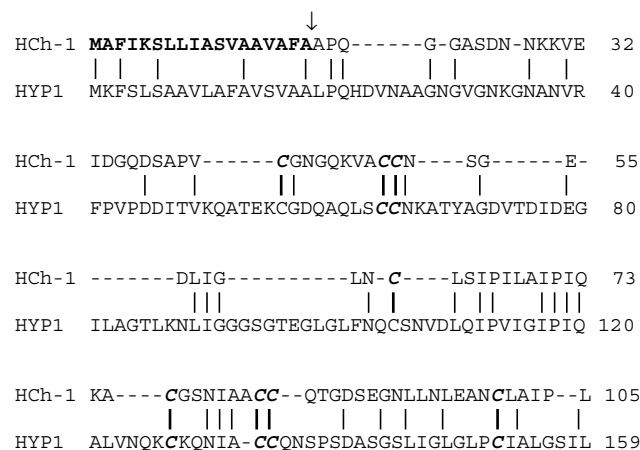


Fig. 1. Alignment of the deduced amino acid sequences of *C. herbarum* HCh-1 and *A. fumigatus* HYP1 hydrophobins. Identical residues are marked by vertical bars, gaps by dashes. Numbers on the right site indicate the residue numbers without gaps starting at the NH₂-terminal methionine residues. The cysteine residues typical for the signature of hydrophobins are shown in bold italics, the putative signal sequence is shown in bold.

Expression and purification of HCh-1 and HYP1

The complete cDNA sequences encoding HCh-1 and HYP1 were expressed in *E. coli* as hexahistidine-tagged proteins and purified by Ni²⁺-affinity chromatography resulting in yields of 9 and 2 mg pure recombinant protein per litre culture. The purified recombinant proteins were found to be free of contaminants as shown by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The estimated molecular masses for the [His]₆-tagged fusions were in good agreement with the calculated values of 10.5 kDa and 16.0 kDa for *C. herbarum* HCh-1 and *A. fumigatus* HYP1, respectively (data not shown).

IgE-specific ELISA, competition ELISA and Western blot

The IgE-binding capacity of the purified recombinant proteins was determined by ELISA. Specific IgE against HCh-1 could be detected in two out of 24 sera from *C. herbarum*-sensitized patients (Table 1, Fig. 3), and in four out of 60 patients suffering from ABPA (Fig. 3). Because patients suffering from allergic bronchopulmonary aspergillosis (ABPA) are sensitized to *A. fumigatus*, we were interested to investigate if the HYP1 hydrophobin from this mould would show IgE binding to the HCh-1-positive sera. Including their hydrophobic signal sequences, HYP1 and HCh-1 share 28% sequence identity (Fig. 1). However, the sera identified to react with HCh-1 showed IgE binding to HYP1 in the range of the control sera of healthy individuals (Fig. 3). Moreover, HYP1 in fluid phase was not able to inhibit IgE-binding to solid phase-coated HCh-1 corroborating the ELISA results (Fig. 4) and demonstrating that these hydrophobins are not cross-reactive. The specificity of the reaction is shown by the lack of IgE-binding of to the structurally unrelated allergen PLA₂ from bee venom used as a control and the results could be confirmed by western blot analysis as shown in Fig. 5.

Skin prick test reactivity

We performed SPTs in 24 patients sensitized to *C. herbarum* with respect to SPT and/or specific IgE and three control individuals to demonstrate the allergenicity of HCh-1 *in vivo*. Recombinant HCh-1 elicited immediate type skin reactions in two *C. herbarum*-sensitized individuals containing HCh-1-specific

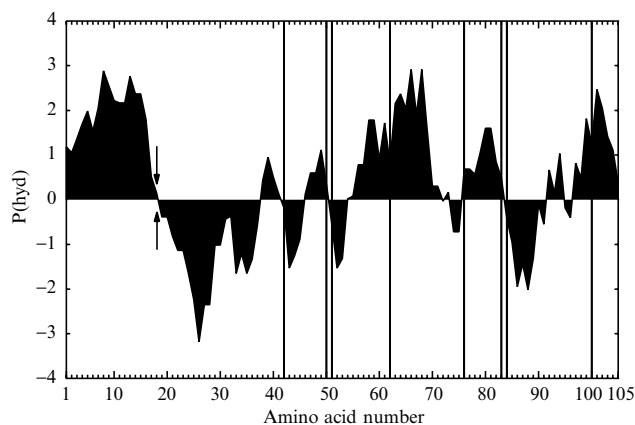


Fig. 2. Hydrophobicity plot of HCh-1 calculated according to ref [22]. Hydrophobic regions show positive, hydrophilic regions-negative P(hyd) values. The positions of the eight cysteines are indicated by vertical lines. The putative cleavage site for of the hydrophobic signal peptide between Ala 18 and Ala 19 is marked with arrows.

IgE antibodies, which is in agreement with the data obtained from ELISA (Table 1). According to the serology, *C. herbarum*-sensitized individuals without IgE to HCh-1 were skin test-negative as well as the no allergic control persons tested. All individuals tested failed to react with the *A. fumigatus* HYP1

Table 1. Induction of immediate skin reactions with recombinant HCh-1 in *C. herbarum* and/or *A. fumigatus*-sensitized individuals with (1, 2) or without (3–24) IgE to HCh-1 and in non-allergic control persons (25–27)*

Individual	<i>C. herbarum</i> extract	<i>A. fumigatus</i> extract	HCh-1	HYP1	IgE HCh-1 [OD ₄₀₅]	IgE <i>C. herbarum</i> [kU/L]
1	4.5	0	12.5	0	0.763	13.1
2	4.5	6.5	7	0	0.360	8.38
3	8.5	0	0	0	0	21.2
4	8	0	0	0	0	2.34
5	7.5	7.5	2	0	0	9.35
6	7	7.5	0	0	0	1.38
7	5	0	0	0	0	4.67
8	6	4.5	0	0	0	3.28
9	4.5	4.5	0	0	0	2.18
10	7	5.5	0	0	0	0.39
11	3.5	4	0	0	0	< 0.35
12	4	2	0	0	0	< 0.35
13	5.5	0	0	0	0	< 0.35
14	5	6	0	0	0	< 0.35
15	4.5	5.5	0	0	0	< 0.35
16	6	4	0	0	0	< 0.35
17	5.5	5	0	0	0	< 0.35
18	4.5	5	0	0	0	< 0.35
19	5.5	4.5	0	0	0	< 0.35
20	4.5	0	0	0	0	< 0.35
21	6	3	0	0	0	< 0.35
22	4.5	0	0	0	0	< 0.35
23	4	5	0	0	0	< 0.35
24	7.5	5.5	0	0	0	< 0.35
25	0	0	0	0	0	< 0.35
26	0	0	0	0	0	< 0.35
27	0	0	0	0	0	< 0.35

* Results represent the mean weal diameters in mm.

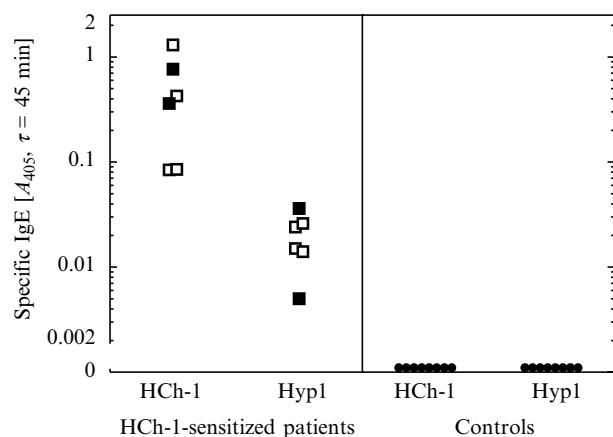


Fig. 3. Specific IgE antibodies to HCh-1 and HYP1 in sera from *C. herbarum*-sensitized patients (■) and in patients suffering from ABPA (□). Sera from healthy control individuals (●) did not show any IgE-binding either to HCh-1 or HYP1.

hydrophobin and to sodium chloride, whereas histamine used as positive control led to immediate type skin reactions in all individual tested (data not shown). These results show a specific, IgE-dependent skin test reactivity to HCh-1 and corroborate the lack of cross-reactivity between the hydrophobins HCh-1 and HYP1.

Discussion

Here we report cloning, production and allergenic characterization of HCh-1, the first described hydrophobin from the deuteromycete *C. herbarum*. Spores of *C. herbarum* belong to the most important fungal allergenic sources, as they occur abundantly and ubiquitously in all continents [1]. The mould is known to cause IgE-mediated diseases such as rhinitis, conjunctivitis and asthma. Several fungal allergens have been cloned

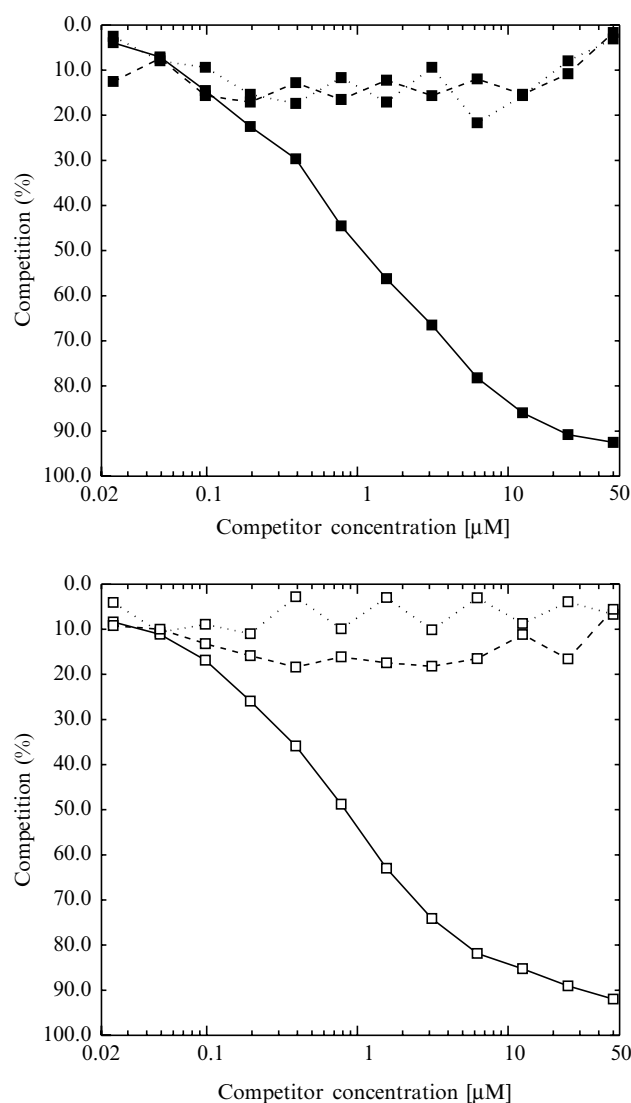


Fig. 4. Inhibition of IgE-binding to HCh-1 coated on a solid phase. Sera of an individual sensitized to *C. herbarum* (■) and of an ABPA patient (□) were pre-incubated with increasing concentrations of HCh-1 (solid lines), HYP1 (dashed lines) and PLA₂ as negative control (dotted lines). Pre-incubated serum samples were transferred to wells coated with HCh-1 and IgE binding was analysed by ELISA.

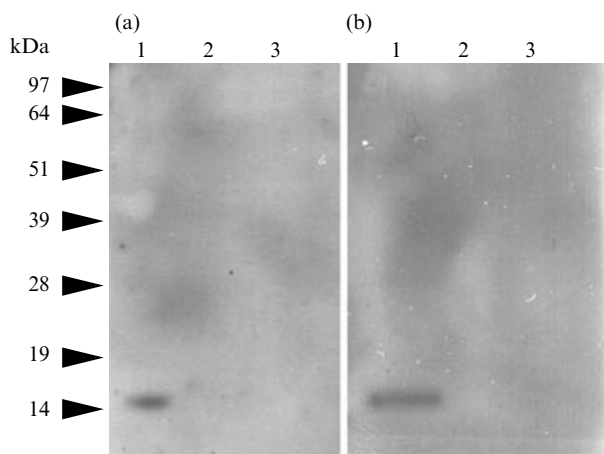


Fig. 5. Immunoblot analysis for specific IgE against recombinant HCh-1 and HYP1 with sera of a *C. herbarum* sensitized individual (a) and of an ABPA patient (b). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with serum of an individual sensitized to *C. herbarum* (a) or of an ABPA patient (b). Lane 1: HCh-1 (1 µg); lane 2: HYP1 (1 µg); lane 3: PLA₂ as negative control (1 µg). The molecular mass standard (kDa) is indicated on the left side.

and characterized [7]; however, all correspond either to secreted or cytoplasmatic proteins [12]. Hydrophobins are members of a family of proteins that is exclusively found in mycelial fungi. They are small secreted proteins, about 100 aa in length, and contain eight conserved cysteine residues that form intramolecular disulphide bridges. Hydrophobins are surface proteins that exert different biological functions during fungal morphogenesis [26, 31]. They are involved in the formation of aerial hyphae, conidia and fruiting bodies [32]. Furthermore, hydrophobins facilitate adhesion to hydrophobic surfaces [33], thereby contributing to the water- and insect-mediated dispersal of conidia into the environment [34]. The property underlying these functions is the ability of hydrophobins to polymerize on air/water as well as on hydrophobic/hydrophilic interfaces to form an amphipathic membrane [26]. The functional role of the disulphide bridges was described recently for the class I hydrophobin SC3 from *Schizophyllum commune*, which is the stabilization of the monomeric form and preventing premature self-assembly of class I hydrophobins [35]. However, after reducing the cysteines and blocking them with iodoacetamide, thereby maintaining the net charge, the reduced SC3 passed from an unfolded conformation through an α -helical to a α -sheet form, which is conformationally comparable to native SC3 assembled at an air–water interface [35]. Therefore it may be assumed that recombinant HCh-1 produced in the cytoplasm of *E. coli* refolds in a similar manner at 25 °C within minutes. Hydrophobins have been divided into two distinct classes according to their chemical properties and hydrophobicity profiles [26, 28]. Aggregates of class I hydrophobins do not dissociate by heating in 2% SDS at 100 °C, but can be dissolved in 100% trifluoroacetic acid or formic acid. The cysteine doublets are followed by some hydrophilic amino acids. In contrast, the cysteine doublets of class II hydrophobins are followed by hydrophobic amino acids, and class II hydrophobin aggregates dissolve readily in 60% ethanol.

HCh-1, which matches the distinct pattern of the arrangement of cysteines and a hydrophobicity profile characteristic for class I hydrophobins [26, 28], represents the only allergenic fungal surface protein described so far.

The clone encoding HCh-1 was isolated by affinity selection of a *C. herbarum* cDNA-library displayed on phage surface using immobilized serum IgE of *C. herbarum*-sensitized individuals as ligand. Phage surface display has been demonstrated to be a powerful technology to directly select cDNAs of interest through gene product/ligand interaction [25, 36] allowing cloning of whole allergen repertoires in high throughput format [37].

The production of recombinant N-terminally [His]₆-tagged HCh-1 protein and affinity purification over Ni²⁺-NTA columns [38] resulted in reasonable yields (9 mg/L culture) of highly pure HCh-1 protein further used for *in vitro* and *in vivo* assays. The purified protein was recognized by serum IgE from individuals sensitized to *C. herbarum* and from patients suffering from ABPA in ELISA (Fig. 3) and Western blots (Fig. 4), and triggered intense type I skin reaction when tested in two of those individuals (Table 1). The high specificity of these reactions was demonstrated by the lack of *in vivo* or *in vitro* reactivity in individuals without serum IgE against HCh-1. The rate of sensitization to HCh-1 seems to be rather low: in a total of 24 patients sensitized to *C. herbarum* in terms of SPT we found only two and in 60 patients sensitized to *A. fumigatus* in terms of RAST we found only four reacting to this newly defined allergen in ELISA. Therefore, HCh-1 fulfils the criteria to be classified as a minor but clinically relevant allergen.

Patients sensitized to *C. herbarum* and HCh-1 all showed multiple sensitizations to various other inhalant allergens and clinically presented with rhinitis as well as asthma. However, no clear clinical differences could be determined in this limited number of patients compared to patients reacting to *C. herbarum* but not HCh-1. In contrast to the newly isolated HCh-1, HYP1, a hydrophobin of *A. fumigatus* sharing 28% sequence identity with HCh-1, lacked to show any allergenicity confirming the observation that proteins with less than 50% sequence identity are rarely cross-reactive [39]. However, it has been shown that other fungi such as *Schizophyllum commune* [40] or *Agaricus bisporus* [41] encode more than one hydrophobin and for the tomato pathogen *Cladosporium fulvum* six different hydrophobin coding sequences have been reported [28, 42]. HCh-1 shows only very limited sequence identity to other hydrophobins and lies at best in the range of 30% when aligned to HYP1 or to Hcf-3 from *C. fulvum*. Nonetheless, it can be assumed that *C. herbarum* produces additional hydrophobins which might be involved in allergic diseases related to fungi.

Acknowledgements

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