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Short communication

Isolation and immunological characterization of a novel *Cladosporium herbarum* allergen structurally homologous to the α/β hydrolase fold superfamily

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ABSTRACT

Because the ascomycete *Cladosporium herbarum* embodies one of the most important, world-wide occurring fungal species responsible for eliciting typical IgE-mediated hypersensitivity reactions ranging from rhinitis and ocular symptoms to severe involvement of the lower respiratory tract, a more comprehensive definition of its detailed allergen repertoire is unquestionably of critical medical as well as therapeutic significance. By screening a *C. herbarum* cDNA library with IgE antibodies pooled from 3 mold-reactive sera, we were able to identify, clone and affinity-purify a novel allergen candidate (29.9 kDa) exhibiting considerable (three-dimensional) homology to the α/β hydrolase fold superfamily. The latter covers a collection of hydrolytic enzymes of widely differing phylogenetic origin as well as catalytic activity (operating in countless biological contexts) that in general exhibit only little sequence similarity yet show a remarkable conservation of structural topology. Our present study (i) characterizes recombinant non-fusion *C. herbarum* hydrolase as a natively folded, minor mold allergen that displays a prevalence of IgE reactivity of approximately 17% in our *in vitro* immunoblot experiments, (ii) proposes the existence of several putative (speculatively cross-reactive) ascomycete orthologues as determined via genome-wide *in silico* predictions, and (iii) finally implies that *C. herbarum* hydrolase could be included in forthcoming minimal testing sets when fungal allergy is suspected.

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1. Introduction

Fungi represent eukaryotic, non-chlorophyllous, cell-wall surrounded, principally spore-bearing organisms that exist as either symbionts, saprophytes or parasites and as such contribute to a complex panel of human diseases ranging from mycotoxicoses following the ingestion of noxious metabolites, rhinitis, fungal sinusitis and allergic asthma to hypersensitivity pneumonitis (also referred to as extrinsic allergic alveolitis), atopic eczema (dermatitis) as well as, although rarely, life-threatening secondary infections like allergic bronchopulmonary aspergillosis within immunocompromised individuals (Bush et al., 2006; Crameri et al., 2006; Horner et al., 1995; Kurup and Banerjee, 2000; Kurup et al., 2000;

Simon-Nobbe et al., 2008). Since airborne mold spores are environmentally omnipresent in concentrations that substantially exceed the average tree or grass pollen intensities, about 6–24% of the common population, nearly 44% among atopics and, remarkably, up to 80% within asthmatic patients are affected by at least one manifestation of IgE-mediated fungal type I hypersensitivity that of course follows the same phenomena as allergies to other commonly encountered, *per se* harmless natural substances (Crameri et al., 2006; Frew, 2004; Jones, 2008; Kurup et al., 2002; Simon-Nobbe et al., 2008; Vijay and Kurup, 2008). Dating back to the period when fungal identification was exclusively or mainly established on their microscopic appearance, allergenic molds without any obvious connection to already known reproductive stages were previously incorporated in the artificial group of Deuteromycetes or “fungi imperfecti”, and only the consequent introduction of standard molecular biology methodologies combined with the analysis of dendrograms derived from rDNA sequence comparisons enabled their final integration into their accurate taxonomic relationships (Crameri et al., 2006; Horner et al., 1995; Simon-Nobbe et al., 2008). *Bona fide* allergens have so far been isolated from 23 fungal genera which can accordingly be categorized into (i) the Ascomycota including, e.g. the families of *Cladosporium*, *Alternaria* and

Abbreviations: AA, amino acid; CD, circular dichroism; *C. herbarum*, *Cladosporium herbarum*; ESTHER, esterases, α/β hydrolase enzymes and relatives; 6xHIS, hexahistidine tag; IPTG, isopropyl- β -D-thiogalactopyranoside; NHS, normal human serum; RAST, radio allergosorbent testing; rnf, recombinant non-fusion; SIT, specific immunotherapy; TEV, tobacco etch virus.

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Aspergillus, (ii) the Basidiomycota and (iii) the Zygomycota (Simon-Nobbe et al., 2008). Differently from most inhaled particles that display a diameter of more than 10 μm and are thus deposited in the nasopharynx where they provoke the typical symptoms commonly recognized as “hay fever”, fungal spores on average are only about 2–3 μm in size, can penetrate the terminal airways and are thus associated with both upper and lower respiratory symptoms (Horner et al., 1995; Kurup et al., 2000, 2002). Whereas *Cladosporium herbarum* (or *Mycosphaerella tassiana* in its teleomorph form) occurs in nearly all climatic zones of the world but demonstrates a seasonal spore release pattern reaching its zenith during summer and autumn months (Burge, 2002; Horner et al., 1995; Vijay and Kurup, 2008), indoor fungi on the other hand are a heterogenous combination of molds growing in indoor locations and those entering from outside. They consequently cause permanent exposure situations, explaining why the majority of fungi-sensitive patients rather suffers from perennial symptoms which can contribute to both the chronicity and severity of asthma (Crameri et al., 2006; Jacob et al., 2002; Portnoy et al., 2004; Terr, 2004).

Screening of fungal cDNA libraries with sera of mold-sensitized patients or the cloning of allergens via phage-display has permitted a continuous advance in the characterization of mold allergens within the last two decades (Achatz et al., 1995, 1996; Breitenbach and Simon-Nobbe, 2002; Crameri, 1998; Rhyner et al., 2004). As far as *C. herbarum* is concerned, several IgE-binding molecules have by now been identified in this way, including acidic ribosomal phosphoproteins P₁ (Cla h 12, Achatz et al., 1995) and P₂ (Cla h 5, Zhang et al., 1995), enolase (Cla h 6, Simon-Nobbe et al., 2000), flavodoxin (YCP4 homologue, Cla h 7, Achatz et al., 1995), NADP-dependent mannitol dehydrogenase (Cla h 8, Simon-Nobbe et al., 2006), aldehyde dehydrogenase (Cla h 10, Achatz et al., 1995), a cold shock factor (Cla h CSP, Falsone et al., 2002), glutathione-S-transferase (Cla h GST, Shankar et al., 2005), type I hydrophobin (Cla h HCh1, Weichel et al., 2003b), heat shock protein 70 (Cla h HSP70, Zhang et al., 1996), a translationally controlled tumour protein homologue (Cla h TCTP, Rid et al., 2008), nuclear transport factor 2 (Cla h NTF2, Weichel et al., 2003a) and a vacuolar serine protease (Cla h 9, Poll et al., 2009). With the exception of the only major allergen Cla h 8 that is recognized by approximately 57% of the *C. herbarum* allergic population, the residual candidates symbolize only minor allergens with a prevalence of less than 20% (Simon-Nobbe et al., 2008) – an observation that is quite widespread within fungal species. In this context, we here present our successful approach of identifying and isolating a novel minor ascomycete allergen, *C. herbarum* hydrolase, which was produced as a pure recombinant non-fusion (rnf) protein and tested for its *in vitro* clinical relevance.

2. Materials and methods

2.1. Reagents, primers and sera

Chemicals were, unless otherwise stated, purchased from Applichem GmbH, Darmstadt, Germany, cloning and restriction enzymes from Fermentas Life Sciences GmbH, St. Leon-Rot, Germany. Primers were synthesized by MWG-Biotech AG, Ebersberg, Germany. Human sera were supplied by the Department of Dermatology, St. Johannis-Spital, Salzburg, Austria.

2.2. Preparation of a crude *C. herbarum* protein extract

A *C. herbarum* (strain collection number 28-0202, Institute of Applied Microbiology, University of Agricultural Sciences, Vienna, Austria) protein extract was prepared as reported before (Achatz et al., 1995; Rid et al., 2008), lyophilized and stored at -20°C .

2.3. Preparation of a *C. herbarum* cDNA library in λ -ZAP

As published previously (Achatz et al., 1995, 1996; Breitenbach and Simon-Nobbe, 2002), a cDNA expression library was constructed in the λ -ZAP® II System (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol with polyA⁺ RNA isolated from the total *C. herbarum* RNA pool.

2.4. Screening of a *C. herbarum* cDNA library with human IgE antibodies

Analogously to the procedure described by Rid et al. (2008), the respective library was screened with a serum pool prepared from 3 *C. herbarum* allergic individuals. In brief, nitrocellulose filters (Schleicher & Schüll, Dassel, Germany) – presoaked with 10 mM IPTG – were layered on top of *Escherichia coli* XL-1 blue host cells infected with 6×10^5 phages, incubated at 41°C for 4.5 h to induce protein synthesis, finally lifted and blocked with Gold Buffer (40 mM Na₂HPO₄, 7 mM NaH₂PO₄, 0.5% BSA and 0.5% Tween-20). After overnight incubation with the serum mixture diluted 1:10 in Gold Buffer, positive IgE-binding plaques were visualized with ¹²⁵I-labelled anti-human IgE (MedPro, Vienna, Austria) by scanning imaging plates (Fujifilm BAS Cassette 2325) with a Fujifilm BAS-1800 II instrument after two days of exposure. Pure phages were subjected to a standard *in vivo* excision protocol to release their cDNA inserts from the λ backbone into pBluescript SK- (Stratagene, La Jolla, CA, USA).

2.5. Rescreening of obtained candidates with radioactively labelled allergen probes

An experimental exclusion of the already known *C. herbarum* allergens Cla h 6, Cla h 10, Cla h 7 and Cla h 5 via hybridization screening was performed as described earlier (Rid et al., 2008). Clones giving no signal to these probes were picked as promising new allergen candidates and their phagemid DNA isolated with the GFX™ Micro Plasmid Preparation Kit (GE Healthcare, Uppsala, Sweden).

2.6. DNA sequencing, database searches and computer-assisted analyses

Respective cDNA inserts were sequenced via the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM™ 310 Genetic Analyzer (Table 1). Homology searches and sequence comparisons were performed through BLAST (<http://www.ncbi.nlm.nih.gov/blast/>), NCBI Conserved Domain Searches (<http://ncbi.nlm.nih.gov/structure/cdd/cdd.html>) and PFAM tools (www.pfam.sanger.ac.uk/). Multiple sequence alignments were obtained using ClustalW (<http://www.ebi.ac.uk/clustalw/>).

Table 1

Oligonucleotides used as primers in PCR and sequencing reactions. EcoRI and XhoI restriction sites are boldfaced, start and stop codons underlined. Fw, forward; rv, reverse; int, internal.

Name	Sequence
T ₃	5'-AATTAACCTCACTAAAGGG-3'
T ₇	5'-GTAATACGACTCACTATAGGGC-3'
Hydrolase-int1 fw	5'-TTGGTATCAACGGCTACA-3'
Hydrolase-int2 fw	5'-GAAGGAGATGGAGCGAAGAA-3'
Hydrolase-int3 rv	5'-CTTATCCCCATCACCACC-3'
Hydrolase-int4 rv	5'-AGGCATCAACCACAGTCC-3'
Hydrolase-HIS fw	5'-GGAATTCAAATGCTGCAGGACTGCAAT-3'
Hydrolase-HIS rv	5'-ACTCTCGAGCTACACGTTTGTCTGGTCTC-3'
pHIS fw	5'-CCATCAGCATTACGATATCCC-3'
pHIS rv	5'-CAACTCAGTTCCTTTCTG-3'

The calculation of protein parameters as well as secondary structure predictions were conducted through the ExPASy Proteomics Server (<http://www.expasy.ch/>), the Interpro (<http://www.ebi.ac.uk/interpro/>), PROSITE (<http://www.expasy.ch/prosite/>), SCAN-PROSITE (<http://www.expasy.ch/tools/scanprosite/>), ProtParam (<http://www.expasy.ch/tools/protparam/>) and European Molecular Biology Laboratory EMBL (<http://www.ebi.ac.uk/embl/>) platforms, the universal protein resource UniProt (<http://www.uniprot.org/>), the MyHits collection (<http://myhits.isb-sib.ch/cgi-bin/index>), the PSIPRED database at www.bioinf.cs.ucl.ac.uk/psipred/ accessible via the BioInfoBank Metaserver (www.meta.bioinfo.pl/), T-COFFEE version 6.85 (<http://www.ebi.ac.uk/t-coffee/>) as well as the SWISS-PROT (<http://expasy.org/sprot/>) knowledge-base. An *ab initio* sequence-based three-dimensional model of *C. herbarum* hydrolase was computed via 3D-JIGSAW (<http://www.bmm.cancerresearchuk.org/~3djigsaw/>), a software which splits the query sequence into small elements and searches for homologous templates in various databases such as PFAM or the RCSB Protein Data Bank PDB at www.rcsb.org/. The resultant structure was analysed via RASWIN version 2.6 (<http://www.openrasmol.org/>) or FATCAT (flexible structure alignment by chaining aligned pairs allowing twists, www.fatcat.burnham.org/). Putative linear B-lymphocyte epitopes were calculated via BepiPred 1.0 (<http://www.cbs.dtu.dk/services/BepiPred/>) or BCPREDS 1.0 (<http://www.ailab.cs.iastate.edu/bcpred/>) and visualized within the respective protein structure using UCSF Chimera (<http://www.cgl.ucsf.edu/chimera/>).

2.7. Isolation of *C. herbarum* genomic DNA

C. herbarum genomic DNA was isolated from 30 mg of mycelium as reported previously (Al-Samarrai and Schmid, 2000), dissolved in 50 μ l 1 $\times$ TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.8) and stored at -20°C .

2.8. Subcloning of *C. herbarum* hydrolase into pHIS parallel 2

The open reading frame encoding *C. herbarum* hydrolase was amplified from the original pBluescript SK- clone with gene-specific oligodeoxynucleotides (Table 1) and ligated into the appropriately restricted, dephosphorylated expression vector pHIS parallel 2 (Sheffield et al., 1999).

2.9. Purification of *C. herbarum* hydrolase via Ni^{2+} affinity chromatography

E. coli BL21(DE3) cells were transformed with 6xHIS-hydrolase whose lacZ-promotor mediated expression was induced by exposure to 0.8 mM IPTG for 4 h, harvested and the resultant pellet resuspended in 1/50 volume of denaturing starting buffer (50 mM Na_2HPO_4 , pH 8.0, 300 mM NaCl, 10 mM imidazole, 8 M urea). *C. herbarum* hydrolase was isolated from the bacterial lysate via addition of 1 mg/ml lysozyme, 10 μ g/ml RNaseA as well as 5 μ g/ml DNaseI and 3 consecutive cycles of freezing and thawing prior to shearing genomic DNA by ultrasonication, a removal of the soluble protein fraction by spinning at $10,000 \times g$ for 30 min and lastly a filtration through a 0.45 μ m low-protein binding Millex-HV filter (Millipore Corporation, Bedford, MA, USA). Protein purification was performed under non-native conditions via competitive imidazole gradient elution utilizing HisTrap-HP columns (GE Healthcare, Uppsala, Sweden) charged with 0.2 volumes 0.1 M NiSO_4 on an Äkta™ Prime Plus chromatography system (GE Healthcare, Uppsala, Sweden). After dialysis against steadily decreasing concentrations of urea (6, 4, 2, and 0 M) for 12 h each and finally against 20 mM phosphate buffer (pH 7.4), the affinity tag was cleaved off with 1 U AcTEV™ protease (Invitrogen, Karlsruhe, Germany) per

4 μ g protein, followed by a second one-step passage over the column in the course of which the native rnf protein was collected in the flowthrough fraction, whereas the enzyme was removed by binding to the sepharose material via a genetically engineered polyhistidine tag at its own N-terminus (Kapust et al., 2001). Protein concentration was measured using a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA) and homogeneity of the samples monitored by Coomassie Brilliant Blue and/or silver (SilverXpress kit, Invitrogen, Carlsbad, CA, USA) staining of 13.5% SDS-PAGE gels.

2.10. Isoelectric focusing and two-dimensional protein gel electrophoresis

Isoelectric focusing was performed via a capillary system (Biometra, Göttingen, Germany) employing soluble carrier ampholytes (Serva, Heidelberg, Germany) which build up a pH gradient between 3 and 10. 3 μ g lyophilized rnf *C. herbarum* hydrolase were dissolved in 20 μ l lysis buffer (9.8 M urea, 2% Triton X-100, 2% ampholines, 0.1 M DTT) and separated by applying constant current (2 mA) as well as power (4 W) and slowly increasing voltage (500–1500 V). The second dimension gel run was performed via conventional 13.5% SDS-PAGE.

2.11. IgE immunoblotting of rnf *C. herbarum* hydrolase

Immunoblot analyses with 1.5 μ g rnf hydrolase per experiment were performed as published formerly (Rid et al., 2009, 2008; Schneider et al., 2006). Cursorily, 28 sera of a collective of patients with a typical case history of immediate type I hypersensitivity to *C. herbarum*, a positive skin prick test response to commercial *C. herbarum* extract or mold mix as well as a corresponding RAST class of greater than 3 were investigated in the course of this preliminary study.

2.12. Circular dichroism (CD) spectroscopy measurements

A CD spectrum of rnf *C. herbarum* hydrolase in the range between 190 and 260 nm was recorded in 10 mM sodium phosphate buffer (pH 7.4) with a J-810 spectropolarimeter (JASCO Inc., Easton, MD, USA) in continuous scanning mode using a 1.0 mm path length quartz cuvette, a 1 nm resolution, a 100 mdeg sensitivity, a 100 nm/min scanning speed and a 1 nm bandwidth (Rid et al., 2009; Schneider et al., 2006). The baseline obtained with pure buffer was subtracted from the sample spectrum. Data were finally expressed as mean residue molar ellipticity $[\Theta]_{\text{MRW}} = \Theta / (10 \times \text{Cr} \times l)$, where Θ is the ellipticity in mdeg, l the cell path length in cm and $\text{Cr} = (n \times 1000 \times c_g) / M_r$ represents the mean residue molar concentration taking into account the number of peptide bonds n , the macromolecule concentration c_g in g/ml as well as the molecular weight M_r in Da, respectively.

3. Results

3.1. Identification of a novel *C. herbarum* allergen homologous to the α/β hydrolase fold superfamily

Because the to date 15 in part already comprehensively characterized and recombinantly available *C. herbarum* allergens are *per se* still not sufficient to account for the heterogenous, rather complex pattern of IgE reactivity observed in *C. herbarum* allergic individuals when analysing the crude fungal protein extract via one- or two-dimensional immunoblots, our research group has put much effort into the molecular cloning and description of novel fungal allergens within the last years (Achatz et al., 1995, 1996; Breitenbach and Simon-Nobbe, 2002; Poll et al., 2009; Rid et al., 2009, 2008; Schneider et al., 2006; Simon-Nobbe et al., 2006,

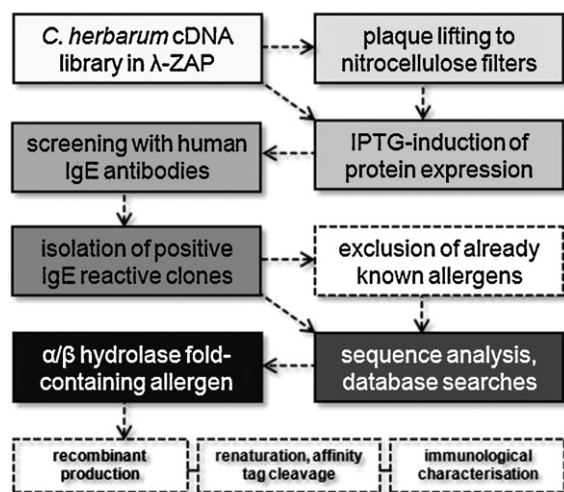


Fig. 1. Short summary of the experimental design that led to the identification, isolation, purification and (immunological) characterization of a novel *C. herbarum* allergen.

2000). By (i) screening a *C. herbarum* cDNA library prepared in the bacteriophage λ with a serum pool combined from 3 ascomycete sensitized patients (a procedure that resulted in 42 plaque-purified clones) and (ii) excluding the well-known allergens enolase, aldehyde dehydrogenase, YCP4 homologue as well as acidic ribosomal phosphoprotein P₂ from subsequent analyses (hence reducing their actual number to 19 potentially interesting candidates), we were able to isolate a previously unknown immunoreactive protein highly homologous to the α/β hydrolase fold superfamily as outlined below. A short graphical impression of the detailed experimental modus operandi is summarized in Fig. 1. As illustrated in Fig. 2, *C. herbarum* hydrolase contains an open reading frame spanning 825 bp that begins with the first methionine initiation codon available at position 164 and ends with a TAG termination triplet at nucleotide location 989, flanked by a 163 bp 5'-UTR and a complete 113 bp 3'-UTR followed by a polyadenylated tail. A PCR amplification of *C. herbarum* hydrolase from fungal genomic DNA using gene-specific primers and the subsequent sequencing of both sense- and antisense strands revealed no evident difference in the definite nucleotide composition compared to the respective cDNA (data not shown), in this way indicating the absence of any introns. The complete cDNA and deduced protein sequence was deposited in the NCBI GenBank database under accession code DQ159861. *C. herbarum* hydrolase possesses a calculated molecular weight of 29.49 kDa, a theoretical isoelectric point (pI) of 5.47 as determined through ExPASy (Gasteiger et al., 2003; Wilkins et al., 1999) and exhibits an aliphatic index of 82.55 (grand average of hydrophaticity: 0.003) as evaluated using ProtParam (Bairoch et al., 2005; Gasteiger et al., 2001). It does not possess any obvious N-terminal leader sequences, nuclear localisation signals, endoplasmic reticulum retention motifs, peroxisomal as well as vacuolar targeting patterns or hydrophobic transmembrane anchors as was determined using SignalP 3.0 (Bendtsen et al., 2004) accessible via <http://www.cbs.dtu.dk/services/SignalP/output.html> and has been predicted to embody a predominantly cytoplasmic molecule (reliability: 94.1%) via the PSORT (Horton et al., 2007) and TargetP (Emanuelsson et al., 2007) portals offered at <http://www.psорт.ims.u-tokyo.ac.jp/> and <http://www.cbs.dtu.dk/services/TargetP/>. The protein harbours several theoretical phosphorylation sites, namely 4 for protein kinase C (T₂₀GK₂₂, S₆₇AK₆₉, T₁₇₅TR₁₇₇, S₂₀₇VR₂₀₉) and 4 for casein kinase II (T₂₀GKE₂₃, T₇₈ADD₈₁, S₁₉₄SLD₁₉₇, S₂₃₈QVD₂₄₁) as determined via NetPhos 2 (Blom et al., 1999) provided by the Technical University of Denmark (<http://www.cbs.dtu.dk/services/NetPhos/>), 1 N-glycosylation pat-

tern (N₂₃₆FSQ₂₃₉) as uncovered via the NetNGlyc 1 Server (Blom et al., 2004) at <http://www.cbs.dtu.dk/services/NetNGlyc/>, as well as 3 N-myristoylation motifs (G₁₂GRLAV₁₇, G₃₃MGDTR₃₈, G₉₉ASFSA₁₀₄), although it remains to be clarified whether these predicted post-translational modifications indeed occur *in vivo*. A supplementary investigation of the *C. herbarum* hydrolase AA sequence with the regular BLASTP algorithm (Altschul et al., 1997) and ultimately a decisive homology search against the ESTHER database (<http://bioweb.enscm.inra.fr/esther>) that again gathers all the published information related to the α/β hydrolase fold superfamily (Cousin et al., 1997; Hotelier et al., 2004; Renault et al., 2005) indicated its plausible relationship to a hypothetical *Giberella zeae* (anamorph: *Fusarium graminearum*) lysophospholipase (274 AA, accession number XP.391338.1, e-value: $2e^{-64}$) implicated in fungal lipid metabolism, to a putative *Streptomyces coelicolor* hydrolase (278 AA, accession number NP.624748, e-value: $5e^{-30}$), to a *Kineococcus radiotolerans* α/β hydrolase fold containing molecule (286 AA, accession number YP.001361968, e-value: $7e^{-24}$), and finally to *Streptomyces lividans* chloroperoxidase L chain A (275 AA, PDB code 1a88, e-value: $3e^{-05}$). The corresponding multiple sequence alignment that compares *C. herbarum* hydrolase with these four α/β hydrolase fold superfamily members is presented in Fig. 3A. Notably, 26 out of the 275 AA (together with the computed catalytic triad involving *C. herbarum* hydrolase S₁₀₁, D₂₂₀ and H₂₅₀) are evolutionarily completely conserved, suggesting their participation in essential physiological functions. By performing further computer-assisted annotations, pattern/profile queries and structure predictions in the InterPro (Apweiler et al., 2001), PROSITE (Hulo et al., 2006), PFAM (Finn et al., 2008), T-COFFEE (Notredame and Suhre, 2004) as well as PSIPRED (McGuffin et al., 2000) databases, it became apparent that *C. herbarum* hydrolase evidently encloses a so-called α/β hydrolase fold-1 (AA 53–155, InterPro: IPR000073, PFAM: PF00561) found in a very wide range of enzymes, an epoxide hydrolase-like domain (AA 52–67, 111–124, 245–267, Interpro: IPR000639, PFAM: PR00412) directly deriving from the former, several currently unintegrated hits, and, even more importantly, actually reveals a considerable three-dimensional homology to *S. lividans* chloroperoxidase L chain A (P-value: $3.66e^{-14}$, raw score: 403.60, 244 equivalent positions) whose overall architecture has in 1998 been resolved by X-ray diffraction (Hofmann et al., 1998). The latter statement is notably reinforced by an *ab initio* sequence-based three-dimensional model of *C. herbarum* hydrolase computed via 3D-JIGSAW (Bates et al., 2001) whose consequential superimposition over *S. lividans* chloroperoxidase L chain A indeed uncovers a substantial overlap – especially in the central 8-stranded (mostly) parallel β -sheet core domain (Fig. 6A), emphasizing once more its presumed membership to the α/β hydrolase fold superfamily. ClustalW (Thompson et al., 1994) was in a last step considered to draw a phylogenetic tree by comparing *C. herbarum* hydrolase with (provisional) homologous α/β hydrolase fold superfamily templates from a selection of both pro- and eukaryotic organisms. The neighbour-joining diagram view in Fig. 3B exemplifies that *C. herbarum* hydrolase undeniably shows significant homology to *G. zeae* lysophospholipase along with which it is clustered in a common branch separated from *S. coelicolor* as well as *K. radiotolerans* α/β hydrolase fold superfamily hydrolases, but is only very distantly related with for instance *Rhizopus* or *Candida* lipases, *Aspergillus* epoxide hydrolase or bacterial haloalkane dehalogenases – a feature that could signify an early evolutionary origin for the *C. herbarum* hydrolase allergen orthologue.

3.2. Expression and purification of *C. herbarum* hydrolase

C. herbarum hydrolase was expressed as a 6xHIS-tagged fusion protein and purified via Ni²⁺ chelate affinity chromatography under

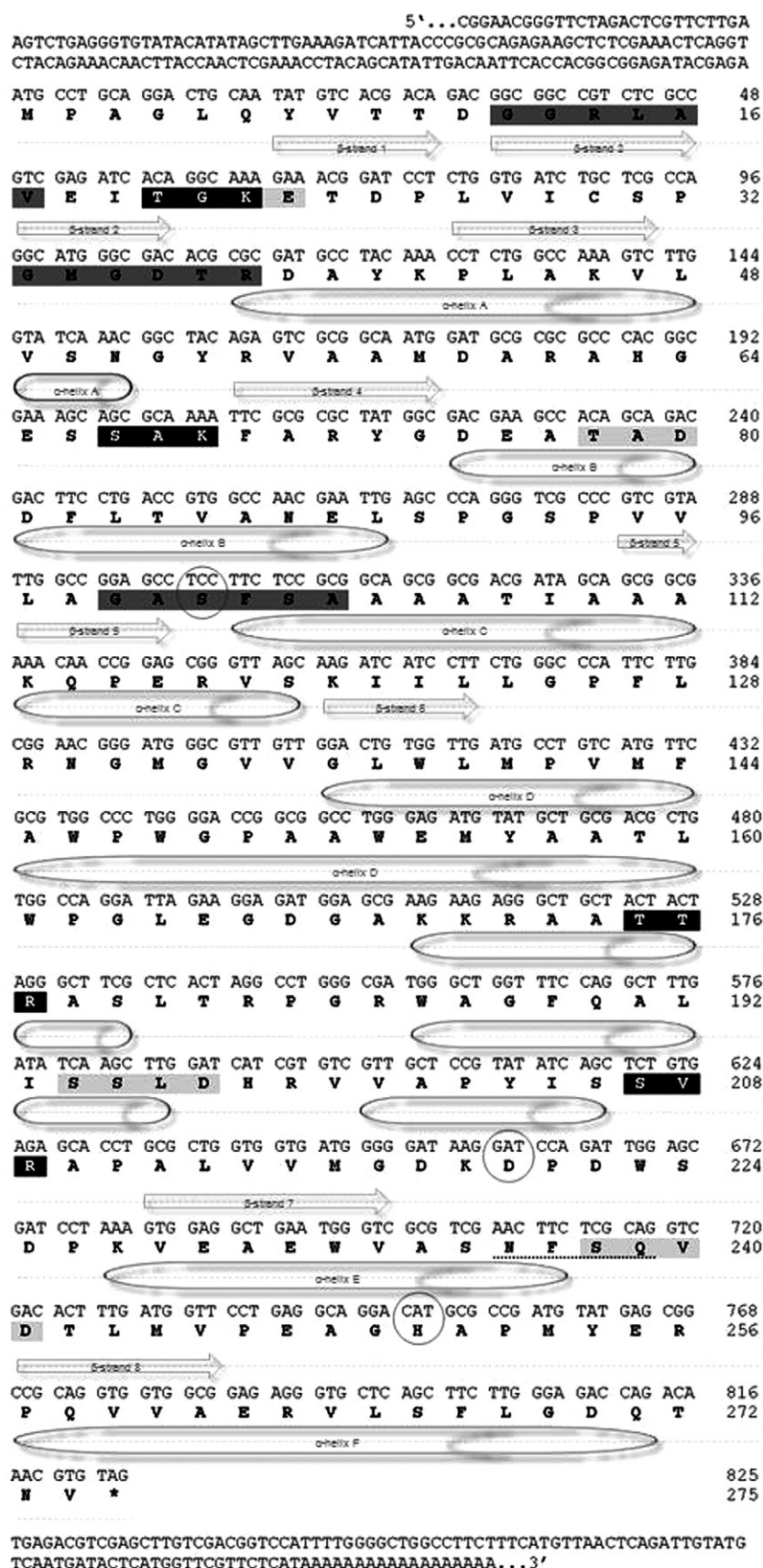


Fig. 2. Nucleotide and deduced AA sequence of full-length *C. herbarum* hydrolase (numbers on the right side denote the actual positions). Transcription is initiated at the first ATG start codon available in the sequence. The termination codon TAG is marked by an asterisk. The predicted sites for protein kinase C phosphorylation (marked in black), casein kinase II phosphorylation (highlighted in light grey), N-glycosylation (underlined with dots) as well as N-myristoylation (dark grey) are indicated as specified in brackets. The highly conserved catalytic triad characteristic for α/β hydrolase fold superfamily members has been circled. Secondary structure elements of *C. herbarum* hydrolase were predicted with high degree of probability (except for the precise position of α helix D) based on a comparison with homologous α/β hydrolase fold superfamily members using various fold recognition and local structure prediction methods. Lines represent coiled regions, arrows indicate β strands and cylinders symbolize α helices.

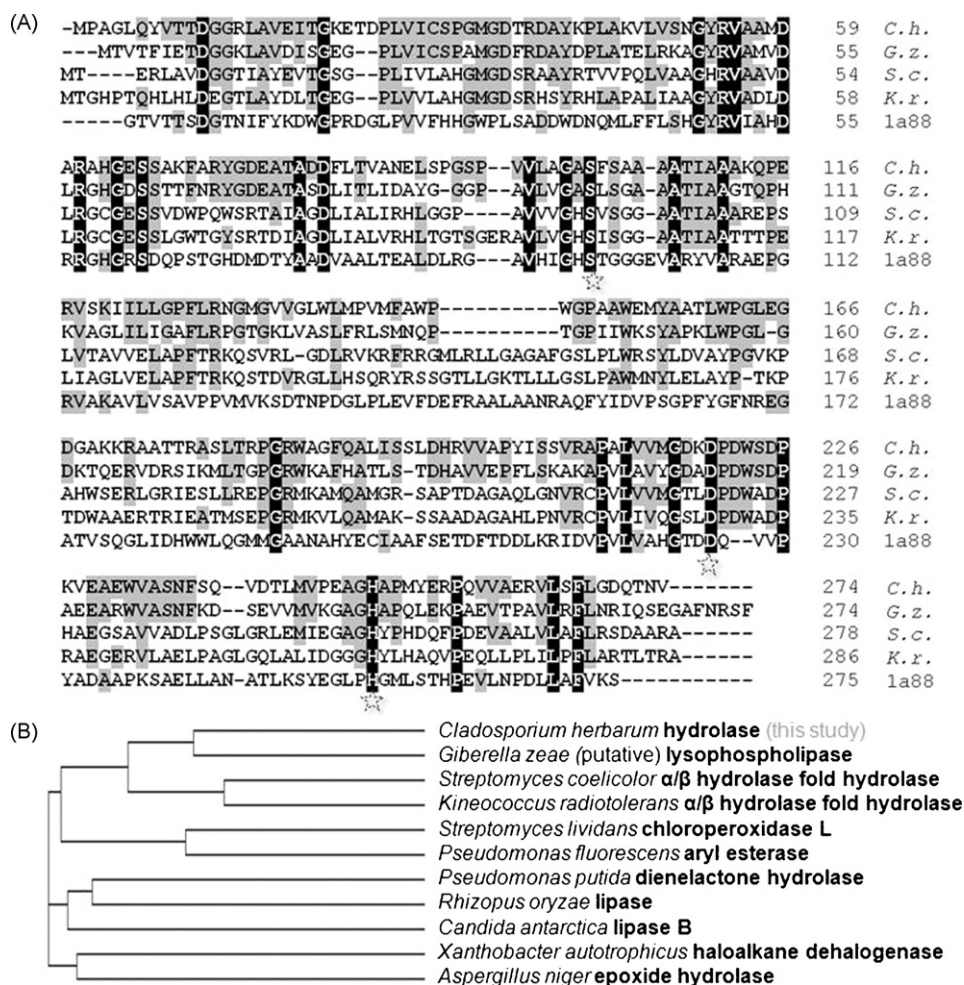


Fig. 3. Multiple sequence alignment. (A) Alignment of *C. herbarum* hydrolase (*C.h.*) with *Giberella zeae* lysophospholipase (*G.z.*) and the phylogenetically distinct α/β hydrolase fold superfamily members *Streptomyces coelicolor* hydrolase (*S.c.*), *Kineococcus radiotolerans* hydrolase (*K.r.*) and *Streptomyces lividans* chloroperoxidase L chain A (1a88) which produced the most significant hits when performing a homology search. AA residues identical in all 5 sequences surveyed are marked with black boxes, residues that *C. herbarum* hydrolase shares with at least one of the proteins in light grey. Gaps are marked by dashes. The highly conserved catalytic triad that is characteristic for the α/β hydrolase fold superfamily and equivalently positioned in all 5 proteins is specified by asterisks. (B) ClustalW was used to construct a phylogenetic tree by comparing *C. herbarum* hydrolase with a selection of α/β hydrolase fold superfamily members as indicated, including the sequences referred to above.

denaturing conditions (Fig. 4A), resulting in a yield of approximately 20.4 mg pure protein per liter culture. Its accumulation in the form of insoluble inclusion body aggregates that was chosen in order to compensate for its poor expression properties demanded, however, a successive multistep *in vitro* refolding procedure to recover its biological activity (Thapa et al., 2008) before its affinity tag was ultimately cleaved off by the extremely site-specific TEV protease (Kapust et al., 2001; Mohanty et al., 2003). The rnf protein, which N-terminally only carries the 5 residues GAMGS that obligatorily derive from the multiple cloning site and are not part of the natural protein, was found to be largely depleted from any *E. coli* contaminants as was determined by one-dimensional (Fig. 4A) as well as two-dimensional (Fig. 4B, resulting in a single spot at a *pI* around 5.6–5.7 which is, nevertheless, somewhat higher than the theoretically calculated value) SDS-PAGE.

A far-UV CD spectrometry measurement – a convenient technique for studying the conformation of polypeptides in solution and for assessing the structural integrity of recombinant proteins (Lees et al., 2006) – finally exposed a typical curve shape for renatured rnf *C. herbarum* hydrolase, consisting of an approximately 45.6% α helical and 16% β strand mixed organization (Fig. 4C). This result indicates that the recombinant protein does not exist as random coils, but is natively like folded at its

secondary structure level and hence suitable for downstream applications.

3.3. Immunoreactivity of rnf *C. herbarum* hydrolase

The importance of *C. herbarum* hydrolase as a novel ascomycete allergen was assessed by subjecting the respective rnf molecule to immunoblotting in the course of which serum IgE obtained from 28 *C. herbarum* allergic individuals (not preselected in any other way) was tested. Parenthetically, the patients' case history showed no particular correlation between RAST class, pattern of IgE reactivity, the patients' symptoms (that were rhinoconjunctivitis and asthma bronchiale) as well as disease severity. Additionally to the routinely performed negative controls where membrane strips were incubated with serum of one non-atopic healthy individual (NHS) or the secondary antibody only, an extra reaction with serum of a birch pollen allergic patient with an extremely high IgE titer was performed (data not shown) in order to exclude any artificial false-positive background reactivity due to unspecific binding of IgE antibodies. As displayed in Fig. 5, the ability of *C. herbarum* hydrolase to bind IgE from 5 out of 28 mold-reactive sera in our preliminary *in vitro* immunoblots provides significant evidence for its allergenicity, albeit its exact prevalence of IgE reactivity is merely

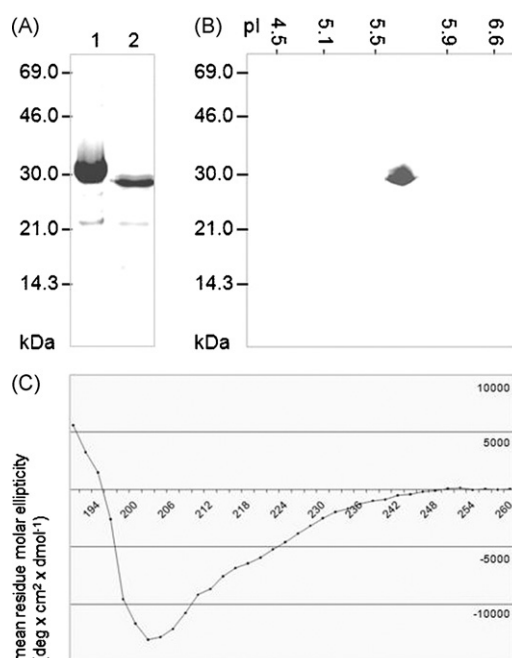


Fig. 4. Purification and renaturation of *C. herbarum* hydrolase. (A) Silver-stained SDS-PAGE gel of 6xHis-hydrolase collected in the 300 mM imidazole fraction containing 8 M urea (lane 1) and of 1.5 µg TEV-digested, renatured rnf hydrolase (lane 2). Molecular mass standards (M) in kDa are indicated on the left side. (B) Coomassie-stain of a two-dimensional approach in the course of which rnf *C. herbarum* hydrolase was separated according to its isoelectric point (pI) and subsequently to its molecular weight. (C) CD spectrum of renatured rnf hydrolase that was recorded in 10 mM sodium phosphate buffer. The final profile represents an average of 5 consecutive scans and was obtained after subtraction of the baseline monitored under identical experimental conditions.

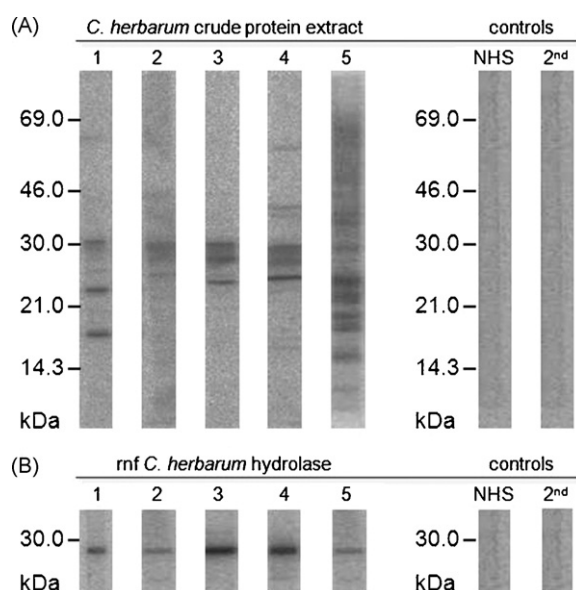


Fig. 5. *In vitro* immunoblot for specific IgE antibodies against *C. herbarum* hydrolase. Five patients (numbered 1–5) with positive IgE reactivity towards the *C. herbarum* crude protein extract (A) recognize rnf *C. herbarum* hydrolase (B) as detected by autoradiography. Molecular mass standards in kDa are given on the left side. Negative control experiments shown include serum of a non-atopic individual (NHS) and usage of 2nd antibody only.

in the order of 17% – a value that classifies it as a novel, but minor fungal allergen.

Advanced computer-assisted statistical investigations that were performed using BepiPred 1.0 (Larsen et al., 2006) and BCPREDS 1.0 (El-Manzalawy et al., 2008) which in short examine actual secondary structure elements including hydrophilic, flexible or surface-protected domains within a given sequence and the frequency of certain AA residues therein that have on the other hand been reported to appear in a variety of known antigenic determinant regions, have in this context located five main putative linear continuous B-cell epitopes on *C. herbarum* hydrolase, namely G₂₁₇DKDPDWSDPKVEAEWVASN₂₃₆ (score: 0.997), K₁₇₀KRAATTRASLTPGRWAGF₁₈₉ (score: 0.952), V₂₄₅PEAGHAPMYERPQVVAERV₂₆₄ (score: 0.94), T₂₀GKETDPLV-CSPGMGDTRD₃₉ (score: 0.907) and M₁₄₀PVMFAWPWGPAAWEM-YAAT₁₅₉ (score: 0.893). As cursorily depicted in Fig. 6B and C, these mapped epitopes marked within the respective three-dimensional protein structure using SWISS-MODEL (Arnold et al., 2006) as well as UCSF Chimera (Pettersen et al., 2004) consist of regions that are to a large extent solvent-exposed and in this way accessible for antigen–antibody interactions within their native microenvironment. Their direct involvement in IgE binding, FcεRI crosslinking as well as mediator discharge by means of for instance immunoblotting, ELISA testings or *in vitro* histamine release studies in comparison to the respective full-length molecule remains, however, to be determined in future studies.

4. Discussion

The main contribution of our manuscript is the description, isolation, purification and further immunological characterization of an up to now unknown ascomycete allergen, i.e. *C. herbarum* hydrolase, which participates in currently still unspecified biological properties within fungal physiology but has been demonstrated to exhibit significant (structural) homology to the so-called α/β hydrolase fold superfamily from which it seems to have arisen in the course of divergent evolution. Since its original identification in 1992 (Ollis et al., 1992) by comparing several deceptively unrelated hydrolytic molecules of widely different activity as well as substrate specificity, the canonical α/β hydrolase fold in question has been promptly recognized as an immensely versatile, widespread tertiary architecture adopted by (i) a broad variety of enzymes such as, e.g. simple esterases or peptidases, epoxide hydrolases, lipases, haloalkane dehalogenases, haloperoxidases, lyases, enolactonases or C–C bond hydrolases (Carr and Ollis, 2009; Heikinheimo et al., 1999; Holmquist, 2000; Hotelier et al., 2004; Nardini and Dijkstra, 1999; Qian et al., 2007) as well as (ii) a restricted number of non-enzymatic members including the synaptic cell adhesion protein neuroligin (Fabrichny et al., 2007) or the N-myc differentiation-related proteins family (Shaw et al., 2002). Summarizing current literature, the α/β hydrolase fold superfamily can be divided into (i) lineages that are related by (moderate) sequence identity across most of or the whole molecule (siblings, such as fungal type B lipases, Schrag and Cygler, 1993), (ii) groups of families with only partial or lower homology (cousins, including *Xanthobacter autotrophicus* haloalkane dehalogenase, Ridder et al., 1999, or *Pseudomonas putida* diene lactone hydrolase, Ollis and Ngai, 1985), (iii) extended categories wherein the AA residues only match around the nucleophile elbow as discussed below, if at all (distant relatives), and finally (iv) enzymes that – continuing the analogy – represent so-called α/β hydrolase fold in-laws and may in time be incorporated into the family tree. Giving the fold its name, the common element in this enzymes is a mostly parallel, eight-stranded central β sheet that displays a superhelical twist (with the first and the last strands crossing each other at an angle of roughly 90°) to form a half-barrel and is surrounded on both sides by α helices αA to

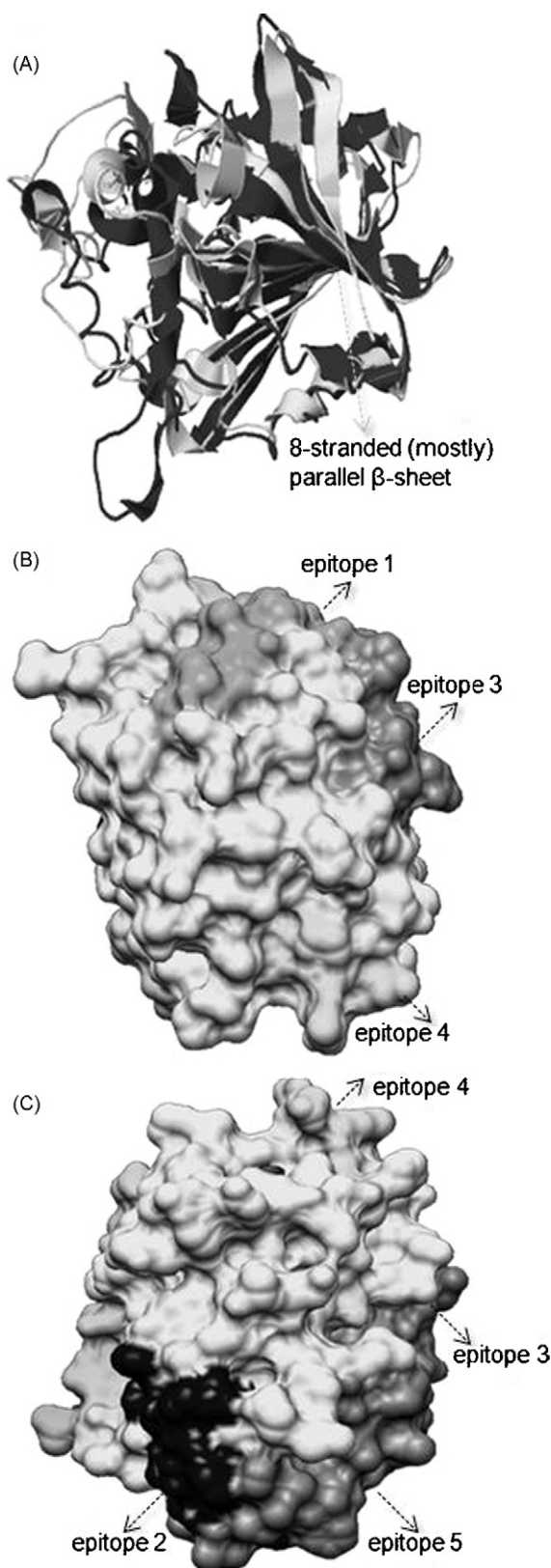


Fig. 6. Structural modelling of *C. herbarum* hydrolase. (A) The three-dimensional configuration of *C. herbarum* hydrolase (grey) that was *ab initio* calculated based on its homology to related proteins of the α/β hydrolase fold superfamily members (exhibiting an already known three-dimensional organization) was superimposed over *Streptomyces lividans* chloroperoxidase L chain A available in the RCSB database under pdb code 1a88 using FATCAT. Both proteins apparently display a rather similar, evolutionarily conserved domain architecture with the first and last helices packing onto one face of the half-barrel, and helices B to E extending from the

αF (whose precise spatial positioning may differ significantly in the individual family members) as can be perceived from Figs. 2 and 6A, in this way providing a stable scaffold for the catalytically active residues (Carr and Ollis, 2009; Heikinheimo et al., 1999; Holmquist, 2000; Hotelier et al., 2004; Nardini and Dijkstra, 1999; Ollis et al., 1992). The latter constitute a highly conserved triad consisting of a nucleophile (serine, cysteine or aspartic acid) directly positioned in a sharp turn after strand $\beta 5$, called the nucleophile elbow, an acidic residue almost always placed in a reverse turn following strand $\beta 7$ and an absolutely conserved histidine located after the last β strand (although the exact shape and length of the histidine-containing loop can differ considerably among the various family members). These evidences are also valid for *C. herbarum* hydrolase, an attribute that in addition to our computer-assisted homology and conserved domain searches further underscores its evident membership to the α/β hydrolase fold superfamily (Carr and Ollis, 2009; Nardini and Dijkstra, 1999). The geometry of the nucleophile elbow, characterized by the consensus sequence R_s (small residue) – X (any AA) – Nu (nucleophile) – X – R_s (*C. herbarum* hydrolase: G₉₉ASFS₁₀₃), allows it to make a close approach to the substrate and in this way contributes to the formation of the oxyanion-binding hole which is needed to stabilize the negatively charged transition state that occurs during hydrolysis (Carr and Ollis, 2009) – it remains to be verified, however, whether our novel *C. herbarum* hydrolase allergen is indeed enzymatically active. The α/β hydrolase fold itself tolerates insertions that may be as long as just a few AA residues (as it is the case in *Fusarium solani* cutinase, Longhi et al., 1997) or may be large enough to create entire extra domains (as in mouse acetylcholinesterase, Bourne et al., 1999), thereby forming movable lids that again serve essential roles in defining the actual design of the substrate-binding pocket and in regulating accessibility to the active site, explaining the extensive variability in fold architecture monitored within the recently solved crystallographic structures of at least 215 α/β hydrolase fold superfamily members (Carr and Ollis, 2009; Heikinheimo et al., 1999; Hotelier et al., 2004; Nardini and Dijkstra, 1999).

We have subsequently been able to reveal that *C. herbarum* hydrolase is recognized by approximately 17% of the respective mold allergic population tested within this initial study and contributes more thoroughly the *C. herbarum* allergen repertoire in the molecular weight range between 18 and 30 kDa – a size interval where many of the affected individuals show a rather versatile, strong pattern of IgE reactivity, but merely the 3 IgE reactive proteins NAPD-dependent mannitol dehydrogenase (29 kDa, Simon-Nobbe et al., 2006), YCP4 homologue (22 kDa, Achatz et al., 1995) as well as translationally controlled tumour protein (18 kDa, Rid et al., 2008) have to date been cloned thereof. Crucially, *C. herbarum* hydrolase presently represents the fourth most important *C. herbarum* allergen in terms of prevalence, following Cla h 8 (57%, Simon-Nobbe et al., 2006) as well as Cla h 6 (22%, Simon-Nobbe et al., 2000) and faintly preceding Cla h 9 (15.5%, Poll et al., 2009). When blasting its deduced AA sequence against the Allergome (Mari and Scala, 2006) or AllFam (Radauer et al., 2008) catalogues accessible via <http://www.allergome.org/> or <http://www.meduniwien.ac.at/allergens/allfam/> (which in brief

opposite surface. The nucleophile is located in a tight turn after $\beta 5$ called the nucleophile elbow which is the best conserved feature and contributes as a peg for overlaying α/β hydrolase structures. (B) Illustration of the putative linear B-lymphocyte epitopes (epitope 1: GDKDPDWSDPKVEAEWVASN; epitope 2: KKRAATTRASLTPGRWAGF; epitope 3: VPEAGHAPMYERPQVVAERV; epitope 4: TGKETDPLVICSPMGMDTRD; epitope 5: MPVMEFAWPWGPAAWEMYAAT) in the ribbon visualization of *C. herbarum* hydrolase applying SWISS-MODEL and UCSF Chimera 1.3. (C) The same model as in (B), but rotated horizontally to give view to the molecule's back side. Areas for which no IgE-binding capacity has been predicted are displayed in white.

Table 2

A selection of hypothetical *C. herbarum* hydrolase orthologues that were *in silico* predicted to exist by performing a translated fungal genomes BLAST search (with decreasing expectation values from the top to the bottom). The databases do currently not include sequences from *Cladosporium* or *Alternaria* species. α/β hydrolase fold family members are not primarily related at their AA level but rather at their tertiary organization, accounting for the only moderate sequence identity.

Organism	Alignment with <i>C. herbarum</i> hydrolase	Alignment statistics	Accession number
<i>Gibberella zeae</i>	AA 7 - 268	53% identity, 64% similarity, 1% gaps	XM.391338.1 NW.060140.1
<i>Aspergillus oryzae</i>	AA 5 - 125 AA 205 - 256 AA 33 - 269	28% identity, 45% similarity, 5% gaps 25% identity, 53% similarity, 3% gaps 24% identity, 36% similarity, 14% gaps	XM.001817901.1 NW.001884682.1 NW.001884672.1
<i>Coccidioides immitis</i>	AA 8 - 270	24% identity, 44% similarity, 8% gaps	XM.001247527.1
<i>Aspergillus flavus</i>	AA 8 - 128 AA 33 - 269	34% identity, 45% similarity, 6% gaps 24% identity, 36% similarity, 14% gaps	AAIH02000046.1 AAIH020000501.1
<i>Schizosaccharomyces japonicus</i>	AA 33 - 268	29% identity, 43% similarity, 4% gaps	XM.002174706.1
<i>Penicillium marneffei</i>	AA 33 - 267	25% identity, 40% similarity, 6% gaps	XM.002144114.1 XM.002152156.1
<i>Aspergillus clavatus</i>	AA 6 - 273	23% identity, 39% similarity, 15% gaps	XM.001276796.1
<i>Aspergillus nidulans</i>	AA 6 - 124 AA 150 - 268	27% identity, 45% similarity, 5% gaps 28% identity, 40% similarity, 10% gaps	XM.653300.1 NW.101319.1
<i>Aspergillus terreus</i>	AA 58 - 267	24% identity, 40% similarity, 7% gaps	XM.001211054.1
<i>Pichia stipitis</i>	AA 27 - 128	31% identity, 44% similarity, 4% gaps	XM.001386940.1
<i>Aspergillus niger</i>	AA 28 - 268	21% identity, 39% similarity, 3% gaps	XM.001391766.1
<i>Neurospora crassa</i>	AA 12 - 89 AA 99 - 271	23% identity, 43% similarity, 8% gaps 21% identity, 39% similarity, 4% gaps	XM.325199.1

proclaim that allergens (i) are generally distributed among an only restricted number of protein families and (ii) are frequently engaged in biochemical processes extending from the hydrolysis of proteins, polysaccharides or lipids as well as further enzymatic activities to the binding of metal ions, storage, regulatory devices and cytoskeleton associations), no hits matching either PF00561 or PR00412 were registered, indicating that both the α/β hydrolase fold-1 and the epoxide hydrolase-like domain have previously not been documented as IgE-binding structures.

More than 80 fungal genomes have, to go one step ahead, recently been fully sequenced or are currently determined (Stajich et al., 2007; Wang et al., 2009), providing us with the possibility to determine whether allergen orthologues are spread among the fungal kingdom. As summarized in Table 2, a first *in silico* screening for putative *C. herbarum* hydrolase homologues via CFGP 2.0 (Park et al., 2008), a web-based comparative fungal genomics platform at www.cfpg.snu.ac.kr, and the Saccharomyces genome database WU-Blast2 algorithm (Dwight et al., 2002) available at www.yeastgenome-org/cgi-bin/blast-fungal/ has in this context revealed the presence of several hypothetical ascomycete counterparts of which especially the predicted *Penicillium* as well as *Aspergillus* (α/β hydrolase fold containing) hydrolases – despite their moderate sequence identities – could represent interesting novel (potentially cross-reactive) candidates as will be tested in future approaches. Although cross-reactivity *per se* is a frequently encountered phenomenon that requires extended similarity in three-dimensional structure (including charge distribution, hydrogen-bonding potential and hydrophobicity patterns) as has, e.g. been demonstrated for fungal aldehyde dehydrogenases, serine proteases, enolases, heat shock proteins, peroxisomal proteins, acidic ribosomal P₂ proteins, translationally controlled tumour proteins or nuclear transport factor 2 – formally termed “pan-allergens” – and contributes to reduce the number of epitopes needed for allergy diagnosis as well as therapy, it has to be admitted, however, that not all proteins belonging to the same fold family are necessarily immunologically cross-reactive (Crameri et al., 2006, 2009; Simon-Nobbe et al., 2008).

Although several longitudinal epidemiologic surveys worldwide have attempted to investigate the overall incidence of sensitization to fungal (especially *C. herbarum* and *A. alternata*) allergens, its exact frequency is still difficult to be reliably established because the data strongly depend on the examined populations and especially the inconsistency of commercial extracts

used within routine assessments often generates false-negative outcomes that most probably underestimate the precise values (Breitenbach and Simon-Nobbe, 2002; Horner et al., 1995; Kurup et al., 2000; Simon-Nobbe et al., 2008). To list a few examples, Broadfield et al. (2002) have randomly tested a group of 1339 adults from the Nottingham area, United Kingdom, and could observe that between the years 1991 and 2000, an unchanged or only slightly increased 31% of the population was sensitized to any allergen as measured by apparent skin prick reactivity defined as a “weal” diameter of larger than 3 mm, among them 0.4% against *C. herbarum* in 1991 and 2.2% in 2000. In a large-scale European multicenter report promoted by the Academy of Allergology and Clinical Immunology in 1997, about 10% of the patients suffering from allergic nasal as well as bronchial symptoms were found to be sensitized to *C. herbarum* and/or *A. alternata*, with rates varying between 3% in Portugal and 20% in Spain (D’Amato et al., 1997). Numbers in a similar scale were presented in a European Community Respiratory Health Evaluation (Zureik et al., 2002) wherein the authors provide evidence for a significant association between exposure to airborne molds (including *C. herbarum*) and life-threatening exacerbations of asthma among 1132 adults – a correlation that has been repeatedly perceived in a variety of (experimental) settings (Black et al., 2000; Denis et al., 2007; Denning et al., 2006; Havaux et al., 2005; O’Driscoll et al., 2009). From the 4962 subjects aged between 3 and 80 years suffering from respiratory symptoms enrolled via the Allergy Unit of Rome, Italy, on the other hand, 19.1% were found to be allergic to either *C. herbarum*, *A. alternata*, *Saccharomyces cerevisiae*, *Aspergillus fumigatus*, *Penicillium notatum* or *Candida albicans*, and again *Cladosporium* and *Alternaria* accounted for the largest number of positive tests, with the prevalence of the latter increasing to nearly 80% in polysensitized patients (Mari et al., 2003). Supplementary publications clearly point out that fungal sensitization is not unique for mild, humid as well as tropical climates (Montealegre et al., 2004), but is also quite common, with rather similar frequencies, in desert environments. 20.9% of a total of 810 patients with allergic respiratory diseases in Saudi-Arabia and Kuwait were, e.g. observed to be sensitized to *Penicillium*, *Cladosporium*, *Aspergillus*, *Candida* or *Alternaria* in contrast to only 5.8% in the control group (Ezeamuzie et al., 2000), again with a striking predominance in children diagnosed with asthma (66.0% in the 7–12-year group). From this selective choice of studies, we can deduce that knowing the detailed allergen complement of *C. herbarum* is undeniably of great medical importance.

Though allergic symptoms can be relieved through antihistaminic medications, the only causative treatment is up to now specific immunotherapy (SIT), a controlled administration of gradually increasing doses of the disease-eliciting allergen by subcutaneous injection or sublingual application to induce a “state of unresponsiveness” to subsequent antigen challenge (Nelson, 2007; Vrtala, 2008). As far as fungal allergy is concerned, SIT is, however, still hampered by the irregularity of the routinely used industrially available crude mold extracts that is due to problems in manufacturing as well as standardizing these solutions, resulting in a highly variable allergen composition (contaminated with a variety of unwanted non-allergenic components) when comparing extracts of different suppliers or distinct batches of the same company that limits their broad application (Cramer et al., 2006; Esch, 2004; Vailes et al., 2001). Despite the fact that SIT with crude fungal extracts is for these reasons problematic, clinically effective (double-blind, placebo-controlled) hyposensitization trials with *A. alternata*, *A. fumigatus* as well as *C. herbarum* extracts have been reviewed (Bonifazi, 1994; Lizaso et al., 2008; Malling, 1992; Tabar et al., 2008), but are in most countries not recommended because (i) an individual might *de novo* develop IgE antibodies against further components present in the crude extract, thus paradoxically broadening his sensitization spectrum, (ii) this treatment cannot be applied according to the patient's individual reactivity profile, and (iii) the required effective therapeutic doses can often not be reached since severe therapy-induced side effects may occur. These complications could, nevertheless, be overcome by the (i) use of ultrapure recombinant allergens that can be produced in unlimited amounts in both pro- and eukaryotic expression systems and reproducibly standardized, as well as by (ii) the administration of genetically engineered hypoallergenic derivatives characterized by reduced IgE-binding reactivity, but preserved antigenicity, thereby opening new vaccination possibilities (Ferreira et al., 2004; Valenta and Kraft, 2002; Valenta and Niederberger, 2007; Vrtala, 2008). A first clinical study (Unger et al., 1999) utilizing Alt a 1 and enolase has in this context already shown that recombinant allergens achieve a higher specificity as well as sensitivity than commercial mold extracts and that a combination of these two molecules, perhaps supplement with a few further allergens, represents a promising instrument for diagnosis and therapy of mold allergy. As trends go noticeably in the direction of component-resolved, patient-tailored approaches (Cromwell et al., 2004; Ferreira et al., 2004), recombinant *C. herbarum* hydrolase could certainly contribute to an improved diagnosis of immediate hypersensitivity reactions in mold allergic patients and offer beneficial immunotherapeutic strategies to better control fungal allergic disease.

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