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The vacuolar serine protease, a cross-reactive allergen from *Cladosporium herbarum*

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ABSTRACT

Subtilisin-like serine proteases make up one of the most important allergen-families regarding the number of individual allergens. Previously, fungal subtilisin-like serine proteases have been identified from *Aspergillus*-, *Penicillium*-, and *Trichophyton*-species having a prevalence of IgE-reactivity between 33% and 80%. Since IgE-cross-reactivity is a common phenomenon within fungal species we wanted to know whether this protein also represents an allergen in *Cladosporium herbarum*. Hence, a screening of a *C. herbarum* cDNA library was performed using the coding sequence of the *Penicillium oxalicum* vacuolar serine protease (Pen o 18) as hybridization probe, ending up with a full-length clone. Biochemical and immunological characterization of this clone revealed that *C. herbarum* vacuolar serine protease most likely is synthesized as a precursor with an N-terminal pro-enzyme sequence and represents a minor allergen (Cla h 9) with a prevalence of IgE-reactivity of 15.5%. Furthermore Cla h 9 specifically reacted with the two monoclonal antibodies FUM20 and PCM39, as do the vacuolar serine proteases from *Aspergillus fumigatus* and *Penicillium* species. Investigation of IgE-cross-reactivity between Cla h 9 and other fungal serine proteases revealed that cross-reactivity is higher between vacuolar than alkaline serine proteases. IgE-epitope mapping of Cla h 9 was done in order to test whether four Cla h 9-peptides having a high sequence homology to previously determined Pen ch 18-IgE-epitopes also harbour IgE-epitopes. Three-dimensional models of the vacuolar serine proteases from *C. herbarum* and *Penicillium chrysogenum* were generated for the three-dimensional localization of the Cla h 9- and Pen ch 18- IgE-reactive and -non-reactive peptides.

Taken together a new *C. herbarum* allergen has been identified, which may be useful in a molecule-based approach of *C. herbarum* allergy-diagnosis and -therapy. Moreover, Cla h 9 represents a further member of the subtilisin-like serine protease allergen-family, which stresses the importance of these proteins with respect to fungal IgE-cross-reactivity.

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1. Introduction

IgE-mediated fungal allergies are well-known medical afflictions affecting 6% (Tariq et al., 1996) to 24% (Salvaggio and Aukrust, 1981) of the general population. Within the group of atopics the

incidence of mould-allergy ranges from 19% (Mari et al., 2003) to 44% (Corey et al., 1997), whereas up to 80% (Lopez and Salvaggio, 1985) of all asthmatics exhibit fungal sensitization. The ascomycete *Cladosporium herbarum* is one of the fungi inducing Type-I allergies. The spores and mycelial cells of *C. herbarum* can be found outdoors as well as indoors and may be responsible for allergic rhinitis and allergic asthma. The prevalence of *C. herbarum* allergy is between 1.7% (Bousquet et al., 2007) and 2.9% (Tariq et al., 1996) within the total population and 18.2% (Nolles et al., 2001) within atopics. In a study of Mari et al. (2003) 4962 patients presenting respiratory symptoms were tested for *C. herbarum* sensitization. Within the

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group of patients being sensitized to more than two fungal species the prevalence of a *C. herbarum* sensitization reached 84%, indicating that *C. herbarum* allergic patients tend to be poly-sensitized to moulds.

Previously, 14 *C. herbarum* allergens have been identified including the acid ribosomal proteins 1 and 2 (Achatz et al., 1995; Zhang et al., 1995), enolase (Achatz et al., 1995; Simon-Nobbe et al., 2000), flavodoxin (Achatz et al., 1995), NADP-dependent mannitol dehydrogenase (MtDH) (Simon-Nobbe et al., 2006), aldehyde dehydrogenase (Achatz et al., 1995), cold shock protein (Falsone et al., 2002), glutathione-S-transferase (Shankar et al., 2005), type I hydrophobin (Weichel et al., 2003b), heat shock protein 70 (Zhang et al., 1996), a TCTP-homologue (translationally controlled tumor protein) (Rid et al., 2008) and the nuclear transport factor 2 (Weichel et al., 2003a). Nearly all of these allergens represent minor allergens, which are recognized by less than 22% of *C. herbarum* allergic patients. The only major allergen with a prevalence of 57% is Cla h 8, a NADP-dependent mannitol dehydrogenase. The fact that there is a high number of minor and a single or very few major allergens is widespread within fungal species.

Cross-reactivity has been described for 17 fungal allergens (Simon-Nobbe et al., 2007). Eight of them were isolated from *C. herbarum*, namely enolase, NTF2, the two ribosomal proteins, flavodoxin, hsp70, mannitol dehydrogenase, and the aldehyde dehydrogenase (Simon-Nobbe et al., 2000, 2006, 2007).

Serine proteases in general and vacuolar serine proteases in particular are prominent allergens in *Aspergillus*, *Penicillium*, *Rhodotorula* and *Trichophyton* species (Chou et al., 2005; Chow et al., 1999; Shen et al., 1999a,b, 2001b, 2003; Woodfolk et al., 1998) with a prevalence of sensitization of up to 77% in case of Pen ch 18 (Shen et al., 2003), the vacuolar serine protease from *Penicillium chrysogenum*. Very recently, a vacuolar serine protease has been isolated from *Cladosporium cladosporioides* (Chou et al., 2008), a mould about whose allergenic potency very little is known up to now (Bouziane et al., 2006). Within the serine proteases isolated so far extensive cross-reactivity on the IgE- as well as on the IgG-level was found. Taking into account that an IgE-reactive protein of about 40 kDa can be seen in *C. herbarum* crude extract, but could not be assigned to the 14 allergens identified previously, we sought to investigate whether the vacuolar serine protease is also an allergen in *C. herbarum*.

Here we present the nucleotide and the deduced protein sequence of a full-length clone of Cla h 9, the *C. herbarum* vacuolar serine protease. Edman-degradation of an excised 2D spot was performed in order to determine the N-terminal sequence of the native protein. Recombinant His₆-fusion-proteins of the vacuolar serine proteases from *C. herbarum*, *Penicillium oxalicum* and *Aspergillus fumigatus* and the alkaline serine protease from *P. chrysogenum* were expressed in *E. coli*, purified to homogeneity and tested for their IgE- and IgG-cross-reactivities. Additionally, an IgE-epitope mapping of the *C. herbarum* vacuolar serine protease was performed. Three-dimensional models of the vacuolar serine proteases from *C. herbarum* and *P. chrysogenum* were generated and used to compare the results of the Cla h 9-IgE-epitope-mapping with the results of two previous studies on *P. chrysogenum*-IgE-epitopes (Yu et al., 2002; Shen et al., 2003).

2. Materials and methods

2.1. Chemicals, primer

Unless otherwise stated all chemicals were obtained from Applichem (Darmstadt, Germany). Enzymes were purchased from Fermentas (St. Leon-Rot, Germany) and columns and chromatographic media from GE-Healthcare (Uppsala, Sweden). Nucleotide

primers were synthesized by HVD-Life Sciences (Vienna, Austria).

2.2. Patients and sera

Sera were collected at the Floridsdorfer Allergiezentrum (Vienna, Austria), and the Center for Clinical and Experimental Allergology (IDI-IRCCS, Rome, Italy). Patients were selected according to a typical clinical case history suggesting mould allergy, a positive skin prick test to a commercial *C. herbarum*- and/or mould extract, along with a positive radio-allergo-sorbent test to *C. herbarum* extract. Sera obtained from the IDI-IRCCS were IgE-reactive to recombinant Alt a 1 and Alt a 6 and subsequently tested for their specific IgE-reactivity to the *C. herbarum* vacuolar serine protease, since co-sensitization to *C. herbarum* and *A. alternata* is a well-known phenomenon. Sensitization to *C. herbarum* was confirmed by IgE-immunoblots with *C. herbarum* crude extract. The serum of a non-atopic person was used as negative control in IgE-immunoblots and inhibition immunoblots. Sera were stored at -20°C .

2.3. Preparation of *C. herbarum* crude extract

C. herbarum crude extract was prepared as described previously (Simon-Nobbe et al., 2006).

2.4. Two-dimensional gel-electrophoresis

Isoelectric focusing was performed using the capillary system of Biometra (Göttingen, Germany) and a pH-gradient of 3–10. Proteins were separated by constant current (2 mA) and power (4 W) and slowly increasing voltage (500–1500 V). The second dimension gel run was performed using a 13.5% SDS-PAGE (Laemmli, 1970). Proteins were visualized with Coomassie Brilliant Blue G-250 and Western blots.

2.5. Edman sequencing

Crude *C. herbarum* extract was brought to 40% saturation at 4°C by the addition of solid ammonium sulphate. Equilibration was performed for 1 h and then the solution was centrifuged at $6000 \times g$ for 30 min. The supernatant was precipitated again with a saturation of 80% ammonium sulphate. After centrifugation the pellet was dissolved in $1 \times \text{PBS}$ and proteins were precipitated with ProteoExtract[®] Protein Precipitation Kit (Calbiochem, EMD Chemicals, Darmstadt, Germany) according to manufacturer's protocol.

After two-dimensional gel-electrophoresis proteins were transferred to a PVDF-membrane and stained with Coomassie Brilliant Blue G-250. In parallel an immunoblot was performed to detect the protein spot corresponding to the putative *C. herbarum* vacuolar serine protease utilizing the monoclonal antibody FUM20, which is directed against fungal serine proteases (Lin et al., 2000). The detected protein spot was excised and used for Edman sequencing. The protein was loaded onto a standard cartridge of an Applied Biosystems Model 492 Procise protein/peptide sequencer (Applied Biosystems, Foster city, CA, USA) with an online Applied Biosystems Model 140C PTH Amino Acid Analyzer.

2.6. Screening of a *C. herbarum* cDNA expression library

A *C. herbarum* cDNA expression library was constructed in the Uni ZAP XR vector from Stratagene (La Jolla, CA, USA). The construction of the library was described previously (Simon-Nobbe et al., 2006). For the hybridization screening a cDNA clone coding for Pen o 18 (GenBank accession number AF243425), the vacuolar serine protease from *P. oxalicum*, was used as a probe. Labelling

Table 1

Oligonucleotides used as primers for PCR and DNA-sequencing. Restriction sites Bam HI, and Not I or Hind III, respectively are displayed in bold.

Name	DNA-sequence
Asp f 18-EDPT-fw	5'-ATA GGA TCC AGC CTC TCC TTT GG-3'
Asp f 18-EDPT-rv	5'-AAA AAG CTT GCC AGT TCC CGA G-3'
pro-Cla h 9-fw	5'-AAA GGA TCC TCG CCC GTG CTT GT-3'
pro-Cla h 9-rv	5'-ATA AGA ATG CCG CCG CTT ACT CGC GCT TGA GC-3'
Cla h 9-SESE-fw	5'-AAA AGG ATC CTC TGA GTC CGA GGT CGA G-3'
Cla h 9-SESE-rv	5'-ATA AGA ATG CCG CCG CTT ACT CGC GCT TGA GC-3'
Cla h 9-P1-fw	5'-AAG TAA GGA TCC GGC GTT GAC GTT TAC GTC-3'
Cla h 9-P1-rv	5'-ATA AGA ATG CCG CCG CTT ACT TGC CGG CGA-3'
Cla h 9-P2-fw	5'-AAA CGC GGA TCC ATT CAG TCC ACC TGG ATT-3'
Cla h 9-P2-rv	5'-ATA AGA ATG CCG CCG CTT AGA GCT TCT TGG-3'
Cla h 9-P3-fw	5'-AAA CGC GGA TCC AAG GCT AAC CTC ATC TCC-3'
Cla h 9-P3-rv	5'-ATA AGA ATG CCG CCG CTT AGT AAC CAC CCT T-3'
Cla h 9-P4-fw	5'-AAA CGC GGA TCC CGC TCC AAC GGC TCT GGC-3'
Cla h 9-P4-rv	5'-ATA AGA ATG CCG CCG CTT AGT CGA GAA TGG G-3'
Pen o 18-EDPA-fw	5'-AAA TAA GGA TCC GAG GAC CCT GCC-3'
Pen o 18-EDPA-rv	5'-ATA AAG AAT GCG GCC GCC TAA GCG CTA AC-3'
Pen ch 13-ANVV-fw	5'-AAA TAA GGA TCC GCC AAC GTG GTT-3'
Pen ch 13-ANVV-rv	5'-ATA AAG AAT GCG GCC GCT CAT TGG GCG TT-3'
T3	5'-AAT TAA CCC TCA CTA AAG GG-3'
T7	5'-GTA ATA CGA CTC ACT ATA GGG-3'

of the probe was carried out using the Prime-a-gene Labelling System (Promega, USA). Plaques showing positive signals were *in vivo* excised as pBluescript SK ($\pm$) phagemids and tested for their insert-size by PCR using the vector specific primers T3 and T7 (Table 1). Several clones were isolated and sequenced on both strands.

2.7. Computer-assisted analyses

The N-terminus of the *C. herbarum* vacuolar serine protease was determined by Edman-Sequencing along with protein sequence alignments of homologous vacuolar serine proteases. Multiple sequence alignments were performed using MultAlin [http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html] (Corpet, 1988). Results were processed using Boxshade V3.21 [http://www.ch.embnet.org/software/BOX.form.html].

2.8. Subcloning, expression and purification of fungal vacuolar serine proteases

Vacuolar serine proteases are pre-pro-enzymes undergoing N-terminal and partially C-terminal processing (Moehle et al., 1987; Nebes and Jones, 1991). The *C. herbarum* vacuolar serine protease without the hydrophobic leader sequence (17 amino acids long), was subcloned as His₆-fusion protein using the primers pro-Cla h 9-fw and pro-Cla h 9-rv (Table 1) and termed His₆-pro-Cla h 9 (Table 2). The N-terminally truncated fragment His₆-Cla h 9-

SESE (S₁₃₉–E₅₁₈) (Table 2) corresponding to the mature protein as determined by Edman-sequencing along with protein-sequence alignments of homologous proteins was constructed with the primers Cla h 9-SESE-fw and Cla h 9-SESE-rv (Table 1). All *C. herbarum* vacuolar serine protease constructs were generated by PCR using the respective cDNA as DNA-template and Pfu-polymerase according to manufacturer's instructions. The PCR products were digested and ligated into the multiple cloning site of the pHIS-Parallel2 vector (Sheffield et al., 1999).

In order to test the IgE-cross-reactivity between the vacuolar serine proteases from *C. herbarum*, *P. oxalicum* and the alkaline serine protease from *P. chrysogenum* (GenBank accession number AF263454) the respective cDNAs corresponding to the mature serine proteases were subcloned as His₆-fusion proteins using the pHIS-Parallel2 vector and termed His₆-Pen o 18-EDPA (E₁₃₆–A₅₀₃) and His₆-Pen ch 13-ANVV (A₁₁₆–Q₃₉₈) (Tables 1 and 2).

Expression of the His₆-fusion proteins was done as described previously (Simon-Nobbe et al., 2006). Purification of the recombinant His₆-fusion proteins was performed using HisTrap-HP columns (GE Healthcare, Uppsala, Sweden) on an Äkta Prime Plus chromatography system (GE-Healthcare) according to manufacturer's recommendations.

2.9. Asp f 18, the vacuolar serine protease from *A. fumigatus*

The *A. fumigatus* vacuolar serine protease (GenBank accession number Y13338) was subcloned into the pQE30-vector (Qia-gen, Hilden, Germany) and termed Asp f 18-EDPT (E₁₃₇–A₄₉₅) (Tables 1 and 2). The expressed protein was purified under denaturing conditions by Ni-NTA affinity chromatography.

2.10. IgE-, IgG- and inhibition immunoblots

Proteins were separated by SDS-PAGE, transferred to a PVDF-membrane and tested with patients' sera as described (Schneider et al., 2006). For the determination of IgE-cross-reactivity between vacuolar and alkaline serine proteases patients' sera were pre-incubated with 15 μ g of purified recombinant protein per ml of patients' serum overnight at 4 °C. The depleted serum then was used to probe a membrane strip containing an electrophoretically separated and blotted different fungal serine protease.

For IgG-immunoblots the two monoclonal antibodies FUM20 and PCM39, were diluted 1:20 in blocking buffer (25 mM Tris pH 7.5, 150 mM sodium chloride, 0.5% (v/v) Tween20, 0.5% (w/v) BSA, and 0.05% (w/v) sodium azide) and incubated for 2 h at RT. After three washing steps with blocking buffer the secondary antibody AP-labelled AffiniPure rabbit-anti-mouse IgG + IgM antibody (Jackson Immuno Research, Suffolk, Great Britain) was diluted 1:10.000 in blocking buffer and incubated for 2 h at RT. After three further washing steps with blocking buffer the blots were developed with the NBT-BCIP solution from Sigma.

2.11. Epitope-mapping of the *C. herbarum* vacuolar serine protease

Based on the results of two studies about IgE-reactive epitopes of the *P. chrysogenum* vacuolar serine protease (Shen et al., 2003; Yu et al., 2002) four peptides of Cla h 9-SESE, termed His₆-Cla h 9-peptide 1–4 (Tables 1 and 2), were tested for their IgE-reactivities. The four peptides were subcloned into the vector pHIS-Parallel2. PCR amplification was performed with primers Cla h 9-P1-fw and -rv, Cla h 9-P2-fw and -rv, Cla h 9-P3-fw and -rv, Cla h 9-P4-fw and -rv (Table 1). The peptides (Table 2) were expressed and purified as described above.

Table 2

Allergen constructs tested in IgE- (inhibition-) immunoblots. The accession numbers refer to the GenBank accession numbers of the corresponding open reading frames used as DNA-template for the PCR-reaction.

Name	Accession number	Amino acid position in the respective coding sequence
His ₆ -pro-Cla h 9	AY787775	S ₁₇ –E ₅₁₈
His ₆ -Cla h 9-SESE	AY787775	S ₁₃₉ –E ₅₁₈
His ₆ -Cla h 9-peptide 1	AY787775	G ₁₇₇ –K ₂₂₇
His ₆ -Cla h 9-peptide 2	AY787775	R ₂₄₄ –D ₂₉₈
His ₆ -Cla h 9-peptide 3	AY787775	I ₃₆₄ –L ₄₁₆
His ₆ -Cla h 9-peptide 4	AY787775	K ₄₁₇ –Y ₄₅₉
His ₆ -Pen o 18-EDPA	AF243425	E ₁₃₆ –A ₅₀₃
His ₆ -Pen ch 13-ANVV	AF193420	A ₁₁₆ –Q ₃₉₈
His ₆ -Asp f 18-EDPT	Y13338	E ₁₃₇ –A ₄₉₅

5' –CGCTCCATCCACCT																				14
CATCGCATCCATCTCAATACCCCCAAACTGTTTGTTCCTCATCTCACCATTACACACTGCCCATC																				81
ATG	CGC	GGC	GCC	CTG	GCT	GGC	CTC	TCG	CTG	GCA	ACA	CTC	GCG	ACA	GCG	TCG				132
M	R	G	A	L	A	G	L	S	L	A	T	L	A	T	A	↓	S			17
CCC	GTG	CTT	GTC	AAC	AGC	ATC	CAC	AAC	GAT	GCT	GCT	CCC	ATC	ATC	TCT	GCC				183
P	V	L	V	N	S	I	H	N	D	A	A	P	I	I	S	A				34
TCC	AAC	GCA	AAG	GAG	ATT	GCA	GAC	AAC	TAC	ATG	ATC	AAG	TTC	AAG	GAC	CAT				234
S	N	A	K	E	I	A	D	N	Y	M	I	K	F	K	D	H				51
GTC	ACC	CAG	AAC	CTG	GCT	GCT	GAG	CAC	CAT	GGC	TGG	GTG	CAG	GAC	CTT	CAC				285
V	T	Q	N	L	A	A	E	H	H	G	W	V	Q	D	L	H				68
GAG	AAG	ACC	CAG	GTC	GCT	AAG	ACC	GAG	CTC	CGC	AAG	CGC	TCG	CAG	TCG	CCG				336
E	K	T	Q	V	A	K	T	E	L	R	K	R	S	Q	S	P				85
ATG	GTT	GAT	GAC	ATC	TTC	AAC	GGC	CTG	AAG	CAC	ACC	TAC	AAC	ATC	GCT	GGC				387
M	V	D	D	I	F	N	G	L	K	H	T	Y	N	I	A	G				102
GGC	CTC	ATG	GGC	TAC	GCT	GGT	CAC	TTC	GAT	GAG	GAT	GTC	ATC	GAG	CAG	ATT				438
G	L	M	G	Y	A	G	H	F	D	E	D	V	I	E	Q	I				119
CGC	AGA	CAC	CCT	GAT	GTC	GAG	CTC	GTT	GAG	AGA	GAT	CAG	GAG	GTC	CAC	GTT				489
R	R	H	P	D	V	E	L	V	E	R	D	Q	E	V	H	V				136
CTC	GGC	TCT	GAG	TCC	GAG	GTC	GAG	AAG	AAC	GCT	CCT	TGG	GGC	CTC	GCC	CGT				540
L	G	↓	S	E	S	E	V	E	K	N	A	P	W	G	L	A	R			153
ATC	TCA	CAC	CGT	GAC	AGC	CTG	AGC	TTC	GGC	ACC	TTC	AAC	AAG	TAC	CTC	TAC				591
I	S	H	R	D	S	L	S	F	G	T	F	N	K	Y	L	Y				170
ACC	GAG	GAC	GGT	GGT	GAG	GGC	GTT	GAC	GTT	TAC	GTC	GTC	GAC	ACC	GGC	ACC				642
T	E	D	G	G	E	G	V	D	V	Y	V	V	D	T	G	T				187
AAC	GTT	GAC	CAC	GTC	GAC	TTC	GAG	GGT	CGC	GCG	TCT	TGG	GGC	AAG	ACC	ATC				693
N	V	D	H	V	D	F	E	G	R	A	S	W	G	K	T	I				204
CCT	CAG	GGT	GAT	GCA	GAT	GAG	GAT	GGC	AAC	GGA	CAC	GGA	ACA	CAC	TGC	TCT				744
P	Q	G	D	A	D	E	D	G	N	G	H	G	T	H	C	S				221
GGC	ACT	GTC	GCC	GGC	AAG	AAG	TAC	GGT	GTC	GCC	AAG	AAG	GCT	CAC	GTC	AAG				795
G	T	V	A	G	K	K	Y	G	V	A	K	K	A	H	V	K				238
GCC	GTC	AAG	GTC	CTC	CGC	TCC	AAC	GGC	TCT	GGC	TCA	ATG	TCC	GAC	GTT	GTC				846
A	V	K	V	L	R	S	N	G	S	G	S	M	S	D	V	V				255
AAG	GGT	GTT	GAG	TAC	GCC	GCC	GAG	TCT	CAC	CTC	GAG	CAG	GTC	AGC	ATC	ACC				897
K	G	V	E	Y	A	A	E	S	H	L	E	Q	V	S	I	T				272
AAG	AAG	GGC	AAG	AGA	AAG	GGC	TTC	AAG	GGC	TCT	ACT	GCC	AAC	ATG	AGC	TTG				948
K	K	G	K	R	K	G	F	K	G	S	T	A	N	M	S	L				289
GGC	GGT	GGC	AAA	TCG	CCC	ATT	CTC	GAC	AAG	GCT	GTC	AAC	GCT	GCC	GTC	GAC				999
G	G	G	K	S	P	I	L	D	K	A	V	N	A	A	V	D				306
GCT	GGT	ATC	CAC	TTC	GCC	GTT	GCC	GCC	GGT	AAC	GAC	AAC	GCT	GAC	TCT	TGC				1050
A	G	I	H	F	A	V	A	A	G	N	D	N	A	D	S	C				323
AAC	TAC	TCT	CCT	GCT	GCC	GCT	GAG	AAC	GCC	GTC	ACC	GTT	GGT	GCC	AGC	ACT				1101
N	Y	S	P	A	A	A	E	N	A	V	T	V	G	A	S	T				340
CTC	GCT	GAC	GAG	CGT	GCC	TAC	TTC	TCC	AAC	TAC	GGC	AAG	TGC	AAC	GAC	ATC				1152
L	A	D	E	R	A	Y	F	S	N	Y	G	K	C	N	D	I				357
TTT	GCT	CCT	GGT	CTT	AAC	ATT	CAG	TCC	ACC	TGG	ATT	GGC	AGC	AAG	TAC	GCC				1203
F	A	P	G	L	N	I	Q	S	T	W	I	G	S	K	Y	A				374
GTC	AAC	ACC	ATC	TCC	GGT	ACC	TCA	ATG	GCC	TCT	CCC	CAC	GTT	GCT	GGT	CTC				1254
V	N	T	I	S	G	T	S	M	A	S	P	H	V	A	G	L				391
CTT	GCC	TAC	CTC	CTT	TCC	CTC	CAG	CCC	GCC	AAG	GAC	TCT	GCC	TTC	GCT	GTC				1305

Fig. 1. Nucleotide and deduced amino acid sequence of the *C. herbarum* vacuolar serine protease. The hydrophobic leader sequence comprising 48 bp is underlined with dots. Amino acids forming the catalytic triad (D₁₈₄, H₂₁₆, S₃₈₂) are highlighted in black boxes. The oxyanion hole residue (N₃₁₇) is displayed in bold and italics. Three cysteines (C₂₂₀, C₃₂₃, C₃₅₄) also depicted in bold and italics may be involved in the formation of disulfide bonds. Vertical arrows indicate the start of the pro-protein and the mature protein, respectively. The sequence corresponding to the putative N-terminal end of the mature protein is underlined. The DNA-sequence of the PCR-primers, which were used for the construction of recombinant His₆-Clp h 9-SESE (Table 1) are displayed in grey boxes. The translation termination site TAA is shown with an asterisk. Numbers to the right denote the nucleotide and amino acid positions, respectively.

L	A	Y	L	L	S	L	Q	P	A	K	D	S	A	F	A	V	408
GCC	GAC	ATC	AGC	CCC	AAG	AAG	CTC	AAG	GCT	AAC	CTC	ATC	TCC	ATT	GCC	ACT	1356
A	D	I	S	P	K	K	L	K	A	N	L	I	S	I	A	T	425
GTT	GGT	GCT	CTG	ACC	GAT	GTC	CCC	AGC	AAC	ACC	GCC	AAC	ATC	CTC	GCC	TGG	1407
V	G	A	L	T	D	V	P	S	N	T	A	N	I	L	A	W	442
AAC	GGT	GGC	GGA	GAG	TCC	AAC	TAC	TCT	GCT	ATT	GTT	GAG	AAG	GGT	GGT	TAC	1458
N	G	G	G	E	S	N	Y	S	A	I	V	E	K	G	G	Y	459
AAG	GCC	ACT	CAC	CGC	CCG	ACC	ATG	CTT	GAG	GAG	ATC	GAG	TCT	GAG	GCC	AAG	1509
K	A	T	H	R	P	T	M	L	E	E	I	E	S	E	A	K	476
GTC	GCC	AGC	AAG	AAG	GTC	TAC	TCT	GAG	GGT	GAC	GAG	CTC	GCC	CAC	AAG	GTC	1560
V	A	S	K	K	V	Y	S	E	G	D	E	L	A	H	K	V	493
GCC	GAG	CTC	ACC	GAG	AAG	GTC	GAG	GAC	CTC	ATC	GCC	GGC	GAG	CTC	AAG	GAC	1611
A	E	L	T	E	K	V	E	D	L	I	A	G	E	L	K	D	510
ATG	TTC	CGT	GAG	CTC	AAG	CGC	GAG	TAA									1638
M	F	R	E	L	K	R	E	*									519
AGAGGAGGCTTGATATTCTACTTGACAAGCATTCTCACTTGGGAAGCCGAGCCCCAGCACAGGTTTG																	1705
GCAATGTGCATAAGACATGTTGGTTCCCTTGCATAGACTGCGCTTAACGATCTGGTTGGATTATTTCG																	1772
GGTAATTGGCCTGACATTTGCGCTCCTTGCAGACGCCCTTTGGCCGACATTATACATGTACGCGAGTA																	1839
GCTTTATGAGAATGCAGATGATTAATTGTAAAAA																	1886

Fig. 1. (Continued).

2.12. Modelling of the vacuolar serine proteases from *C. herbarum* and *P. chrysogenum*

Three-dimensional models of the vacuolar serine proteases from *C. herbarum* and *P. chrysogenum* (previously known as *P. notatum*) were generated by using MODELLER 9v2 [<http://salilab.org/modeller/>], a program for comparative protein structure modelling. The model of Cla h 9 was based on the structure of the homologous proteinase K from *Tritirachium album* (strain limber) (Protein Data Bank code: 1IC6) (Betzel et al., 2001) exhibiting subtilisin-like specificity and belonging to the subtilisin family. The model of Pen ch 18 was based on the structure of the homologous *T. album* (strain limber) proteinase K (PDB: 2PRK) (Betzel et al., 1988).

The visualization of the models was done with the program UCSF Chimera [<http://www.cgl.ucsf.edu/chimera/>] (Pettersen et al., 2004).

3. Results

3.1. Cloning and sequencing of the *C. herbarum* vacuolar serine protease

In order to identify whether a member of the subtilisin-like serine protease allergen-family represents an allergen in *C. herbarum*, a *C. herbarum* λ -ZAP cDNA expression library was screened using the coding sequence of the mature *P. oxalicum* vacuolar serine protease (Pen o 18-EDPA (E₁₃₆–A₅₀₃)) (Table 2) as DNA-probe. *P. oxalicum* vacuolar serine protease was chosen since it is one of the fungal members of the subtilisin-like serine protease allergen-family. Several positive clones were isolated, sequenced on both strands with identical results and deposited at GenBank with the accession number AY787775.

The full-length cDNA (Fig. 1) spans 1889 bp. The open reading frame has a length of 1557 bp encoding an 519 amino acids long polypeptide and is flanked by an untranslated 5' region of 81 bp preceding the start methionine and an untranslated 3' region of 248 bp. Translation of the transcript starts at the first AUG codon available. The typical polyadenylation signal (AATAAA) is missing in the 3' untranslated region.

Fungal vacuolar serine proteases are known to be pre-pro-proteins undergoing N-terminal cleavage resulting in mature proteins of about 38 kDa (Chou et al., 2005; Shen et al., 2001b). Analyzing the coding sequence of *C. herbarum* vacuolar serine protease with the SignalP 3.0 software [<http://www.cbs.dtu.dk/services/SignalP/>] (Bendtsen et al., 2004) a hydrophobic leader sequence of 16 amino acids was localized at the N-terminal end. The three catalytic residues are D₁₈₄, H₂₁₆, S₃₈₂ and three cysteines (C₂₂₀, C₃₂₃, C₃₅₄) are present, which may be involved in the formation of disulfide bonds. The conserved oxyanion hole-residue is at position N₃₁₇. Amino acid G₃₈₀ is totally conserved in all subtilases (Siezen and Leunissen, 1997).

3.2. N-terminal sequence of the mature *C. herbarum* vacuolar serine protease

Based on the fact that homologous vacuolar serine proteases from *Penicillium* and *Aspergillus* species perform auto-proteolysis resulting in a mature protein, we attempted to define the N-terminus of the native *C. herbarum* vacuolar serine protease by Edman-degradation. Determination of the N-terminal end of the mature protein was important since for all subsequent immunological tests among others the mature protein was used. In order to determine the first amino acid of the mature *C. herbarum* vacuolar serine protease, *C. herbarum* crude extract was precipitated with ammonium sulphate and enriched by a ProteoExtract Protein Precipitation Kit. Subsequently, the extract was separated by 2D-gel electrophoresis, blotted onto a PVDF-membrane and stained with Coomassie Blue. The protein spot corresponding to *C. herbarum* vacuolar serine protease was identified by an IgG-immunoblot of a 2D-gel using the monoclonal antibody FUM20, which is known to specifically bind to vacuolar and alkaline serine proteases (Lin et al., 2000). The respective protein spot corresponding to *C. herbarum* vacuolar serine protease was excised from the membrane and subjected to Edman-degradation. The N-terminal sequence we obtained from *C. herbarum* vacuolar serine protease was EVEKNAP, but due to a shortage of protein this result remained somewhat equivocal.

In order to clarify the N-terminal end of *C. herbarum* vacuolar serine protease a multiple sequence alignment of homologous

Cla h 9	MRCALAGLSLATLATASPVLVNSIHNDAAPIISASNAKEIADNYMIKFKDHVTQNLAAEH	60
Cla c 9	-----	
Pen o 18	MKGFLSLTLPLLAASPTWSETIHNGAAPLISSTSAKEIPDSYIVVFKKHV GASAAASAH	60
Asp f 18	MKGFLSLTLPLLAASPTWSETIHNGAAPLISSTSAKEIPDSYIVVFKKHVNAESAAAH	60
Pen ch 18	MKGFLSLTLPLLAASPTWSETIHNGAAPLISSTSAKEIPDSYIVVFKKHVDPSSASAH	60
Pen ch 13	-----MGFLKVLATSLATLAVDAGTLTASNDAVIESSYIVVMNDVSTAEFSTH	52
Cla h 9	HGWVDLHEKTQVAKTELKRKRSQSPMVDIENGLKHTYNIAAGLMGYAGHFEDVIEQIR	120
Cla c 9	-----	
Pen o 18	HSWVDIHS--DNVRMELKKRSLFGFSEEPYLGKHTFHVAGSLMGYAGHFHEDVIEQVR	118
Asp f 18	HSWVDIHS-AQNERVELKRKSLFGFSEEPYLGKHTFHVAGSLMGYAGHFHEDVIEQVR	119
Pen ch 18	QSWIQEVHT-AHTGRMELKKRSLFGFSEEPYLGKHTFHVAGSLMGYAGHFHEDVIEQIR	119
Pen ch 13	REWATNVH-----ARLSRRKNGETSPG-----KH-FEING-LKGYTASDENTAKDIA	98
Cla h 9	RHPDVELVERDQEVHVLGSESEVEKNAP-WGLARISHRDSLSFGTFNKLYTEDGGEGVD	179
Cla c 9	-----DQEVHVLKSESEVEKNAP-WGLARISHRDSLSFGTFNKLYTEDGGEGVD	49
Pen o 18	RHPDVEYIEKDSEVHHF-EDPAIEKNAP-WGLARISHRDSLSFGTFNKLYAEDGGEGVD	176
Asp f 18	KHPDVEYIEKDSEVHTM-EDPTVEKSAWGLARISHRDSLSFGTFNKLYASEGGEGVD	177
Pen ch 18	RHPDVYIEKDSEVHTM-SEGSVEKNAP-WGLARISHRDSLSFGTFNKLYAEGGGEGVD	177
Pen ch 13	NDEAVKYIEPDMIVNA--IANVVQSNVPSWGLARISSKRT---GT-TSYTYDSTAGEGVV	152
Cla h 9	▼	
Cla c 9	▼	
Pen o 18	AYVDTGTNVHDVDFEGRASWGKTIPOGDADEDGNGHGTHCSGTIAGKKYGVAKKANVYA	236
Asp f 18	AYVDTGTNVHDVDFEGRASWGKTIPTDDEDGNGHGTHCSGTIAGKKYGVAKKANVYA	237
Pen ch 18	AYVDTGTNVHDVDFEGRASWGKTIPOGDADEDGNGHGTHCSGTIAGKKYGVAKKANVYA	237
Pen ch 13	FYGVDTGIDISHSDFGGRKNGTNVVDND-NTDNGHGHTASTAGSKYGVAKKANVYA	211
Cla h 9	VKVLRSNGSGMSDVVKGVEYAAESHLEQVSITKKGKRKGFKGSTANMSLGGGKSPILDK	299
Cla c 9	VKVLRSNGSGMSDVVKGVEYAAESHLEQVSITKKGKRKGFKGSTANMSLGGGKSPILDK	169
Pen o 18	VKVLRSNGSGMSDVVKGVEYAAESHLEQVSITKKGKRKGFKGSTANMSLGGGKSPILDK	296
Asp f 18	VKVLRSNGSGMSDVVKGVEYAAESHLEQVSITKKGKRKGFKGSTANMSLGGGKSPILDK	297
Pen ch 18	VKVLRSNGSGMSDVVKGVEYAAESHLEQVSITKKGKRKGFKGSTANMSLGGGKSPILDK	293
Pen ch 13	VKVLGADSGTNSGVISGMDWAVKDAKSRGANGK-----YVMTSLGGEFSKAVND	262
Cla h 9	●	
Cla c 9	●	
Pen o 18	AVNAAVDAGIHFAVAAGNDNADSCNYSPPAAENAVTVGASTLADERAYFSNYGKNDIFA	356
Asp f 18	AVNAAVDAGIHFAVAAGNDNADSCNYSPPAAENAVTVGASTLADERAYFSNYGKNDIFA	357
Pen ch 18	AVNAAVDAGIHFAVAAGNDNADSCNYSPPAAENAVTVGASTLADERAYFSNYGKNDIFA	353
Pen ch 13	AAANVVKSGIFLSVAAGNEAENASNSPPAAEACTLAASTDGSASFNTGSSVVDLYA	322
Cla h 9	▼	
Cla c 9	▼	
Pen o 18	PGLNISTWIGSKYAVNTISGTSMASPHVAGLLAYLLSLQPAKDSAFVADISPKKLLKAN	416
Asp f 18	PGLNISTWIGSKYAVNTISGTSMASPHVAGLLAYLLSLQPAKDSAFVADISPKKLLKAN	417
Pen ch 18	PGLNISTWIGSKYAVNTISGTSMASPHVAGLLAYLLSLQPAKDSAFVADISPKKLLKAN	413
Pen ch 13	PGQSIITAAIPGG--CSKTLISGTSMASPHVAGLLAYLLSLQPAKDSAFVADISPKKLLKAN	370
Cla h 9	LISIATVGALTDVPSNTANILAWNGGGESNYSAIVEKGGYKATHREPTMLEETISEAKVAS	479
Cla c 9	LISIATVGALTDVPSNTANILAWNGGGESNYSAIVEKGGYKATHREPTMLEETISEAKVAS	349
Pen o 18	LITITATSGALSDIPSDTPNLLAWNGGGSSNYTEIVSKGGYKAGASESMKKHLDLVGKVE	476
Asp f 18	LITITATSGALSDIPSDTPNLLAWNGGGSSNYTEIVSKGGYKAGASESMKKHLDLVGKVE	468
Pen ch 18	LISVATEGTLTDIPSDTPNLLAWNGGGSSNYTKIADGGYKAHNAETT-----VEDRIG	467
Pen ch 13	IVQLAT-SSISRAPSGTTSKLLYNGINV-----	397
Cla h 9	KKVYSEGDELAHKVAELTEKVEDLISGDLKDMFRELKRE	518
Cla c 9	KKVYSEGDELAHKVAELTEKVEDLISGDLKDMFRELKRE	388
Pen o 18	EVIATKEQKVLSEELGAIYSEIKDAVSA-----	503
Asp f 18	GLVHKAELKLTTELGAIEYSEIHDAVA-----	495
Pen ch 18	GIIDSAEKAFHKLGAIEYSEIKDAVSA-----	494
Pen ch 13	-----	

Fig. 2. Protein sequence alignment of vacuolar- and alkaline serine proteases. The coding sequences of the vacuolar serine proteases from *C. herbarum*, *C. cladosporoides*, *P. oxalicum*, *A. fumigatus*, *P. chrysogenum*, and the *P. chrysogenum* alkaline serine proteases were aligned. Identical amino acids are shown in black boxes whereas homologous residues are in grey boxes. Gaps are marked by dashes and the residue positions are given on the right. The first four amino acids of the mature proteins are framed. The three amino acids forming the catalytic triad are marked with a black triangle (▼), oxyanion hole residue N is marked with a black circle (●).

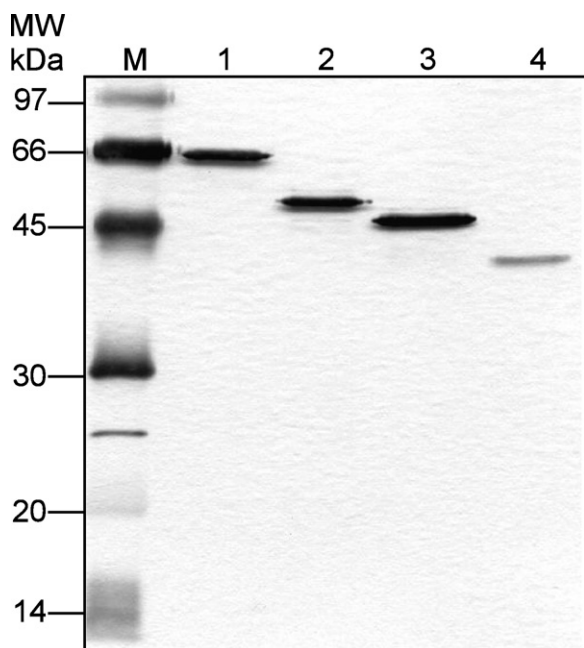


Fig. 3. SDS-PAGE of purified vacuolar- and alkaline serine proteases from *C. herbarum*, *P. oxalicum*, and *P. chrysogenum*. Two constructs of *C. herbarum* vacuolar serine protease, as are His₆-pro-Cla h 9 (lane 1) and His₆-Cla h 9-SESE (lane 2) along with the *P. oxalicum* vacuolar serine protease (His₆-Pen o 18-EDPA) (lane 3) and the *P. chrysogenum* alkaline serine protease (His₆-Pen ch 13-ANVV) (lane 4) were purified by Ni-NTA affinity chromatography. From each protein 0.5 µg were separated by SDS-PAGE and silver-stained.

fungal proteases was performed. In Fig. 2, the vacuolar serine proteases from *C. herbarum* (Cla h 9), *Cladosporium cladosporioides* (Cla c 9) (Chou et al., 2008), *P. oxalicum* (Pen o 18), *P. chrysogenum* (Pen ch 18), *A. fumigatus* (Asp f 18) and the alkaline serine protease from *P. chrysogenum* (Pen ch 13) are aligned showing regions of high homology in the centre flanked by low homology regions at the N- and C-terminal ends. For the vacuolar serine proteases from *C. cladosporioides*, *P. oxalicum*, *P. chrysogenum*, and *A. fumigatus* the N-terminus was shown to be SESE..., EDPA..., SEGs..., and EDPT..., respectively (Chou et al., 2008; Reichard et al., 2000; Shen et al., 1999a). Due to the high sequence identities of the mature vacuolar serine proteases from *P. oxalicum*, *P. chrysogenum*, *A. fumigatus* (64–78%), and a sequence identity of 97% to the *C. cladosporioides* vacuolar serine protease we used the amino acids ₁₃₉SESEVEKNAP₁₄₈ (Fig. 1) as N-terminal sequence for our recombinant N-terminally truncated construct “His₆-Cla h 9-SESE”, which most likely corresponds to the mature protein. The calculated molecular weight and pI of His₆-Cla h 9-SESE are 39.9 kDa and 5.86, respectively.

3.3. Immunological characterization of the *C. herbarum* vacuolar serine protease

IgE-reactivity of the *C. herbarum* vacuolar serine protease was tested with two constructs. On the one hand, the N-terminally truncated mature “His₆-Cla h 9-SESE” (S₁₃₉–E₅₁₈), whose N-terminal end corresponds to the N-terminal ends of the homologous mature vacuolar serine proteases from *P. oxalicum*, *P. chrysogenum* and *A. fumigatus* was cloned and expressed (Tables 1 and 2) (Fig. 3). On the other hand, the pro-protein without the hydrophobic leader sequence, termed “His₆-pro-Cla h 9” (S₁₇–E₅₁₈), was constructed (Tables 1 and 2) (Fig. 3) in order to investigate, whether there exist differences in the IgE-reactivity pattern between pro-Cla h 9 and the mature Cla h 9. Investigation of the IgE-reactivity of His₆-pro-Cla h 9 and His₆-Cla h 9-SESE revealed that 17 out of 110 *C. herbarum*

sensitized patients reacted with the two vacuolar serine protease constructs (data not shown). Thus, the vacuolar serine protease represents a minor allergen with a prevalence of 15.5% and was given the abbreviation “Cla h 9” by the World Health Organization, International Union of Immunological Societies allergen nomenclature subcommittee [<http://www.allergen.org/>]. Since there were no differences in the IgE-reactivity pattern of pro-Cla h 9 and mature Cla h 9 only the mature Cla h 9 construct, His₆-Cla h 9-SESE, was used for further immunological experiments. In Fig. 4A, *C. herbarum* crude extract is tested with the sera of five *C. herbarum* allergic patients being IgE-reactive with the vacuolar serine protease.

Previously, five monoclonal antibodies were generated against *Penicillium citrinum* and *A. fumigatus* culture medium and crude extract (Lin et al., 2000). Two of these antibodies (PCM39 and FUM20) reacted with the vacuolar serine proteases from *P. notatum*, *P. oxalicum*, *P. citrinum* and *A. fumigatus*. We could show that Cla h 9-SESE is also reactive with PCM39 and FUM20 (Fig. 5) indicating that the epitopes of these monoclonal antibodies are cross-reactive.

3.4. IgE-cross-reactivity between fungal serine proteases

Serine proteases in general and vacuolar serine proteases in particular are known for their pronounced IgE-cross-reactivity. In order to test the IgE-cross-reactivity of *C. herbarum* vacuolar serine protease inhibition immunoblots of Cla h 9 with two vacuolar and one alkaline serine protease(s) were performed. The mature vacuolar serine proteases from *P. oxalicum* (His₆-Pen o 18-EDPA (E₁₃₆–A₅₀₃)), *C. herbarum* (His₆-Cla h 9-SESE (S₁₃₉–E₅₁₈)), and the alkaline serine protease from *P. chrysogenum* (His₆-Pen ch 13-ANVV (A₁₁₆–Q₃₉₈)) were cloned as His₆-fusion proteins and purified to homogeneity by affinity chromatography (Fig. 3). Five sera from *C. herbarum* sensitized patients were incubated with *C. herbarum* extract in order to prove their specific IgE-reactivity. All sera tested reacted with a protein in the molecular weight range of 45 kDa presumably corresponding to Cla h 9 and several additional allergens (Fig. 4A, lanes 1–5). Investigation of IgE-cross-reactivity between Cla h 9-SESE and Pen o 18-EDPA (Fig. 4B) revealed that Pen o 18-EDPA-pre-incubated serum of patient 1 (Fig. 4B, panel III) showed residual IgE-binding to Cla h 9-SESE and the same serum pre-incubated with Cla h 9-SESE still bound to Pen o 18-EDPA (data not shown). From these experiments it may be deduced that both vacuolar serine proteases share most of their IgE-epitopes although there exist mould-specific epitopes as can be seen in case of patient 1 (Fig. 4B, panel III). Analysis of IgE-cross-reactivity between Cla h 9-SESE and Asp f 18-EDPT disclosed that in case of Asp f 18-EDPT-pre-incubated serum only a very faint IgE-binding to Cla h 9-SESE (Fig. 4C, panel IV) remained indicating that Cla h 9 and Asp f 18 share most of their IgE-epitopes. Studying the cross-reactivity between Cla h 9-SESE and Pen ch 13-ANVV showed that Pen ch 13-ANVV-depleted serum (Fig. 4D, panel V) exhibited only a very faint residual IgE-reactivity to Cla h 9-SESE indicating that Cla h 9 and Pen ch 13 also share several IgE-epitopes. Investigation of the IgE-cross-reactivity between Pen o 18-EDPA and Pen ch 13-ANVV revealed that Pen o 18-EDPA-depleted serum did hardly show any residual IgE-binding to Pen ch 13-ANVV indicating that these serine proteases share most of their IgE-epitopes (data not shown). Taken together the serine proteases Cla h 9, Asp f 18 and Pen ch 13 share most of their IgE-epitopes, but additionally all three allergens also harbour a few mould-specific epitopes.

3.5. Epitope mapping of the *C. herbarum* vacuolar serine protease

On the basis of our results of IgE-cross-reactivity between Cla h 9 and the serine proteases of *P. oxalicum*, *P. chrysogenum*, and *A. fumigatus* we wanted to know whether the Cla h 9-regions being homologous to the IgE-epitopes of *P. chrysogenum* (Yu et al., 2002;

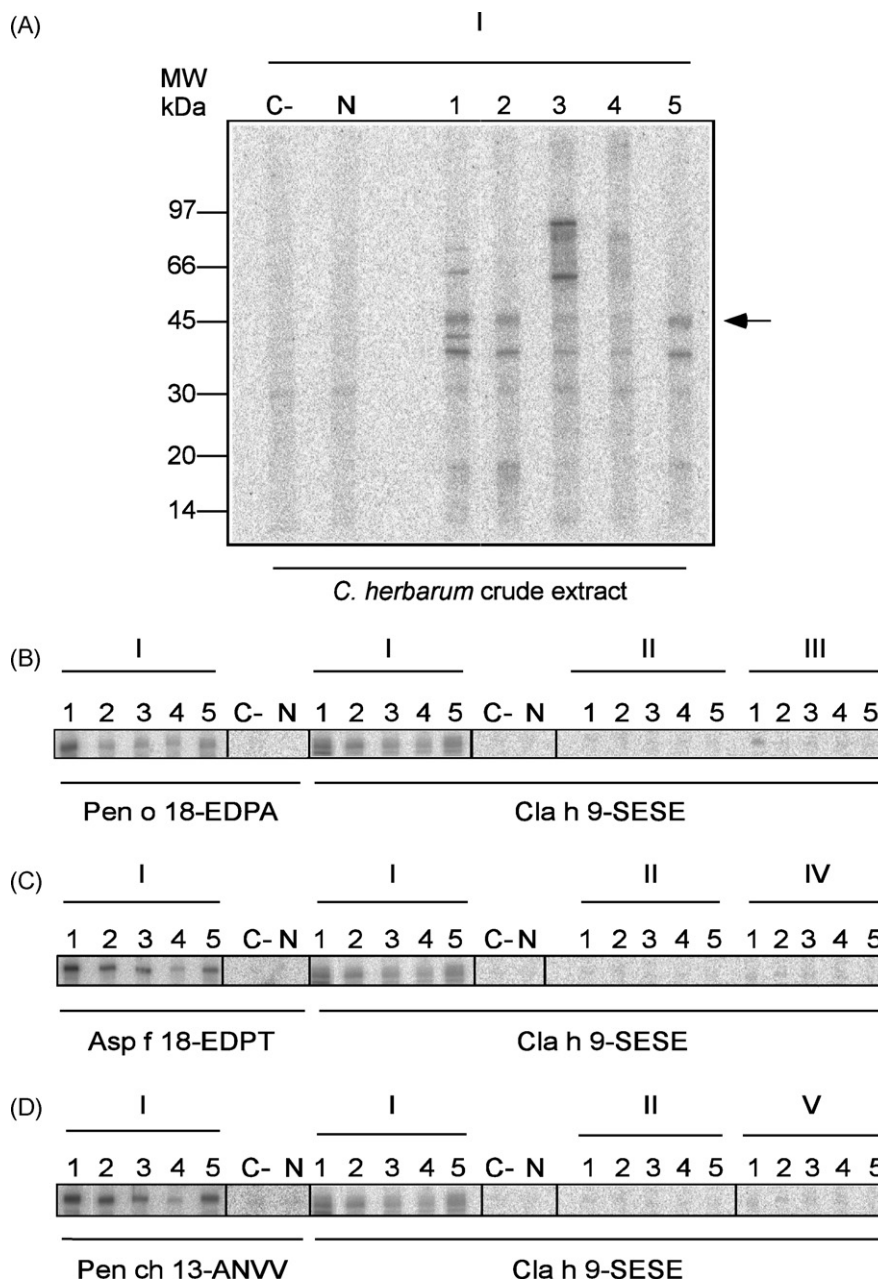


Fig. 4. IgE-cross-reactivity between fungal serine proteases. In panels (A–D) *C. herbarum* extract or the respective proteins were tested with the sera of five *C. herbarum* allergic patients (lanes 1–5). In lanes C- the membrane is solely incubated with the secondary antibody, whereas in lanes N the membrane was first incubated with a normal human serum of a non-atopic person followed by the secondary antibody. In panel (A) *C. herbarum* extract is tested with the five sera of *C. herbarum* allergic patients (lanes 1–5). The arrow marks the protein band presumably corresponding to Cla h 9. In panels (B–D) the lines below the immunoblot-lanes along with an allergen name refer to the protein bound to the membrane. In the lanes (1–5) outlined with I the sera were incubated without any pre-incubation. In panel B-II sera 1–5 were pre-incubated with His₆-Cla h 9-SESE and in B-III sera were pre-incubated with His₆-Pen o 18-EDPA. In panel C-II sera 1–5 were pre-incubated with His₆-Cla h 9-SESE and in C-IV sera were pre-incubated with His₆-Asp f 18-EDPT. In panel D-II sera 1–5 were pre-incubated with His₆-Cla h 9-SESE and in D-V sera were pre-incubated with His₆-Pen ch 13-ANVV.

Shen et al., 2003) also harbour IgE-epitopes and thus account for IgE-cross-reactivity between these vacuolar serine proteases.

IgE-reactive B-cell epitopes from the *P. chrysogenum* vacuolar serine protease have been analyzed by two groups (Shen et al., 2003; Yu et al., 2002) (Table 3). Please note that in Yu et al. (2002) this fungus is still named *P. notatum* and the allergen accordingly Pen n 18, while in Shen et al. (2003) the modern name *P. chrysogenum* and Pen ch 18 are used.

For the analysis of Cla h 9-IgE-reactive epitopes we constructed four His₆-fusion-peptides distributed along the entire protein-sequence, covering the regions being homologous to the *P. chrysogenum* B-cell epitopes, comprising 42–54 amino acids. The

four Cla h 9-peptides were purified by Ni-NTA affinity chromatography, separated by SDS-PAGE and tested with ten sera of *C. herbarum* sensitized patients. All 10 sera reacted with *C. herbarum* extract, the full-length Cla h 9 without the hydrophobic leader sequence (pro-Cla h 9) and the putative mature Cla h 9-SESE, respectively. The reactivity pattern of the ten sera with pro-Cla h 9 and Cla h 9-SESE were comparable. Regarding IgE-epitope-mapping one patient reacted with peptide 2 but none of the sera reacted with peptides 1, 3 and 4 (Fig. 6).

In Fig. 7, a protein-sequence alignment of pre-pro Pen ch 18, Pen n 18 and Cla h 9, respectively is shown. IgE-binding areas are highlighted in white, Cla h 9-amino acids with a grey background

Table 3

IgE-reactive epitopes of the vacuolar serine proteases from *P. chrysogenum* (Pen ch 18, Pen n 18) and *C. herbarum* (Cla h 9).

Allergen name	GenBank Acc. no.	Peptide	Position in ORF	IgE-reactivity in %	Reference
Pen n 18	AF264027	V7	G ₁₇₅ –E ₁₉₃	37.5 (N = 8)	Yu et al. (2002)
		C12	V ₁₈₀ –W ₁₉₈	75.0 (N = 8)	Yu et al. (2002)
		L3	T ₂₀₁ –K ₂₂₅	37.5 (N = 8)	Yu et al. (2002)
		C13	R ₂₄₂ –W ₂₅₈	12.5 (N = 8)	Yu et al. (2002)
		V3	A ₂₆₂ –D ₂₉₂	12.5 (N = 8)	Yu et al. (2002)
		C11	I ₃₅₈ –A ₃₇₈	25.0 (N = 8)	Yu et al. (2002)
		C10	A ₄₀₁ –L ₄₁₀	25.0 (N = 8)	Yu et al. (2002)
		C15	K ₄₁₁ –L ₄₃₄	12.5 (N = 8)	Yu et al. (2002)
		V11	G ₄₂₁ –Y ₄₅₃	12.5 (N = 8)	Yu et al. (2002)
Pen ch 18	AF263454	PN1	S ₁₃₇ –K ₂₃₉	100.0 (N = 11) ^a	Shen et al. (2003)
		PN1-a	S ₁₃₇ –S ₁₅₉	16.7 (N = 6) ^b	Shen et al. (2003)
		PN1-d	D ₁₉₁ –G ₂₁₃	16.7 (N = 6) ^b	Shen et al. (2003)
		PN1-e	E ₂₀₉ –K ₂₃₁	66.7 (N = 6) ^b	Shen et al. (2003)
		PN2	K ₂₃₂ –S ₃₃₃	18.2 (N = 11) ^a	Shen et al. (2003)
		PN2-b	S ₂₅₀ –K ₂₇₂	50.0 (N = 2) ^c	Shen et al. (2003)
		PN3	A ₃₁₄ –Y ₄₅₃	18.2 (N = 11) ^a	Shen et al. (2003)
		Peptide 2	R ₂₄₄ –D ₂₉₈	10.0 (N = 10) ^d	
Cla h 9	AY787775				

The peptides were tested for their IgE-reactivities with ^asera from asthmatics with IgE-reactivity to a 32 kD component of the *P. chrysogenum* extract ^bPN1-positive asthmatic patients ^cPN2-positive asthmatic patients ^d*C. herbarum* allergic patients with IgE-reactivity to Cla h 9.

are not IgE-binding, and amino acids underlined in black are either not IgE-binding or were not tested. On the right of the multiple sequence alignment the abbreviations of the respective peptides are given. Finally, the 3-dimensional localization of the IgE-binding and non-binding areas is shown in Fig. 8 with the same colour code as was used for Fig. 7.

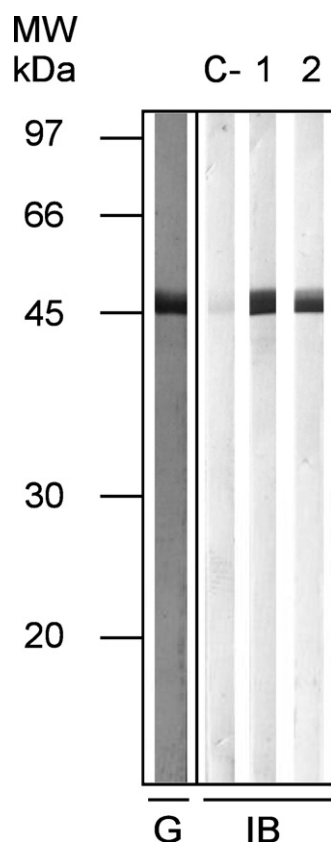


Fig. 5. IgG-blot of His₆-Cla h 9-SESE with two monoclonal antibodies. His₆-Cla h 9-SESE was separated by SDS-PAGE and stained with Coomassie-Brilliant Blue (lane G). In parallel an immunoblot (IB) of His₆-Cla h 9-SESE was performed, where the protein on the one hand was incubated with the secondary antibody alone (C-) and additionally with the two monoclonal antibodies FUM20 (lane 1) and PCM39 (lane 2).

Additionally, the four peptides were also tested with the monoclonal antibodies FUM20 and PCM39. Although Cla h 9-SESE is reactive with FUM20 (Fig. 5) none of the four peptides bound FUM20. In contrast, PCM39 specifically bound to *C. herbarum* extract, pro-Cla h 9 and Cla h 9-SESE and peptide 2 (data not shown) indicating that Cla h 9-peptide 2 harbours the PCM39-IgG-epitope. The moAb PCM39 originally was generated against *P. citrinum* and *A. fumigatus* culture medium and crude extract (Lin et al., 2000). From these experiments it may be deduced that Cla h 9-peptide 2 harbours at least one cross-reactive epitope, but the other Pen ch 18-IgE-epitopes seem to be mould-specific.

3.6. 3D model of the vacuolar serine proteases from *C. herbarum* and *P. chrysogenum*

Previously, Yu et al. (2002) have generated a three-dimensional model of Pen ch 18 based on the X-ray structure of the *Tritirachium album* proteinase K (PDB: 2PRK) (Betzel et al., 1988).

Pen ch 18 and Cla h 9 are highly homologous with a sequence identity of 69.1% for the mature protein sequences. Due to the high sequence identity and their extensive IgE- and IgG-cross-reactivity we attempted to perform a 3D-modeling of Cla h 9 based on the crystal structure of the *Tritirachium album* serine protease proteinase K (PDB: 1IC6) (Betzel et al., 2001). The two proteinases K –

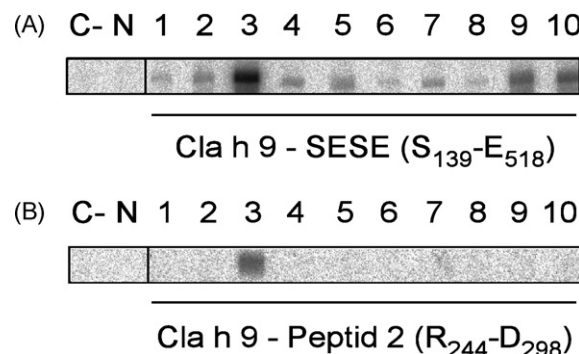


Fig. 6. IgE-reactivity of Cla h 9-peptide 2. His₆-Cla h 9-SESE (panel A) and His₆-Cla h 9-peptide 2 (R₂₄₄–D₂₉₈) (panel B) were tested with ten sera (lanes 1–10) of *C. herbarum* allergic patients. In lane C- the membrane is solely incubated with the secondary antibody, and in lane N the membrane was first incubated with a normal human serum of a non-atopic person followed by the secondary antibody.

Pen ch 18	1	MKGFLSLTLLPLLVAASPVAVNSIHNDAAPIILSSMTSKDIPDSYIVVFKKHVDPPSSASAH	
Pen n 18	1	MKGFLSLTLLPLLVAASPVAVNSIHNDAAPIILSSMTSKDIPDSYIVVFKKHVDPPSSASAH	
Cla h 9	1	MKGALAGLSLATLATASPVLVNSIHNDAAPIISASNAKEIADNYMIKF KDHVTONLAAEH	
Pen ch 18	61	QSWLQEVHT-AHTGRMELKKRSLFGFDFAFMGLKHTFHIAGSLLGYAGHFHEDVIEQIR	
Pen n 18	61	QSWLQEVHT-AHTGRMELKKRSLFGFDFAFMGLKHTFHIAGSLLGYAGHFHEDVIEQIR	
Cla h 9	61	HGWVQDLHEKTQVAKTELKRKRSQSPMVDDIFNGLKHTYNIAGGLMGYAGHFDEEDVIEQIR	
Pen ch 18	120	RHPDVDYIEKDSEVRTM-SEGSVEKNAPWGLARISHRESLSFGNFNKYLYAEEGGEGVDA	PN1 (a) v7
Pen n 18	120	RHPDVDYIEKDSEVRTM-SEGSVEKNAPWGLARISHRESLSFGNFNKYLYAEEGGEGVDA	Peptide 1
Cla h 9	121	RHPDVELVERDQEVHVLGSESEVEKNAPWGLARISHRDSLSFGTFNKLYTEDGGE	
Pen ch 18	179	YVIDTGANVKHVD FEGRANWGKTI PQGDAEDGNGHGTHCSGTIAGKKFGVAKKANVYAV	PN1 (d,e) v7, C12, L3
Pen n 18	179	YVIDTGANVKHVD FEGRANWGKTI PQGDAEDGNGHGTHCSGTIAGKKFGVAKKANVYAV	Peptide 1
Cla h 9	181	YVVDGTGNVDHVD FEGRASWGKTI PQGDAEDGNGHGTHCSGTIAGKKFGVAKKANVYAV	
Pen ch 18	239	KVLRSNGSGTMSDVVKGV EWAEEAHIKKSKKGD----KKFKGSVANMSLGGGSSRTLDLA	PN2 (b) C13, V3
Pen n 18	239	KVLRSNGSGTMSDVVKGV EWAEEAHIKKSKKGD----KKFKGSVANMSLGGGSSRTLDLA	Peptide 2
Cla h 9	241	KVLRSNGSGTMSDVVKGV EWAEEAHIKKSKKGD----KKFKGSVANMSLGGGSSRTLDLA	
Pen ch 18	295	VNAAVDAGIHF AVAAGNDNADACNYS PAAAEKAITVGASTLADERAYFSNYGKCTDIFAP	PN3
Pen n 18	295	VNAAVDAGIHF AVAAGNDNADACNYS PAAAEKAITVGASTLADERAYFSNYGKCTDIFAP	
Cla h 9	301	VNAAVDAGIHF AVAAGNDNADSCNYS PAAAEKAITVGASTLADERAYFSNYGKCTDIFAP	
Pen ch 18	355	GLNISTWVGS DHATNTISGTSMA SPHIA GLLAYVSLAPAKDSAYAVADVTPKQLKAAL	C11, C10, C15
Pen n 18	355	GLNISTWVGS DHATNTISGTSMA SPHIA GLLAYVSLAPAKDSAYAVADVTPKQLKAAL	Peptide 3,4
Cla h 9	361	GLNISTWVGS DHATNTISGTSMA SPHIA GLLAYVSLAPAKDSAYAVADVTPKQLKAAL	
Pen ch 18	415	ISVATEGTLTDIPSDTPNLLAWNGGGSANYTKILADGGYKAHNAETT-----VEDRIGG	C15, V11
Pen n 18	415	ISVATEGTLTDIPSDTPNLLAWNGGGSANYTKILADGGYKAHNAETT-----VEDRIGI	Peptide 4
Cla h 9	421	ISIATVGALTDVPSNTANILAWNGGGSANYSAIVEKGGYKATHRPTMLEEIESEAKVASK	
Pen ch 18	469	IIDSAEKAFHKELGAIYSEIKDAVSA-----	
Pen n 18	469	IIDSAEKAFHKELGAIYSEIKDAVSV-----	
Cla h 9	481	KVYSEGDELAKHVAELTEKVEDLIAGELKDMFRELKRE	

Fig. 7. Sequence alignment and IgE-epitope mapping of the vacuolar serine proteases from *C. herbarum*, *P. chrysogenum*, and *P. notatum*. In this sequence alignment IgE-reactive epitopes of Pen ch 18 and Pen n 18 and Cla h 9 are displayed with a white background. Those Cla h 9-peptides which were not IgE-binding are shown in a grey box, whereas Cla h 9 areas given in black boxes were not tested. Pen ch 18- and Pen n 18-residues in black boxes are either not IgE-binding or were not tested for their IgE-reactive properties. To the right of the sequence alignment the abbreviations of the peptides, as used in the publications about Pen ch 18 and Pen n 18, respectively (Shen et al., 2003; Yu et al., 2002), and as obtained in the present work are listed.

1IC6 and 2PRK – only differ in 1 out of 279 amino acids, but nonetheless 3D-modelling of Cla h 9 on basis of 1IC6 provided energetically better results compared to 2PRK.

The 3D model (Fig. 8) of Cla h 9 was generated from amino acid N₁₄₆–G₄₄₄, corresponding to those areas where a defined structure was calculated. At the N-terminal and C-terminal ends of Cla h 9 the sequence similarity to 1IC6 was too low in order to deduce a defined 3D-structure. The sequence identity between Cla h 9 (N₁₄₆–G₄₄₄) and 1IC6 (N₅–N₂₇₆) is 46.8%.

In parallel, a three-dimensional model of Pen ch 18 (N₁₄₄–G₄₃₈) was generated based on the X-ray structure of the *T. album* proteinase K (PDB: 2PRK) as published by Lee et al. (2007). The sequence identity between Pen ch 18 (N₁₄₄–G₄₃₈) and 2PRK (N₅–N₂₇₆) is 47.3%.

The model of Pen n 18 (Fig. 8–Pen n 18) is the same as Pen ch 18 since these two allergen names refer to the same protein, but in the Pen n 18-model those IgE-epitopes are highlighted in white, which were published by Yu et al. (2002).

4. Discussion

Serine endo- and exopeptidases are extremely widespread and fulfil diverse functions. Serine proteases are grouped into six clans, among which the (chymo)trypsin-like and the subtilisin-like are the two largest ones. Both clans are characterized by a highly similar arrangement of the catalytic residues histidine, aspartic acid, and serine, but differ in their protein scaffold which either is β/β ((chymo)trypsin-like proteases) or α/β (subtilisin-like pro-

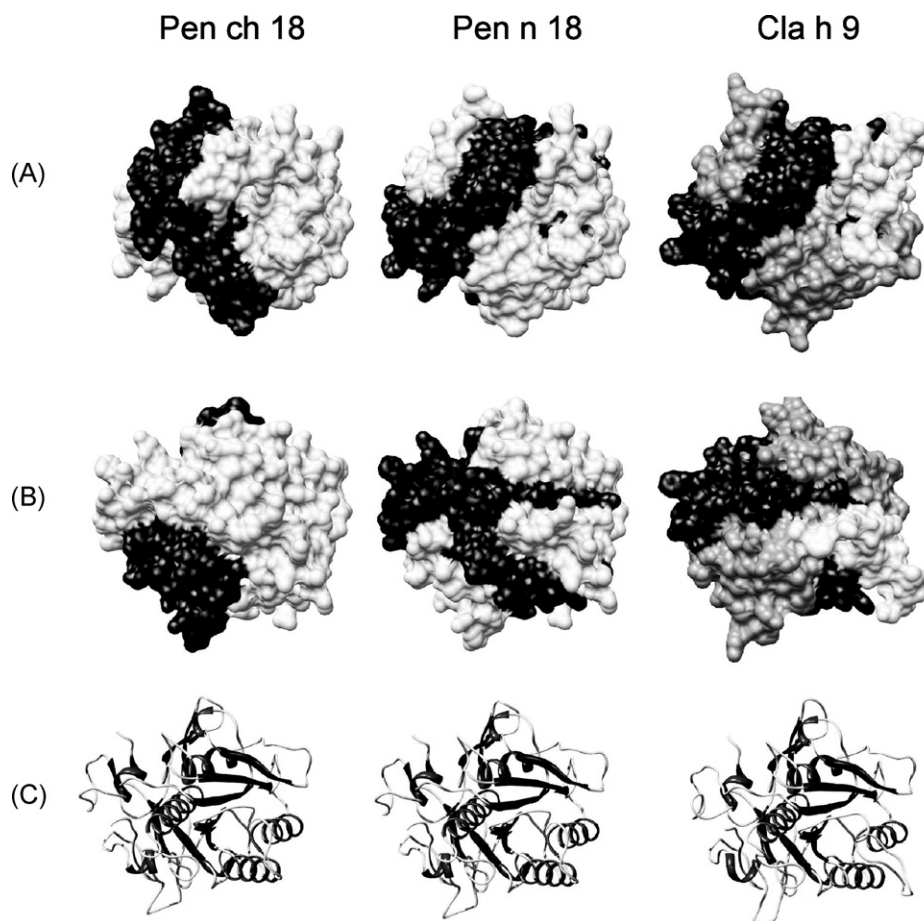


Fig. 8. 3D-model of Cla h 9 and Pen ch 18. The models were generated by the program MODELLER [<http://salilab.org/modeller/>], which generates a 3D model of a given protein on the basis of a sequence alignment with a homologous protein with a known 3D structure. In case of Pen ch 18 and Pen n 18, respectively, *Tritirachium album* proteinase K (PDB: 2PRK) was used as template, whereas *T. album* peptidase K (PDB: 1iC6) was the template for Cla h 9. The models were generated from N₁₄₆ to G₄₄₄ (Cla h 9) and N₁₄₄ to G₄₃₈ (Pen ch 18 and Pen n 18), respectively. In row A, the molecular surface model is shown from the front whereas in row B the models are turned by 180° giving view to the back-side. Row C shows a ribbon visualization of the models. White residues represent IgE-binding areas, whereas black residues are either not IgE-binding or were not tested for their IgE-reactivity (Pen ch 18, Pen n 18). In case of Cla h 9 black residues were not tested and grey residues are not IgE-binding.

teases) (Barrett and Rawlings, 1995). The subtilisin-like proteases, like the vacuolar serine proteases, frequently are also termed “subtilases” and occur in archaea, bacteria, yeasts, fungi and higher eukaryotes (plants, insects, amphibian, fish, mammals) (Siezen and Leunissen, 1997). The majority of the subtilases are synthesized as precursor-molecules with a pre- and pro-sequence at their N-terminal ends (Bryan et al., 1995). The pre-sequence serves as signal peptide for the translocation via a cellular membrane. The pro-sequence on the one hand acts as a protease self-inhibitor and on the other hand as an intra-molecular chaperone being responsible for the correct folding of the mature protein. The pro-sequence usually is removed from the mature protein by autoproteolysis (Yabuta et al., 2001). The subtilase superfamily itself may be divided into six families as are subtilisin, thermolysin, proteinase K, lantibiotic peptidases, kexin, and pyrolysins. The fungal serine proteases belong to the proteinase K family having sequence-identities of more than 37% among each other. Only few members of this family have a significant C-terminal extension beyond the catalytic domain (Siezen and Leunissen, 1997). Subtilases in general have a high degree of sequence variability even within the catalytic domain, with the exception of the catalytic residues. Nearly every other residue may be replaced by one or more amino acids (Siezen and Leunissen, 1997). The overall sequence variability along with highly conserved amino residues forming the catalytic triad and the oxyanion hole are general char-

acteristics of proteinases K, and are found in the *C. herbarum* vacuolar serine protease, Cla h 9. The Cla h 9 - open reading frame encodes a 519 amino acids long polypeptide (Fig. 1). A multiple sequence alignment of Cla h 9 with homologous vacuolar serine proteases from *C. cladosporioides*, *P. oxalicum*, *A. fumigatus* and *P. chrysogenum* and the alkaline serine protease from *P. chrysogenum* (Fig. 2) clearly show that the three amino acids Asp (D), His (H), Ser (S) forming the catalytic triad, are conserved as well as N₃₁₇ (Cla h 9) representing the oxyanion hole residue. In contrast, the protein-sequence throughout the remaining domains is much more variable.

Analysis of the native Cla h 9-N-terminal sequence by Edman-degradation of protein-spots excised out of 2D-immunoblots along with multiple sequence alignments of homologous vacuolar serine proteases (Fig. 2) revealed that the N-terminus of the mature *C. herbarum* vacuolar serine protease most likely starts with S₁₃₉. The localisation of the N-terminal end at S₁₃₉ is supported by the fact that in case of the homologous allergen of *C. cladosporioides* (Cla c 9), having a sequence identity of 97.6%, the N-terminal end of the mature protein was determined to be S₉. Since the Cla c 9 cDNA-clone was N-terminally truncated the majority of the pre-pro-sequence was missing except eight amino acids (Chou et al., 2008). Additionally, the respective protein, His₆Cla h 9-SESE (S₁₃₉–E₅₁₈), has a calculated molecular weight of 39.9 kDa and migrates in SDS-PAGE at about 45 kDa (Fig. 3, lane2), which is

in accordance with the IgE-reactive protein band at 45 kDa in *C. herbarum* protein extract (Fig. 4, panel A).

The allergen family of the “Subtilisin-like serine proteases” is one of the most important allergen-families judged by the number of allergens in this family. The route of exposure within this allergen family is focused on inhalation (Radauer et al., 2008).

Cla h 9 was produced as recombinant His₆-fusion protein with and without N-terminal leader sequence and tested with 110 sera of *C. herbarum* allergic patients. IgE-immunoblots revealed that 17 out of 110 sera (15.5%) reacted with recombinant Cla h 9. In comparison to the vacuolar serine proteases of *P. oxalicum*, *A. fumigatus*, and *P. chrysogenum*, having a prevalence of IgE-reactivity of 89% (Shen et al., 1999b), 79% (Shen et al., 2001a), and 77% (Shen et al., 2003), respectively, Cla h 9 is a minor allergen. Nonetheless, in terms of incidence Cla h 9 ranks within all cloned *C. herbarum* allergens at the third position directly after the major allergen Cla h 8, a NADP-dependent-Mannitol Dehydrogenase (prevalence of 57.1%) (Simon-Nobbe et al., 2006) and Cla h 6, an enolase (prevalence of 22%) (Breitenbach and Simon-Nobbe, 2002).

IgE-cross-reactivity of allergens in general and fungal allergens in particular is a well-known phenomenon, which immunologically is based on shared B-cell epitopes among homologous proteins. Cross-reactivity along with co-sensitization represents the major reason for the fact that a vast majority of mould allergic patients reacts with several fungal species (Horst et al., 1990). Up to now IgE-cross-reactivity has been described for 17 fungal allergens. Approximately one-third of them exclusively can be found within a single fungal phylum, two thirds belong to different fungal phyla, whereas members of 10 allergen families partly also exhibit cross-reactivity with non-fungal species. All allergenic vacuolar serine proteases which have been isolated so far, exclusively originate from fungal phyla making it a prominent fungal allergen (Simon-Nobbe et al., 2008). IgE-cross-reactivity between vacuolar serine proteases previously has been described for *Penicillium*-, *Aspergillus*-, and *Rhodotorula*-species (Chou et al., 2005; Lin et al., 2000). Based on our experiments we could show that the *C. herbarum* vacuolar serine protease is IgE-cross-reactive with the *P. oxalicum* (Fig. 4, panel B) and *A. fumigatus* (Fig. 4, panel C) vacuolar serine proteases and the *P. chrysogenum* alkaline serine protease (Fig. 4, panel D). Despite extensive IgE-cross-reactivities between these allergens each fungus additionally contained mould-specific-IgE-epitopes. Investigation of the IgE-cross-reactivity between Pen o 18 and Pen ch 13 (data not shown) revealed that both allergens share most of their IgE-epitopes.

Previously, five monoclonal antibodies against vacuolar and/or alkaline serine proteases have been generated. FUM20, one of these moAbs, was generated through the immunization of BALB/c mice with *A. fumigatus* crude extract and culture medium, and reacted with a 34-kDa component in the *A. fumigatus* culture medium (Lin et al., 2000). PCM39, another moAb, originated from the immunization of BALB/c mice with *P. citrinum* culture medium and was reactive with a 33-kDa component in *P. citrinum* crude extract (Lin et al., 2000). In order to investigate cross-reactivity of these moAbs several serine proteases have been tested for specific reactivity. Previously, FUM20 was shown to be reactive with the vacuolar serine proteases from *P. chrysogenum*, *P. citrinum*, *P. oxalicum*, *R. mucilaginosa*, *A. fumigatus* (Lee et al., 2007), and *C. cladosporioides* (Chou et al., 2008) and the alkaline serine proteases from *A. fumigatus* and *A. flavus* (Lee et al., 2007). In the present work (Fig. 5), we now could demonstrate that Cla h 9 is also reactive with FUM20, which is in concordance with the postulated FUM20-linear epitope encompassing 9 amino acids (Lee et al., 2007), as are Cla h 9-₁₄₂EKNAPWG₁₅₀. Cla h 9-peptide 2 did not react with FUM20 since the FUM20-epitope is located upstream of Cla h 9-peptide 2. In previous works, PCM39 was shown to be IgG-reactive with the alkaline serine proteases from *A. flavus* (Chou et al., 1999), *A.*

fumigatus, *P. citrinum*, *P. chrysogenum* (Lin et al., 2000), and the vacuolar serine proteases from *A. fumigatus*, *P. citrinum*, *P. chrysogenum*, and *P. oxalicum* (Lin et al., 2000). The PCM39-IgG-epitope of Pen ch 18 is located within residues ₂₆₈KKGDKKFKGSVANMSLGGSSRT₂₉₀ (Shen et al., 2003). In this work, we demonstrated that Cla h 9 is also reactive with PCM39 (Fig. 5). Cla h 9-peptide 2 was also shown to be IgG-reactive with PCM39 (data not shown). This result is in concordance with the data of Shen et al. (2003) since Cla h 9-peptide 2 corresponds to the homologous region of the Pen ch 18-PCM39-IgG-epitope. In contrast to our results Cla c 9 is not reactive with PCM39 (Chou et al., 2008), which may be due to the difference in the amino acid sequence within this IgG-epitope.

Investigation of IgE-reactive epitopes of an allergen may help understanding the molecular features of an allergen. Additionally, information about the localization of cross-reactive IgE-epitopes may be gained.

Previously, the vacuolar serine protease of *P. chrysogenum* was investigated for its IgE-reactive epitopes by two groups. Yu et al. (2002) performed proteolytic digestions of rPen n 18 (S₁₃₇–Y₄₅₃) (Pen n 18 is an old synonym for Pen ch 18) ending up with 19 peptides covering most of the protein sequence. Nine of these peptides, V7, C12, L3, C13, V3, C11, C10, C15, and V11, partly overlapping in their primary sequence, turned out to be IgE-binding (Table 3). The prevalence of IgE-reactivity of these nine peptides ranged from 12.5% to 75% (N=8), with peptide C12 near to the N-terminal end showing the highest IgE-reactivity. Fig. 7 gives a protein-sequence alignment of the vacuolar serine proteases of *P. chrysogenum* and *C. herbarum* indicating the localization of the respective IgE-reactive epitopes on the right. Shen et al. (2003) constructed three overlapping fragments (PN1–PN3) of rPen ch 18 (S₁₃₇–Y₄₅₃). Additionally, six overlapping synthetic peptides were generated within the Pen ch 18-PN1-fragment (PN1-a–PN1-f) and the Pen ch 18-PN2-fragment (PN2-a–PN2-f), respectively. Analysis of IgE-reactivity revealed that Pen ch 18-PN1 is recognized by 100% of the patients (N=11), and Pen ch 18-PN2 and 3 have a prevalence of 18% (N=11) (Table 3). Within the synthetic peptides Pen ch 18-PN1-e turned out to be a dominant linear IgE-epitope with a prevalence of 67% (N=6).

In the present study, IgE-binding epitopes of Cla h 9 were analyzed and visualized in their three-dimensional context performing modelling of Cla h 9 on the basis of the known crystal structure of the homologous *T. album* proteinase K (PDB: 1IC6). Since we could prove that the vacuolar serine proteases from *C. herbarum*, *A. fumigatus*, and *P. oxalicum* are highly IgE-cross-reactive (Fig. 4) we wanted to test whether the Cla h 9-regions, being homologous to the Pen ch 18-IgE-epitopes, also represent IgE-epitopes. Therefore, four peptides with a length of 42–54 amino acids (Table 2) corresponding to the IgE-reactive peptides of Pen ch 18 (Shen et al., 2003; Yu et al., 2002) (Table 3), were cloned as His₆-fusion proteins. The four peptides were purified to homogeneity and subsequently tested with ten sera of *C. herbarum* sensitized patients, which specifically react with His₆-Cla h 9-SESE (Fig. 6A). From these peptides only Cla h 9-peptide 2 (R₂₄₄–D₂₉₈) was recognized by one out of ten sera (Fig. 6A).

Comparing the results of the IgE-epitope-mapping of Cla h 9 with Pen n 18 (Yu et al., 2002) and Pen ch 18 (Shen et al., 2003) revealed that the regions homologous to Cla h 9-peptide 2 are Pen ch 18-PN2, Pen n 18-C13, and Pen n 18-V3 (Fig. 7). The prevalence of IgE-reactivity for these peptides is 10% (N=10) for Cla h 9-peptide 2, 18.2% (N=11) for Pen ch 18-PN2 (K₂₃₂–S₃₃₃) (Shen et al., 2003), and 12.5% (N=8) in case of Pen n 18-C13 (R₂₄₂–W₂₅₈) and Pen n 18-V3 (R₂₆₂–W₂₉₂) (Yu et al., 2002). In Fig. 8, the three-dimensional structures of Cla h 9 and Pen ch 18 were modelled based on the X-ray structures of two homologous proteinase K sequences from the mould *T. album* (PDB: 1IC6, 2PRK) (Betzel et al., 1988, 2001), which only differ in one amino acid. IgE-reactive epi-

topes of Cla h 9 and Pen ch 18 and Pen n 18 are displayed in white. As expected most of the amino acids corresponding to Cla h 9-peptide 2 and Pen ch 18-PN2 and Pen n 18-C13, -V3 are located on the surface and therefore are accessible for IgE-antibodies. Analysing Cla h 9-Peptide 2 with respect to linear B-cell-epitopes with the BepiPred-method [<http://www.cbs.dtu.dk/services/BepiPred/>] (Larsen et al., 2006) revealed that peptides ${}_{245}\text{SNGSGSMDS}_{253}$ and ${}_{271}\text{ITKKGKRKGFKGSTANMSLGGGKSPIL}_{297}$ have a high probability to form linear B-cell epitopes. Taken together the region of Cla h 9-peptide 2 harbours a minor, putatively cross-reactive IgE-epitope. Comparing the highly IgE-reactive fragment of Pen ch 18-PN1e ($\text{E}_{209}\text{--K}_{231}$) with Cla h 9-peptide 1 ($\text{G}_{177}\text{--K}_{227}$), applying the BepiPred-method reveals that Pen ch 18 amino acids ${}_{209}\text{EDGNGHGTHCSGT}_{221}$ and the corresponding Cla h 9 amino acids ${}_{211}\text{EDGNGHGTHCSGT}_{223}$ are part of a predicted epitope. Having a look at the surface accessibility of these amino acids in the 3D-models of Cla h 9 and Pen ch 18 reveals that there is a minor difference in position E_{209} (Pen ch 18) and E_{211} (Cla h 9), respectively, which could be responsible for an altered IgE-reactivity.

Comparing the area of Pen n 18-C12 ($\text{V}_{180}\text{--W}_{198}$) with the homologous amino acids of Cla h 9-peptide 1 discloses that the difference in the amino acid sequence is relatively high, which may explain the differences in IgE-reactivity.

5. Conclusion

Summing up, we could isolate a vacuolar serine protease from *C. herbarum*, which was termed Cla h 9 by the WHO-IUIS allergen nomenclature sub-committee [<http://www.allergen.org/Allergen.aspx>]. This allergen is recognized by 15.5% of the *C. herbarum* sensitized patients tested and it is highly IgE-cross-reactive with other vacuolar and alkaline serine proteases. Moreover, it reacts with the two moAbs FUM20 and PCM39, which were shown to be reactive with a broad panel of fungal vacuolar and alkaline serine proteases. Epitope-mapping of Cla h 9 along with modelling of the three-dimensional structure disclosed that Cla h 9-peptide 2 is IgE-reactive, and that Cla h 9-peptide 2 and the homologous highly IgE-reactive Pen ch 18-peptide PN1e have a short peptide (EDGNGHGTHCSGT) in common, which is located on the protein surface and predicted to be a linear epitope. Cla h 9-peptide 2 is the second identified IgE-binding epitope of a *C. herbarum* allergen besides the 69 amino acids long peptide of *C. herbarum* enolase, which was shown to be a major IgE-epitope (Simon-Nobbe et al., 2000). Since Cla h 9 is the third most important allergen in terms of prevalence beside Cla h 8 (NADP-dependent mannitol dehydrogenase) (Simon-Nobbe et al., 2006) and Cla h 6 (enolase) (Breitenbach and Simon-Nobbe, 2002; Breitenbach et al., 1997) it represents an interesting candidate molecule for a molecule-based approach of *C. herbarum* allergy diagnosis. Additionally, a further analysis of Cla h 9-peptide 2 and Pen ch 18-peptide PN1e, respectively, may disclose a cross-reactive IgE-binding epitope.

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