

Partial Amino Acid Sequence of a Cellulase-Like Component with IgE-Binding Properties from *Stachybotrys chartarum*

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Key Words

Amino acid sequence · Cellulase · Glycoside hydrolase · Immunoglobulin E · *Stachybotrys chartarum*

Abstract

Background: The aim of this study was to characterize the amino acid sequence of a selected *Stachybotrys chartarum* component and to investigate human IgE reactivity against components of *S. chartarum* and nine other fungal species. **Methods:** Human IgE reactivity against *S. chartarum* and nine other fungal extracts was investigated by the immunoblotting method. For automated amino acid sequencing analyses, the *S. chartarum* extract was purified by ion exchange chromatography prior to in-gel alkylation and digestion with modified trypsin. **Results:** Human IgE reactivity was detected against eight components in the *S. chartarum* extract. Over 80% of the sera from the exposed subjects and less than 50% of the control sera recognized the 33-, 48- and 50-kD *S. chartarum* components. The human sera detected a 48- to 50-kD component from the extracts of eight fungal species. Nineteen peptide sequences were identified from the 48-kD component of *S. chartarum*. An analysis of the peptide sequences revealed homology

with known fungal glycoside hydrolase enzymes (cellulases). **Conclusions:** The data showed human IgE reactivity against several *S. chartarum* components, including one at 48 kD. On the other hand, the human sera recognized 48- to 50-kD components from seven other fungal species, suggesting shared antigenic components (e.g. enolase) between the fungi. Thus, to our knowledge, this is the first antigen identified from *S. chartarum*.

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Introduction

Stachybotrys chartarum is a saprophytic greenish-black fungus found worldwide and known to grow well on cellulose-based materials such as straw, hay and paper and cellulose-containing building materials like gypsum board or wallpaper [1–3]. *S. chartarum* is assumed to be a rare contaminant in buildings [4], since it requires high-moisture conditions to grow [5]. Water leaks, infiltration, condensation or flooding may, however, provide a good starting point for *S. chartarum* contamination in cellulose-based building materials [6–8].

S. chartarum is known to produce several potential toxins, especially when growing on cellulose-based mate-

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rials [2, 3, 7, 9]. Thus, the risk of exposure to stachybotryotoxins among occupants is usually a concern when *S. chartarum* contamination is detected in wet building materials and constructions [10–12]. Much less attention has been paid to the antigenic and allergenic properties of this fungus. Even though the health-related impact of *S. chartarum* is believed to be derived mainly from toxin exposure, immunological responses to the fungus should be studied further.

Only a few cases of elevated *Stachybotrys*-specific antibodies or other allergic responses to *Stachybotrys* exposure in humans have been published so far [13–15]. Korpi et al. [16] investigated the effects of inhalation exposure to *S. chartarum* extract on the serum total immunoglobulin (Ig) E and *S. chartarum*-specific IgA, IgG1 and IgG2a antibody levels in mice. *S. chartarum* exposure increased the antibody levels except for those of IgA. The human IgE reactivity against the 34- and 52-kD components of *S. chartarum* was recently reported [17]. In our previous study [18], we characterized an antigen profile for *S. chartarum* and reported the influence of culture media and incubation time on the antigenic composition of *S. chartarum* extracts. We also described the role of carbohydrate moieties in the IgE-binding capacity of *S. chartarum* proteins. Additionally, cross-reactivity between *S. chartarum* and ten other fungal extracts was demonstrated using rabbit IgG antibodies. Based on the results, a 50-kD component in 15% acrylamide gel (shown in the present study as a 48-kD component in 10% acrylamide gel) was proved to be the most characteristic immunologically reactive component of *S. chartarum* [18]. This component was tentatively shown to possess human IgE reactivity in immunoblotting analyses [19].

The aim of this study was to determine partial amino acid sequences of the 48-kD *S. chartarum* component, to further investigate human IgE reactivity against *S. chartarum* components and to compare the human IgE reactivity detected with reactivity against nine other fungal species to which serum donors might have been exposed.

Materials and Methods

Crude Fungal Antigen Preparation

Fungal antigen crude extracts (CE) were prepared substantially as described earlier [18]. Briefly, *Alternaria alternata* [Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) 62006], *Aspergillus versicolor* [University of Kuopio (UKU) 3], *Cladosporium cladosporioides* (DSMZ 62121), *Memnoniella echinata* (Centraalbureau voor Schimmelcultures 304.54), *Penicillium brevicompactum* [American Type Culture Collection (ATCC) 58606], *Phialophora rebens* (National Public Health Institute RE27), *Phoma* sp. (UKU 8),

S. chartarum (ATCC 208877, a nontoxic strain), *Trichoderma viride* (DSMZ 63065) and *Ulocladium botrytis* (UKU 11) were cultured in cellulose broth for 10 days at 25°C. Mycelium was separated from the broth by centrifugation, lyophilized and resuspended in phosphate-buffered saline (PBS) with 0.02% (w/v) phenylmethyl sulfonyl fluoride and sodium azide. The mycelium suspension was homogenized mechanically and extracted in a shaker at 4°C overnight. After centrifugation, the supernatant was dialyzed (molecular weight cutoff 14.5 kD) against ultrapure water, sterile-filtered and lyophilized. Lyophilized extract was stored at –70°C and resuspended in ultrapure water for use.

Separation of *S. chartarum* Protein Components by Ion Exchange Chromatography

For separation of *S. chartarum* components, the extracted proteins were precipitated overnight at –20°C using 3 × volume of ice-cold acetone. A Mono Q HR 5/5 anion exchange column (Amersham Biosciences, UK) was preequilibrated with 20 mM Tris-HCl buffer (pH 7.5), and the precipitated *S. chartarum* proteins were dissolved in the same buffer and sterile-filtered. ÄKTApurifier 10 (Amersham Biosciences) with a linear gradient from 0 to 1 M NaCl in 45 min with a flow speed of 1 ml/min was used for separation of the purified *S. chartarum* protein extract. Fractions were collected and identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described below and concentrated by ultrafiltration. Fractions containing the 48-kD component were used for sequencing analyses.

Human Sera

Human serum samples were collected from 23 Danish teachers working in two schools where *S. chartarum* was found in indoor air samples ('exposed subjects') and 23 colleagues working in a school building where no indoor air complaints or water damage had been reported ('control subjects'). All three of the schools were primary schools with about 500–600 pupils and 50–60 teachers each, and all were located in southern Denmark within a radius of 25 km. Nineteen of the exposed subjects worked in the basement of a 100-year-old brick school building, where there had been water infiltration through the outer brick wall. The floor covering was linoleum carpet and the brick walls were decorated with wood chip paper. The ceiling was made of cardboard plates. An additional four exposed teachers worked in a 43-year-old brick school building with flat roofs and damp ceiling structures. The building was built on flagstone. The walls were made of bricks, plastered with sand material. The roof was composed of big plates of rough organic material and there were holes in the membrane resulting in condensation of water in the roof construction. In both the water-damaged schools, in addition to *S. chartarum*, *Penicillium* spp., *A. versicolor* and *T. viride* were detected in the indoor air in the winter. The teachers in the former school building had been exposed for about 1 year, and those in the latter building for about 2 or 3 years before the collection of sera. No renovations had been performed in either school building in the meantime. The main structure of the school building where the control subjects worked was 83 years old, with additional constructions to increase the space available. The building was made of brick and had gable roofs. No water damage or indoor air complaints were reported in the school, and indoor air fungal flora was shown to reflect the normal outdoor air flora.

The exposed subjects (n = 23) were between 26 and 57 years of age; 35% of them were smokers and 17% had self-reported asthma. The controls were matched with the exposed subjects regarding their age, sex, smoking habits and atopy. In the control group, the age distribution was from 26 to 58 years; 22% were smokers, 8.6% had self-reported asthma and 13% had hay fever. In both groups, 20 subjects were females and 3 males.

A written and signed consent for participation in this study was obtained from each subject. The scientific ethical committee of the South Danish University had approved the wording of the consent form and the use of human subjects in the study. The sera were stored temporarily at -20°C, transported to Finland in dry ice and stored at -70°C before analyses.

SDS-PAGE and Immunoblotting

SDS-PAGE, electrophoretic transfer and immunoblotting analyses were carried out substantially as described previously [18]. In the SDS-PAGE analysis, 10% acrylamide gel was used under reducing conditions. The amount of fungal protein (CE) loaded in the gel was 500 µg. A low molecular weight electrophoresis calibration kit and Low-Range Rainbow Molecular Weight Markers (Amersham Pharmacia Biotech Inc., UK) were used as molecular weight standards. Molecular weights were determined using GS-710 Calibrated Imaging Densitometer and Quantity One Software (BioRad Laboratories, USA).

Human IgE reactivity (n = 46) to *S. chartarum* components was studied by the immunoblotting method. To study the human IgE reactivity to different fungal species, the sera of five exposed subjects who had shown an intense reaction to the 48-kD component of *S. chartarum* and their matched control sera were used. In the analyses, the dilution used for human sera was 1:5, for the *S. chartarum*-specific rabbit polyclonal antiserum [18] 1:10,000, for the rabbit anti-human Ig (P0295, Dako, Denmark) 1:1,000 and for the peroxidase-conjugated swine anti-rabbit Ig (P0399, Dako) 1:10,000. Only the reactions clearly exceeding the intensity of the negative control (serum was replaced with a blocking agent, gelatine-PBS-Tween) were regarded as positive.

Enzyme-Linked Immunosorbent Assay

The levels of IgE and IgG in human sera were analyzed by enzyme-linked immunosorbent assays (ELISA). Microtiter plates were coated with the *S. chartarum* CE at a protein concentration of 1 µg/ml. One hundred microliters of human sera, at dilutions of 1:10 (IgE) or 1:400 (IgG), were added in duplicate into the wells of the plates and the plates were incubated at 37°C for 1 h. PBS (pH 7.4) containing 0.1% (w/v) casein and 0.5% (v/v) nonionic detergent, Nonidet P40, was used as diluent and for washes. After three washes, 100 µl of rabbit anti-human IgE (A0094, Dako) or peroxidase-conjugated rabbit anti-human IgG (P0214, Dako) were added at dilutions of 1:500 or 1:2,000 and incubated at 37°C for 1 h. Rabbit anti-human IgE was detected with biotinylated goat anti-rabbit IgGs (E0432, Dako; 100 µl, 1:1,000, 1 h at 37°C) followed by Vectastain Elite ABC reagent (PK-6100, Vector Laboratories, USA; 100 µl, 1:500). Color reactions were developed with 1% (w/v) tetramethylbenzidine and 0.05% urea hydrogen peroxide in 0.1 M sodium acetate buffer (100 µl, pH 6.0). The reaction was stopped after 15 min (2 M H₂SO₄), and optical density at 450 nm was measured.

Statistical differences in the Ig levels between the subject groups were tested with the Mann-Whitney U test.

In-Gel Alkylation and Digestion with Modified Trypsin for Sequencing Analyses

The purified *S. chartarum* protein extract was run on 10% SDS-PAGE and stained with Coomassie brilliant blue. The 48-kD protein band and a blank region of the gel (used as a negative control) were then excised from the gel. The gel bands were cut into small (1 × 1 mm) pieces and prewashed twice with 200 µl of 200 mM NH₄HCO₃-acetonitrile (ACN) (1:1) to remove the Coomassie stain. After washing, the gel pieces were shrunk by dehydration in acetonitrile, which was then removed, and the gel pieces were dried in a vacuum centrifuge for about 5 min. A volume of 20 mM dithiothreitol in 100 mM NH₄HCO₃ sufficient to cover the gel pieces was added, and the proteins were reduced for 30 min at 56°C. After incubation, the reducing reagent was removed and the gel pieces were shrunk by dehydration in acetonitrile. A volume of 55 mM iodoacetamide in 100 mM NH₄HCO₃ sufficient to cover the gel pieces was added, and the proteins were alkylated for 15 min at ambient temperature in the dark. After incubation, the alkylating reagent was removed and the gel pieces were washed once with 100 mM NH₄HCO₃ and shrunk by dehydration in acetonitrile, which was then removed. The washing and shrinking steps were repeated using milliQ water instead of NH₄HCO₃ prior to drying the gel pieces in a vacuum centrifuge for about 5 min. Finally, the gel pieces were swollen in 10 µl of digestion buffer containing 100 mM NH₄HCO₃-10% ACN with trypsin (0.05 mg/ml; Promega, USA). After 10 min of incubation, the buffer solution (100 mM Tris-HCl (pH 9.2)-10% ACN) was added to cover the gel pieces during enzymatic cleavage (37°C overnight). The buffer solution was recovered and the peptides further extracted twice with 150 µl of 0.1% trifluoroacetic acid (TFA)-60% ACN at 37°C for 30 min. The extracts were added to the first solution and the samples were concentrated using a vacuum centrifuge to a volume of about 50 µl, then 100 µl of 0.1% TFA was added and concentrated again to the final volume of about 50 µl.

Amino Acid Sequence Analysis

For sequence analysis by Edman chemistry, the tryptic peptides from the *S. chartarum* 48-kD protein band were separated by reversed-phase chromatography using a 0.3 × 150 mm PepMap (RP18, LC-Packings, The Netherlands) column. The peptides were eluted by a linear gradient of acetonitrile (0–40% in 100 min) in 0.07% formic acid using a flow of 2 µl/min. The elution was monitored at 214 nm and the peptides were collected manually into 10 µl of 25% acetonitrile in 0.07% formic acid. Before sequence analysis, the peptide size and purity was analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (Bruker Biflex, Bruker Daltonics, Bremen, Germany). Amino acid sequencing of selected peptides by Edman chemistry was performed using a Procise 494A sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, Calif., USA).

For de novo sequence analysis by LC-electrospray tandem mass spectrometry, the peptides were separated on a 0.075 × 150 mm PepMap (RP18, LC-Packings) column by using a linear gradient of acetonitrile (0–50% in 50 min) in 0.07% formic acid at a flow rate of 0.2 µl/min.

The liquid flow was directly sprayed into a quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK) and the fragment spectra were analyzed using the BioLynx sequencing program (Micromass). The samples were analyzed also with a LCQ ion trap mass spectrometer (Finnigan, San Jose, Calif., USA).

Fig. 1. Graph of 33- to 70-kD *S. chartarum* components detected by the sera from exposed (n = 23) and control (n = 23) subjects in the IgE immunoblotting analyses.

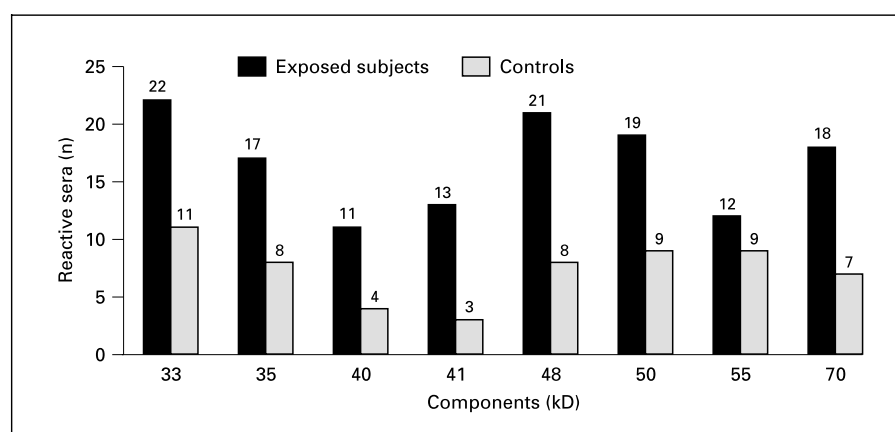


Table 1. *S. chartarum* IgG and IgE antibody levels among the exposed and control subjects

	Mean	Standard deviation	Median	Maximum	Minimum
IgG, OD (optical density)					
Exposed subjects (n = 23)	1.227	0.509	1.186	1.999	0.375
Control group (n = 23)	1.295	0.407	1.137	1.888	0.564
IgE, OD (optical density)					
Exposed subjects (n = 23)	1.107	0.327	1.161	1.615	0.253
Control group (n = 23)	0.973	0.371	0.899	1.655	0.287

Results

Human IgE Reactivity against S. chartarum and Other Fungi

In the IgE immunoblotting analyses, the sera of the exposed and control subjects detected eight components in the *S. chartarum* CE within the molecular weight range of 30–70 kD. The results are presented in figure 1. Over 90% of the exposed sera recognized the 33- and 48-kD components (the 48-kD component formerly estimated as 50 kD [18]), >80% of the sera detected the 50-kD component and >70% of the sera recognized the 35- and 70-kD components. In the control group, the most frequently detected component was about 33 kD (48% of the sera). In the ELISA analyses, no significant differences in the *S. chartarum*-specific IgE and IgG antibody levels were detected between the exposed and control groups ($p > 0.1$, table 1).

The components (within 30–70 kD) in the extracts of ten fungal species detected by the sera of five exposed and five control donors in the human IgE immunoblotting analyses are presented in table 2. The 35- and 55-kD com-

ponents were detected from eight fungal extracts, and the 48-kD component was detected from the extracts of six fungal species studied. All ten sera recognized the 35-, 50- and 70-kD components from *A. alternata* and the 48-kD component from *C. cladosporioides*. The 50-kD component of *M. echinata* and the 55-kD component of *A. alternata* were recognized by nine sera, and the 41-kD component of *U. botrytis* by eight sera. In the exposed group, the IgE reactivity to all *S. chartarum* components and to several *A. versicolor* (35-, 40- and 70-kD) and *M. echinata* (47- and 48-kD) components was distinctly more prevalent than in the control group (table 2).

Purification and Identification of S. chartarum Protein Components

After the purification of the *S. chartarum* CE by acetone precipitation and anion exchange chromatography, the number of the components in the protein extract was reduced to about one fourth compared to the CE, as shown in figure 2, and the 48-kD protein could be easily isolated from the 10% SDS-PAGE gel for the in-gel digestion analysis.

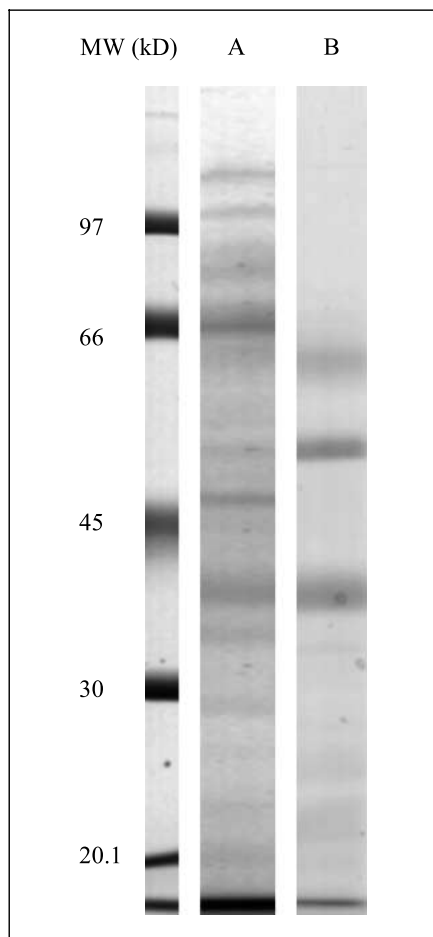


Fig. 2. SDS-PAGE analyses. Left lane: molecular weight (MW) standards (Amersham Pharmacia Biotech); lane A: crude *S. chartarum* extract; lane B: purified *S. chartarum* extract.

The partial amino acid sequence of the 48-kD protein component was analyzed from the in-gel digested samples by Edman degradation and MS/MS mass spectrometry amino acid sequencing methods. Altogether, 19 peptide sequences were identified, covering about one third of the total amino acid structure of the 48-kD *S. chartarum* protein component (table 3). The peptide sequences analyzed were homologous with various glycoside hydrolase enzymes (cellulases) produced by many fungal species. Only peptides number 12 and 13 showed no homology to glycoside hydrolase enzymes by homology comparison (NCBI/BLASTP). Since slightly different sequencing methods were used during the work, a few analyzed peptide sequences were very similar or nearly the same. However, at least 47% homology was found between analyzed peptides and the same amino acid regions in previously identified glycosyl hydrolases (fig. 3).

Discussion

Barnes et al. [17] reported the human IgE reactivity against the *S. chartarum* components of 34 and 52 kD. In this study, eight IgE-reactive components within 30–70 kD were detected in the *S. chartarum* CE. The components of about 33, 48 and 50 kD were most often recognized by both the exposed and control subjects. A higher number of exposed subjects produced IgE antibodies against those components compared to the control subjects (83–96% versus 35–48%). However, no differences in the IgE or IgG antibody levels were detected between

Table 2. The IgE reactivity of sera from five exposed subjects that showed an intense reaction to the 48-kD component of *S. chartarum* and of their matched control subjects against components of ten fungal species

Fungal species	Component, kD											
	31	33	35	40	41	47	48	50	55	60	65	70
<i>Stachybotrys chartarum</i>	1/0	5/2	5/2	4/0	5/0	0/0	5/1	5/1	5/0	0/0	0/0	3/0
<i>Ulocladium botrytis</i>	2/4	2/3	0/1	0/0	5/3	1/0	1/2	0/2	0/1	5/0	0/0	2/3
<i>Alternaria alternata</i>	2/1	1/2	5/5	0/3	0/0	0/0	0/0	5/5	4/5	0/0	4/1	5/5
<i>Memnoniella echinata</i>	0/0	0/0	3/0	0/0	0/0	5/0	5/1	4/5	4/2	4/3	0/0	0/0
<i>Cladosporium cladosporioides</i>	0/0	0/0	1/0	1/4	0/0	0/0	5/5	0/0	5/1	2/3	0/0	0/0
<i>Aspergillus versicolor</i>	0/0	0/0	4/0	4/0	0/0	0/0	0/0	3/4	2/0	0/0	0/0	5/0
<i>Penicillium brevicompactum</i>	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
<i>Phialophora rebens</i>	3/3	3/0	1/1	0/2	5/0	0/0	1/4	0/0	1/0	0/0	1/3	0/0
<i>Phoma</i>	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
<i>Trichoderma viride</i>	2/4	3/1	3/1	0/0	0/0	0/0	1/2	0/0	2/0	1/0	0/0	0/0
All reactions	10/12	14/8	23/9	9/9	15/3	6/0	18/15	22/17	23/9	12/6	5/4	15/8

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CLUSTAL W (1.82) multiple sequence alignment

1_4-BETA-D-GLUCAN  MQIKSYIQYLAAALPLLSSVAAQQAGTITAENHPRMTWKRCSGPGNCQTVQGEVVIDANW 60
EXOGLUCANASE      MQIKSYIQYLAAALPLLSSVAAQQAGTITAENHPRMTWKRCSGPGNCQTVQGEVVIDANW 60
CELLULASE         ----MFHKAVLVAFSLVTIVHGQQAGTQTAENHPQLSSQKCTAGGSCTSASTSVVLDSNW 56
PEPTIDES 1/18, 17 -----AGTNTAETHPR-----PGSDGEVVIDSNW

1_4-BETA-D-GLUCAN  RWLHNNG--QNCYEGNKWTS-QCSSATDCAQRCALDGANYQSTYGASTSGDSLTLKFVTK 117
EXOGLUCANASE      RWLHNNG--QNCYEGNKWTS-QCSSATDCAQRCALDGANYQSTYGASTSGDSLTLKFVTK 117
CELLULASE         RWVHTTSGYTNCYTGNTWDASICSDPVSCAQNCALDGADYAGTYGITTSGDALTLKFVT- 115
PEPTIDES          -----

1_4-BETA-D-GLUCAN  HEYGTNIGSRFYLMANQNKYQMFTLMNNEFAFDVDLSKVECGINSALYFVAMEEDGGMAS 177
EXOGLUCANASE      HEYGTNIGSRFYLMANQNKYQMFTLMNNEFAFDVDLSKVECGINSALYFVAMEEDGGMAS 177
CELLULASE         ---GSNVGSRVYLMEDETNYQMFKLMNQEFTFDVSNLPCGLNGAVYFVQMDQDGGTSK 172
PEPTIDES 10, 6    -----GFNNSVVQSE-----AK

1_4-BETA-D-GLUCAN  YPS-RAGAKYGTGYCDAQCARDLKFIGGKANIEGWRPSTNDPNAGVGPMGACCAEIDVWE 236
EXOGLUCANASE      YPSNRAGAKYGTGYCDAQCARDLKFIGGKANIEGWRPSTNDPNAGVGPMGACCAEIDVWE 237
CELLULASE         FPNKAGAKFGTGYCDSQCPQDIKFINGEANIVDWTASAGDANS GTGSFGTCCQEMDIWE 232
PEPTIDES 6,15,4,19 YPTNEAGARFGTGYCDAQ-----FWGGQANR---EPSDNDPNAGVGPR-----
PEPTIDES 5,3      YPTNEAGVK-----DGMEPSDNXPNA-----

1_4-BETA-D-GLUCAN  SNAYAYAFTPHACGSKNRYHICETNNCGGTYSDDRFAGYCDANGCDYNPYRMGNKDFYGK 296
EXOGLUCANASE      SNAYAYAFTPHACGSKNRYHICETNNCGGTYSDDRFAGYCDANGCDYNPYRMGNKDFYGK 297
CELLULASE         ANSISAAYTPHPCTVTEQTRCSGSDCGQGS---DRFNGICDPDGCDFNSFERMGNTEFYGK 289
PEPTIDES 7        -----MGNTGFYGK
PEPTIDES 8        -----MLNVYFYGK

1_4-BETA-D-GLUCAN  GKTVDTNRKFTVVSFRERN-----RLSQFFVQDGRKIEVPPPTWPGLPNSADITPE 347
EXOGLUCANASE      GKTVDTNRKFTVVSFRERN-----RLSQFFVQDGRKIEVPPPTWPGLPNSADITPE 348
CELLULASE         GLTVDTSQKFITVTQFISDDGTADGNLAEIRRFVYQNGKVIPNSVQITGIDPVNSITED 349
PEPTIDES 9        -----FTVVSQFAQNR-----
PEPTIDES 11       -----TVVSQFASVGR-----

1_4-BETA-D-GLUCAN  LCDAQFRVFDDRNRF AETGGFDALNEALTIPMVLVMSIWDDHHSNMLWLDSSYPPEKAG- 406
EXOGLUCANASE      LCDAQFRVFDDRNRF AETGGFDALNEALTIPMVLVMSIWDDHHSNMLWLDSSYPPEKAG- 407
CELLULASE         FCTQQKT VFGDTNNFAAKGGLKQMG EAVKNGMVLALS LWD DYA QMLWLDSDYPTTADPS 409
PEPTIDES          -----

1_4-BETA-D-GLUCAN  LPGGDRGPCPTTSGVPAEVEAQYPNA-VVWSNIRFGPIGSTVNV----- 449
EXOGLUCANASE      LPGGDRGPCPTTSGVPAEVEAQYPDAQV VWSNIRFGPIGSTVNV----- 451
CELLULASE         QPGVARGTCPTTSGVPSQVEGQEGSSSVIYSNIKFGDLNSTFTGTLTNPSSPAGPPVTSS 469
PEPTIDES 16       -----FGPIGSTVR-----

1_4-BETA-D-GLUCAN  -----
EXOGLUCANASE      -----
CELLULASE         PSEPSQSTQPSQPAQPTQPAGTAAQWAQCGMGFTGPTVCASPFTCHVLNPPYYSQCY 526
PEPTIDES          -----

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Fig. 3. CLUSTAL W (1.82) multiple sequence alignment between sequenced peptides of the 48-kD *S. chartarum* component and the three best scored glycosyl hydrolases (*Humicola grisea* 1,4-beta-D-glucan cellobiohydrolase; *Humicola grisea* exoglucanase; *Irpex lacteus* cellulase, Cel3).

Table 3. The amino acid sequence of 19 in-gel digested peptides from the *S. chartarum* 48-kD protein component

Peptide No.	Sequence	Number of amino acids
1	AGTNTAETHPR	11
2	LTWSAGT	7
3	DGMEPSDNXP	12
4	FWGGQANR	8
5	YPTNEAGVK	9
6	AKYPTNEAGAR	11
7	MGNTGFYVK	9
8	MLNVYFYVK	9
9	FTVVSQFAQNR	11
10	GFNNSVQSE	10
11	TVVSQFASVGR	11
12	VMEDVYL/IEQR	10
13	WSVTDWVAL/IK	10
14	STDWVVL/IDN	9
15	FGTGYCDAQ	9
16	FGPL/IGSTVR	9
17	PGSTDGEVVL/IDSNW	14
18	AGTNTAETHPR	11
19	EPSDNDPNAGVGPR	14

All peptides were sequenced in the Institute of Biotechnology, University of Helsinki, except peptide number 8, which was sequenced in the Department of Biochemistry, University of Kuopio, and peptide number 9, which was sequenced in the Department of Pharmaceutical Chemistry, University of Kuopio.

the groups in this study. Likewise, in the study of Barnes et al. [17], 49% of the healthy blood donors showed *S. chartarum* IgG reactivity. These findings suggest the possibility of fungal cross-reactivity, i.e. that several fungal species share similar or even the same antigenic components [20]. About 70 fungal allergens have been identified and approved by the international Allergen Nomenclature Committee [21]. Most allergens are hydrolytic enzymes (EC group 3), such as proteases or enolases, enzyme inhibitors, regulatory proteins or proteins involved in transport identified in many fungal species [22]. The slight discrepancy between the results of the immunoblotting and ELISA analyses can be explained by the fact that the serum antibody reactivity against the total antigen extract was measured in the ELISA, while in the immunoblotting analyses, the reactivity against each protein component was detected separately.

In this study, the human sera recognized two components (35 and 55 kD) in the CE from eight of the ten fungal species studied in the IgE immunoblotting analyses.

Three components of *A. alternata* (35, 50 and 70 kD) and one component of *C. cladosporioides* (48 kD) were detected by all ten sera. This was logical because *A. alternata* and *C. cladosporioides* are common outdoor air fungi. In the literature, several 34-kD components of *Aspergillus* and *Penicillium* are described as serine proteases [23]; 53-kD components of *A. alternata* and *Cladosporium herbarum* are described as aldehydedehydrogenases [24]; and a 48-kD component of *C. herbarum* is described as an enolase [24]. On the other hand, the sera of the subjects who were known to be exposed to *S. chartarum*, *A. versicolor*, *Penicillium* sp. and *T. viride* in their work environment showed more prevalent reactions to several *S. chartarum*, *A. versicolor* and *M. echinata* components compared to the control group. Antigenic components of *M. echinata* have not been reported in the literature, but the morphology of the fungus closely resembles *S. chartarum* [25], and thus they may also have similar antigenic components.

In our previous study [18], the 50-kD component (now described as a 48-kD component in 10% acrylamide gel) was shown to be the most characteristic component of *S. chartarum*. In this study, the human serum IgE recognized the components of about 48 or 50 kD in the extracts from eight of the ten fungal species. Several allergens of *A. alternata*, *C. herbarum*, *Aspergillus fumigatus* and *Penicillium citrinum* have been identified as enolases with a molecular weight of about 47–48 kD [24, 26, 27]. Nevertheless, no signs of homology to enolases were seen in the amino acid structure of the sequenced peptides of the 48-kD component isolated from *S. chartarum* in this study. Instead, homology in the amino acid sequence between 17 out of 19 peptides of the 48-kD *S. chartarum* component and fully identified glycosyl hydrolases (also known as cellulases) was observed, suggesting that the 48-kD *S. chartarum* component is a glycosyl hydrolase, more accurately a cellobiohydrolase type of cellulose-degrading enzyme. On the other hand, it is also possible that the sequenced peptides consist of several cellulose-like or other components with a similar molecular weight. However, only two of the analyzed peptides showed no direct homology to fully identified cellulases, supporting the assumption that the 48-kD component is a cellulase.

S. chartarum is known to be a cellulolytic fungus [1–3]. Endo-1,4- β -glucanase (cellulase) production by *Stachybotrys elegans* has been reported by Tweddell et al. [28]. A few fungal cellulases have been identified as allergens, e.g. 1,4-(1,3;1,4)- β -D-glucan glucanhydrolase from *T. viride* and *Aspergillus niger* [22]. In the literature, several identified glycosyl hydrolases have a molecular weight between 45 and 55 kD, consisting of about 450–550 amino acid

moieties [29–35]. Glycoside hydrolases are a widespread group of enzymes that hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety/ies [36]. Cellulolytic enzymes have traditionally been classified as exoglucanases or cellobiohydrolases, endoglucanases and β -glucosidases. Classification of glycoside hydrolases into families is based on amino acid similarity [37].

There is very limited knowledge of identified amino acid sequences of proteins and/or enzymes produced by *S. chartarum*. Only a short N-terminal sequence of a chymotrypsin-like serine protease from *S. chartarum* was reported by Kordula et al. [38]. Furthermore, tentative

characterization of the hemolysin stachylysin has been undertaken by mass spectrometry [39]. Thus, to our knowledge, this is the first antigen identified from *S. chartarum*.

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