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Molecular cloning and immunological characterisation of potential allergens from the mould *Fusarium culmorum*

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Abstract

Background: High quality and stability are essential requirements of commercial allergen preparations. Recently we have demonstrated the very low stability of protein allergens in an extract of the ubiquitous mould *Fusarium culmorum*.

Objective: The present study was performed to identify, isolate and characterise allergens of *F. culmorum* as a basis for a stable allergenic reference material. In addition, the significance of IgE binding to carbohydrate structures in the natural allergen source was investigated.

Methods: Sera of 52 subjects with suspected mould allergy were used to determine the IgE binding capacity of a commercial *F. culmorum* extract and an in-house extract by immunoblotting and enzyme allerge sorbent test (EAST). Binding of IgE-antibodies to putative carbohydrate structures located on glycoproteins was verified by periodate treatment of blot strips prior to immunodetection. A complementary (c)DNA expression library of *F. culmorum* was prepared and screened for IgE-binding clones using sera from *F. culmorum*-sensitive individuals. Positive clones were isolated, and the open reading frames were subcloned into expression vectors to produce recombinant proteins in *E. coli*. The recombinant proteins were tested for their IgE reactivity by immunoblotting and EAST.

Results: Using the in-house extract for EAST and immunoblot experiments 44% (23/52) of the sera were found to contain *F. culmorum*-specific IgE antibodies. Compared to the in-house extract, nearly all IgE-reactivities in the range of 15–30 kD were lacking in the commercial preparation as examined by immunoblot analysis and only 10% (5/52) of the sera were found to contain *F. culmorum*-specific IgE by EAST. IgE binding to putative carbohydrate structures was observed in the high molecular weight range in approximately 50% (12/23) of the IgE-positive sera by both extracts. Three IgE binding clones were isolated from the cDNA-library. One clone (Fus c 1) is homologous to the highly conserved 60S acidic ribosomal protein P2 described as minor allergen in other moulds. The second (Fus c 2) shows high similarity (64%) to a respiratory allergen from the basidiomycete *Coprinus comatus* (Cop c 2). The third clone (Fus c 3) was not related to known proteins. With sera from 26 individuals sensitised to *F. culmorum* the IgE prevalence of recombinant proteins rFus c 1, rFus c 2 and rFus c 3 was found to be 35, 50, and 15%, respectively.

Conclusions: *F. culmorum* may represent an underestimated source of aeroallergens. In contrast to highly labile and poorly standardised *F. culmorum* extracts, the new recombinant allergens may serve as stable allergenic reference material. A combination of rFus c 1 and rFus c 2 is suitable to diagnose 81% of *F. culmorum*-sensitised subjects. IgE reactivity to putative carbohydrate structures is relatively frequent, and can not be detected by these allergens.

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Keywords: *Fusarium culmorum*; Mould allergy; cDNA-library; Recombinant allergens; Cross-reactivity; Carbohydrate structures

1. Introduction

Fungal spores and airborne fragments of mycelium as omnipresent atmospheric components indoors and outdoors

are recognised as important causes of respiratory allergies. The world-wide prevalence of fungal allergy among atopic subjects is estimated from 3 to 40% depending on the allergic population studied, the test system and extract used and mould species tested (Kersten and Hoek, 1980; Horner et al., 1995).

The medical history of the patient, skin prick tests and in vitro test systems for measuring allergen-specific IgE

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antibodies in the patients' serum are the basis of allergy diagnosis. Unfortunately, most commercial mould allergen extracts used for in vivo and in vitro diagnostics and specific immunotherapy (SIT) are neither well characterised nor standardised. Recently we have demonstrated the very low stability due to proteolytic activity of an allergen extract prepared in-house from *Fusarium culmorum* representing a potential cause of false negative diagnostic results (Hoff et al., 2002). An optimal panel of pure fungal allergens which is currently not available for all allergenic species would be an important tool for allergy research (Horner et al., 1995). In particular, the replacement of labile allergen extracts due to proteolytic activity derived from several mould species by carefully characterised recombinant allergens may lead to a significant improvement of both diagnosis and SIT of mould allergy. The clinical relevance of IgE-binding carbohydrate structures which have been found on many glycoprotein allergens from plants or hymenoptera venom (Vieths et al., 2002a,b; Ballmer-Weber et al., 2001; Fötisch and Vieths, 2001; Kimura et al., 2001; Okano et al., 2001; Di Felice et al., 2001; Kwaasi et al., 2002; Hamilton, 2002) is still unknown but should be considered also for mould allergens.

To date *Alternaria alternata*, *Aspergillus fumigatus* and *Cladosporium herbarum* are recognised as major causes of fungal allergies and many allergens of these species have been characterised at the molecular level (Achatz et al., 1995; Cramer, 1998, 2002; Mason et al., 1993; Zang et al., 1995).

Airborne spores of *Fusarium* spp. are also widespread and especially common in agricultural areas (Morrow et al., 1964; VonWahl and Kersten, 1991). Approximately 24% of atopic individuals in one study were skin test and RAST positive to *F. solani* extracts (O'Neil et al., 1986). Fourteen of 18 allergens of *F. solani* share epitopes with either *F. equiseti* or *F. moniliforme* as indicated by immunoblot inhibitions (Verma and Gangal, 1994). A 65 kDa glycoprotein of *F. solani* was described as allergen cross-reactive with fungi responsible for allergic manifestations (Verma et al., 1995). Furthermore, there is evidence that *F. culmorum* may be an important source of aeroallergens. Kersten and Hoek (1980) showed by provocation tests of 276 mould-sensitised patients that respiratory allergy to *F. culmorum* is approximately 50% less prevalent than *A. alternata* allergy, but more frequent than allergy to *C. herbarum*. The present study was performed to identify and characterise potential allergens from the mould *F. culmorum*, to obtain data on IgE binding to carbohydrate structures on natural mould allergens, and to produce recombinant allergens as stable reference material for allergy diagnosis and standardisation of allergen extracts.

2. Methods

2.1. Identification of *F. culmorum*-sensitised subjects

A number of 52 sera of subjects with suspected mould allergy as indicated by positive CAP/RAST (CAP/RAST

class ≥ 2) or positive SPT to *F. culmorum* were tested for specific IgE-antibodies directed against *F. culmorum* by enzyme allerge sorbent test (EAST) and Western blot analysis using an in-house *F. culmorum* extract (described further). All *F. culmorum*-positive sera were also tested for specific IgE to common moulds by the CAP-fluorescence enzyme immunoassay (FEIA) system (Pharmacia Diagnostics, Uppsala, Sweden).

Three sera containing *F. culmorum*-specific IgE antibodies were selected for screening a cDNA expression library (described further). All 52 sera and a serum of a non allergic control subject were used to determine the IgE reactivity of the recombinant allergens. *F. culmorum*-positive sera were used to determine the IgE reactivity of a commercial allergen extract of *F. culmorum*.

2.2. Allergen extracts

The allergen extracts derived from *F. culmorum* and *A. alternata* were prepared in-house by a low temperature "acetone powder" method (Vieths et al., 1994) using viable freeze-dried source material purchased from Allergon AB (Ängelholm, Sweden). The extracting solution was 15 ml sterile PBS per g acetone powder and the protein concentration of the extracts was adjusted to 1 mg/ml. An *F. culmorum* extract for research purposes (not intended for use in humans) was a kind gift from HAL Allergen Laboratorium B.V. (Haarlem, The Netherlands).

2.3. Immunoblot analysis

F. culmorum extract as well as purified proteins (see further) were subjected to SDS-PAGE under reducing conditions followed by transfer onto a 0.45 μ m nitro-cellulose membrane (Schleicher und Schüll, Dassel, Germany) by tank blotting (Hoefer Pharmacia Biotech Inc., San Francisco, CA, USA). Blots probed with patient's sera (1:7.5) were detected with alkaline phosphatase (AP)-conjugated monoclonal mouse anti-human IgE (1:1000; PharMingen, San Diego, CA, USA). The binding patterns were visualised using NBT/BCIP as substrate (Alkaline Phosphatase Conjugate Substrate Solution, Bio-Rad Laboratories, Hercules, CA, USA). All incubation steps were carried out at RT. The diluent for all reagents was 50 mM Tris-buffered saline, pH 7.4 (TBS), containing 0.05% (v/v) Tween 20 and 0.1% BSA (w/v). Prior to immunodetection, blots were blocked with TBS containing 0.3% (v/v) Tween 20 for 30 min. Washing solution was 50 mM TBS, 0.05% (v/v) Tween 20.

2.4. Immunoblot inhibition analysis

For blot inhibitions, sera were diluted 1:7.5 in incubation buffer and incubated for 15 min with 100 μ g protein of mould or skim milk extract prior to immunodetection. Incubation of the blot strips and immunodetection were performed as described above.

2.5. Enzyme allerge sorbent test and EAST inhibition experiments

Self-prepared solid phases were used together with a commercial EAST according to the instructions of the manufacturer (Allergopharma, Reinbek, Germany). Therefore, the antigens were coupled to cyanogen bromide-activated filter paper disks (Hycor, Kassel, Germany) at optimised concentrations of 10 µg protein from *F. culmorum* extract or 250 ng (rFus c 1) and 1 µg (rFus c 2) recombinant protein per disk as described (Vieths et al., 1995). The results were expressed as EAST classes using reference sera provided with the EAST kit. For EAST inhibition 50 µl of diluted (1:2) serum pool was incubated with 50 µl of various dilutions of inhibitor (various extracts: 500, 50, 5, 0.5, 0.05, and 0.005 µg protein/ml; recombinant proteins: 100, 10, 1, 0.1, 0.01, and 0.001 µg/ml diluted in incubation buffer). Inhibition was calculated as follows:

$$\text{inhibition(\%)} = \frac{\text{OD}_{\text{seven}} - \text{OD}_{\text{seven+inhibitor}}}{\text{OD}_{\text{seven}} - \text{OD}_{\text{unspecific binding}}} \times 100$$

(with OD = extinction of 405 nm).

2.6. Establishment of an unidirectional cDNA expression library

Total RNA was isolated from freeze-dried source material of *F. culmorum* (Allergon AB, Ängelholm, Sweden) according to a modified method of Glišin et al. (1974). Therefore, the mould powder was well suspended in 5.5 M guanidinium-isothiocyanate solution containing 25 mM sodium citrate, 0.5% *N*-lauroylsarcosine, and 0.2 M β-mercaptoethanol (pH 7.0) and incubated at 60 °C for 10 min. Cell debris was removed by centrifugation at 7600 × *g* for 10 min at RT. The supernatant was carefully layered on top of 5.7 M CsCl, 0.01 M EDTA (pH 7.5) and subjected to ultracentrifugation at 175,000 × *g* for 18 h at RT. The RNA pellets were washed three times with 70% ethanol, dissolved in water and precipitated with 3 M sodium acetate (pH 5.0) and 96% ethanol. After recentrifugation and washing the pellet was dissolved in a small volume of water.

A cDNA expression library was constructed in λSCREEN vector (Novagen, Madison, WI, USA) with poly(A)⁺RNA isolated from total RNA by the Oligotex mRNA Midi Kit from Qiagen (Hilden, Germany). First and second strand cDNA synthesis was performed using the OrientExpress Kit (Novagen, Madison, WI, USA) with Oligo(dT) primer and carried out in the presence of 5-methyl dCTP, which protects any internal *Eco*RI and *Hind*III restriction sites from digestion. *Eco*RI/*Hind*III adaptors were ligated to the cDNA molecules followed by digestion with *Eco*RI and *Hind*III and ligation into *Eco*RI/*Hind*III digested λSCREEN vector. A primary library of 4.52 × 10⁵ recombinants was amplified to generate a high titer phage stock (1 × 10⁸ pfu/ml). The *E. coli* strain ER1647 was used as host for amplification.

2.7. Screening of the cDNA library with human IgE antibodies

The cDNA expression library was screened with three sera from *F. culmorum*-sensitised subjects (A, G and R, Fig. 4). Serum from a non-atopic individual served as negative control. *E. coli* BL21(DE3)pLysE cells were transfected with the phage particles and plated on Luria-Bertani (LB) agar Petri plates. Each plate (90 mm diameter) was plated with approximately 2 × 10⁴ phage particles and incubated at 37 °C for 5–7 h. Hybond C nitro-cellulose round-filters (Amersham Biosciences Europe GmbH, Freiburg, Germany) were layered on the top agar, and incubation was continued for 4 h. The filters were lifted and washed for 3 × 10 min with Tris-buffered saline containing 0.1% Tween 20 (TBST). After saturation with TBST + 1% gelatine (typ A porcine skin, 300 Bloom, Sigma, St. Louis, MO, USA) at RT for 30 min, the filters were incubated at RT overnight with a 1:10 dilution of human serum pre-incubated with a lysate of *E. coli* in TBST. The IgE-binding clones were detected as described for immunoblot analysis above. Positive plaques were isolated, replated, and rescreened until pure clones were obtained.

2.8. Rapid PCR analysis of phage plaques and DNA sequencing

Clones were screened for insert size by direct PCR using vector-specific primers (SP6 promotor and T7 terminator primers). An agar plug containing a single plaque was transferred into 50 µl SM buffer (0.1 M NaCl, 0.01 M MgSO₄, 0.05 M Tris-HCl, 0.01% gelatine; pH 7.5) and incubated for 1 h at RT. An amount of 5 µl of the eluted phages were boiled at 99 °C for 5 min and used as template. PCR cycling was performed at 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 2 min, with a total of 35 cycles. The insert size was determined by agarose gel electrophoresis. DNA sequencing was performed with the PCR product as template and SP6/T7 primers using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and a 373-A DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were analysed using the Lasergene program package version 5 (DNASTAR, Madison, WI, USA). All amino acid sequence similarity searches were done with the NCBI standard protein-protein BLAST program (Altschul et al., 1990). For alignment of amino acid sequences the MultAlin Web server of the Institut National de la Recherche Agronomique (Toulouse, France) was used (Corpet, 1988). Secondary structure prediction analysis was performed by the predict protein server from the Columbia University Bioinformatics Center (New York, USA).

2.9. Rapid amplification of cDNA Ends (5'RACE)

5'RACE was performed using the GeneRacer Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturers

instructions to assure getting the complete 5'-regions of the transcripts. Therefore, poly(A)⁺RNA from *F. culmorum* was first treated with calf intestinal phosphatase to eliminate free 5'-phosphates from truncated and non-mRNA. Thereafter, the dephosphorylated RNA was treated with tobacco acid pyrophosphatase to remove the 5'-cap structure from intact full-length mRNA which then provides a phosphate for GeneRacer RNA-Oligonucleotide ligation using T4 RNA ligase. Reverse transcription reaction with gene-specific primers Fusc1-GSP1 (5'-CTCGTCAGCGTCAAT-3'), Fusc2-GSP1 (5'-CAGTGATACCATATT-3') and Fusc3-GSP1 (5'-CAGC-ATCAGAAACA-3') was performed using SuperScript II reverse transcriptase (all listed primers were purchased from SIGMA-ARK, Darmstadt, Germany). The amplification of the 5'-ends was performed using reverse gene-specific primers Fusc1-GSP2 (5'-CCGACTGAGGTAAGGACGGC-CTTGA-3'), Fusc2-GSP2 (5'-GCGTCCTGGACATGGTC-CACGTTGACTT-3'), and Fusc3-GSP2 (5'-GCTCGTTTAT-CTCAGCCCAAGGATT-3') and the GeneRacer 5'-Primer provided with the kit. The PCR conditions were: hot start (94 °C, 2 min); 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min; 35 cycles. After agarose gel electrophoresis, the products were excised from the gel, purified by the QIAEX II gel extraction kit (Qiagen, Hilden, Germany), and subjected to TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA, USA). Sequencing was executed as mentioned above using T3 and T7 sequencing primers.

2.10. Allergen expression and purification

To amplify the complete open reading frames from IgE-binding clones forward primers (Fusc1-NdeI: 5'-AAT-ATTATCATATGAAGCACCTCGC-3'; Fusc2-NdeI: 5'-AT-TATCATATGGTTTACCACATTAC-3'; Fusc3-NcoI: 5'-A-ATTTCCATGGCAGGGGATCACGCTG) and reverse primers (Fusc1-BamHI: 5'-ATATAGGATCCTTTTTTTTTT-TTTTTTTT-3'; Fusc2-BamHI: same as Fusc1-BamHI; Fusc3-BamHI: 5'-TAATTGGATCCTCAAGTTCCCAG-CTGG-3') were designed on the basis of the sequences received by 5'-RACE and PCR was executed (94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min; 25 cycles) using *F. culmorum*-cDNA which was obtained by using the cDNA-Synthesis kit (Amersham-Pharmacia, Freiburg, Germany) as template. After amplification the PCR products were purified as mentioned above. Fus c 1- and Fus c 2-cDNAs were digested with NdeI/BamHI and subcloned into expression vector pET11a (Novagen, Madison, WI, USA) to produce non-fusion proteins. Fus c 3-cDNA was digested with NcoI/BamHI and subcloned into expression vector pIVEX2.4a (Roche, Mannheim, Germany) to produce a His-tag-fusion protein. Protein expression was carried out in BL21(DE3) competent cells (Novagen, Madison, WI, USA) by induction with 0.1 M IPTG at OD 0.8 for 5 h. *E. coli* cells were pelleted by gentle centrifugation at 5000 × g. The supernatant was decanted and the pellets resolved in a small volume of 0.01 M phosphate buffered saline (PBS).

The cells were cracked by freezing in liquid nitrogen three times followed by centrifugation at 8000 × g for 20 min. Cleared lysates containing the expressed non-fusion proteins were separated by SDS-PAGE followed by negative reversible staining using 0.3 M copper(II)chloride according to the method of Lee et al. (1987). The desired protein bands were excised from the gels, destained with 0.25 M Tris/0.25 M EDTA, pH 7.4 and the proteins eluted using the centrilotor system (Millipore, Bedford, MA, USA) following the manufacturers instructions. The His-tagged rHis-Fus c 3 protein was purified through nickel nitrilotriacetic agarose (Ni-NTA) resin (Qiagen, Hilden, Germany) following the instructions of the manufacturer. Prior to N-terminal microsequencing (see further) the histidin-tag was removed from 50 µg of the purified molecule by protease cleavage using 2 µg factor Xa in incubation buffer (20 mM Tris-HCl, 100 mM NaCl, and 2 mM CaCl₂, pH 8.0) at RT over night according to the method of Nagai et al. (1985).

2.11. N-terminal amino acid sequencing

Microsequencing of rFus c 1, rFus c 2, and rFus c 3 was performed according to the method of Matsudaira (1987) by using a Procise 492 protein sequencer in pulsed liquid mode (Applied Biosystems, Foster City, CA, USA). All chemicals used were from Applied Biosystems.

2.12. Periodate oxidation

F. culmorum extract as well as purified allergen rFus c 1 were subjected to SDS-PAGE and blotted onto a nitro-cellulose membrane as described above. According to the method of Woodward et al. (1985) the blots were cut into strips followed by a washing step with 50 mM sodium acetate buffer (pH 4.5) after which control strips were incubated with the same buffer for 1 h. Experimental strips were exposed to varying concentrations of sodium periodate (0.1–20 mM) in the pH 4.5 buffer for 1 h in the dark at RT. Control and experimental strips were then rinsed with sodium acetate buffer and incubated with 50 mM sodium borohydride dissolved in TBS for 30 min at RT. After three washes with TBST, the strips were incubated in patients' serum over night at RT. Detection of bound IgE-antibodies was carried out as described for immunoblot analysis above.

3. Results

3.1. Library synthesis, screening, and sequence analysis of Fus c 1, Fus c 2 and Fus c 3

An unidirectional *F. culmorum*-cDNA library was constructed using the λSCREEN vector system that yielded a primary size of 4.52×10^5 plaque forming units (pfu) which were amplified up to 1×10^8 pfu. In the present

Table 1
Molecular features of *F. culmorum* allergens and identities to known allergens

Systematic name	Biological function	cDNA (bps)	aa	MW (kD)	Identity with known allergens	Accession no.
Fus c 1	Ribosomal protein P2	653	109	11	86% Alt a 6 (P42037, X78227); 78% Asp f 8 (Q9UUZ6); 78% Cla h 4 (P42039, X78223)	AAL79930
Fus c 2	Thioredoxin-like protein	700	121	13	36% Cop c 2 (Q9UW02)	AAL79931
Fus c 3	Not known	2601	450	49	No identity	AY167564

Fus c 1 and Fus c 2 are registered at the “List of Allergens” of the IUIS Allergen Nomenclature Subcommittee (<http://biobase.dk/pub/who-iuis/allergen.list>).

study, three single sera of *F. culmorum*-sensitised subjects (A, G and R, see Fig. 4) were used to selectively enrich IgE binding clones out of approximately 2×10^4 pfu/serum. After four rounds of panning all clones were reactive with the atopic sera but not with the control serum (data not shown). PCR analysis of randomly picked clones enriched by sera A, G and R yielded identical insert sizes of approximately 560, 660, and 2250 bp, respectively. Sequencing of the cDNA-inserts indicated that clones enriched by sera A and R coded for full-length proteins implying complete 3′ untranslated regions (UTRs) and polyA tails. The sequence of the cDNA-clone enriched by serum G was obviously incomplete at the 5′-region. By the technique of rapid amplification of 5′-cDNA ends (5′-RACE) and subsequent sequencing three full-length cDNAs with a total of 653 bp (further called Fus c 1), 700 bp (Fus c 2), and 2601 bp (Fus c 3) were obtained, respectively. In the case of Fus 1 and Fus c 2 only short 5′-UTRs were found to be accessorially present in the full-length transcripts whereas 351 bps were additionally found for the 5′-region of Fus c 3. The nucleotide and deduced amino acid sequences of the isolated clones are presented in Fig. 1A–C. The particular features of each clone and sequence identities with known allergens are summarised in Table 1. Similarity searches with the NCBI BLAST program showed that Fus c 1 is homologous to the 60s acidic ribosomal protein P2 which is highly conserved among several species. Fus c 2 shows high similarity to members of the thioredoxin family with the typical redox active site composed of two cysteines flanking two proline molecules (Fig. 1B). Fus c 3 has no significant similarity with any known protein but contains a conserved helix-loop-helix domain structure at the C-terminal end in combination with a nuclear localisation signal which addresses it as potential DNA-binding protein (Fig. 1C). No potential N-glycosylation sites were predicted for Fus c 1 and Fus c 2, but four possible N-glycosylation sites are located within the Fus c 3-sequence as shown in Fig. 1C.

3.2. Expression, purification and immunological characterisation of rFus c 1, rFus c 2 and rHisFus c 3

The coding sequences of Fus c 1, Fus c 2 and Fus c 3 were subcloned into pET11a expression vector and expressed as non-fusion proteins in *E. coli* strain BL21(DE3). The resulting proteins rFus c 1 and rFus c 2 were soluble

and purified from the bacterial lysate by electro-elution. N-terminal microsequencing of the first 11 amino acids by Edman degradation confirmed the identity of the purified proteins (data not shown). Because of the very low expression level of the non-fusion protein rFus c 3 the Fus c 3 sequence was expressed as His-tag-fusion protein (rHisFus c 3) and purified by affinity chromatography. Identity of the purified protein was confirmed by removing the histidin-tag using protease factor Xa and the first 10 amino acids of the cleaved protein were microsequenced as mentioned above (data not shown). Molecular weight and purity of the recombinant proteins were further analysed by SDS-PAGE under reducing conditions and subsequent silver-nitrate staining (Fig. 2). rFus c 1, rFus c 2 and rHisFus c 3 showed apparent molecular weights of about 15, 13, and 50 kD, respectively. Contaminations with other proteins were not observed.

The IgE-binding capacities of rFus c 1, rFus c 2, and rHisFus c 3 were analysed by EAST and immunoblotting with 52 sera from subjects with suspected mould allergy. Nine out of 52 sera (17%), 13 out of 52 sera (25%), and 4 out of 52 sera (8%) showed significant IgE binding to rFus c 1, rFus c 2, and rHisFus c 3 in the enzyme allergosorbent test, respectively (data not shown). Taken together 37% (19/52) of sera from patients with suspected mould allergy reacted with the three recombinant molecules. Using the Western blot technique it was possible to detect specific IgE antibodies directed against rFus c 1 in 11 out of 52 sera (21%) but none of the sera detected rFus c 2 in this assay which was obviously due to misfolding of the protein on the membrane during the blotting procedure. Three sera (6%) reacted with rHisFus c 3 in this experiment (data not shown). The results of the positively tested sera are summarised in Table 2.

3.3. EAST inhibition experiments

To verify the presence of natural homologues of the two most relevant recombinant allergens rFus c 1 and rFus c 2 in *F. culmorum* extract dose-dependent EAST inhibition experiments were performed using rFus c 1 or rFus c 2 coupled to the solid phase and *F. culmorum* extract as inhibitor. Inhibition with the recombinant proteins was used as positive control and skim milk extract was applied as a negative control. EAST inhibitions were carried out with pooled sera with EAST classes ≥ 2 against the recombinant allergens (sera Q, R for rFus c 1 and sera R, T for

(A)

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1   GT CAT ACC GAA CAA CCT TCA CCA CCG AAC TCG AGG TTT CAG TCT CCG CCC AAC CTG CGG TTA AAG TGA TTG ATT TCT CTT GTT
84  CTC GTC GAC TCC AAA CAA ACC ACA ATC AAA M K H L A A Y L L L G L G G N T S P
1   1
168 TCT GCC GCC GAT GTC AAG GCC GTC CTT ACC TCA GTC GGT ATT GAC GCT GAC GAG GAC CGC CTG AAC AAG CTT ATC TCT GAG CTC
19  S A A D V K A V L T S V G I D A D E D R L N K L I S E L
252 GAG GGC AAG GAC ATC CAG CAG CTC ATC GCT GAG GGT TCC GAG AAG CTC GCT TCC GTT CCT TCT GGT GGT GCC GGT GGC GCT TCC
47  E G K D I Q Q L I A E G S E K L A S V P S G G A G G A S
336 GGT GGT GCC GCC GCT GGT GGT GCC GCT GAG GAG GCC AAG GAG GAG AAG GAG GAG AAG GAG TCC GAC GAG GAT
75  G G A A A A G G A A E E A K E E E K E E E K E E S D E D
420 ATG GGC TTC GGT CTT TTC GAC TAA GCG ATC TCT GTC CAG AGA TTC GCA AAG TAA CGA TAC CTC ACG AAT TTC GAA GAT GCT ACG
103 M G F G L F D -
504 CAC GGG TTG TTC ACC AGT TCT TAC ATG TCT TAT GCG TGG ATG GAT TGG CCG GTC ATG GCT GGA TTG GGT CTC TCT CAG GAT GGG
588 ATA ACA TAC GGT TAT GGA AAT TAA AAA AAA AGG CCG TTC GGC TCT TCG AAA AAA AAA AAA AAA AAA

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(B)

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1   ATC AAA AAA GCC ACA TCT AAA ATC AGA TCA ATA CCA CCC AAC ACA TTA CGC AAA ATG GTT CAC CAC ATT ACT TCA AAC GAC GAG
1   1
85  CTA CAG AAG CTC CTC TCA TCA ACA ACA TAC GTC GTC GTT GAC TTC TTC GCA GAC TGG TGT CCT CCC TGC AAG GCC ATT GCC CCC
11  L Q K L L S S T T Y V V V D F F A D W C P P C K A I A P
168 CAA CGC TAG ACA TCT AAC GCA AGA GCC GAA CGA ATA CTC CAG AGA CGA CCA GTG CCC CAG ATC CTA CTG CAG CCA CGG ATT CGA
39  V Y E Q L S T K H S V P D V L A F A K V N V D H V Q D A S
253 GCA CAA CAA TAT GGT ATC ACT GCC ATG CCC ATT TTG ATG TTC AAG GAA GGA AAG CAG GTT GCC GTC AAG GCC GAC GGC GTG
67  A Q Q Y G I T A M P T F M F F K E G K Q V A V N G Q A V
337 ATC AAG GGT GCT GAT CCC AGA ACG CTG GGA GCG GCA GCT GAG AAG CTG GGC GGT CTA GCA CAA AAG CGA GTT GCT GGC GCT TAA
95  I K G A D P R T G A A A A E K L G G L A Q K R V A G A -
421 GGA AAT GAG AGG TTT AAA AGT ACG GCC GGG AAT AGA AAG GAC AAA AAG AAA GAG AGT TCG GAG AAT TTA CTC CAG GGT TCA TTG
505 CAG CAT TTG TCA TGG CAC CTA AAT TAG AGG CAT TTT GTG TGC CTG CAT TTG GGA GTT AAG GTT AGA GTT TTC CAG AGG AAT GAT
589 GTT GCA CGT GGA GGA AAT GAA GGA TCA ACG GTA CTG GCT TAG ATG ACC GGG ATC GAG AAC AAG CTA CGG GAT ATA AGA CAT GAC
673 TGT TTG CCG AAA AAA AAA AAA AAA AAA A

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(C)

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1   GA AGC GCG GTT CCG GCC GTG GGC CCA GGG CGA GTG GCT CAG CAG AAG CTG TTT ATC AAG ACG TTC ACA GAT CGA AAC TCA GTC
84  GCT CGT GGT ATC CGA TAC CAT CCA AAT CCT CAT ACT CAA GCG ACA ATA AGT TGC TGC CAA AGT GGC ACC TCC CAT CGA TAC TCG
168 CAA CGC TAG ACA TCT AAC GCA AGA GCC GAA CGA ATA CTC CAG AGA CGA CCA GTG CCC CAG ATC CTA CTG CAG CCA CGG ATT CGA
252 ATC CTT ACT ATG GCA GGG GAT CAC GCT GGA GAC CAG TCA TTC TAT GAC TTT CTC ATA GAA GAG CCA GAG ATG ATA GCG CCA ACG
1   1
336 CCA CCC GGC CAA TTC CCG CAT CAA CAG CCC ATT TCA TCG CCG AAT CGT ACC AGT CGC AAC ACG CCG CTG CCG CCA GAA TCG ACC
26  P P G Q F P H Q Q P I S S P N R T S R N T P L R P E S T
420 GAA ATC GAG ACC CAC CAT GCG AAC CCG CCA GCT TTG CCG GTA CTC GGA ATG CAA CTA CCG GTT CCA GGG ACG GTA CCC
54  E I E T H H A N H P P A L P V L G M Q L P V P G T V P
504 GAG TCA TCA CGT GCT CAG TCA CGA GCT TCC CTC AAC CTC GAC ATC GAC CTC GAC TTA CAC GCG CAC TCT CAC CCA TCA CAT CTC
81  E S S R A Q Q S R A S L N L D I D L D L H A P S H P S H L
588 TCG CAT GGA GCT CCC CAT GAG CAA GAG CAC GCC CAC GAA ATT CAG AGG CAC AGA GCT CAT TCA CAG AGG TCA CGC GGT TTG
110 S H G A P H E Q E H A H E I Q R H R A H S A Q S S A G L
672 CCA CCC ACT GGG TTT GCA AGT CAT CTC CCT CCT GCA TCA TCA GGT CCC GTG TCG TTG GGT TGG AAC ATG TAC CAT GTC CCT CCA
178 P P T G F A S H L P P A S S G P V S L G W N M Y H V P P
756 AAT CTT CAG CTA AAC GCA AAT CAG TTT AAT TTC GAG GTT CCA GGA CAT ATG AAT GTA TCT GGG CAC CCA ACC CAT CTG GAA CAT
166 N L H L A N A N Q F N F E V P G H M N V S G H P T H L E H
840 TCA TCC ACA AAT CCA AAC TCG TTC CAC TAC GAA CAC AAC AAT GTT TCA CCG TCA AGC ATA CAT CCA AGC ACT GCC CAC TTT GAC
194 S T N P N S T F H Y E H N I V S P S S I H P S A H F D
924 GGA GAA GTT CCA AGC CAA TGG GAT GAC TCC CTA GGT CAT GGC GCG AGC ACT CCA AAA GTG CGG ACT CCC TCG CAT CAT GTC TCG
222 G E V P S Q W D D S L G H G A S T P K V R T P S H H V S
1008 AGC AAT CTT TGG GCT GAG ATA AAC GAG CCC ACA GGC GCA GAC AAC GAC AAC TTG GCC GTT ACC CGG CCA GAA AGC CCC GAT
250 S N P W A E I N E P T G G D N D N L A P V T R P R K P A
1092 CGA GCA AGA CCG CAA AAG AAA GAG CCT CGC AAG CTC TCA GAT GCT TCT CAG GGA GCA AGG AGC AGC AGT ACA GGT GGC ACC GCT
278 R A R R Q K K E P R K L S D A S Q G A R S S S T G G T A
1176 CAT TCT GTT TCT GAT GCT GCA AGT CCA AGT TCT ACA TCC CAG CAG AGC GGT GCC AGT CTC ACC TCC AAA TCT GCC TCA ATG ACG
306 H S V S D A A S P S S T S H Q S R A S L T S K S A S M T
1260 TCC GCA GCC TCG ACA GCT TCG AGC CGA AAA AGC AAG CTC CGA AGC GCC TCT AGA ACT TCC AAG AAT ACT CTA GAT AAA CCA AAC
334 S A A S T A S S S R K S K L R S A S R T S K N T L D K F N
1344 GAC ACA GCC GAA GAC CGT CGA ACC CGA GCA TCG CAT AAT CTT GTT GAA AAA CAA TAT CGA AAC CGA CTC AAC GCC CAA TTT GAA
362 D T A E D R R T R A S H N L V E K Q Y R N R L N A Q F E
1428 TCG CTC CTA CAT GCA CTA CCC GAA CAA ATA CGA CAC GGT GAT AAT GGA GGT GGC AAT GGG AAC GTG GAC AAC GAA TCC CAA CAG
390 S L L H A L P E Q I R H G D N G G G N G N V D N E S E Q
1512 GCG AAC GAT CTT GAC CGA CGA GTC AGT AAG GGC GAG GTG CTT GAA ATG GCT CGC AGG CAT ATC GAA GCA CTA GAG CGT GAA CGA
418 A N D L D R R V S K G E V L E M A R R H I E A L E R E R
1596 AAC CAG CTG GGA ACT TGA GAA CCT TGA GCT CCA CGG AAA TAT ACG AAA CTC AAA GGA TCG GGG TCC GAA AAT ATG AGC AGG TCT
446 N Q L G T -
1680 GGT CAG GAG ATT TCT GTC GAG TCC GAC ATT ATC AAG AAA GAG GCG AAG ACA GGC GAG GAA GAT GAA GGC GAA AAT CCA TAA CTC
1764 AGA TCG GCA TTT TCG ACA AGA AAG AGT TGG ATG CAG AAT AAG TTT CAG TCA AAC GGA CTG CTC GTG GCA ATA TGC TCT CCA ATG
1848 TGG GCT TCC GTT CTT CCC AAG AGT CAT ATT TTG CGT CCA GCT TGT TCC CTT TCA TTG CAT TGT TCC CAT TCC CAT TTG ATA
1932 CAA TCC CTT TTT CAA ACC TCG GCT TCA TCA TGA CGA CAG ATC AAC CAT CAA TCG CAC CCT ATT CAT ATA TCA TCC GCA GCC GAC
2016 CCA TTA ACT TGT CCA TCA TAT TCT AGC TCG AGG CTT TTG ATG TTC ATG ACC CAG AAA ATA TTG GGA CTT TGG CTC ACT CTC
2100 GAT ATG CTT GCT TGT CTC CCT GTC TTT CTT CTT CCT TAT ATA CTC TTC TAT ATT TAT ACT TCG ACA TCC TGT TTT ATA CTT CCT
2184 CTC TTC ACG ACC TCC TGC CCG TAC CTT CCT TCC CTT CAC ATC AAC TCA CAC TAT TCA CAA GCC CTG CCA AAA GAA AAG AAC ATA
2268 GAG TTT TGG GTC GAG ACA TAC TGC ATT GAT GCC CTG TTG TAC TTT ATA TCA ACA CGT CTC TTT CTA CCT TAG TTT GCT
2352 TCA TTG TTT CAT GAT GAG CCC CTT CAC ATT CCA CAG ATG GGA ATC GCG GTT TAG GAT TTA GGA TTT TAC AAT GCA CAA TGG AGA
2436 CAC AAT GAG GGG ATA TGA AAT GGT TTA AAG CAG AGA GAC AAT AGA TGG TGG ACT TCA ACA TGG ACT AAT GGT ATT ATC TTT
2520 GGA AGG GTT GCC TTA TTG GTG GGC ATA TGA ATA TAA TAG ACA TTT GAT TGC GGT TTG AAA AAA AAA AAA AAA AAA AAA A

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Fig. 1. Full-length cDNA with deduced amino acid sequences of recombinant *F. culmorum* allergens. (A) rFus c 1; (B) rFus c 2, redox active site at position 30–33 (bold and shaded); (C) rFus c 3, helix-loop-helix motif ranging from the residues 370–442 (shaded in grey) and a nuclear localization signal ranging from residue 274–289 (underlined) are located within the molecule. Four potential N-glycosylation sites are marked in bold letters and underlined.

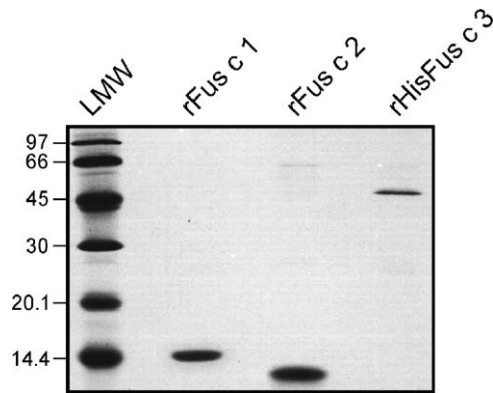


Fig. 2. SDS-PAGE and subsequent silver staining of the recombinant *F. culmorum* allergens rFus c 1, rFus c 2, and rHisFus c 3. An amount of 200 ng of each protein were loaded per lane.

rFus c 2). The results are presented in Fig. 3A and B. A dose-dependent decrease of IgE reactivity to rFus c 1 up to 67% occurred with increasing amounts of *F. culmorum* extract as inhibitor, whereas no inhibition was caused by skim milk extract (not shown). Self-inhibition with rFus c 1 was strongly dose-dependent and reached 98% inhibition at a concentration of 100 µg/ml. For rFus c 2 we found dose related self-inhibition up to nearly 100% at a concentration of 10 µg/ml. A dose-dependent decrease of IgE reactivity to rFus c 2 up to 99% occurred with increasing amounts of *F. culmorum* extract as inhibitor. Again, there was no inhibition caused by skim milk extract (data not shown). The evaluation of the effective dose 50 (ED₅₀) values showed that an approximately 250-fold amount of *F. culmorum* extract was needed to obtain the same inhibition as with rFus c 1 itself. In the case of rFus c 2 ca. the 30-fold amount of *F. culmorum* extract gave the same inhibition as with rFus c 2 as inhibitor.

3.4. Sensitisation to *F. culmorum*: comparison of two allergen extracts

Fifty-two sera of subjects with suspected mould allergy were analysed for specific IgE-antibodies directed against *F. culmorum* by EAST and immunoblotting using our in-house extract. Twenty-three subjects (44%) were found to be sensitised to *F. culmorum* (Table 2 and Fig. 4A, sera A–Z except of V, W, X which exclusively reacted with rFus c 2). In 18 sera, specific IgE antibodies were detected by both EAST and immunoblot analysis whereas five sera showed merely weak IgE-reactivity in the immunoblot. Approximately 50% (12/23) of the positive sera (e.g. serum E and S) gave strong signals at the high molecular weight level (30–80 kD) whereas sera such as A, C, and R exclusively reacted with distinct protein bands (around 15–30 kD).

Performing the same experiments using the commercial *F. culmorum* extract we found only five sera (10%) to be reactive by EAST (Table 2). All reactivities of this extract

observed by immunodetection were exclusively at the high molecular weight level except of one distinct band around 27 kD detected by serum U (Fig. 4B).

In conclusion we found 26 out of 52 mould sensitised subjects (including the sera V, W, X) sensitised to *F. culmorum* (50% prevalence) when we used our in-house extract in combination with our recombinant allergens (Table 2). In comparison we found only 10% of mould-sensitised subjects to contain *F. culmorum*-specific IgE-antibodies by EAST by using the commercial extract.

In addition, all *F. culmorum*-positive sera were tested for specific IgE to the common mould species *A. alternata*, *C. herbarum*, *A. fumigatus*, *Penicillium notatum*, and *F. moniliforme* using the CAP-FEIA System (Table 2). The following prevalences of specific IgE antibodies were determined: 96% *F. moniliforme*, 96% *P. notatum*, 96% *A. fumigatus*, 92% *A. alternata*, and 92% *C. herbarum*.

3.5. Verification of IgE-reactive carbohydrate epitopes of *F. culmorum* glycoproteins

The immunoblot data (Fig. 4) suggest that some of the strong IgE-reactivities detected at the higher molecular weights (e.g. sera G, K, S) may result from binding of specific IgE antibodies to carbohydrate structures as similar patterns were reported for IgE binding to glycans in plant and hymenoptera extracts (Vieths et al., 2002a,b; Ballmer-Weber et al., 2001; Fötisch and Vieths, 2001; Kimura et al., 2001; Okano et al., 2001; Di Felice et al., 2001; Kwaasi et al., 2002; Hamilton, 2002). To verify this assumption we used the technique of periodate oxidation. Following the blotting procedure and prior to standard immunodetection the *F. culmorum* blot was cut into strips. These strips were incubated with increasing concentrations of sodium periodate (0.1–20 mM) in order to destroy carbohydrate structures by oxidation. All strips and untreated controls were used for immunodetection with two patients' sera; serum G (Fig. 4) recognised multiple bands and thus suspected to react with carbohydrate structures, and serum A which recognised two distinct protein bands at 20 and 25 kD (Fig. 4A). To control whether the oxidation procedure affected IgE binding to protein epitopes the same experiment was performed with the recombinant allergen rFus c 1. The results are presented in Fig. 5. A strongly decreasing IgE reactivity to *F. culmorum* extract with increasing periodate concentration was observed with serum G. Detection with serum A showed only a very weak decrease of signal intensity at high periodate concentrations (5–20 mM). No reduced signal intensity was observed even at the highest periodate concentration in the control experiment with rFus c 1.

3.6. Cross-reactivity of *F. culmorum* with *A. alternata*

To examine whether *F. culmorum* shares IgE-binding epitopes with the well characterised allergens from *A. alternata*, immunoblot inhibition experiments were carried out

Table 2

Sensitization profile of 26 *F. culmorum*-sensitised subjects as measured by EAST (0–4, EAST classes); immunoblot analysis (–, negative; –/+, weak signal; +, strong signal; ++, very strong signal) and CAP–FEIA (0–6, CAP-classes; nt, not tested)

Serum	<i>F. culmorum</i> (in-house)		<i>F. culmorum</i> (commercial)		rFus c 1		rFus c 2		rFus c 3		<i>A. alternata</i>	<i>C. herbarum</i>	<i>A. fumigatus</i>	<i>P. notatum</i>	<i>F. moniliforme</i>
	EAST	Blot	EAST	Blot	EAST	Blot	EAST	Blot	EAST	Blot	CAP	CAP	CAP	CAP	CAP
A	1	+	0	–	2	+	0	–	0	–	4	3	3	3	4
B	0	–/+	0	–	0	–/+	0	–	0	–	3	3	1	1	2
C	1	+	0	–	3	++	0	–	0	–	6	5	5	4	5
D	1	+	0	–	1	+	0	–	0	–	2	5	4	4	3
E	3	++	2	+	0	–	1	–	3	+	3	2	3	4	3
F	3	++	2	+	0	–	1	–	3	+	3	2	3	4	3
G	3	++	2	+	0	–	1	–	3	+	3	2	3	4	3
H	2	+	0	–/+	0	+	2	–	0	–	3	3	3	3	2
I	1	–/+	0	–	2	+	0	–	0	–	3	3	2	3	3
J	0	+	0	–/+	0	–/+	0	–	0	–	4	3	2	3	3
K	0	–/+	0	–	0	–	3	–	0	–	2	2	2	1	3
L	3	++	1	–/+	0	–	0	–	0	–	1	1	2	3	3
M	2	–/+	0	–	1	–/+	3	–	0	–	3	3	3	3	4
N	2	+	0	–/+	0	–	0	–	0	–	2	3	3	3	3
O	1	–/+	0	–	0	–	1	–	0	–	3	4	4	5	3
P	1	+	0	–	0	–	0	–	0	–	0	0	1	3	2
Q	0	–/+	0	–	3	+	0	–	0	–	3	3	2	1	2
R	0	+	0	–	3	+	3	–	0	–	3	4	2	2	3
S	1	+	0	–	0	–	0	–	0	–	nt	nt	nt	nt	4
T	2	–/+	0	–	0	–	4	–	0	–	4	4	2	3	2
U	2	+	0	–/+	0	–	0	–	0	–	0	1	1	2	2
V	0	–	0	–	0	–	2	–	0	–	3	0	1	0	0
W	0	–	0	–	0	–	2	–	0	–	4	2	2	3	2
X	0	–	0	–	0	–	2	–	0	–	3	3	0	2	1
Y	1	–/+	0	–	1	–	0	–	0	–	4	3	3	3	2
Z	2	–/+	2	–/+	2	–/+	1	–	1	–	4	3	3	3	3
Σ	18/52 (35%)	23/52 (44%)	5/52 (10%)	9/52 (17%)	9/52 (17%)	11/52 (21%)	13/52 (25%)	0/52 (0%)	4/52 (8%)	3/52 (6%)	23/25 (92%)	23/25 (92%)	24/25 (96%)	24/25 (96%)	25/26 (96%)

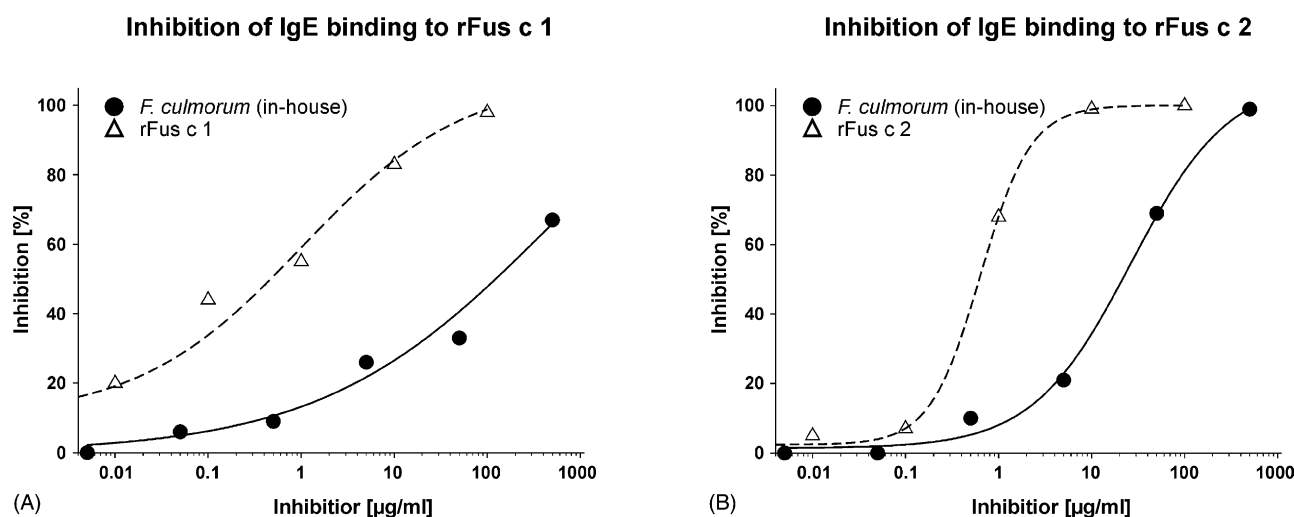


Fig. 3. EAST inhibition. (A) The patient's serum pool was pre-incubated for 1 h with increasing amounts of in-house *F. culmorum* extract or rFus c 1. After subsequent incubation with rFus c 1 immobilised on paper disks the residual IgE-binding capacity was measured. (B) Experiment was performed as described above using the same extract and rFus c 2 as inhibitors and rFus c 2 immobilised on paper disks.

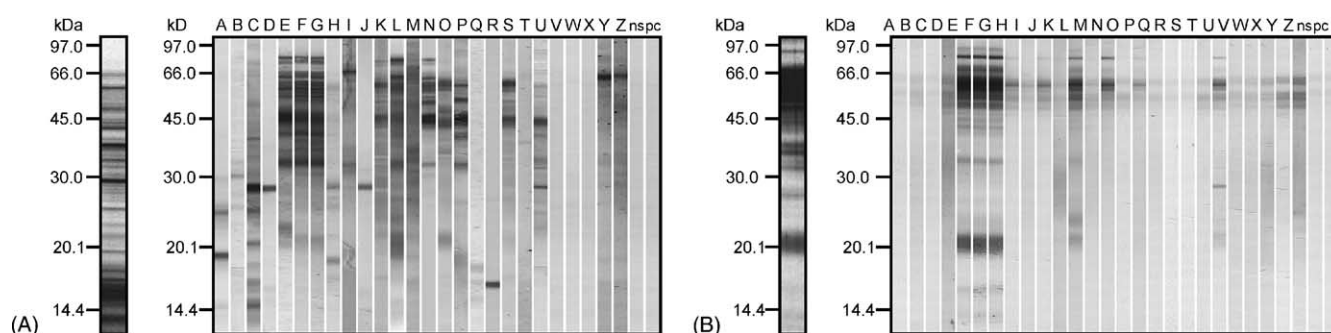


Fig. 4. IgE antibody reactivities of 26 sera from *F. culmorum*-sensitised subjects by immunoblot analysis of either an in-house (A) or commercial (B) *F. culmorum* extract. *F. culmorum* extracts separated by SDS-PAGE and stained with silver nitrate are presented beneath the blots.

using the same sera as in the periodate oxidation experiment. Pre-incubation of serum A with 100 µg protein of *A. alternata* prior to immunodetection led to a total disappearance of the two IgE reactive bands around 18 and 27 kDa, but there was still weak activity to the putative carbohydrate epitopes left when we used serum G for detection (Fig. 6). As expected total inhibition was observed using 100 µg protein of *F. culmorum* and no inhibition was found by the skim milk extract.

4. Discussion

The diagnosis and treatment of mould allergy is still hampered by the lack of standardised allergen extracts with high biologic activity. The lability of allergen extracts derived from moulds as shown for *A. fumigatus* (Hansen et al., 1994) and our *F. culmorum* extract (Hoff et al., 2002) as well as heterogeneity in allergen composition due to genetic variation (DeVouge et al., 1996), or different growth conditions

(Breitenbach et al., 2002) are main problems that generally affect studies of mould allergy. Attempts to produce a standardised *A. alternata* extract have been made (Helm et al., 1988; Aden et al., 1999), but problems with stability of the extract arose that further emphasise the difficulty of working with these crude biologic preparations. The comparison of in-house with industrially prepared *F. culmorum* extract is further proof that this problem is still unsolved.

Recombinant allergens are considered as an alternative to crude extracts. To date many mould proteins with specificity to human IgE antibodies have been molecularly cloned (Achatz et al., 1995; Cramer, 1998, 2002; Mason et al., 1993; Zang et al., 1995) especially from the species *A. alternata*, *C. herbarum*, and *A. fumigatus*.

In the present study, the evaluation of 52 sera from subjects with suspected mould allergy for IgE antibodies specific to the common mould *F. culmorum* indicated that 50% of the patients were sensitised to this species. This result is in accordance with the findings of Kersten and Hoek (1980) who described the high relevance of this species

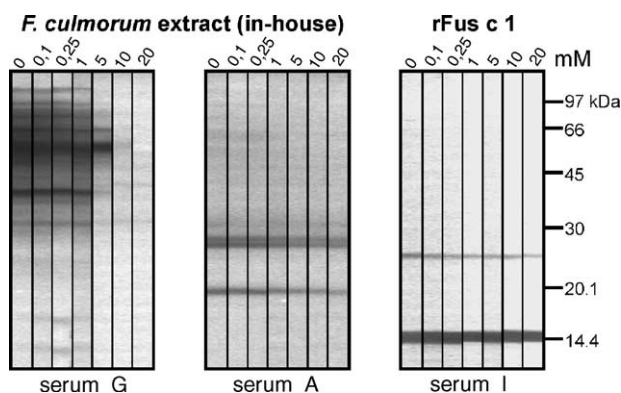


Fig. 5. Influence of peridate treatment on the IgE antibody reactivity to *F. culmorum* allergens of sera G and A. Blot-strips were treated with increasing amounts of peridate (0–20 mM) to destroy putative carbohydrate epitopes and the residual IgE-reactivity was measured.

as source of aeroallergens. As expected, all these subjects were also sensitised to other widespread moulds as indicated by immuno-CAP analysis (Table 2). As confirmed by our immunoblot inhibition experiment with *A. alternata* (Fig. 6) cross-reactivity is most likely the reason for this finding which contradicts the statement of Kersten and Hoek (1980) that *A. alternata* represents an allergen with no cross-reactivity to other moulds. From this experiment we have the indication that cross-reactivity is at least partially mediated due to similar epitopes on homologous protein allergens in *A. alternata* and *F. culmorum* because we found total inhibition of the corresponding reactivity by both *F. culmorum* and *A. alternata*. However, indirect information that *Fusarium* may directly sensitise a subgroup of patients results from the CAP data: two of the 26 *F. culmorum* sensitised patients (P and U, see Table 2) were IgE negative to *Alternaria*, and seven sera had significantly higher specific IgE levels against *Fusarium* than against *Alternaria*. This finding gives also a hint that a significant proportion of the IgE

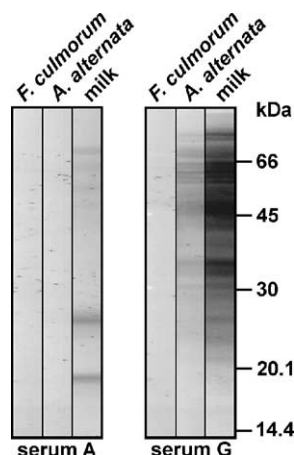


Fig. 6. Immunoblot inhibition of binding to *F. culmorum* extract protein of serum A and G IgE-antibodies using 100 μg of either *F. culmorum*- or *A. alternata*-extract as inhibitors. An amount of 100 μg of skim milk extract were used as negative control.

binding to *F. culmorum* is independent from cross-reactivity to *A. alternata*. Hence, we believe that *F. culmorum* may be a clinically important species. In addition, carbohydrate structures with unclear clinical relevance appear to play a major role in IgE cross-reactivity to other mould species (Fig. 6).

By screening the cDNA expression library with sera from atopic subjects, one IgE-binding clone, Fus c 1, encoding the ribosomal protein P2 and another, Fus c 2, encoding a thioredoxin-like protein were isolated. Both deduced amino acid sequences showed very high identity to sequences from proteins of well known allergen families (Table 1). The third protein Fus c 3 showed no identity to any known allergen. Due to the four potential *N*-glycosylation sites located within the molecule it might be very interesting to perform further studies, e.g. synthesis of rFus c 3 in an eucaryotic expression system in order to generate a glycosylated recombinant allergen. Further studies are required to clarify whether natural Fus c 3 is glycosylated and thus contributes to the carbohydrate-mediated IgE reactivity of *F. culmorum*.

The prevalence of IgE-reactivity to our recombinant allergens regarding the 26 *F. culmorum*-sensitised subjects is as follows: rFus c 1 (35%), rFus c 2 (50%), and rHisFus c 3 (15%). Using the recombinant allergens rFus c 1 and rFus c 2 in the EAST it was possible to identify 81% of *F. culmorum*-sensitised subjects. Beyond this no degradation of the recombinant proteins was observed during 6 month of storage at 4 °C (results not shown) indicating no stability problem compared to the crude extract. In addition, the EAST inhibition studies showed that there are actually natural homologues to rFus c 1 and rFus c 2 in our in-house *F. culmorum* extract although they were not detectable by immunoblot. Besides this, the recombinant Fus c 2 was also undetectable by the immunoblot but showed a strong IgE reactivity in the native EAST (Table 2). This finding indicates that unfolding of the recombinant protein caused by the denaturing conditions of the SDS-PAGE but not misfolding during expression in *E. coli* is the reason for this lack of IgE reactivity on nitro-cellulose membranes.

Hence, the advantage of screening a cDNA expression-library with sera of atopic subjects is to isolate and characterise allergens which may be unrepresented in a crude extract due to proteolytic activity. Our recombinant proteins could be very helpful to investigate the clinical relevance of allergy to *F. culmorum* in subsequent clinical studies for instance by using them for nasal or bronchial provocations. Our results further indicate that IgE-binding to carbohydrate epitopes frequently occurs in subjects sensitised to *F. culmorum*. Since the biological activity of anti-carbohydrate IgE is a matter of controversial discussion, further studies of this phenomenon are required.

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