

An allergenic polypeptide representing a variable region of *hsp* 70 cloned from a cDNA library of *Cladosporium herbarum*

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Summary

Background Extracts of *Cladosporium herbarum*, a major source of fungal aeroallergens, exhibit a complex profile of IgE-binding proteins. Yields of conventionally purified allergens from this mold have been insufficient to permit further molecular analyses.

Objective To enhance and simplify the purification of allergens from *C. herbarum*, we have sought to use recombinant DNA techniques to clone, identify and bacterially express immunoselected *C. herbarum* allergens.

Methods We constructed a cDNA library in λ ZAP II using mRNA isolated from *C. herbarum*. From this library, phage clones encoding a new allergen were immunoselected using pooled human atopic IgE. The cloned cDNA was excised from the phage vector as a recombinant pBluescript II SK-phagemid and sequenced. Expression of the recombinant allergen was carried out in *E. coli* XL1-blue transformants of the phagemid. Bacterial lysates from cells induced to express the cloned allergen were immunoblotted and probed with individual human atopic IgEs.

Results The cDNA clone encodes a 278 amino acid polypeptide homologous to the C-terminal portion of 70 kDa heat shock protein (*hsp* 70). The polypeptide possesses features common to other *hsps* 70, i.e. a similar hydropathic profile and a variable C-terminal region with conserved sequence at the very C-terminus. Binding of the recombinant peptide to IgE from 38% of atopic sera or plasma from individuals allergic to *C. herbarum* was demonstrated.

Conclusion These results indicate that amino acid substitutions are relatively conserved even in the variable C-terminal regions of *hsp* 70 species. Thus, this study should draw attention to the possibility of induction of anaphylactic responses in a sensitized individual when *hsp* 70 from any pathogenic species is administered for vaccination.

Keywords: *Cladosporium herbarum*, fungi, allergen, IgE, *hsp* 70, molecular cloning, cDNA sequence, immunoblotting

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Introduction

Cladosporium herbarum (*C. herbarum*) is the most abundant fungus found in our environment and a

major source of fungal inhalant allergens [1–3]. The allergenic composition of the extracts from this organism is complex and difficult to characterize [4,5]. Our recent studies demonstrated that there are at least 17 different allergens in an extract of a *C. herbarum* isolate, IMI 96220 [6]. Availability of adequate amounts of the pure allergens is essential to further understand their molecular structures, which is a pre-requisite for the development of more efficacious immunotherapy.

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However, our attempts to purify the allergens from the extract by conventional physicochemical methods have yielded insufficient amounts for further molecular analysis of these allergens.

Recently, recombinant DNA techniques have been employed in molecular characterization of allergens, which resulted in availability of sequence data and biological functions of a number of allergenic proteins or polypeptides from a variety of sources [7–13]. A few mould allergens have been cloned and characterized recently, including a dehydrogenase from *Candida albicans* [14], a cytotoxic mytogillin and a hsp 90 fragment from *Aspergillus fumigatus* [15,16], and a ribosomal P₂ protein from *C. herbarum* [7]. In this study, we report a new allergen cloned from a *C. herbarum* cDNA library. Sequence analysis led to a conclusion that this allergenic polypeptide is a C-terminal fragment of a protein belonging to the heat shock protein (hsp) 70 family.

Materials and methods

Establishment of a unidirectional cDNA library

An isolate, IMI 96220, of *C. herbarum* was cultured on liquid synthetic revised tobacco medium at 22°C [17]. The mycelia were harvested on days 7, 15, and 23. After three washes with sterile ice-cold phosphate buffer saline (PBS) the mycelia were dried and stored at –70°C. The mycelial mass was ground in a mortar and pestle under liquid nitrogen. The powder was suspended in 4.7 mol/L guanidine isothiocyanate containing 1% β -mercaptoethanol and homogenized with a Polytron homogenizer, followed by addition of N-lauroylsarcosine to a final concentration of 0.1%. The debris was removed by centrifugation at 10 000 rpm for 10 min. The supernatant was layered on top of 5.7 mol/L cesium chloride and subjected to ultracentrifugation at 25 000 rpm with an SW28 rotor for 24 h. The RNA pellets were extracted twice with phenol/chloroform and precipitated with sodium acetate and ethanol. After quantitation of the RNA, equal amounts of the three different time cultures were pooled.

A cDNA expression library was constructed in Lambda ZAP II vector (Stratagene, CA) with Poly A⁺ RNA isolated from the total RNA pool by oligo(dT) cellulose chromatography [18]. The first strand DNA molecules were synthesized using primer *Xho*I(dT)₁₅ and reverse transcriptase, followed by second strand cDNA synthesis using DNA polymerase and RNase H. Potential internal *Eco*RI sites in the cDNA molecules were methylated before ligation to *Eco*RI adaptors. Both the cDNA molecules and the vector were digested with *Eco*RI and *Xho*I. The cDNA was ligated into Lambda

ZAP II vector. The *E. coli* strain XL1-blue was used as host for amplification and screening of the library.

Screening of the cDNA library with human IgE antibodies

The cDNA library was screened using a human serum pool from six *C. herbarum* atopic individuals, as determined by skin test and direct RAST [6]. Sera from six non-atopic individuals were also pooled and used as a negative control. *E. coli* XL1-blue cells were transfected with the phage particles and plated on Luria-Bertani (LB) agar in Petri plates. Each plate (90 mm diameter) was plated with approximately 5×10^3 phage particles and incubated at 42°C for 4 hours. Nitrocellulose filters (Schleicher & Schuell, NH) presoaked with 10 mmol/L isopropyl- β -D-thiogalactopyranoside (IPTG, BRL, ON) were layered on the top agar and incubated at 37°C for 3.5 hours. The filters were lifted and washed for 3×5 minutes with Tris-buffered saline containing 0.5% Tween 20 (TBST). After saturation with 3% blocking reagent (Boehringer Mannheim, QC) at room temperature for three hours, the filters were incubated at room temperature overnight with a 1 in 5 dilution of the serum pool preabsorbed with an extract of *E. coli*. The IgE-bound plaques were detected using alkaline phosphatase conjugated goat anti-human IgE antibodies (Sigma) and substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

DNA sequencing

The positive clones were isolated, replated, and rescreened until pure clones were obtained. Overnight cultures of *E. coli* XL1-blue cells were co-infected by the cloned Lambda phage and helper phage *Exassist* (Stratagene, CA) to convert the phage into phagemid pBlue-script SK-. The double stranded plasmid DNA was used as a template for DNA sequencing. The plasmid was isolated from an overnight culture of the XL1-blue cells using the Magic Miniprep Kit (Promega, WI). Asymmetric PCR was performed to produce different lengths of single strand DNA using 5'-fluorescein-labelled M13 reverse and -40 primers, Tth DNA polymerase, and dNTP and ddNTP [19] provided in the Pharmacia Auto-Cycle Kit (Pharmacia, QC). PCR cycling was at 95°C for 36 seconds, 50°C for 36 seconds, and 72°C for 84 seconds, with a total of 25 cycles. A primer walking strategy was employed for continuation of sequencing from both ends of the cDNA insert. The primers, besides M13 reverse and -40, were designed according to the obtained cDNA sequence and synthesized using a Cyclone Plus DNA Synthesizer (Millipore, MA). Sequences were obtained and recorded using an automatic

ALF DNA sequencer (Pharmacia, QC). The cDNA sequence and deduced amino acid sequence were analyzed using DNAsis and Prosis programs (Hitachi, CA), respectively.

Allergen expression and reactivity with individual atopic IgE

The purified positive phage clone was used to co-infect *E. coli* SOLR cells with helper phage, *Exassist* (Stratagene, CA). The phage clones were converted into the phagemid by incubation at 37°C for 4 hours with vigorous shaking. The culture supernatant containing phagemid particles was used to infect overnight cultures of XL1-blue cells and the cells were cloned on ampicillin-containing LB agar. Single colonies were picked to inoculate LB-

ampicillin media and grown overnight at 37°C. Fresh LB media was inoculated with the overnight growth culture at a ratio of 1:10 (v/v) and incubated at 37°C for 1.5 hours. IPTG was added to induce synthesis of the fusion protein when the cell density reached 0.5 A₆₀₀ units, followed by further incubation at 37°C for 3 hours. The cells were harvested and lysed using SDS-PAGE sample buffer and boiling. The recombinant allergen was detected by SDS-PAGE and IgE immunoblot [20].

Human atopic IgE-containing sera or plasmas (PlasmaLab International, WA) from individuals allergic to *C. herbarum* (as determined by RAST > 10%) were assayed for reactivity with rCh3.1 protein in immunoblots. Sera were diluted 1:2 with TBST and pre-incubated with bacterial lysate to reduce background. Blots were incubated with sera at room temperature

1	G	GCT	GTT	GCC	TAC	GGT	GCT	GCC	GTC	CAG	GCC	GCT	ATC	CTC	TCT	GGT	GAC
1	A	V	A	Y	G	A	A	V	Q	A	A	I	L	S	G	D	
50	ACT	TCT	TCC	AAG	TCC	ACC	AAA	GAG	ATC	CTT	CTT	CTC	GAC	GTC	CGC	CCT	CTC
17	T	S	S	K	S	T	K	E	I	L	L	L	D	V	A	P	L
101	TCC	CTC	GGC	ATT	GAG	ACC	GCT	GGT	GTC	ATG	ACC	GCC	CTC	ATC	AAG	CGC	
34	S	L	G	I	E	T	A	G	G	V	M	T	A	L	I	K	R
152	AAC	ACC	ACC	ATC	CCC	AAG	AAG	TCC	GAG	ACC	TTC	TCC	ACC	TTC	TCC	GAC	
51	N	T	T	I	P	T	K	K	S	E	T	F	S	T	F	S	D
203	AAC	CAG	CCT	GGT	GTC	CTC	ATC	CAG	GTC	TTC	GAA	GGC	GAG	CGC	GCT	CGC	ACG
68	N	Q	P	G	V	L	I	Q	V	F	E	G	E	R	A	R	T
254	AAG	GAC	ATC	AAC	CTT	ATG	GGC	AAG	TTC	GAG	CTC	TCC	GGC	ATC	CGG	CCT	CGC
85	K	D	I	N	L	M	G	K	F	E	L	S	G	I	R	P	A
305	CCC	CGT	GGT	GTT	CCT	CAG	ATC	GAG	GTC	ACC	TTC	GAC	CTT	GAC	GCC	AAC	GGA
102	P	R	G	V	P	Q	I	E	V	T	F	D	L	D	A	N	G
356	ATC	ATG	AAC	GTC	TCC	GCC	CTT	GAG	AAG	GGT	ACC	GGC	AAG	ACC	AAC	AAG	ATT
119	I	M	N	V	S	A	L	E	K	G	T	G	K	T	N	K	I
407	GTC	ATC	ACC	AAC	GAC	AAG	GGC	CGC	CTT	TCC	AAG	GAG	GAG	ATC	GAG	CGC	ATG
136	V	I	T	N	D	K	G	R	L	S	K	E	E	I	E	R	M
458	CTT	GCT	GAC	GCC	GAG	AAG	TAC	AAG	GAG	GAG	GAT	GAG	GCC	GAG	GCT	GGC	CGC
153	L	A	D	A	E	K	Y	K	E	E	D	E	A	E	A	G	R
509	ATC	CAG	GCC	AAG	AAC	GGC	CTC	GAG	TCC	TAC	GCC	TAC	TCT	CTC	AAG	AAC	ACC
170	I	Q	A	K	N	G	L	E	S	Y	A	Y	S	L	K	N	T
560	GTC	TCC	GAC	CCC	AAG	GTT	GAG	GAG	AAG	CTG	TCC	GCT	GAG	GAC	AAG	GAG	ACC
187	V	S	D	P	K	V	E	E	K	L	S	A	E	D	K	E	T
611	CTC	ACT	GGC	GCC	ATC	GAC	AAG	ACT	GTC	GCC	TGG	ATC	GAC	GAG	AAC	CAG	ACC
204	L	T	G	A	I	D	K	T	V	A	W	I	D	E	N	Q	T
662	GCC	ACC	AAG	GAG	GAG	TAC	GAG	GCT	GAG	CAG	AAG	CAG	CTC	GAG	AGC	GTT	GCC
221	A	T	K	E	E	Y	E	A	E	Q	K	Q	L	E	S	V	A
713	AAC	CCC	GTC	ATG	ATG	AAG	ATC	TAC	GGC	GCT	GAG	GGT	GGT	GCT	CCC	GGT	GGC
238	N	P	V	M	M	K	I	Y	G	A	E	G	G	A	P	G	G
764	ATG	CCC	GGC	CAG	GGT	GCC	GGC	GCT	CCC	CCG	CCC	GGT	GCT	GGC	GAT	GAC	GGC
255	M	P	G	Q	G	A	G	A	P	P	P	G	A	G	D	D	G
815	CCC	ACC	GTC	GAG	GAG	GTC	GAC	TAA	GCG	TCT	CGC	CCC	TCT	ACG	ATA	CCC	AAC
272	P	T	V	E	E	V	D	*									
866	CTG	CAT	CGA	CAA	CAT	GGC	GTT	AAC	GAT	TAG	AAG	GTC	TTT	TCT	GCT	TTG	CTT
917	TGC	TTT	GGT	TTC	AAA	CTT	GAT	GAT	ACA	ACG	ACT	TGG	TGG	ACA	CAA	AAA	GCA
968	TCC	CCC	TGT	AAT	GTA	GCT	TGC	CTC	CTT	TTG	AGG	CTT	TTT	CCA	AAT	TAA	AAC
1019	TTT	CTG	TTT	AAC	TTT	GAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	A		1061

Fig. 1. DNA and deduced amino acid sequences of cDNA clone Ch3.1. Nucleotide sequence shown is the sequence of the *EcoRI* and *XhoI* cDNA insert. The deduced rCh3.1 amino acid sequence starts after the *EcoRI* site (not shown) and is in frame with the β -galactosidase ORF in the vector; the Ch3.1 sequence ends with the TAA stop codon denoted by asterisk.

overnight, washed with TBST for 3×5 min, and incubated with alkaline phosphatase-conjugated or ^{125}I -labeled goat anti-human IgE for 1 h at room temperature. After washing as before, blots were either exposed to X-ray film or incubated briefly with alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl_2) and stained with NBT and BCIP.

Results

Identification and isolation of a cDNA clone

The cDNA library was screened with the serum pool from the atopic individuals. One clone, designated

Ch3.1, was found to react with human IgE antibodies. The pure clone was obtained by three rounds of rescreening of the allergen-producing phages. The IgE reactivity of the clone was further examined with the serum pool from non-atopic individuals. The results indicated that the cloned phage plaques reacted with the atopic but not with the normal serum pool (unpublished data). Using the helper phage, the clone was converted into phagemid, which was purified for further analysis.

Nucleotide sequence of the cDNA clone

The DNA sequence of Ch3.1 is shown in Fig. 1. The cDNA insert is 1061 base pairs in length with a translated

HOMOLOGY ANALYSIS 70 kDa hsp

Ch3.1	AVAYGAAVQAAILSGDTSSKSTKEILLLDVAPLSLGIETAGGVMTALIKR
yeast	AVAYGAAVQAAILtGdESSK-Tqd1LLLDVAPLSLGIETAGGVMTkLIpR
human	AVAYGAAVQAAILmGDkSeK-vqdlLLLDVAPLSLGL1ETvGGVMTALIKR
maize	AVAYGAAVQAAILSGegnersS--dlLLLDVtPLSLGL1ETAGGVMTvLIpR
mouse	AVAYGAAVQAAILmGD-kSenvqdlLLLDVAPLSLGL1ETAGGVMTALIKR
bovine	AVAYGAAVQAAILSGD-kSenvqdlLLLDVtPLSLGIETAGGVMTvLIKR

Ch3.1	NTTIPTKKSETFSTFSDNQPGVLIQVFEGERARTKDINLMGKFELSGIRP
yeast	NsTIPTKKSEiFSTyaDNQPGVLIQVFEGERAKTKDnNL1GKFELSGIpP
human	NsTIPTKKqTqiFtTySDNQPGVLIQVFEGERAmTKDnNL1GrFdLtGIpP
maize	NTTIPTKKeqvFSTySDNQPGVLIQVFEGERARTKDnNL1GKFELSGIpP
mouse	NsTIPTKKqtTfTySDNQPGVLIQVFEGERAmTrDnNL1GrFELSGIpP
bovine	NTTIPTKKqtTfTySDNQPGVLIQVFEGERAmTKDnNL1GKFELtGIpP

Ch3.1	APRGVPQIEVTFDLdANGIMNVSALEKGTGKTNKIVITNDKGRLSKEEIE
yeast	APRGVPQIEVTFDvDsNGILNVSAveKGTGKsNKItITNDKGRLSKedIE
human	APRGVPQIEVTFDiDANGILNVtAtdKsTGKvNKItITNDKGRLSKEEIE
maize	APRGVPQItvTFDiDvNnILNVSAedKtTGqkNKItITNDKGRLSKEEIE
mouse	APRGVPQIEVTFDiDANGILNVtAtdKsTGKANKItITNDKGRLSKEEIE
bovine	APRGVPQIEVTFDiDANGILNVSAvdKsTGKeNKItITNDKGRLSKedIE

Ch3.1	RMLADAEKYKEEDEAEAGRIQAKNGLESYAYSLKNTVSDPKVEEKLSAED
yeast	KMvAeAEKfKEEDEkEsqRIAsKNqLESiAYSLKNTiS--eagdkLeqaD
human	RmvlDAEKYKaEDEvqrekIaAKNaLESYAfmKsvVSDeglkqKisEsD
maize	kMvqeAEKYKaEDEevkKkvdaKNaLEnYAYnmrNTikDdKiasKLpAED
mouse	RMvqeAErYKaEDEvqrdRvaAKNaLESYAfmKsaVeDeglkqKLSead
bovine	RMvqeAEKYKaEDEkqrkvssKNsLkSYAfmKatVeDeKlqgKindED
	* * * * *
Ch3.1	KETLTGAIDKTVAWIDENQTATKEEYEAQKQLESVANPVMKIY---GA
yeast	KdTvTkkaeeTisWlDsNtAsKEEfdkklKeLqdiANPiMsKlyqagGA
human	KnkildkcneLLsWlEvNqlAeKdEfdhkrKeLEqmcNPiitKIY-----
maize	KkkiedAvDgaisWlDsNqlAevEEfEdkmKeLEgicNPiiaKmY---xg
mouse	KkkvldkcqevisWlDsNtlAdKEEfVhkreeLErVcsP---iIs---Gl
bovine	KqkildkcneiInWlDkNQTAeKEEfEhQqKELEKvCNpiitKly---qs
	* * * * *
Ch3.1	EGGAPG-GMPGQ-----GAGAPPPGAGDDGPTVEEVD
yeast	pGGAaG-GaPGG-----fpGgaPPapeaeGPTVEEVD
human	qGGctG-pacGt-----G---yVpGrpatGPTiEEVD
maize	EGagmG-aaaGm-----dedAPsgGsG-agPkiEEVD
mouse	yqGAgapGagGf-----GAqAPPkGAsGsGPTiEEVD
bovine	aGGmPG-GMPGgmpgGfpGgGAPPsGgassGPTiEEVD
	* * * * *

Fig. 2. Homology analysis of rCh3.1 allergenic polypeptide with hsp70. Sequences of rCh3.1 and the C-terminal portions of other hsp70 are aligned and have gaps aligned to show maximum homology. The non-matched and perfectly matched amino acids are shown as lower case and capital letters respectively. The asterisks under the sequences indicate the perfect matches among all the sequences.

region of 839 base pairs, which is in phase with the open reading frame (ORF) of the β -galactosidase gene in λ -ZAP II vector. This clone has an ORF of 838 nucleotides and a complete 3' non-translated region with a poly(A) tail. The canonical polyadenylation motif AATTAA [20] is found 25 bases upstream of the poly(A). The polypeptide encoded by the gene is 278 amino acid residues in length. Possible gene expression enhancers were observed in the gene sequence, such as a CACA box at position of 78 bases upstream of the poly(A).

Homology analysis

Sequence homology analysis of the polypeptide with all known sequences in the Swiss-Pro data bank revealed that the polypeptide belongs to the heat shock protein 70 family. The amino acid sequence of this polypeptide was compared with the corresponding *hsp* 70 C-terminal fragments of different species. The sequence homologies of the polypeptide to that of yeast [21], human [22], maize [23], mouse [24], and bovine species [25] are 71%, 62%, 63%, 63%, and 67%, respectively (Fig. 2). It was observed from the sequence comparisons that most of the sequence divergence observed was confined to a 140 amino acid region at the C-terminus and that the amino terminal sequences of these *hsp* 70 are highly conserved. However, the very C-terminal eight amino acids, in particular the last four amino acids, are also highly conserved.

The hydrophatic profile of the cloned polypeptide was analysed using the Kyte and Doolittle method [26] with a window of six amino acids. The hydrophatic patterns of the *hsp* 70 fragments of the above species were compared with that of the polypeptide (Fig. 3). The N-terminal portions (1–120) of these fragments are predominantly amphipathic. The next 120 amino acids show a highly hydrophilic character in all *hsp* 70 species, indicating that amino acid substitutions are conserved in different *hsp*s 70, even though the sequence in this region of a given *hsp* 70 is most variable. The very C-termini of these molecules seem to be relatively neutral.

Expression of the recombinant allergen in *E. coli*

To further study the allergenicity of the recombinant *hsp*70, the recombinant polypeptide was synthesized in *E. coli* XL1-blue cells using the phagemid expression system. The bacterial lysate was analysed by SDS-PAGE and IgE immunoblot (Fig. 4). A recombinant protein was visualized by Coomassie Blue staining at 36 kDa in the lysate of the recombinant phagemid-transformed cells but not in the non-transformed cells (Fig. 4A). The recombinant protein accounted for about 20% of

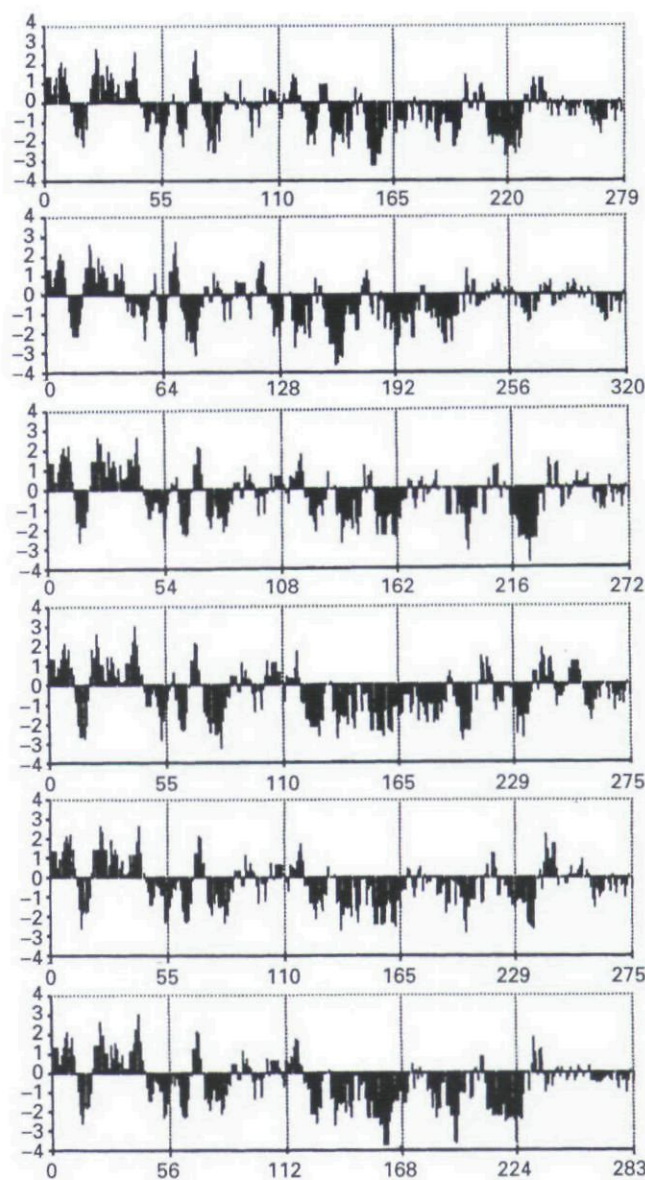


Fig. 3. Hydrophatic profiles of the compared sequences as presented in Fig. 2. The profiles of the amino acid sequences were obtained using the Kyte and Doolittle method with a window of six amino acids. The six profile blocks (from top to bottom) are of rCh3.1, yeast, human, maize, mouse, and bovine *hsp*s 70.

the total bacterial protein according to scanning densitometry of the gel. Immunoblot analysis demonstrated that the recombinant protein was recognized by the human IgE antibodies, whereas the control bacterial proteins showed no reactivity to the IgE antibodies (Fig. 4B). This recombinant allergenic polypeptide is termed rCh3.1 allergen.

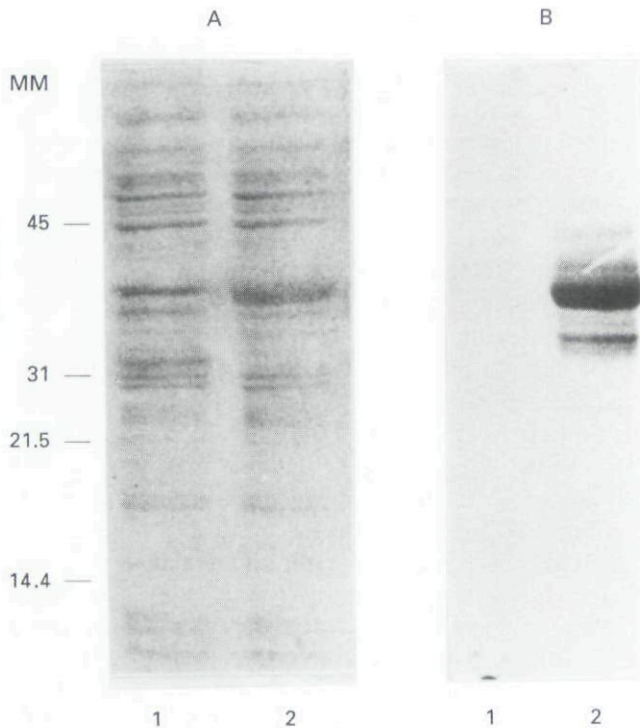


Fig. 4. Expression of recombinant allergen. The fusion protein was expressed in *E. coli* and analysed by SDS-PAGE and immunoblot. Lane 1, non-transformed cells; lane 2, the vector containing cDNA insert-transformed cells. The recombinant polypeptide, rCh3.1, is indicated by the arrow. A, Coomassie blue stain of the gel, B, IgE-immunoblot.

Reactivity of rCh3.1 allergen with individual human atopic IgEs

To assess the reactivity of rCh3.1 allergen with atopic IgEs from *Cladosporium herbarum*-sensitive individuals, Western blots of bacterial lysates from induced XL1-blue transformants of clone Ch3.1 were incubated with human atopic IgE-containing serum or plasma, followed

by ^{125} I-labeled goat anti-IgE. The results in Fig. 5 illustrate that 5 of 13 individual sera tested (38%) were reactive with rCh3.1 allergen. In particular, sera in lanes 3 (a component of the pooled atopic serum) and 12 reacted strongly with the recombinant peptide, but with relatively high background at the dilutions used in this experiment. Other antisera reacted less strongly, but with greater specificity than sera #3 and 12 (#1,5,13). Generally, the degree of reactivity with rCh3.1 allergen did not correlate with RAST values, as positive sera had RAST values ranging from 10–25%, whereas sera with 40–45% reactivity were negative for rCh3.1 allergen. Two sera (#9,10) reacted specifically with proteins whose molecular masses did not correspond to the 36 kDa rCh3.1. These were not considered positive. Similar results were also observed in experiments utilizing alkaline phosphatase-conjugated anti-human IgE and chromogenic substrates NBT and BCIP (data not shown).

Discussion

A cDNA clone encoding an allergenic polypeptide of *Cladosporium herbarum* was isolated and characterized in the present study. Sequence analysis and comparison led to a conclusion that the open reading frame (ORF) of the gene and its deduced amino acid sequence are correct. The ORF of the clone is in phase with the ORF of the β -galactosidase in λ ZAP II vector, which encodes a fusion protein recognized by IgE antibodies.

The polypeptide shares about 70% sequence homology with the C-terminal fragments of the other *hsp* 70 of eukaryotic cells [21–25]. In addition to highly conserved sequence features of the *hsp* 70 family, it also diverges in the C-terminal portion whereas the very C-terminus possesses a striking conserved sequence. Thus we can conclude that this cloned polypeptide belongs to the heat shock protein 70 family. To the best of our knowledge, it

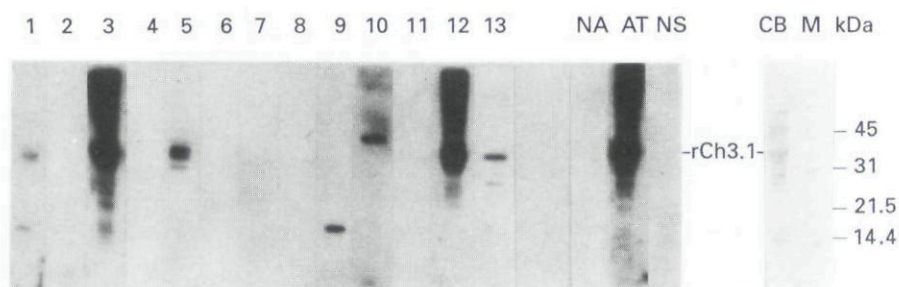


Fig. 5. Reactivity of rCh3.1 protein with individual human atopic IgEs. Blots of bacterial lysate containing rCh3.1 allergen were incubated with IgEs and ^{125} I-labeled goat anti-human IgE as described in *Materials and methods*. Lanes 1–13: individual IgEs; lane NA: non-atopic pooled human sera; lane AT: pooled human IgE; lane NS: no human serum control; lane CB: Coomassie blue R250-stained blot; lane M: molecular size standards, with sizes in kDa indicated at right.

appears that this is the first report implicating *hsp* 70 as a mould allergen.

The *hsps* 70 are one of the most highly conserved protein families. Homologous proteins are found in both eukaryotes and prokaryotes, showing 60–78% sequence identity among the eukaryotic proteins and 40–60% identity between the eukaryotic and prokaryotic proteins [27]. The conserved sequences of the proteins ensure that their essential functions, such as interactions with other proteins or peptides, are common to a variety of species. The diversity of the sequences, however, may account mainly for their immunogenicity among different species.

In fact, these proteins from various pathogens have been shown to be one of the major antigens involved in human and animal immune responses [28]. Therefore, recent interest has been focused on the potential use of the proteins for vaccination. Indeed, great endeavour has been made to develop safe and efficacious vaccines using the *hsp* proteins against a variety of pathogens [29,30]. However, the present study cautions against the use of such proteins as vaccines in the absence of further studies of the allergenicity of the proteins. Because of the high percentage of sequence identity among the *hsps* 70, some IgE-binding epitopes of the reported allergen may be shared with the other *hsps* 70. Hence, one can not exclude a risk of anaphylactic response when a *hsp* 70 from any given species is introduced into a previously sensitized individual, even though the IgE antibodies involved in the response may not be directly induced by the given *hsp* 70. This argument also has its support from recent studies of the distribution of epitopes on the *hsps* 70 [31,32]. It has been shown that the majority of the antibody-binding epitopes are located on the conserved N-terminal portion of a *hsp* 70 [31], although the C-terminal portions of the proteins were found to be the most important antigenic region [32] on the basis of their antibody binding properties and sequence variabilities.

Hydropathic profiles of the proteins were compared in this study. The conserved N-terminal portions have conspicuous amphipathic features, i.e. sequence features of T-cell epitopes [33]. The C-terminal portions of the proteins are consistently hydrophilic, although this region shows the most variable amino acid sequences. Thus, this region would be likely exposed to the outside of the proteins and are possibly recognized by antibodies [34]. Further analysis of epitopes of the cloned protein using synthetic peptides or recombinant fragments will facilitate an understanding of its allergenicity.

The recombinant allergenic polypeptide, rCh3.1, had a relatively high expression level in the XL1-blue and pBluescript SK system. This system developed by Stratagene is used frequently for the purposes of cloning and subcloning of DNA fragments. However, it is seldom

used to generate a large amount of fusion protein since there is usually a relatively low expression level of the cloned gene in the system, which we have experienced in our previous studies. The availability of relatively high expression levels of the recombinant polypeptide in this study may be due to the existence of gene expression enhancers in the cloned gene, which remains an interest to be pursued further.

We have also determined that the recombinant rCh3.1 protein binds to IgE in 5 of 13 sera or plasmas from *C. herbarum*-sensitive individuals. This classifies *hsp*70 as a minor allergen from *Cladosporium herbarum*. However, this allergen remains of potential importance, as no major allergens to this mold have as yet been characterized. Moreover, the strong immunoreactivity of two samples to *hsp*70 suggests that this allergen may be the dominant allergen in at least a small proportion of individuals. The proportion of patients recognizing this allergen is also likely to be a conservative estimate using this assay, given that the allergen is bacterially expressed and partially denatured in immunoblots. Examination by RAST-inhibition using highly purified allergen (preferably expressed in a mammalian expression system) remains the most accurate means of determining the utility of this allergen. Efforts to express the *C. herbarum* *hsp* 70 in the yeast, *Pichia pastoris*, are ongoing.

In general, fungal allergens tend to be more difficult to isolate and characterize than those from plant pollens, as molds adapt to their environment at a rapid rate; thus, different isolates from the same species may exhibit different allergen profiles. Efficient immunotherapy against *Cladosporium herbarum* may ultimately depend on the application of TH₁ response-inducing peptides representing a spectrum of minor allergens, rather than relying on a single major allergen. In addition, peptide fragments from cloned *hsp*70 of *C. herbarum* have the potential to provide efficient immunotherapy against more than one mould species, as we have recently cloned a ribosomal P₂ protein allergen from *C. herbarum* [7], and both ribosomal P₂ protein and a C-terminal fragment from *hsp* 70 of *Alternaria alternata* have also been identified as minor allergens in our laboratory (De Vouge *et al.*, unpublished data).

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