

Molecular cloning and expression of a *Penicillium citrinum* allergen with sequence homology and antigenic crossreactivity to a hsp 70 human heat shock protein

H.-D. SHEN*†, L.-C. AU*, W.-L. LIN*, S.-F. LIAW*, J.-J. TSAI§ and S.-H. HAN†‡

*Department of Medical Research, Veterans General Hospital, §Section of Allergy and Immunology, Cathay General Hospital, †Center for Immunology and ‡Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan, Republic of China

Summary

Background *Penicillium citrinum* has been identified as the most prevalent airborne *Penicillium* species in the Taipei area. However, detailed studies on allergens of this ubiquitous *Penicillium* species are still lacking.

Objective For the characterization of allergens of this prevalent *Penicillium* species, molecular cloning and expression of the allergen genes of *P. citrinum* were performed in the present study.

Methods Molecular cloning of the allergen genes was performed by using a λUni-Zap XR cDNA library of *P. citrinum* and serum from an asthmatic patient. The cloned cDNA was excised from the phage vector as a recombinant pBluescript phagemid and sequenced. The cDNA of the IgE-binding clone was expressed as fusion protein with the glutathione-S-transferase. The corresponding natural allergen was analysed by absorption immunoblotting using monoclonal antibody and serum from asthmatic patient. The frequency of IgE-binding to the allergen cloned was analysed by dot immunoassay using recombinant allergen and by immunoblotting using the whole extract of *P. citrinum*.

Results In the screening of cDNA library of *P. citrinum* using serum from an asthmatic patient, IgE-binding cDNA clones designated SC4 and XL were obtained. The 5'-truncated, 0.7-kb and 1.7-kb cDNA inserts of clones SC4 and XL contained open reading frames of 163 and 503 amino acids, respectively. On alignment, the deduced amino acid sequences showed that 97 (60%) of the 163 amino acids and 376 (75%) of the 503 amino acids were identical to the corresponding amino acid sequence of the human heat shock protein in the hsp70 family. Both recombinant SC4 and XL showed positive SDS-PAGE-immunoblot reactivity to a monoclonal antibody MA3-006 against the human hsp 70 protein. For characterization of the corresponding natural allergen, immunoblotting reactivities of MA3-006 and IgE antibodies to the 70 kDa component of *P. citrinum* have been shown to be disappeared after absorption of these antibodies with the recombinant SC4 protein. Sera from 14 (41%) of 34 *Penicillium*-allergic patients showed IgE-binding to the recombinant XL protein and the 70 kDa component in the extract of *P. citrinum*.

Conclusion Results obtained suggest that hsp 70 is an allergen of *P. citrinum* and that clones SC4 and XL contain partial cDNAs of this allergen gene.

Keywords: allergen, hsp 70, molecular cloning, *Penicillium citrinum*

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Introduction

Penicillium has been identified as one of the fungal genera

responsible for extrinsic bronchial asthma [1–3]. More than 150 different species have been discovered in the genus *Penicillium* [4]. However, little was known about allergens of these ubiquitous fungal species. Recently, through molecular cloning using MoAbs against allergens, the 68 kDa allergen of *P. notatum* has been identified as a beta-N-acetylglucosaminidase [5]. Results obtained from a recent survey conducted by our laboratories showed that *P. citrinum* is the most prevalent airborne *Penicillium* species in the Taipei area [6]. Furthermore, *P. citrinum* has also been shown to be one of the five most frequently recovered species of *Penicillium* indoors and out in Topeka KS [7]. For characterization of allergens of this prevalent *Penicillium* species, molecular cloning of the allergen genes using λ Uni-Zap XR cDNA library of *P. citrinum* and serum from an asthmatic patient was performed in the present study. Results obtained suggest that the heat shock protein in the hsp70 family may be one of the allergens of *P. citrinum*.

Materials and methods

Allergenic extract of *Penicillium citrinum*

For preparation of the allergenic extract, *P. citrinum* strain 52-5 isolated from the air in the Taipei area [6] was used. The species was identified by us and verified by the American Type Culture Collection. Fungal mats of *P. citrinum* 52-5 containing both spores and mycelia were collected after its growth in a liquid medium containing Czapek concentrate [4] (10 mL/L), K_2HPO_4 (1.0 g/L) and sucrose (30 g/L) for 9 days at 25°C. The crude extract of *P. citrinum* was prepared by extracting the fungal mats (1:10, v/v) with phosphate-buffered saline (85 mM of NaCl, 2.6 mM of KH_2PO_4 , and 49 mM of Na_2HPO_4 , pH 8.0) for 4 h at 40°C with constant shaking. After centrifugation, the supernatant was dialysed against 50 mM of NH_4HCO_3 , pH 8.0 for 24 h at 40°C. After sterile filtration (0.2 μ m; Sartorius, Hayward, CA, USA), the extract was lyophilized in aliquots and stored at 4°C. The protein content of the extract was determined by the dye-binding method of Bio-Rad (Richmond, CA, USA).

Cloning and molecular techniques

In order to construct a cDNA library of *P. citrinum*, total RNA was extracted from an 8-day-old fungal matt culture of *P. citrinum* strain 52-5. About 8 g of the fungal matt was ground under liquid nitrogen with mortar and pestle and was then resuspended in 20 mL of the guanidinium thiocyanate-containing RNAzol™ B solution (Biotecx Laboratories, Inc., Houston, TX, USA). The suspension solution was homogenized by using the MSK cell homogenizer

(B. Braun, Melsungen, Germany) for a total of 10 min with intervening cooling on ice for 1 min after each 1 min homogenization. After filtration, the filtrate was divided into 1 mL aliquots in Eppendorf tubes. After the addition of 100 μ L of $CHCl_3$, the mixture was vortexed thoroughly and kept on ice for 5 min. After centrifugation, the aqueous fraction was mixed with an equal volume of isopropanol and left on ice for 15 min. Following the centrifugation procedure, the pellet was washed once with 75% alcohol and vacuum dried. A lambda-uni-ZAP™ XR-based cDNA library of *P. citrinum* was custom-made by Stratagene, La Jolla, CA, USA. The library contained 4.5×10^6 independent clones with the size of the cDNA insert greater than 400 bp. The cDNA clones containing DNA sequences of allergens were screened by plaque immunoassay of the IPTG-induced library essentially as described previously [8,9]. IgE plaque immunoassay was conducted by using 10000 pfu on 15 cm petri dishes and serum from an asthmatic patient diluted 1/2 in *E. coli* lysate. IgE reactivity was detected with alkaline phosphate-labelled monoclonal anti-human IgE (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) and developed using the alkaline phosphate conjugate substrate kit (Bio-Rad, catalog number 170-6432). The serum sample used was obtained from an asthmatic patient (Table 1, no. 33) with positive immediate skin test to an extract of *Penicillium* species supplied by Taiwan Allergy Center, Taipei. The cDNA insert-containing pBluescript phagemid of IgE-binding cDNA clones (SC4 and XL) obtained was excised from the ZAP vector *in vivo* by superinfection of the *E. coli* SOLR™ strain with helper phage (ExAssist™ helper phage, M13, Stratagene) according to the protocol supplied by the Stratagene Corporation. The excised phagemid replicated in the *E. coli* SOLR strain host was purified by using the Wizard™ Minipreps DNA purification system of Promega (Madison, WI, USA). The nucleotide sequences were determined by the dideoxy sequencing procedure as described previously [8,9].

Preparation of recombinant SC4 and recombinant XL

SC4 and XL were expressed as fusion proteins with the glutathione-S-transferase (GST) essentially as described [9]. The cDNA insert derived from clone SC4 was obtained through polymerase chain reaction (PCR). The 5' primer used was an oligonucleotide containing the *Bam*HI-recognizing site and the 5'-end sequence of the cDNA insert (5'ATGGATCCCAGATTGAGGTTACC3'). The 3' reverse primer used was an oligonucleotide containing the *Eco*RI-recognizing site and the sequences based on the 3'-end noncoding region (after the TAA stop codon) sequence of the cDNA insert (5'ATGAATTCGCAAGGCGATACGT3'). The PCR product was separated by agarose gel

Table 1. Characterization of patients whose serum samples have been used in dot immunoassay and SDS-PAGE-immunoblotting for the detection of specific IgE antibodies against recombinant proteins and components in the extract of *P. citrinum*

Serum no.	Patient history	Skin-prick test	CAP test
1	BA*	+	2+
2	BA	ND	1+
3	BA	—	1+
4	BA	—	1+
5	BA	+	1+
6	BA & AR**	ND	1+
7	BA	—	1+
8	BA & AR	ND	1+
9	BA	—	2+
10	BA	ND	2+
11	BA & AR	+	2+
12	BA & AR	—	2+
13	BA & AR	+++	4+
14	BA	+++	ND
15	BA & AR	+++	ND
16	BA & AR	+++	0
17	BA	++	ND
18	BA	++	0
19	BA	+	ND
20	BA	+	ND
21	BA	+	ND
22	BA	+	ND
23	BA	+	ND
24	BA	++	0
25	BA	+	ND
26	BA	++	ND
27	BA & AR	+	ND
28	BA	ND	3+
29	BA	ND	3+
30	BA	ND	2+
31	BA	ND	2+
32	BA	ND	1+
33	BA	++#	0
34	BA	+	0
35	Atopic Dermatitis	+	0

*and **: BA and AR represent bronchial asthma and allergic rhinitis, respectively. #Serum no. 33 was also used in cDNA cloning in the present study and the skin test of this patient was performed by using an extract of *Penicillium* species supplied by Taiwan Allergy Center, Taipei.

electrophoresis and purified by using the GeneClean II kit (Bio 101 Inc., La Jolla, CA, USA). The purified PCR product was then digested with *Bam*HI and *Eco*RI and was purified as mentioned above. The *Bam*HI-cDNA-*Eco*RI fragment was then ligated into pGEX-2T [10] which has been digested with *Bam*HI and *Eco*RI. The

recombinant vector was used to transform *E. coli* TG1. The polypeptide fused to glutathione-S-transferase encoded by the Sj26 gene of the pGEX was affinity-purified from glutathione agarose column [10]. Recombinant XL-GST fusion protein was obtained according to the same procedure as described above except that the 5'-end primer used in PCR was an oligonucleotide containing the *Bam*HI-recognizing site and the 5'-end sequence of the cDNA insert (5'ATGGATCCGCCTACCTGGGTGGTA3') of clone XL.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The reactivity between the recombinant protein or components of the crude fungal extract with commercial MoAb or IgE antibodies in sera of allergic patients was analysed by SDS-PAGE and immunoblotting essentially as described [11]. Briefly, the recombinant protein or components of the crude allergenic extract were separated by SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membrane (0.45 µm, Millipore, Bedford, MS, USA). After blocking with 1% skim milk, the blots were incubated with monoclonal anti-heat shock protein 70 antibody (MA3-006, Affinity BioReagents, Inc., Neshanic Station, NJ, USA) at room temperature for 1 h or with diluted human serum samples (1:5 dilution in Tris-buffered saline, pH 7.5 containing 0.05% Tween 20 [TTBS] and 1% skim milk) for 16 h at 4°C. Following washing procedures, the blots were incubated with affinity-purified, horseradish peroxidase-labelled goat anti-mouse IgG (H + L) (Bio-Rad) or with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for 1 h at room temperature. After washing, the blots were developed in a substrate solution of acetate buffer (50 mM, pH 5.0) containing 1 mM 3-amino-9-ethyl-carbazole (Sigma) and 0.015% (v/v) hydrogen peroxide for about 5 min or in a substrate solution of carbonate buffer (0.1 M NaHCO₃, 1.0 mM MgCl₂, pH 9.8) containing 0.35 M BCIP (5-bromo-4-chloro-3-indoyl-phosphate *p*-toluidine salt) and 0.37 M NBT (p-nitro blue tetrazolium chloride) for about 30 min. After washing with water, the deposition of the red or the purple stain which demonstrated antigen-antibody binding was recorded by photography. The MoAb WH9 against the house dust mite allergen Der p 7 [11] and the cord serum sample were used as negative control. For absorption immunoblotting, MoAb MA3-006 or human serum sample was mixed separately with 100 µg of recombinant SC4 or with 100 µg of Der p 7-GST fusion protein [9] at room temperature for 1 h. The mixtures were then incubated individually with PVDF membranes blotted with components in the crude extract of *P. citrinum*. The immunoblotting was then performed as described above.

Dot immunoassay

To determine the number of serum samples with IgE binding to recombinant SC4 and recombinant XL, dot immunoassay was performed. Ten microlitre of a recombinant SC4 or a recombinant XL solution in a concentration of 100 µg/mL were dotted onto the nitrocellulose membrane (0.45 µm, Bio-Rad). The membrane was air-dried for 1 h and blocked with 1% skim milk at room temperature for another hour. After washing with TTBS, the recombinant protein-containing membranes were incubated individually at 4°C overnight with different serum samples (1:5 diluted with TTBS containing 1% skim milk) from asthmatic patients (Table 1) with positive immediate skin test to *P. notatum* obtained from Greer Laboratories, Inc. (Lenoir, NC, USA) or with positive CAP test (Kabi Pharmacia Diagnostics AB, Uppsala, Sweden) to *P. notatum*. The membranes were washed again with TTBS and incubated with alkaline phosphate-conjugated monoclonal anti-human IgE antibodies and developed in an enzyme substrate solution as described in immunoblotting. For control, one µg of GST alone and 1 µg of another *P. citrinum*-GST fusion protein (SC3) were also included and reacted at the same time with all the asthmatic serum samples tested. Furthermore, cord serum and serum sample from an atopic dermatitis patient were also used as a control.

Results

cDNA cloning and sequencing

The lambda-Uni-ZAPTM XR-derived clones SC4 and XL from the *P. citrinum* cDNA library were isolated because they showed a high IgE-binding activity to a *Penicillium*-allergic serum by plaque immunoassay. The cDNA-containing pBluescript phagemid was excised from the Uni-ZAP vector of clones SC4 and XL individually by the ExAssist/SOLR system. The nucleotide sequence of the cDNA inserts was determined by direct dideoxy sequencing. The nucleotide sequence (1739 bp) of cDNA clone XL was deposited in GenBank under the accession number U64207. The amino acid sequence deduced from an open reading frame of cDNA clone XL is shown in Fig. 1. The insert DNA encoding a 503 amino acid residue protein is truncated at the 5' end. The protein contained two cysteines and five putative N-glycosylation sites (Fig. 1). The typical 'AATAAA' polyadenylation signal sequence for mammalian genes is not found within 10–30 bases upstream from the polyA tail in clone XL. Instead, AT-rich sequences are observed as in other yeast and mold genes. The 0.7 kb cDNA insert of clone SC4 showed a nucleotide sequence identical to the corresponding sequence of XL starting from nucleotide no. 1021 except that the last 7 nucleotides are

AAAAAAA rather than the last 21 nucleotides of XL. The first amino acid deduced from an open reading frame of SC4 is Gln which corresponds to the amino acid numbered 341 of XL in Fig. 1. When the deduced amino acid sequence was compared with the protein database of the IntelliGenetics, Release 5.4 (IntelliGenetics, Inc., Mountain View, CA, USA), 376 of the 503 (75%) amino acids of XL were found to be identical to the amino acid sequence of the hsp 70 human heat shock protein (accession no. M11711 and M15432 (Fig. 1). Four corresponding potential N-glycosylation sites were found in both sequences of HUMHSP 70 (at amino acid residues 151–153, 360–362, 417–419 and 486–488) and XL (at amino acid residues 19–21, 227–229, 285–287 and 355–357) (Fig. 1). Besides, amino acid sequence identities of 48% (59/124) and 77% (207/269) were also found as compared with the sequences of clones Mag29 and rCh3.1 (Fig. 1), respectively which contained partial cDNAs coding for allergens related to a heat shock cognate protein (hsc71) in the hsp 70 family of the house dust mite *D. farinae* [12] and the hsp 70 allergen of *Cladosporium herbarum* [13].

Characterization of recombinant SC4 and recombinant XL

Complementary DNA fragments of SC4 and XL produced by PCR were cloned individually into the expression vector pGEX 2T (see Materials and methods). The fusion proteins from these constructs were produced at a yield of 0.5–1.0 mg/L and 0.05 mg/L for recombinant SC4 and XL, respectively. Analysed by SDS-PAGE, the recombinant SC4 and XL showed MW of about 44 kDa and 80 kDa, respectively (Fig. 2, panel A). Both recombinant SC4 and XL but not GST alone showed immunoblotting reactivity to IgE antibodies in the same serum sample used in the cDNA screening (Fig. 2, panel B). Besides, recombinant SC4 and XL also showed immunoblotting activity to a commercially available MoAb (MA3-006) against the epitope located between amino acids 504–617 of human hsp 70 (Fig. 2, panel C).

The reactivity of IgE antibodies in sera of asthmatic patients to recombinant SC4 and XL was analysed by dot immunoassay. Twenty (serum nos. 1, 3–5, 8–9, 13–15, 17, 21–27, 30–31 and 33) of the 34 sera (Table 1) tested showed positive IgE binding to the XL-GST fusion protein (Fig. 3). However, weak IgE-binding reactivity to the glutathione-S-transferase protein has also been observed for seven of these positive serum samples (nos. 1, 9, 13–15, 17 and 30–31) (Fig. 3). Strong IgE-binding reactivity to recombinant SC4 was observed for serum samples 15 and 33. For controls, enzyme colour reactivity was not detected when a cord serum was used (Fig. 3, serum no. 36). In the same dot immunoassay, another GST-*P. citrinum* fusion protein SC3 containing sequences unrelated to XL was also included.

XL	1	AYLG	TVNN	AVIT	VPAY	<u>FND</u>	SQR	QATK	DAGL	IAGL	NVLR	IINE	PTAA	AIAY	GLDK	55		
HUMHSP70	133		YP:T				V:.....									187		
XL	56	KTEG	ERNV	LIFD	LGGG	TFDV	SLLT	IEEG	IFEV	KSTAG	DTHL	GGED	FDNR	LVNH	FV	110		
HUMHSP70	188	TGK:					I::	DD:		A:.....						242		
XL	111	NEFK	RKHK	KDLT	TNAR	LRRL	RTAC	ERAK	RTL	SSAA	QTSI	EID	SLFE	GIDF	YTSI	165		
HUMHSP70	243	E:.....	ISQ:	K::	V:.....		ST:	A:	L:.....							297		
XL	166	TRAR	FEEL	CQDL	FRGT	MPEV	ERVLR	DAKID	KSSV	HEIV	LVGG	STRIP	KIQK	LVSD		220		
HUMHSP70	298			S:::	S:::	L:::	KA:::	L::	AQI:	DL:		V:::	LQ:			352		
XL	221	FFN-KDANK	SINP	DEAV	AYGA	AVQA	AIL	SGDT	SSK	STNE	ILL	LDV	APLS	SLGI	ETA	274		
HUMHSP70	353	:::GR:L		G:.....		M:-	K:ENV	QDL:		L:::						406		
rCh3.1	1									K:.....						40		
XL	275	GGVM	TPLIK	RNTT	TIPT	TKK	SETF	STYS	DNQ	PGVL	IQVF	EGER	ARTK	DN	LLGKFEL	329		
HUMHSP70	407		A:.....	S:.....	QTQI:	T:.....		Y:.....	M:.....	R:::						461		
rCh3.1	41		A:.....		F:.....		I::	M:.....								95		
XL	330	TGIP	PAPR	GV	PQIE	VTFD	LDANG	IMNV	SASE	KGTG	KSNK	ITIT	TNDK	GRLS	KEEIE	384		
HUMHSP70	462	S:.....	-:.....	I:.....	L::	T:	TD:	S::	A:.....							515		
rCh3.1	96	S::	R:.....		L:.....	T::	V:.....									150		
Mag29	1														DD::	5		
XL	385	RMLA	EAEK	YKA	EDEA	EASRI	QAKN	GLES	YAYS	SLKN	TITE	GKL--	QMS	DDDK	KKKIE	437		
HUMHSP70	516	::	VQ:.....		VQRE:	VS::	A:::	FNM:	SAVE	DEG:	KGKI:	EA:::	VL			570		
rCh3.1	151	::::	D:.....		E:.....	G:.....		VSDP:	VEEK	L:AE:	ETLT					205		
Mag29	6	::	VK:::	S::	E::	DKQR	D::	A:::	S::	G::	FQM:	A:LD:	EAIK	SKV:	EE:	R:::	L	60
XL	438	DKIS	EIIS	WLDN	<u>NOTA</u>	EKDE	YESQ	QKE	LEA	IANP	IMQA	AYGA	AGG	APPQ	QORADGE	492		
HUMHSP70	571	::	CQ:.....		A:TL:		F:HKR:		QVC:	ISGL:	QG::	PG:	GGFG	AAQG		625		
rCh3.1	206	GA:	DKTVA:	I:E:::	T:E::	AE::	Q::	SV::	V:MKI:	E:::	GGMP	GQ:A				260		
Mag29	61	::	VD:VLK:	A:AL:		F:H:	R:::	SVC:	ITKL:	QQ:::	GAGG	MPG:	F			115		
XL	493	TEE-----														503		
HUMHSP70	626	PKGGSGS-----														640		
rCh3.1	261	GAPPPGAGDD-----														278		
Mag29	116	PGGFPGTDGSGGGAAGDGGKSGPTI::V:														145		

Fig. 1. Composite alignment of the amino acid sequences of clone XL, the human heat shock protein in the hsp 70 family (HUMHSP 70, see reference 14) and the sequences of clones Mag 29 (ref 12) and rCh3.1 [13] representing partial cDNA clones of the hsp 70 allergen of house dust mite *D. farinae* and the fungus *Cladosporium herbarum*, respectively. The HUMHSP 70 amino acid sequence shown is from residues 133-640. Identity to the XL sequence is indicated by (:). Dashes denote spaces introduced to optimize the alignment. Underlined sequences are the potential N-glycosylation sites.

Sera 1, 9, 13, 15, 17 and 31 showed weak and serum 33 showed strong enzyme colour reactivity. Serum no. 35 obtained from an atopic dermatitis patient with positive skin test to *P. notatum* showed strong IgE-binding to recombinant XL and SC4 (Fig. 3).

Nature of the corresponding allergen in the *P. citrinum* extract

To identify the natural allergen corresponding to the SC4 gene, absorption immunoblotting was performed using MoAb MA3-006 against the human hsp 70 protein. Strip

A in Fig. 4 shows the Coomassie Blue-stained PVDF membrane blotted with components in the crude extract of *P. citrinum* separated by electrophoresis in a 12.5% SDS-PAGE. An immunoblotting reactivity of MoAb MA3-006 to the component of *P. citrinum* with an MW of about 70 kDa was observed (Fig. 4, B-1). The immunoblotting reactivity was not detected when MoAb MA3-006 was absorbed with 100 µg of recombinant SC4 (Fig. 4, B-2). However, the immunoblotting reactivity was retained when MoAb MA3-006 was absorbed with 100 µg of a control Der p 7-GST fusion protein (Fig. 4, B-3). A similar result was observed when the same serum sample used in the molecular cloning

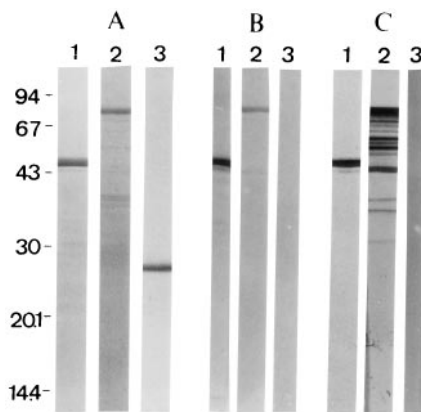


Fig. 2. SDS-PAGE-immunoblotting reactivity of recombinant SC4, XL and the glutathione S-transferase protein (GST) to IgE and MoAb MA3-006. Panel A shows the Coomassie blue-stained SDS-PAGE protein profile of the affinity-purified SC4-GST (A-1), XL-GST (A-2) and the GST protein (A-3). Panels B and C show the immunoblotting reactivity of recombinant SC4 (strips 1), XL (strips 2) and GST (strips 3) to human IgE antibodies in serum from an asthmatic patient (panel B) and MoAb MA3-006 against human hsp 70 (panel C), respectively.

experiment was used in the absorption immunoblotting as shown in panel C. The IgE-immunoblotting reactivity to the 70 kDa component of *P. citrinum* (Fig. 4, C-1) was not detected when the serum sample was absorbed with 100 μ g of recombinant SC4 (Fig. 4, C-2). The IgE-immunoblotting reactivity was still observed when the serum sample was absorbed with 100 μ g of Der p 7-GST fusion protein (Fig. 4, C-3).

To analyse IgE reactivity to the corresponding 70 kDa

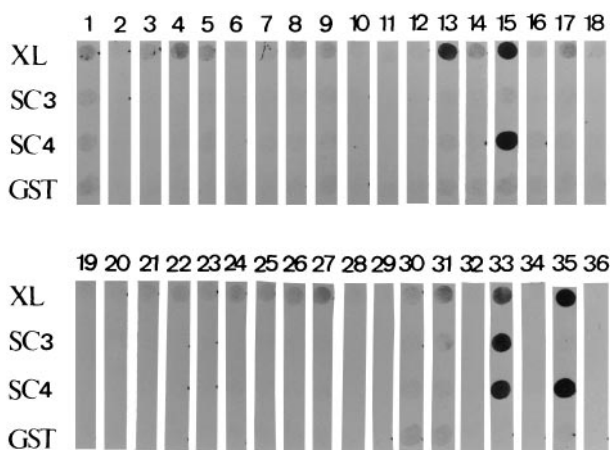


Fig. 3. The reactivity of recombinant XL, SC3, SC4 and GST to IgE antibodies in sera from 34 *Penicillium*-allergic patients (Table 1) analysed by dot immunoassay. Serum 35 was obtained from an atopic dermatitis patient and serum 36 was a cord serum sample.

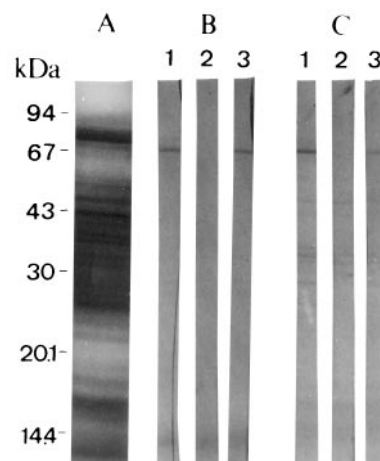


Fig. 4. Reactivity of MoAb MA3-006 and IgE antibodies to the extract of *P. citrinum*. Panel A shows the Coomassie blue-stained protein profile of the *P. citrinum* extract on a PVDF blot. Strips 1 of both panels B and C show the immunoblotting reactivity of MA3-006 (panel B) and IgE antibodies in serum of an asthmatic patient (panel C) to components in the extract of *P. citrinum*. Strips 2 and 3 show the results of immunoblotting after absorption of the antibodies with the recombinant SC4 (strips 2) and with the control Der p 7-GST (strips 3), respectively.

component in the extract of *P. citrinum*, immunoblotting was performed using the same asthmatic serum samples (Table 1) which have been used in the dot immunoassay. Results in Fig. 5 showed that sera 1, 4-7, 13-15, 17, 19-20, 22-24, 26-27, 30 and 32-35 showed IgE-immunoblotting reactivity to the *P. citrinum* component with a molecular weight of about 70 kDa. Strip M represents the result of immunoblotting by using MoAb MA3-006 for marking the position of the corresponding 70 kDa component of *P. citrinum*. The 33 kDa component which showed strong IgE-binding reactivity to 11 of the 34 asthmatic serum samples tested may be considered as an important allergen of *P. citrinum*. Enzyme colour reactivity was not observed when cord serum sample (no. 36) was used. Thus, results obtained showed that fourteen (sera 1, 4-5, 13-15, 17, 22-24, 26-27, 30 and 33) of the 34 (41%) asthmatic sera tested showed IgE reactivity to XL-GST fusion protein and the 70 kDa component of *P. citrinum*.

Discussion

Molecular cloning of allergen genes using sera from asthmatic patients has been shown to be one of the successful approaches on characterization of allergens. In the present study, by using a similar approach, IgE-binding cDNA clones SC4 and XL of *P. citrinum* were isolated. Although cDNA inserts of SC4 and XL were truncated at the 5' end,

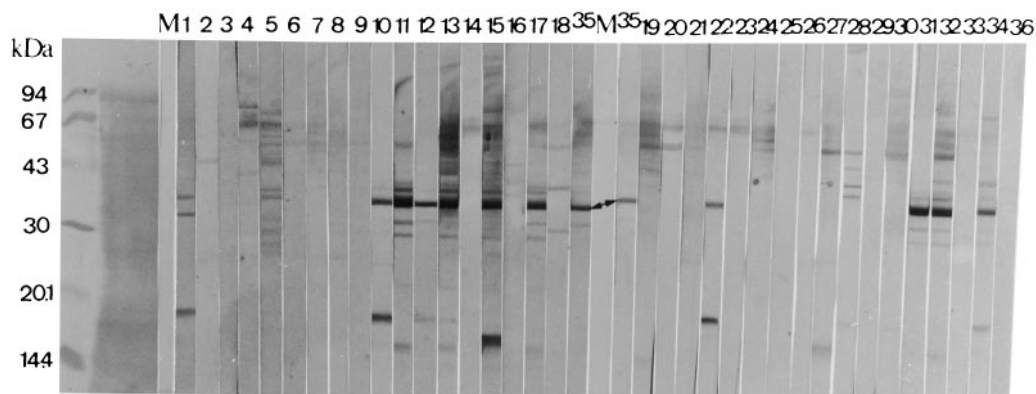


Fig. 5. Immunoblotting analysis of the reactivity of IgE antibodies in sera obtained from 34 *Penicillium*-allergic patients (Table 1) to components in the extract of *P. citrinum*. Serum no. 35 was obtained from an atopic dermatitis patient and serum no. 36 was a cord serum sample. Strip M represents the result of immunoblotting by using MoAb MA3-006 against the human hsp 70.

the observation that SC4 and XL share amino acid sequence identity of 60% and 75%, respectively to the corresponding regions of the amino acid sequence of human hsp 70 [14] suggests that SC4 and XL may be cDNA clones of the heat shock protein in the hsp 70 family of *P. citrinum*. Results from immunological characterization showed that the monoclonal antibody MA3-006 against human hsp 70 reacted with both the recombinant protein encoded by SC4 and XL and a component of *P. citrinum* with a MW of about 70 kDa. The fact that the immunoblotting reactivity of both IgE and MoAb MA3-006 against the 70-kDa component of *P. citrinum* was absorbed by recombinant SC4 but not by an unrelated GST-fusion protein confirms further that SC4 is a partial cDNA clone of a hsp 70 family allergen of *P. citrinum*.

When organisms are submitted to a heat shock treatment and to other stressful physiological conditions, a family of proteins, termed heat-shock proteins (hsps), are actively synthesized [15]. Members of the 70 kDa heat shock gene family are highly conserved across a wide range of organisms. Although the mechanism of hsp 70 action is still poorly understood, the hsp 70s have been implicated in a variety of essential cellular processes. They assist in the proper folding of polypeptides, inhibit protein aggregation, target misfolded protein for degradation, facilitate polypeptide translocation, and participate in disassembling oligomeric structures [16–18]. In addition to functioning as molecular chaperones, a member of the hsp 70 heat shock family has also been shown to play a role in antigen processing [19]. Furthermore, hsp 70 proteins have also been identified to be immunodominant antigens in a wide variety of bacterial, fungi and parasitic infections [20–21]. Other observation has also suggested a role of stress proteins in different autoimmune diseases [20, 22]. The significance of stress proteins as potential allergens in human beings is

still unclear. By screening of a *Dermatophagoides farinae* cDNA library with rabbit antiserum and sera from patients with mite allergy, the 67 kDa allergen of *D. farinae* has been shown to be related to a heat shock cognate protein (hsc71) in the hsp 70 family [12]. Recently, an allergenic polypeptide representing a variable region of hsp 70 cloned from a cDNA library of *Cladosporium herbarum* has also been reported [13]. Furthermore, Kumar *et al.* reported the isolation of an *Aspergillus fumigatus* cDNA coding for a 65-kDa IgE-binding protein, which is homologous to hsp 90 [23]. Burnie and Matthews also demonstrated elevated levels of IgM, IgG and IgE antibodies against *Aspergillus* hsp 90 protein in patients with allergic bronchopulmonary aspergillosis [24].

The hsp 70s have two domains. The highly conserved amino-terminal ATPase domain and a variable carboxy-terminal peptide-binding domain, connected by a proteolysis-sensitive region [25]. The highly conserved amino-terminal ATPase domain of hsp 70 was not found in clones SC4 and XL isolated in the present study. Analysed by dot immunoassay and immunoblotting using sera from *Penicillium*-sensitive patients, the frequency of IgE-binding to recombinant XL and the 70 kDa component of *P. citrinum* was about 41%. Although most of the positive sera showed relatively weak IgE reactivity, sera 13, 15 and 33 showed strong IgE-binding to the XL-GST fusion protein. It suggests that the 70 kDa component may be a significant allergen at least for these three patients. In dot immunoassay, sera 15 and 33 showed IgE-binding to recombinant XL and SC4, while serum no. 13 showed strong IgE-binding to recombinant XL only. This finding suggests that there may be at least two different IgE-binding epitopes on the 70 kDa heat shock protein of *P. citrinum*. The binding frequency of IgE in 41 mite allergic sera to a blot of Mag29 coding for 145 amino acids corresponding to

the C-terminal, about one-fifth of the entire hsp 70 allergen of *D. farinae* was 9.8% [12]. Five of the 13 (38%) *C. herbarum*-sensitive sera tested were reactive with rCh3.1 allergen which contained a 278 amino acid polypeptide homologous to the C-terminal portion of 70 kDa heat shock protein [13]. The clinical significance of these hsp 70 allergens may be assessed more accurately by using recombinant molecules encoded by the full-length cDNAs or the corresponding natural allergens purified from their respective crude allergenic extracts.

A major immunoregulatory mechanism in inflammatory infections and autoimmune and allergic diseases is the control of the balance of cytokines secreted by TH₁/TH₂ subsets of T helper (TH) cells [26]. Heat shock proteins have been shown to be one of the major antigens involved in human and animal immune responses against various pathogens and be antigens involved in autoimmune diseases [20–22]. Results obtained also showed that T-cell clones obtained from tuberculoid leprosy patients which were reactive with mycobacterial heat shock proteins exhibited a TH₁-like cytokine secretion pattern with very high levels of IFN γ [27]. It has also been considered that protective immunity to *Mycobacterium tuberculosis* is mediated by a TH₁ response [28]. Patients with progressive tuberculosis seems to have a TH₂ component to their response [29]. Furthermore, TH₁ response has also been shown to be associated with the causes of many of the autoimmune diseases known [26]. However, results obtained from the present study and those from Aki *et al.* and Zhang *et al.* [12, 13] showed that the heat shock protein may also responsible for the TH₂-associated allergic disorders. It is interesting to define further epitopes on heat shock proteins as well as the possible regulation mechanism(s) responsible for different immune responses associated with heat shock proteins. Recently, due to its adjuvant-free helper effect, heat shock proteins have also been considered as carrier molecules in the design of vaccine constructs [30]. The fact that the heat shock protein may also be a potential allergen as shown in the present study should draw an attention on such strategies.

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