



MOLECULAR CLONING OF MAJOR AND MINOR ALLERGENS OF *ALTERNARIA ALTERNATA* AND *CLADOSPORIUM HERBARUM*

GERNOT ACHATZ,* HANNES OBERKOFER,* ERICH LECHENAUER,*
 BIRGIT SIMON,* ANDREA UNGER,* DORIS KANDLER,*
 CHRISTOPH EBNER,† HANSJÖRG PRILLINGER,‡ DIETRICH KRAFT†
 and MICHAEL BREITENBACH*§

*Institute of Genetics and General Biology, University of Salzburg, Austria; †Institute of General
 and Experimental Pathology, AKH, University of Vienna, Austria; and

‡Institute of Applied Microbiology, University of Bodenkultur, Vienna, Austria

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Abstract—The two moulds, *Alternaria alternata* and *Cladosporium herbarum*, are recognized as major causes of fungal allergies. Cloning, sequencing and heterologous expression of the allergens of the two moulds is a necessary step in understanding fungal allergy and in the development of new and improved methods of diagnosis and therapy. The seven new mould allergens presented here represent four new allergen proteins: aldehyde dehydrogenase (ALDH), enolase, YCP4 (previously found as a *Saccharomyces cerevisiae* protein of unknown function), and the acidic ribosomal protein, P2. Three of them (ALDH, YCP4 and P2) were found to be allergens in both fungi, *Alternaria* and *Cladosporium*. All allergens found so far are cytoplasmic proteins and are rather well conserved in evolution even when comparing distant species. Most of the allergens have “household” functions (ALDH, enolase). One allergen (P2) is a homolog of a very highly conserved human lupus erythematosus (LE) antigen. None of the fungal allergens is clearly related to other known non-fungal allergens.

Key words: *Alternaria*, *Cladosporium*, recombinant allergens, aldehyde dehydrogenase, enolase, ribosomal protein P2.

INTRODUCTION

The number of fungal species occurring in the environment is estimated to be at least 100,000 (Ainsworth, 1971). Most of these fungal species can be propagated by airborne spores and therefore constitute potential respiratory allergens. However, only about 10–20 fungal species have been shown to be allergenic (Karlsson-Borgå *et al.*, 1989). For most of those, diagnostic kits are available (Karlsson-Borgå *et al.*, 1989). The two deuteromycetes (fungi imperfecti), *Alternaria alternata* and *Cladosporium herbarum*, belong to the most important known allergenic moulds, because they occur ubiquitously on all continents (Webster, 1986) and because patients have been found to be sensitized to these moulds most frequently (Al-Doory and Domson, 1984). The two moulds differ in that *Alternaria alternata* is predominant in warm and humid climates (for instance, in the southern parts of the U.S.A.) while *Cladosporium herbarum* is the leading allergenic mould in cooler climates, for instance, in Scandinavia (Al-Doory and Domson, 1984; Karlsson-Borgå *et al.*, 1989). The incidence of mould sensitization was reported to be about 10% relative to the total number of type-I-allergic

patients (Karlsson-Borgå *et al.*, 1989 and literature cited therein). This will be compared to the numbers found in our patient collective.

Surprisingly, characterization of allergenic proteins from both *Alternaria alternata* and *Cladosporium herbarum* up to now has not led to a universally accepted picture of the major and minor allergens. The problems leading to this situation are: (i) fungus identification (several geographical subtypes of each species may exist, Karlsson-Borgå *et al.*, 1989); (ii) standardization of growth conditions (to be dealt with in the results section of this paper); and (iii) standardization of extraction procedures. Especially, no complete protein or cDNA sequence of any allergen of the two moulds has been published. Alt I, the major allergen of *Alternaria alternata*, has been purified by several research groups (Yunginger, 1980; Paris *et al.*, 1991; Deards and Montague, 1991) and a partial N-terminal protein sequence has been published in abstract form (Portnoy *et al.*, 1994).

Given the importance of the two moulds mentioned above and the unclear situation with respect to classical allergen characterization, it seemed highly desirable to us to clone and sequence cDNAs coding for the major and minor allergens of the two moulds. Cloning, sequencing and recombinant production of allergens seems to be a prerequisite to improvements in both diagnosis and

§Author to whom correspondence should be addressed.

therapy. We are reporting here seven complete sequences of cDNAs coding for major and minor allergens of *Cladosporium herbarum* and *Alternaria alternata*. These are the first complete cDNA and derived protein sequences of *Cladosporium* and *Alternaria* allergens. We are presenting evidence that the seven fungal allergens are very probably cytoplasmic, non-glycosylated proteins.

MATERIALS AND METHODS

Chemicals

All chemicals used, unless otherwise stated, were obtained from Sigma (St Louis, MO, U.S.A.) and were of the highest available purity. Enzymes were obtained from Boehringer (Mannheim, Germany). Radiochemicals were obtained from NEN (Vienna, Austria) unless otherwise stated.

Sera

Sera from 194 patients allergic to moulds were selected for this study. About 60% of them had positively tested to *Alternaria* or *Cladosporium* in RAST, the others had positively tested to a mould allergen mixed RAST (Pharmacia, Uppsala, Sweden). The sera were supplied by three Austrian allergy clinics: (i) Allergieambulatorium Reumannplatz, Vienna: 133 sera; (ii) Lungenambulanz Krankenhaus Lainz, Vienna: 38 sera; (iii) Allergieambulanz Landeskrankenhaus Salzburg: 23 sera. None of the patients had received hyposensitization treatment before testing was performed. For cloning purposes, a mixture of sera from two patients (T.G., G.K.) was used, who recognized the desired major and minor allergens of both *Cladosporium* and *Alternaria*. Sera from two healthy persons with negative case history of any type I allergy served as controls.

Cultivation of *Cladosporium herbarum*

Cladosporium herbarum (strain collection number: 28-0202) was obtained from the microbiological strain collection of the Institute of Botany, University of Regensburg, Germany (courtesy of Prof. Helmuth Bezel), and was cultivated either on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) at 23°C for 5 days or on liquid YPD media for 7 days. The identity of this material was verified using standard microbiological criteria (Brandenburger, 1985). Protein extracts obtained after both cultivation methods yielded identical immune blots (data not shown). Liquid cultivation was preferable for the isolation of mRNA due to absence of interference by agar particles. Fungal mats were inspected by light microscopy to ensure the presence of both spores and mycelia and were harvested by transferring the fungal mat to a falcon tube, or by centrifugation of the liquid culture at 5000 rpm for 10 min.

For comparison, lyophilized *Cladosporium* mycelium (containing spores) was obtained from Allergon (Ängelholm, Sweden), extracted and analysed for the presence of mRNA and allergenic proteins as above.

Cultivation of *Alternaria alternata*

Alternaria alternata was obtained from the Institut für Gärungsgewerbe, Technical University of Berlin (strain collection number: 08-0203, courtesy of Prof. Ulf Stahl) and was cultivated on YPD plates for 3 days at 28°C. After 3 days on YPD, both protein and mRNA extractions were performed. Cultures older than 3 days gradually became pigmented and only degraded mRNA could be isolated. Growth of *Alternaria alternata* on liquid YPD was found to be very slow and was therefore not used for the present work. Inspection of the fungal mat and harvesting procedures were comparable to the methods described for *Cladosporium herbarum*. Again, the strain was typed by standard microbiological criteria (Brandenburger, 1985).

The fungal material of *Alternaria alternata* as obtained from Allergon yielded nearly no mRNA. Therefore, it was not used for the present study. However, in one experiment the IgE-reactive proteins present in the commercial material were directly compared with those of the material grown in our laboratory.

Protein extraction

After determination of the wet weight, the fungal material was ground in a mortar at liquid nitrogen temperature (*Alternaria alternata*) or under addition of dry ice (*Cladosporium herbarum*). Ground mycelium (100 mg) and spores were then mixed with 1 ml extraction buffer (10 mM K_2HPO_4 , 2 mM EDTA, 3 mM NaN_3 , 10 μ l/ml PIM); (PIM: 0.1 M benzamidine, 0.2 mg/ml aprotinin, 0.2 mg/ml leupeptin, 10 mg/ml bacitracin, 0.1 mg/ml antipain, 70 μ g/ml pepstatin A) and stirred overnight at 4°C. After centrifugation, the supernatant was used for SDS-PAGE (*Cladosporium herbarum*). The supernatant obtained from *Alternaria alternata*, before SDS-PAGE, was dialysed overnight against 10 mM K_2HPO_4 (pH = 7.0) containing 3 mM NaN_3 . Protein concentration was measured with the Bio-Rad protein assay according to Bradford (described in Ausubel *et al.*, 1990).

Immune blots

Protein extract (total 10 μ g) was loaded on a 12% or 15% SDS-polyacrylamide gel (Ausubel *et al.*, 1990), electrophoresed for 1 hr at 150 mA, and stained with Coomassie brilliant blue (Ausubel *et al.*, 1990). For immunoblot analysis, the same amount of protein was loaded on the gel, electrophoresed as above and transferred to Immobilon-P (Millipore Corp., Bedford, MA) by electroblotting (Bio-Rad) according to the manufacturer's instructions. After blotting, the air-dried membranes were cut into 5 mm strips and blocked with buffer A (50 mM sodium phosphate buffer pH = 7.5, 0.5% (w/v) Tween 20, 0.5% (w/v) BSA, 0.05% (w/v) NaN_3) for 1 hr. The strips were then incubated overnight at 4°C in patients' sera diluted 1:10 with buffer A. After washing three times for 15 min with buffer A, the strips were incubated in 1 ml of buffer A containing 2 nCi ^{125}I -labeled rabbit anti-human IgE antibodies (Phadebas RAST,

Pharmacia, Uppsala, Sweden) overnight. After RAST incubation, the blots were washed with buffer A three times for 15 min. The blot was then exposed for 3 days to Hyperfilm (Amersham, U.K.).

Isolation of poly(A)⁺ mRNA from Cladosporium herbarum

After centrifugation, the mycelium was resuspended in homogenization buffer (4.2 M guanidinium isothiocyanate, 100 mM HEPES pH=8.3, 2% β -mercaptoethanol, 5 mM EDTA) and homogenized using an Ultra-Turrax apparatus (Janke and Kunkel KG, Staufen, Germany). The homogenate was centrifuged and extracted with PCI (phenol/chloroform/isoamyl alcohol; 24/24/1; v/v/v) to remove lipids, the aqueous phase was again extracted with water-saturated phenol, and finally with chloroform/isoamyl alcohol. RNA was precipitated with isopropanol, centrifuged, and the pellet was resuspended in homogenization buffer, layered over a 5.7 M cesium chloride solution and subjected to ultracentrifugation at 120,000g for 16 hr. Poly(A)⁺ RNA was isolated from total RNA using oligo(dT)-cellulose chromatography (Ausubel *et al.*, 1990).

Isolation of poly(A)⁺ RNA from Alternaria alternata

The fungal mat was lifted from the Petri plate and agar particles were carefully removed. The material was resuspended in homogenization buffer and homogenized with an UltraTurrax apparatus as described above. After PCI extraction, phenol extraction, chloroform extraction, and isopropanol precipitation, the pellet was resuspended in DEPC (diethyl pyrocarbonate)-water. The pellet always contained a large amount of carbohydrates. Therefore, before precipitation of the total RNA, the carbohydrates were precipitated by addition of two volumes of ethanol and 1/10 volume of 3M NaCl for 15 sec at room temperature. After centrifugation (10 sec, 14,000 rpm) the RNA was precipitated overnight at -20°C by adding another three volumes of ethanol. Poly(A)⁺ RNA was isolated from total RNA by use of oligo(dT)-cellulose chromatography.

cDNA library construction

The cDNA library was prepared from *Cladosporium herbarum* and *Alternaria alternata* mRNA in the λ -ZAP (Stratagene, La Jolla, CA) expression vector (Short *et al.*, 1988). The library was constructed according to the standard Stratagene protocol. A primary library of 1×10^6 recombinant phages was amplified to generate a higher titer of 10^9 pfu.

Immunoscreening of the library

The libraries were screened with a mixture of two patients' sera (see above). About 1×10^5 phages were used to infect the *E. coli* strain XL-1blue, plated on LB-medium plus 10 mM MgSO₄ and incubated for 4 hr at 42°C. Nitrocellulose filters pre-soaked with 10 mM IPTG (isopropyl thiogalactoside) were used to induce the synthesis of the fusion proteins. The proteins were

allowed to bind to the membranes by a further incubation at 37°C for 3 hr. The filters were blocked for 45 min with buffer A. After overnight incubation of the filters at 4°C with the serum mixture mentioned above (diluted 1:10 in buffer A), positive plaques were visualized by binding of ¹²⁵I-labeled rabbit anti-human IgE antibody (diluted 1:6 with buffer A) and autoradiography as described above for the immune blots.

Plaque purification, in vivo excision and expression of recombinant fusion allergens

Positive plaques were picked with a Pasteur pipette and purified by repeating the above procedure. Pure phages obtained after the secondary screening were treated according to the *in vivo* excision protocol to subclone them into pBluescript SK+ (Stratagene). To induce gene expression of the cDNA insert, the *E. coli* cells at O.D.₆₀₀ = 0.2 were treated with 5 mM IPTG and grown for 5 hr. Subsequently, *E. coli* cells were isolated by centrifugation and the pellet was boiled in SDS-buffer (62 mM Tris, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 25 mM bromophenol blue) for 10 min. After centrifugation, the supernatant was separated by SDS-PAGE and immune blots were performed as described above.

Sequencing of the cDNA inserts

The pBluescript SK+ plasmids containing the recombinant cDNA inserts were isolated and purified according to a standard plasmid preparation protocol (Ausubel *et al.*, 1990). The inserts were sequenced by dideoxy chain termination method (Sanger *et al.*, 1977) using the T7-sequencing kit (Pharmacia).

RESULTS

Incidence of allergies against Cladosporium and Alternaria

The total number of patients' sera used in this study was 194 (pre-selected according to RAST as mentioned above), of which 128 originated from one allergy clinic in Vienna. Among those, the number of patients with IgE reactivity towards *Alternaria* (as tested in immune blots) was 98 and towards *Cladosporium*, 62 (48 patients reacted to both *Cladosporium* and *Alternaria*). Therefore, 112 of 128 sera reacted to either *Cladosporium* or *Alternaria*, and 16 sera were unreactive in immune blots. The 112 patients represent roughly 1% of all patients treated in the allergy clinic during the course of one year. This estimate is probably a serious underestimate, since no systematic testing with *Alternaria* or *Cladosporium*-specific RAST was undertaken. Rather, these tests were performed only if the case history indicated a possibility of allergy to moulds.

Construction and screening of the cDNA expression libraries

Isolation and purification of mRNA, cDNA synthesis and library construction were all described in the Materials and Methods section. About 1×10^6 plaques (200,000 plaques per large Petri dish) were screened out

of each of the two amplified libraries. A total of 150 positive clones were identified by screening each of the two libraries with a serum mixture of two patients (see Materials and Methods). After *in vivo* excision of 12 randomly chosen clones, plasmids were isolated and digested with EcoRI/XhoI, and insert sizes were analysed on an agarose gel (data not shown). In the case of *Cladosporium herbarum* four different types of inserts could be detected. In the case of *Alternaria alternata* five different inserts were cloned. In order to confirm that the recombinant phages isolated in this way indeed expressed fusion proteins reactive with the IgE used for screening of the libraries, the fusion proteins were induced, separated by SDS-PAGE and tested in immune blots as described in the Materials and Methods section. All 24 recombinant phage clones could be induced to produce a recombinant fusion protein reactive to patients' IgE (data not shown).

Analysis of the cDNA inserts

Seven different inserts (four of *Cladosporium* and three of *Alternaria*) were sequenced (for the most part on both strands) and shown to contain the complete open reading frames and in addition varying amounts of 5'-untranslated sequence (10–100 nt) as well as 3'-"tails" (70–200 nt). In those cases where the primary cDNA clones were incomplete at their 5'-ends (two of the seven clones), the primary cDNAs were used to screen the libraries again by hybridization and additional clones were isolated that contained at least the complete reading frame of the corresponding protein. The biochemical identity of all seven encoded proteins was deduced by

sequence comparisons as shown in detail below. To be able to correlate the cloned allergens with bands recognized by patients' sera in raw extracts, all seven open reading frames were expressed in *E. coli* as recombinant non-fusion proteins (to be published elsewhere) and their electrophoretic mobilities were compared to those of the natural allergens. The seven allergens were named according to the accepted rules for allergen nomenclature and will be described in the following paragraphs. Basically, the allergens are numbered (e.g. Alt a 1) according to the frequency of patients recognizing the specific allergen. A prerequisite for using this type of allergen nomenclature is that the allergen be sufficiently well characterized by protein purification or sequence analysis. Not all of the *Alternaria* and *Cladosporium* allergens (including the most prominent allergens of both fungi) have been cloned. In total, nine protein bands binding patients' IgE in *Cladosporium* extracts and ten protein bands in *Alternaria* extracts were recognized. Table 1 shows an overview of all the cloned and not yet cloned allergens. We are including the not yet cloned allergens in the table for two reasons: to document the percentage of the patients who recognize a specific allergen and to explain the rationale for the allergen numbers we are using in the present communication.

The allergen, Alt a 1, has been well characterized previously (Yunginger, 1980; Paris *et al.*, 1991; Deards and Montague, 1991; Portnoy *et al.*, 1994). In our collective of patients allergic to *Alternaria*, this was found to be the most frequently recognized allergen. A protein band of about 30 kDa was seen on immune blots of unreduced

Table 1. Summary of all allergens of *Alternaria alternata* and *Cladosporium herbarum* that are recognized by our patients.

kD (biological function)	<i>Alternaria alternata</i>			<i>Cladosporium herbarum</i>		
	Allergen	% of Patients	Cloned	Allergen	% of Patients	Cloned
110 red.	—	—	—	Cla h 8	11	—
100 red.	—	—	—	Cla h 9	6	—
85 red.	Alt a 3	42	—	—	—	—
63 red.	Alt a 4	37	—	—	—	—
53 red.	Alt a 10	2	+	Cla h 3	36	+
(ALDH)						
48 red.	—	—	—	Cla h 6	20	+
(enolase)						
45 red.	Alt a 2	47	—	Cla h 2	43	—
42 red.	Alt a 9	5	—	Cla h 7	17	—
39 red.	Alt a 8	7	—	—	—	—
30 non-red	Alt a 1	80	—	—	—	—
30 red.	Alt a 5	33	—	Cla h 1	61	—
22 red.	Alt a 7	7	+	Cla h 5	22	+
(homolog to YCP4 yeast protein)						
11 red.	Alt a 6	8	+	Cla h 4	22	+
(P2 ribosomal protein)						

With the exception of Alt a 1, all allergens can be detected in an immune blot under reducing conditions. In total, ten IgE-reactive proteins could be detected in *Alternaria*, three of which have been cloned. Nine IgE-reactive proteins could be detected in *Cladosporium*, four of which have been cloned. Five allergens co-migrate and could be closely related in both moulds. Three of them (ALDH, YCP4 and P2) not only co-migrate in *Alternaria* and *Cladosporium*, but could be shown by cDNA cloning and sequencing to be very probably functionally closely related.

Alternaria extracts separated on SDS-PAGE. However, most of these patients (including the two patients whose sera were used for cloning) did not recognize the expected doublet of bands at around 15 kDa in immune blots of reduced protein extracts. This would point to (a) conformational epitope(s) relevant for IgE binding to Alt a 1 and would explain why our patients sera did not recognize recombinant plaques which express, most probably only one of the two subunit chains. This might be the reason why the two subunits of Alt a 1 were not cloned in the present work.

The allergens Alt a 10 (M_r =53 kDa, EMBL accession number: X78227) of *Alternaria alternata* and Cla h 3 (M_r =53 kDa, EMBL accession number: X78228) of *Cladosporium herbarum* are aldehyde dehydrogenases (ALDHs). The cDNA of Alt a 10 has a length of 1640 bp (Fig. 1). The correct open reading frame was identified by start and stop codons in the nearly correct consensus context (Kozak, 1984; Birnstiel *et al.*, 1985) and by sequence comparison with the highly conserved sequence of *Aspergillus fumigatus* ALDH (Fig. 3). The deduced protein sequence spans 496 amino acids corresponding to a calculated M_r of 53 kDa. The cDNA of Cla h 3 (Fig. 2) has a length of 1661 nt. The open reading frame (identified as above) spans a region of 1491 nt corresponding to a protein of 497 amino acids corresponding to 53 kDa. The two proteins co-migrate and show the expected electrophoretic mobility in SDS-PAGE (see Figs 12 and 13). On protein level, Alt a 10 and Cla h 3 are 77% identical and share a homology of 86%. Sequence comparisons of the two cDNAs with the SWISS-PROT database revealed significant homologies with ALDHs of a wide variety of species using the Bestfit program of the GCG software package. *Aspergillus fumigatus* ALDH yielded the highest score of identity and homology. A triple sequence comparison is shown in Fig. 3. Alt a 10 (ALDH) was recognized by IgE of 2% of the patients with positive immune blots of *Alternaria alternata* extracts. The corresponding figure of Cla h 3 was 36%. Cla h 3 can therefore be classified as an important allergen of *Cladosporium herbarum*. The consensus sequences of eukaryotic protein coding genes in their 5'-non-coding region (CCAGCCATG) as described by Kozak (1984) were compared with the 5'-non-coding regions of Alt a 10 and Cla h 3. A weak to moderate conformity was found. The plant consensus (GCCATCATG; Lütcke *et al.*, 1987) cannot be found in the case of AltA53 and ClaH53. The sequences also contain a polyadenylation signal (Birnstiel *et al.*, 1985) which is in good agreement with the universal consensus sequence (AATAAA).

The allergens Alt a 6 (M_r =11 kDa, EMBL accession number: X78222) of *Alternaria alternata* and Cla h 4 (M_r =11 kDa, EMBL accession number: X78223) of *Cladosporium herbarum* are ribosomal proteins (P2). The cDNA of Alt a 6 has a length of 557 bp (Fig. 4). The correct open reading frame of 113 amino acids was identified as above by sequence comparisons (Fig. 6). As above, comparisons with the accepted consensus sequences in the 5'- and 3'-untranslated regions were included. The cDNA of Cla h 4 has a length of 640 bp and

encodes a protein of 111 amino acids (Fig. 5). Alt a 6 and Cla h 4 are 75% identical (85% homologous) at the protein level. Multiple sequence alignment shows that the two sequences share high homology with the human acidic ribosomal phosphoprotein P2 (Rich and Steitz, 1987; see Fig. 6). P2 is a member of a family of three human acidic ribosomal phosphoproteins (P-proteins), P0, P1 and P2, which are well known lupus erythematoses antigens (see Discussion). Cla h 4 was recognized by 22% of the patients showing positive immune blots with *Cladosporium* extracts, Alt a 6 was recognized by 8% of the patients showing positive immune blots with *Alternaria* extracts. The allergens Alt a 7 (M_r =22 kDa, EMBL accession number: X78225) of *Alternaria alternata* and Cla h 5 (M_r =22 kDa, EMBL accession number: X78224) of *C. herbarum* show significant homologies to the YCP4 gene of *Saccharomyces cerevisiae*. The cDNA of Alt a 7 has a length of 940 bp (Fig. 7). The correct open reading frame of 205 amino acids was identified as above by sequence comparisons. The cDNA of Cla h 5 has a length of 892 bp and encodes a protein of 205 amino acids (Fig. 8). Alt a 7 and Cla h 5 are 65% identical (81% homologous) at the protein level. Multiple sequence alignment shows that the two sequences share high aa sequence homology (70%) with the YCP4 protein of *Saccharomyces cerevisiae*. YCP4 in *Saccharomyces cerevisiae* was identified by genome sequencing (Biteau *et al.*, 1992) and has no known function. Nevertheless, the sequences of the three known members of the YCP4 "family" do not show any hydrophobic domains or targeting signals making it very probable that YCP4 is a cytoplasmic protein like the other allergens presented here. It is a non-essential yeast gene. Alt a 7 was recognized by 7% of our patients with positive immune blots to *Alternaria* extracts, whereas Cla h 5 was recognized by 22% of the respective patients. The allergen Cla h 6 (M_r =47 kDa, EMBL accession number: X78226), of *C. herbarum* is an enolase. The cDNA of Cla h 6 has a length of 1451 bp (Fig. 10). The correct open reading frame of 441 amino acids was identified as above by sequence comparisons (Fig. 11). The best match with the deduced amino acid sequence of Cla h 6 was *Saccharomyces cerevisiae* enolase 1 (73% amino acid identity and 83% amino acid similarity). In *Saccharomyces cerevisiae*, two very closely related enolases exist, one of which (enolase 1) is a heat shock protein (Iida and Yahara, 1984). It is impossible to decide by sequence comparison alone whether Cla h 6 is also a heat shock protein. Further experiments are clearly needed to clarify this point. The enolases of several mammals and of *Drosophila* also are highly homologous to Cla h 6 (not shown). Cla h 6 is a typical minor allergen of *C. herbarum* as it is recognized by IgE of 20% of all patients with a positive *Cladosporium* immune blot. Enolase (47 kDa) migrates slightly faster than ALDH corresponding to about 53 kDa (Fig. 13). The situation is complicated by the fact that in *Cladosporium* extract another major allergen that migrates at 45 kDa (Cla h 2) is also present. Despite the very high homology of all known eukaryotic enolases, we were not able to show that *Alternaria* enolase

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-58
GGCACGAGGAGAACTAGTCTCGAGTTTCTTCATATACCCCAATTGTTTTCACAGCT
1 / 1 31 / 11
ATG ACA TCT GTA AAG CTC TCA ACC CCT CAG ACG GGC GAG TTC GAG CAG CCC ACC GGA CTC
Met Thr Ser Val Lys Leu Ser Thr Pro Gln Thr Gly Glu Phe Glu Gln Pro Thr Gly Leu
61 / 22 91 / 31
TTC ATC AAC AAT GAG TTC GTA AAG GCT GTT GAC GGC AAG ACC TTT GAT GTT ATC AAC CCC
Phe Ile Asn Asn Glu Phe Val Lys Ala Val Asp Gly Lys Thr Phe Asp Val Ile Asn Pro
122 / 41 151 / 51
TCC ACT GAG GAG GTC ATC TGC AGT GTT CAG GAG GCC ACC GAG AAG GAT GTT GAC ATT GCT
Ser Thr Glu Glu Val Ile Cys Ser Val Gln Glu Ala Thr Glu Lys Asp Val Asp Ile Ala
181 / 61 221 / 71
GTT GCT GCC GCC CGC AAA GCC TTC AAC GGC CCA TGG gCA AAG GAG ACA CCA GAG AAC AGG
Val Ala Ala Ala Arg Lys Ala Phe Asn Gly Pro Trp Ala Lys Glu Thr Pro Glu Asn Arg
241 / 81 271 / 91
GGA AAG CTG CTT AAC AAG CTT GCC GAT CTG TTC GAG AAG AAT GCC GAC CTC ATT GCT GCT
Gly Lys Leu Leu Asn Lys Leu Ala Asp Leu Phe Glu Lys Asn Ala Asp Leu Ile Ala Ala
301 / 101 331 / 111
GTC GAG GCT CTC GAC AAC GGC AAG GCC TTC AGC ATG GCC AAG AAC GTC GAT GTT CCC GCC
Val Glu Ala Leu Asp Asn Gly Lys Ala Phe Ser Met Ala Lys Asn Val Asp Val Pro Ala
361 / 122 391 / 131
GCC GCT GGT TGC CTG AGG TAC TAC GGA GGA TGG GCC GAC AAG ATT GAG GGC AAG GTC GTC
Ala Ala Gly Cys Leu Arg Tyr Tyr Gly Gly Trp Ala Asp Lys Ile Glu Gly Lys Val Val
422 / 141 451 / 151
GAC ACA GCA CCC GAC AGC TTC AAC TAC ATC cGC AAG AGC CTA TTG GTG TTT GCG GTC AGA
Asp Thr Ala Pro Asp Ser Phe Asn Tyr Ile Arg Lys Ser Leu Leu Val Phe Ala Val Arg
481 / 161 511 / 171
TCA TCC ATG GAA CTT CCT ATT CTC ATG TGG TCA TGG AAG ATT GGT CCT GCC ATC GCC ACT
Ser Ser Met Glu Leu Pro Ile Leu Met Trp Ser Trp Lys Ile Gly Pro Ala Ile Ala Thr
541 / 181 571 / 191
GGT AAC ACC GTC GTC CTG AAG ACT GCT GAG CAG ACA CCT CTC TCC GCA TAC ATT GCC TGC
Gly Asn Thr Val Val Leu Lys Thr Ala Glu Gln Thr Pro Leu Ser Ala Tyr Ile Ala Cys
601 / 201 631 / 221
AAG CTG ATC CAG GAG GCC GGT TTC CCA CCA GGT GTC ATC AAC GTC ATC ACT GGT TTC GGA
Lys Leu Ile Gln Glu Ala Gly Phe Pro Pro Gly Val Ile Asn Val Ile Thr Gly Phe Gly
661 / 222 691 / 231
AAG ATC GCC GGT GCT GCC ATG TCC GCT CAC ATG GAC ATT GAC AAG ATT GCC TTT ACT GGT
Lys Ile Ala Gly Ala Ala Met Ser Ala His Met Asp Ile Asp Lys Ile Ala Phe Thr Gly
722 / 241 751 / 251
TCA ACC GTT GTC GGC CGT CAA ATC ATG AAG TCT GCC GCT GGC TCC AAC TTG AAG AAG GTC
Ser Thr Val Val Gly Arg Gln Ile Met Lys Ser Ala Ala Gly Ser Asn Leu Lys Lys Val
781 / 261 811 / 271
ACT CTT GAG CTC GGA GGC AAG AGC CCC AAC ATT GTC TTC GCC GAC GCA GAT CTT GAC GAG
Thr Leu Glu Leu Gly Gly Lys Ser Pro Asn Ile Val Phe Ala Asp Ala Asp Leu Asp Glu
841 / 281 871 / 291
GCT ATC CAC TGG GTC AAC TTT GGT ATT TAC TTC AAC CAC GGA CAG GCT TGT TGT GCT GGT
Ala Ile His Trp Val Asn Phe Gly Ile Tyr Phe Asn His Gly Gln Ala Cys Cys Ala Gly
901 / 301 931 / 311
TCG CGT ATC TAC GTC CAA GAA GAG ATC TAC GAC AAG TTC ATC CAG CGC TTC AAG GAG CGG
Ser Arg Ile Tyr Val Gln Glu Glu Ile Tyr Asp Lys Phe Ile Gln Arg Phe Lys Glu Arg
961 / 322 991 / 331
GCT GCT CAG AAC GCT GTT GGT GAC CCA TTC GCC GCG ACA CTC CAG GGT CCT CAA GTC TCG
Ala Ala Gln Asn Ala Val Gly Asp Pro Phe Ala Ala Thr Leu Gln Gly Pro Gln Val Ser
1022 / 341 1051 / 351
CAG CTC CAG TTC GAC CGT ATC ATG GGC TAC ATC GAG GAG GGC AAG AAG TCT GGC GCG ACC
Gln Leu Gln Phe Asp Arg Ile Met Gly Tyr Ile Glu Glu Gly Lys Lys Ser Gly Ala Thr
1081 / 361 1111 / 371
ATC GAG ACT GGT GGC AAC CGT AAG GGT GAC AAG GGT TAC TTC ATC GAG CCC ACA ATC TTC
Ile Glu Thr Gly Gly Asn Arg Lys Gly Asp Lys Gly Tyr Phe Ile Glu Pro Thr Ile Phe
1141 / 381 1171 / 391
TCC AAC GTA ACC GAG GAC ATG AAG ATT CAG CAA GAA GAG ATC TTC GGC CCC GTC TGC ACA
Ser Asn Val Thr Glu Asp Met Lys Ile Gln Gln Glu Glu Ile Phe Gly Pro Val Cys Thr
1201 / 401 1231 / 411
ATC TCC AAG TTC AAG ACA AAG GCC GAC GTC ATC AAG ATT GGC AAC AAC ACC ACA TAC GGT
Ile Ser Lys Phe Lys Thr Lys Ala Asp Val Ile Lys Ile Gly Asn Asn Thr Thr Tyr Gly
1261 / 422 1291 / 431
Ctt tcG GCC GCT GTA CAC ACA TCC AAC CTC ACC ACT GCC ATC GAA GTT GCC AAC GCG CTC
Leu Ser Ala Ala Val His Thr Ser Asn Leu Thr Thr Ala Ile Glu Val Ala Asn Ala Leu
1322 / 441 1351 / 451
CGT GCA GGA ACT GTC TGG GTC AAC TCC TAC AAC ACT CTT CAC TGG CAG CTT CCC TTC GGA
Arg Ala Gly Thr Val Trp Val Asn Ser Tyr Asn Thr Leu His Trp Gln Leu Pro Phe Gly
1381 / 461 1411 / 471
GGG TAC AAG GAG TCT GGT ATT GGG CGC GAG TTG GGA GAG GCG GCG CTG GAC AAC TAC ATC
Gly Tyr Lys Glu Ser Gly Ile Gly Arg Glu Leu Gly Glu Ala Ala Leu Asp Asn Tyr Ile
1441 / 481 1471 / 491
CAG ACC AAG ACC GTG TCT ATT CGT CTT GGC GAT GTT CTG TTC GGT TAA
Gln Thr Lys Thr Val Ser Ile Arg Leu Gly Asp Val Leu Phe Gly OCH
1489
GCGCTATGCTGGTTTTTTAGCGAAGGATCTCATAAGTACTATGTATAGCTCAAGTAGTCATGA GGATCTAAATGAATGT
AAAGAATTATCAACAn 1582

```

Fig. 1. Complete cDNA sequence and derived protein sequence of Alt a 10 (ALDH). EMBL Accession number: X78227.

-69
CACGAGGTTGAGGTTGTGCCATTGCCCTCTCTGTTGATACCCCAACAACGAGACACATCAC TTCAAT
1 / 1 31 / 11
ATG ACT TCC GTC CAG CTT GAG ACG CCG CAC TCC GGC AAG TAC GAG CAG CCG ACC GGC CTC
Met Thr Ser Val Gln Leu Glu Thr Pro His Ser Gly Lys Tyr Glu Gln Pro Thr Gly Leu
61 / 21 91 / 31
TTC ATC AAC AAC GAG TTC GTC AAG GGC CAA GAA GGC AAG ACC TTC GAT GTC ATC AAC CCC
Phe Ile Asn Asn Glu Phe Val Lys Gly Gln Glu Gly Lys Thr Phe Asp Val Ile Asn Pro
121 / 41 151 / 51
TCC GAC GAG AGC GTG ATC ACC CAG GTC CAC GAG GCC ACC GAG AAG GAT GTC GAC ATC GCC
Ser Asp Glu Ser Val Ile Thr Gln Val His Glu Ala Thr Glu Lys Asp Val Asp Ile Ala
181 / 61 211 / 71
GTC GCC GCC GCC CGC CAA GCC TTC GAG GGC TCA TGG AGA CTG GAG ACA CCC GAG AAC CGT
Val Ala Ala Ala Arg Gln Ala Phe Glu Gly Ser Trp Arg Leu Glu Thr Pro Glu Asn Arg
241 / 81 271 / 91
GGC AAG CTG CTC AAC AAC CTC GCC AAC CTG TTC GAG AAG AAC ACT GAC CTC CTT GCT GCC
Gly Lys Leu Leu Asn Asn Leu Ala Asn Leu Phe Glu Lys Asn Thr Asp Leu Leu Ala Ala
301 / 101 331 / 111
GTT GAG TCG CTC GAC AAC GGC AAG GCC ACT TCC ATG GCA AGG GtG ACA TCA GCA TGT GCG
Val Glu Ser Leu Asp Asn Gly Lys Ala Thr Ser Met Ala Arg Val Thr Ser Ala Cys Ala
361 / 121 391 / 131
TCC GGC TGC CTC AGA TAC TAC GGT GGT TGG GCG GAC AAG ATC ACC GGC AAG GTC ATC GAC
Ser Gly Cys Leu Arg Tyr Tyr Gly Gly Trp Ala Asp Lys Ile Thr Gly Lys Val Ile Asp
421 / 141 451 / 151
ACT ACG CCC GAC ACT TTC AAC TAC GTC AAG AAG GAG CCC ATT GGT GTT TGC CGG TCA GAT
Thr Thr Pro Asp Thr Phe Asn Tyr Val Lys Lys Glu Pro Ile Gly Val Cys Arg Ser Asp
481 / 161 511 / 171
CAT TCC CTG GAA CTT CCC CTT CTC ATG TGG GCA TGG AAG ATC GGC CCG GCC ATT GCT TGC
His Ser Leu Glu Leu Pro Leu Leu Met Trp Ala Trp Lys Ile Gly Pro Ala Ile Ala Cys
541 / 181 571 / 191
GGT AAC ACT GTC GTC CTG AAG ACT GCT GAG CAG ACC CCT CTT GGT GGT CTC GTC GCT GCC
Gly Asn Thr Val Val Leu Lys Thr Ala Glu Gln Thr Pro Leu Gly Gly Leu Val Ala Ala
601 / 201 631 / 211
AGC CTC GTC AAG GAG GCC GGT TTC CCT CCT GGTGGTC ATC AAC GTC ATT TCC GGT TTC GGC
Ser Leu Val Lys Glu Ala Gly Phe Pro Pro Gly Val Ile Asn Val Ile Ser Gly Phe Gly
661 / 221 691 / 231
AAG GTC GCT GGT GCC GCT CTC TCT TCT CAC ATG GAC GTC GAC AAG GTG GCC TTC ACC GGT
Lys Val Ala Gly Ala Ala Leu Ser Ser His Met Asp Val Asp Lys Val Ala Phe Thr Gly
721 / 241 751 / 251
TCC ACC GTT GTC GGC CGC ACA ATC CTC AAG GCT GCT GCC TCT TCC AAC TTG AAG AAG GTC
Ser Thr Val Val Gly Arg Thr Ile Leu Lys Ala Ala Ala Ser Ser Asn Leu Lys Lys Val
781 / 261 811 / 271
ACC CTC GAG CTC GGT GGC AAG TCA CCA AAC ATT GTC TTC GAG GAC GCC GAT ATT GAC AAC
Thr Leu Glu Leu Gly Gly Lys Ser Pro Asn Ile Val Phe Glu Asp Ala Asp Ile Asp Asn
841 / 281 871 / 291
GCC ATC TCA TGG GTC AAC TTC GGT ATC TTC TTC AAC CAC GGC CAG TGC TGC TGT GCT GGT
Ala Ile Ser Trp Val Asn Phe Gly Ile Phe Phe Asn His Gly Gln Cys Cys Cys Ala Gly
901 / 301 931 / 311
TCG CGT GTG TAC GTT CAG GAG AGC ATC TAC GAC AAG TTC GTC CAG AAG TTC AAG GAG CGC
Ser Arg Val Tyr Val Gln Glu Ser Ile Tyr Asp Lys Phe Val Gln Lys Phe Lys Glu Arg
961 / 321 991 / 331
GCA CAG AAG AAC GTT GTT GGC GAC CCC TTC GCC GCC GAC ACA TTC CAG GGT CCT CAG GTT
Ala Gln Lys Asn Val Val Gly Asp Pro Phe Ala Ala Asp Thr Phe Gln Gly Pro Gln Val
1021 / 341 1051 / 351
TCC AAG GTT CAG TTC GAC CGC ATT ATG GAG TAC ATC CAG GCC GGC AAG GAC GCC GGT GCC
Ser Lys Val Gln Phe Asp Arg Ile Met Glu Tyr Ile Gln Ala Gly Lys Asp Ala Gly Ala
1081 / 361 1111 / 371
ACC GTC GAG ACC GGT GGA AGC CGT AAG GGT GAC AAG GGC TAC TTC ATT GAG CCC ACC ATC
Thr Val Glu Thr Gly Gly Ser Arg Lys Gly Asp Lys Gly Tyr Phe Ile Glu Pro Thr Ile
1141 / 381 1171 / 391
TTC TCC AAC GTC ACA GAG GAC ATG AAG ATC GTG AAA GAG GAG ATC TTC GGC CCC GTC TGC
Phe Ser Asn Val Thr Glu Asp Met Lys Ile Val Lys Glu Glu Ile Phe Gly Pro Val Cys
1201 / 401 1231 / 411
TCG ATC GCC AAG TTC AAG ACC AAG GAG GAC GCC ATC AAG CTC GGC AAC GCC AGC ACA TAC
Ser Ile Ala Lys Phe Lys Thr Lys Glu Asp Ala Ile Lys Leu Gly Asn Ala Ser Thr Tyr
1261 / 421 1291 / 431
GGT CTC GCC GCC GCC GTC CAC ACC AAG AAC CTC AAC ACC GCC ATC GAG GTC TCC AAC GCT
Gly Leu Ala Ala Ala Val His Thr Lys Asn Leu Asn Thr Ala Ile Glu Val Ser Asn Ala
1321 / 441 1351 / 451
CTC AAG GCG GGC ACC GTC TGG GTC AAC ACT TAC AAC ACC CTC CAC CAC CAG ATG CCG TTC
Leu Lys Ala Gly Thr Val Trp Val Asn Thr Tyr Asn Thr Leu His His Gln Met Pro Phe
1381 / 461 1411 / 471
GGT GGC TAC AAG GAG TCC GGT ATT GGC CGT GAG CTC GGC GAG GAT GCG TTG GCC AAC TAC
Gly Gly Tyr Lys Glu Ser Gly Ile Gly Arg Glu Leu Gly Glu Asp Ala Leu Ala Asn Tyr
1441 / 481 1471 / 491
ACT CAG ACC AAG ACT GTT AGC ATT CGT CTC GGT GAC GCC CTC TTC GGT TAG
Thr Gln Thr Lys Thr Val Ser Ile Arg Leu Gly Asp Ala Leu Phe Gly AMB
1492
GCGTGTGAGGAGATGGATTAATGGGTGTGAAGCGGTTGAAGATCGTTTCGGTGTAAATT ACGTATGACGTTATGA
AATGAATAACAATGTGTACTGA_n 1592

Fig. 2. Complete cDNA sequence and derived protein sequence of Cla h 3 (ALDH). EMBL Accession number: X78228.

	1				50	
Alt a 10	MTSVKLS	TPq	TG...eFEQPT	GLFINNEFVK	aVDGKTDFVI	NPSLeEVIcs
Cla h 3	MTSVGL	TPh	sG...KYEQPT	GLFINNEFVK	GqEGKTDFVI	NPSdesVITq
AspALDH	.msdIfT	Tie	TpviKYEQPI	GLFINNEFVK	GVEGKTfqi	NPSnEkVITS
Consensus	--S----	T--	-----EQP-	GLFINNEFVK	---GKTF-VI	NPS-E-VI--
	51				100	
Alt a 10	VgEATEKD	VD	IAVAAARKAF	nGPWakETPE	NRGKLLNKLA	DLFEKNadLi
Cla h 3	VHEATEKD	VD	IAVAAARqAF	EGSWRIETPE	NRGKLLNnLA	nLFEKNdDL
AspALDH	VHEATEKD	VD	VAVAAARaAF	EGPWrgvTPS	erGiLiNKLA	LmErdidTL
Consensus	V-EATEKD	VD	-AVAAAR-AF	-G-W---TP-	-RG-L-N-LA	-L-E---D--
	101				150	
Alt a 10	AAVEaLDNGK	AFSMAKnVDv	paAaGCLRY	YGWADKIEGK	VVDtAPdsFN	
Cla h 3	AAVESLDNGK	AtSMAR.Vts	AcAsGCLRY	YGWADKIEGK	VIDTcPDFTN	
AspALDH	AAIESLDNGK	AftMAK.VDI	AnsiGCLRY	aGWADKIhGq	tIDTnPETLt	
Consensus	AA-E-LDNGK	A--MA--V--	----GCLRY	-GWADKI-G-	--DT-P----	
	151				200	
Alt a 10	YIRK.sliVf	avrsSMELPi	LMwSWKIGPA	IatGNTVVLK	TAEQTPLSAy	
Cla h 3	YVKEPIGVC	rsdhSLELPL	LMwWKIGPA	IACGNTVVLK	TAEQTPLggL	
AspALDH	YtRhEPVVC	gqiipwnFPL	LMwSWKIGPA	VaAGNTVVLK	TaQTPLSAL	
Consensus	Y-----V-	-----P-	LMW-WKIGPA	-A-GNTVVLK	TA-QTPL--	
	201				250	
Alt a 10	IaCKLIqEAG	FPPGVINVI	GFGKIAGAAM	SaHMDIDKIA	FTGSTVVGRRq	
Cla h 3	VAAslVKEAG	FPPGVINVIS	GFGKVAGAAL	SSHMDVDKVA	FTGSTVVGRR	
AspALDH	yAAKLIKEap	FPaGVINVIS	GFGrtAGAAi	SSHMDIDKVA	FTGSTVVGpT	
Consensus	-A--L--EA-	FP-GVINVI	GFG--AGAA-	S-HMD-DK-A	FTGST-VG--	
	251				300	
Alt a 10	IMKsAAgSNL	KKVTLELGGK	SPNIVFaDAD	lDeAihWVNF	GIYFNHQaC	
Cla h 3	ILKAAASNL	KKVTLELGGK	SPNIVFEDAD	IDNAISWVNF	GIFFNHQqCC	
AspALDH	ILqAAAKSNL	KKVTLELGGK	SPNIVFDDAD	IDNAISWanF	GIFFNHQqCC	
Consensus	I---AA-SNL	KKVTLELGGK	SPNIVF-DAD	-D-AI-W-NF	GI-FNHQ-QC	
	301				350	
Alt a 10	CAGSRIVQVE	eIYDKFIQRF	KERAaQNaVG	DPFAA.TLQG	PQVSQQLQFDR	
Cla h 3	CAGSRVYVQVE	sIYDKFVQKF	KERAQKNvVG	DPFAADITQG	PQVskvQFDR	
AspALDH	CAGSRILVQVE	giYDKFVaRF	KERAQKNkVG	nPFeqDTFQG	PQVSQQLQFDR	
Consensus	CAGSR--VQVE	-IYDKF--F	KERA--N-VG	-PF--T-QG	PQVS--QFDR	
	351				400	
Alt a 10	IMgYIeeGKK	sGATIEtGcN	RKGDKGYFIE	PTIFSNVTED	MKIqQEEIFG	
Cla h 3	IMEYIqaGKd	AGATVETGGS	RKGDKGYFIE	PTIFSNVTED	MKIvKEEIFG	
AspALDH	IMEYInhgKK	AGATVaTGGd	RhGneGYFIq	PTVftdVTsD	MKIaQEEIFG	
Consensus	IM-YI--GK-	-GAT--TGG-	R-G--GYFI-	PT-F--VT-D	MKI--EEIFG	
	401				450	
Alt a 10	PVCTISKEFKT	KADvIKIGNn	TTYGLsAAVH	TsNLtTAIEV	aNALrAGTVW	
Cla h 3	PVCsIaKFKT	KeDAIKIGNa	sTYGLAAAVH	TKNLNTAIEV	SNALKAGTVW	
AspALDH	PVvTIqKFKd	vAEAIKIGNs	TdYGLAAAVH	TKNvNTAIV	SNALKAGTVW	
Consensus	PV--I-KFK-	----IK-GN-	--YGL-AAVH	T-N--TAI-V	-NAL-AGTVW	
	451				500	
Alt a 10	VNsYNtLHWQ	LPFGGYKESG	IGRELGEaAL	DNYiQTKTVS	IRLGDvLFGZ	
Cla h 3	VNLYNtLhHQ	MPFGGYKESG	IGRELGEaAL	anyTQTKTVS	IRLGDALFGZ	
AspALDH	INhYNhisYQ	aPFGGFkqSG	IGRELGSyAL	ENYtQIKTVh	yRLGDALFa.	
Consensus	-N-YN---Q	-PFGG-K-SG	-GRELG--AL	-NY-Q-KTV-	-RLGD-LF--	

Fig. 3. Protein sequence alignment between Cla h 3, Alt a 10 and ALDH from *Aspergillus fumigatus*. Alt a 10 and Cla h 3 are 77% identical and share a homology of 86%. *Aspergillus* ALDH is about 80% homologous to the other two sequences.

is an *Alternaria* allergen. *Alternaria* enolase has not been cloned up to now.

Comparison of the recombinant allergens with the native allergens

We cloned all seven cDNAs as recombinant non-fusion allergens (to be described in detail elsewhere) in the expression vector pMW172, transformed the recombinant plasmids in the *E. coli* strain BL21 and induced with IPTG. The recombinant allergens are under control of the T7 promoter. IPTG induces the recombinant T7-polymerase in the *E. coli* strain BL21. The unpurified *E. coli* protein extracts after SDS-PAGE and blotting on nitrocellulose were incubated with the serum mixture of the two patients mentioned in the Materials and Methods section. Figures 12 and 13 show these immune blots in comparison with immune blots of *C. herbarum* and *Alternaria alternata* extracts, respectively. Exact co-migration and similar IgE binding of recombinant and

natural allergens was observed (in some lanes, additional bands are seen that may represent degradation products or aggregation products of the recombinant non-fusion allergens). The co-migration indicates the correspondence between cloned allergens and bands in immune blots of fungal raw extracts and very probably means that the seven allergens we are dealing with here are not post-synthetically modified (see also Discussion). More specifically, these results show that large glycoprotein moieties (as in N-glycosylation of secreted proteins) are not added onto these proteins. The immune blots were aligned with Coomassie-stained *Alternaria* and *Cladosporium* extracts showing that the seven cloned allergens are not prominent protein bands in the extracts.

DISCUSSION

We are presenting here our successful attempt to characterize the allergens of *Alternaria alternata* and

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-10
GGCAGGAGCA
1 / 1
ATG AAG CAC CTC GCG GCA TAC CTC CTC CTC GGC CTT GGT GGC AAC ACC TCG CCC TCC GCT
Met Lys His Leu Ala Ala Tyr Leu Leu Leu Gly Leu Gly Gly Asn Thr Ser Pro Ser Ala
61 / 22
GCC GAC GTC AAG GCC GTC CTT GAG TCC GTT GGT ATC GAG GCT GAC TCC GAC CGT CTT GAC
Ala Asp Val Lys Ala Val Leu Glu Ser Val Gly Ile Glu Ala Asp Ser Asp Arg Leu Asp
122 / 41
AAG CTG ATC TCC GAG CTT GAG GGC AAG GAC ATC AAC GAG CTC ATC GCT TCC GGT TCC GAG
Lys Leu Ile Ser Glu Leu Glu Gly Lys Asp Ile Asn Glu Leu Ile Ala Ser Gly Ser Glu
181 / 61
AAG CTT GCT TCC GTT CCC TCC GGT GGT GCC GGT GGT GCT GCC GCT TCC GGT GGT GCT GCT
Lys Leu Ala Ser Val Pro Ser Gly Gly Ala Gly Gly Ala Ala Ala Ser Gly Gly Ala Ala
241 / 81
GCC GCT GGT GGC TCC GCT CAG GCT GAG GCC GCT CCT GAG GCC GCC AAG GAG GAG GAG AAG
Ala Ala Gly Gly Ser Ala Gln Ala Glu Ala Ala Pro Glu Ala Ala Lys Glu Glu Glu Lys
301 / 101
GAG GAG TCT GAC GAG GAC ATG GGT TTC GGT CTC TTC GAC TAA
Glu Glu Ser Asp Glu Asp Met Gly Phe Gly Leu Phe Asp OCH
343
GCGCATCACCATCGCCAAACAAACACCTTCACCTACCTTTACGAAGTATGATCCGGCCAATCT CTCGACTATCAGAGA
ATCATGCGCAATGAGAACATTGCGGAGTGTGCGAGCTGCACTGCAAGGGGCTTGGAGAAGG ATACCGCGCAATAGT
AGAATAGTTGCTGCTGAGATACCCGGAATGAAGCCATGCACCTCCAN 547

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Fig. 4. Complete cDNA sequence and derived protein sequence of Alt a 6 (P2 ribosomal protein).
EMBL Accession number: X78222.

C. herbarum by cDNA cloning, sequencing and expression in *E. coli* of recombinant non-fusion proteins. This approach seems to be the only one that leads to a reproducible, well characterized and pure source of allergens for diagnosis and therapy (Kraft and Schon, 1993).

A comparison of commercial protein extracts of *Alternaria* and *Cladosporium* with the extracts described here shows that most of the protein bands in immune blots co-migrate. However, our extracts do show additional bands, especially in the high *M_r* range (data not shown). This is important, because it shows, in addition to morphological and physiological criteria, that we are dealing with biological material that is the correct species and is comparable and in part, superior to the commercially available material.

It might be worthwhile to consider the prevalence of spores of the two fungi investigated here in the air in Austria, where all of our patients live. It was found that

spores of *Cladosporium* are more numerous and occur for an extended part of the year while spores of *Alternaria* were only found for a short period of the year when the temperature was highest (Ebner *et al.*, 1988).

To date, only two mould allergen sequences have been published: one (Asp f 1) is a homolog of the highly cytotoxic fungal protein, mitogillin (Moser *et al.*, 1992), and the other one, also from *Aspergillus fumigatus*, is Hsp70 (Kumar *et al.*, 1992).

The seven new mould allergens presented here represent four new allergen proteins: ALDH, enolase, YCP4, and the acidic ribosomal protein P2. Three of them (ALDH, YCP4 and P2) were found to be allergens in both fungi, *Alternaria* and *Cladosporium*. We did not investigate in detail a possible immunological cross-reactivity between homologous proteins of the fungal species. However, many of the patients' IgE recognize, for instance, only the YCP4 homologue of *Alternaria*, but not the one of *Cladosporium*, or *vice versa*. The same is true

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-105
CACGAGCCACCACCCATCCAGGACATTGTAGCTTCAGTCTGCGCTTGGTCTAAACGTGATTGA TTCTCCTGTCTTTAAA
CCAACCAACCCCAACACCGTCAAG
1 / 1
ATG AAG TAC CTC GCA GCT TTC CTC CTC CTC GGC TTG CCG GGC AAC TCC TCC CCC TCT GCT
Met Lys Tyr Leu Ala Ala Phe Leu Leu Leu Gly Leu Pro Gly Asn Ser Ser Pro Ser Ala
61 / 22
GAG GAC ATC AAG ACC GTC CTC AGC TCC GTT GGC ATC GAC GCC GAC GAG GAG CCG TCT CAG
Glu Asp Ile Lys Thr Val Leu Ser Ser Val Gly Ile Asp Ala Asp Glu Glu Pro Ser Gln
122 / 41
CTC CTC CTT AAG GAG CTC GAG GGC AAG GAC ATC AAC GAG CTG ATC TCT TCC GGC TCT GAG
Leu Leu Leu Lys Glu Leu Glu Gly Lys Asp Ile Asn Glu Leu Ile Ser Ser Gly Ser Glu
181 / 61
AAG CTC GCC TCC GTT CCC TCT GGT GGT GCT GGT GCC GCC TCT GCC GGT GGT GCT GCC GCC
Lys Leu Ala Ser Val Pro Ser Gly Gly Ala Gly Ala Ala Ser Ala Gly Gly Ala Ala Ala
241 / 81
GCT GGT GGT GCC GCT GAG GCC GCC CCC GAG GCC GAG AAG GCT GAG GAG GAG AAG GAG GAG
Ala Gly Gly Ala Ala Glu Ala Ala Pro Glu Ala Glu Lys Ala Glu Glu Glu Lys Glu Glu
301 / 101
TCT GAC GAC GAC ATG GGC TTC GGT CTC TTC GAC TAA
Ser Asp Asp Asp Met Gly Phe Gly Leu Phe Asp OCH
337
GCGGCGAATCCGGCAATGGATGTTTGTATGCGAAATCCTGGTCAAGGGATGGCGTTTGGTGA GGAAGGGTGCCAGCGA
TCTGCCCCAAGAAATGCGGAGGACGAGAGACATGACTGTGTCGTGGACTTGTCTGTCGCGAGA GCGGAAGCGCAGCGCC
TGTTTATACGTGAAATGAACCAACCTTCATCAGCCAACGAN 535

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Fig. 5. Complete cDNA sequence and derived protein sequence of Cla h 4 (P2 ribosomal protein).
EMBL Accession number: X78223.

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1
Alt a 6 MKhLAAYLLL GLGGNTSPSA aDVkaVLESV GIEADsDRLd KLISELEGKD
Cla h 4 MKYLAaFLLL GLpGNSSPSA eDIKtVLsSV GIDADEEpsq lllKELEGKD
humrla2 MrYVAsYLLa aLGGNSSPSA kDIKKILDSV GIEADDDRln KvISELnGKn
Consensus M---A--LL- -L-CN-SPSA -D-K--L-SV GI-AD----- ---EL-GK-

51
Alt a 6 INELIASGSE KLASVPSGGA Gg.AAAsGgA AAAGGSaQAE AAPEAAK.EE
Cla h 4 INELIsSGSE KLASVPSGGA GA.AsAgGaA AAgGa...AE AAPEAEKaEE
humrla2 IeDvIaGig KLASVPaGGA vAvsAaPgsA ApAaGSaPaa AeekkDekke
Consensus I---I--G-- KLASVP-GGA -----A-G-A A-----A- A-----E

101 116
Alt a 6 EKEESDEDMG FGLFDZ
Cla h 4 EKEESDDDMG FGLFDZ
humrla2 ESEESDDDMG FGLFD-
Consensus E-EESD-DMG FGLFD-

```

Fig. 6. Sequence alignment between Cla h 4, Alt a 6 and human ribosomal protein P2. Alt a 6 and Cla h 4 are 75% identical (85% homologous) at the protein level. The two fungal sequences are on average 79% homologous to the human ribosomal protein P2 sequence.

for the P2 ribosomal protein. A comparison of these allergens with the known allergens of pollens, house dust mites, and foodstuffs allows for a few generalizations to be made:

(i) None of the mould allergens that are now known with respect to sequence and/or enzymatic function correspond to a previously known non-fungal allergen, although many of the known fungal allergens do occur as homologous allergens in more than one fungal species. A detailed sequence comparison of the new fungal allergen sequences with 25 previously known allergen sequences (data not shown) yielded no detectable similarities. The identity scores were below 10% and no common sequence motifs of more than three to four amino acids were found.

Also, we found no evidence that IgE recognizing a certain fungal allergen in Western blots cross-react with, say, a certain pollen allergen. A polyvalent patient who recognizes numerous protein bands in IgE Western blots of pollens, foodstuffs and even *E. coli* proteins, was unreactive towards the two fungi investigated here (data not shown). All this circumstantial evidence further underlines the notion that common allergenic epitopes occurring in a wide variety of different allergens probably do not exist. To a limited degree, such commonalities have been found indeed, the most well known of which is the similarity of Bet v 1 with allergens of apple (Ebner *et al.*, 1991) and other fruits (Mohapatra, 1993).

(ii) We notice that all the newly sequenced fungal

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-22
GGCACGAGCCAATTCTGTTCAAG
1 / 1
ATG GCT CCC AAG ATC GCG ATT GTG TAC TAC TCC ATG TAC GGC CAC ATC AAG AAG ATG GCC
Met Ala Pro Lys Ile Ala Ile Val Tyr Tyr Ser Met Tyr Gly His Ile Lys Lys Met Ala
61 / 22
GAT GCT GAG TTG AAG GGT ATC CAA GAG GCT GGC GGT GAT GCC AAG CTC TTC CAA GTC GCC
Asp Ala Glu Leu Lys Gly Ile Gln Glu Ala Gly Gly Asp Ala Lys Leu Phe Gln Val Ala
122 / 41
GAG ACC CTG CCT CAG GAA GTC CTC GAC AAG ATG TAC GCG CCC CCC AAG GAC TCA TCG GTC
Glu Thr Leu Pro Gln Glu Val Leu Asp Lys Met Tyr Ala Pro Pro Lys Asp Ser Ser Val
181 / 61
CCC GTC CTC GAG GAC CCA GCC GTC CTC GAA GAA TTT GAC GGC ATC CTC TTC GGC ATC CCC
Pro Val Leu Glu Asp Pro Ala Val Leu Glu Glu Phe Asp Gly Ile Leu Phe Gly Ile Pro
241 / 81
ACC GCG TAC GGC AAC TTC CCC GCA CAA TTC AAG ACC TTC TGG GAC AAG ACA GGC AAG CAA
Thr Arg Tyr Gly Asn Phe Pro Ala Gln Phe Lys Thr Phe Trp Asp Lys Thr Gly Lys Gln
301 / 101
TGG CAA CAA GGC GCC TTT TGG GGA AAG TAC GCC GGT GTC TTC GTT TCG ACG GGC ACC CTG
Trp Gln Gln Gly Ala Phe Trp Gly Lys Tyr Ala Gly Val Phe Val Ser Thr Gly Thr Leu
361 / 122
GGC GGT GGC CAG GAG ACG ACT GCC ATT ACC AGC ATG AGC ACG CTT GTC GAC CAC GGT TTC
Gly Gly Gly Gln Glu Thr Thr Ala Ile Thr Ser Met Ser Thr Leu Val Asp His Gly Phe
422 / 141
ATC TAC GTT CCC CTT GGC TAC AAG ACT GCG TTT AGC ATG TTG GCC AAC TTG GAC GAG GTC
Ile Tyr Val Pro Leu Gly Tyr Lys Thr Ala Phe Ser Met Leu Ala Asn Leu Asp Glu Val
481 / 161
CAC GGT GGA AGC CCA TGG GGT GCT GGT ACC TTC TCT GCC GGC GAT GGA TCG AGG CAG CCC
His Gly Gly Ser Pro Trp Gly Ala Gly Thr Phe Ser Ala Gly Asp Gly Ser Arg Gln Pro
541 / 181
AGT GAG CTT GAG CTC AAC ATT GCG CAG GCT CAG GGT AAG GCT TTC TAC GAG GCT GTT GCC
Ser Glu Leu Glu Leu Asn Ile Ala Gln Ala Gln Gly Lys Ala Phe Tyr Glu Ala Val Ala
601 / 201
AAG GCG CAT CAG TGA
Lys Ala His Gln OPA
616
CTAGATGATGGAAGACTGCTGGGCCTTGTGTGCTGAGAACAAGACGGTGGAGAATGGGAATGG AGCAACGACAAATGGT
AGCACTGCCCTGGCCGCTCTACTTCTACTCTGCTCGATCTTCTTCGCAACCCAGCAGAAGA CCGGAAGAGGACAAGG
ATAAGAGGGCGGTCATGTGGATTGCCCTGCAAGTGGCATATCATGTAGACTTTAGCCCTTC TTTTCTACCTCTTCT
TTTCTACTTGTGATATTTTGGATGAAATGAAACGAAATGAAATATTTAGCCATCATACT ACAn 918

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Fig. 7. Complete cDNA sequence and derived protein sequence of Alt a 7. EMBL Accession number: X78225.

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-21
GCACGAGAACCAACCATCACA
1 / 1 31 / 11
ATG GCT CCC AAG ATC GCC ATC ATC TTC TAC TCC ACC TGG GGA CAC GTC CAG ACC CTC GCC
Met Ala Pro Lys Ile Ala Ile Ile Phe Tyr Ser Thr Trp Gly His Val Gln Thr Leu Ala
61 / 22 91 / 31
GAG GCC GAG GCC AAG GGC ATC CGC GAG GCC GGC GGT TCC GTC GAC CTC TAC CGC GTC CCC
Glu Ala Glu Ala Lys Gly Ile Arg Glu Ala Gly Gly Ser Val Asp Leu Tyr Arg Val Pro
122 / 41 151 / 51
GAG ACC CTC ACC CAG GAG GTT CTG ACC AAG ATG CAC GCT CCT CCT AAG GAC GAC TCC ATC
Glu Thr Leu Thr Gln Glu Val Leu Thr Lys Met His Ala Pro Pro Lys Asp Asp Ser Ile
181 / 61 221 / 71
CCT GAG ATC ACC GAC CCT TTC ATT CTC GAG CAG TAC GAT CGC TTT CCT CAT GGG CAT CCC
Pro Glu Ile Thr Asp Pro Phe Ile Leu Glu Gln Tyr Asp Arg Phe Pro His Gly His Pro
241 / 81 271 / 91
ACC CGC TAC GGC AAC TTC CCC GCG CAG TGG AGG ACC TTC TGG GAC CGC ACC GGC GGC CAA
Thr Arg Tyr Gly Asn Phe Pro Ala Gln Trp Arg Thr Phe Trp Asp Arg Thr Gly Gly Gln
301 / 101 331 / 111
TGG CAG ACC GGT GCC TTC TGG GGC AAG TAC GCC GGC CTG TTC ATC AGT ACT GGT ACC CAG
Trp Gln Thr Gly Ala Phe Trp Gly Lys Tyr Ala Gly Leu Phe Ile Ser Thr Gly Thr Gln
361 / 122 391 / 131
GGT GGT GGA CAG GAG AGC ACT GCT CTC GCT GCC ATG AGC ACT CTC TCC CAC CAC GGC ATT
Gly Gly Gly Gln Glu Ser Thr Ala Leu Ala Ala Met Ser Thr Leu Ser His His Gly Ile
422 / 141 451 / 151
ATC TAC GTT CCT CTT GGC TAC AAG ACC ACA TTC CAC CTC CTC GGC GAC AAC AGC GAG GTC
Ile Tyr Val Pro Leu Gly Tyr Lys Thr Thr Phe His Leu Leu Gly Asp Asn Ser Glu Val
481 / 161 511 / 171
CGC GGT GCC GCA GTG TGG GGC GCT GGT ACC TTC TCC GGT GGT GAC GGT TCC CGC CAG CCC
Arg Gly Ala Ala Val Trp Gly Ala Gly Thr Phe Ser Gly Gly Asp Gly Ser Arg Gln Pro
541 / 181 571 / 191
TCC CAG AAG GAA TTG GAG CTC ACC GCC CAG GGT AAG GCC TTC TAT GAG GCT GTC GCC AAG
Ser Gln Lys Glu Leu Glu Leu Thr Ala Gln Gly Lys Ala Phe Tyr Glu Ala Val Ala Lys
601 / 201
GTC AAC TTC CAG TGA
Val Asn Phe Gln OPA
616
CTGCAAGGTGCGGAAGAAGTCAACAACAGCAAGCCCAAACTACGGCTGAGCCTACCCAGCC ATCTGCTGGCAACGGA
AGCGCAAAGGAGACTCTCTGCGCACACCGTGCCACGACGACGACGCTCCACTCAGCCCCAAC CCAGAGACGAGAACAA
GAGCGAAGCCCATGTGGCTCGCGTCAAGTCTCTGTCTCATAGCGATACCTACACCTTTAT TAGAAACCTTATGACA
ATTTCATTTCACAATTCA 871

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Fig. 8. Complete cDNA sequence and derived protein sequence of Cla h 5. EMBL Accession number: X78224.

allergens presented here are soluble cytoplasmic proteins (as judged from their function; only for YCP4 is a function unknown). For comparison, both soluble cytoplasmic proteins (Breiteneder *et al.*, 1989; Valenta *et al.*, 1991; Grote, 1991; Jilek *et al.*, 1993) and secreted proteins (Knox *et al.*, 1993; Nilsen *et al.*, 1991; Batanero *et al.*, 1994) have been found among cloned and

sequenced pollen allergens. The cytoplasmic allergens are very probably non-glycosylated. Indeed, the seven allergens presented here were produced as recombinant non-fusion proteins and exhibited exactly the same electrophoretic mobility and IgE-binding properties in Western blots as the natural allergens of the mould extracts. This would be highly improbable if the latter

```

1 50
Alt a 7 MAPKIAIVVY SmYGHlKkMA DAElKGiQEA GGdAkLFqVa ETLPOEVLGK
Cla h 5 MAPKIAIIFY STWGHVqtLA EAeAkGirEA GGsvDLyRVp ETLtQEVLTk
YCP4Scer .mvKIAIItY STYGHIdvLA qAvkKGveaA GGKADiYRve ETLpEVLTK
Consensus ---KIAI--Y S--GH----A -A--KG----A GG-----V- ETL--EVL-K

51 100
Alt a 7 MyAPPKDsSV PVleDPaVLE eFDgILFGIP TRYGNFPAQF kTFWDKTGkQ
Cla h 5 MhAPPKDDSI PeiTDPfILE qYDrFphGhP TRYGNFPAQW rTFWDrTGGQ
YCP4Scer MnAPqKpEdI PVaTEktILE .YDaFLFGVP TRFGNLPAQW saFWDKTGGL
Consensus M-AP-K---- P-----LE --D----G-P TR-GN-PAQ- --FWD-TG--

101 150
Alt a 7 WQqGAFWgKY AGVFVSTGTl GGGQEtTAit sMSTLvdHGF IYVPLGYKta
Cla h 5 WQtGAFWgKY AGIFISTGTq GGGQESTAla aMSTLsHHGI IYVPLGYKtC
YCP4Scer WakGsLnGKa AGIFVSTssy GGGQESTvka cLSyLaHHGI IFIPLGYKns
Consensus W--G---GK- AG-F-ST--- GGGQE-T--- --S-L--HG- I--PLGYK--

151 200
Alt a 7 FsMLAnlDEV HGGSPWGAGT FSaGDGSRQP SeLELnIAqA QGKAfYEAVA
Cla h 5 PhLLgdnSEV rGaavWGAGT FSGGDGSRQP SqkELelt.A QGKAfYEAVA
YCP4Scer FaeLAsiEEV HGGSPWGAGT LaPgDGSRTa SpLELrIAei QGKtFYEtak
Consensus F--L----EV -G---WGAGT ----DGSR-- S--EL----- QGK-FYE--

201 249
Alt a 7 KahqZ.....
Cla h 5 KvmfQZ.....
YCP4Scer Klfpakeakp stekkttttsd aakrqtcpaa attaekkedk gllscctvm
Consensus K-----

```

Fig. 9. Sequence alignment between Cla h 5, Alt a 7 and YCP4 of *Saccharomyces cerevisiae*. Alt a 7 and Cla h 5 are 65% identical (81% homologous) at the protein level. The homology of both sequences on protein level to YCP4 is on average 70%.

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-18
GCACGAGAAACCGCCAAC
1 / 1
ATG CCT ATC TCC AAG ATC CAC TCC CGC TAC GTC TAC GAC TCC CGT GGA AAC CCC ACC GTC
Met Pro Ile Ser Lys Ile His Ser Arg Tyr Val Tyr Asp Ser Arg Gly Asn Pro Thr Val
61 / 21
GAG GTC GAC ATT GTC ACC GAG ACC GGT CTT CAC CGC GCC ATT GTC CCC TCT GTT GCC TCT
Glu Val Asp Ile Val Thr Glu Thr Gly Lhu His Arg Ala Ile Val Pro Ser Val Ala Ser
121 / 41
ACC GGC TCC CAC GAG GCT TGC GAG CTC CGT GAT GGT GAC AAG AGC AAG TGG GCT GGC AAG
Thr Gly Ser His Glu Ala Cys Glu Leu Arg Asp Gly Asp Lys Ser Lys Trp Ala Gly Lys
181 / 61
GGT GTG ACC AAG GCT GTT GCC AAC GTC AAC GAG ATC ATT GCT CCC GCT CTC ATC AAG GAG
Gly Val Thr Lys Ala Val Ala Asn Val Asn Glu Ile Ile Ala Pro Ala Leu Ile Lys Glu
241 / 81
AAC CTC GAC GTC AAG GAC CAG GCC GCC GTT GAC GCT TTC CTC AAC AAG CTC GAT GGC ACC
Asn Leu Asp Val Lys Asp Gln Ala Ala Val Asp Ala Phe Leu Asn Lys Leu Asp Gly Thr
301 / 101
ACC AAC AAG ACC AAG ATT GGT GCC AAC GCC ATC CTC GGT GTC TCC ATG GCT GTG GCC AAG
Thr Asn Lys Thr Lys Ile Gly Ala Asn Ala Ile Leu Gly Val Ser Met Ala Val Ala Lys
361 / 121
GCT GCC GCC GCC GAG AAG CGT GTC CCT CTC TAC GCC CAC ATC AGC GAC CTT TCC GGC ACC
Ala Ala Ala Ala Glu Lys Arg Val Pro Leu Tyr Ala His Ile Ser Asp Leu Ser Gly Thr
421 / 141
AAG AAG CCC TTC GTT CTC CCC GTT CCC TTC ATG AAC GTC GTC AAC GGT GGC TCC CAC GCg
Lys Lys Pro Phe Val Leu Pro Val Phe Met Asn Val Val Asn Gly Gly Ser His Ala
481 / 161
GGT GGC CGT CTT GCC TTC CAG GAG TTC ATG ATC GTT CCC AGC Ggg GCT CCC TCC TTC ACC
Gly Gly Arg Leu Ala Phe Gln Glu Phe Met Ile Val Pro Ser Gly Ala Pro Ser Phe Thr
541 / 181
GAG GCC ATG CGC CAG GGT GCT GAG GTC TAC CAG AAG CTG AAG TCC CTC ACC AAG AAG AGG
Glu Ala Met Arg Gln Gly Ala Glu Val Tyr Gln Lys Leu Lys Ser Leu Thr Lys Lys Arg
601 / 201
TAC GGC CAG TCT GCC GGC AAC GTC GGT GAC GAG GGT GGT GTC GCT CCC GAT ATT CAG ACC
Tyr Gly Gln Ser Ala Gly Asn Val Gly Asp Glu Gly Gly Val Ala Pro Asp Ile Gln Thr
661 / 221
GCT GAG GAG GCT CTT GAC CTC ATC ACC GAC GCC ATT GAG GAG GCC GGC TAC ACT GGC CAG
Ala Glu Glu Ala Leu Asp Leu Ile Thr Asp Ala Ile Glu Glu Ala Gly Tyr Thr Gly Gln
721 / 241
ATC AAG ATC GCC ATG GAC gtt GCC TCC TCC GAG TTC TAC AAG GCC GAC GAG AAG AAG TAC
Ile Lys Ile Ala Met Asp Val Ala Ser Ser Glu Phe Tyr Lys Ala Asp Glu Lys Lys Tyr
781 / 261
GAC Ctt GAC TTC AAG AAC CCC GAC TCT GAC AAG AGC AAG TGG ATC ACC TAC GAG CAG CTT
Asp Leu Asp Phe Lys Asn Pro Asp Ser Asp Lys Ser Lys Trp Ile Thr Tyr Glu Gln Leu
841 / 281
GCC GAC CAG TAC AAC GAG CTC GCT GCC AAG TAC CCC ATT GTC TCC ATT GAG GAC CCC TTC
Ala Asp Gln Tyr Asn Glu Leu Ala Ala Lys Tyr Pro Ile Val Ser Ile Glu Asp Pro Phe
901 / 301
GCT GAG GAT GAC TGG GAG GCC TGG AGC TAC TTC TAC AAG ACC TCT GGC TCT GAC TTC CAG
Ala Glu Asp Asp Trp Glu Ala Trp Ser Tyr Phe Tyr Lys Thr Ser Gly Ser Asp Phe Gln
961 / 321
ATC GTC GGT GAT GAC CTT ACC GTC ACC AAC CCC GAG TTC ATC AAG AAG GCC ATC GAG ACC
Ile Val Gly Asp Asp Leu Thr Val Thr Asn Pro Glu Phe Ile Lys Lys Ala Ile Glu Thr
1021 / 341
AAG GCC TGC AAC GCC CTC CTC CTC AAG GTC AAC CAG ATC GGT ACC ATC ACC GAG GCC ATC
Lys Ala Cys Asn Ala Leu Leu Lys Val Asn Gln Ile Gly Thr Ile Thr Glu Ala Ile
1081 / 361
AAC GCC GCC AAG GAC TCC TTC GCC GCT GGC TGG GGT GTC ATG GTC Tct caC CGT TCC GGT
Asn Ala Ala Lys Asp Ser Phe Ala Ala Gly Trp Gly Val Met Val Ser His Arg Ser Gly
1141 / 381
GAG ACC GAG GAC GTC ACC atc gcc gac ATC GTT GTC GGT CTC CGT GCC GGC CAG ATC AAG
Glu Thr Glu Asp Val Thr Ile Ala Asp Ile Val Val Gly Leu Arg Ala Gly Gln Ile Lys
1201 / 401
ACC GGT GCC CCT GCC CGT TCC GAG CGT CTC GCC AAG CTC AAC CAG ATC CTC CGT ATC GAG
Thr Gly Ala Pro Ala Arg Ser Glu Arg Leu Ala Lys Leu Asn Gln Ile Leu Arg Ile Glu
1261 / 421
GAG GAG CTC GGT GAC AAG AGG CTC TAC GCC GGT GAC AAC TTC CGC ACT GCC ATC AAC TTG
Glu Glu Leu Gly Asp Lys Arg Leu Tyr Ala Gly Asp Asn Phe Arg Thr Ala Ile Asn Leu
1321 / 441
TAA
OCH
1324
GCTATCTCATAGCTTGATGGCTACGAGTAGTAATTTCTTTATCATGAGGATGACGAGACGG ATGCATGGCTAGCGTT
TAGTCGGATGAACAAAAGTATTCAAATGTAn 1433

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Fig. 10. Complete cDNA sequence and derived protein sequence of Cla h 6 (enolase). EMBL Accession number: X78226.

ones were typical fungal N-glycosylated (Barnes *et al.* 1990) proteins. However, this rule is not generally true for fungal allergens, as Alt a 1, the major *Alternaria* allergen, consists of two peptide chains joined by disulfide bonds (Deards and Montague, 1991). It probably has to pass through the secretory pathway, and may be glycosylated. The major allergens of dust mites are

excreted proteases of the digestive tract (Chua *et al.*, 1988).

(iii) All the allergens presented here are rather highly conserved proteins. At least one of them, enolase, has been shown to be an important allergen of *Candida albicans* (Franklyn *et al.*, 1990; Ishiguro *et al.*, 1992). ALDH, enolase and P2 proteins occur in all eukaryotic

	1				50
eno1_yeast	.AVSKVYARS	VYDSRGNPTV	EVELTTEKGV	FRSIVPSGAS	TGVHEALEMR
eno2_yeast	.AVSKVYARS	VYDSRGNPTV	EVELTTEKGV	FRSIVPSGAS	TGVHEALEMR
Cla h 6	mpISKIhsRy	VYDSRGNPTV	EVDivTetG1	hRaIVPSvAs	TGsHEAcELR
Consensus	---SK---R-	VYDSRGNPTV	EV---TE-G-	-R-IVPS-AS	TG-HEA-E-R
	51				100
eno1_yeast	DGDKSKWMGK	GVLhAVkNVN	DVIAPAFVKA	NiDVKDQKAV	DDFLiSLDGT
eno2_yeast	DeDKSKWMGK	GVmAVhNVN	nVIAaAFVKA	NLDVKDQKAV	DDFLiSLDGT
Cla h 6	DGDKSKWaGK	GVtkAVaNVN	EITAPALiKe	NLDVKDQaAV	DaFLnKLDGT
Consensus	D-DKSKW-GK	GV--AV-NVN	--IA-A--K-	N-DVKDQ-AV	D-FL--LDGT
	101				150
eno1_yeast	ANKSKLGANA	ILGVSLAAaR	AAAAEKNVPL	YkHLADLSKS	KTSPYVLPVP
eno2_yeast	ANKSKLGANA	ILGVSMaAAR	AAAAEKNVPL	YqHLADLSKS	KTSPYVLPVP
Cla h 6	tNKtKiGANA	ILGVSMaVAK	AAAAEKrVPL	YaHisDLsgt	K.kPFVLPVP
Consensus	-NK-K-GANA	ILGVs-A---	AAAAEK-VPL	Y-H--DLS--	K--P-VLPVP
	151				200
eno1_yeast	FLNVINGGSH	AGGALALQEF	MIAPTGAkTF	AEALRIGSEV	YHNLSLTKK
eno2_yeast	FLNVINGGSH	AGGALALQEF	MIAPTGAkTF	AEAMRIGSEV	YHNLSLTKK
Cla h 6	FMNVINGGSH	AGGrLAFQEF	MIvPsGapsF	tEAMRqGaEv	YqkLSLTKK
Consensus	F-NV-NGGSH	AGG-LA-QEF	MI-P-GA--F	-EA-R-G-EV	Y--LKS LTKK
	201				250
eno1_yeast	RYGASAGNVG	DEGGVAPNIQ	TAEeALDLIV	DAIKAAGHDG	KVKIGLDCAS
eno2_yeast	RYGASAGNVG	DEGGVAPNIQ	TAEeALDLIV	DAIKAAGHDG	KVKIGLDCAS
Cla h 6	RYGqSAGNVG	DEGGVAPDIQ	TAEeALDLit	DAIeeAGytG	qIKIaMDvAS
Consensus	RYG-SAGNVG	DEGGVAP-IQ	TAEeALDLI-	DAI--AG-G	--KI--D-AS
	251				300
eno1_yeast	SEFFKDG..K	YDLDFKNPnS	DKSKWLTGpQ	LADLYHSLMK	RYPIVSIEDP
eno2_yeast	SEFFKDG..K	YDLDFKNPES	DKSKWLTGve	LADMYHSLMK	RYPIVSIEDP
Cla h 6	SEFYKadekK	YDLDFKNPDS	DKSKWiTyeQ	LAdqYneLaa	KYPIVSIEDP
Consensus	SEF-K---K	YDLDFKNP-S	DKSKW-T---	LAD-Y--L--	-YPIVSIEDP
	301				350
eno1_yeast	FAEDDWEAWS	HFFKTAG..I	QIVADDLTVT	NPKRIATAIE	KKAADALLLK
eno2_yeast	FAEDDWEAWS	HFFKTAG..I	QIVADDLTVT	NPaRIATAIE	KKAADALLLK
Cla h 6	FAEDDWEAWS	yFYKtsGsdif	QIVgDDLTVT	NPeIfkKAIE	tKAcnALLLK
Consensus	FAEDDWEAWS	-F-KT-G---	QIV-DDLTVT	NP--I--AIE	-KA---ALLLK
	351				400
eno1_yeast	VNQiGTLSES	IKAAQDSFAA	GWGMVSHRS	GETEDTFIAD	LVVGLRTGQI
eno2_yeast	VNQiGTLSES	IKAAQDSFAA	nWGMVSHRS	GETEDTFIAD	LVVGLRTGQI
Cla h 6	VNQiGTLtEa	InAAKDSFAA	GWGMVSHRS	GETEDvtIAD	iVVGLRaGQI
Consensus	VNQiGT--E-	I-AA-DSFAA	-WGVMVSHRS	GETED--IAD	-VVGLR-GQI
	401				442
eno1_yeast	KTGAPARSER	LAKLNQLLRI	EEELGDnAVF	AGENFHHGDK	L.
eno2_yeast	KTGAPARSER	LAKLNQLLRI	EEELGDkAVY	AGENFHHGDK	L.
Cla h 6	KTGAPARSER	LAKLNQLLRI	EEELGDkRly	AGDNFrtain	Lz
Consensus	KTGAPARSER	LAKLNQ-LRI	EEELGD----	AG-NF-----	L-

Fig. 11. Sequence alignment between Cla h 6 and ENO1 and ENO2 (the two isoforms of enolase) of *Saccharomyces cerevisiae*. The averaged homology on the protein level of Cla h 6 to the yeast enolases is 83%.

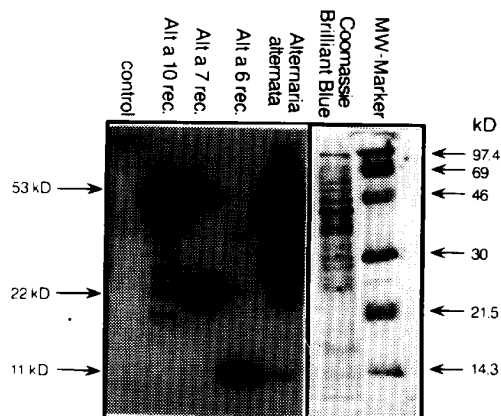


Fig. 12. IgE immune blot, showing a comparison between *Alternaria alternata* protein extract and the three cloned *Alternaria* allergens (Alt a 6, Alt a 7 and Alt a 10) expressed as non-fusion proteins in the *E. coli* strain BL21. Note the co-migration of natural and recombinant allergens. The Coomassie brilliant blue-stained membrane shows that none of the cloned allergens are abundant proteins in the extract. The buffer control confirms the specificity of the IgE immune blot.

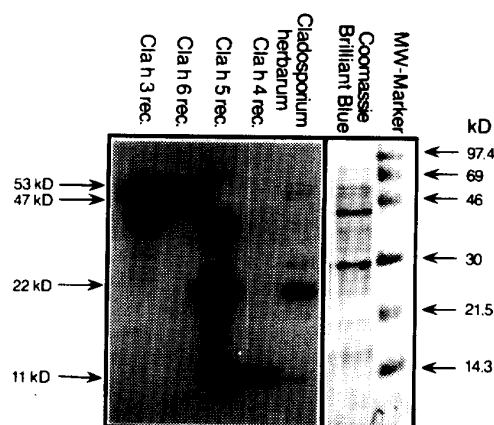


Fig. 13. IgE immune blot showing a comparison between *Cladosporium herbarum* protein extract and the four cloned *Cladosporium* allergens (Cla h 3, Cla h 4, Cla h 5 and Cla h 6) expressed as non-fusion proteins in the *E. coli* strain BL21. The electrophoretic mobility of native and recombinant allergens is identical. The Coomassie brilliant blue-stained membrane shows that none of the cloned allergens are abundant proteins.

cells with significant sequence identity and similarity. The human P2 ribosomal protein is a well known lupus erythematoses antigen, the relevant epitope for LE being the highly conserved C-terminal sequence KEESESD/EDMGFGLFD. Two other highly conserved ribosomal proteins (P0 and P1) also share this sequence. We have not yet tested immunological cross-reactivity between the mould and human ribosomal proteins with either SLE patients or allergics. Therefore, we do not know whether the P2 epitope relevant for autoimmunity is identical to the epitope relevant for allergy. However, it is tempting to speculate that the allergic reaction in the patients that do recognize this minor allergen might be stimulated by an internal boost, especially in inflamed tissue. A similar conclusion was drawn concerning profilin, a minor pollen allergen that is highly conserved in all eukaryotes (Valenta *et al.*, 1991).

(iv) Most of the seven allergens presented here are "household" proteins needed for basic metabolism like glycolysis or protein synthesis. This fact makes it unlikely that these allergenic proteins be spore-specific. However, the fungal material used for the present study contained vegetative cells as well as conidiospores and cells and conidiospores were not separated prior to allergen extraction. Future investigations will show whether some of the not yet cloned and sequenced allergens of *Alternaria* and *Cladosporium* are indeed spore-specific (Paris *et al.*, 1990). The household nature of the seven allergens presented here also makes it unlikely that a majority of them are "stress" proteins, like, for instance, heat shock proteins. A possible example of a fungal allergen that is indeed a heat shock protein, is hsp70 of *Aspergillus fumigatus* (Kumar, 1992).

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