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Enolases Are Highly Conserved Fungal Allergens

Abstract

Background: Lack of knowledge of the identity of fungal allergens still is a major obstacle for improvement of diagnosis and therapy of allergies to moulds. We have therefore further analyzed the allergens of the two moulds, *Alternaria alternata* and *Cladosporium herbarum* and found that enolases (EC 4.2.1.11) are major allergens, at least of the two fungal species just mentioned. **Methods:** The enolases of *Alternaria* and *Cladosporium* were cloned from cDNA libraries constructed from vegetative cells of the two moulds by immunological screening with sera from selected patients allergic to the moulds. The two enolases were expressed as recombinant nonfusion proteins and used for determination of the incidence of allergy to enolase among a cohort of patients. **Results:** Sequencing of the two enolases showed very close relationships with other known fungal enolase sequences. Competition experiments using immunoblots of the recombinant nonfusion proteins showed nearly complete identity of the epitopes on both enolases. Serum from a patient reactive to *Cladosporium* enolase reacted equally well with the enolases of *Alternaria*, *Saccharomyces* and *Candida*. About 50% each of the sera from patients reactive to *Cladosporium* and *Alternaria* were strongly reactive to the recombinant enolases. **Conclusions:** Enolases are therefore considered to be highly conserved major fungal allergens.

Introduction

Enolase (EC 4.2.1.11) is a highly expressed key enzyme of glycolysis and gluconeogenesis in all prokaryotic and eukaryotic cells that is needed for the conversion of glyceralate-2-phosphate to phosphoenolpyruvate and vice versa. It is therefore a cytoplasmic household enzyme. Interestingly, enolases have been recognized as fungal allergens before. The enolase from *Cladosporium herbarum* was previously cloned, sequenced and expressed as a recombinant protein by us [1]. The two enolases of *Saccharomyces cerevisiae* [2, 3] and the single enolase of *Candida albicans* [4, 5], a well-known human pathogen, were also identified as allergens by the IgE immunoblot technique. The enolase of *C. albi-*

cans was recently cloned and sequenced [6]. In addition, location of *Candida* enolase in a cell wall fraction and in the periplasmic space (or culture medium), respectively, has also been reported [7, 8], which could be important for the sensitization of patients.

We are presenting here the sequence of the newly cloned *A. alternata* enolase that has been sequenced and expressed as a nonfusion recombinant protein. We show that the epitopes on the two enolases of *Cladosporium* and *Alternaria* are nearly identical and that the recombinant enolases of *Cladosporium* and *Alternaria* and the enolases in extracts from *Saccharomyces* and *Candida* cross-react with patients' IgE. Immunological cross-reactivity of enolases seems to be well correlated with sequence similarity.

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	1	50	100
<i>Alternaria alternata</i>	-mtitKIhAR SVYDSRGNPT VEVDivTtEtG LhRaIVPSGA STGshEAcEL RDGDKSKWGG KGVtKAVANV NdtIAPrLiK eKLDVKDQaA VDaFLnKLDG		
<i>Cladosporium herbarum</i>	-mpISKIHsR yVYDSRGNPT VEVDivTtEtG LhRaIVPSvA STGshEAcEL RDGDKSKWag KGVtKAVANV NeIAPALIK eNLDVKDQaA VDaFLnKLDG		
<i>Saccharomyces c. - 1</i>	--AvSKvYAR SVYDSRGNPT VEVelTTEKG vFRSIVPSGA STGVHEALEm RDGDKSKWmG KGVlhAVKNV NDvIAPafvK ANIDVKDQKA VddFLlSLDG		
<i>Saccharomyces c. - 2</i>	--AvSKvYAR SVYDSRGNPT VEVelTTEKG vFRSIVPSGA STGVHEALEm RDeDKSKWmG KGVmAVnNV NnvIAaAfVK ANLDVKDQKA VddFLlSLDG		
<i>Candida albicans</i>	MsyatKIhAR yVYDSRGNPT VEVDfTTdKG LFRSIVPSGA STGVHEALEL RDGDKSKWlG KGVlKAVANV NdiIAPALIK AkiDVvDQak iDeFLlSLDG		
Consensus	--A-SKIhAR SVYDSRGNPT VEVD-TTEKG LFRSIVPSGA STGVHEALEL RDGDKSKW-G KGV-KAVANV ND-IAPALIK ANLDVKDQ-A VD-FL-SLDG		
	101	150	200
<i>Alternaria alternata</i>	TtNKtnLIGAN AILGVSMaIA KAAAAEKgVP LYaHIsDLag tKK-PYVLfV PFqNVLNGGS dAGGrLAPQe FMiVpCeApT FseAMRQGaE VyqkLKaLaK		
<i>Cladosporium herbarum</i>	TtNKtKiGAN AILGVSMaVa KAAAAEKrVP LYaHIsDLsG tKK-PfVLfV PFMNVVNGGS HAGGrLAPQe FMiVpSGaPs FteAMRQGaE VyqkLKSltK		
<i>Saccharomyces c. - 1</i>	TaNKSKlGAN AILGVSLAAs rAAAAEKnVP LYkHlADLSK sKtsPYVLfV PFfNLVNGGS HAGGALALQe FMiAPTgAKT FaEALRIGSE VYHNLKSLtK		
<i>Saccharomyces c. - 2</i>	TaNKSKlGAN AILGVSMAAA rAAAAEKnVP LYqHlADLSK sKtsPYVLfV PFfNLVNGGS HAGGALALQe FMiAPTgAKT PaEAMRIGSE VYHNLKSLtK		
<i>Candida albicans</i>	TpNKSKlGAN AILGVSLAAA nAAAAagqPi LYKHIANIsN aKKgkfVLfV PFqNVLNGGS HAGGALAPQe FMiAPTgVsT FseALRIGSE VYHNLKSLtK		
Consensus	T-NKSKlGAN AILGVSMAAA -AAAAEK-VP LY-HIADLS- -KK-PYVLfV PF-NVLNGGS HAGGALAPQe FMiAPTGA-T F-EAMRIGSE VYHNLKSLtK		
	201	250	300
<i>Alternaria alternata</i>	KtYQGSAGNV GDEGGVAPDI QTAEeALDLI tKaIeeAGYT GKlKIAMDVA SSeFYKadeK KYDLDFKNPd SDKSKWlTye QLaemYkSLa eKYPIVSIED		
<i>Cladosporium herbarum</i>	KRYGGSAGNV GDEGGVAPDI QTAEeALDLI tDAIeeAGYT GqIKIAMdVA SSeFYKadeK KYDLDFKNPd SDKSKWlTye QLaDqYneLa aKYPIVSIED		
<i>Saccharomyces c. - 1</i>	KRYGGSAGNV GDEGGVAPnI QTAEeALDLI vDAIkaAGhd GKVKIglDcA SSeFfK--DG KYDLDFKNPn SDKSKWlTgP QLaDlYhSLn kRYPIVSIED		
<i>Saccharomyces c. - 2</i>	KRYGGSAGNV GDEGGVAPnI QTAEeALDLI vDAIkaAGhd GKVKIglDcA SSeFfK--DG KYDLDFKNPe SDKSKWlTgV eLADmYhSLn kRYPIVSIED		
<i>Candida albicans</i>	KkYQGSAGNV GDEGGVAPDI kTpKEALDLI mDAIdkAGYk GKVGlAMDVA SSeFYK--DG KYDLDFKNPe SDpSKWlSGp QLaDlYeqLi seYPIVSIED		
Consensus	KRYGGSAGNV GDEGGVAPDI QTAEeALDLI -DAI--AGY- GKVKIAMdVA SSeFYK--DG KYDLDFKNP- SDKSKWlTG- QLaD-Y-SL- --YPIVSIED		
	301	350	400
<i>Alternaria alternata</i>	PFAEDDWEAW SyFFKTydgq --IVGDDLTV TNpEfIKkAI ElKscNALLL KVNqIGtITE aIqAAkDaFg AGWGMVSHR SGeTEDvtIA DiVVGLRsGQ		
<i>Cladosporium herbarum</i>	PFAEDDWEAW SyFyKtSGsd fQIVGDDLTV TNpEfIKkAI EtKacNALLL KVNqIGtITE aInAAkDSFA AGWGMVSHR SGeTEDvtIA DiVVGLRaGQ		
<i>Saccharomyces c. - 1</i>	PFAEDDWEAW SHFFKtAg-- IQIVaDDLTV TNpKRIATAI EKKAADALLL KVNqIGTLsE SIkAAqDSFA AGWGMVSHR SGeTEDTFIA DLVVGLRtGQ		
<i>Saccharomyces c. - 2</i>	PFAEDDWEAW SHFFKtAg-- IQIVaDDLTV TNpRIATAI EKKAADALLL KVNqIGTLsE SIkAAqDSFA AdWGMVSHR SGeTEDTFIA DLVVGLRtGQ		
<i>Candida albicans</i>	PFAEDDWEAW vHFFervGdk IQIVGDDLTV TNpTRIATAI EKKAANALLL KVNqIGTLTE SIqAAndSyA AGWGMVSHR SGeTEDTFIA DLVVGLRsGQ		
Consensus	PFAEDDWEAW SHFFKT-G-- IQIVGDDLTV TNp-RIKTAI EKKAANALLL KVNqIGTLTE SI-AA-DSFA AGWGMVSHR SGeTEDTFIA DLVVGLR-GQ		
	401	440	
<i>Alternaria alternata</i>	IKTGAPARSE RLAKLNQILR IEEELGdkrl YAGnNfRtAv nLZ		
<i>Cladosporium herbarum</i>	IKTGAPARSE RLAKLNQILR IEEELGdkrl YAGnNfRtAI nLZ		
<i>Saccharomyces c. - 1</i>	IKTGAPARSE RLAKLNQILR IEEELGdnAv fAGeNfhgd kLZ		
<i>Saccharomyces c. - 2</i>	IKTGAPARSE RLAKLNQILR IEEELGdkav YAGeNfhgd kLZ		
<i>Candida albicans</i>	IKTGAPARSE RLAKLNQILR IEEELGseAI YAGkdFqkAs qLZ		
Consensus	IKTGAPARSE RLAKLNQILR IEEELGD-A- YAG-NF--A- -LZ		

Fig. 1. Multiple amino acid sequence alignment of five fungal enolases. The single letter code for the standard proteinogenic amino acids is used. The consensus indicates that the amino acid residue is identical in at least three sequences.

Materials and Methods

Cloning and Expression of the Enolase of *A. alternata*

The enolase of *C. herbarum* was cloned as previously described [1]. Using the *Cladosporium* enolase-encoding cDNA as a probe, we screened an *A. alternata* cDNA expression library in the phage vector, lambda-ZAP. After isolation of a positive clone, the insert was re-cloned by the PCR into the vector pMW172 [1] and sequenced by the dideoxy method. For immunoblot analysis, *Alternaria* enolase was expressed in *Escherichia coli* [1], separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane and tested with patients' serum.

Immunological Cross-Reactivity between *Cladosporium* and *Alternaria* Enolase

After separation and blotting of an extract of *E. coli* cells expressing *Cladosporium* enolase, the protein band corresponding to the recombinant enolase was cut out and incubated with patients' serum. The depleted serum was then used to decorate an *A. alternata* protein

blot. A similar procedure was performed with recombinant *Alternaria* enolase and the depleted serum was applied to blotted protein extract of *Cladosporium*.

Enolase, an Allergen in Different Fungi

For testing IgE reactivity to enolase in different moulds, protein extracts of *A. alternata*, *C. albicans*, and *S. cerevisiae* and purified *Cladosporium* enolase were electrophoresed, blotted and incubated with patient serum according to standard immunological procedures.

Alignment and Dendrogram of Enolase Sequences of Various Species

The enolase sequences used for the alignment were obtained from the SWISS-PROT databank. For carrying out the alignment the GCG (Genetics Computer Group) software package was used. The underlying algorithm is a simplification of the progressive alignment method of Feng and Doolittle. The alignment and consensus sequence for the five fungal enolases was improved manually. The dendrogram was calculated using the PileUp program in the GCG software package.

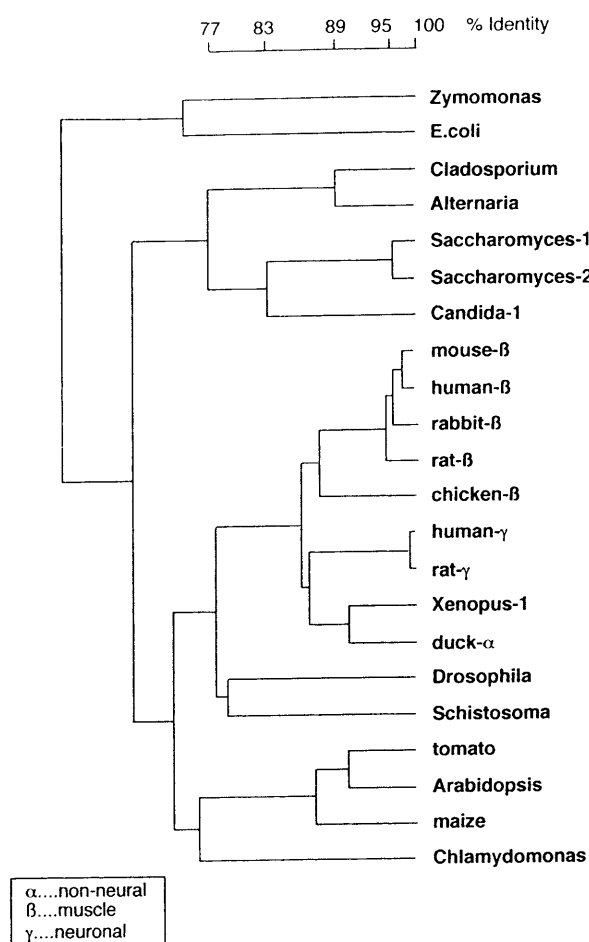


Fig. 2. Unrooted dendrogram of known enolase sequences. The scale designates average percent identity between two sequences or groups of sequences as indicated by the bifurcations.

Results and Discussion

Figure 1 shows the multiple sequence alignment and the consensus sequence of the five presently known fungal enolase sequences. Protein sequences derived from the experimentally determined cDNA sequences are shown. It is obvious that the fungal sequences show a very high degree of amino acid identity, even among fungi that are not close relatives according to current systematics (like ascomycetes on the one hand and fungi imperfecti on the other hand). Any pairwise comparison of these sequences reveals at least 77% amino acid identities (compare fig. 2).

Figure 2 shows the dendrogram calculated for the known enolase protein sequences. The fungi form a group well separated from the three other major kingdoms (prokaryotes,

plants and animals). In the animals, the enolase family splits into three iso-families, so that, for instance, the beta-enolases of different species cluster together and are more closely related than, say, human beta- and gamma-enolase. Based on these sequence comparisons, we do not expect the human enolases to react with patients' IgE. However, this has not been tested experimentally.

We present here two immunological experiments that seem to agree very nicely with the above sequence considerations (data not shown in detail):

First, serum from a typical patient displaying allergic reactions to both *Cladosporium* and *Alternaria* was depleted by pre-incubation with recombinant *Cladosporium* enolase bound to PVDF strips. This treatment resulted in complete disappearance of the enolase-specific band (but no other bands) from an IgE immunoblot of a total protein extract of *Alternaria*. The reverse experiment was also performed and resulted in substantial reduction of the enolase-specific band from an extract of *Cladosporium*. These results indicated nearly complete identity of the IgE-binding epitopes on the two enolases which is not surprising as their amino acid sequence identity is 89%.

The second experiment was blotting protein extracts from *Candida* (a clinical isolate from a patient with a skin infection), *S. cerevisiae* (a standard laboratory strain), *A. alternata* (our previously described strain) [1] and as a control, pure recombinant *Cladosporium* enolase onto PVDF and decorating it with IgE from the same allergic patient as above, who was known to recognize the enolases from *Alternaria* and *Cladosporium*. The resulting immunoblot showed bands of about equal intensity at exactly the same molecular weight (46–48 kD) in all four fungi.

We tested the purified recombinant *Cladosporium* enolase in IgE immunoblots with about 50 patients sensitized to *Cladosporium*. Thus it was revealed that enolase is the second most important allergen of this mould, with more than 50% of those patients reacting positively. Similar experiments with pure recombinant enolase of *Alternaria* are under way.

In conclusion, enolase was found to be a highly conserved major allergen of all fungi investigated here. If it can be confirmed that enolase in *Candida* [7, 8], and possibly in several allergenic fungi, is not exclusively located in the cytoplasm but also in the periplasmic space, this could among other factors explain the dominant role of this allergen.

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References

- 1 Achatz G, Oberkofler H, Leehenauer E, Simon B, Unger A, Kandler D, Ebner C, Prillinger HJ, Kraft D, Breitenbach M: Molecular cloning of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. *Mol Immunol* 1994;32:213-227.
- 2 Iida H, Yahara I: Yeast heat shock protein of M 48,000 is an isoprotein of enolase. *Nature* 1985;315:688-689.
- 3 Baldo BA, Baker RS: Inhalant allergies to fungi: Reactions to bakers yeast (*Saccharomyces cerevisiae*) and identification of bakers yeast enolase as an important allergen. *Int Arch Allergy Appl Immunol* 1988;86:201-208.
- 4 Ishiguro A, Homma M, Torii S, Ianaka K: Identification of *Candida albicans* antigens reactive with immunoglobulin E antibody of human sera. *Infect Immun* 1992;60:1550-1557.
- 5 Ito K, Ishiguro A, Kanbe T, Tanaka K, Torii S: Detection of IgE antibody against *Candida albicans* enolase and its cross-reactivity to *Saccharomyces cerevisiae* enolase. *Clin Exp Allergy* 1995;25:522-528.
- 6 Mason AB, Buckley HR, Gorman JA: Molecular cloning and characterization of the *Candida albicans* enolase gene. *J Bacteriol* 1993;175:2632-2639.
- 7 Angiolella L, Facchin M, Stringaro A, Maras B, Simonetti N, Cassone A: Identification of a glucan-associated enolase as a main cell wall protein of *Candida albicans* and an indirect target of lipopeptide antimycotics. *J Infect Dis* 1996;173:684-690.
- 8 Sundstrom P, Aliaga GR: A subset of proteins found in culture supernatants of *Candida albicans* includes the abundant, immunodominant, glycolytic enzyme enolase. *J Infect Dis* 1994;169:452-456.