

Characterization of IgE-binding epitopes on *Candida albicans* enolase

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Summary

Candida albicans enolase is one of the important allergens in *Candida* allergy. We isolated and purified 46 kDa *C. albicans* enolase (CAE) from *C. albicans* and characterized epitopes for IgE antibody by lectin-blotting and enzymatic digestion followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Lectin blotting and deglycosylation indicated that this protein did not contain polysaccharide side chains. The purified CAE and recombinant fusion protein produced from CAE gene possessed common epitopes for IgE antibody. We estimated IgE binding epitopes on the basis of reported amino acid sequences from the analysis of cDNA encoding CAE. V8 protease digestion of CAE gave six polypeptide fragments (A–F). The N-termini of each fragment were confirmed by amino acid sequence and the C-termini were estimated by molecular weights of each fragment and the specific cutting site of V8 protease. Fragment C (25.0 kDa; F-171–I-399) reacted to 90% IgE antibodies examined, whereas fragments D (21.0 kDa; F-171–I-360), E (16.2 kDa; F-171–D-317) and F (13.0 kDa; A-47–E-170) showed no IgE binding. Our results suggest that epitopes for IgE antibodies exist near the C-terminal of the protein.

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Introduction

Candida albicans is a common commensal of the gastrointestinal tract in man but may also cause a disseminated infection in immunocompromised hosts [1] or an allergic disease such as bronchial asthma [2] or atopic dermatitis [3]. Several studies have described antigenic and allergenic components of *C. albicans* for diagnosis of fungal infection or allergy [4,5]. Enolase, an enzyme that catalyses the reversible dehydration of 2-phosphoglycerate to phosphoenolpyruvate, is an immunodominant antigenic component in disseminated *C. albicans* infection. *C. albicans* enolase (CAE) antigen or CAE-specific IgG antibody, in addition to cell wall mannoprotein, were detected specifically in the serum of patients with systemic *C. albicans* infections [6,7]. CAE is also an important

allergen. Ishiguro *et al.* [8] reported that the 46 kDa component of *C. albicans* antigen frequently reacted to IgE antibody of allergic patients, and demonstrated that the protein was an enolase due to the similarity of its partial amino acid sequence to *Saccharomyces cerevisiae* enolase (SCE) as well as by antigenic crossreactivity with SCE by rabbit antiserum. The importance of *C. albicans* 46 kDa protein as an allergen was first suggested by Savolainen *et al.* [9]. SCE was also suspected to be the immunodominant component of *S. cerevisiae* allergen [10]. Thus, these reports identified enolase as an important antigen and allergen.

Sundstrom and Aliaga [11] cloned and sequenced a 1320 bp cDNA encoding CAE and predicted 440 amino acid sequence with a molecular weight 47 kDa. Subsequently, Mason *et al.* [12] and Frnklyn *et al.* [13] reported similar results on molecular cloning of CAE gene. Genes that encode enolase proteins have been cloned in several species spanning from lower eukaryotes to humans and are known to be highly conserved. For example, the

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amino acid sequence predicted from CAE gene showed 65.1 and 65.6% identity with that of human α - and γ -enolase, respectively [11].

Several studies attempted to identify IgE binding epitopes of allergens at a molecular level [14–16]. For example, B cell epitopes of *Der p* I, a major allergen of *Dermatophagoides pteronyssinus* was analysed using histamine release capacity of four synthesized peptides with 11–21 amino acid composition [14]. In addition, the amino terminal peptide of birch pollen allergen (*Bet v* I 23–38) was found to have IgE-binding capacity [15]. As for eight different vespid venom antigen 5 molecules, the primary structure of IgE-binding epitopes was analysed and the structural basis for the antigenic crossreactivity was demonstrated [16]. However, only a few studies have analysed the epitopes of fungal allergens [17]. The difficulty of studying fungal allergens may be related to their 'batch to batch' variety of crude antigens [18] and their highly glycosylated properties in nature. These difficulties seem to be due to the use of a crude antigen for analysis. Epitope analysis of a well characterized fungal antigen may be necessary in order to clarify the immune mechanism of fungal infection and allergy, as well as the structural basis of crossreactivity of fungal allergens. In the present series of experiments, we purified enolase from *C. albicans*, characterized CAE and its IgE binding epitopes, and predicted epitopes on the amino acid sequence by enzymatic cleavage of the antigen.

Materials and methods

Organism

C. albicans strain C9 (serotype A) [8,19] was used in the present experiments. Strain C9 was maintained at -80°C in 10% DMSO. Yeast cells were pre-incubated in 100 ml of GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) for 24 h at 37°C under constant shaking. Then yeast form cells at stationary phase were inoculated into 10 l of fresh GYEP and cultured for 24 h at 37°C with continuous shaking.

Isolation and purification of CAE

Yeast cells grown in GYEP for 24 h at 37°C were washed in distilled water and treated, at room temperature for 30 min, with 0.1 M EDTA (pH 9.0) containing 0.3 M 2-mercaptoethanol. After centrifugation, the pellet was suspended in 50 mM phosphate-buffer (pH 7.0) containing 12.5 U/ml zymoylase 100 T (Seikagaku Kogyo Co., Tokyo, Japan) and incubated at 37°C for 60 min. Spheroplasts were harvested by centrifugation and homogenized in 50 mM phosphate-buffer (pH 7.0) using a glass

homogenizer. Saturated NH_4SO_4 solution was added to the supernatant, and the precipitated material between 50 and 75% saturation was collected. The sediment was dissolved in 10 mM Tris-HCl (pH 7.8) and dialysed against the buffer for 48 h at $5-8^{\circ}\text{C}$. The sample was applied to a DEAE Sephacel (Pharmacia LKB, Uppsala, Sweden) column and eluted with a linear gradient of 0–0.2 M NaCl in 10 mM Tris-HCl (pH 7.8). Each fraction was analysed for protein contents by SDS-PAGE, and fractions corresponding to the 46 kDa component were pooled. The pooled fraction was dialysed against 10 mM Tris-HCl (pH 8.3) for 24 h at $5-8^{\circ}\text{C}$ and applied to a DEAE Sephacel column. The 46 kDa protein was eluted with a linear gradient of 50–200 mM NaCl in 10 mM Tris-HCl (pH 8.3). The eluent was dialysed against 20 mM phosphate-buffer containing 0.15 N NaCl (PBS) and applied to a Concanavalin A (ConA)-agarose column (Honen Co., Tokyo, Japan) [20]. PBS-eluted fractions were pooled and dialysed against distilled water. The 46 kDa protein was stored at -80°C until used. The enolase activity of the 46 kDa protein was determined according to the method of Westhead [21], using *Saccharomyces cerevisiae* enolase (SCE) (Sigma Co., St Louis, USA) as a control.

Recombinant CAE

The protein product of 1.4-kbp cDNA encoding *C. albicans* enolase, fused to the carboxyl-terminal end of glutathione S-transferase, was kindly provided by Dr G. R. Aliaga, Department of Microbiology and Immunology, Texas College of Osteopathic Medicine, University of North Texas, USA. Characterization of this fusion protein (FP) has been previously reported by Sundstrom and Aliaga [11].

Patient sera

Sera from 10 atopic patients, reacting to purified 46 kDa protein (CAE) in IgE immunoblotting, were used in the present study. All of them showed positive IgE to crude *Candida albicans* antigen with a score of three or more by CAP-RAST FEIA kit (Pharmacia, Uppsala, Sweden). The experimental protocol was approved by the Human Ethics Review Committee of our institution.

SDS-PAGE and immunoblotting

Experiments using SDS-PAGE and immunoblotting were conducted using a method previously described by Ishiguro *et al.* [8]. Briefly, the electrophoresed gel was stained with Coomassie brilliant blue R250 (CBB) or electroblotted onto polyvinylidene difluoride (PVDF)

sheets (Immobilon, Millipore, Bedford, MA, USA). Blocking was performed by soaking 3% skim milk in TBS (20 mM Tris-HCl, pH 7.5, 0.5 N NaCl) for 30 min. For IgE immunoblotting, the sheet was allowed to react overnight with the serum (1:10) at 20–25°C. After washing, the sheet was incubated with affinity-purified alkaline phosphatase-conjugated goat anti-human IgE antibody (Kirkegaard & Perry Laboratories Inc. Gaithersburg, MD, USA) (1:2000) for 3 h at 20–25°C. Bands were visualized with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate disodium salt as substrate. For immunoblotting with anti-46 kDa protein (CAE) rabbit antiserum [8], the sheet was incubated overnight with anti-46 kDa polyclonal rabbit serum (1:2000) at 20–25°C, followed by horseradish peroxidase conjugated affinity purified goat anti-rabbit IgG (Bio Rad, Hercules, CA, USA) (1:2000) for 3 h at 20–25°C. The sheet was later soaked in TBS and incubated at 20–25°C in a mixture of 50 ml of TBS, 30 μ l H₂O₂ and 30 mg of 4-chloro-1-naphthol in 10 ml methanol. Molecular weight markers used in the present study (Sigma) were as follows: α -lactalbumin (14.2 kDa) from bovine milk, a trypsin inhibitor (20.1 kDa) from soy bean, trypsinogen (24.0 kDa) from bovine pancreas, carbonic anhydrase (29.0 kDa) from bovine erythrocytes, glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa) from rabbit muscle, egg albumin (45.0 kDa), and bovine albumin (66.0 kDa).

Immunoblot inhibition test

The immunoblot inhibition test was used to examine whether CAE and the recombinant FP share IgE binding epitopes. Patient sera diluted to 1:10 with TBS containing 1% skim milk were incubated overnight in the presence of 5 or 10 μ g/ml CAE or FP at 4°C. Electroblotted sheets were later allowed to react with the pretreated sera, followed by a second incubation process, as described above.

Lectin blotting

Electrophoresed gels were blotted onto nitrocellulose sheets [Cellulosenitrat(E)BA85; Schleicher & Schnell, Dassel, Germany] and blocked with TBS containing 3% BSA and 0.1% Tween-20. After washing with TBS containing 1% BSA and 0.05% Tween-20, each sheet was incubated with 10 μ g/ml peroxidase-conjugated lectins [Concanavalin A (ConA), phytohemagglutinin (PHA), *Ricinus communis* agglutinin (RCA), peanut lectin (PNA), wheat germ agglutinin (WGA)] for 60 min. After the second washing, peroxidase staining was completed as described above.

Endo- and exoglycosidase treatment

CAE (300 μ g/ml) was incubated for 1 h at 37°C with 40 or 400 U/ml endoglycosidase H (Biolabs, New England, USA) in 0.2 M citrate buffer (pH 5.0). For exoglycosidase treatment, CAE (300 μ g/ml) was incubated with 0.5 or 5.0 U/ml α -mannosidase from Jack beans (Sigma) and 1 mM ZnSO₄ in 50 mM sodium acetate buffer (pH 4.4). Finally, following glycosidase treatment, IgE immunoblotting was carried out using ovalbumin as a control for enzymatic digestion.

Protease treatment

CAE (200 μ g/ml) was digested with 5 μ g/ml of *Staphylococcus aureus* V8 protease (Sigma), which is specific for glutamyl and some aspartyl peptide bonds [22], in 125 mM Tris-HCl (pH 6.8), 0.1% SDS and 1 mM EDTA. The mixture was incubated at 37°C for 1 h and proteolysis was terminated by the addition of 100 μ l of 20% SDS/1000 μ l sample. The product was resolved on 12% polyacrylamide gel followed by amino acid sequencing or immunoblotting. The molecular weight of each fragment was determined from their relative mobility compared with molecular weight markers.

Amino acid sequence

Separated polypeptides were transferred onto PVDF (Immobilon) sheets by using a Tris-borate buffer system according to the method described by Matsudaira [23] using a semi-dry electroblotter (Sartorius, Gettingen, Germany). Polypeptide bands detected by CBB-R250 staining were cut out, destained in 90% methanol and analysed with an automatic peptide sequencer (ABI 473A, Applied Biosystems, CA, USA).

Results

Characterization of CAE

The 46 kDa protein was purified from extract of *C. albicans* spheroplasts by sequential fractionation with DEAE and ConA-agarose column chromatography. The PBS-eluted fraction on ConA-agarose chromatography contained a major component of 46 kDa and a few minor components on SDS-PAGE and immunoblotting with anti-CAE rabbit antiserum (Fig. 1, lane 1). Enolase activity of this fraction was 4262 U/mg protein, more than ten times higher than that of SCE (406 U/mg protein). This fraction was designated as CAE and used in the following experiments.

CAE testing for carbohydrate moiety by lectin



Fig. 1. Coomassie brilliant blue-R250 (CBB) staining of SDS-PAGE (A) and immunoblotting profile (B) of *C. albicans* enolase (CAE, lane 1) and V8 protease digested CAE (lane 2). Immunoblotting was performed with anti-CAE rabbit serum. Molecular weight standards are shown on the left. Six polypeptide fragments obtained by V8 protease digestion are indicated on the right (A–F).

blotting with ConA, PHA, RCA, PNA and WGA demonstrated that all lectins failed to bind to CAE. We also examined the existence of carbohydrates in the CAE by enzymatic digestion using both endoglycosidase H

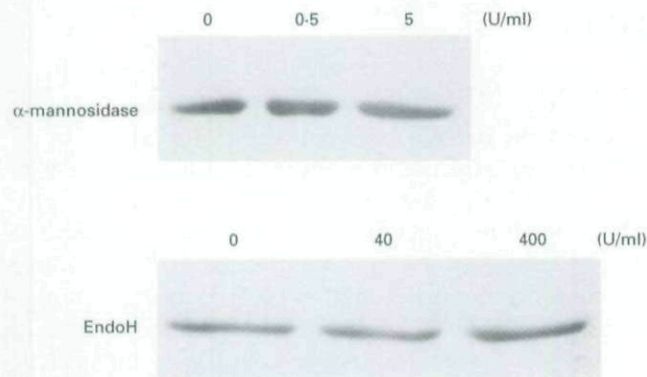


Fig. 2. Effects of deglycosylation on IgE immunoblotting of CAE. The antigen was treated with α -mannosidase or endoglycosidase H and allowed for IgE immunoblottings.

and α -mannosidase. Although deglycosylation of ovalbumin was observed at 0.5 U/ml α -mannosidase or 40 U/ml endoglycosidase H, the use of higher concentrations of both enzymes did not affect the molecular weight of CAE (Fig. 2). These results indicated that CAE did not contain a polysaccharide moiety.

We also examined the chemical properties of IgE-binding epitopes on CAE. Reducing the condition of CAE with 2-mercaptoethanol and boiling for 10 min did not influence the binding to IgE antibody. Furthermore, IgE binding revealed by immunoblotting was similar to that by dot-blotting without denaturation by SDS. These results suggested that IgE-binding epitopes were mostly related to the sequential basis rather than the conformational nature of the molecule. These results prompted us to characterize IgE-binding epitopes on the primary amino acid sequence of the molecule.

Immunoblotting inhibition test between CAE and recombinant FP

Immunoblotting inhibition test examined antigenic crossreactivity between purified CAE and recombinant FP, a product of CAE gene. Simultaneous IgE binding between CAE and FP was detected in sera of two atopic patients during IgE immunoblotting. Pre-treatment of sera with CAE or FP completely inhibited the binding of IgE to FP as well as to the homologous protein (Fig. 3). These results indicated common epitopes for human IgE antibody are present in both CAE and recombinant FP.

V8 protease digestion of CAE

In order to examine the sequential epitopes of CAE for IgE antibody, CAE was digested with *S. aureus* V8 protease and polypeptide fragments were separated by SDS-PAGE. Six major bands (A–F), detected by CBB staining of SDS-PAGE, reacted to polyclonal anti-CAE rabbit antibody (Fig. 1). The molecular weights and N-terminal amino acid sequences of each fragment are shown in Table 1. Fragment A (46 kDa) was found to be the original CAE and fragment B (42.0 kDa) was thought to lack a small polypeptide on the N- or C-terminal of CAE. Fragments C (25.0 kDa), D (21.0 kDa) and E (16.2 kDa) had similar N-terminal amino acid sequences. The N-terminal six amino acid sequence was identical to that from Phe-171 to Thr-176 (FMIAPT) except one amino acid on fragment E (Arg instead of Met) reported by Sundstrom and Aliaga [11]. The N-terminal amino acid sequence of fragment F (13.0 kDa) was identical to that of Ala-47 to Asp-52 of CAE. The C-termini of each fragment on CAE were accurately predicted using the molecular weights of each fragment

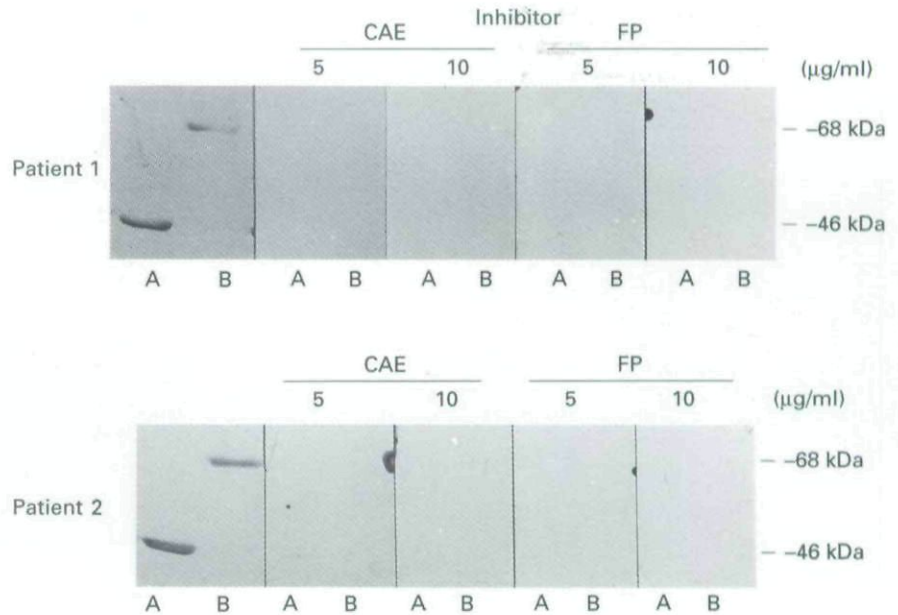


Fig. 3. IgE immunoblotting inhibition test of CAE (A) and recombinant fusion protein (FP) of CAE gene (B) in two patients. For immunoblotting inhibition assay, sera (1 : 10 dilution) were pre-incubated overnight at 4°C with each concentration of the inhibitor and followed by immunoblotting. The molecular weight of each antigen is shown on the right.

and specificity of V8 protease. The estimated C-termini and calculated molecular weights of amino acid composition of each fragment are summarized in Table 1.

IgE immunoblotting of protease-digested CAE

Figure 4 illustrates two examples of IgE immunoblotting profiles. All 10 sera reacted to fragments A and B, while nine (90%) reacted to fragment C. On the other hand, no IgE binding was observed to fragments D, E and F (Table 1). Figure 5 is a schematic drawing of peptide fragments and their IgE-binding activity. A comparison of IgE-binding activity of fragments C and D suggested that IgE-binding epitopes are present near the C-terminal of the protein, particularly between Gln-361 and Ile-399.

Discussion

We were able in the present study to isolate and purify *C. albicans* enolase from spheroplasts of *C. albicans* and characterize the epitope(s) for IgE. The purified CAE did not contain a polysaccharide moiety as lectin binding and molecular weight change after enzymatic deglycosylation were not observed. This finding is similar to *S. cerevisiae* enolase which is also known to contain no polysaccharide moiety [24]. These results suggest that epitopes of CAE for IgE antibody are present in protein moiety.

Reduction with 2-mercaptoethanol and boiling did not influence IgE binding since CAE contains no cysteine residues [11]. Furthermore, degeneration of

CAE with SDS also failed to affect IgE-binding capacity. These findings indicate that IgE-binding activity of CAE is related to the primary structure rather than conformational epitopes. Characterization of IgE-binding epitopes according to the primary structure of CAE [11], suggested existence of common epitopes for human IgE between CAE and recombinant FP of *C. albicans* enolase. Enolase is biologically active only as a dimer, and in general, the enzyme subunits are products of different enolase genes [12]. However, *C. albicans* possesses a single enolase gene per haploid genome, indicating that the enzyme must exist as homodimer of a single gene product [12,13].

IgE immunoblotting analysis of V8 protease digested fragments of CAE in the present study suggested that IgE binding epitopes may exist near the C-terminal of the

Table 1. Properties of fragments obtained from V8 proteinase digested CAE

Fragment (kDa)	N-terminal amino acid	Estimated peptide (kDa)
A (46.0)	Blocked	Original CAE protein
B (42.0)	Not done	
C (25.0)	FMIAPT- - -	F(171)-I (399) (25.05)
D (21.0)	FMIAPT- - -	F(171)-I (360) (20.97)
E (16.2)	FRIAPT- - -	F(171)-D (317) (16.23)
F (13.0)	ALELRD - - -	A (47)-E (170) (12.70)

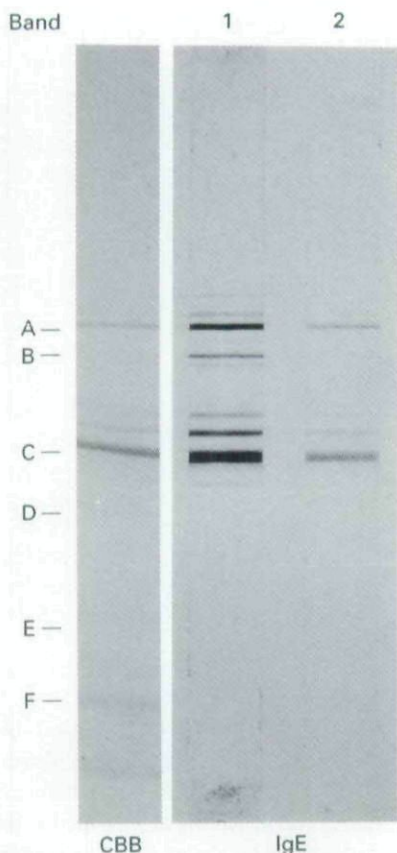


Fig. 4. IgE immunoblotting profiles of V8 protease-digested CAE. A CBB-R250 staining of electroblotted PVDF sheet and designations of each fragment are shown on the left.

protein, especially between Gln-361 and Ile-399. Determined C-terminal residues in the present study may, however, be imprecise since they were only estimated from molecular weights measured by SDS-PAGE.

In general, continuous antigenic determinants on a globular protein are hydrophilic and surface-located

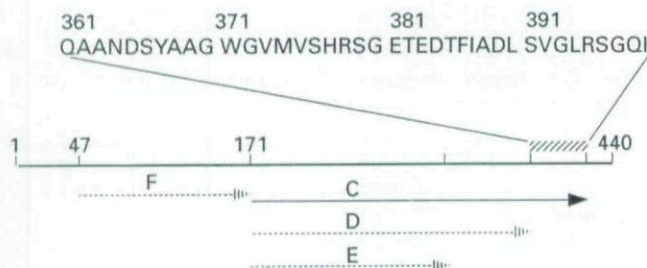


Fig. 5. Schematic illustration of the fragments (C-F) on 440 amino acid sequence of CAE. Solid line: a fragment that reacted with IgE, dotted line: a fragment that did not react with IgE. The estimated IgE-binding epitope is represented by oblique lines and the amino acid sequence of that region is shown according to Sundstrom and Aliaga [11].

residues [25]. Hoop and Wood prediction for hydrophilicity suggests that the Gln-361 to Ile-399 region contains some hydrophilic residues. On the other hand, Chou-Fasman analysis for predicting the secondary structure of a molecule suggests that this region consists of α -helices and small β -sheets [11]. The crystal structure of SCE has been determined to be an irregular $\alpha\alpha\beta\beta(\alpha + \beta)_6$ TIM barrel structure in which α -helices are located outside the molecule [26,27]. The Lys-352 to Ala-365 of *S. cerevisiae* enolase 1, corresponding to CAE 361–399, are the seventh α -helix of the TIM barrel structure. Furthermore, computer graphic analysis of the three-dimensional structure of SCE revealed that Gln-361 to Arg-378 exists on the surface of the molecule. Such data suggest that Gln-361 to Ile-399 residues could possibly be the antigenic determinant of CAE.

CAE shows only a partial crossreactivity to *S. cerevisiae* enolase and no crossreactivity with human enolase or rabbit muscle enolase (unpublished observation). In contrast to 371–399 of CAE, those of 361–370 are not identical to the corresponding residues of enolases from *S. cerevisiae* rabbit muscle and human [12].

In the present study, we reported some preliminary findings related to epitopes for IgE antibody on CAE, which was a highly conserved protein in yeast cells. The possibility that tertiary structure on the molecule is related to the IgE binding remains unclear. Confirmation of IgE epitopes requires more direct evidence through the use of synthetic polypeptides [28]. Studies determining epitopes for IgG antibody and T cell receptors may be of interest since CAE acts as an immunodominant antigen in systemic *C. albicans* infection and represents an important allergen in *Candida* allergy.

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