

Purification of native enolase from medically important *Candida* species

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The 48 kDa glycolytic enzyme, enolase, has been identified as an immunodominant antigen in *Candida albicans* infections. It has also been identified as an important fungal allergen. Enolase from a number of medically important *Candida* species has been purified using a two-step anion- and cation-exchange chromatography method that was preceded by an organic extraction. The enolases purified by this method have a high specific activity and the procedure is 40% efficient, with an average of 5 mg of enolase/g of *Candida* cells. The purification of native enolase from medically important *Candida* species will enable the immunological significance and interspecies relationships of this major fungal antigen to be investigated.

Introduction

Candida species are major fungal pathogens that are associated with a high rate of mortality in hospitalized patients. The most frequent cause of *Candida* infections is *Candida albicans*; however, other *Candida* species such as *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. lusitanae* and *C. krusei* now account for an increasing proportion of *Candida* infections [1–4]. One of the major reasons for this increase has been the introduction of azole drugs, such as fluconazole for prophylaxis and treatment of candidiasis [4–8]. Given the fulminant and rapidly fatal outcome of acute disseminated candidiasis, rapid and reliable identification of infection by the newly emerging *Candida* species is critical to the implementation of early and appropriate antifungal therapy. However, the identification of invasive or disseminated candidiasis is frequently based on clinical suspicion as clinical symptoms are diffuse and not easily differentiated from those manifested by other infectious agents [9]. There is therefore considerable interest in the identification and characterization of antigens that may be useful in the diagnosis and treatment of *Candida* infections.

Recent studies with the *Candida* species, *C. albicans* and *C. tropicalis* have identified the cytosolic enzyme enolase as an immunodominant antigen [10,11]. Antibody to the enolase antigen is considered more predictive and specific

for invasive candidiasis as the cytoplasmic antigen is exposed only during invasive infection. Patients with localized candidiasis express only weak positive antibody titre [12].

To investigate further the immunological relevance of enolase from other pathogenic *Candida* species we have developed a rapid and efficient method to purify *Candida* enolases. The method has been applied successfully to purify enolase from a number of medically important *Candida* species, namely, *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. lusitanae* and *C. krusei*. These enolases can now be studied to determine their immunological significance. The extent of antibody cross-reactivity between the enolases of the medically important *Candida* species can also be investigated.

Materials and methods

Yeast identification and growth conditions

Clinical isolates of *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. lusitanae* and *C. krusei* were obtained from Dr Rose McAleer of the Mycology section of the PathCentre (QEII Medical Centre, Nedlands, WA, Australia). The identity of the *Candida* species was confirmed with the use of an API 20C Auxonagram strip (API System S. A., Marcy l'Étoile, France). A starter culture was inoculated to YEPD culture medium (1% yeast extract, 2% peptone and 2% D-glucose) and incubated for 24 h at 30 °C with aeration. A 200 ml starter culture provided the basis for 10 litre *Candida* cultures to be grown under similar conditions in a 23 litre Bio-Flo Fermenter IV System (New Brunswick Scientific, Edison, NJ, U.S.A.).

Harvest and preparation of yeast cells

The *Candida* cultures were harvested from the Bio-Flo fermenter system and separated from culture medium with the use of a Pellicon filtration cassette (Millipore, Bedford,

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Abbreviations used: PGA, D-(+)-2-phosphoglyceric acid; PEP, phosphoenolpyruvate.

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MA, U.S.A.). Concentrated cells were separated from residual medium by centrifugation in 500 ml centrifuge flasks for 15 min at 1660 g and 4 °C. The supernatant was discarded and the pelleted cells were resuspended in protein-extraction buffer (20 mM Bis-Tris, pH 6.5). The yeast cells were centrifuged as described previously then resuspended and pooled for further processing.

Cell lysis and preparation of crude lysate

Candida cell walls were ruptured mechanically with the use of a Dymill® (WAB, Basel, Switzerland). Milling was maintained until 99% cell disruption was obtained. The crude *Candida* cytoplasmic extracts were collected and dispensed into 50 ml centrifuge tubes. The extracts were centrifuged for 12 h at 8517 g and 4 °C to precipitate particulate matter and cellular debris. The supernatants containing the soluble protein fraction were recovered and passed through a 0.45 µm filter membrane. The filtrates were then extracted with an equal volume of chilled chloroform. Following centrifugation at 4 °C for 15 min at 1036 g the upper aqueous phase was aspirated and transferred to a dialysis tube. The crude protein fractions were dialysed in column binding buffer (20 mM Bis-Tris, pH 6.5) for 12 h in preparation for chromatography.

Anion-exchange chromatography

The crude *Candida* cytoplasmic extracts were recovered from dialysis and filtered through a 0.2 µm syringe filter (cellulose acetate). The filtered extracts were applied to a Pharmacia Biotech XK 50/20 chromatography column packed with Pharmacia Biotech Source 15Q quaternary ammonium anion exchanger (Pharmacia LKB, Uppsala, Sweden). The column was equilibrated prior to chromatography with column binding buffer A (20 mM Bis-Tris, pH 6.5). Anion-exchange chromatography of the crude extracts was controlled and recorded using the Bio-Rad Econo® system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Bound protein was eluted from the column with a salt gradient of buffer B (1 M NaCl in buffer A, pH 6.5). The recovered fractionated proteins were analysed by an enzyme-activity assay. Eluate fractions containing enolase activity were selected and dialysed in preparation for cation-exchange chromatography.

Cation-exchange chromatography

Fractions having enolase enzyme activity were dialysed for 12 h at 25 °C in high-pure H₂O. The dialysed fractions were recovered and filtered through a 0.2 µm syringe filter. The filtrate was concentrated 10-fold by evaporation under

vacuum for 5 h. The concentrated samples were dialysed with binding buffer C (10 mM sodium acetate, pH 4.7) immediately prior to application to a Pharmacia Biotech Mono S HR10/10 chromatography column packed with methyl sulphonate cation exchanger (Pharmacia LKB). Cation-exchange chromatography was performed using the Bio-Rad Biologic® system. Bound protein fractions were eluted from the column with a salt gradient of buffer D (1 M NaCl in buffer C, pH 4.7). Fractions containing enolase activity were identified by enzyme-activity assay.

Enzyme-activity assay

The active enzyme enolase will hydrolyse D-(+)-2-phosphoglyceric acid (PGA) to phosphoenolpyruvate (PEP). The production of PEP can be monitored by spectrophotometry at 240 nm. Protein solution (20 µl) was combined with 1 ml of enolase substrate solution (50 mM Tris/HCl, pH 7.4/2.7 mM magnesium acetate/1.0 mM EDTA/1.2 mM PGA) in a quartz cuvette and the change of absorbance recorded at 1 min intervals. The specific activity is defined as the conversion of 1 µmol of PGA to PEP/min per mg of protein [13]. An estimate of the quantity of protein in solution was performed using the Bio-Rad (Bradford) microassay procedure.

SDS/PAGE and immunoblot analysis

Sample loading buffer was added to the enolase samples which were then boiled for 5 min prior to SDS/PAGE [12% (w/v) gel concentration]. Following electrophoresis the fractionated proteins were transferred by Western blot to PVDF transfer membrane (PolyScreen®, NEN® Research, Boston, MA, U.S.A.) for immunoscreening.

Free binding sites were blocked with 2% BSA (ICN) in a solution of PBS/Tween 20 (PBS, pH 7.3/0.01% Tween 20) at 25 °C for 90 min. Hyperimmune sera raised in rabbit against recombinant *C. albicans* enolase [14] diluted 1/100 in PBS-Tween 20 was then added and incubated at 25 °C for 90 min. After extensive washing in PBS-Tween, anti-rabbit IgG alkaline phosphatase conjugate (Silenus) diluted 1/2000 in PBS-Tween 20 was applied and incubated at 25 °C for 90 min. Following further washing, the detection substrates [5-bromo-4-chloroindol-3-yl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT)] were applied.

Results

Fractionation of crude cytoplasmic extract

The elution profile recorded during fractionation of the *C. krusei* crude extract on the Pharmacia Biotech XK 50

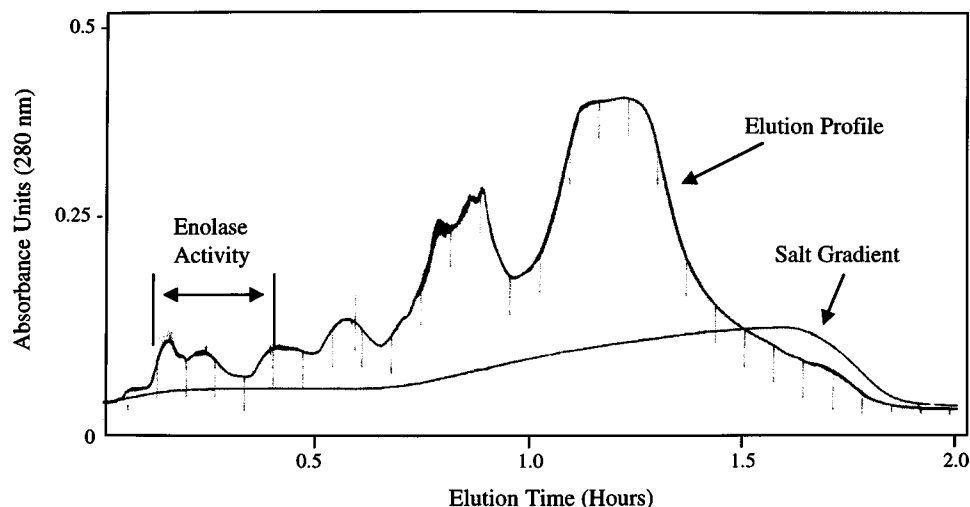


Figure 1 Elution profile for fractionation of crude *C. krusei* cytoplasmic extract by anion-exchange chromatography

Crude *C. krusei* cytoplasmic extract was applied to a Pharmacia Biotech XK 50 chromatography column containing Source Q anion-exchange resin. Bound proteins were eluted using a 0–1 M NaCl salt gradient. Eluate fractions (10 ml) were collected and assayed for enolase enzyme activity. Fractions containing activity were pooled and dialysed.

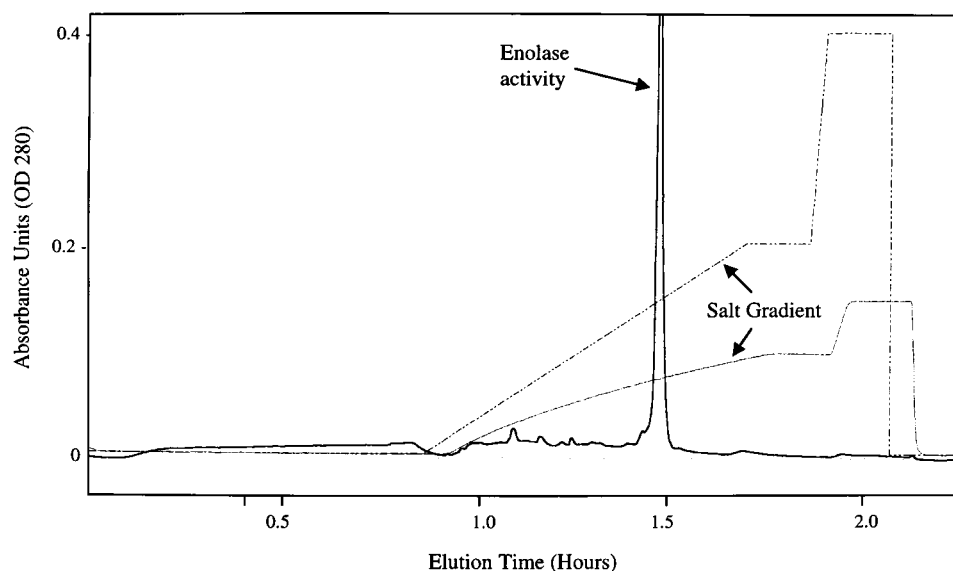


Figure 2 Elution profile recorded during cation-exchange purification of *C. krusei* enolase

Following dialysis, fractions containing enolase activity were applied to a Pharmacia Biotech Mono S HR10/10 chromatography column. Bound proteins were eluted using a 0–1 M NaCl salt gradient. The enolase activity was eluted in a single 4 ml fraction that corresponded to the main absorbance peak.

chromatography column using Source Q anion-exchange resin is presented in Figure 1. Eluate fractions were analysed for enolase enzyme activity, the enolase activity was contained within five 10 ml fractions. Similar elution profiles were obtained for extracts of the other *Candida* species (results not shown). The absorbance profile (Figure 1) of the fractions containing enolase activity and SDS/PAGE (results

not shown) indicated that further purification of the enolase samples was required.

Cation-exchange chromatography

Fractions recovered from anion-exchange chromatography with significant enolase activity were applied to the

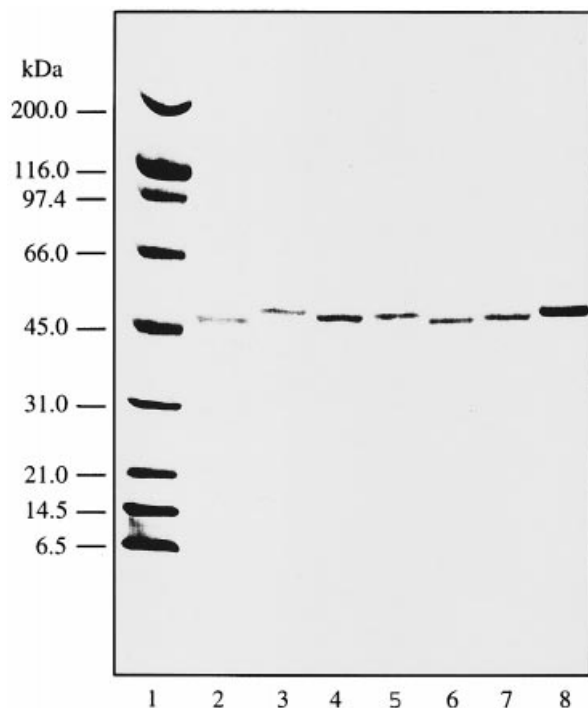


Figure 3 SDS/PAGE analysis of purified enolase enzyme recovered from cation-exchange chromatography

Extracts (2 µg) of each *Candida* enolase sample recovered from cation-exchange chromatography (Mono S) were applied to separate wells of a homogeneous SDS/PAGE (12%, w/v) gel. Following electrophoresis the gel was stained with Coomassie Brilliant Blue. Lane 1 contains molecular-mass markers (Bio-Rad). Lanes 2–7 contain enolases from *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis* and *C. lusitanae* respectively. Lane 8, commercial preparation of Baker's yeast enolase (Sigma).

Pharmacia Biotech Mono S HR 10/10 cation-exchange column. Figure 2 presents the elution profile recorded during the isolation of *C. krusei* enolase. The enolase enzyme was eluted by salt gradient into a single 4 ml fraction, which was characterized by a well-defined spike. Similar elution profiles were observed for the enolases of other *Candida* species using identical elution conditions (results not shown).

SDS/PAGE and immunoblotting

Aliquots (2 µg) of the purified *Candida* enolases were applied to separate lanes in a 12% polyacrylamide gel in a Bio-Rad mini-protean electrophoresis system. Figure 3 represents the results observed when the purified fractions were electrophoresed and stained with Coomassie Brilliant Blue. Each purified *Candida* enolase appears as a single band of approximately 46–48 kDa.

To confirm that the Coomassie Brilliant Blue staining bands seen on the SDS/PAGE gel were enolase, the samples were immunoblotted and reacted with rabbit hyperimmune antisera raised against a recombinant *C. albicans* enolase. The

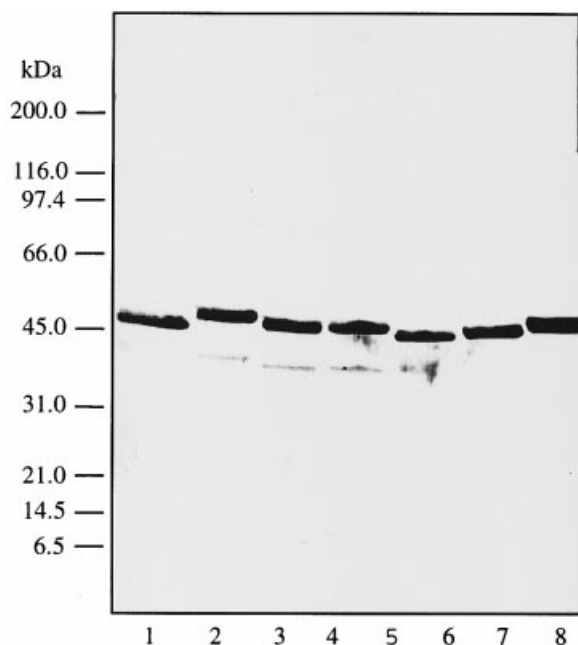


Figure 4 Immunoblot of purified *Candida* enolases reacted with anti-*C. albicans* enolase hyperimmune rabbit antisera

Extracts (2 µg) of each *Candida* enolase sample recovered from cation-exchange chromatography (Mono S) were applied to separate wells of a homogeneous SDS/PAGE (12%, w/v) gel. Following electrophoresis the gel was immunoblotted and reacted with anti-*C. albicans* enolase hyperimmune rabbit antisera. Lane 1 contains molecular-mass markers (Bio-Rad). Lanes 2–7 contain enolases from *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis* and *C. lusitanae* respectively. Lane 8, commercial preparation of Baker's yeast enolase (Sigma).

anti-enolase antisera reacted with each of the seven 46–48 kDa protein bands (Figure 4).

Specific activity and enolase recovery

The activity of the enolases was measured by an enzyme-activity assay, using the commercially available Baker's yeast enolase (Sigma) as a standard. The concentration of protein in the preparations was determined using a protein assay kit (Bio-Rad). The specific activity for each *Candida* enolase was estimated to be approximately 65–70 units/mg of protein. The yield of enolase was about the same for each *Candida* culture, at 160 mg/litre of culture or 5 mg/g (wet weight) of cells. The overall efficiency of the purification process was about 40% for each *Candida* species.

Discussion

The enolase from the pathogenic yeast *C. albicans* has been identified as an immunodominant antigen in disseminated infection [10,15] and in allergic disease [16,17]. The enolases

from the moulds *Alternaria* and *Cladosporium* have been identified as major fungal allergens [18]. As well as binding antibody, *C. albicans* enolase is also capable of stimulating cell-mediated immunity [19,20]. The majority of nosocomial fungal infections are due to *C. albicans*. However, other *Candida* species such as *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis* and *C. lusitanae* are increasingly being identified as significant causes of infection [1–4]. The immunological relevance and extent of antibody cross-reactivity of enolases from other medically important *Candida* species should be investigated. To this end, we have developed a reliable and efficient method to obtain highly purified native enolase from the medically important *Candida* species.

Chromatography protocols for the purification of native Baker's yeast enolase [13,21] were adapted for the purification of the *Candida* enolases. The chromatography conditions were established on the assumption that the pI values of the enolases from the non-*albicans* *Candida* species would be similar to that of *C. albicans*. The enolase gene of *C. albicans* has been sequenced and the amino acid sequence deduced [22–24]. Using the deduced amino acid sequence, the theoretical pI value for the *C. albicans* enolase was calculated as 5.42 using the MacVector program (Oxford Molecular, Oxford, U.K.). Based on the theoretical pI of the *C. albicans* enolase, the pH of the anion-exchange (Source Q) elution buffer was set at pH 6.5 and that of the cation-exchange (Mono S) elution buffer set at pH 4.7. At these pH values it was possible to reproducibly purify the enolases from all the other *Candida* species tested.

While centrifugation and filtration was adequate for the removal of cellular debris and other particulate matter, soluble contaminants remained in the lysate, increasing viscosity and diminishing the resolution of the Source 15Q ion-exchange chromatography column. The use of chloroform to remove the lipid component from the crude lysate in an organic extraction procedure prior to dialysis enhanced resolution by approximately 75%.

Analysis of the fractions eluted from the Mono S column revealed that 80% of the original enzyme activity applied to the column was eluted in a single fraction. SDS/PAGE analysis of this fraction displayed a single band of protein around 46–48 kDa. The observed molecular masses for the protein bands obtained from the different *Candida* species were consistent with those of the enolases of *C. albicans* and *Saccharomyces cerevisiae* [24]. In each case, the *Candida* enolases seemed to have been purified.

The specific activities of the *Candida* enolases were determined and ranged from 65 to 70 units/mg of protein. This compares favourably with commercially available preparations of Baker's yeast enolase (Sigma), which have a specific activity of 45–50 units/mg of protein.

Rabbit hyperimmune antisera raised against purified recombinant *C. albicans* enolase reacted with all the puri-

fied proteins, including the Baker's yeast enolase standard, indicating that there are conserved or cross-reactive IgG antibody-binding epitopes in all the *Candida* enolase molecules.

The availability of purified enolases from other non-*albicans* species of *Candida* will enable the immunological cross-reactivity of the enolases to be investigated. This is important in the development of serological assays using the enolase antigens. Also, the immunological relevance and interspecies relationship of the *Candida* enolase antigens can be investigated.

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