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Cloning and Expression of Isocitrate Lyase, a Key Enzyme of the Glyoxylate Cycle, of *Candida albicans* for Development of Antifungal Drugs

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Abstract This paper describes the development of an enzymatic assay system for the identification of inhibitors of isocitrate lyase (ICL), one of the key enzymes of the glyoxylate cycle that is considered as a new target for antifungal drugs. A 1.6 kb DNA fragment encoding the isocitrate lyase from *Candida albicans* ATCC10231 was amplified by PCR, cloned into a vector providing His-Patch-thioredoxin-tag at the N-terminus, expressed in *Escherichia coli,* and purified by metal chelate affinity chromatography. The molecular mass of the purified ICL was approximately 62 kDa, as determined by SDS-PAGE, and the enzyme activity was directly proportional to incubation time and enzyme concentration. The effects of itaconate-related compounds on ICL activity were also investigated. Among them, itaconic acid, 3-nitropropionate, and oxalate had strong inhibitory activities with IC_{50} values of 5.8, 5.4 and 8.6 μg/ml, respectively. These inhibitors also exhibited antifungal activity on YPD agar media containing acetate as a sole carbon source, albeit at high concentration. The results indicate that the *C. albicans* ICL may be a regulatory enzyme playing a crucial role in fungal growth and is a prime target for antifungal agents.

Key words: *Candida albicans*, glyoxylate cycle, isocitrate lyase, gene expression, *in vitro* assay, inhibitors

Since the vast majority of life-threatening mycoses occur in immunocompromised patients, the importance of broad-spectrum, fungicidal agents of acceptable toxicity can not be overemphasized. The discovery of novel antifungal drugs by the pharmaceutical industry has been motivated by several factors: (i) an increasing number of fungal infections has been observed worldwide in the past decade [9], (ii) the number of antifungal drugs available to treat fungal infection in humans remains limited to a few agents, including principally amphotericin B, azoles, and 5-fluocytosine for invasive fungal infections [4], and (iii) antifungal drugs have a limited spectrum of activity and often have toxic side effects, and are increasingly encountering natural or acquired resistance in fungal pathogens [16]. The last factor is particularly troubling, because conventional screening procedures have resulted in the discovery and commercialization of very few effective antifungal compounds with unique modes of action.

The glyoxylate cycle is required by fungi or many other microorganisms for growth on C_2 carbon sources such as fatty acid or acetate [10, 19]. This cycle is a sequence of anaplerotic reactions catalyzed by the following key enzymes: isocitrate lyase (ICL) (EC 4.1.3.1), cleaving isocitrate to glyoxylate and succinate; and malate synthase (MLS) (EC 4.1.3.2), condensing glyoxylate with acetyl-CoA to malate. Together, catalysis by these enzymes ensures the bypass of two oxidative steps of the tricarboxylic acid cycle (TCA cycle) in the synthesis of succinate. Thus, the glyoxylate cycle can conserve carbons and adequately supply TCA cycle intermediates for biosynthesis when microorganisms grow on C_2 carbon sources as the sole carbon sources [1, 3, 8, 18]. Recently, it has been reported that the genes of the glyoxylate cycle are highly induced when *Candida albicans*, an important pathogenic fungus of humans, is phagocytized by macrophage [13]. The inside environment of phagolysosome, abundant in fatty acids or their breakdown products as carbon sources, makes *C. albicans* utilize the enzymes of the glyoxylate cycle to permit to use the C_2 carbon sources in this environment. In addition, mutant strains of *C. albicans* lacking isocitrate lyase are markedly less virulent than wild-types [13]. Because this pathway is thought not to operate in humans, the key enzymes of the glyoxylate cycle could be promising targets for the control

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of fungal infection and to develop antifungal drugs [1, 13]. In this work, as a part of our studies to search for new inhibitors of ICL, the *ICL* gene was cloned from *C. albicans* ATCC10231 and expressed in *Escherichia coli*. The inhibitory activities in itaconate-related compounds and TCA cycle metabolites were also investigated.

Genomic DNA of *C. albicans* ATCC10231 was used as a template for the amplification of the *ICL* gene by PCR. The expression vector pBAD/TOPO-Thio® (Invitrogen, The Netherlands) was used for the cloning procedures and *E. coli* TOP10 strain (Invitrogen) was used as a host for plasmid manipulation and gene expression. *C. albicans* and *E. coli* strains were grown in YPD medium (1% Yeast extract, 2% Bacto peptone, and 2% Dextrose) and LB medium (1% Bactotryptone, 0.5% Yeast extract, and 1% NaCl, pH 7.0), respectively. Highly purified genomic DNA from *C. albicans* was prepared with a Wizard® genomic DNA purification kit (Promega, U.S.A.). *C. albicans* homologue of *ICL1* (GenBank accession number AF222905) was identified by searching currently available *C. albicans* genome sequence data from the Stanford DNA Sequencing and Technology Center. Based on the nucleotide sequence of *ICL1*, two synthetic primers (5-A**GAATTC**CTACCATGCCTTACACTCC-3, forward primer; 5'-CTTC**GTCGAC**TCAAAATTAAGCCTTG-3', reverse primer) were designed to carry the *Eco*RI and *Sal*I recognition sites, respectively (bold). PCR was performed in the following conditions on a thermal cycler: an initial denaturation at 94°C for 3 min, then 30 cycles consisting of 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min, followed by a final extension at 72°C for 10 min. The amplified PCR fragment (1.6 kb) was purified with a SUPREC-02™ purification column (Takara Shuzo Co., Japan), ligated into the pBAD/Thio-TOPO vector for TA cloning, and transformed into *E. coli* TOP10. Positive clones were selected on LB plates containing ampicillin (100 μg/ml). The construct was confirmed by digestion with the restriction enzyme and subjected to nucleotide sequencing with an ABI PRISM 377 DNA sequencer using two synthetic primers: 5'-TCACAACCCGGGCACTGCGCCG-3', forward primer; 5'-AGACCCCACACTACCATCGGCG-3', reverse primer. The sequence of the *C. albicans* ATCC10231 *ICL* clone showed 99% identity with the registered *ICL1* at the amino acid level (data not shown).

The expression of ICL in *E. coli* TOP10 is driven by the *ara*BAD promoter. The AraC encoded on the pBAD/Thio-TOPO® positively regulates this promoter by forming a complex with L-arabinose. Therefore, expression of the *ICL* gene in PCR-positive *E. coli* clones was induced by addition of arabinose [12, 14, 17]. In our first attempt to express the ICL proteins at 37°C, a substantial fraction of the expressed ICL protein remained in the insoluble fraction, although the overexpressed ICL protein was obtained. One approach to reduce protein aggregation is to reduce the culture temperature [5]. Thus, the positive transformants were grown in LB medium containing ampicillin (100 μg/ml) at 37°C for 13 h (OD_{600}=1~2). The pre-incubated cells (50 ml) were inoculated into 1 l of LB broth containing ampicillin and incubated with shaking at 25°C until OD_{600} reached approx. 0.5. At this point, L-arabinose was added to the medium to a final concentration of over 0.02%, and an additional incubation was performed at 25°C for 8 h to induce the expression of ICL proteins. Growth at the low temperature resulted in improved solubility of the recombinant protein.

The proteins expressed in this bacterial expression system are fused to His-Patch (HP)-Thioredoxin at the N-terminal for the simplified purification. Since HP-Thioredoxin has a Ni-binding property at pH 7.2, the expressed ICL could easily be purified by a Ni-NTA affinity column chromatography. Thus, after harvested by centrifugation at 3,000 ×*g* for 10 min, cells were resuspended in an ice-cold 20 mM phosphate buffer (pH 7.0) and lysed by lysozyme treatment and sonication [15]. Cell debris was removed by centrifugation and the supernatant was applied on an Xpress™ protein purification system (Invitrogen) equilibrated with 20 mM phosphate buffer and 500 mM NaCl (pH 7.0) containing 12 mM imidazole. After the unbound proteins had been washed out, the bound proteins were eluted with the same buffer containing 100 mM imidazole. Fractions containing the N-terminal fused protein (HP-Thioredoxin) were collected, and the N-terminal HP-tag was cleaved with EnterokinaseMax™ (Invitrogen) under the following conditions: 50 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$, and 0.1% Tween-20, and incubation for 16 h at 37°C. After cleavage, the reaction mixture was dialyzed by using Centricon YM-50 (Millipore, U.S.A.) against an enzyme reaction buffer and then subjected to an AKTA purifier (Amersham Pharmacia Biotech, Uppsala, Sweden) to purify the ICL protein. By SDS-PAGE analysis, the molecular masses of N-terminal-fused ICL and the enterokinase-treated ICL were calculated to be 78 kDa and 62 kDa, respectively (Fig. 1) [11].

The enzyme activity of the purified ICL was determined by the method of Dixon and Kornberg [6, 7]. A basic concept of this method is to measure the formation of glyoxylate phenylhydrazone in the presence of phenylhydrazine and isocitrate at 324 nm. One ml of the reaction mixture contained 20 mM sodium phosphate buffer (pH 7.0), 1.27 mM *threo*-DL (+) isocitrate, 3.75 mM $MgCl_2$, 4.1 mM phenylhydrazin, and various amounts of ICL enzyme suspension. The reaction was carried out at 37°C for 30 min. Protein concentration was determined by the method of Bradford using the Bio-Rad protein assay kit (Bio-Rad, U.S.A.) and bovine serum albumin as a standard [2]. The enzyme activity of the purified ICL showed dependence on the amount of enzyme and incubation time (data not shown). Since glycolysis and TCA cycle

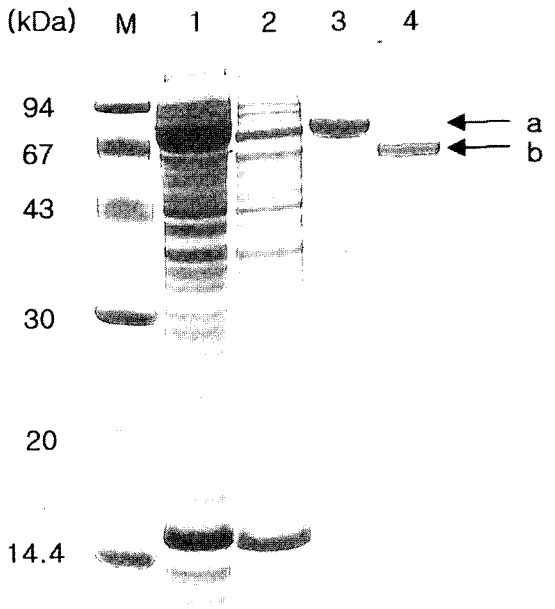

Fig. 1. SDS-PAGE analysis of the isocitrate lyase (ICL) expressed in *E. coli* TOP10.
The samples were loaded on a 15% polyacrylamide gel. M, protein molecular weight standard; Lane 1, total protein of host with ICL-pBAD/Thio-TOPO; lane 2, unbound fraction of Ni-NTA affinity column; lane 3, eluted fraction containing the fused ICL; lane 4, enterokinase-treated recombinant ICL. Label "**a**" indicates the overexpressed recombinant ICL (ca. 78 kDa) and "**b**" indicates enterokinase-treated (cleaved) recombinant ICL (ca. 62 kDa).

intermediates have frequently been reported to inhibit the ICL activity [7, 13, 18, 20], the inhibitory effects of the related metabolites and their analogues were also examined in this experiment. Among the compounds tested (Table 1), itaconate (an analogue of succinate), 3-nitropropionate (an analogue of succinate), and oxalate (an analogue of glyoxylate) were found to be the strongest inhibitors, with IC_{50} values of 5.8, 5.4, and 8.6 μg/ml, respectively. However, the TCA cycle intermediates, including citrate, succinate, and malate, were weak inhibitors, and the structural analogues of isocitrate had no inhibitiory acitivity (Table 1). As reviewed by Vanni *et al.* [20], the potent inhibition of the ICL activity by oxalate and itaconate is common to most of the reported ICLs. Similar ICL inhibition by 3-nitropropionate has also been reported for *Mycobacterium tuberculosis* [18].

The strategy for survival during chronic stages of infection entails a metabolic shift in the yeast's carbon source to C_2 substrates generated by β-oxidation of fatty acids [13]. Under these conditions, glycolysis is decreased and the glyoxylate shunt is significantly upregulated to allow anaplerotic maintenance of TCA cycle and assimilation of carbon via gluconeogenesis. However, screening for

Table 1. Inhibition of ICL activity by several metabolites and related compounds.[a]

Compound	IC_{50} (μg/ml)
Known inhibitors	
Itaconate	5.8
Itaconic anhydride	11.3
3-Nitropropionate	5.4
Related metabolites	
Citrate	383.2
Succinate	134.4
Malate	450.1
Glycolate	410.5
Malonate	302.7
Oxalate	8.6
Structural analogues	
2,5-Dichlorobenzoquinone	>500
Quisqualic acid	>500
Hydroxyectonine	>500
3-Hydrophenylglycine	>500
Isoascorbic acid	>500
2-Pyrrolidons-5-carboxylate	>500
Imidazole-4,5-dicarboxamide	>500

[a]Reactions were carried out in 1 ml incubation mixture containing 20 mM sodium phosphate buffer (pH 7.0), 1.27 mM *threo*-$_{DL}$-isocitrate, 3.75 mM $MgCl_2$, 4.1 mM phenylhydrazin, and ICL (100 μg/ml) at 37°C for 30 min. Appropriate blank contained all of the above-mentioned compounds, except test sample. The IC_{50} values represent the concentration giving 50% inhibition relative to the blank.

inhibitors against ICL is extremely complex in infected macrophages or mice [18]. Our approach has been to screen compounds, first for inhibitory activity against the enzyme and then for fungal survival when the fungi are grown on a C_2 carbon source. Thus, *C. albicans* (10^3 cells/ml) was plated on YPD agar plates containing either acetate (2%) or glucose (2%) as a limiting carbon source. Discs of filter paper soaked with various concentrations of ICL inhibitors were added to the plates, and inhibition was graded based on the radius of the inhibition zone. As shown in Fig. 2, itaconate, oxalate, and 3-nitropropionate, which are potent ICL inhibitors *in vitro* (Table 1), had no effect on the *C. albicans* grown on glucose, but were inhibitory to *C. albicans* grown on acetate, albeit at high concentration. These results indicate that itaconate, oxalate, and 3-nitropropionate are good starting candidates for structure-based drug design.

In conclusion, the results obtained for the metabolite inhibition (Table 1 and Fig. 2) indicate that the *C. albicans* ICL may be a regulatory enzyme playing a crucial role in fungal growth, in good agreement with the recent finding that ICL plays a pivotal role in the coupling system of the TCA and the glyoxylate cycles in oxalate biosynthesis [13]. In addition, as the enzymes of the glyoxylate cycle are not found in mammals, they are prime targets for antimicrobial agents. Further research is underway to

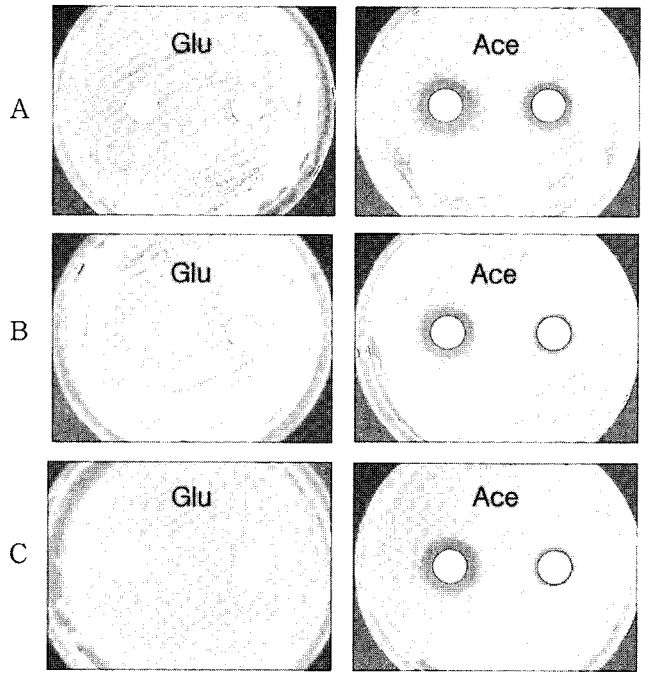

Fig. 2. Inhibition of isocitrate lyase (ICL) activity.
C. albicans (10^3 cells/ml) was plated on YPD agar plates containing either 2% glucose (Glu) or 2% acetate (Ace) as the limiting carbon source. Discs of filter paper soaked with 80 mM (left) and 40 mM (right) of each ICL inhibitor (A, itaconate; B, oxalate; C, 3-nitropropionate) were added to the plates.

control the medically important fungi and yeasts with specific inhibitors, based on this investigation.

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