Role of Osmotic and Salt Stress in the Expression of Erythrose Reductase in Candida magnoliae

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The osmotolerant yeast, Candida magnoliae, which was isolated from honeycomb, produces erythritol from sugars such as fructose, glucose, and sucrose. Erythrose reductase in C. magnoliae (CmER) reduces erythrose to erythritol with concomitant oxidation of NAD(P)H. Sequence analysis of the 5'-flanking region of the CmER gene indicated that one putative stress response element (STRE, 5'-AGGGG-3'), found in Saccharomyces cerevisiae, exists 72 nucleotides upstream of the translation initiation codon. An enzyme activity assay and semiquantitative reverse transcription polymerase chain reaction revealed that the expression of CmER is upregulated under osmotic and salt stress conditions caused by a high concentration of sugar, KCl, and NaCl. However, CmER was not affected by osmotic and oxidative stress induced by sorbitol and H₂O₂, respectively. The basal transcript level of CmER in the presence of sucrose was higher than that in cells treated with fructose and glucose, indicating that the response of CmER to sugar stress is different from that of GRE3 in S. cerevisiae, which expresses aldose reductase in a sugar-independent manner. It was concluded that regulation of CmER differs from that of other aldose reductases in S. cerevisiae.

Keywords: Candida magnoliae, erythrose reductase, aldose reductase, Saccharomyces cerevisiae, stress reponse

Erythritol [1,2,3,4-butanetetrol, molecular weight (MW) 122.12], a four-carbon sugar alcohol, naturally occurs in a variety of foods such as fruits, mushrooms, and fermented foods including cheese, wine, and soy sauce [6]. Owing to its excellent taste and lack of insulin-stimulating properties, erythritol has been used as a low-calorie sweetener and pharmaceutical excipient [7, 19, 25]. Most ingested erythritol are not metabolized by the human body and are excreted unchanged in the urine without altering blood glucose and insulin levels. It also prevents dental caries because the bacteria that cause dental caries are not able to utilize erythritol as a carbon source [19].

Both chemical and fermentation processes have been introduced for the large-scale production of erythritol. Erythritol can be produced from periodate-oxidized starch or dialdehyde starch by a high-temperature chemical reaction in the presence of a nickel catalyst [22, 24]. The chemical process has not been industrialized because it involves complicated steps and the starting material is expensive. Industrial production of erythritol is achieved by a fermentation process with glucose from hydrolyzed wheat and corn starches, fructose, and sucrose [2, 7]. It has been reported that osmophilic yeast and bacteria are able to produce erythritol [9, 10, 15, 26], and Trichosporonoides megachilensis SNG-42 has been used for commercial production of erythritol in Japan [9]. Candida magnoliae JH110, a yeast strain isolated from honeycomb [10], is also able to produce erythritol with high yield, and its mutant has been used to further enhance erythritol productivity [12, 23, 27].

Erythrose reductase (ER) catalyzes the final step of erythritol biosynthesis, which converts erythrose to erythritol with concomitant oxidation of NAD(P)H [13, 14, 25]. ER from a couple of microbial sources has been purified and characterized [16, 17, 25], and the genetic sequence and functional characteristics of ER from C. magnoliae (CmER) were recently reported [13].

In this study, the nucleotides in the 5'-flanking region were sequenced and analyzed in order to elucidate the regulatory expression of CmER. In addition, the effects of
a variety of stress conditions on its expression and ER enzyme activity in *C. magnoliae* were examined.

**MATERIALS AND METHODS**

**Strains, Plasmid, and Culture Conditions**

*C. magnoliae* JH110 [13] grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) was stored at −80°C. *Escherichia coli* TOP10 [F- merA Δ(mrr-hadRMS-merBC) 980lacZAM15 ΔlacX74 recA1 araD139 Δ ara-leu 7697 galU galK rpsL. (Stir) endA1 napG] (Invitrogen, Carlsbad, CA, USA) was used for plasmid DNA preparation and was routinely grown in LB medium (0.5% yeast extract, 1% peptone, 1% NaCl) at 37°C. LB plates supplemented with 50 mg/l ampicillin were used to select for plasmid-harboring *E. coli*. The pGEM-T plasmid (Promega, Madison, WI, USA) was used for DNA walking.

For the ER enzyme activity assay and reverse transcription polymerase chain reaction (RT-PCR) analysis, *C. magnoliae* was grown in YEPD medium at 30°C to an optical density (OD₆₀₀) of 1.0, harvested by filtration to remove medium, transferred to YEPD peptone, 2% glucose) was stored at 10°C. *M. luteus* 7697 (Strir M15 M15 lacY1 [F- lac PSR) was stored at 4°C for 10 min. One unit of ER activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of NADPH per minute at 37°C. Specific ER activity was expressed as units per milligram of cellular protein [13].

**RESULTS AND DISCUSSION**

**Sequence Analysis of the 5’-Flanking Region**

A 450 bp genomic DNA sequence containing the 5’-upstream flanking region of the *CmER* gene was identified, aligned, and compared with the promoter sequences of the stress-responsive genes in *S. cerevisiae* (Fig. 1). As with other stress-controlled genes in *S. cerevisiae* such as *CTT1* or *HSP104*, one putative stress-response element (STRE) sequence, 5’-AGGGG-3’ [11], was found 72 nucleotides upstream of the initiation codon, indicating that *CmER* might be regulated in a similar manner to the *GRE3* gene in *S. cerevisiae*, which has one STRE 144 nucleotides upstream of the translation initiation codon.

Although the exact biological roles of yeast aldol-keto reductases (AKRs) are largely unknown, it has been suggested that AKRs such as Gre3, Gey1, and Ypr1 play crucial roles in the stress response of the budding yeast *S. cerevisiae* [4, 20, 21]. *ScGRE3* is upregulated by a wide variety of stress conditions including osmotic stress, heat-shock, and oxidative stress [4]. Transcription of *ScGRE3* is positively regulated by Msn2 and Msn4, two well-known stress-responsive transcription factors in *S. cerevisiae* [5].

**PREPARATION OF CELL EXTRACTS**

Harvested cells were broken by vortexing with acid-washed glass beads (Sigma-Aldrich) in 1 ml of 50 mM phosphate buffer (pH 6.5) for 3 min. Cell homogenates were centrifuged at 15,000 ×g for 20 min at 4°C, and the supernatants were used as cell extracts. Cell extracts were prepared from three independent cultures. Protein concentrations were determined by the Bio-Rad assay using bovine serum albumin as the standard.

**ER Enzyme Activity Assay**

The ER assay mixture (250 µl) contained crude enzyme, 12 mM D-erythrose, and 4 mM NADPH in 50 mM phosphate buffer (pH 6.5). ER activity was determined by measuring the decrease in OD₆₅₀ at 37°C for 10 min. One unit of ER activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of NADPH per minute at 37°C. Specific ER activity was expressed as units per milligram of cellular protein [13].

**RNA Isolation and RT-PCR**

Total RNA was prepared using TRIzol reagent (Invitrogen). RT-PCR was performed using the cDNA EcoDry Premix (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. Total RNA (0.5 µg) and primers CmER-F (5’-ATGTCTTCGACCTACCC-3’) and CmER-R (5’-CGTCGGCTAGTCACTATG-3’) were used in 30 cycles of RT-PCR amplification. Aliquots of the PCR product were separated on a 0.8% agarose gel.

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Regulatory Expression of the CmER Gene Under Stress Conditions

Expression of the CmER gene was examined in the presence of different carbon sources. In addition, the effects of a variety of stress conditions induced by NaCl, KCl, sorbitol, H\textsubscript{2}O\textsubscript{2}, and methylglyoxal on gene transcription and ER enzyme activity were investigated.

As concentrations of fructose and glucose increased, ER activity was notably enhanced (Fig. 2). ER enzyme activity was enhanced more than 2-fold as the sucrose concentration increased from 2% to 10% without a drastic change in transcript level. The basal transcript level of ER in the presence of 2% sucrose was higher than that observed after treatment with 2% glucose or 2% fructose, indicating that expression of the CmER gene might be carbon-source-dependent. Based on Duncan’s Multiple Range Test (p<0.05), a different letter indicates a significant difference between means.

![Fig. 2](image-url)

**Fig. 2.** Level of erythrose reductase transcripts and enzyme activity under a variety of stress conditions.

Total RNA (0.5 µg) was used for a semiquantitative RT-PCR reaction comprised of 30 cycles. PCR products were separated on a 0.8% agarose gel, and ethidium-bromide-stained rRNA for each sample is shown as the loading control. Enzyme assay results were obtained from three independent experiments, and averages and standard errors are shown. Based on Duncan’s Multiple Range Test (p<0.05), a different letter indicates a significant difference between means.

A significant increase in the transcript level of ER was also observed when the cells were grown under stress conditions induced by KCl and NaCl, indicating that salt stress as well as sugar stress is involved in transcription of the CmER gene. Treatment with 0.5 M KCl exerted a profound effect on both the transcript level and ER enzyme activity, which may support the finding that the erythritol production rate is significantly enhanced by supplementation of KCl into the cultivation medium [3, 18]. Since both enzyme activity and transcript level were enhanced in the presence of 0.5 M KCl and 0.5 M NaCl, it was thought that the CmER gene is upregulated under salt stress. However, neither transcript level nor enzyme activity was significantly affected by oxidative stress caused by H\textsubscript{2}O\textsubscript{2} or 1.0 M sorbitol. It should be mentioned that CmER expression was not affected by oxidative stress induced by H\textsubscript{2}O\textsubscript{2}, which is not the case for ScGRE3 expression [1]. Treatment of the cells with methylglyoxal did not result in an increase in ER enzyme activity.

The ER enzyme activity and transcript level were further examined in the presence of a variety of sugar concentrations. As shown in Fig. 3, the ER enzyme activity was closely related to sugar concentration; that is the increase in ER enzyme activity with increased sugar concentration was nearly linear. The highest ER enzyme activity was obtained at 40% sucrose, which is partially
consistent with the results shown in Fig. 2. A clear increase in the ER transcript level was observed with increases in the concentrations of fructose and glucose, although there were some different transcription patterns between fructose and glucose. When the cells were grown in fructose, the increase in ER transcription level was not as significant as that in glucose.

The results of this study revealed that CmER transcription is upregulated in order to increase the ER enzyme activity under osmotic and salt stress conditions. The upregulation of CmER transcription under osmotic and salt stress conditions was consistent with the expression trends of GRE3 in S. cerevisiae. However, unlike ScGRE3, the expression of CmER was found to be carbon-source-dependent in this study. Moreover, CmER did not respond to oxidative stress induced by H$_2$O$_2$.

This is the first report of the regulatory expression of erythrose reductase in C. magnoliae. Future studies should explore the genetic circuit underlying the stress response in C. magnoliae.

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References


