酵母Gcy1pはエリスロースとエリスロース−4−リン酸を還元する

研究ノート

Yeast Gcy1p Reduces Erythrose and Erythrose-4-phosphate

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Abstract

Erythrose reductase reduces D-erythrose into erythritol, a low calorie sweetner. Erythrose redutases have been identified in erythritol-producing fungi (*Trichosporonoides megachiliensis*), while yeast orthologues are still unknown. Gcy1p, known as a *Saccharomyces cerevisiae* aldose-reductase, possessed significant homology with the fungal erythrose reductases. The recombinant Gcy1p, expressed in *Escherichia coli*, showed similar substrate specificity towards D-erythrose and glycerol using NADPH as a reductant. Moreover, the Gcy1p reduced D-erythrose-4-phosphate as efficiently as D-erythrose. These facts suggest that *S. cerevisiae* has the potential to produce erythritol.

Key words: erythritol, pentose-phosphate shunt, erythrose reductase, Gcy1p

Introduction

Meso-erythritol, a natural sugar alcohol, is currently used in reduced calorie foods in the United States and Japan. Erythritol has attracted attention for its extremely low digestibility and approved safety for diabetics. Industrially, erythritol is produced by fermentation with an erythritol producing fungi (*Trichosporonoides megachiliensis*),[1,2] while *Saccharomyces cerevisiae* does not produce erythritol.

Erythrose reductases (ERs) convert D-erythrose to erythritol with NADPH. We previously reported on the molecular cloning of ERs from *T. megachiliensis*.[3] Sequence analysis revealed that ERs shared significant homology (36~38% identity) with Gcy1p of *S. cerevisiae*. This fact raised the question of whether the Gcy1p can have erythrose reductase activity.

Here we report that Gcy1p, expressed in *Escherichia coli*, showed similar substrate specificity towards D-erythrose and glycerol as did ERs using NADPH as a reductant. Moreover, the Gcy1p reduced D-erythrose-4-phosphate as efficiently as D-erythrose. These facts suggest that *S. cerevisiae* has the potential to produce erythritol.

Materials and Methods

Isolation of yeast genomic DNA.

Yeast genomic DNA was purifed from *Saccharomyces cerevisiae* s288c (Invitrogen, Carlsbad, CA) with the standard protocol.

Functional expression of Gcy1p in E. coli.

Coding region for the yeast gcy was amplified with each of the primers [sense;5'-cacggatcc CCTGCTACTTTACATGATTCT-3', antisense; 5'-agtctcgag TTACTTGAATACTTCGAAAGG-3'] and Pfu DNA polymerase (Stratagene, La Jolla, CA) at 30 thermal cycles of 94℃ 1 min, 50℃ 1 min, and 68℃ 1 min. DNA sequencing was confirmed with

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an ABI 310A genetic analyzer with BigDye Terminator (Applied Biosystems Japan, chiba). A 936 bp fragment was ligated in the frame with N-terminal His-tag of a pRSETA (Invitrogen) plasmid after restriction enzyme digestion. pRSETA plasmids for ER1, ER2 and ER3 were obtained as previously described. [3] The pRSETA plasmids were transformed into BL21 (DE3) pLysS (Novagen, Madison, WI). Transformed cells were cultured at 25℃ for 20 hrs, then 0.1mM IPTG was added and further cultured for 48 hrs. Recombinant Gcy1p and ERs were recovered from cells by sonication and centrifugation (26,400 g x 15 min, 4℃). Recombinant Gcy1p and ERs were purified with a nickel-chelated agarose (Qiagen, Tokyo) from supernatants. Extra-tags were removed from recombinant Gcy1p and ERs with enterokinase treatment (Invitrogen).

Measurement of protein concentration and reductase activity.

Protein quantity was measured by Protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin (Sigma, St. Louis, MI) as a standard. Erythrose reductase activity for reduction was measured with 12 mM D-erythrose and 0.2 mM NADPH in 50 mM phosphate buffer (pH 6.5) at 37℃. One unit (U) of reductase activity was defined as the consumption of 1 μmole NADPH min^{-1}. Km values for D-erythrose was calculated from initial rates of 0.5 to 12 mM D-erythrose.

Polyacrylamide gel electrophoresis (PAGE) and western blot analysis

Recombinat proteins were resolved with 8-25% SDS-PAGE gradient gels (Atto, Tokyo) and stained with Coomassie brilliant blue R 250. For the immuno-blot experiment, gels were electrically transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA) as previously described.[3] Antibodies against native ER3 raised in mice were used at 1:2,000 dilution as described.[3] Immuno-reactivity of ER1, ER2, and ER3 on PVDF membrane was visualized on a MR-X-ray film with Renaissance western blot kit (Dupont, Johnston, IA) after primary antibody and HRP labeled anti-mouse-IgG (GE Healthcare Bio-sciences, Piscateway, NJ) treatment.

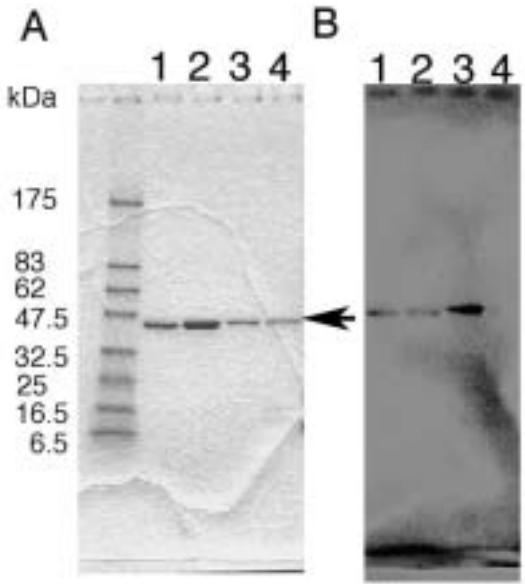

Figure 1. SDS-PAGE analysis of recombinant Gcy1p and ERs.

Recombinant ER1 (lane1), ER2 (lane2), ER3 (lane 3) and Gcy1p (lane 4) were resolved with SDS-PAGE (A) and analyzed by western blotting with antibodies against native ER3. Expected molecular weight size (35 kDa) was indicated by an arrow.

Results and Discussion

Functional expression of Gcy1p

We functionally expressed recombinant Gcy1p and ERs in *Escherichia coli*. Extra-tags removed Gcy1p and ERs were resolved by SDS-PAGE. Fig 1A shows that the recombinant Gcy1p (expected size 35079Da) had bands at 35 kDa. Figure 1B shows that antibodies against native ER3 did not recognize Gcy1p (lane 4). This fact suggests that the structure of Gcy1p is different from those of ERs.

Substrate specificity

We analyzed Km values and substrate specificity of the recombinant Gcy1p. The specific activity for recombinant Gcy1p with NADPH (0.2 mM) and D-erythrose (12 mM) was 4.7 units/mg. Recombinat Gcy1p did not reduce D-erythrose and glycerol with NADH as a reductant. The Km value of the Gcy1p against D-erythrose was 3.4 mM. This obtained value was comparable to recombinant ERs (2.2~8.6 mM).

Native and recombinant ERs were reported to have substrate specificity towards C3- and C4- aldehydes. We analyzed substrate specificity of the recombinant Gcy1p. Table 1 shows that the recombinant Gcy1p reduced D-erythrose as efficiently as glyceraldehydes, but not galactose, glucose,

Table 1. Substrate specificity of recombinant of Gcy1p, ER1, ER2 and ER3.

	recombinant			
substrate	Gcy1p	ER3	ER1	ER2
dihydroxyacetone	6.7	20.3[a]	11.4[a]	11.2[a]
D-gylceraldehyde	80	98.2[a]	102.0[a]	107.8[a]
D-erythrose	100	100[a]	100	100
D-erythrose-4-phosphate	87.8	18.4	12.7	20
D-xylose	3.5	4.5[a]	3.2[a]	2.4[a]
D-glucose	0.0	0.0[a]	0.0[a]	0.0[a]
D-galactose	0.0	0.0[a]	0.0[a]	0.0[a]
D-sorbose	0.0	0.0[a]	0.0[a]	0.0[a]
D-ribose	0.0	0.0[a]	0.0[a]	0.0[a]
D-arabinose	0.0	0.0[a]	0.0[a]	0.0[a]
D-glucuronate	126.7	0.8[a]	0.7[a]	0.0[a]
p-nitrobenzaldehyde	146.7	66.3[a]	11.5	8.8[a]

The data ([a]) was from Biosci. Biotechnol. Biochem. 69(5), 944-951, (2005).

The initial rate of each enzyme with D-erythrose (12 mM) an Period NADPH (0.2 mM) was set at 100.

Period Concentration of the other substrates was 12 mM.

ribose, sorbose and xylose. This observation was consistent with the substrate specificity of ERs.[4] Interestingly, the Gcy1p reduced D-erythrose-4-phosphate, an intermediate of pentose-phosphate shunt, more efficiently than ER1, ER2 and ER3. This fact may reflect a difference in the substrate binding sites between the Gcy1p[5] and ERs. In *Leuconostoc oenos*, D-erythritol-4-phosphate production was suggested by the reduction of D-erythrose-4-phoshate. [6] The same reaction might occur with Gcy1p.

D-erythrose-4-phosphate and D-erythritol-4-phosphate could be converted into erythrose and erythritol by phosphatases. The TM1254 phosphatase from *Thermotoga maritima*, which is homologous to yeast YKL033W-A (31% identities, 50% similarity), RHR2p (26% identities, 42% similarity) and HOR2p (27% identities, 43% similarity), was reported to possess substrate specificity towards D-erythrose-4-phosphate (Km; 0.15mM).[7] RHR2p and HOR2p, induced under hyperosmotic stress, are DL-glycerol-3-phosphatase. [8] These facts indicate that baker yeasts possess enzymes to produce erythritol in an osmotic stress condition.

D-erythrose-4-phosphate is an intermediate for pentose-phosphate shunt and aromatic amino acid synthesis. In an L-phenylalanine producing *E. coli*, D-erythrose-4-phosphate was not detectable. [9] This fact led us speculate that the D-erythrose-4-phosphate level in yeast might be low. Metabolic engineering to enhance D-erythorse-4-phosphate concentration would lead to erythritol production in yeast.

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酵母Gcy1pはNADPH依存性のアルドースリダクターゼである．大腸菌で発現させた組換え型Gcy1pは，トリコスポロノイデス メガチリエンスのエリスロースリダクターゼと同様にC-4及びC-3アルデヒドに対する基質特異性を示した．さらに，酵母Gcy1pはエリスロース-４-リン酸も還元した．このことは，エリスリトールを産生しないとされてきた酵母においても，Gcy1pとホスファターゼ存在下では，エリスリトールが産生できる可能性を示している．