Purification and Characterization of Nonmitochondrial Citrate Synthase from *Saccharomyces cerevisiae*

Cho, Nam Seok, Kwang Soo Kim*, and Pil Jae Maeng

Department of Microbiology, College of Natural Sciences, Chungnam National University, Daejeon 305-764, Korea
*Laboratory of Molecular Neurobiology, The W. M. Burke Medical Research Institute, Cornell University Medical College, White Plains, New York 10605, U. S. A.

*Saccharomyces cerevisiae*의 Nonmitochondrial Citrate Synthase 분리 및 특성

조남석 · 김광수* · 맹필재
충남대학교 자연과학대학 미생물학과
*Laboratory of Molecular Neurobiology, The W. M. Burke Medical Research Institute, Cornell University Medical College, White Plains, New York 10605, U. S. A.

ABSTRACT: Citrate synthase 1 (mitochondrial) and citrate synthase 2 (nonmitochondrial) were purified from *Saccharomyces cerevisiae*. The physical and enzymatic characteristics of citrate synthase 2 were analyzed in comparison with citrate synthase 1. Both isoenzymes were shown to be dimeric proteins of identical subunits, and the molecular weights of the subunits were estimated to be 48.3 kDa for citrate synthase 1 and 47.0 kDa for citrate synthase 2, respectively. The optimal pH value for enzyme activity was pH 7.5 for both isoenzymes. However, the optimal temperature for the activity was strikingly different; while the activity of citrate synthase 1 reached its peak at 65°C, that of citrate synthase 2 was maximal at 40°C. Citrate synthase 2 showed much lower thermal and pH stability than citrate synthase 1. In addition, citrate synthase 2 was affected much more by the metal ions such as Zn^{2+}, Mn^{2+} and Co^{2+} than citrate synthase 1. Among the several possible regulatory metabolites tested, ATP showed the strongest inhibitory effect on both enzymes. ADP and NADH were found to have greater effect on citrate synthase 2 than on citrate synthase 1. Kinetic analysis revealed that citrate synthase 2 has approximately 7- and 3.5-fold lower affinity to acetyl CoA and to oxaloacetate, respectively, than citrate synthase 1.

KEY WORDS ☐ Nonmitochondrial citrate synthase, Mitochondrial citrate synthase, Purification, *Saccharomyces cerevisiae*

Citrate synthase (oxaloacetate lyase; EC 4.1.3.7) catalyzes the aldol condensation reaction of acetyl coenzyme A (acetyl-CoA) and oxaloacetate to form citrate (Krebs and Lowenstein, 1960). In eucaryotic cells, citrate synthase is primarily involved in the first step of tricarboxylic acid

(TCA) cycle which produces NADH used for ATP generation via electron transport system and provides carbon skeletons necessary for various biosynthetic reactions. Citrate synthase also participates in the glyoxylate cycle which serves as an anaplerotic pathway necessary for the metabolism of certain nonfermentable carbon sources that are degraded via acetyl Co-A (Duntze *et al.*, 1969).

In *Saccharomyces cerevisiae*, the gene for citrate synthase, *CIT1*, was first cloned by Suissa *et al.*

(1984), and the amino acid sequence of the enzyme deduced from the DNA sequence was shown to have considerable homology to that of pig heart citrate synthase. Rickey and Lewin (1986) reported that destroying the citrate synthase gene by inserting the yeast *LEU2* gene within its reading frame reduced the mitochondrial citrate synthase (citrate synthase 1, CS 1) activity more than 50-fold, and that the loss of the mitochondrial activity resulted in an increase in the extramitochondrial activity. This revealed the existence of a second, nonmitochondrial citrate synthase (citrate synthase 2, CS 2) in *S. cerevisiae*, and Kim *et al.* (1986) isolated the second gene, *CIT2*, encoding CS 2. Only after both *CIT1* and *CIT2* genes were disrupted, the classical citrate synthase phenotype (i.e., glutamate auxotrophy and inability to grow on nonfermentable carbon sources) appeared. While the amino acid sequences of the *CIT1* and *CIT2* products are highly homologous, they diverge strikingly at their amino termini (Rosenkrantz *et al.*, 1986), which provides a possible explanation for why the CIT2 product, unlike the *CIT1* product, fails to be imported into mitochondria.

Lewin *et al.* (1990) fractionated the cell lysate of *S. cerevisiae* by ultracentrifugation, showed that the CS 2 activity cosedimented with peroxisomes, and concluded that the product of the *CIT2* gene of *S. cerevisiae* is peroxisomal. This conclusion, however, involves some suspicion in that it is simply based on the finding that the CS 2 activity in postmitochondrial supernatant was extremely sensitive to preincubation at pH 8.1 (Rosenkranz *et al.*, 1986). Beside the fact that the CS 2 activity is unstable at alkaline pH, its physicochemical and enzmatic properties have not been fully characterized.

In the present report, we describe the purification of CS 1 and 2 from genetically engineered *S. cerevisiae* strains which contain either *CIT1* or *CIT2* gene on a high-copy number plasmid and analyses of the physical and enzymatic properties of CS 2 in comparison to CS 1.

MATERIALS AND METHODS

Organisms and media

S. cerevisiae PSY40 (*cit2::URA3, CIT1, leu2*) transformed with plasmid pLCS1 (*CIT1, LEU2*) and *S. cerevisiae* PSY38 (*cit1::LEU2, CIT2, ura3*) with plasmid pUCS2 (*CIT2, URA3*) were used for purification of CS 1 and 2 from the cell lysate, respectively. Transformation was carried out by the lithium acetate method (Ito *et al.*, 1983).

The parental strains, *S. cerevisiae* PSY38 and PSY40, were grown on YEPD complete medium and the transformed strains on YNB minimal medium supplemented with 0.01% of lysine for 2-3 days at 30°C, and stored at 4°C. YEPD contained 1 % Bacto-yeast extract (Difco), 2 % dextrose, and 2 % Bacto-peptone (Difco); and YNB medium contained 0.67% Bacto-yeast nitrogen base w/o amino acid (Difco) and 2% dextrose (Sherman *et al.*, 1983). Solid media were prepared by adding 2% agar to the liquid media.

Preparation of the crude enzymes

S. cerevisiae PSY40 carrying pLCS1 and *S. cerevisiae* PSY38 carrying pUCS2 were grown in a 5 *l* fermentor jar containing 3.5 *l* of YEPD medium at 30°C under vigorous aeration, respectively. After 24 hours at the end of the logarithmic growth phase, the cultures were harvested by centrifugation (2,500×g, 10 min) and the cell pellets were washed twice with buffer A (10 mM Tris/acetate buffer containing 0.5 mM β-mercaptoethanol, pH 7.4). The washed pellet was resuspended in buffer A and disrupted with a French press under 3,000 psi. The suspension was centrifuged at 5,000×g for 10 min to remove cell debris, treated with a sonicator for 30 sec, and centrifuged at 30,000×g for 10 min. In the case of the cell lysate of *S. cerevisiae* PSY40 with pLCS 1, the supernatant was used as a crude enzyme preparation of CS 1. The sonicated lysate of *S. cerevisiae* PSY 38 carrying pUSC2, on the other hand, was supplemented with 0.2% Triton X-100 and 200 mM KCl, and gently stirred for 1 hour at 4°C. After the lysate was centrifuged at 30,000×g for 10 min, the supernatant was collected. Triton X-100 and KCl in the supernatant were removed by ultrafiltration through a Diaflo membrane, type PM 10, and the concentrate was used as a crude enzyme preparation of CS 2.

Enzyme assay

Citrate synthase was assayed by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) method (Srere, 1969). The reaction mixture (1 m*l*) contained 100 mM Tris-HCl (pH 8.0), 0.25 mM DTNB, 0.2 mM oxaloacetate, 0.1 mM acetyl-CoA, and 10 μl of enzyme solution. The reaction was followed by measuring the change in A_{412} at 30°C. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmole min^{-1} of CoA under the conditions of assay.

Purification of the enzymes

Each of the crude enzyme preparations was loaded onto a column (1.5×15 cm) that had been packed with Reactive Blue 4 agarose gel (Sigma) in buffer A. The column was eluted consecutively with buffer A, 0.5 mM oxaloacetate in buffer A, buffer A, 0.5 mM CoA in buffer A, 0.5 mM oxaloacetate and 0.5 mM CoA in buffer A, and finally with 1 M KCl. The fractions of 2 m*l* were collected at the flow rate of 0.4 m*l* min^{-1}.

To purify CS 2, the citrate synthase fractions eluted from the affinity column were concentrated by ultrafiltration and fractionated on a Mono Q HR 5/5 FPLC (Fast protein liquid chroma-

tography) column (Pharmacia) with a KCl gradient (0-400 mM) in 50 mM Tris-HCl buffer (pH 7.4) at a flow rate of 0.5 m*l* min^{-1}.

Determination of protein concentration

Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970) with some modification. The samples dissolved in sample buffer (10 mM Tris-HCl buffer pH 8.0, 1.0 mM EDTA, 1.0% SDS, 5% β-mercaptoethanol) were heated for 5 min at 100 °C. The mixture was then supplemented with a small amount of 10 % sucrose solution containing, 20 ppm bromophenol blue and loaded on a SDS-polyacrylamide gel (T=10%, C=2.7%). A constant voltage of 100 V was applied until the samples had run into the stacking gel. Then for the next 8 hours the run remained at 160 V. The proteins were stained with 0.25% Coomassie blue R250 solution in methanol/acetic acid/water (40:10:50, by volume) mixture for 2 hours. Destaining was performed in methanol/acetic acid/water (30:10:60) mixture.

The molecular weights of the citrate synthases were determined by measuring the mobilities on a 10% SDS-Polyacrylamide gel with standard proteins as molecular weight markers.

Study of the characteristics of the enzymes

The molecular weights of the native forms of the purified enzymes were determined by gel filtration on a Superose 12 HR 10/30 FPLC column (Pharmacia). The column was equilibrated and eluted with buffer A at a flow rate of 0.5 m*l* min^{-1}.

The isoelectric point (pI) values of the purified enzymes were measured by chromatofocusing through a Mono P HR 5/5 FPLC column (Pharmacia). The column was initially equilibrated with 25 mM Tris-HCl buffer (pH 7.1) and eluted with 50 m*l* of 10-fold diluted Polybuffer 74-HCl buffer (pH 4.0) at a flow rate of 0.5 m*l* min^{-1}.

The effect of temperature on citrate synthase activity was examined at the temperatures between 10 and 80°C at pH 7.4, and the effect of pH in the range of pH 6-9 at 30°C. To study the thermal stability of the purified enzymes, the residual activity after preincubation of the enzymes at the temperatures between 20 and 60°C in 100 mM Tris-HCl buffer (pH 7.4) in the absence of substrates was measured. The pH stability was similarly examined except that the enzymes were pretreated at the pH values between 6.0 and 9.0 at 30°C.

To test the effect of metal ions on the activity of citrate synthase, the enzyme was assayed in the reaction mixture containing MnSO$_4$, MnCl$_2$,
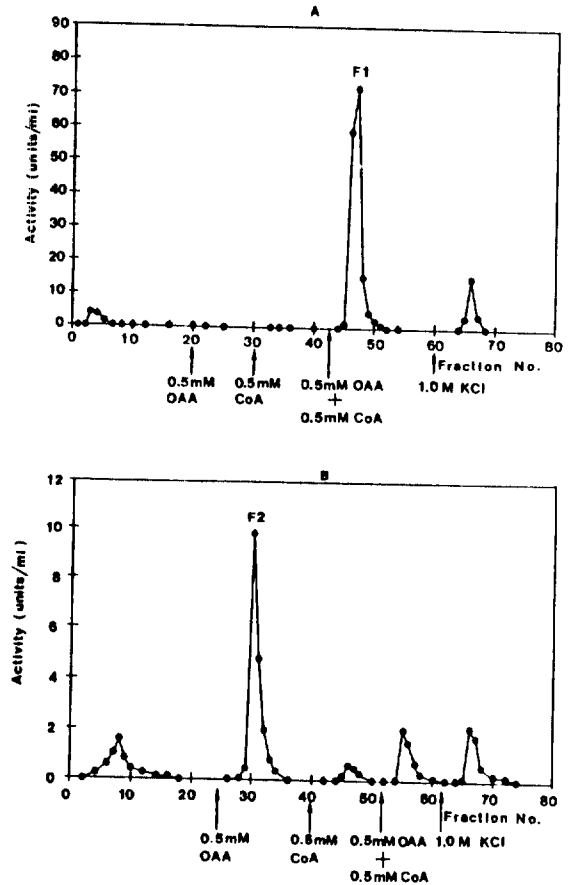

Fig. 1. *Affinity chromatography of crude enzyme from S. cerevisiae PSY40 carrying pLCS1 plasmid (A) and PSY38 carrying pUCS2 plasmid (B) on Reactive Blue 4-Agarose. Column dimensions: 1.5×15 cm. Fraction volume: 2 ml.*

ZnSO$_4$, MgCl$_2$, CuSO$_4$ or CoCl$_2$ at the concentrations of 0.1, 0.5, or 1 mM under standard assay conditions.

The effect of several plausible regulatory metabolites, ATP, ADP, NADH, NAD$^+$ and NADPH, was also examined by the similar method as described above except that the concentrations of the metabolites added to the reaction mixtures were 1, 5, or 10 mM.

Km values of the citrate synthases for acetyl-CoA and for oxaloacetate were determined respectively by measuring the variation of reaction rate with the concentration of one rate-limiting substrate.

RESULTS AND DISCUSSION

Purification of the enzymes
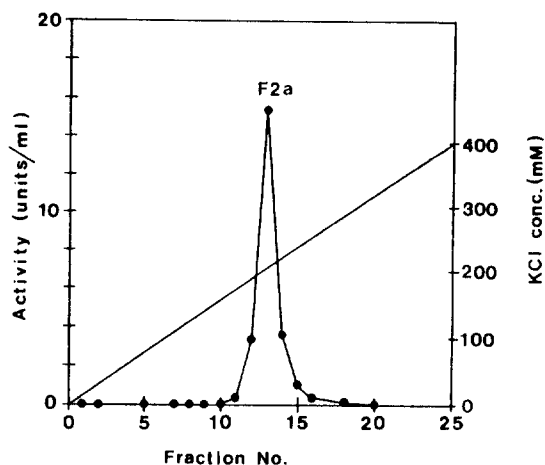
Each of the crude enzymes prepared from *S.*

Fig. 2. *Ion exchange chromatography of partially purified CS 2 (F2) on a Mono Q HR 5/5 column (Pharmacia) using FPLC system. Column dimensions: 0.5×5 cm. Fraction volume: 1 ml.*

Fig. 3. *SDS-polyacrylamide gel electrophoretic analysis of CS 1 and CS 2. (A) Lanes: 1, molecular weight markers; 2, crude enzyme from S. cerevisiae PSY40 carrying pLCS1; 3, purified CS 1 (F1). (B) Lanes: 1, molecular weight markers; 2, crude enzyme from S. cerevisiae PSY38 carrying pUCS2; 3, partially purified CS 2 (F2); 4, purified CS 2 (F2a).*

cerevisiae PSY40 strain carrying pLCS1 or PSY38 strain carrying pUCS2 was fractionated through a Reactive Blue 4 agarose gel column. Fig. 1A. shows the elution profile for CS 1 from the affinity column, and Fig. 1B. for CS 2. CS 1 showed strong affinity for the gel and eluted with buffer A containing both 0.5 mM oxaloacetate and 0.5 mM CoA (F1). On the other hand, CS 2 showed somewhat weaker affinity than CS 1 and was mainly eluted with buffer A containing only 0.5 mM oxaloacetate (F2). The selectivity of oxaloacetate for the elution of CS 2, however, was not very sufficient. This result implies that the two isoenzymes have considerable difference in the affinity for their substrates which possibly arises from the different structures of their active sites. As seen in Fig. 3, while a single protein band was observed in the lane for F1 fraction, some impurities were found in F2 fraction.
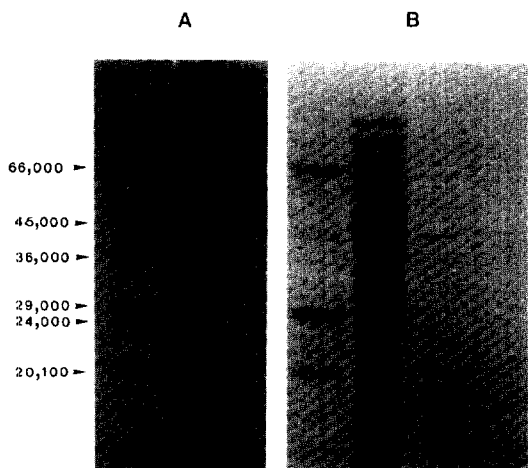
Further purification of F2 was achieved by ion exchange chromatography on a Mono Q HR 5/5 column. As shown in Fig. 2, citrate synthase activity was detected in a symmetrical peak, F2a, and elimination of the impurities was confirmed by SDS-PAGE (Fig. 3).

A quantitative evaluation of the results obtained from the purification steps is given in Table 1. The specific activities of the purified CS 1 and 2 were estimated to be 162.4 and 146.7 units mg^{-1}. They are about 148- and 1,467-fold higher than those of the crude enzyme preparations, respectively. The yields of purified enzymes were 33.5% for CS 1 and 11.7% for CS 2. The poorer yield of the latter is probably due to its lower stability and weaker affinity for the affinity gel

Table 1. *Purification of CS 1 and CS 2.*

| Enzymes	Procedures	Volume (ml)	Activity (units/ml)	Total activity (units)	Protein conc. (mg/ml)	Specific activity (units/mg protein)	Yield (%)
CS1	Crude enzyme	100.0	22.1	2,210	19.40	1.1	100.0
	Affinity chromatography; OAA-CoA eluate (F1)	6.0	123.4	740	0.76	162.4	33.5
CS2	Crude enzyme	100.0	7.5	750	70.10	0.1	100.0
	Affinity chromatography; OAA eluate (F2)	6.0	24.2	145	0.46	52.6	19.3
	Ion exchange chromatography; (F2a)	1.0	88.0	88	0.60	146.7	11.7
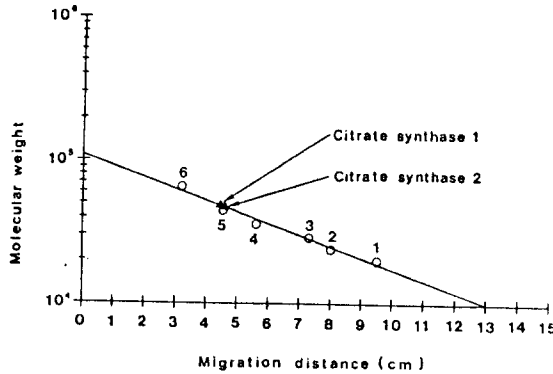
Fig. 4. *Determination of subunit molecular weight of CS 1 and CS 2 by SDS-polyacrylamide gel electrophoresis. Molecular weight markers: 1, trypsin inhibitor (20,100); 2, trypsinogen (24,000); 3, carbonic anhydrase (29,000); 4, G3P dehydrogenase (36,000); 5, ovalbumin (45,000); 6, bovine serum albumin (66,000).*
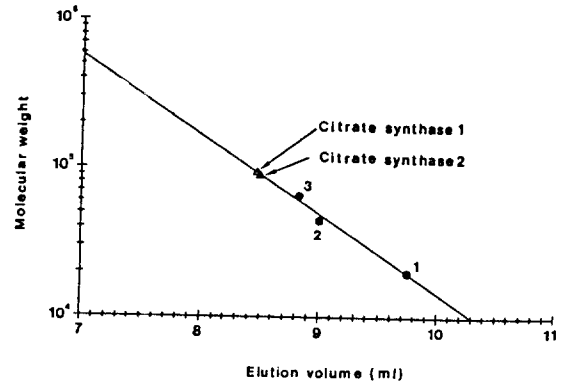

Fig. 5. *Determination of native molecular weight of CS 1 and CS 2 by gel filtration on a Superose 12 HR 10/30 column (Pharmacia) using FPLC system. Column dimensions: 1×30 cm. Molecular weight markers: 1, trypsin inhibitor (20,100); 2, ovalbumin (45,000); 3, bovine serum albumin (66,000).*

than the former.

It was noticed that purified CS 2 was considerably labile in buffer A even at 4°C. The purification of this enzyme was carried out promptly, and glycerol was added up to 50% to the enzyme solution to stabilize the enzyme.

Molecular weights of the enzymes

The molecular weights of CS 1 and 2 subunits were determined by SDS-PAGE analyses with molecular weight standard proteins (Fig. 3). A linear relationship was obtained when the relative mobility of the standard proteins was plotted against the logarithmic values of the molecular weights (Fig. 4). The molecular weights of the enzyme subunits were estimated to be about 48.3 kDa for CS 1 and 47.0 kDa for CS 2, respectively, that are considerably smaller than the values, 53.5 and 51.4 kDa, calculated from the open reading frames of corresponding genes (Rosenkrentz et al., 1986). This result suggests that the enzymes are translated as precusor molecules in cytoplasm and subjected to posttranslational processing, i.e., removal of N-terminal signal sequences during the process of intracellular transport to their own target organelles. Lewin et al. (1990) reported that the CS 2 activity cosedimented with peroxisomes, and concluded that the product of the *CIT2* gene of *S. cerevisiae* is peroxisomal. In addition, they suggested that C-terminal SKL (serine-lysine-leucine) tripeptide of CS 2 might play a role as peroxisomal targeting signal in *S. cerevisiae.* This view was based on the demonstration of Gould et al. (1988, 1989) that the SKL sequence is one type of the signals that can act to target proteins to mammalian peroxisomes *in vivo.* However, the peroxisomal catalase A from *S. cerevisiae* does not
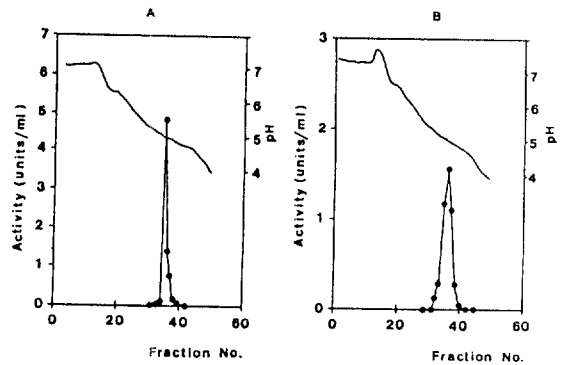

Fig. 6. *Chromatofocusing of CS 1 (A) and CS 2 (B) on a Mono P HR 5/5 column (Pharmacia) using FPLC system. Column dimensions: 0.5× 5 cm. Fraction volume: 1 ml.*

have the C-terminal SKL sequence (Cohen et al., 1988), which indicates that the process of targeting proteins to peroxisomes can occur via some other types of signals.

The purified enzymes were subjected to FPLC gel filtration and both the molecular weights of the native enzymes were estimated to be 90-100 kDa (Fig. 5). This result suggests that both the isoenzymes are dimeric proteins assembled with two identical subunits.

Isoelectric points of the enzymes

The pI values of CS 1 and 2 were both determined to be 5.0 by chromatofocusing (Fig. 6). In contrast, CS 1 and 2 purified from castor bean seeds differ greatly in their pIs: 5.9 for the former and 9.1 for the latter (Zehler et al., 1984)
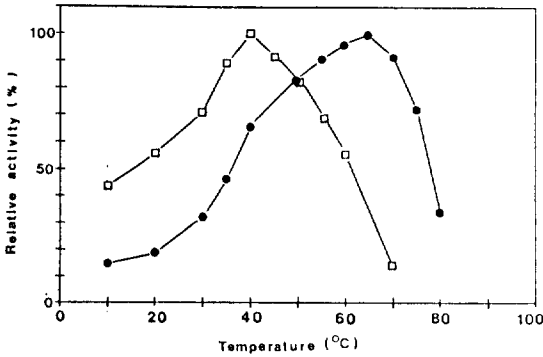
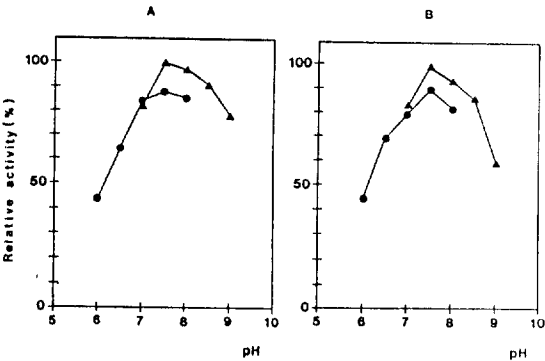Fig. 7. Effect of reaction temperature on the activity of CS 1 and CS 2. Enzyme activity of: CS 1, ●; CS 2, □.

Fig. 9. Thermal stability of CS 1 (A) and CS 2 (B). Residual enzyme activity after incubation at: 20 ℃, ■; 30℃, ▲; 40℃, △; 50℃, ●; 60℃, ○.

Fig. 8. Effect of reaction pH on the activity of CS 1 (A) and CS 2(B). Enzyme activity in: phosphate buffer, ●; Tris-HCl buffer, ▲.

Fig. 10. pH stability of CS 1 (A) and CS 2 (B). Residual enzyme activity after incubation at: pH 6.0, ○; pH 6.5, ●; pH 7.0, △; pH 7.5, ▲; pH 8.0, □; pH8.5, ■; pH 9.0, ▽.

Effect of temperature and pH on the enzyme activity

The activity of CS 1 and 2 was assayed at various temperatures between 10 and 80℃. Strikingly, the isoenzymes demonstrated quite different responses to the reaction temperature. As seen in Fig. 7, the optimal temperatures for the enzyme activity were 65℃ for CS 1 and 40℃ for CS 2. Thus, CS 1 appeared to be more active at higher temperatures than CS 2.

The effect of pH on the enzyme activity was examined (Fig. 8). The optimal pH for both CS 1 and 2 was pH 7.5. CS 1 showed more than 75% of the maximal activity in the range of pH 7.0-9.0 and CS 2 in the range of pH 7.0-8.5, which suggests that there is little difference between the effect of pH on the activity of the two isoenzymes.

Thermal and pH stability of the enzymes

Each purified enzyme was incubated at various temperatures in the absence of substrates, and the residual enzyme activity was measured at intervals under standard assay conditions. As presented in
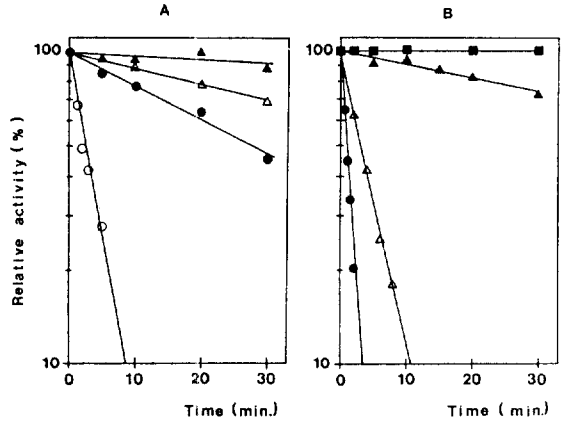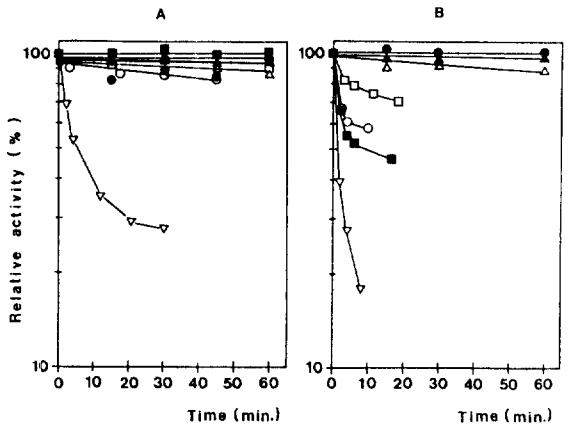
Fig. 9, CS 2 lost 50% of its activity in 8 min at 40℃ and lost 80% in 2 min at 50℃. On the other hand, CS 1 lost 30 % and 55 % of its activity in 30 min at 40℃ and 50℃, respectively. This result revealed that CS 2 is markedly more labile than CS 1 especially at the temperatures above 40℃.

As shown in Fig. 10, CS 2 retained its activity for 1 hour at pH 6.5-7.5 and was labile beyond this range, while CS 1 showed considerable stability at pH 6.0-8.5. This result is in accordance with the finding that CS 2 activity in postmitochondrial supernatant was sensitive to pretreatment at pH 8.1 (Rosenkranz et al., 1986).

Effect of metal ions on the enzyme activity

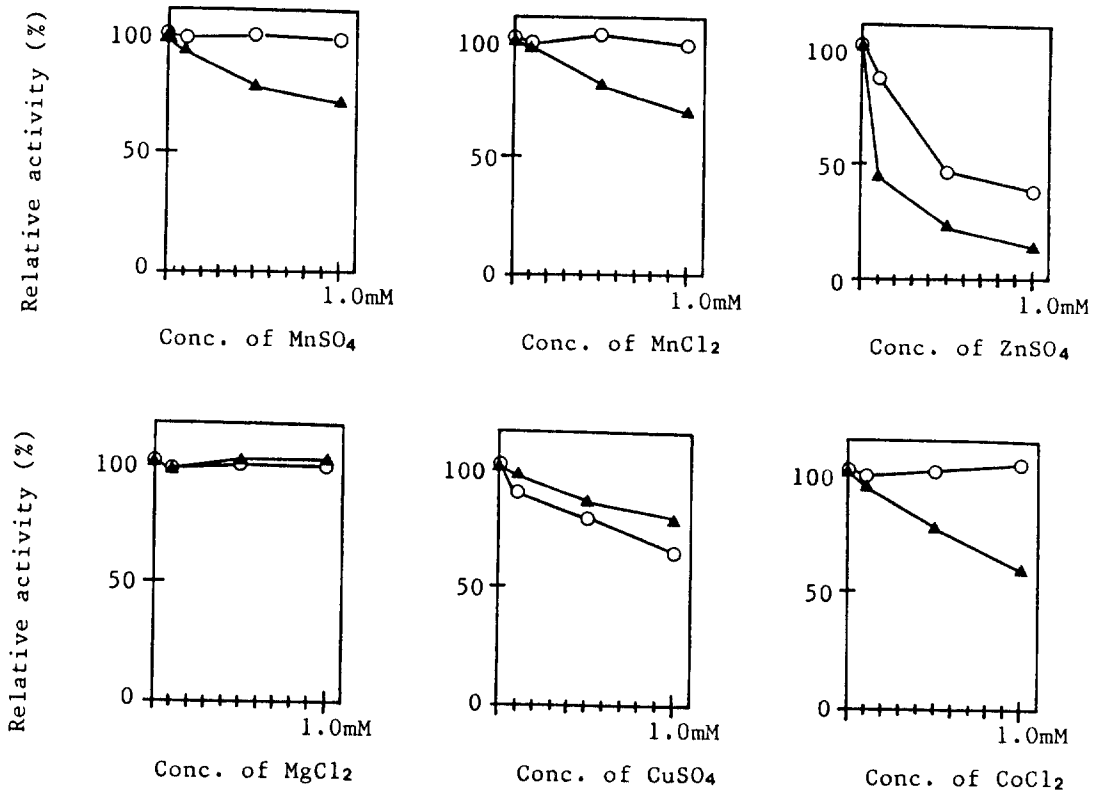The inhibitory effect of several metal ions on

Fig. 11. Effects of different metal ions on the citrate synthase activity. Enzyme activity of: CS 1, ◯; CS 2, ▲
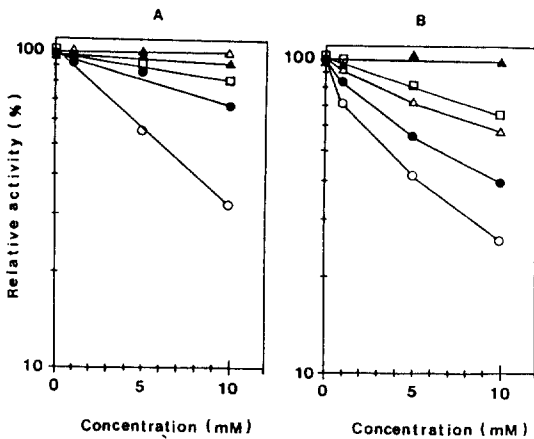

Fig. 12. Effects of different metabolites on the activity of CS 1 (A) and CS 2 (B). Metabolites: ATP, ◯; ADP, ●; NADH, △; NAD, ▲; NADPH, ▢

Table 2. Km values of CS 1 and CS 2.

Enzymes	Km (μM)	
	For Acetyle Co-A	For Oxaloacetate
CS 1	5.2	22.5
CS 2	35.4	79.3

the citrate synthase activity was examined, and the results are presented in Fig. 11. While Mg^{2+} ion showed no effect on any of the two isoenzymes, Zn^{2+} ion showed most severe effect on both enzymes, especially on CS 2, among the metal ions tested. Cu^{2+} ion also exhibited some inhibitory effect, which was much weaker than that of Zn^{2+}, on both enzymes. On the other hand, Mn^{2+} and Zn^{2+} ions inhibited CS 2 to a considerable extent, but did not affect the activity of CS 1 at all. This result indicates that the activity of CS 2 is generally more sensitive to metal ions than that of CS 1 is.

Effect of some metabolites on the enzyme activity

As illustrated in Fig. 12, ATP showed the strongest inhibitory effect on both enzymes among the possible regulatory metabolites tested, for about 80% of CS 1 and 2 activity was inhibited in the presence of 10 mM of ATP. ADP (10 mM) inhibited the activity of CS 1 by approximately

30% and CS 2 by 60%. NADH showed quite a distinguishing result that 10 mM of this metabolite reduced about 45% of CS 2 activity but did not affect the activity of CS 1 at all. $NADP^+$ exhibited some, but not very strong, inhibitory effect on both enzymes, and NAD^+ showed negligible inhibition.

Kinetics

Km values of the enzymes were determined using Lineweaver-Burk plot (not shown) and listed in Table 2. The Km values of CS 1 and 2 for acetyl-CoA were 5.2 and 35.4 μM, respectively. For oxaloacetate, the Km of the former was 22.5 μM and that of the latter was 79.3 μM. This result indicates that CS 2 has approximately 7- and 3.5-fold lower affinity to acetyl CoA and to oxaloacetate, respectively, than CS 1.

적 요

Saccharomyces cerevisiae 세포로부터 citrate synthase 1 (mitochondrial) 및 citrate synthase 2 (nonmitochondrial)를 각각 분리 · 정제하고 citrate synthase 2의 물리학적 및 효소학적 특징을 citrate synthase 1에 대하여 비교 · 분석하였다. 정제된 두 동위효소는 모두 동일한 하부단위체로 구성된 dimer 단백질임이 확인되었으며, 각 하부단위체의 분자량은 citrate synthase 1의 경우 48.3 kDa, citrate synthase 2의 경우 47.0 kDa으로 각각 나타났다. 최적 반응 pH는 7.5로서 서로 유사하였으나, 최적 반응 온도는 citrate synthase 2의 경우 40℃로서 citrate synthase 1의 경우보다 25℃나 낮게 나타났다. 또한, citrate synthase 2는 열 및 pH에 대하여 citrate synthase 1보다 크게 떨어지는 안정성을 보였으며, Zn^{2+}, Mn^{2+}, Co^{2+} 등의 금속 이온에 의한 활성의 저해도 citrate synthase 1의 경우보다 더 크게 받는 것으로 밝혀졌다. 두 효소의 활성을 조절할 것으로 기대되는 몇몇 대사산물들의 영향을 조사한 결과, 그들 중 ATP는 두 효소 모두에 대하여 가장 강력한 저해효과를 보였으며, ADP와 NADH는 citrate synthase 1보다 citrate synthase 2에 대하여 더 강한 저해효과를 나타내었다. 두 동위 효소의 kinetics를 조사한 결과, citrate synthase 2의 acetyl CoA 및 oxaloacetate에 대한 친화력은 각각 citrate synthase 1의 1/7 및 1/3.5 정도에 불과한 것으로 확인되었다.

REFERENCES

1. **Cohen, G., W. Rapatz, and H. Ruis,** 1988. Sequence of CTA1 gene and amino acid sequence of catalase A derived from it. *Eur. J. Biochem.* **176,** 159-163.
2. **Duntze, W., D. Neumann, J.M. Gancedo, W. Atzpodien, and H. Holzer,** 1969. Studies on the regulation and localization of the glyoxylate cycle enzymes in Saccharomyces cerevisiae. *Eur. J. Biochem.* **10,** 83-89.
3. **Gould, S.J., G.-A. Keller, N. Hosken, J. Wilkinson, and S. Subramani,** 1989. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell. Biol.* **108,** 1657-1664.
4. **Gould, S.J., G.-A. Keller, and S. Subramani,** 1988. Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. *J. Cell. Biol.* **107,** 897-905.
5. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura,** 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153,** 163-168.
6. **Kim, K.S., M.S. Rosenkrantz, and L. Guarente,** 1986. *Saccharomyces cerevisiae* contains two functional citrate synthase genes. *Mol. Cell. Biol.* **6,** 1936-1942.
7. **Krebs, H.A. and J.M. Lowenstein,** 1960. The tricarboxylic acid cycle, pp. 129-203. In D. M. Greenberg (ed.), Metabolic pathways. Academic Press, Inc., New York.
8. **Laemmli, U.K.,** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.
9. **Lewin, A.S., V. Hines, and G.M. Small,** 1990. Citrate synthase encoded by the CIT2 gene of *Saccharomyces cerevisiae* is peroxisomal. *Mol. Cell. Biol.* **10,** 1399-1405.
10. **Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall,** 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.
11. **Rickey, T.M. and A.S. Lewin,** 1986. Extra-mitochondrial citrate synthase activity in baker's yeast. *Mol. Cell. Biol.* **6,** 488-493.
12. **Rosenkrantz, M., T. Alam, K.S. Kim, B.J. Clark, P.A. Srere, and L.P. Guarente,** 1986. Mitochondrial and nonmitochondrial citrate synthase in *Saccharomyces cerevisiae* are encoded by distinct homologous genes. *Mol. Cell. Biol.* **6,** 4509-4515.
13. **Sherman, F., G.R. Fink, and J.B. Hicks,** 1983. Methods in yeast genetics. Cold Spring Harbor Laborytory. Cold Spring Harbor, N. Y.
14. **Srere, P.A.,** 1969. Citrate synthase. *Methods Enzymol.* **13,** 3-26.
15. **Suissa, M., K. Suda and G. Schatz,** 1984. Isolation of the nuclear genes for citrate synthase and fifteen other mitochondrial proteins by a new screening method. *EMBO. J.* **3,** 1773-1781.
16. **Zehler, H., K.-S. Thomson, C. Schnarrenberger,** 1984. Citrate synthase from germinating caster bean seeds - I. Purification and properties. *Physiol. Plant.* **60,** 1-8.