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Study on the Biosynthesis on Neomycin: Characterization of Isocitrate Dehydrogenase of the Neomycin Producer, *Streptomyces fradiae* and its Possible Relation to the Regulation of Biosynthesis of Neomycin

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S. fradiae showed very high activity of isocitrate dehydrogenase compared to other microorganisms. The activity of this enzyme was increased with the growth of the organism. But the increase might not imply its involvement in the growth. Rather its increased activity seemed to have a connection with the biosynthesis of neomycin. The enzyme showed high specificity toward $NADP^+$ and D-isocitrate with Km values of 5.75 and 6.74 μM, respectively. It was activated by Mn^{2-}. Its molecular weight was estimated from its gel retardation coefficient to be in the range of 61,000-63,000 daltons and its optimum pH was 8.0. The enzyme was thermally unstable.

Introduction

In many antibiotic producing *Streptomyces* sp. it has been known that when glucose is abundant in a medium, antibiotics are scarcely produced although growth of microorganisms is dominant[1]. However as glucose in the medium is consumed, growth of the organisms is terminated and the production of antibiotics is initiated. A similar regulation has also been observed in the neomycin producer[2]. Thus it is interesting to figure out how *S. fradiae* controls the biosynthesis of neomycin.

We have been assumed that not only glucose itself had a regulation role in the biosynthesis of antibiotics, but also a metabolite which may be produced from glucose during catabolism and accumulated in the cell can have a role initiating the biosynthesis of many antibiotics[2]. As the ratio of the metabolite to glucose intaken from a medium exceeds certain value, the growth of the microorganism is terminated and the production of neomycin may be initiated. When we examined the production of neomycin by culturing *S. fradiae* in a chemically defined medium supplemented with various metabolites, fumarate was found to activate the biosynthesis of neomycin[2,3]. Furthermore, the production of neomycin in the presence of fumarate was found further activated by a small amount of glucose and repressed by a large amount of glucose[2,4]. Thus it was interesting to examine the enzymes involved in the citric acid cycle, in the metabolism of glucose or in gluconeogenesis to find out which is involved in the termination of growth, or in the initiation of biosynthesis of neomycin.

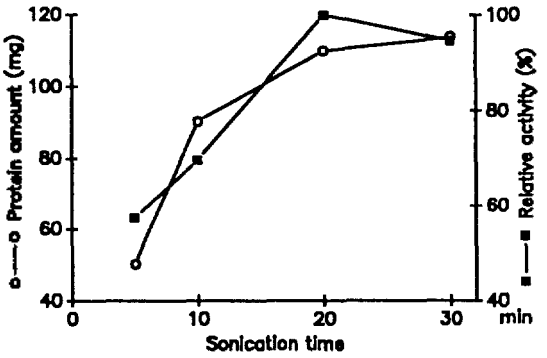

Figure 1. The release of protein (○) and isocitrate dehydrogenase (■) from *S. fradiae* (NRRL B-1195) after sonication.

Examination of literature indicates that a lot of studies[5-7] have been carried out on the enzymes of *E. coli* that are concerned with the metabolism of glucose, in the gluconeogenesis, or in the citric acid cycle, but very limited studies have been reported in the case of streptomycetes[8,9]. Thus we have examined several enzymes of *S. fradiae* which are known to be involved in the metabolism or the biosynthesis of glucose, or in the citric acid cycle to see whether any of these enzymes are activated by fumarate[10]. We found that *S. fradiae* had a very high activity of isocitrate dehydrogenase. To the best of our knowledge, no reports have been appeared on this enzyme in streptomycetes. So we characterized the isocitrate dehydrogenase in *S. fradiae*, examined its role on the biosynthesis of neomycin, and compared it with those of other organisms. In this paper we describe the results from these studies.

Results and Discussion

Optimum Condition for Sonication. *Streptomyces* sp. are generally grown in a mycelial form and careful preparation of cell free extract is necessary. As we increased the sonication time for *S. fradiae* grown for 40 hours in TSB medium, the amount of isocitrate dehydrogenase and the total protein released in PED buffer was increased with sonication time up to 20 min as shown in Figure 1. But maximum activity of isocitrate dehydrogenase was observed in the sample sonicated for 20 min although more protein was obtained at 30 min's sonication. This result seemed to imply the destruction of the enzyme at longer exposure on ultrasonic wave.

Examination of the Activities of Enzymes. *S. fradiae* showed extraordinary strong activity of isocitrate dehydrogenase which possessed exceptionally high specificity on $NADP^+$. The enzyme showed very low activity with NAD^+ which was about 1.8% of that observed with $NADP^+$. NADPH oxidase which oxidizes NADPH to NADP might interfere in the assay of isocitrate dehydrogenase. But we observed that its activity was negligibly law (only 1.2% of that of isocitrate dehydrogenase). Thus, we carried out the assay of isocitrate dehydrogenase without correcting the effect of NADPH oxidase. The activity of NADH oxidase in the cell free extract was 8.5% of that of NADP-specific isocitrate dehydrogenase, which was about 7 times stronger than the NADPH oxidase activity.

Table 1. Activities of Enzymes in *S. fradiae*

Enzyme	Relative activity(%)
Isocitrate dehydrogenase ($NADP^+$ specific)	100
Isocitrate dehydrogenase (NAD^+ specific)	1.81
Glutamate dehydrogenase (NAD^+ specific)	5.45
Glutamate dehydrogenase ($NADP^+$ specific)	1.09
NADH oxidase	8.55
NADPH oxidase	1.22

The activity of isocitrate dehydrogenase at 25℃ was taken as 100% and activities of other enzymes were expressed as percent ratio of this activity.

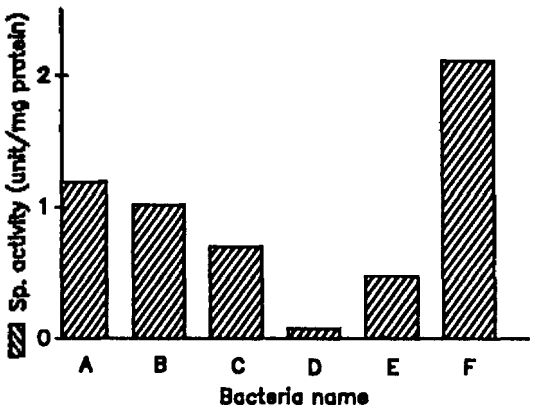

Figure 2. The activities of isocitrate dehydrogenase in the cell free extracts of *E. coli* (A), *S. kanamyceticus* (B), *S. griseus* ATCC 23345 (C), *Micromonospora spectinosus* (D), *S. griseus* ATCC 12475 (E), and *S. fradiae* (F).

Microorganisms are known to produce two distinct glutamate dehydrogenase, one specific for NAD^+ and the other specific for $NADP^+$. Examination of glutamate dehydrogenase in the cell free extract of *S. fradiae* indicated that it had NAD-specific glutamate dehydrogenase, the activity of which was equivalent to 5.45% of that of the total isocitrate dehydrogenase. The enzyme showed 5 times lower activity with $NADP^+$ than with NAD^+ (Table 1).

Examination of several microorganisms for isocitrate dehydrogenase activity revealed that *S. fradiae* showed the highest activity of isocitrate dehydrogenase (Figure 2). Other streptomycetes such as *S. kanamyceticus* and *S. griseus*, showed low activity for the enzyme as *E. coli*. *Micromonospora spectinosus* showed very low activity.

Change of Isocitrate Dehydrogenase Activity with Growth of *S. fradiae*. When *S. fradiae* was cultured in tryptic soy broth (TSB), the dry cell weight in the broth was increased 3.4 times at 48 hrs culture as shown in Figure 3. Glucose was added in the medium at the concentration of 27.7 mM, but its concentration was decreased to 0.68 mM after 96 hrs culture. The pH of the medium was 7.31 at the beginning of the culture but it was slowly increased to 8.54 at 120 hrs culture. The production of neomycin was observed at 12 hrs culture and its quantity in the medium was increased to the maximum 36.4 μg/mg mycelia at 120 hrs culture. The activity of isocitrate dehydrogenase in the cells harvested at various stage of growth was increased dur-

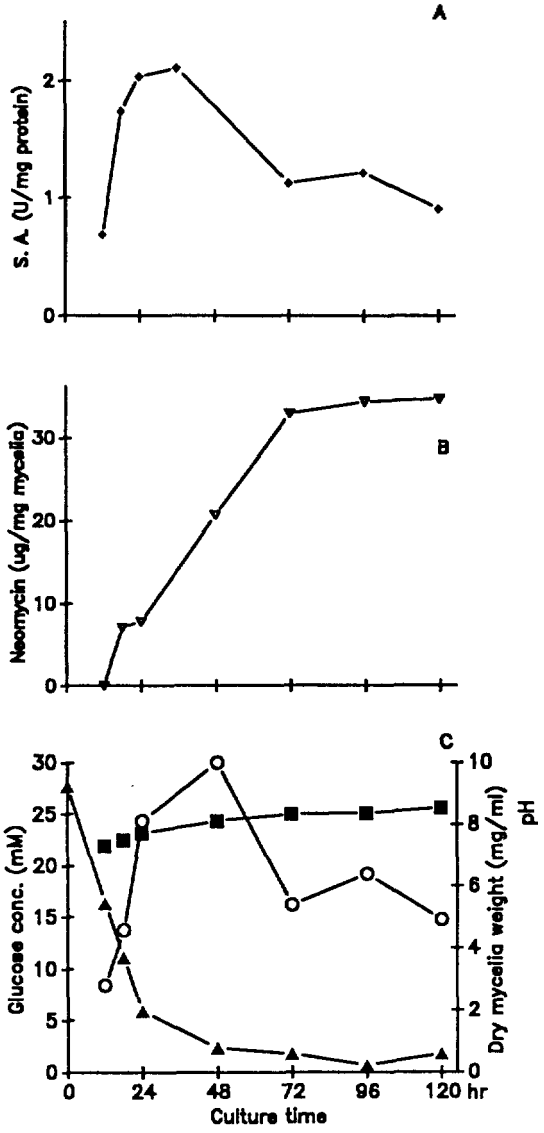

Figure 3. A. The specific activity (◆) of isocitrate dehydrogenase in the cell free extract of *S. fradiae* harvested at the various stage of growth. B. The amount of neomycin (▽) in the cultured broth. C. The dry cell weight (○), the amount of glucose (▲) in the broth and pH (■) of the medium. The organism was cultured in tryptic soy broth and the broth was sampled at regular intervals and analyzed.

ing the first 36 hrs culture and decreased afterwards (Figure 3a). Apparently, the high increase of the activity of isocitrate dehydrogenase was not related to the amount of glucose present in the medium. Currently we could not find any other evidences that isocitrate dehydrogenase has any regulatory role on the growth of the organism or on the production of neomycin. Although high increase of this enzyme was noticeable during the growth of the organism, the increase of the activity of the enzyme seemed not to be related to the growth of the organism. But the increase of the amount of the antibiotic accumulated in the medium about 24 hrs later would imply the possibility that the enzyme had some regulatory role on the biosynthesis of the antibiotic.

The activity of isocitrate dehydrogenase did not show any change in the minimal salt medium supplemented with glucose, but an abrupt increase of the enzyme in the organism in the same medium supplemented with fumarate was observed[10]. This result also supported the possibility that the increase of the activity of isocitrate dehydrogenase was not connected to the growth of the organism but was implying the involvement of this enzyme in the regulation of the biosynthesis of neomycin. Also some evidences obtained from feeding experiments with ^{14}C-fumarate implied transformation of fumarate to glutamate at early stage of biosynthesis of neomycin from fumarate[4,17]. Fumarate was found not only incorporated into neomycin, but also it certainly activated the biosynthesis of neomycin[4]. For the confirmative evidences we should need to establish a better system using an isocitrate dehydrogenase blocked mutant and need more works on this enzyme.

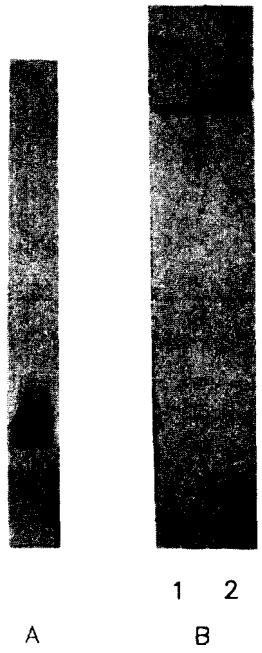

Figure 4. The band of isocitrate dehydrogenase obtained by active staining with 7.5% (A) or 15% (B) polyacrylamide gel slice after electrophoresis of the cell free extract of *S. fradiae*. The cell free extract (0.05 ml) was loaded on the top of the polyacrylamide (7.5% and 15% for A and B lanes, respectively) high pH-discontinuous slab gel (15×16×1.5 mm) and was analyzed by electrophoresis. The gel slices was treated with active staining mixture consisted of 2.5 mM NAD^+ (lane 1 in B lane) or $NADP^+$ (lane A and lane 2 in lane B) and other reagents as described in the experimental section.

Examination of Isocitrate Dehydrogenase by Active Staining. Electrophoresis of the cell free extract obtained from *S. fradiae* on 7.5%, 10%, or 15% nondenaturing high pH-discontinuous polyacrylamide gel and active staining of the gel showed the band of isocitrate dehydrogenase which was specific to $NADP^+$ (Figure 4). In this analysis a band of isocitrate dehydrogenase which was specific to NAD^+ was also observed at the same position with much lower activity. This gel electrophoresis confirmed as well that the isocitrate dehydrogenase was specific to $NADP^+$. The observation of a thin band of NAD^+-specific isocitrate

Table 2. R_f Values of Proteins in Electrophoresed Acrylamide gels

Gel %		7.5%	10%	15%
Protein	MW(daltons)			
Albumin (chicken egg)	45 k	0.958	0.616	0.289
Albumin (bovine serum)	66 k	0.851	0.493	0.181
Albumin (bovine serum, dimer)	132 k	0.532	0.246	0.072
Isocitrate dehydrogenase	61-63 k	0.883	0.551	0.207

Table 3. Requirement of Divalent Cations for Isocitrate Dehydrogenase

Divalent cation	Activity of isocitrate dehydrogenase(%)
Mn^{2+}	100
Mg^{2+}	21.6
Co^{2+}	15.0
Cu^{2+}	10.9
Fe^{2+}	6.96
Ni^{2+}	6.59
Ca^{2+}	5.10

The activity of isocitrate dehydrogenase was obtained in assay mixtures containing 0.33 mM $NADP^+$, 0.167 mM DL-isocitrate, 0.1 M *tris*-HCl (pH 7.5). 1 mM of metal salts (Cl^- as the counter ion). The relative activity to that of Mn^{2+} is presented.

dehydrogenase activity by the active staining might not imply the existence of a NAD^+-specific enzyme; the same $NADP^+$-specific isocitrate dehydrogenase might show activity in the presence of NAD^+ with a much lower activity. The active staining of the electrophoresed gel with $NADP^+$ showed by active staining one more small band containing isocitrate dehydrogenase which was active in the presence of $NADP^+$ only. This band might imply the existence of an isozyme, but currently it is hard to deduce any conclusion. The band might be due to the existence of an enzyme having a different, or partly denatured conformation, or of a phosphorylated enzyme. In *E. coli* the activity of isocitrate dehydrogenase was controlled by phosphorylation and dephosphorylation[11]. We have not studied further the regulatory mechanism of the activity of isocitrate dehydrogenase in *S. fradiae*.

The major band of isocitrate dehydrogenase on 7.5%, 10% and 15% acrylamide gel showed R_f values at 0.88, 0.55, and 0.21 by active staining. Other marker proteins, chicken egg albumin (MW. 45,000 daltons), bovine serum albumin monomer (MW. 66,000 daltons), and bovine serum albumin dimer (MW. 132,000 daltons) were electrophoresed under the same condition and stained with Coomasie blue. From the R_f values (Table 2), the retardation coefficients were obtained from a Ferguson plot in which log ($R_f \times 100$) was plotted against gel percentage. When the log values of the molecular weights were plotted against the retardation coefficients, the molecular weight of isocitrate dehydrogenase in *S. fradiae* was found to be in the range of 61,000-63,000 daltons, which was quite different from those of isocitrate dehydrogenase

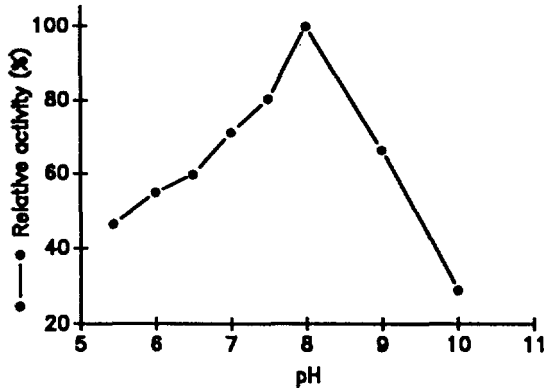

Figure 5. Activity of isocitrate dehydrogenase in the cell free extract of *S. fradiae* at various pH.

reported from *E. coli, Stearothermophilus* sp. and *Vibrio* sp., which were reported to have a molecular weight in the range of 90,000-98,000 daltons[12]. But this range of the molecular weight is very similar to those isolated from *Thermus aquaticus*, and pig heart, which are also $NADP^+$ specific and are reported to have a molecular weight in the range of 60,000-70,000 daltons[13].

The Requirement of Divalent Metal Ions for the Activity of Isocitrate Dehydrogenase. We examined the effects of divalent cations on the activity of isocitrate dehydrogenase and the results are shown in Table 3. The metal ions were added in the reaction mixture at the concentration of 1 mM. Among many divalent cations Mn^{2+} showed the highest activity of isocitrate dehydrogenase. The effects of other ions were described as the percentage of this value in Table 3. When Mg^{2+}, Co^{2+}, and Cu^{2+} were added in the assay mixture, about 22%, 15% and 11% of the activity of Mn^{2+}, respectively, were observed. From this study, the isocitrate dehydrogenase in *S. fradiae* was found to require Mn^{2+} for the maximum activity. When three times more amount of Mg^{2+} than that of Mn^{2+} was added in the assay mixture, the same activity of isocitrate dehydrogenase was observed.

Determination of the apparent values of Km. The isocitrate dehydrogenase of *S. fradiae* showed Km values 10.2 μM, 6.74 μM, and 5.75 μM against DL-isocitrate, D-isocitrate, and NADP, respectively. The Km values for the isocitrate dehydrogenase isolated from *Bacillus* sp. in purified form showed 9.9 μM, 5.4 μM, and 7.3 μM against DL-isocitrate, D-isocitrate, and NADP, respectively and the enzyme in *Thermus thermophilus* 6.3 μM and 8.8 μM against NADP and DL-isocitrate, respectively. The Km value of isocitrate dehydrogenase in *S. fradiae* against DL-isocitrate was approximately two times higher than that of D-isocitrate. Thus it seems that the isocitrate dehydrogenase in *S. fradiae* is pretty highly specific to D-isocitrate.

The Effect of pH. Isocitrate dehydrogenase showed the highest activity at pH 8.0 as shown in Figure 5. This value is very similar to those obtained from *Thermus thermophilus*[14] (pH 8.0) and from alkalophilic *Bacillus* sp.[12] (pH 7.8).

The Effect of Temperature. When the activity of isocitrate dehydrogenase by incubation of the reaction mixture at various temperature, the highest activity was observed

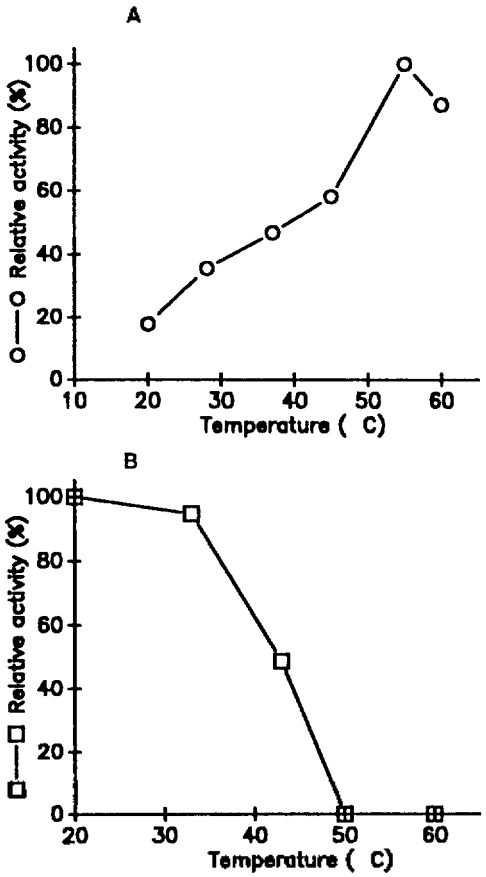

Figure 6. Effect of temperature on the activity of isocitrate dehydrogenase. A. Activity of isocitrate dehydrogenase at different temperature. B. Thermal stability of isocitrate dehydrogenase. The activity of the enzyme in the cell free extract was assayed after it was incubated for 10 min in the water bath maintained at the temperature shown in the figure.

at 55℃ (Figure 6a). The apparent activation energy obtained from Arhenius equation was 7.3 kcal/mole. The activation energy of isocitrate dehydrogenase in *Thermus thermophilus, Bacillus subtilis, Thermus aquaticus, Chlamydomonas reinharti* were reported to be 10.5, 11, 12.5 and 10 kcal/mole, respecitively[12-14]. The stability of the enzyme on the temperature was studied by examining the remaining activity of isocitrate dehydrogenase after incubation of the cell free extract at several different temperatures for 10 min. The activity of the enzyme was found to be retained almost perfectly at 33℃ but to be completely lost at 50℃ (Figure 6b). These results indicated that the enzyme was thermally unstable.

Conclusion

We found that the biosynthesis of neomycin was activated by fumarate, an intermediate of the citric acid cycle, and was further activated by the presence of a small amount of glucose. But the production of neomycin was completely depressed by a large amount of glucose. Feeding experiments with ^{14}C-fumarate in *S. fradiae* implied the formation of glutamate at the early stage of the biosynthesis of neomycin from fumarate[4]. Furthermore, examination of various enzymes in the citric acid cycle and in the glycolysis and gluconeogenesis revealed that *S. fradiae*, a neomycin producing organism contained higher activity of isocitrate dehydrogenase compared to other organisms. Thus we have examined the enzyme in detail and tried to characterize it and wanted to correlate the increase in its activity with the biosynthesis of neomycin. Although, at the present time, it is difficult to conclude that the increase of its activity is connected to the increased activity of biosynthesis of neomycin, the increase of neomycin at 24 hrs later than that of the activity of enzyme implies that it has some connections to the regulation of the biosynthesis of neomycin. Although *S. fradiae* showed higher activity of isocitrate dehydrogenase compared to other organisms, its activity was not increased by the presence of glucose and its high activity seemed not to be related to the growth of the organism.

The molecular weight of the enzyme was found to be in the range of 61,000-63,000 daltons by active gel staining. The enzyme was highly specific for NADP and D-isocitrate with Km values of 5.75 and 6.74 μM, respectively. It was activated by Mn^{2+} and its optimum pH was pH 8.0, its apparent activation energy E_a was 7.3 kcal. Its catalytic activity was maximum at 55℃ but completely destroyed at 50℃ within 10 min.

Experimental

Microorganisms. *Streptomyces fradiae* NRRL B-1195 was kindly donated by A.J. Lyons in Northern Regional Research Laboratory.

Medium and Culture of Microorganisms. Microorganisms were stocked on ISP No.4. For the study of isocitrate dehydrogenase, *S. fradiae* was seed-cultured in tryptic soy broth at 28℃ for 28 hrs. The seed culture was added to the same broth to give 4% (v/v) of an inoculum size. The microorganism was harvested by centrifugation at 16,000 g for 25 min and washed with saline two times.

Preparation of Cell Free Extract. The harvested cells was suspended in PED buffer[9] (1 g/2 m*l*; 75 mM KH_2PO_4, 2 mM dithiothreitol, 1 mM EDTA, pH 7.0) and sonicated 20 times (30 μ) for 30 sec with 30 sec's pause. The sonicated mixture was centrifuged at 16,000 g for 20 min and the supernatant was used as cell free extract.

Examination of the Optimum Condition for the Sonication. *S. fradiae* was cultured in 500 m*l* of TSB for 40 hrs. Cells were harvested from the culture (50 m*l*) by centrifugation, washed with saline two times. The cells were suspended in PED buffer (1 g/2 m*l*), sonicated at 30 μ for 30 sec with 30 sec's pause. The sonicated solution was centrifuged at 16,000 g to give cell free extract. The cell free extract was assayed for isocitrate dehydrogenase. The amount of the total protein was analyzed by the Lowry method[15].

Assay of Isocitrate Dehydrogenase. The method developed by Kornberg[16] was modified and employed for the assay of isocitrate dehydrogenase. The cell free extract (20 μ*l*) diluted 5 times with PED buffer was added to the assay mixture (1.0 m*l*) which contained 1.00 mM $MnCl_2$, 0.33 mM $NADP^+$, 0.167 mM DL-isocitrate in *tris*-HCl buffer (pH 7.0, 0.10 M). The assay mixture was incubated at 37℃ and the production of NADPH was monitored by measuring the absorbance at 340 nm.

Assay of Glutamate Dehydrogenase[18]. The *tris*-HCl

buffer (0.10 M; pH 7.5) which contained 0.1 M sodium L-glutamate, 2 mM NAD (or 2 mM NADP) added with cell free extract (100 μl) and incubated at 25℃. The production of NADH or NADPH was monitored.

Assay of NADH or NADPH Oxidase[19]. NADH oxidase or NADPH oxidase was assayed by measuring the decrease of the absorbance of the assay mixture at 340 nm. The assay mixture contained 3.33 mM $MgCl_2$, 3.33 mM NADH or NADPH.

Polyacrylamide Gel Electrophoresis and Active Staining. Polyacrylamide gel electrophoresis was carried out with a discontinuous nondenaturing slab gel by the method described by Hames and Rickwood[14]. Polyacrylamide gel (7.5%) was prepared in a gel preparation plate by polymerization of the solution which was made of acrylamide-bisacrylamide (30 : 0.8) solution (7.5 ml), a resolving buffer (3.0 M *tris*-HCl, pH 8.8, 3.75 ml) and distilled water (14.75 ml). Polymerization was initiated by mixing the solution with ammonium persulfate solution (1.5%, 1.5 ml), and then with TEMED (15 μl). The 15%-polyacrylamide gel was prepared similarly by polymerization of the solution which contained acrylamide-bisacrylamide (30 : 0.8) solution (15.0 ml), a resolving buffer (3.0 M *tris*-HCl, pH 8.8, 3.75 ml) and distilled water (9.75 ml). Ammonium persulfate solution (1.5%, 1.5 ml), and TEMED (15 μl) were added successively to the solution just before polymerization as well. Stacking gel was also prepared similarly with the solution which contained acrylamide-bisacrylamide (30 : 0.8) solution (2.5 ml), a stacking gel buffer (0.5 M *tris*-HCl, pH 6.8, 3.75 ml) and distilled water (10.0 ml) by initiating polymerization with ammonium persulfate solution (1.5%, 1.0 ml) and TEMED (15 μl). The stacking gel was prepared on the top of the resolving gel carefully. A comb was placed on the top of the stacking gel to make wells before polymerization was completed. The *tris*-HCl (pH 6.8, 0.0625 M) buffer containing 10% glycerol and 0.002% bromophenol blue was mixed with the same volume of cell free extract and 50 μl of the sample solution was loaded in the well. Electrophoresis was carried out at 4℃ by applying 150 V and 200 V on the electrophoresis unit as the sample was passing through the stacking and the resolving gels, respectively. Staining of proteins in polyacrylamide gel was carried out by shaking the gel slice in methanol-acetic acid-water (5 : 2 : 5, 500 ml) containing Coomassie blue (0.25 g) for 1 hr. The stained gel was washed with methanol-acetic acid-water (1 : 1 : 8, 2 l) several times and further decolorized by keeping the gel slice in the same solution overnight.

Active Staining. Localization of isocitrate dehydrogenase on the resolved gel was carried out by adapting the method reported by Reeves[11]. The discontinuous nondentauring electrophoresed slab gel (7.5%, 10% or 15%) was cut by the lane with a blade and immersed in a buffer solution (*tris*-HCl, 50 mM, pH 8.0, 5 ml) which contained 10 nM DL-isocitrate, 2.5 mM $NADP^+$, 1 mM $MgCl_2$, 8.1 mM phenazine methosulfate, 9.6 nM 3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyltetrazolium bromide and shaken smoothly for 10 min at 37 ℃. The reaction was stopped by washing the gel with water.

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