Journ. Fac. Sci., Hokkaido Univ. Ser. V (Botany), **11** (3, 4): 259–267, 1979.

Purification and some properties of $NADP^+$-specific isocitrate dehydrogenase from a psychrotrophic bacterium, *Flavobacterium* sp. strain G–3

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$NADP^+$-specific isocitrate dehydrogenase (ICDH) (EC 1.1.1.42) from a psychrotrophic bacterium, *Flavobacterium* sp. strain G-3, has been purified to a nearly homogeneous state by means of protamine sulfate, DEAE-cellulose, hydroxyapatite, and Sephadex G-200 gel filtration. As a mesophilic enzyme, $NADP^+$-ICDH from *Proteus vulgaris* has been also purified 78-fold by similar procedure. About one half of the activity of G-3 ICDH was lost by incubation at 40°C for 20 min, while the activity of *P. vulgaris* ICDH was quite stable. The inactivation of G-3 ICDH was completely protected in the presence of Mn^{2+} and isocitrate. At low temperature (4°C), G-3 ICDH was more active and stable than *P. vulgaris* ICDH.

The abrupt change of the inhibition by glyoxylate plus oxalacetate was observed above 15°C in G-3 ICDH and above 37°C in *P. vulgaris* ICDH. Arrhenius plots of these enzyme activities between 2°C and 40°C indicated that some conformational changes were occurred at 25°C in G-3 ICDH, but not in *P. vulgaris* ICDH.

Microorganisms can be roughly divided into three classes based on their growth temperature, namely thermophiles, mesophiles, and psychrophiles. Usually mesophiles have been used extensively for biochemical studies. Recently the researches on thermophiles have made progress in an effort to elucidate the biochemical mechanisms of biological processes under extremely high temperature (reviewed by SINGLETON and AMELUNXEN, 1973; OSHIMA, 1974; OSHIMA, 1976). Although there are many and wide cold-environments on the earth which are favorable to psychrophiles, the investigations on psychrophiles have not yet been numerous, and most of these works are concerned with the effect of higher temperature. An important question in psychrophily is why psychrophiles can grow at 0°C or below, and the question has not been solved, though there are some reports concerning with membraneous fatty acid composition (CULEEN *et al.*, 1971; and so on) and a proteinous factor activating protein synthesis under low temperature (SZER, 1970). We are interested to investigate whether psychrophilic enzyme has any different nature from the homologous mesophilic enzyme, because any comparative studies on the purified enzymes from psychrophiles

and mesophiles have not been reported.

In this paper, purification of $NADP^+$-specific isocitrate dehydrogenases from *Flavobacterium* sp. strain G-3, a psychrotrophic bacterium, and from *Proteus vulgaris*, and some properties of these enzymes are reported.

Materials and Methods

Bacteria: A psychrotrophic bacterium, *Flavobacterium* sp. strain G-3 isolated by us (SARUYAMA *et al.*, 1978), was grown at 20°C by shaking in the ordinary nutrient medium consisting of 1% each of pepton and meat extract. A mesophilic bacterium, *Proteus vulgaris*, was grown by the same way as the psychrotroph except the temperature at 37°C.

Assay of isocitrate dehydrogenase activity: The activities of isocitrate dehydrogenase were determined spectrophotometrically at 340 nm with a Hitachi Perkin-Elmer type 139 spectrophotometer. The standard reaction mixture (2 m*l*) consisted of 33 mM Tris-HCl (pH 8.4 for psychrotroph and pH 7.8 for mesophile), 0.67 mM $MnCl_2$, 2 mM DL-isocitrate, 0.12 mM $NADP^+$ and appropriate amount of enzyme. The reaction temperature was 20°C unless otherwise stated. The unit of the activity was defined as the amount of enzyme that reduced 1 μmole of $NADP^+$ per min. Protein concentration was determined by the method of LOWRY *et al.* (1951) with bovine serum albumin as a standard.

Polyacrylamide disc gel electrophoresis: Polyacrylamide disc gel electrophoresis was carried out with 7.5% acrylamide as a separate gel at pH 8.3 under current intensity of 2 mA per tube. After electrophoresis protein was stained with Coomassie Brilliant Blue.

Chemicals: $NADP^+$ was obtained from Boehringer Mannheim, and DL-isocitrate was from Sigma. DEAE-cellulose was a product of Brown, and Sephadex G-25 and G-200 were Pharmasia's. Hydroxyapatite was prepared by the method of TISELIUS *et al.* (1956). All other reagents were of analytical grade.

Experimental Procedures

Purification of $NADP^+$-isocitrate dehydrogenase from Flavobacterium *sp. strain G-3*

Protamine sulfate treatment and ammonium sulfate fractionation: About 30 g of washed cells suspended in 150 m*l* of 20 mM Tris-HCl (pH 7.4) were disrupted by sonic vibration, and cell debris was removed by centrifugation at 11,000 r. p. m. for 20 min. To the supernatant (165 m*l*) 50 m*l* of 1% protamine sulfate solution was added with stirring and the precipitate

obtained by centrifugation was discarded. Then the enzyme was purified by ammonium sulfate fractionation between 0.55 and 0.9 saturation with addition of solid ammonium sulfate. The enzyme solution was adjusted to pH 7.4 by addition of 1 M NH_4OH during this fractionation.

DEAE-cellulose column chromatography: The precipitate formed by ammonium sulfate fractionation was collected by centrifugation (11,000 r.p.m., for 20 min) and dissolved in 12 m*l* of 20 mM Tris-HCl (pH 7.4) containing 0.5 mM $MnCl_2$ and 0.67 mM dithiothreitol. This enzyme solution was dialyzed against 2 *l*. of the same buffer and then charged onto a DEAE-cellulose column (1.4×25 cm) previously equilibrated with the same buffer. After washing of the unadsorbed material with the same buffer, enzyme was eluted with linear gradient of NaCl (0−0.4 M) in the same buffer.

Hydroxyapatite column chromatography: Active fractions eluted from the DEAE-cellulose column were combined, loaded onto a Sephadex G-25 column (2.4×27 cm) equilibrated with 20 mM phosphate buffer (pH 7.4) containing 0.6 mM dithiothreitol, and eluted with the same buffer. Then the combined active fraction was applied to a hydroxyapatite column (1.4×13 cm) and eluted with linear gradient of phosphate (20 mM−300 mM).

Sephadex G-200 gel filtration: Enzyme solution obtained by hydroxyapatite chromatography was dialyzed against 2 *l*. of 20 mM Tris-HCl (pH 7.4) containing 0.5 mM $MnCl_2$ and 0.6 mM dithiothreitol, and then concentrated to about 4 m*l* with polyethylene glycol No. 6000. The sample was

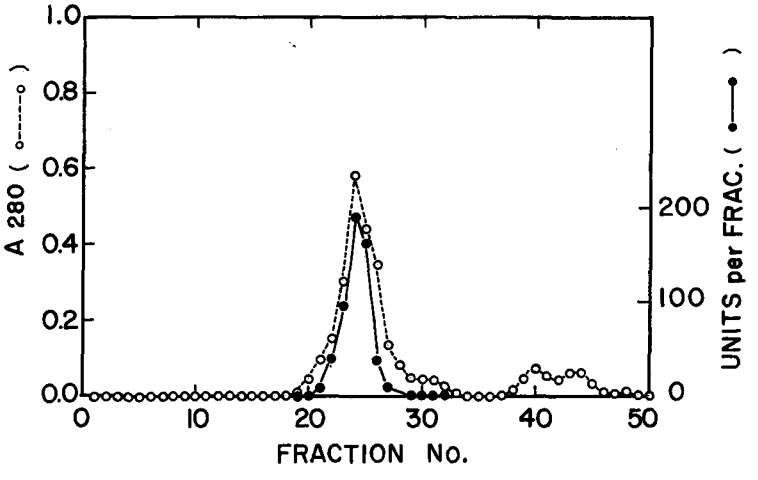

Fig. 1. Sephadex G-200 gel filtration of G-3 ICDH. Sample was run through a column with flow rate of 2.0 m*l*/h. Details are described in the Experimental Procedures.

TABLE 1 Purification of isocitrate dehydrogenase from G-3

Purification step	Volume (m*l*)	Total activity (units)	Specific activity (units/mg prot.)	Purification (-fold)
Crude extract	165	1531.9	0.31	1
Protamine sulfate	185	1040.7	0.51	1.7
Ammonium sulfate	18	1003.3	2.82	9.1
DEAE-cellulose	20	935.5	14.26	45.9
Hydroxyapatite	20	593.5	52.06	167.6
Sephadex G-200	4	114.5	54.52	175.5

Details are given in the Experimental Procedures.

divided into two fractions each of which was subjected to gel filtration. Two m*l* of this concentrated solution was filtrated through a Sephadex G-200 column (1.4×42 cm) equilibrated with 20 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 0.5 mM $MnCl_2$, and 0.6 mM dithiothreitol. A profile of the gel filtration is shown in Fig. 1, and the results of the purification are summarized in Table 1. The purity of G-3 enzyme was more than 90% as judged by polyacrylamide disc gel electrophoresis (Fig. 2). Fig. 2 shows that the purified G-3 ICDH contains very small amount of contaminant.

Purification of NADP$^+$-ICDH from Proteus vulgaris: The purification procedure of isocitrate dehydrogenase from *P. vulgaris* was essentially the same as G-3, except that one fifth volume of 1% protamine sulfate was used. 1.4 mg of partially purified enzyme was obtained from about 14 g of washed cells. The specific activity of purified enzyme was 43.52 units/mg protein. The enzyme was about 70% pure as judged by polyacrylamide disc gel electrophoresis (Fig. 2).

Preparation of metal-free ICDH: For examination of the effect of metal ions on the enzyme, metal-free ICDH was prepared as follows: About 1.6 units of

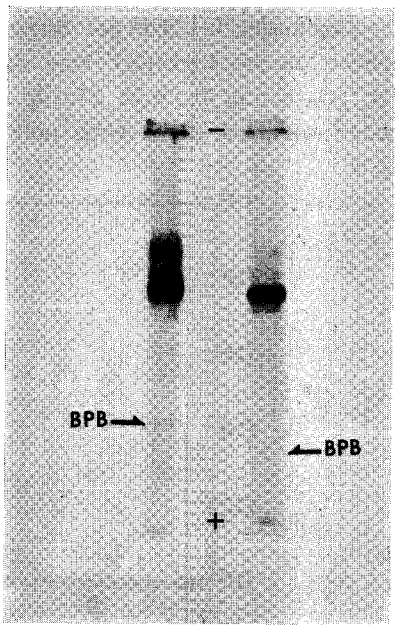

Fig. 2. Disc gel electrophoretic patterns of the purified ICDH's. Left, *P. vulgaris* ICDH, right, G-3 ICDH.

the purified enzyme in 0.4 m*l* of 20 mM Tris-HCl (pH 7.4) containing 10 mM EDTA was incubated for 30 min at 4°C. Then the metal-free enzyme was obtained by filtration through a Sephadex G-25 column (0.5×7 cm) which was thoroughly washed with 10 mM EDTA and equilibrated with 20 mM Tris-HCl (pH 7.4). The enzyme had no activity unless Mn^{2+} or Mg^{2+} was added.

Results

Cofactor requirement: Both enzymes were $NADP^+$-specific ones, and required Mn^{2+} for their full activities. Mn^{2+} could be partially replaced with Mg^{2+} and Co^{2+}, but Ba^{2+}, Ca^{2+}, and Cd^{2+} were rather inhibitory at the same concentration as Mn^{2+} (0.67 mM). Km value of G-3 ICDH for Mn^{2+} was 2.3×10^{-6} M at 20°C

Optimum pH: The pH-activity profiles of both the enzymes are shown in Fig. 3. pH optimum for the activity of G-3 ICDH was about 8.5 in Tris-HCl, but it was shifted to about 10 in glycine-NaOH. This result suggested that G-3 ICDH might be susceptible to some salts. In *P. vulgaris* ICDH, any difference of the activities was not observed between these buffers, and pH optimum for the activity showed rather wide range, between pH 7.4 and 8.4.

Effect of temperature on the enzyme stability: Stabilities of both the enzymes were examined at high (40°C) or low (4°C) temperature. One half

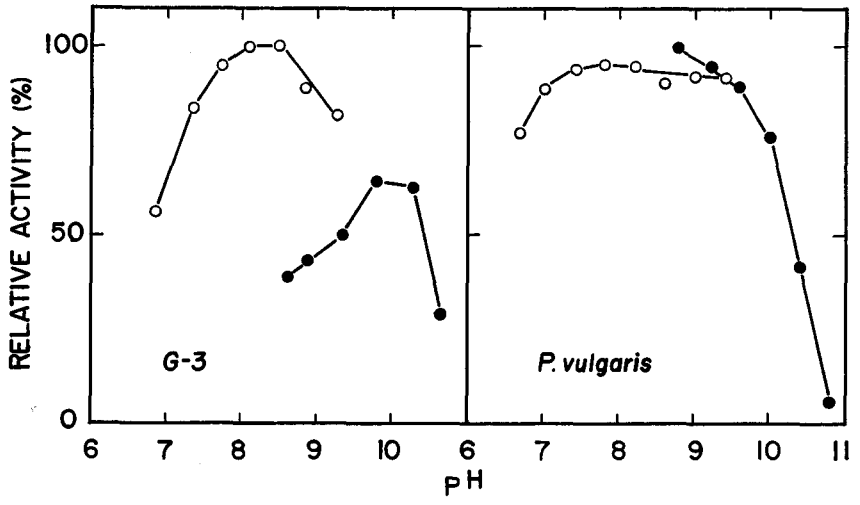

Fig. 3. pH-activity profiles of the ICDH's. The enzyme activities were determined in 33 mM Tris-HCl (○), or 50 mM glycine-NaOH (●). Other conditions were the same as the standard assay system.

of the original activity of G-3 ICDH was lost by incubation at 40°C for 20 min (Fig. 4), whereas no change was observed in *P. vulgaris* ICDH by the same treatment. Fig. 4 also shows the protective effect of isocitrate and/or Mn^{2+} on G-3 ICDH. In contrast with at 40°C, G-3 ICDH was quite stable at 4°C for 3 weeks, but 21.2% of the activity was lost in *P. vulgaris* ICDH after only one week.

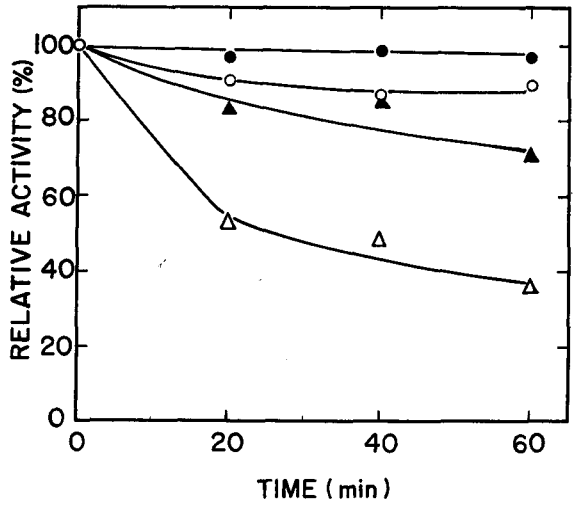

Fig. 4. Thermostabilitiy of the purified G-3 ICDH. All incubation mixtures contained 20 mM Tris-HCl (pH 7.4) and 0.4 units of the metal-free enzyme in a final volume of 1.0 m*l*. The concentrations of Mn^{2+} and isocitrate were 0.67 mM and 1 mM, respectively. Incubation was performed at 40°C in the presence of Mn^{2+} (○), isocitrate (▲), Mn^{2+} and isocitrate (●), and none (△).

Concerted inhibition by glyoxylate plus oxalacetate: Since SHIIO and OZAKI (1968) reported the concerted inhibition, isocitrate dehydrogenases from a variety of sources had been found to be inhibited as this manner. In the presence of 0.25 mM each of glyoxylate and oxalacetate both the enzymes were inhibited severely, but abrupt changes of the inhibition were observed at about 15°C and above in G-3 ICDH and at 37°C and above in *P. vulgaris* ICDH, and the gradual release from the inhibition was observed with elevating temperature (Fig. 5).

Effect of temperature on the reaction velocities: The reaction velocities of both the enzymes were maximum at 50°C. The activity of *P. vulgaris* ICDH at low temperature (2°C) was negligible, but in G-3 ICDH 12% of the activity at 50°C remained at 2°C. Arrhenius plots of both the enzyme

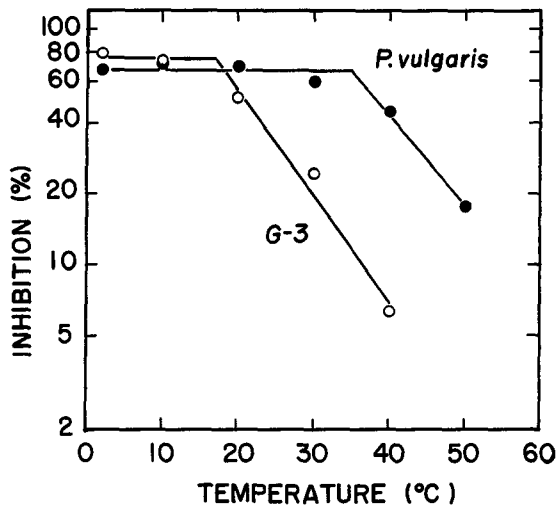

Fig. 5. Effect of temperature on the inhibition by glyoxylate plus oxalacetate. The activites were determined in the presence or absence of 0.25 mM each of glyoxylate and oxalacetate at various temperatures.

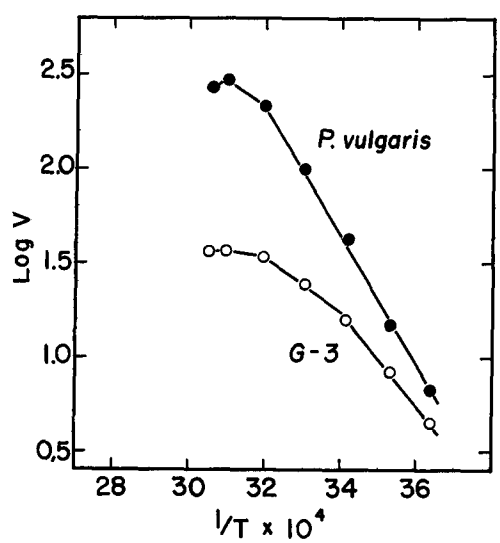

Fig. 6. Arrhenius plots of the activities of ICDH's. The activities were determined in the standard assay mixture at various temperatures.

activities are shown in Fig. 6. In *P. vulgaris* ICDH it showed straight line in the range of temperature from 2°C to 40°C, while a break was observed at 25°C in G-3 ICDH. The activation energies of G-3 ICDH were calculated to be 11.1 kcal per mole in the range from 2°C to 25°C, and 7.1 kcal in the range from 25°C to 40°C. In *P. vulgaris* ICDH it was 15.5 kcal.

Discussion

$NADP^+$-specific isocitrate dehydrogenase was purified from a psychrotrophic bacterium, *Flavobacterium* sp. strain G-3. This is the most purified enzyme among psychrophilic enzymes reported within our knowledge. It had been reported that psychrophilic enzymes were generally thermolabile (LANGRIDGE and MORITA, 1966; UPAHYAN and STOKES, 1963). This is the case in our purified enzyme, too. G-3 ICDH was labile at 40°C at which *P. vulgaris* ICDH was quite stable. At low temperature, however, G-3 ICDH was more stable than *P. vulgaris* ICDH. The inactivation of *P. vulgaris* ICDH at low temperature would not be due to the proteolytic effect by any contaminants in the enzyme preparation, since the stabilities of these enzymes at high temperature were reversed each other completely.

The biological significance of the concerted inhibition with glyoxylate plus oxalacetate was obscure, but then it was very interesting that the inhibition changed markedly by temperature. Since that glyoxylate plus oxalacetate inhibited ICDH competitively with isocitrate was confirmed in this study (data not shown) and by SHIIO and OZAKI (1968) and SAIKI and ARIMA (1975), some conformational changes of enzyme which was resulted in selective binding of substrate or decrease of binding with inhibitor might be induced at higher temperature. The break points of the inhibition-temperature relations in both the enzyme (Fig. 5) seemed to be reasonable temperature so as to occur conformational changes of proteins, because a little conformational change was occurred at 25°C in G-3 ICDH, while no change was observed in *P. vulgaris* ICDH up to 40°C from the Arrhenius plots (Fig. 6). Anyway isocitrate dehydrogenase from G-3 was more thermolabile than that from *P. vulgaris*. This thermolabile conformation of G-3 ICDH seemed to be closely connected with its rather high activity at low temperature.

From this concept more detailed studies on molecular structure and functions of the enzyme are in progress in our laboratory.

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