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Purification and Some Properties of Glycerol Dehydrogenase from *Erwinia aroideae*[1]

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Glycerol dehydrogenase was purified from *Erwinia aroideae* IFO 3830 by precipitation of acetone and ammonium sulfate, and chromatographies on diethylaminoethyl (DEAE)-cellulose, Sephadex G-200 and DEAE-Sephadex A-50. The purified enzyme was demonstrated to be homogeneous by disc electrophoresis. Optimum pH and temperature of this enzyme for glycerol oxidation was 10.5 and 50°, respectively. The glycerol dehydrogenase was stable over a pH between 6 and 9 at 5° for 15 hr, and showed more than 90% activity of the original under the conditions of pH 7.0 and 70° for 20 min. It was clarified that glycerol, glycerol-α-monochlorohydrin, 1,2-propanediol and 2,3-butanediol were good substrates for glycerol dehydrogenase from *Erwinia aroideae*. From the result of effect of sulfhydryl agents, it is suggested that this enzyme has a catalytic sulfhydryl group.

Keywords——glycerol dehydrogenase; *Erwinia aroideae*; purification; properties; NAD

Glycerol dehydrogenase [glycerol: NAD oxidoreductase, E.C. 1.1.1.6] which has the ability of catalyzing glycerol into dihydroxyacetone in the presence of NAD, has found in bacteria and animal tissues. In 1953, Burton and Kaplan[3] first reported that glycerol dehydrogenase was partially purified from *Aerobacter aerogenes* as a microbial enzyme. Afterwards, it has been described that a number of bacteria have produced this enzyme, including *Aerobacter aerogenes*,[4] *Eschericia coli*,[5] *Bacillus subtilis*,[6] *Streptococcus faecalis*.[7] Also, glycerol dehydrogenase activity was observed in rabbit tissues such as liver[8] and skeletal muscle[9]. However, the enzymic properties of the glycerol dehydrogenases reported hitherto were examined, employing not with the homogeneous preparations but with the partially purified ones or the tissue homogenates.

In the present paper, for the purpose of developing new bacteria which produce a lot of glycerol dehydrogenase and applying this enzyme to the determination of serum triglyceride, screening of it was carried out. Consequently, it was found out that bacteria which belong to *Erwinia aroideae* produced a lot of glycerol dehydrogenase, from which the enzyme was isolated and purified. In addition, its enzymic properties was investigated and compared with those of glycerol dehydrogenase from other sources reported.

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3) R.M. Burton and N.O. Kaplar, *J. Am. Chem. Soc.*, **75**, 1005 (1953).
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Materials and Methods

Materials——DEAE-cellulose was obtained from Brown Co. Sephadex G-200 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals. All other chemicals were of reagent grade quality.

Culture Condition and Preparation of Crude Extract with Glycerol Dehydrogenase Activity——Bacteria used were gifts from Institute for Fermentation Osaka. The culture medium was composed of 1% of meat extract, 1% of polypeptone, 0.5% of NaCl and 2% of glycerol, pH 7.5. After cultivating in 20 l of the above medium at 30—37° for 30—35 hr using a 30 l of fermentor, the cells obtained by centrifugation were suspended in 10 mM phosphate buffer (pH 7.0) and sonicated for 10 to 15 min with 250 watt sonic oscillator (Kaijo Denki Co.). The resulting supernatant was used as the crude extract with glycerol dehydrogenase activity.

Enzyme Assay——After a mixture consisting of 0.1 ml of 10 mM NAD, 0.1 ml of 1 M $(NH_4)_2SO_4$, 0.6 ml of 0.5 M carbonate buffer (pH 10.5), 1.7 ml of distilled water and 0.5 ml of the enzyme solution was added in a 10 mm light path 4 ml quartz cuvette, 0.3 ml of 1 M glycerol was added to it. The enzyme reaction was carried out at 25° for several min. One unit of glycerol dehydrogenase was defined as the amount which was able to produce 1 μmol of NADH per min under the above conditions.

Determination of Protein——Protein content was determined by measuring the absorbance at 280 nm with Hitachi spectrophotometer Model 323 or by the method of Lowry *et al.*[10] with bovine serum albumin as standard.

Disc Electrophoresis——In order to clarify the homogeneity of the purified enzyme, disc electrophoresis was performed using 7.5% polyacrylamide gel with 50 mM Tris-glycine buffer (pH 9.4) at 5 mA constant current per tube and 4° for 90 min according to the method of Davis.[11] The gels were stained for protein with Amido Black 10B.

Results and Discussion

Purification of Glycerol Dehydrogenase from *Erwinia aroideae*

Crude extract obtained from *Erwinia aroideae* was brought to 65% $(NH_4)_2SO_4$ saturation by addition of the solid salt, followed by centrifugation. The resulting precipitate was dissolved in a small volume of cold distilled water and centrifuged to remove insoluble materials. To the supernatant obtained above, 2 volumes of cold acetone was slowly added with stirring. Also, the precipitate obtained was dissolved in a minimum volume of cold water, followed by centrifuging it to take off insoluble substances. The enzyme solution obtained by the previous step was fractionated with solid ammonium sulfate and glycerol dehydrogenase activity was almost recoveried in the fraction of 40—50% $(NH_4)_2SO_4$ saturation. This fraction was dissolved in 10 mM phosphate buffer (pH 7.0), dialyzed against the same buffer for 24 hr and then applied onto a column (2.5×70 cm) of DEAE-cellulose, previously

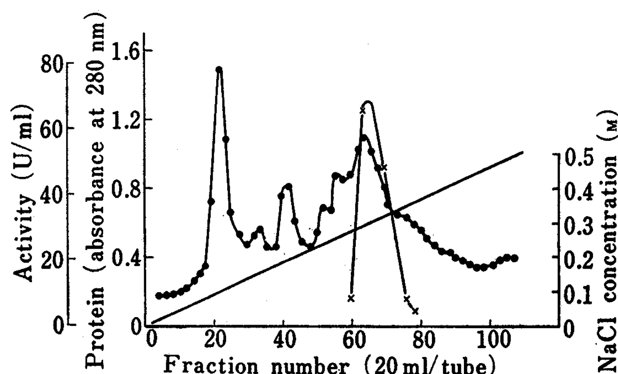

Fig. 1. Column Chromatogram of Glycerol Dehydrogenase on DEAE-cellulose

Flow rate: 80 ml/hr. For details see text. Activity: —×—; protein: —●—; concentration of NaCl:——.

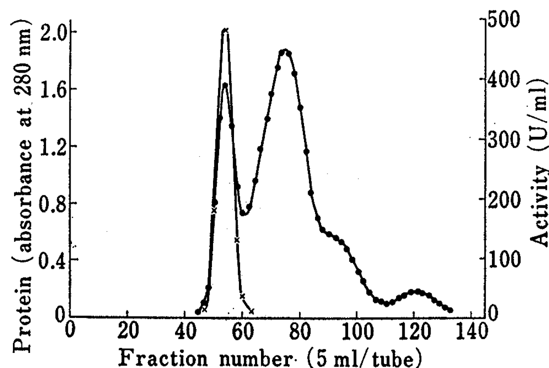

Fig. 2. Column Chromatogram of Glycerol Dehydrogenase on Sephadex G-200

Flow rate: 20 ml/hr. For details see text. Activity: —×—; protein: —●—.

10) O.H. Lowry, N.J. Rosenbrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).

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equilibrated with the buffer dissolved above. After washing with the same buffer, the adsorbed enzyme was eluted with a continuous NaCl gradient from 0 to 0.5 M as shown in Fig. 1. The high enzyme activity were pooled and precipitated with adding of $(NH_4)_2SO_4$ to give a final concentration of 60% saturation. The precipitate collected by centrifugation was dissolved in a minimum volume of 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl and applied to a column (2.6×100 cm) of Sephadex G-200. Equilibration of the column and elution of the enzyme was performed with the same buffer containing 0.1 M NaCl. The chromatogram is shown in Fig. 2.

Sephadex G-200 eluates having glycerol dehydrogenase activity were precipitated with the same manner described above using $(NH_4)_2SO_4$ and dialyzed against 10 mM phosphate buffer (pH 7.0) for 24 hr. The dialyzed solution was adsorbed on DEAE-Sephadex A-50 column (1.6×37 cm) equilibrated with 10 mM phosphate buffer (pH 7.0). After washing the column with the same buffer, the adsorbed enzyme was eluted with a linear gradient of NaCl (0—0.5 M) as shown in Fig. 3. The fractions showing the enzymic activity were collected and concentrated with membrane filter (Ulvac MC-2). The above purification procedures are summarized in Table I.

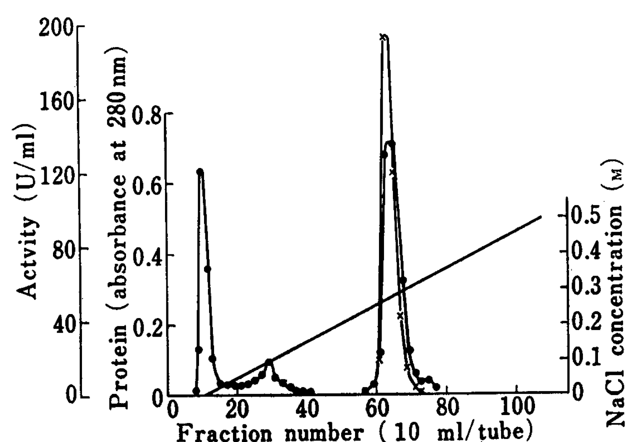

Fig. 3. Column Chromatogram of Glycerol Dehydrogenase on DEAE-Sephadex A-50

Flow rate: 40 ml/hr. For details see text. Activity: —×—; protein: —●—; concentration of NaCl: ——.

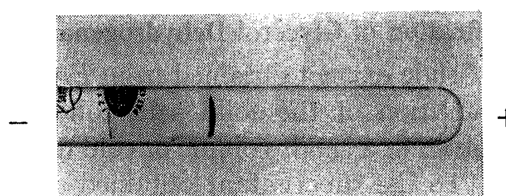

Fig. 4. Disc Electrophoresis of Purified Glycerol Dehydrogenase

For details see text.

Table I. Purification Procedures of Glycerol Dehydrogenase from *Erwinia aroideae*

Procedure	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield of activity (%)
Crude extract	85600	15600	0.182	100
Precipitation by acetone	67600	13700	0.203	87.5
Fractionation by ammonium sulfate (40—50% saturation)	3560	9530	2.68	60.9
DEAE-cellulose elute	67.9	9060	133	57.9
Sephadex G-200 elute	47.8	6800	142	43.5
DEAE-Sephadex A-50 elute	33.1	5230	158	33.4

Glycerol dehydrogenase was purified approx. 1000-fold from crude extract with a recovery activity of 33%. The purified enzyme has 158 units/mg of protein of specific activity. This value seems to be higher than those of the enzymes from other sources, including *Aerobacter aerogenes* (35 units/mg) reported by McGregor *et al.*[4a)] As shown in Fig. 4, the purified glycerol dehydrogenase showed a single protein band on a polyacrylamide gel, indicating that it was homogeneous.

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Effect of pH on Activity and Stability

The optimum pH for oxidation of glycerol was investigated. As shown in Fig. 5, the optimum pH was about 10.5, and more than 80% of the maximum activity was observed over a pH range of 10.0 to 12.0. This was similar to those of the enzymes from *Eschericia coli*[5] and *Aspergillus niger*.[12]

For the purpose of examing pH stability, the enzyme was incubated with buffers having various pH values at 5° for 15 hr and found to be stable in a pH range of 6.0 to 9.0, but inactivated below pH 4.5 and above pH 11.0.

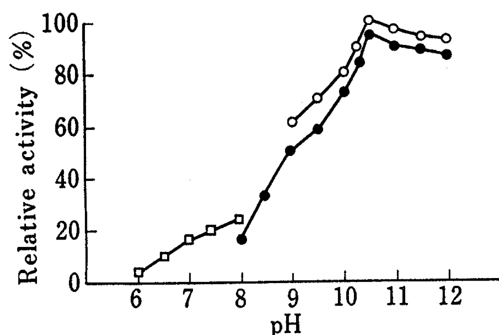

Fig. 5. Effect of pH on the Activity of Glycerol Dehydrogenase

Buffer solutions used for the study were 0.1 M phosphate (□), 0.1 M glycine-NaOH (●) and 0.1 M carbonate (○).

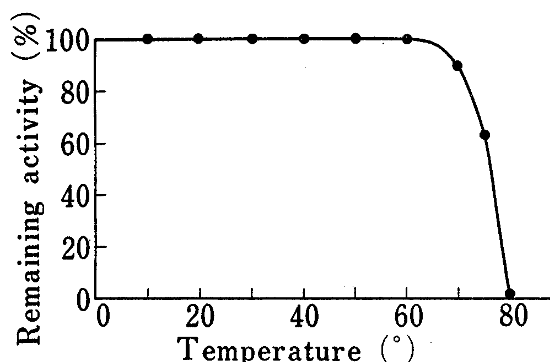

Fig. 6. Effect of Temperature on the Stability of Glycerol Dehydrogenase

For details see text.

Effect of Temperature on Activity and Stability

The optimum temperature for glycerol dehydrogenase was examined and the highest was observed at 50°. Karmann *et al.*[9] previously reported that glycerol dehydrogenase from rabbit skeletal muscle was rapidly inactivated with the treatment of 55° or 60°. However, as shown in Fig. 6, our enzyme retained more than 90% of the original activity under the condition of 70° and pH 7.0 for 20 min, which was similar to the result obtained from that of *Eschericia coli*.[5]

Substrate Specificity

Oxidation of various substrates by glycerol dehydrogenase was examined in 0.1 M carbonate buffer (pH 10.5). The result is presented in Table II. It was observed that the enzyme

Table II. Substrate Specificity of Glycerol Dehydrogenase

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Glycerol	100	D-Sorbitol	0
Glycerol α-monochlorohydrin	136	1,3-Propanediol	59
Isopropyl alcohol	0	1,3-Dichloro-2-propanol	0
Ethylene glycol	8	D-Ribose	0
β-Glycerophosphate	0	D-Glucose	0
meso-Inositol	0	Ethanol	0
1,4-Butanediol	0	*n*-Propyl alcohol	0
1,2-Propanediol	271	Ascorbic acid	0
2,3-Butanediol	102		

The concentration of each substrate was 30 mM in the assay system.
Assay was done in the standard conditions.

12) B.S. Baliga, G.M. Bhatnagar, and V. Jagannathan, *Biochim. Biophys. Acta*, **58**, 384 (1962).

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showed the high activity on some polyhydric alcohol containing 3 or 4 carbon atoms, but no activity on the other polyols having 2,5 and 6 carbon atoms, and that 1,2-propanediol was the best substrate for this enzyme. This finding was agree with those of the enzymes from *Aerobacter aerogenes*[4a)] and rabbit skeletal muscle.[9)]

Effect of Various Metal Salts and Reagents on the Activity

Oxidation of glycerol was measured in the presence of metal salts. The enzymic activity was strongly inhibited by $CuSO_4$ and $ZnSO_4$, and partial inhibition was observed with $HgCl_2$ as shown in Table III.

TABLE III. Effect of Various Compounds on Glycerol Dehydrogenase

Compound	Concentration (mM)	Remaining activity (%)
None		100
$FeSO_4$	0.2	133
$MgCl_2$	0.2	100
$CuSO_4$	0.2	0
$ZnSO_4$	0.2	4
$SnCl_2$	0.2	96
$HgCl_2$	0.2	19
$MnCl_2$	0.2	104
LiCl	0.2	63
$CoCl_2$	0.2	71
$CdCl_2$	0.2	80
$Ca(CH_3COO)_2$	0.2	107
EDTA	10	101
Ascorbate	10	101
Cysteine	10	95
Iodoacetic acid	100	86
	10	98
	0.1	84
p-Chloromercuribenzoate	0.01	100

It was presented that glycerol dehydrogenase from other bacteria,[4d,5)] and animal tissues[9)] were inactivated by sulfhydryl agents such as *p*-chloromercuribenzoate. Therefore, it was investigated whether glycerol dehydrogenase from *Erwinia aroideae* was inhibited or not by them. It was clarified that this enzyme was inactivated by them, but the inhibited enzyme was reversed to some degree by the addition of cysteine. From these results, this glycerol dehydrogenase is seemed to be the enzyme having a catalytic SH group.

Michaelis Constant

The effect of different concentration of substrate and the cofactor on the velocity of enzyme was studied and the K_m values were calculated graphically according to the

TABLE IV. Michaelis Constants for Various Substrates

Substrate	K_m (M)
Glycerol	1.2×10^{-2}
Glycerol α-monochlorohydrin	9.6×10^{-3}
1,2-Propanediol	7.2×10^{-4}
2,3-Butanediol	1.2×10^{-2}
1,3-Propanediol	2.7×10^{-2}
NAD	2.8×10^{-4}

13) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

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line weaver-burk method.[13] These results are shown in Table IV. In the standard assay system, the apparent K_m values for glycerol and 1,2-propanediol were 1.2×10^{-2} M and 7.2×10^{-4} M, respectively. These values are lower than those observed in glycerol dehydrogenase from *Aerobacter aerogenes* (3.9×10^{-2} M and 5.1×10^{-3} M).[4a]

13) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, **658** (1934).

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