Glycerol Dehydrogenase and Dihydroxyacetone Reductase of a Methyilotrophic Yeast, *Hansenula ofunaensis*

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NAD⁺-Linked glycerol dehydrogenase (GDH) activity in *Hansenula ofunaensis* grown on glycerol was separated into three enzymes by a procedure involving DEAE-cellulose and DEAE-Sephadex A-50 column chromatographies. The oxidative activities toward glycerol of GDH I-1 and GDH I-2 were only detected when the concentration of glycerol was high, while GDH II showed significant activity with a low concentration of glycerol. The *K_m* values for glycerol of GDH I-1, GDH I-2 and GDH II were 0.56 M, 2.9 M and 0.16 M, respectively. The *K_m* values for dihydroxyacetone of GDH I-1, GDH I-2 and GDH II were 0.40 M, 0.36 M and 1.7 mM, respectively. The oxidative activities toward 1,2-propanediol of GDH I-1 and GDH II were 0.37- and 1.5-fold those toward glycerol, respectively. GDH I-2 showed significant oxidative activity toward ethanol, while GDH I-1 and GDH II showed no such activity. GDH I-2 was identical to dihydroxyacetone reductase induced in methanol-grown cells.

Isozymes of glycerol dehydrogenase (GDH) have been demonstrated in *Geotrichum candidum*¹⁾ and *Klebsiella pneumoniae*.²⁾ It was also found that *Schizosaccharomyces pombe* produced four pyridine nucleotide-linked GDHs (or triose reductases).³⁾ *S. pombe*^{4,5)} and *Candida valida*⁶⁾ are known to utilize glycerol through NAD⁺-linked GDH. GDH of *K. pneumoniae* has been used for anaerobic glycerol dissimilation.⁷⁾

Previously, we classified methyilotrophic yeasts into three groups with regard to the primary enzyme involved in glycerol dissimilation.^{8,9)} *Hansenula ofunaensis* has the oxidative pathway involving GDH, but not the phosphorylative pathway involving glycerol kinase. The activity to oxidize glycerol to dihydroxyacetone was induced only by glycerol, i.e., not by methanol. On the other hand, an enzyme which preferably reduced dihydroxyacetone to glycerol was detected in the methanol medium and designated as dihydroxyacetone reductase.^{10,11)} Dihydroxyacetone is an intermediate in the methanol metabolism of methyilotrophic yeasts.¹²⁾ Thus,

GDH could be a key enzyme between the metabolic pathways for methanol and glycerol. In the present paper, a comparative study of GDHs in *H. ofunaensis* grown on glycerol will be reported.

MATERIALS AND METHODS

Cultivation and enzyme assay. *H. ofunaensis* was cultivated in the glycerol medium and harvested as described previously.¹⁰⁾ Enzyme activity and protein determinations through out the enzyme preparation were carried out under the same conditions as given previously.¹⁰⁾ The conductivity of the eluate on column chromatography was determined with a CD-35MII conductivity meter (M&S Instruments) at 10°C. For measuring the *K_m* values for dihydroxyacetone and NADH, an assay system containing 100 mM potassium phosphate buffer (KPB), pH 6.0, 0.5 mM dihydroxyacetone for GDH I-1 and GDH I-2 or 1.3 mM dihydroxyacetone for GDH II, 0.1 mM NADH for GDH I-1 or 0.2 mM NADH for GDH I-2 and GDH II, and the enzyme, in a total volume of 0.6 ml, was used, the concentrations of dihydroxyacetone and NADH being varied. To measure the *K_m* values for glycerol and NAD⁺, an assay system containing 100 mM NH₄Cl-NH₄OH, pH 9.0, for GDH I-1 or 100 mM KPB, pH 8.0, for GDH I-2 and GDH II, 2 mM NAD⁺, 0.5 M glycerol and the enzyme, in a total volume of 0.6 ml, was used, the concentrations of

glycerol and NAD^+ being varied.

RESULTS

Separation of GDH in glycerol-grown cells

All operations were performed at $0 \sim 10^\circ\text{C}$, and KPB, pH 7.0 (buffer A), and Tris-HCl buffer, pH 7.0 (buffer B) and pH 7.7 (buffer C),

containing 1 mM dithiothreitol, were used.

Glycerol-grown cells from 5-l of culture broth were suspended in about 630 ml of 0.01 M buffer A. The suspended cells were disrupted and the supernatant solution was obtained as described previously.⁸⁾

The cell-free extract was then applied onto a DEAE-cellulose column (5.0×20 cm) equili-

FIG. 1. Separation of GDH on DEAE-Cellulose Column Chromatography.

Elution was performed as described in text at flow rates of 130 ml/hr (A) and 180 ml/hr (B). Eluates were collected in 10-ml fractions.

FIG. 2. Separation of GDH on DEAE-Sephadex A-50 Column Chromatography.

Elution was performed as described in text at flow rates of 120 ml/hr (A), 91 ml/hr (B) and 100 ml/hr (C). Eluates were collected in 5-ml fractions.

brated with 0.01 M buffer A. The enzymes were eluted with a concentration gradient of buffer A (0.03~0.07 M) after washing with 0.03 M buffer A (Fig. 1A), and then with one of from 0.1 M buffer A containing 0.1 M KCl to 0.1 M buffer A containing 0.2 M KCl (Fig. 1B) after washing with 0.1 M buffer A containing 0.1 M KCl. As shown in Fig. 1A, GDH activity was separated into two fractions, which were designated as GDH I-1 and GDH I-2, in order of elution. In Fig. 1B, one peak of GDH activities of GDH I-1, GDH I-2 and GDH II. The conductivities at the peaks of the activities of GDH-I-1, GDH I-2 and GDH II were 9.9 mmho, 13 mmho and 36 mmho, respectively. Each fraction was concentrated by ultrafiltration with a collodion bag.

The fractions containing GDH I-1 and GDH I-2 obtained on DEAE-cellulose chromatography were applied onto a DEAE-Sephadex A-50 column (1.8 × 20 cm), respec-

tively, which had been equilibrated with 0.01 M buffer B. After washing with 0.1 M buffer B, GDH I-1 was eluted with a gradient system

TABLE I. PURIFICATION OF GDH

An assay system containing 100 mM KPB (pH 6.0), 0.17 mM NADH, 0.1 M dihydroxyacetone and each enzyme fraction was used for measuring NADH-dependent dihydroxyacetone reduction.

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Fold
Cell-free extract	1,300	3,500	0.37	100	1
DEAE-Cellulose					
GDH I-1	32	22	1.5	2.5	4.1
GDH I-2	30	45	0.67	2.3	1.8
GDH II	620	79	7.8	48	21
DEAE-Sephadex A-50					
GDH I-1	9.4	2.2	4.3	0.72	12
GDH I-2	12	14	0.86	0.90	2.3
GDH II	180	5.7	32	14	86

FIG. 3. Effect of pH on GDH Activity.

For measuring the oxidation of glycerol, the assay mixture contained 100 mM buffer, 1 mM NAD⁺, 0.5 M glycerol and the enzyme. The reduction was measured with an assay mixture containing 100 mM buffer, 0.1 mM NADH for GDH I-1 and GDH II or 0.17 mM NADH for GDH I-2, 0.5 M dihydroxyacetone for GDH I-1 and GDH I-2 or 0.0013 M dihydroxyacetone for GDH II, and the enzyme. NH₄Cl-NH₄OH (pH 8~11) (□); K₂HPO₄-KH₂PO₄ (pH 6~8) (△); Tris·HCl (pH 7.2~9.0) (●); and Tris-maleate (pH 4.7~9.4) (▲) were used as buffers.

from 0 to 0.1 M KCl in 0.1 M buffer B, and GDH I-2 with one from 0 to 0.16 M KCl in 0.1 M buffer B, respectively (Figs. 2A and 2B). GDH II was applied onto a DEAE-Sephadex A-50 column (1.8 × 20 cm) equilibrated with 0.01 M buffer C. After washing with 0.1 M buffer C containing 0.2 M KCl, GDH II was eluted with a gradient from 0.2 M to 0.8 M KCl in 0.1 M buffer C (Fig. 2C). The peaks of the activities of GDH I-1, GDH I-2 and GDH II were eluted at conductivities of 11.6 mmho, 16 mmho and 35 mmho, respectively, under the conditions employed. The two peaks in Fig. 2B consist of GDH I-2, the main component, and GDH I-1, presented the minor component. A summary of the purification procedure is presented in Table I.

After DEAE-Sephadex A-50 column chromatography, GDH I-1, GDH I-2 and GDH II were found to have been purified 12-fold, 2.3-fold and 86-fold, compared with the cell-free extract, respectively, and were used for the following experiments.

Characterization of GDH I-1, GDH I-2 and GDH II

1) *Effect of pH on GDH activity.* Variation in the GDH activity was investigated between pH 5.2 and 11 (Fig. 3). The maximum activity

of GDH I-1 in the forward reaction was observed at pH 8.0~9.0, while that in the reverse reaction was observed at pH 6.0~7.0 (Fig. 3A). The type of buffer used had little effect on the activities.

The optimum pH of GDH I-2 for the oxidation of glycerol was 8.0 and that for the reduction of dihydroxyacetone was 6.0, as shown in Fig. 3B. GDH I-2 activity was inhibited by Tris·HCl buffer.

The oxidation of glycerol to dihydroxyacetone by GDH II was highest at pH 9.0, whereas the reduction of dihydroxyacetone was highest at pH 6.0 (Fig. 3C). Tris·HCl and Tris-maleate buffers inhibited both the oxidation of glycerol and the reduction of dihydroxyacetone by GDH II.

2) *Effect of temperature on GDH activity.* The activity was measured at various temperatures between 5°C and 65°C at pH 6.0 (Fig. 4). The optimum temperature of GDH I-1 was 35°C (Fig. 4A). GDH I-1 was incubated at various temperatures for 20 min and then its residual activity was determined. GDH I-1 did not retain any activity at 45°C (Fig. 5A).

The reaction rate of GDH I-2 was maximum at 55°C (Fig. 4B). The residual activity of GDH I-2 was 0% after incubation at over 55°C and 90% at 45°C (Fig. 5B). The activity

FIG. 4. Temperature Dependency.

An assay system containing 100 mM KPB, pH 6.0, 0.1 mM NADH, 0.5 M dihydroxyacetone for GDH I-1 and GDH I-2 or 0.013 M dihydroxyacetone for GDH II, and the enzyme was used at various temperatures.

FIG. 5. Thermal Stability.

The enzyme activity was determined after incubation at each temperature for 20 min. An assay system containing 100 mM KPb (pH 6.0), 0.1 mM NADH for GDH I-1 or 0.17 mM NADH for GDH I-2, 0.5 M dihydroxyacetone for GDH I-1 or 0.1 M dihydroxyacetone for GDH I-2, and the enzyme was used. An assay system containing 100 mM KPb (pH 8.0), 1 mM NAD⁺, 0.5 M glycerol and the enzyme was used for GDH II.

TABLE II. SUBSTRATE SPECIFICITIES OF GDH I-1, GDH I-2 AND GDH II IN THE FORWARD AND REVERSE REACTIONS

For measuring the oxidation of various substrates, an assay system containing 100 mM KPb (pH 8.0), 1 mM NAD⁺, 0.5 M substrate and the enzyme was used for GDH I-1, GDH I-2 and GDH II. No significant activity toward 0.5 M ethylene glycol, 0.5 M lactic acid and 0.17 M glycerol 3-phosphate was observed. For measuring the reduction of various substrates, an assay system containing 100 mM KPb (pH 6.0), 0.1 mM NADH, 0.13 M substrate for GDH I-1 and GDH I-2 or 0.013 M substrate for GDH II, and the enzyme was used. These GDHs did not act on DL-glyceraldehyde.

	Relative activity (%)		
	GDH I-1	GDH I-2	GDH II
Oxidation			
Glycerol	100	<1	100
1,2-Propanediol	37	100	150
Ethanol	0	20	0
Reduction			
Dihydroxyacetone	100	100	100
Methylglyoxal	0	180	48
Acetol	0	230	33

of GDH I-2 was more stable at 15 to 40°C than at 2.5 to 5°C.

As shown in Fig. 4C, GDH II showed the highest activity at 35°C. The residual activity of GDH II was 13% at 45°C (Fig. 5C).

3) *Substrate specificity.* GDH I-1 was found to exhibit a rather narrow substrate specificity (Table II). 1,2-Propanediol was dehydrogenated at 37% the rate for glycerol by GDH I-1. Other substrates tested were not oxidized by GDH I-1. GDH I-1 did not reduce acetol, DL-glyceraldehyde or methylglyoxal.

GDH I-2 showed a broad substrate specificity. Not only glycerol and 1,2-propanediol but also ethanol were active substrates for GDH I-2. When the activity toward 1,2-propanediol was taken as 100%, those toward glycerol and ethanol were under 1% and about 20%, respectively. GDH I-2 could reduce methylglyoxal and acetol more rapidly than dihydroxyacetone. DL-Glyceraldehyde was inert as a substrate.

In the elution patterns of GDH II on DEAE-cellulose and Sepharose 6B column chromatographies, the peak corresponding to the dehydrogenase activity toward glycerol overlapped with that corresponding the activity toward 1,2-propanediol. The activity of GDH II toward 1,2-propanediol was 1.5-fold that toward glycerol. GDH II did not oxidize other compounds tested than glycerol and 1,2-propanediol, as in the case of GDH I-1. Methylglyoxal and acetol were reduced by GDH II at 48% and 33% the rate for dihy-

FIG. 6. Relationship between the Initial Reaction Velocities of GDH I-1 (A) and GDH II (B) and the Glycerol Concentration.

The enzyme activity was determined as described under MATERIALS AND METHODS.

FIG. 7. Relationship between the Initial Reaction Velocities of GDH I-1 (A) and GDH II (B) and the NAD^+ Concentration.

The enzyme activity was determined as described under MATERIALS AND METHODS.

droxyacetone, respectively.

4) *K_m values.* The *K_m* values of GDH I-1 for glycerol and NAD^+ were 0.56 M and 0.17 mM, respectively, at pH 9.0 (Figs. 6A and 7A). The *K_m* values of GDH I-1 for dihydroxyacetone

and NADH were 0.40 M and 0.025 mM, respectively, at pH 6.0 (Figs. 8A and 9A).

The *K_m* values of GDH I-2 for glycerol and NAD^+ were 2.9 M and 0.029 mM. As shown in Figs. 8B and 9B, the *K_m* values of GDH I-2 for

FIG. 8. Relationship between the Initial Reaction Velocities of GDH I-1 (A), GDH I-2 (B) and GDH II (C) and the Dihydroxyacetone Concentration.

The enzyme activity was determined as described under MATERIALS AND METHODS.

FIG. 9. Relationship between the Initial Reaction Velocities of GDH I-1 (A), GDH I-2 (B) and GDH II (C) and the NADH Concentration.

The enzyme activity was determined as described under MATERIALS AND METHODS.

FIG. 10. Effect of the Concentration on the Reduction of Dihydroxyacetone by GDH II.

The enzyme activity was determined with an assay system containing 100 mM KPB (pH 6.0), 0.17 mM NADH, dihydroxyacetone at various concentrations and the enzyme.

dihydroxyacetone and NADH were 0.36 M and 0.033 mM, respectively.

The apparent K_m values of GDH II for glycerol and NAD^+ were 0.16 M and 0.28 mM, respectively (Figs. 6B and 7B). The apparent K_m values for dihydroxyacetone and NADH were 1.7 mM and 0.063 mM, respectively, at pH 6.0 (Figs. 8C and 9C). Dihydroxyacetone caused inhibition at a concentration of more than 10 mM (Fig. 10). When another system containing 100 mM NH_4Cl – NH_4OH , pH 9.0, 1 mM NAD^+ and 0.5 M glycerol, and the enzyme, was used, and the concentrations of glycerol and NAD^+ were varied, the K_m values for glycerol and NAD^+ were 83 mM and 0.034 mM, respectively. The activity under the latter conditions was unstable.

DISCUSSION

H. ofunaensis grown on glycerol was found to produce three types of GDH. These three enzymes were characterized in this study.

GDH I-2 and GDH II were inhibited by Tris. Such inhibition has been shown for 1,2-propanediol dehydrogenase from cells of a methanol-utilizing bacterium, *Micrococcus eburneus*, grown on 1,2-propanediol,¹³⁾ and GDH of *C. valida*.¹⁴⁾ GDH I-2 showed the highest activity after treatment at 35°C (Fig. 5). It was reported that a rat liver acetyl-CoA hydrolase shows the extreme cold lability, i.e., the enzymatically active dimeric and tetrameric forms dissociate into the inactive monomer at low temperature, and the activity is recovered on treatment at 37°C.¹⁵⁾ GDH I-2 could be considered to have such cold lability, although activation at 35°C is also possible.

The activities of GDH I-1, GDH I-2 and GDH II in the forward and reverse reactions with other substrates than glycerol and dihydroxyacetone were different. The activity against each substrate in Table II shows the same elution pattern in DEAE-cellulose column chromatography. GDH I-1 and GDH II did not oxidize ethanol. The enzymes of *G. candidum* are also specific for 2-hydroxy compounds.¹⁾ GDHs of *K. pneumoniae*, *Mucor*

*javanicus*¹⁶⁾ and *Cellulomonas* sp. NT 3060¹⁷⁾ showed detectable activities toward ethylene glycol. Glycerol 3-phosphate was a substrate for GDH of *Cellulomonas* sp. NT 3060.¹⁷⁾ Our enzymes did not oxidize ethylene glycol or glycerol 3-phosphate. Acetol was reduced by GDH I-2 and GDH II 230% and 33% the rate for dihydroxyacetone, respectively. GDH I-1 did not reduce acetol. Acetol was active as dihydroxyacetone in an *Escherichia coli* mutant.¹⁸⁾ Acetol is an oxidized product of 1,2-propanediol. GDH I-2 and GDH II oxidized 1,2-propanediol more rapidly than glycerol. The oxidation rate for 1,2-propanediol of GDH I-1 was 37% that for glycerol. *G. candidum* utilized 1,2-propanediol,¹⁹⁾ and its GDH activity was induced by 1,2-propanediol. *H. ofunaensis* produced GDH II on the addition of 1,2-propanediol to the methanol medium, in spite of there being no growth on 1,2-propanediol as a carbon source.

Each enzyme showed different K_m values for glycerol and dihydroxyacetone, and these K_m values were high. *C. valida*⁶⁾ and *S. pombe*,^{4,5)} are known to utilize glycerol through GDH. The K_m values for glycerol were 58 mM for *C. valida*¹⁴⁾ and 0.5 mM for *S. pombe*.²⁰⁾ The K_m values for dihydroxyacetone were 0.77 mM for *C. valida*¹⁴⁾ and 0.06 mM for *S. pombe*.²⁰⁾ The K_m values of GDH for glycerol and dihydroxyacetone were 3.7 mM and 1.2 mM, while those of another GDH, of *G. candidum* were 3.4 mM and 1.0 mM, respectively.¹⁾ The K_m values for glycerol of GDH from *K. pneumoniae* were 1.7×10^{-2} M and 5.6×10^{-3} M in the presence of 3.3 mM and 33 mM NH_4Cl , respectively.²¹⁾ The K_m values for glycerol of GDH II were 1.6×10^{-1} M and 8.3×10^{-2} , in the absence and presence of 100 mM NH_4^+ , respectively. The K_m value for dihydroxyacetone of GDH of *K. pneumoniae* was 0.13 mM. The reduction of dihydroxyacetone was inhibited by the substrate at concentrations of above 0.4 mM.²²⁾ The reduction by GDH II of *H. ofunaensis* was inhibited by dihydroxyacetone at a concentration of more than 10 mM, while the K_m value for dihy-

droxyacetone of GDH II was 1.7 mM.

Gartner and Kopperschlager determined that the low affinity for glycerol of GDH of *C. valida* prevented the excessive formation of dihydroxyacetone phosphate which could lead to the toxic production of methylglyoxal.¹⁴⁾ *K. pneumoniae* had two types of GDH, one of which may be responsible for anaerobic glycerol dissimilation. The physiological function of the other minor GDH is unknown. The oxidative pathway of glycerol dissimilation in *K. pneumoniae* was induced irrespective of the glycerol concentration in the growth medium under anaerobic conditions. Under aerobic conditions the oxidative pathway was induced when glycerol was abundant, and the phosphorylative pathway was induced when glycerol was limited. The *K_m* for glycerol was about 10^{-3} mM for glycerol kinase and 20 mM for GDH. Huetting *et al.* speculated that the organism possesses this type of regulatory mechanism in order to prevent traumatic effects due to unrestricted uptake of glycerol and intracellular accumulation of phosphorylated derivatives.²³⁾ Methylglyoxal was also found in methylotrophic yeasts.²⁴⁾ *H. ofunaensis* grown on glycerol of methanol showed high dihydroxyacetone kinase activity. *H. ofunaensis* might dissimilate glycerol and control the level of a toxic compound, by using these GDHs which have various affinities for glycerol and dihydroxyacetone.

H. ofunaensis produced three types of GDH when grown on glycerol. Dihydroxyacetone reductase from methanol-grown cells gave the same experimental data as GDH I-2 from glycerol-grown cells.¹⁰⁾ The *K_m* values for glycerol of dihydroxyacetone reductase from *H. ofunaensis*¹⁰⁾ and *Dunaliella parva*²⁵⁾ were 2.9 M and 1.4 M, respectively. Dihydroxyacetone reductase of *Dunaliella parva* is used for glycerol production.²⁶⁾ The role of this enzyme in methylotrophic yeasts will be elucidated in a further study.

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