Enzymatic Production of D-Glycerate from L-Tartrate

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Received February 13, 1989

D-Glyceric acid is a useful chiral synthon in synthetic organic chemistry. To produce D-glycerate cheaply, microorganisms that convert L-tartrate into D-glycerate with good yields and selectivity were isolated from soil samples. One microorganism obtained was identified as a strain of *Pseudomonas* sp. group Ve-2 and found to produce a new enzyme, L-tartrate decarboxylase. This enzyme catalyzes the direct conversion of L-tartrate into D-glycerate, with almost 100 % selectivity. In the presence of a cell-extract of *Pseudomonas* sp. group Ve-2, the amount of D-glycerate produced from L-tartrate reached 53 g/l under the best conditions examined, with a molar yield of almost 100 % and an optical purity of more than 92 % e.e.

D-Glyceric acid is a useful chiral synthon in synthetic organic chemistry.^{1,2)} The best known method for the preparation of optically pure D-glyceric acid is the oxidation of α -methyl-D-glucopyranoside, followed by hydrolysis.³⁾ However, it is rather complicated. D-Glyceric acid is alternatively obtained by optical resolution of its racemic form with penicillia or aspergilli.⁴⁾

D-Glycerate is also synthesized in many microorganisms. In *Pseudomonas putida*,^{5,6)} and *Rhodopseudomonas sphaeroides*,⁷⁾ D-glycerate is formed from L-tartrate by a coupled enzymatic system in the presence of catalytic amounts of NAD⁺ and Mn²⁺. The first enzyme in this system, L-tartrate dehydrogenase (EC 1.1.1.93), catalyzes the NAD⁺-linked oxidation of L-tartrate to oxaloglycolate.⁸⁾ The formation of D-glycerate from oxaloglycolate and the oxidation of NADH are mediated by the second enzyme, oxaloglycolate reductive decarboxylase (EC 1.1.1.92).⁹⁾ D-Glycerate is also produced from tartronic

semialdehyde in *P. ovalis*,¹⁰⁾ and from hydroxypyruvate in *Pseudomonas* sp.¹¹⁾ in the presence of equivalent amounts of NADH. However, D-glycerate production with microbial enzymes has not been developed industrially.

L-Tartrate is a cheap by-product of the wine industry and, therefore, is expected to be a good starting material for D-glycerate production. To produce D-glycerate cheaply, we have screened microorganisms that convert L-tartrate into D-glycerate with good yields and selectivity. One microorganism obtained was found to produce a new enzyme, L-tartrate decarboxylase, which catalyzes the direct conversion of L-tartrate into D-glycerate, with almost 100% selectivity.¹²⁾ It seems reasonable to produce D-glycerate from L-tartrate using this enzyme. In this paper, we describe the isolation of a microorganism that produces L-tartrate decarboxylase, the formation of the enzyme and the simple enzymatic production of D-glycerate from L-tartrate, with emphasis

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on the reaction conditions.

Materials and Methods

Materials. NAD⁺ and NADH were purchased from Kohjin Co., and glyoxylate reductase (EC 1.1.1.26, from spinach leaves) and diaphorase (EC 1.8.1.4, NADH:dye oxidoreductase, from *Clostridium kluyveri*) from Sigma Chemical Co. Other chemicals used were the best available commercial products.

Screening of glycerate-producing microorganisms. L-Tartrate-assimilating microorganisms were obtained by using a medium containing 1.0% potassium L-tartrate, 0.3% (NH₄)₂SO₄, 0.3% K₂HPO₄, 0.01% MgSO₄·7H₂O and 0.02% yeast extract. The pH was adjusted to 7.5 with 3N HCl. This medium was used as the basal medium. A small amount of a soil sample was inoculated in 5 ml of the basal medium. After aerobic cultivation at 25°C until growth was detected visually, the microorganisms were isolated on agar plates of the basal medium. The isolated bacteria were maintained on the basal medium supplemented with 2% agar.

Cells of a microorganism obtained were inoculated into 200 ml of the basal medium in a 500 ml-flask. After aerobic cultivation at 25°C for 12 hr, the cells were harvested by centrifugation and then washed twice with 0.85% NaCl. The washed cells, about 1 g (wet weight), were suspended in 1 ml of 0.1M Tris-HCl buffer (pH 7.5) and then subjected to sonication at 4°C for 2 min with a Seiko Instruments model 7040 ultrasonic disintegrator. After centrifugation (40,000 × *g* for 20 min at 4°C), the supernatant was used for glycerate production and the assay of L-tartrate dehydrogenase.

Standard conditions for D-glycerate production. The standard reaction mixture for the production of glycerate contained 100 μmol of Tris-HCl buffer (pH 7.5), 1 μmol of MgCl₂, 1 μmol of NAD⁺, 50 μmol of potassium L-tartrate and an appropriate amount of the cell-extract, in a final volume of 1.0 ml. After incubation at 30°C for 1 hr, 0.2 ml of 3N HCl was added. Tartrate and glycerate were determined by liquid chromatography using a TOSOH HLC-803D with an ULTRON PS-80H column (0.8 × 30 cm; Shinwa Kako Co.). Elution was carried out with 0.126% HClO₄ at the flow rate of 0.8 ml/min. The elution times of tartrate and glycerate were 9.2 min and 11.1 min, respectively.

Analytical methods. The experimental conditions for the assay of L-tartrate decarboxylase were identical to those for glycerate production. One unit of the enzyme was defined as the amount of enzyme that catalyzed the appearance of 1 μmol of D-glycerate per min.

L-Tartrate dehydrogenase activity was assayed with a Shimadzu UV-3000 spectrophotometer, with the cell com-

partment thermostatically maintained at 30°C. The standard assay mixture for the measurement of L-tartrate dehydrogenase was the same as the standard reaction mixture for glycerate production, in a total volume of 1.0 ml. The reaction was started by the addition of enzyme to the reaction mixture previously incubated at 30°C. The quantity of the enzyme used was such that there would be a linear change in the absorbance at 340 nm for at least 2 min. One unit of the enzyme was defined as the amount of enzyme that catalyzed the appearance of 1 μmol of NADH per min. Specific activity was expressed as units per mg of protein.

Protein was determined by the method of Lowry *et al.*¹³⁾ with crystalline bovine serum albumin as a standard.

Results and Discussion

Screening of glycerate-producing microorganisms

To produce D-glycerate cheaply, initially, microorganisms that convert L-tartrate into glycerate in good yields were screened. Among the L-tartrate-assimilating microorganisms obtained, cells of 7 strains were found to produce enzyme(s) that convert L-tartrate into glycerate. Although L-tartrate dehydrogenase activity was found in cell-extracts of the 7 microorganisms, no relationship was found between the L-tartrate dehydrogenase activity and the glycerate-producing activity (Table I). Since the best yield of glycerate and the best

Table I. SCREENING OF GLYCERATE-PRODUCING MICROORGANISMS

The reaction was carried out in the standard reaction mixture at 30°C for an hour. The reaction mixture contained 0.1 ml of the cell extract obtained, in a total volume of 1 ml.

Strain	Cell yield (wet weight, g/200 ml)	Glycerate-producing activity		Total L-tartrate dehydrogenase activity (milliunits)
		Glycerate formed (μmol)	Tartrate consumed (μmol)	
507B	0.66	29	30	8
509B	0.69	21	21	8
5D1A	0.49	38	38	16
5D8A	0.56	23	37	21
5D13B	0.70	13	13	6
704A	0.52	23	27	21
7D10C	0.20	Trace	Trace	10

Table II. TAXONOMIC CHARACTERISTICS OF STRAIN 5D1A

Flagella	Polar 1
Motility	Motile by polar flagella
Shape	Rod, 0.8 ~ 1.2 × 2.0 ~ 3.2 μ m
Gram stain	Negative
Oxidase test	Negative
Catalase test	Positive
Growth	
anaerobic	Negative
37/41°C	Positive/negative
Pigments	Yellow-orange (non-diffusible)
O-F test	Oxidative
Gas from glucose	Negative
β -Galactosidase test	Negative
Indol production	Negative
Nitrate reduction	Negative
Denitrification	Negative
Phenylalanine deaminase	Positive
Levan from sucrose	Negative
Lecithinase test	Negative
Urease test	Negative
Hydrolysis of	
starch	Positive
gelatin	Negative
casein	Negative
DNA	Negative
Tween 80	Negative
esculin	Negative
Tyrosine degradation	Negative
Growth factor requirements	None
Carbon source utilization test	
Utilized: acetate, caprate, citrate, malate, malonate, L-arabinose, fructose, glucose, mannose, maltose, xylose, mannitol, gluconate, 2-ketogluconate, L-serine.	
Not utilized: adipate, glycolate, levulinate, phenylacetate, N-acetylglucosamine.	

selectivity, almost 100%, were obtained with the cell-extract of strain 5D1A, this strain was used for further experiments. The taxonomic characteristics of the strain are shown in Table II. Although this strain did not correspond to any species described in the 8th edition of Bergey's Manual of Determinative Bacteriology,¹⁴⁾ it was identified as a strain of *Pseudomonas* sp. group Ve-2 according to the 4th edition of the Manual of Clinical Microbiology.¹⁵⁾

Identification of the product

To identify the reaction product and to determine its steric configuration, the product

was isolated from the reaction mixture. The cell-extract of strain 5D1A (containing 938 mg of protein) was added to 1 l of 20 mM Tris-HCl buffer (pH 7.5) containing 100 mmol of potassium L-tartrate, 2 mmol of NAD⁺ and 0.2 mmol of MgCl₂. On incubation at 30°C for 24 hr, L-tartrate was converted into glycerate completely. Then the reaction mixture was applied to an Amberlite IRA-400 (OH form) column (5 × 21 cm). The column was washed with 1 l of H₂O and then the glycerate was eluted with 1 l of 1 N HCl. The solution was concentrated *in vacuo* and the glycerate hemicalcium salt (dihydrate) was obtained according to the method of Baer *et al.*¹⁶⁾ The purity of the glycerate hemicalcium salt obtained was estimated to be almost 100% by high-performance liquid chromatography.

The reaction product and authentic DL-glycerate showed the same retention time on high-performance liquid chromatography. The nuclear magnetic resonance and infra-red spectra of the product also showed the same patterns as those of the authentic DL-glycerate hemicalcium salt. The specific optical rotation value ($[\alpha]_D^{20}$) of the product was +13.3 (reported $[\alpha]_D^{20}$ values for D-glycerate hemicalcium, +12.9 ~ +15.5¹⁷⁾). About 96% of the reaction product reacted with glyoxylate reductase and NAD⁺ to yield NADH on 24 hr incubation at 25°C. Glyoxylate reductase, from spinach leaves, catalyzes the NAD⁺-dependent oxidation of D-glycerate but does not act on the L-isomer.¹⁸⁾ The formation of NADH was followed at 546 nm as the formation of formazan dye of *p*-nitrotetrazolium blue by diaphorase.⁷⁾ Because the equilibrium of D-glycerate oxidation lies so far to the side of formation of D-glycerate,¹⁸⁾ it is difficult to oxidize D-glycerate into β -hydroxypyruvate completely. Therefore, the optical purity of the product was estimated to be more than 92% *e.e.* Thus, the reaction product was identified as D-glycerate.

Enzyme participating in the conversion of L-tartrate into D-glycerate

In *P. putida*, D-glycerate is formed from L-

Table III. SUBSTRATE SPECIFICITY OF THE CELL-EXTRACT

Substrate (50 mM)	Relative value (%)
L-Tartrate	100
meso-Tartrate	12
D-Tartrate	0
DL-Malate	0
Dihydroxyfumarate ^a	0

^a NADH (10 mM) was used in the place of NAD⁺.

tartrate through the actions of two enzymes, L-tartrate dehydrogenase and oxaloglycolate reductive decarboxylase.^{5,6)} To elucidate whether the enzyme(s) participating in D-glycerate production is the same as that of *P. putida* or a new one, we investigated the substrate specificity of the cell-extract of strain 5D1A (Table III). D-Glycerate was also formed from meso-tartrate, but not from D-tartrate. Although L-tartrate dehydrogenase activity was found in the cell-extract (Table I), dihydroxyfumarate, the enolic isomer of oxaloglycolate, was not converted into D-glycerate in the presence of 10 mM NADH. Thus the enzyme participating in the conversion of L-tartrate into D-glycerate from *Pseudomonas* sp. 5D1A was different from that of *P. putida*.⁹⁾ We have purified the enzyme to homogeneity and proved that it is a new enzyme, L-tartrate decarboxylase which catalyzes the direct conversion of L-tartrate into D-glycerate, through additional experiments.¹²⁾ The L-tartrate dehydrogenase activity observed with the cell-extract of strain 5D1A was due to a side reaction catalyzed by L-tartrate decarboxylase. The detailed properties of the enzyme will be described elsewhere.

Formation of L-tartrate decarboxylase

To establish the most effective medium for the enzyme formation, the effects of several carbon sources on the formation of the L-tartrate decarboxylase by strain 5D1A cells were examined using the basal medium. The best cell yield and the highest L-tartrate decarboxylase activity were observed with L-tartrate as a sole carbon source (Table IV).

Table IV. EFFECTS OF CARBON SOURCES ON THE FORMATION OF L-TARTRATE DECARBOXYLASE

Carbon source (0.5%)	L-Tartrate decarboxylase (units/mg protein)
L-Tartrate	0.21
meso-Tartrate	0.063
D-Tartrate	0.029
Glycerol	0

Table V. EFFECTS OF VARIOUS DIVALENT CATIONS ON D-GLYCERATE PRODUCTION

The reaction was carried out at 30°C for 1 hr as described in the text except that MgCl₂ was replaced by other metals, as indicated. The reaction mixtures contained 3.2 mg of protein, in a total volume of 1 ml.

Metal (1 mM)	Relative activity (%)
None	100
MgCl ₂	158
MnCl ₂	145
CoCl ₂	101
CaCl ₂	92
NiSO ₄	86
FeSO ₄	34
CuCl ₂	15
ZnSO ₄	2
EDTA-treated	0

Thus L-tartrate is the best inducer of L-tartrate decarboxylase.

Establishment of the optimal reaction conditions for D-glycerate production

To produce D-glycerate most economically, the optimal conditions for D-glycerate production were examined with the cell-extract of *Pseudomonas* sp. strain 5D1A. The optimal pH and temperature for the D-glycerate production from L-tartrate were 7.0 to 8.0 and around 30°C, respectively, on 3 hr incubation.

The L-tartrate decarboxylase was affected by the addition of various metal ions. The enzyme was activated by the addition of Mg²⁺ or Mn²⁺, and inactivated by Fe²⁺, Zn²⁺ or Cu²⁺ (Table V). The cell-extract of strain 5D1A completely lost its L-tartrate decarboxylase activity on incubation with 1 mM EDTA at 30°C for 1 hr. The inactivated L-tartrate de-

Fig. 1. Time Course of D-Glycerate Production.

The reaction mixture contained, in 2 ml: Tris-HCl buffer (pH 7.5), 200 μ mol; $MgCl_2$, 0.4 μ mol; cell-extract (protein, 2.37 mg); NAD^+ , 2 μ mol (○), 4 μ mol (△, □) or 20 μ mol (▲, ■, ◆); and potassium L-tartrate, 200 μ mol (○), 400 μ mol (△, ▲), 1 mmol (□, ■) or 2 mmol (◆). The D-glycerate formed and residual L-tartrate were assayed in 0.05 ml aliquots.

carboxylase was nearly reactivated by the addition of 1 mM $MgCl_2$ or $MnCl_2$. The effect of $MgCl_2$ was almost constant at concentrations of 0.2 mM and over.

Time course of D-glycerate production

Figure 1 shows the time course of D-glycerate production from L-tartrate with various concentrations of L-tartrate and NAD^+ . The reaction rates and the final yields of D-glycerate increased with increasing concentrations of NAD^+ . The yields of D-glycerate produced under the optimal reaction conditions from 100 and 200 μ mol/ml of L-tartrate in the presence of 1 and 2 μ mol/ml of NAD^+ , respectively, reached nearly 100%. However, only 220 μ mol/ml of D-glycerate was formed from 500 μ mol/ml of L-tartrate in the presence of 2 μ mol/ml of NAD^+ in 120 hr. The reaction had ceased by that time. However, it recommenced, at half the initial rate, on the addition of 2 μ mol/ml of NAD^+ . In the presence of 10 μ mol/ml of NAD^+ , 500 μ mol/ml of L-tartrate was converted completely into D-glycerate. Although the L-tartrate decarboxylase requires NAD^+ as a cofactor,¹²⁾ no net reduction was observed through the reaction. Thus the dependence of the final yield on the concentration of NAD^+ may be due to decom-

position of NAD^+ . When 1 mmol/ml of L-tartrate was used as a substrate, strong substrate inhibition was observed (Fig. 1).

We described here the simple enzymatic production of D-glycerate from L-tartrate by the cell-extract of *Pseudomonas* sp. 5D1A. We are currently developing a new system for preparing D-glycerate with immobilized cells.

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