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Characterization of a Lactate Oxidase from a Strain of Gram Negative Bacterium from Soil

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ABSTRACT

A lactate oxidase was purified about 36-fold from a newly screened strain KY6 of gram negative bacterium from soil to yield a homogeneous protein. The native enzyme had a molecular mass of 204 kDa measured by Sephadex G-200 and that of subunit on the SDS-PAGE was found to be 45 kDa. The enzyme was optimally active at pH 7.7 and showed stability at pH range of 5.7 to 9.5 for 24 h at 4°C. The optimum temperature was 70°C and the enzyme activity was stable for 10 min up to 45°C. The half-life of the enzyme activity was about 10 min at 55°C. The best substrate of the enzyme was D-lactate and Km value for D-lactate was 0.14 m*M*. The Km value for DL-lactate was 0.20 m*M*. Substrate inhibition of the enzyme was observed at higher concentrations than 20 m*M* of DL-lactate and 10 m*M* of D-lactate.

Index Entries: Lactate oxidase; characterization; bacterium; pyruvate preparation.

INTRODUCTION

Biochemically, pyruvate is formed and converted by many reactions. Compared with other small nonchiral building blocks, pyruvate is relatively expensive. In the last 6 yr, more than 30 patents dealt with the

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preparation of pyruvate. Chemical as well as biochemical procedures were developed. This could be seen from a paper in which pyruvate was produced from gluconate (*1*). In many living organisms, lactate can be transformed to pyruvate by lactate dehydrogenases through the reversed reactions. The lactate dehydrogenase is well known as a kind of 2-oxo-acid oxidoreductase, which requires NADH and/or NADPH as an electron donor in lactate forming reaction. Then, the formation of pyruvate from lactate by this enzyme requires NAD^+ and/or $NADP^+$. It was reported that some lactate dehydrogenases require ferricytochrome c and others as acceptors in pyruvate forming reaction. During the 1950s and 1960s, some investigations (*2*) reported the finding of a lactate oxidase from *Mycobacterium phlei,* which catalyzed the following reaction:

$$CH_3CHOHCOOH + 1/2O_2 = CH_3COCOOH + H_2O$$

The lactate oxidase catalyzes the direct formation of pyruvate from lactate. This kind of enzyme seems to be very useful for formation of pyruvate in preparative scale. However, the enzyme sources, i.e., *Mycobacterium,* have a problem about cultivation because the microorganisms are pathogenic. Then, we attempt to seek for a lactate oxidase that catalyzes the similar reaction with the lactate oxidase from *Mycobacterium* without requirement of the expensive NAD^+ and/or $NADP^+$.

Recently, we described the screening for the lactate oxidase producer. A bacterial strain was found able to oxidize lactate to pyruvate with higher activity. The cofactors such as FAD, NAD^+, NADH, $NADP^+$, and NADPH had no effect of reactivation on the enzyme. Here we describe the purification and characterization of the enzyme.

MATERIALS AND METHODS

Chemical

All of the chemicals used in this work were commercial products of reagent grade quality.

Microorganism and Enzyme Preparation

The lactate producer, a not yet fully identified gram-negative bacterium (KY6), was screened by us from soil as described elsewhere (*3*). The strain was cultivated under static condition in the medium composed of 1.0% DL-lactate. 0.5% $(NH_4)_2SO_4$, 0.03% KH_2PO_4, 0.03% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.5% NaC,l 0.5% peptone, 0.5% yeast extract, 0.5% meat extract, and 10^{-5}% riboflavin in tap water, at pH 7.0 and 28°C with 10% inoculation size amount for 5 d.

Enzyme Assay for Lactate Oxidase

Reduction of the dye, 2,6-dichlorophenolindo phenol (DCPI) found by Robinson et al. (4) was used as a rapid method measuring the oxidation of lactate by more purified enzyme preparation. To the spectrophotometer cuvet, the following standard components were added; 1.8 mL of 10 m*M* potassium phosphate buffer (pH 7.0) containing 5 m*M* EDTA and 1.8 mg of bovine serum albumin, 30 μL of 1*M* of DL-lactate, 0.1 mL of 1 m*M* DCPI, and sufficient water to bring the volume to 2.5 mL. After incubation at 37°C for 15 min, 0.5 mL of a suitable dilution of enzyme was added to the cuvet and reaction was started in the spectrophotometer (HITACHI Model 200-10) equipped with thermospace. The absorbance at 600 nm was measured every 15 or 30 s and the rate of the reaction was determined from the initial linear plot relating absorbance to time. Control lacking the enzyme or substrate were routinely included in the assays.

Determination of Protein

Protein was assayed by the method of Lowry et al. (5) with bovine serum albumin as the standard.

Purification

All the procedures were carried out at 0–4°C. Potassium phosphate buffer (pH 7.0) was used throughout the purification.

Step 1: Preparation of Cell-Free Extract

The cells were harvested after cultivation, washed with distilled water, and suspended in 10 m*M* of potassium phosphate buffer, pH 7.0. The cell suspension was disrupted with a Kaijo Denki ultrasonic oscillator (20 kHz) for 10 min below 15°C, and then the supernatant was obtained by centrifugation at 10,000*g* for 30 min. The cell-free extract was obtained after dialysis for 24 h against 10 m*M* phosphate buffer (pH 7.0) and the following centrifugation. The solution as the crude enzyme preparation for purification was obtained from the cells (110 g of wet weight) harvested from 20 L of broth.

Step 2: Ammonium Sulfate Fractionation

Solid ammonium sulfate was added to the cell-free extract to 0.3 saturation. After standing overnight, the precipitate was removed by centrifugation. The ammonium sulfate concentration of the supernatant was brought to 0.70 saturation. After standing overnight, the precipitate was collected by centrifugation and dissolved in a small amount of 10 m*M* buffer. The enzyme was dialyzed for 48 h against four changes of 3 L of the same buffer. The supernatant was obtained after centrifugation at 10,000*g* for 30 min.

Step 3: DEAE-Cellulose Column Chromatography

The supernatant was applied to a DEAE-cellulose column (5.5 × 55 cm) previously equilibrated with 10 m*M* buffer. The enzyme solution was eluted with the same buffer at a rate of 1 mL/min. Most of the enzyme was not absorbed and eluted. The active fractions were combined and concentrated with ammonium sulfate (0.75 saturation). The precipitate was dissolved in 30 mL of 10 m*M* buffer. The enzyme suspension was divided into ten portions and each portion was proceeded to the following steps.

Step 4: Sephadex G-150 Filtration

Three milliliters of the enzyme solution were applied to a Sephadex G-150 column (2.8 × 150 cm) previously equilibrated with 10 m*M* buffer, and eluted with the same buffer at a flowrate of 10 mL/h. The active fractions were pooled and concentrated with ammonium sulfate (0.75 saturation). The precipitate was dissolved in a minimum volume of 10 m*M* buffer, then dialyzed against the same buffer.

Step 5: CM-Cellulose Column Chromatography

The enzyme solution was charged on a CM-cellulose column (2.8 × 45 cm) previously equilibrated with 10 m*M* buffer, then eluted with the same buffer at a flowrate of 1 mL/min. The active fractions were pooled and concentrated by the addition of ammonium sulfate to 0.75 saturation. The precipitate was dissolved in a minimum volume of 10 m*M* buffer.

Step 6: Sephadex G-200 Filtration

The dialyzed enzyme solution was applied to a Sephadex G-200 column (1.2 × 120 cm) previously equilibrated with 10 m*M* buffer, then eluted with the same buffer at a flowrate of 5 mL/h. The active fractions were pooled and concentrated by Amicon ultrafiltration with a PM-10 membrane. The concentrated enzyme solution was dialyzed against 1 m*M* buffer.

Step 7: Hydroxylapatite Column Chromatography

The concentrated enzyme solution was charged on a hydroxylapatite column (1.2 × 5 cm) previously equilibrated with 1 m*M* buffer. After washing with the same buffer, the column was developed by stepwise elution with increasing the buffer concentration (1, 5, 10, 15, and 20 m*M*). The activity was found in the 10 m*M* fractions. The active fractions were pooled and concentrated by Amicon ultrafiltration with a PM-10 membrane. The concentrated enzyme solution was used as the purified enzyme (3 mg protein/mL).

Table 1
Purification of the Lactate Oxidase from Strain KY6

Step	Total protein, mg	Total units, U	Specific activity, U/mg	Yield, %
Crude extract	11,400	386,000	33.9	100
Ammonium sulfate	10,900	372,000	34.1	96
DEAE-cellulose	5370	278,000	51.8	72
Sephadex G-150	3900	265,000	68.0	69
CM-cellulose	1020	196,000	192	51
Sephadex G-200	300	96,000	320	25
Hydroxylapatite	21	25,000	1220	7

Determination of Molecular Weight of Enzyme

The molecular weight of the enzyme was determined by gel filtration on Sephadex G-200 by calibration of the standard proteins. Elution was carried out with 10 m*M* potassium phosphate buffer (pH 7.0). Molecular weight markers used were ovalbumin (mol wt 43 kDa), bovine serum albumin (mol wt 66 kDa), catalase (mol wt 232 kDa), ferritin (mol wt 440 kDa), and blue dextrane 2000 (mol wt 2000 kDa).

SDS-PAGE

Homogeneity of the oxidase was determined by sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis. The electrophoresis was performed in 12.5% acrylamide gel and 0.1% SDS with a discontinuous Tris-glycine buffer system by the method of Laemmli (*6*). The molecular mass of subunits was estimated by SDS-PAGE (*6*). Molecular weight markers used were lactalbumin (mol wt 14 kDa), soybean trypsin (mol wt 20.1 kDa), carbonic anhydrase (mol wt 30 kDa), ovalbumin (mol wt 43 kDa), bovine serum albumin (mol wt 67 kDa), and phosphorylase b (mol wt 94 kDa). Protein was stained with Coomassie brilliant blue R.

RESULTS

Purification

The result of the lactate oxidase purification are summarized in Table 1. The enzyme was purified about 36-fold with a recovery about 7%.

Enzyme Purity and Molecular Weight

The purified enzyme preparation showed an almost single protein band on SDS-PAGE (Fig. 1). The molecular weight of the protein was

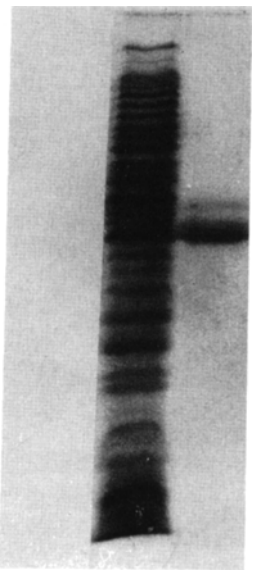

Fig. 1. SDS-polyacrylamide gel electrophoresis of the purified enzyme. The purified oxidase and crude enzyme preparation were subjected to SDS-polyacrylamide slab gel electrophoresis with 12.5% acrylamide. Left: cell free extract (30 μg of protein). Right: purified enzyme (10 μg of protein).

estimated to be 45 kDa using the standard mol-wt markers. As judged from the results of gel filtration, the molecular weight of the enzyme was estimated to be about 204 kDa. The results indicate that the enzyme is a polymeric protein.

Effects of pH and Temperature on the Activity and Stability

As shown in Fig. 2, the enzyme was optimally at pH 7.7 and showed stability at pH range of 5.7–9.5 when kept for 24 h at 4°C. The stability of enzyme was determined after incubation at different temperatures from 40 to 80°C for 10 min by assaying. Figure 3 shows effects of temperature on activity and stability of the enzyme. The optimum temperature for initial rate of the enzyme reaction was 70°C and the enzyme activity was stable for 10 min up to 45°C. The half-life of the enzyme activity was about 10 min at 55°C.

Effect of Substrate Concentration

The effects of substrate concentrations on the initial velocity of the enzyme reaction were examined at 37°C, pH 7.7, using DL-lactate and D-lactate as substrates. The Michaelis apparent constants (Km) for DL-lactate and D-lactate were 0.20 and 0.14 m*M*, respectively, and Vmax values for these two substrates were the same of 0.26 A600 min^{-1} mg^{-1},

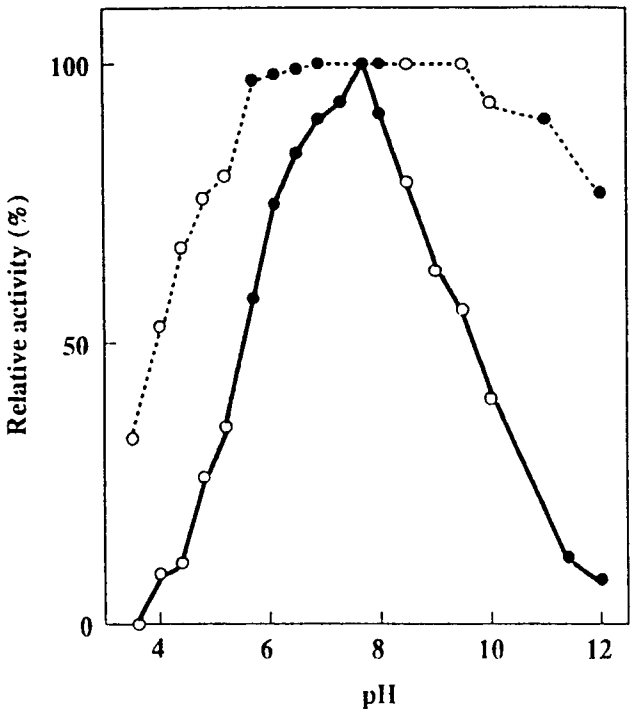

Fig. 2. Effects of pH on activity (solid line) and stability (dotted line) of the enzyme. 0.05*M* CH_3COOH-CH_3COONa for pH 3.6–5.6; 0.05*M* NaH_2PO_4-Na_2HPO_4 for pH 5.7–8.0; 0.05*M* glycine-NaOH for pH 8.5–10.0; 0.05*M* Na_2HPO_4 NaOH for pH 11–12.

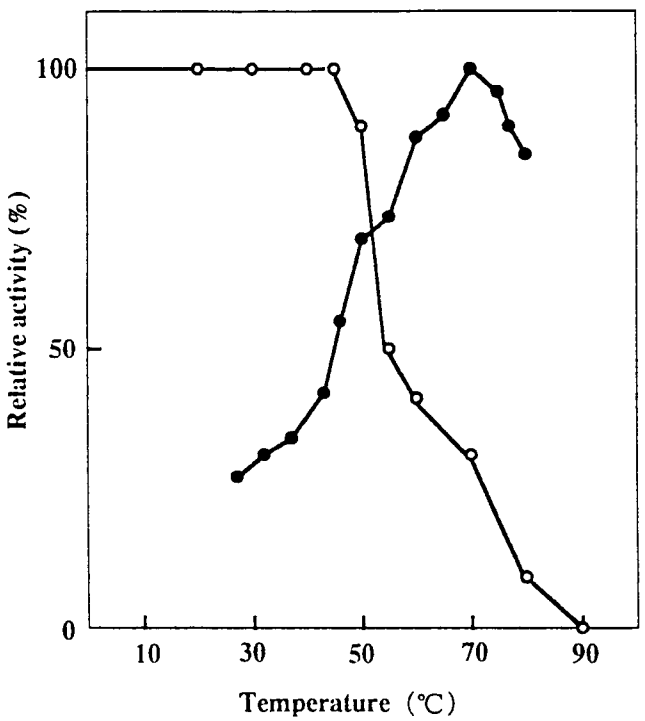

Fig. 3. Effects of temperature on activity (●) and stability (○) of the enzyme.

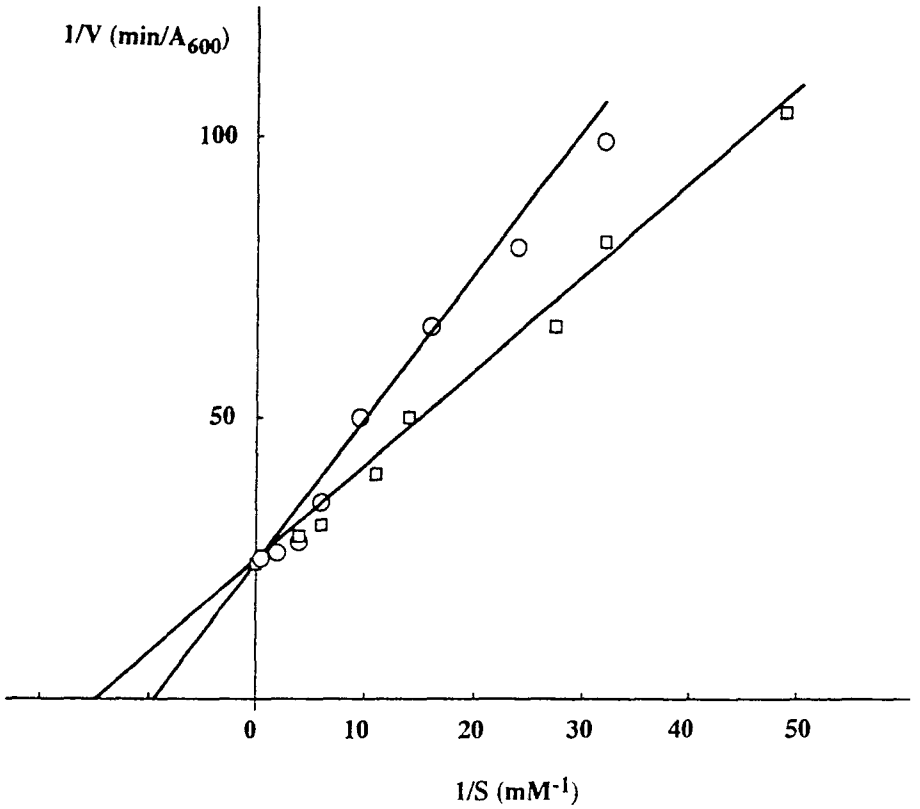

Fig. 4. Lineweaver-Burk plots of the enzyme reactions. ○, DL-lactate; □, D-lactate.

which were calculated with Lineweaver-Burk plot of the enzyme reaction (Fig. 4). As shown in Fig. 5, higher concentrations of above 20 m*M* of DL-lactate and 10 m*M* of D-lactate caused the substrate inhibition of the enzyme activity.

Substrate Specificity of the Enzyme

Table 2 shows the substrate specificities of the enzyme. Various hydroxy acids (0.3 m*M*) were assayed at 37°C, pH 7.7. The results indicated that the best substrate of the enzyme was D-lactate.

Effects of Varies Compounds on the Enzyme Activity

Table 3 shows the effects of metallo-ions and some inhibitors on the enzyme activity at 37°C, pH 7.7, without EDTA. EDTA enhanced the enzyme activity and Fe^{2+} strongly inhibited the enzyme activity.

Requirement of Cofactor

According to the method described by Horecker (7), the apoenzyme was prepared with modification. About 80 mL of the purified enzyme

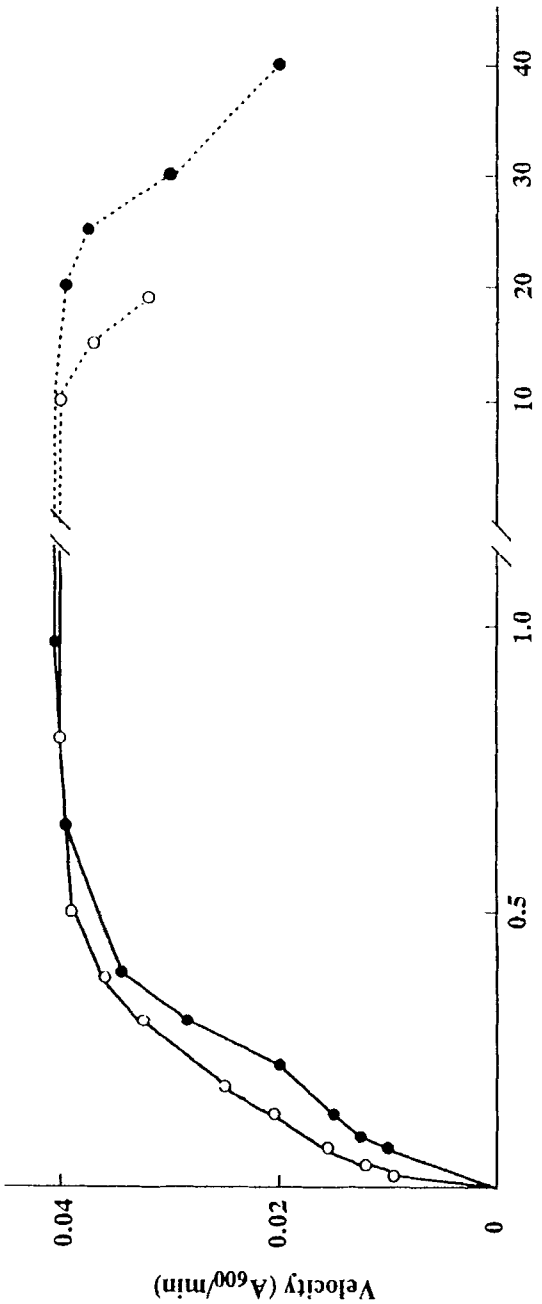

Fig. 5. The effects of concentrations of substrates (DL-lactate and D-lactate) on the reaction velocity of the enzyme. ●, DL-lactate; ○, D-lactate.

Table 2
Specifities of Various Substrates on the Enzyme Activity

Substrate	Relative activity, %
DL-Sodium lactate	100
D-Sodium lactate	117
L-Sodium lactate	67
Lithium-L-lactate	40
L-Hydroxyisocaproate	34
Sodium glycolate	5
L-Malic acid	52

Table 3
Effects of Various Compounds on the Enzyme Activity

Chemical, 1 m*M*	Relative activity, %
None	100
NaCl	100
KCl	100
$MgSO_4$	88
$CoCl_2$	98
$CuSO_4$	100
$FeSO_4$	14
$CaCl_2$	94
$MnSO_4$	53
$ZnSO_4$	72
$HgCl_2$	72
EDTA	140
p-Chloromercuribenzoate	79
Iodoacetate	67
L-Phenylalanine	72
L-Leucine	71

solution were added at 0°C by 18 g of ammonium sulfate and 8 mL of 1*M* H_2SO_4 containing 2.2 g of ammonium sulfate. After 5 min, the mixture was centrifuged. The colorless protein precipitate was dissolved in 5 mL of 0.1*M* potassium phosphate buffer (pH 7.0). The suspension after neutralization with 10% of NH_4OH was dialyzed against a changes of 2.5 L of the buffer (10 m*M*) for 48 h. The apoenzyme prepared above was added with different cofactors, then reacted at 37°C, pH 7.7, overnight. Enzyme activities were assayed in absence of FMN. As shown in Table 4, interestingly, the activity of the apoenzyme was reactivated only by the addition of FMN. The other cofactors such as FAD, NAD^+, NADH, $NADP^+$, and NADPH, had no effect on reactivation. Holoenzyme did not require the additional FMN and any cofactors.

Table 4
Reactivation of Apoenzyme by Various Cofactors

Enzyme, 3 mg/mL	Cofactor added to the assay system	Relative activity, %
Holoenzyme	None	100
Apoenzyme	None	19
Holoenzyme	FMN (0.25 m*M*)	96
Apoenzyme	FMN (0.25 m*M*)	98
Apoenzyme	FAD (0.25 m*M*)	25
Apoenzyme	NAD^+ (0.5 m*M*)	20
Apoenzyme	NADH (0.5 m*M*)	21
Apoenzyme	$NADP^+$ (0.5 m*M*)	19
Apoenzyme	NADPH (0.5 m*M*)	18
Apoenzyme	Methylene blue (0.25 m*M*)	25
Apoenzyme	Anthraquinone-2,6-disulfonate (0.75 m*M*)	30

DISCUSSION

Lactate dehydrogenase could be used for the preparation of pyruvate in the presence of lactate and NAD^+ and/or $NADP^+$. However, on a preparative scale, the enzyme cannot be used because stoichiometric amounts of expensive pyridine nucleotides are needed for the oxidative reaction (*8*). In regard of the regeneration of $NAD(P)^+$, enzymatic methods are available. However, such systems have some intrinsic problems and complications because two substrates, two products, and the labile coenzymes $NAD(P)^+$/NAD(P)H are required for the recycling reaction.

It is known that there are some enzymes possessing prosthetic groups and catalyzing the oxidative reaction by means of direct electron transfer from a hydroxy group. The lactate oxidase (II) described by Yamamura et al. (2) and Sutton (*9*) was an enzyme of such oxidase, which did not require $NAD(P)^+$ as a cofactor. However, the enzyme was produced by pathogenic *Mycobacteria*. Our bacterium KY6 obtained from soil produced the lactate oxidase, which was similar with the lactate oxidase with the direct enzymatic reaction. This bacterium apparently does not belong to the genus *Mycobacterium*, which does not grow under anaerobic culture condition and is gram positive (*10*). The lactate oxidase purified by us from strain KY6 was not the same as the lactate oxidase (II) from *Mycobacteria* reported by Yamamura et al. (2) and Sutton (*9*). The lactate oxidase (II) from *Mycobacteria* was activated with the addition of methylene blue and diphosphopyridine nucleotide, and flavin cofactors did not activate the enzyme activity (2). Our enzyme in the form of apoenzyme was reactivated only by FMN. Glycolate oxidase [EC 1.1.3.1] (*11*) and L-

hydroxy acid oxidases (4) containing flavin cofactors might slightly catalyze the reaction of lactate to pyruvate, but these oxidases were from the animal sources.

The lactate oxidase of strain KY6 could be purified by comparatively simple procedures. The low yield of the enzyme may be owing to that it was not absorbed by DEAE-cellulose and CM-cellulose under the operation conditions unexpectedly although much inert protein was separated from the column chromatographies. These might have led to a loss of enzyme activity and a low recovery.

From the results of the substrate specificity, the enzyme should be designated as D-lactate oxidase. L-lactate inhibited the enzyme reaction rate with D-lactate competitively because both Vmax values of DL-lactate and D-lactate were the same and Km value for DL-lactate was larger than that for D-lactate.

REFERENCES

1. Yanase, H., Mori, N., Masuda, M, Kita, K., Shimao, M., and Kato, N. (1992), *J. Ferment. Bioeng.* **73,** 287–291.
2. Yamamura, Y., Kusunose, M., and Kusunose, E. (1952), *Nature* **170,** 207–208.
3. Xu, P., Yano, T., Yamamoto, K., Suzuki, H., and Kumagai, H. (1996), *J. Ferment. Bioeng.* (in press).
4. Robinson, J. C., Keay, L., Molinari, R., and Sizer, I. W. (1962), *J. Biol. Chem.* **237,** 2001–2010.
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265–275.
6. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
7. Horecker, B. L. (1950), *J. Biol. Chem.* **183,** 593–605.
8. Thanos, I., Bader, J., Gunther, H., Neumann, S., Krauss, F., and Simon, H. (1987), *Methods in Enzymology* **136,** 302–317.
9. Sutton, W. B. (1954), *J. Biol. Chem.* **210,** 210–320.
10. Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Polt, J. G. (1986), *"Bergey's Manual of Systematic Bacteriology"* **2,** 1436.
11. Tokushige, M. and Sizer, W. (1967), *J. Biochem.* **62,** 719–725.