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Purification and Characterization of Cold-Active L-Glutamate Dehydrogenase Independent of NAD(P) and Oxygen

Akira Yamamura,[1] Toshifumi Sakaguchi, Yuji Murakami, Kenji Yokoyama, and Eiichi Tamiya

School of Materials Science, Japan Advanced Institute of Science & Technology, 1-1 Asahidai, Tatsunokuchi, Ishikawa 923-1292

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L-Glutamate dehydrogenase (GLDH) independent of NAD(P) and oxygen was first obtained from the psychrotrophic bacterium *Aeromonas* sp. L101, originally isolated from the organs of salmon (*Oncorhynchus keta*). GLDH was purified by a series of chromatography steps on DEAE-Sepharose, Superdex 200pg, Q-Sepharose, CM-Sepharose, and Phenyl-Sepharose. The purified protein was determined to have a molecular mass of 110 kDa and a pI of 5.7. Maximum activity was obtained at 55°C and pH 8.5. The activity of GLDH at 4 and 20°C was 38 and 50%, respectively, of that at 50°C. GLDH was coupled to cytochrome *c* and several redox dyes including 1-methoxy-5-methylphenazinium methylsulfate (1-Methoxy PMS), 2,6-dichlorophenylindophenol (DCIP), 9-dimethylaminobenzo[*a*]phenoxazin-7-ium chloride (meldola's blue), 3,3′-[3,3′-dimethoxy-(1,1′-biphenyl)-4,4′-diyl]-bis[2-(4-nitrophenyl)-5-phenyl-2*H* tetrazolium chloride] (nitroblue tetrazolium; NBT), and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H* tetrazolium (INT). The presence of NAD(P) and oxygen gave no oxidation activity to GLDH. Spectroscopic profile and ICP data indicated a b-type cytochrome containing iron.

Key words: *Aeromonas* sp., cold-active enzyme, L-glutamate dehydrogenase, heme *b*, heme-containing enzyme.

So far, the enzymatic oxidation of L-glutamate has mainly been classified into two broad groups. Glutamate dehydrogenases (GLDH) can catalyze the oxidative deamination of L-glutamate to 2-oxoglutarate, using the nicotinamide coenzymes $NAD(P)^+$ as follows:

$$\text{L-glutamate} + NAD(P)^+ + H_2O \rightleftharpoons \text{2-oxoglutarate} + NH_4^+ + NAD(P)H.$$

This oxidation is generally considered to be a dehydrogenase type reaction [EC 1.4.1.2-4] (*1*). The reaction can be distinguished from the oxidase type reaction by L-glutamate oxidase (GLOX) in the utilization of molecular oxygen as given below [EC 1.4.3.11] (*2*):

$$2\ \text{L-glutamate} + O_2 + H_2O \longrightarrow 2\ \text{2-oxoglutarate} + 2\ NH_4^+ + H_2O.$$

Stoichiometrically, one molecule oxygen can oxidize two molecules of L-glutamate. Oxygen atoms in the reactant water are replaced by oxygen in the product water, whereas hydrogen peroxide is generated during the oxidation of D-glutamate [EC 1.4.3.7] (*3*):

$$\text{D-glutamate} + \text{oxygen} + H_2O \longrightarrow \text{2-oxoglutarate} + NH_4^+ + H_2O_2.$$

In the oxidation of L-glutamate, a flavoenzyme, purified from a Gram positive bacteria, *Streptomyces endus*, forms hydrogen peroxide as a product (*4*).

These reactions comprise one of most important metabolic pathways in higher organisms, which need to dispose of excess nitrogen, and many lower organisms, which are able to assimilate nitrogen in the form of ammonia. In most microorganisms, such L-glutamate oxidation is usually performed by soluble or the membrane-bound dehydrogenases that require $NAD(P)^+$ or molecular oxygen. $NAD(P)^+$-dependent L-glutamate oxidoreductases have been found in various eucaryotes, *Saccharomyces cerevisiae* (*5*), and *Candida utilis* (*6*), and procaryotes such as *Bacillus subtilis* (*7*), *Escherichia coli* (*8*), *Bacteroides thetaiotaomicron* (*9*), *Bacillus licheniformis* (*10*), *Bacillus megaterium* (*11*), *Pseudomonas aeruginosa* (*12*), *Salmonella typhimurium* (*13*), *Clostridium* SB_4 (*14*), the photosynthetic bacterium *Rhodospirillum rubrum* (*15*), *Cyanobacterium synechocystis* sp. (*16*), and *Mycoplasma laidawii* (*17*). However, there is no report of the presence of a glutamate dehydrogenase independent of $NAD(P)^+$ or molecular oxygen (acceptor type GLDH). In addition, some $NAD(P)^+$-dependent glutamate dehydrogenases that are labile at low temperatures have been detected in *Azospirillum brasilense* (*18*) and *Bacillus* spp. (*19, 20*). These reports suggest the loss of glutamate dehydrogenase activity due to exposure to a cold environment in some mesophilic bacteria. From these suggestions, questions have been raised as to what kind of glutamate dehydrogenase cold-active (psychrophilic and psychrotrophic) microorganisms contain, and whether or not the enzyme is active in cold environments.

[1] To whom correspondence should be addressed. Tel: +81-761-51-1660, Fax: +81-761-51-1665, E-mail: yamamura@jaist.ac.jp

Cold-adapted (psychrophilic and psychrotrophic) microorganisms are distinguished from mesophiles by their ability to grow at low temperature (*21-23*). They have specific mechanisms for energy transduction and for regulating the intracellular environment and metabolism, functional cell membranes, cell walls, and enzymes stable and/or active in the cold environments (*21-23*). In particular, their cold-active enzymes have attracted much attention as low energy biocatalysts, because not only is there higher activity at low temperature but also a lower activation energy than mesophilic enzymes from general microorganisms (*22*). Cold-active enzyme is therefore expected to be applicable to various industrial processes such as waste treatment, biosensing, fermentation, food processing, and medicine manufacture. So far, various cold-active enzymes, such as proteases (*24-27*), α-amylase (*28*), β-galactosidase (*29*), triosephosphate isomerase (*30*), and a few dehydrogenases (*31-36*), have been found from psychrophiles or psychrotrophes. We have accomplished the purification and characterization of novel cold-active serine protease from *Flavobacterium balustinum* (*26*) and demonstrated its possible usage in laundry detergent. However, the genetic, protein structural, and configurational bases of the stable activity at low temperature are not understood, and the practical uses of cold-active enzymes or microorganisms have not been realized. Further studies and investigations on cold-active enzymes and microorganisms are required to obtain definite information concerning cold-adaptation. Especially, there are no reports of cold-active redox enzymes except isocitrate dehydrogenase (*31-33*), histidinol dehydrogenase (*34*), and lactate dehydrogenase (*35, 36*).

Here, we first report the purification and characterization of a novel cold-active glutamate dehydrogenase independent of $NAD(P)^+$ and oxygen, extracted from a psychrophilic bacterium, *Aeromonas* sp. L101.

MATERIALS AND METHODS

Isolation of Psychrotrophic Bacteria—Psychrotrophiles were obtained from the organs of salmon (*Oncorhynchus keta*) and crab fish (*Chinoecetes opilio*) living in cold habitats, and from soil in cold places. Cold-adapted bacteria were isolated by picking up a single colony formed on an agar (1.5%) plate in isolation medium at 10°C. The isolation medium contained the following (in grams per liter of water): polypeptone (Wako Pure Chemical) (1.0), yeast extract (Difco) (2.5), NaCl (0.5), glucose (0.1), and $MgSO_4 \cdot 7H_2O$ (0.1). Soluble starch, olive oil, or casein (0.5 gram per liter medium) was added to the isolation medium as an extra conspicuous carbon source. The isolates were restreaked on an agar (1.5%) plate in nutrient broth containing (in grams per liter of medium) glucose (0.1) and $MgSO_4 \cdot 7H_2O$ (0.1). The plates were incubated at 10°C for 2 weeks. After precultivation in liquid medium, strain L101 was kept in modified nutrient broth containing 20% glycerol at −20°C as a single pure strain for the subsequent experiments.

Activity Staining for the Detection of GLDH from Psychrotrophiles—For the detection of GLDH activity, isolated psychrotrophic bacteria were cultured in 15-ml test tubes containing 5 ml of modified nutrient broth on a rotary shaker (150 rpm) at 10°C for 72 h. Cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C, and then washed twice in 0.5% NaCl solution. The cells were then suspended in 200 μl of 20 mM sodium phosphate buffer (pH 7.0) and disrupted with an ultrasonic processor (Heat Systems, Astrason model XL2020) for 5 min (cycling 10 s on and off), with cooling in ice water. Cells and debris were first removed by centrifugation at 10,000 × *g* for 10 min at 4°C, and then 10 μl of marker solution (0.001% bromophenol blue) and 8 mg of sucrose was added to 30 μl of supernatant. Native polyacrylamide gel electrophoresis was carried out on a vertical stab gel in Tris-glycine buffer (pH 8.0) at 30 mA for 5 h at 5°C (*37*). After electrophoresis, the gels were washed with 20 mM phosphate buffer (pH 7.0) and incubated in phosphate buffer (pH 7.0) containing 1 mM 3,3′-[3,3′-dimethoxy-(1,1′-biphenyl)-4,4′-diyl]-bis[2-(4-nitrophenyl)-5-phenyl-2*H* tetrazolium chloride] (NBT) and 100 mM L-glutamate for 24 h at 10°C. GLDH activity was detected by the appearance of a blue band on the gel by the reduction of NBT.

Measurements of GLDH Specific Activity—GLDH activity was measured spectrophotometrically at 20°C as the increase in the absorbance (530 nm) due to the reductant (formazan) of NBT. Enzyme solution (0.1 ml) was added to 0.8 ml of Tris-HCl buffer (pH 9.0), and mixed with 0.1 ml of assay mixture containing 1 mM NBT and 100 mM L-glutamate. This mixture was placed directly into a glass microcuvette (1-cm light path), and incubated to measure the increase in the absorbance (ΔA/min) at 20°C for 2 min in a thermo-controlled spectrophotometer (Beckman DU 640). One unit of enzyme activity was defined as the amount of enzyme capable of reducing 1 μmol of NBT per min. By using the extinction coefficient of formazan (36,000 $M^{-1} \cdot cm^{-1}$), it was determined that one absorbance unit at 530 nm is equivalent to 28 nmol of formazan. For the determination of the specific activity, the total protein concentration was measured by the method of Bradford (*38*) with a commercial assay kit (Bio-Rad Laboratories). Bovine serum albumin was used as a standard protein for calibration.

Bacterial Culture and Mass Cultivation—To investigate the optimum conditions for GLDH production from isolated bacteria (strain L101), growth and GLDH activity were measured at various temperatures (5, 10, 15, and 20°C). Cells were precultivated in nutrient broth at 10°C to an optical density at 660 nm of 1.0, and then 1 ml of the culture was inoculated into a 100-ml flask containing 20 ml of nutrient broth. Growth (OD_{660nm}) and GLDH activity were measured at various temperatures.

Mass cultivation was carried out in 3 liters of nutrient broth in a jar fermentor under the optimum conditions (15°C) for GLDH production as determined from these investigation. The culture was agitated at 150 rpm under a 3 liters/min stream of filtrated air until they reached stationary phase.

Purification Steps—GLDH from *Aeromonas* sp. L101 was purified by the procedures described below.

Step 1. Crude extract: The harvested cells were washed with cool 0.5% NaCl and then frozen at −20°C. Frozen cells (about 40 g) were thawed and suspended in 100 ml of 20 mM Tris-HCl buffer (pH 9.0), and disrupted by repeated 10 s bursts of ultrasonic vibration for a total of 2 h with continuous cooling in ice-water. The crude extract (supernatant) was obtained by centrifugation at 20,000 × *g* for 30

min at 4°C.

Step 2. DEAE-Sepharose chromatography: The crude extract was loaded onto a column of DEAE-Sepharose Fast Flow (Pharmacia Biotech.) packed in an INdEX 100 column (10×25 cm; 2 liters; Pharmacia Biotech.) equilibrated with 3 column volumes of 20 mM Tris-HCl buffer, pH 9.0, (buffer A). The column was then washed with buffer A, and GLDH was eluted gradually in 3 steps with buffer A containing NaCl concentrations of 0.2, 0.4, and 0.6 M (4 liters of each) at a flow rate of 220 ml/min. Fractions containing high GLDH activity were collected.

Step 3. Ammonium sulfate fractionation: Ammonium sulfate fractionation (0.5–0.9 saturation) was performed by adding solid ammonium sulfate to the collected supernatant from Step 2. The precipitate formed by at 90% ammonium sulfate saturation was collected by centrifugation (20,000×*g*, 20 min, 4°C) and dissolved in 5 ml of 50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl.

Step 4. Superdex 200 pg chromatography: The enzyme solution from Step 3 was loaded onto a column of Superdex 200 prep grade (2.6×60 cm; Pharmacia Biotech.) equilibrated with 50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 1 ml/min. The protein containing GLDH activity was eluted with the same buffer, and the active fractions were collected. The supernatant was subjected to concentrating ultrafiltration in an Amicon stirred cell unit (model 8400) fitted with a Diaflo ultrafilter PM30 (30,000-molecular-weight cutoff) under 5 kgf/cm^2 pressure.

Step 5. Q-Sepharose chromatography: After desalination by ultrafiltration with three exchanges of buffer A, the supernatant from Step 4 was loaded onto a column of Q-Sepharose (1.6×10 cm; Pharmacia Biotech.) equilibrated with buffer A. The column was washed with buffer A, and GLDH activity was eluted with a linear gradient of NaCl (0 to 500 mM in a total volume of 200 ml). Fractions with high activity were combined. For concentration, desalination, and buffer exchange, the supernatant was subjected to ultrafiltration as described in Step 4 with 20 mM acetate buffer, pH 5.0 (buffer B).

Step 6. CM-Sepharose chromatography: The enzyme solution from Step 5 was loaded onto a column of CM-Sepharose (1.6×2.5 cm; Pharmacia Biotech.) equilibrated with buffer B. The column was washed with buffer B, and then GLDH activity was eluted with a linear gradient of NaCl (0 to 600 mM in a total volume of 200 ml). Fractions with high GLDH activity were combined and desalted by ultrafiltration with 20 mM phosphate buffer (pH 7.0) containing 1 M ammonium sulfate (buffer C).

Step 7. Phenyl-Sepharose chromatography: The desalted enzyme solution was applied to a column of Phenyl-Sepharose (0.7×5 cm; Pharmacia Biotech.) equilibrated with buffer C. The column was washed with buffer C. GLDH activity was eluted with a linearly decreasing gradient of ammonia sulfate (1 to 0 M in a total volume of 200 ml). Fractions with high GLDH activity were combined. For concentration and desalination, the supernatant was subjected to ultrafiltration with 20 mM phosphate buffer (pH 7.0) as described above.

Molecular Mass and Isoelectric Point—Gel filtration and SDS electrophoresis were carried out to estimate of the molecular mass of the purified enzyme. Gel filtration was performed on a Superdex 200 pg column (1.6×60.0 cm; Pharmacia Biotech.) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl. The purified enzyme was loaded onto the column, and the retention time was compared with those of the calibration samples in the kits (LMW and HMW gel filtration calibration kits; Pharmacia Biotech.), and the molecular mass was estimated.

To investigate enzyme purity in the non-denatured form, the purified enzyme was subjected to electrophoresis in a native homogeneous polyacrylamide (7.5%) slab gel (PhastGel, Homogeneous 7.5; Pharmacia Biotech.) at a constant current of 20 mA for 25 min at 15°C with the exclusive buffer (Native buffer strip; Pharmacia Biotech.) by an automatic electrophoresis system (PhastSystem; Pharmacia Biotech.). In addition, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under the same conditions with a ready-made gradient (8 to 25%) gel (PhastGel Gradient 8-25; Pharmacia Biotech.) and the exclusive buffer strip (SDS buffer strip; Pharmacia Biotech.) using the automatic electrophoresis system. The molecular weight and subunits changes in the presence of SDS were investigated. Protein bands were stained with 0.05% Coomassie Brilliant Blue R-250 (CBB) solution, and standard molecular mass markers (LMW and HMW electrophoresis calibration kits; Pharmacia Biotech.) were used for the estimation of molecular mass.

Analytical isoelectric focusing was carried out in a ready-made gel (pH 3 to 9) for pI measurements (PhastGel IEF 3-9; Pharmacia Biotech.) for 80 min using an automatic electrophoresis system (PhastSystem; Pharmacia Biotech.). Proteins were stained with 0.05% CBB solution containing 0.1% $CuSO_4$. Standard pI marker proteins (Isoelectric focusing calibration, Broad pI kit; Pharmacia Biotech.) were used for pI determination.

Detection of Enzymatic Products—The enzymatic products were confirmed by measuring ammonia by the indophenolblue reaction (*39*) with an ammonia test kits (Wako ammonia test kit; Wako Pure Chemical) and 2-oxoglutarate by high performance liquid chromatography (SC-8020; TOSOH) on a TSK-GEL ODS-80Ts prepacked column (4.6×150 mm; TOSOH) and a model UV-8020 UV detector (TOSOH) at an absorbance of 240 nm. 2-Oxoglutarate was eluted at 40°C with 0.1 M potassium phosphate buffer (pH 2.0) at a flow rate of 1.2 ml/min. The retention time, estimated from the standard, was used for a qualitative identification. The enzymatic determination of hydrogen peroxide was carried out spectrophotometrically with 3,3′-dimethoxybenzidine and horseradish peroxidase at an absorbance of 460 nm.

Effects of pH and Temperature—For the determination of the effect of pH on activity, two buffers [0.1 M sodium phosphate buffer (pH 5.5–8.0) and 0.1 M borate buffer (pH 8.0–9.7)] covering the range between pH 5.5 and 9.7 were prepared. The enzyme was incubated at varying pH at 20°C in buffer containing 50 mM L-glutamate and 1 mM NBT. GLDH activity was measured spectrophotometrically as described above. To determine the pH stability, the enzyme was preincubated at 20°C for 20 min in various buffers [20 mM sodium phosphate buffer (pH 5.5–8.0) or 20 mM borate buffer (pH 8.0–12.0)]. The enzyme solutions were diluted 50-fold with 0.5 M sodium phosphate buffer (pH 7.0) containing L-glutamate and NBT, and the residual activity was determined.

To investigate the effect of temperature on enzymatic activity, the enzyme solution was added to 50 volumes of 20 mM sodium phosphate buffer (pH 7.0) containing L-glutamate and NBT. The enzymatic reaction was carried out in a thermo-controlled spectrophotometer (DU 640; Beckman) for 2 min. The activity was measured spectrophotometrically. For the determination of enzyme thermostability, the enzyme solution was preincubated for 20 min at various temperatures in 20 mM sodium phosphate buffer (pH 7.0). Heating was stopped by cooling in an ice bath. Measurement of the residual activity was performed spectrophotometrically at 20°C in 20 mM phosphate buffer (pH 7.0) containing L-glutamate and NBT as described above. The activity is represented as the relative value against the original activity.

Substrate Specificity—For the determination of substrate specificity, the enzyme solution was incubated at 20°C in 20 mM Tris-HCl buffer (pH 9.0) containing 1 mM NBT and 50 mM amino acid. The activity was measured spectrophotometrically as described above.

The utilization of electron acceptors was investigated as follows using: 2,6-dichlorophenolindophenol (DCIP; $\varepsilon_{600} = 17{,}500\ M^{-1}\cdot cm^{-1}$), 1-methoxy phenazine methosulfate (1-methoxy PMS; $\varepsilon_{505} = 2{,}840\ M^{-1}\cdot cm^{-1}$), 9-dimethylaminobenzo[$\alpha$]phenoxazin-7-ium chloride (meldola's blue; $\varepsilon_{570} = 29{,}000\ M^{-1}\cdot cm^{-1}$), nitrobluetetrazolium (NBT; $\varepsilon_{530} = 36{,}000\ M^{-1}\cdot cm^{-1}$), 2-(4-indophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H* tetrazolium (INT; $\varepsilon_{490} = 15{,}000\ M^{-1}\cdot cm^{-1}$), nicotinamide adenine dinucleotide (NAD^+; $\varepsilon_{340} = 6{,}620\ M^{-1}\cdot cm^{-1}$), nicotinamide adenine dinucleotide phosphate ($NADP^+$; $\varepsilon_{340} = 6{,}000\ M^{-1}\cdot cm^{-1}$), and oxygen. The enzyme was incubated at 20°C in 20 mM Tris-HCl buffer (pH 9.0) containing 50 mM L-glutamate in the presence of 1 mM of an electron acceptor reagent other than oxygen. Electron acceptor utilization was estimated by measuring the absorption change for 2 min. The estimation of oxygen consumption was carried out with an oxygen electrode (Model BO-G, ABLE, Japan) in a measuring cell. Twenty millimolar Tris-HCl buffer (pH 9.0) contained 50 mM L-glutamate was added to the cell and saturated with a stream of oxygen (pO_2: 0.28 mM). After the addition of enzyme, the solution was incubated at 20°C for 2 h with stirring, and the amperometric determination of oxygen concentration was performed.

Determination of K_m and V_{max}—Because GLDH catalyzes a two substrate reaction (L-glutamate and NBT), V_{max} and K_m parameters for each substrate were determined by Lineweaver-Burk analysis. The steady state kinetics were done using a saturating concentration of one substrate and varying the other substrate concentration. The rates of L-glutamate oxidation were measured by monitoring the NBT reduction spectrophotometrically at 530 nm in 20 mM sodium phosphate buffer (pH 7.0) at 20°C. Saturating concentrations were 50 mM for L-glutamate and 1 mM for NBT.

Spectral Properties—The ultraviolet-visible absorption spectrum was recorded in 0.1 M phosphate buffer (pH 7.0) at 20°C with a Beckman DU-640 spectrophotometer at a scan speed of 240 nm per min. The difference spectrum (reduced minus oxidized) was obtained by recording the spectrum of the enzyme reduced by adding a few crystals of solid sodium dithionite, and the spectrum of the enzyme was oxidized by adding a few crystals of potassium ferricyanide. The pyridine ferrohemochrome of this enzyme was formed in 0.15 M NaOH, and 0.2 M pyridine by adding a few crystals of solid sodium dithionite.

Iron Content—The iron content of the enzyme was determined by emission spectrography using inductively coupled plasma spectroscopy (ICP) (FISONS; Applied Research Laboratories, Switzerland). Quantitative analysis was performed by measuring the specific spectrum for Fe (248 nm).

RESULTS AND DISCUSSION

Screening and Characterization of Psychrotrophic Bacteria Producing GLDH—In total, 136 strains of cold-adapted bacteria were isolated from salmon, crab, and habitats, and their GLDH activities were tested using NBT without $NAD(P)^+$. Four strains (L101, P105, L301, and A105) showed high activity, as shown in Table I. The best cold-active GLDH producer, strain L101, isolated from the internal organs of a salmon (*Oncorhynchus keta*), was selected for further characterization. This bacterium is Gram-negative and facultatively anaerobic. It is rod-shaped, and has a size approximately $0.8 \times 2.0\ \mu m$ and a single polar flagellum for motility, based on observations with a scanning electron microscope. It also has cytochrome *c* oxidase and catalase, and excretes protease, lipase, and DNase. It shows no growth on TBS agar, and is resistant to the vibriostatic agent O/129. Previous results suggested that strain L101 belongs to *Aeromonas* sp. (*40*). Therefore, this bacterium was named *Aeromonas* sp. L101 in this study.

Figure 1A shows that *Aeromonas* sp. L101 grows fastest at 15°C. It is able to grow between 5 and 20°C, and growth is completely suppressed at 30°C. Final growth at 20°C was less than at 10 or 15°C. From these observations, strain L101 was confirmed to be a psychrotrophile. The highest amount of GLDH activity was produced at 15°C, and the GLDH activity increased remarkably after 7 h incubation, as shown in Fig. 1B. About 4-fold greater activity was seen at 20°C. Based on these results, the conditions for the mass cultivation of L101 were determined at 15°C.

Purification of GLDH—The GLDH enzyme produced in *Aeromonas* sp. L101 was purified by several chromatographic steps as described in "MATERIALS AND METHODS." A typical overall purification is summarized in Table II. The final enzyme preparation obtained was purified about 70-fold over the crude extract, however, the overall yield was only 0.8%. The reason for the decrease in the specific activity after ammonium sulfate precipitation and gel filtration may be that an isozyme was removed when the

TABLE I. **Specific activities of L-glutamate oxidation in crude extracts of isolated cold-active bacteria.**

Strain	Specific activity (unit/mg protein)*
L101	0.170
P105	0.098
L301	0.089
A105	0.045

*Activity was measured at 20°C by the spectrophotometric method. One unit was defined as the amount of enzyme that reduced 1 μmol of nitroblue tetrazolium (NBT) per min under the conditions of assay. Amount of total protein was determined by the method of Bradford (*38*).

enzyme was solubilized. The GLDH activity decreased following the adsorption chromatographies (DEAE-, Q-, CM-, and Phenyl-Sepharose) because the cold-active enzyme is generally labile (*41*). The purified enzyme was observed as a nearly single band on native-PAGE, as shown in Fig. 2A. The molecular mass of the native GLDH was determined to be around 110 kDa by gel filtration (data not shown). The chromatogram suggested that the active fraction consists of a single enzyme because a symmetrical peak appeared at the location of the active fraction corresponding to a molecular mass of 110 kDa (data not shown). On the other hand, two different protein bands were observed on SDS-PAGE, as shown in Fig. 2B, with apparent molecular masses of about 76 and 34 kDa, respectively. This result suggests that the enzyme consists of two hetero-subunits. The isoelectric point of the GLDH from strain L101 was estimated to be 5.7-5.8, as shown in Fig. 2C.

Reported L-glutamate dehydrogenases consists of homo-subunits, such as the hexamer GLDH from porcine liver (M.W. of subunits, 52,000) (*42*) and the tetramer GLDH from *Neurospora crassa* (M.W. of subunits, 111,000-121,000) (*43*). The reported L-glutamate oxidases are also dimers, such as the enzyme from *Streptomyces edus* (M.W. of subunits, 50,000) (*4*). However, the GLDH from psychrotrophic strain L101 consists of two heterosubunits (76 kDa, 34 kDa).

Enzymatic Products and Electron Acceptors—Neither hydrogen peroxide production nor changes in the dissolved oxygen concentration were observed during the enzyme reaction. The indophenol blue test and HPLC indicated

TABLE II. **Purification of L-glutamate dehydrogenase from *Aeromonas* sp. strain L101.**

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extracts	3,300	91.25	0.028	100.0	1.0
DEAE-Sepharose	1,267	45.55	0.036	49.9	1.3
Ammonium sulfate	1,050	30.16	0.029	33.1	1.0
Superdex 200pg	776	19.13	0.025	21.0	0.9
Q-Sepharose	120	10.56	0.088	11.6	3.1
CM-Sepharose	5	1.73	0.346	1.92	12.4
Phenyl-Sepharose	0.37	0.73	1.960	0.80	70.0

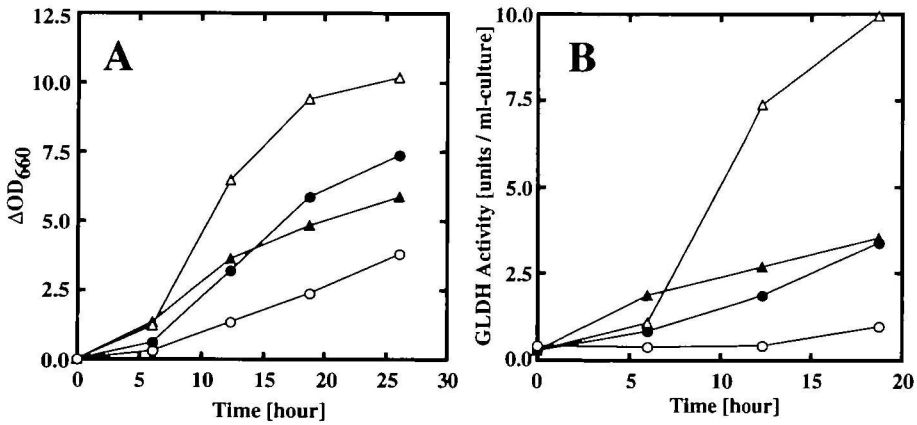

Fig. 1. **Growth of strain L101 (A) and the production of L-glutamate dehydrogenase (B) at 5°C (○), 10°C (●), 15°C (△), and 20°C (▲).** Strain L101 was cultured with shaking in a 100-ml flask in 20 ml of nutrient broth medium. ΔOD_{660} indicates the change in OD_{660} (optical density 660 nm) with time.

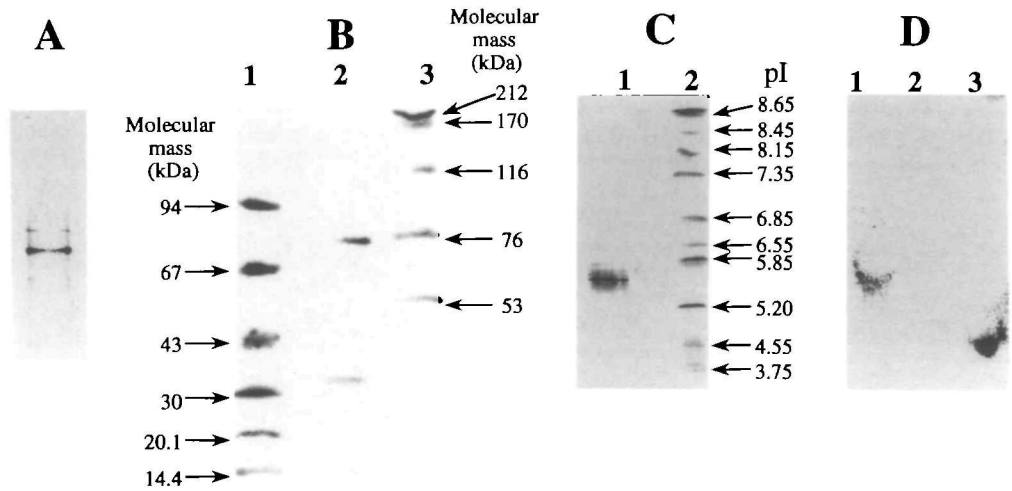

Fig. 2. **Electrophoresis of the GLDH.** (A) Native-PAGE (7.5%) of L-glutamate dehydrogenase. (B) SDS-PAGE (8-25%) of L-glutamate dehydrogenase. Lane 2, purified enzyme. Lane 1, low molecular mass markers: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). Lane 3, high molecular mass markers: myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), glutamic dehydrogenase (53 kDa). (C) Isoelectric point of L- glutamate dehydrogenase. Lane 1, purified enzyme. Lane 2, isoelectric point markers; amyloglucosidase (3.50), methyl red (3.75), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin-acidic (6.85), horse myoglobin-basic (7.35), lentil lectine-acidic (8.15), lentil lectine-middle (8.45), lentil lectine-basic (8.65), trypsinogen (9.30). (D) Activity staining with L-glutamate and NBT. Lane 1, purified enzyme. Lane 2, L-glutamate dehydrogenase from bovine liver. Lane 3, L-glutamate oxidase from *Streptomyces* sp. X119-6.

ammonia and 2-oxoglutarate respectively, as the products of the oxidation of L-glutamate. Two moles of ammonia and 2-oxoglutarate were produced stoichiometrically with the use of 1 mol NBT as an electron acceptor. GLDH catalyzes the oxidative deamination of L-glutamate as shown by the following enzymatic reaction:

$$2\ \text{L-glutamate} + \text{NBT}^{2+} + 2\ \text{H}_2\text{O} \longrightarrow 2\ \text{2-oxoglutarate} + 2\ \text{NH}_4^+ + \text{formazan}.$$

Figure 2D shows the activity staining for GLDH separated by native gel electrophoresis, NAD-dependent GLDH from bovine liver and the FAD type of GLOX from *Streptomyces* sp. X119-6. Our GLDH and GLOX showed activity staining with NBT, however, GLDH from bovine liver was inactive in the absence of $NAD(P)^+$.

Our GLDH was not coupled with NAD^+ or $NADP^+$, however, it reacted with several redox dyes including 1-methoxy PMS, DCIP, meldola's blue, NBT, and INT. The activity with 1-methoxy PMS was 8 times higher than with NBT, as shown in Table III. The redox potential is insufficient for a discussion of the reduction rate, because conformational affinity is thought to be more important between GLDH and the electron acceptor.

The purified enzyme is brown in color. The visible and ultraviolet absorption spectra of the purified enzyme are shown in Fig. 3A. In the visible range, an absorption maximum at 407 nm can be seen. Reduction of the enzyme with sodium dithionite or L-glutamate resulted in the appearance of a peak at 556 nm and a shift in the 407 nm peak to 416 nm was observed. The maximum at 556 nm in the reduced enzyme corresponded to the position of the α-band of *b*-type or *c*-type cytochrome. However, the reduced enzyme showed no peak around 520 nm corresponding to the position of the β-band of cytochrome.

The iron content of the enzyme was measured by inductively coupled plasma. The iron concentration of the enzyme solution was 22.2 μM, and the protein concentration of the enzyme solution was 22.6 μM. A difference spectrum of the reduced minus oxidized forms of the purified enzyme showed α, β, and γ peaks at 556, 524, and 418 nm, respectively (Fig. 3B). The pyridine ferrohemochrome spectrum of the enzyme showed α and β peaks at 556 and 523 nm, respectively (Fig. 3C). This indicates that the enzyme contains heme *b* (*44-46*). The enzyme has one iron atom per enzyme molecule, and also shows peroxidase activity like a heme-containing protein. This indicates that the enzyme has heme *b* as a prosthetic group.

The enzyme reacts with cytochrome *c* and redox dye as an electron acceptor, while no reaction is observed with oxygen, NAD^+, and $NADP^+$. This indicates that the enzyme is an acceptor type of enzyme. NAD^+ or/and $NADP^+$ as electron acceptors are used in the oxidation of L-glutamate by reported GLDH. Oxygen is used in the oxidation of L-glutamate through FAD of L-glutamate oxidase. No acceptor type of L-glutamate dehydrogenase has been reported.

Strain L101 also shows high cytochrome *c* oxidase activity. Cytochrome *c* is oxidized by this oxidase and used as an electron acceptor by GLDH. The acceptor types of enzyme include flavoproteins (*47, 48*), heme-containing enzymes (*48-50*), quinoproteins (*50*) and so on.

Effect of Temperature and pH—The optimal temperature for the reaction was determined to be 55°C in 20 mM

TABLE III. **Electron acceptors for L-glutamate dehydrogenase from the strain L101.**[a]

Electron acceptor	Reduction rate (%)	Redox potential (V)
1-Methoxy PMS	851	+0.06
DCIP	212	+0.67
Meldola's blue	191	+0.13
INT	141	−0.09
Cytochrome *c*	134	+0.22
NBT	100	−0.05
NAD^+	0	−0.32
$NADP^+$	0	−0.32
Oxygen[b]	0	+0.82

[a]L-Glutamate dehydrogenase was estimated spectrophotometrically using 50 mM L-glutamate and 1 mM of electron acceptor in 20 mM Tris-HCl buffer (pH 9.0) at 20°C. The linear absorbance increase was followed for ~2 min with a recording spectrophotometer, and enzyme activity was calculated from the ΔA/min. 100% activity is 1 μmol of electron acceptor reduced per min per mg of protein. [b]The reduction rate was detected by the decrease in the current of an oxygen electrode per min per mg of protein. The initial concentration of oxygen was 0.28 mM, a saturating concentration at 20°C in 20 mM Tris-HCl buffer (pH 9.0).

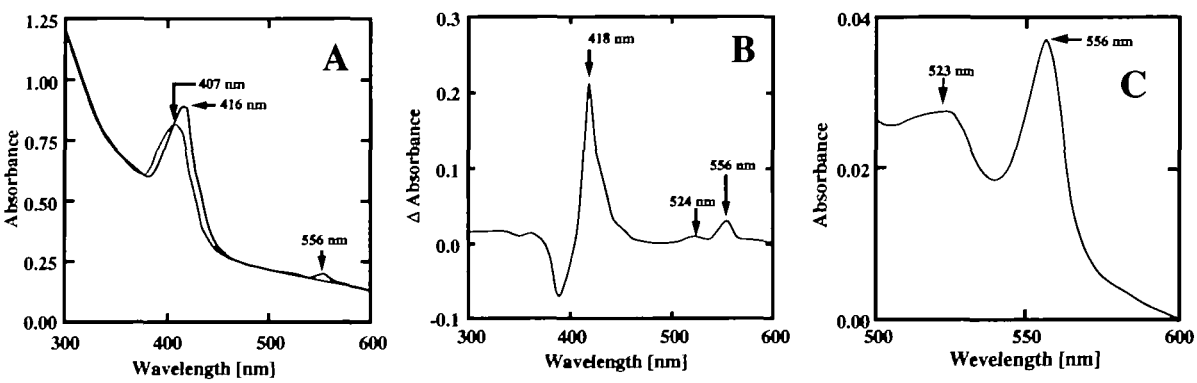

Fig. 3. **Ultraviolet/visible absorption spectra of reduced and oxidized GLDH in 50 mM phosphate buffer (pH 7.0), and the pyridine ferrohemochrome spectrum.** (A) Ultraviolet/visible spectra of (—) oxidized and (—) reduced GLDH. The concentration of the enzyme was 1.5 mg/ml. Reduction of GLDH was performed by adding a few crystals of sodium dithionite. Oxidation of GLDH was performed by adding a few crystals of potassium ferricyanide. (B) Difference spectrum between reduced and oxidized GLDH spectrum minus the oxidized GLDH spectrum. (C) The pyridine ferrohemochrome spectrum. Pyridine ferrohemochrome was formed in 0.15 M NaOH and 0.2 M pyridine by the addition of dithionite.

phosphate buffer, pH 7, as shown in Fig. 4A. The optimal temperature for GLDH from mesophilic bacteria is around 60°C (*51, 52*). Thus, a shift toward a lower temperature of at least 10°C was observed for our GLDH. Our GLDH has the characteristics of a typical psychrotrophic enzyme. Similar properties, including optimal activity at low temperature, have been observed for other reported enzymes from psychrophiles. For example, histidinol dehydrogenase from *Bacillus psychrophilus* has an optimal temperature of 25°C at pH 9.8 (*34*); a psychrophilic *Bacillus psychrosaccharolyticus* produces lactate dehydrogenase with an optimal temperature of 25°C (*35*); a psychrotroph, *Pseudomonas fluorescens* 114, produces a protease with an optimal temperature of 35°C (*25*); an Antarctic yeast, *Candida humicola*, produces a protease with an optimal temperature of 37°C (*53*). Figure 4B shows Arrhenius plots of our GLDH. The plots show a decrease at higher temperatures due to protein denaturation, while they show linearity at lower temperatures. The activation energies of cold-active and mesophilic GLDH were calculated from their slopes ($E_a = -\text{slope} \times 8.314$ J/mol·K) as 7.4 kJ/mol for cold-active GLDH, 12.9 kJ/mol for mesophilic GLDH from bovine liver, and 21.3 kJ/mol for thermophilic GLDH from *Phormidium laminosum* (*54*). According to this result, cold-active GLDH is a good catalyst with a lower activation energy than mesophilic GLDH. The thermal stability of our GLDH was assessed in 20 mM phosphate buffer, pH 7, after heating for 20 min at various temperatures (5-50°C). The enzyme was stable at temperatures below 30°C, as shown in Fig. 4C. However, mesophilic GLDH from beef liver was stable at 60°C. Thus, Our GLDH is thermolabile, compared with its mesophilic counterparts. The thermolability of the enzyme is assumed to be caused by its loose structure, which is easily denatured by changes in external parameters, such as temperature. The enzyme is also stable at low temperature. The enzyme is not a cold-labile enzyme such as NAD-dependent L-glutamate dehydrogenase from *Bacillus* species (*9, 21*). Thus, our GLDH is tolerant to a cold environment. To be cold-active, an enzyme must have a temperature-dependent activity curve that is shifted toward low temperatures and limited thermal stability due to its fast denaturation at moderate temperature (*42*). Because we found these properties in our GLDH, we conclude that it is a cold-active enzyme.

The pH characteristics of our GLDH were determined using NBT and L-glutamate as substrates. Two buffers covered the range between pH 6 and 11. The highest enzyme activity was observed at pH 8.5 (in 100 mM borate buffer) at 20°C, as shown in Fig. 5A. The enzyme retained ~80% of its activity when stored at 20°C for 20 min over a pH range of 6.0-11.0, as shown in Fig. 5B. When stored below pH 5.0 or above pH 12, the enzyme activity was

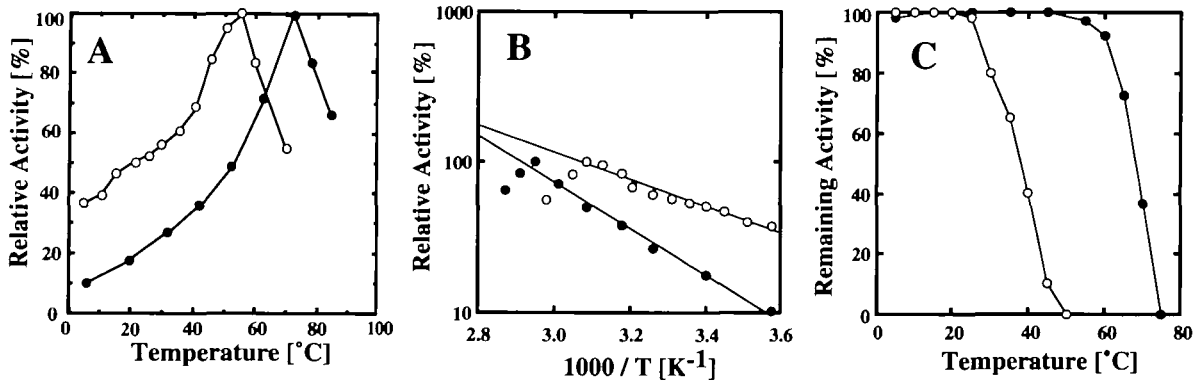

Fig. 4. **Effect of temperature on enzyme activity and stability.** (A) The enzyme activity of GLDH from strain L101 was measured by following the rate of reduction of NBT in 20 mM phosphate buffer (pH 7) using 50 mM L-glutamate and 1 mM NBT for 2 min (○). The enzyme activity of GLDH from beef liver was measured by following the rate of reduction of NAD^+ in 20 mM phosphate buffer (pH 7) using 50 mM L-glutamate and 1 mM NAD^+ for 2 min (●). (B) Arrhenius plots were obtained by plotting the logarithm of the relative activity for the reciprocal of the absolute temperature. The activation energy was 7.4 kJ/mol for GLDH from strain L101 (○) and 12.9 kJ/mol for GLDH from bovine liver (●). (C) Enzyme stability was determined by measuring the remaining activity of GLDH from strain L101 (○) and GLDH from bovine liver (●) after incubation for 20 min in 20 mM phosphate buffer (pH 7).

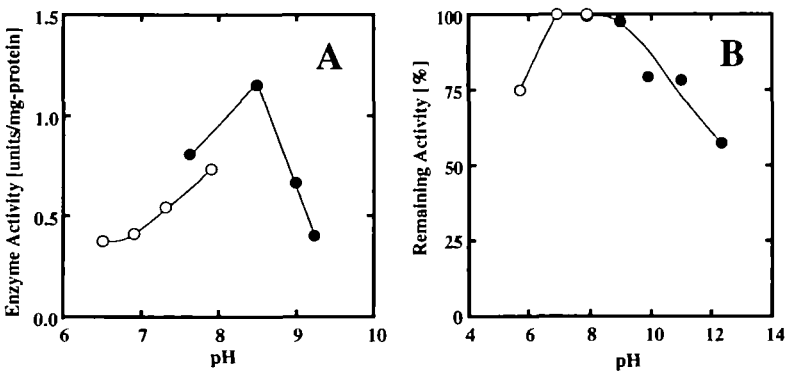

Fig. 5. **Effect of pH on the enzyme activity and stability of GLDH from strain L101.** (A) Enzyme activity was measured at 20°C in 100 mM buffer (phosphate buffer (pH 5.5-8) (○) or borate buffer (pH 8-12) (●)) containing 50 mM L-glutamate and 1 mM NBT for 2 min. (B) The purified enzyme was incubated for 20 min at 20°C in each buffer. After incubation, the remaining activity was determined in 500 mM phosphate buffer (pH 7) containing 50 mM L-glutamate and 1 mM NBT.

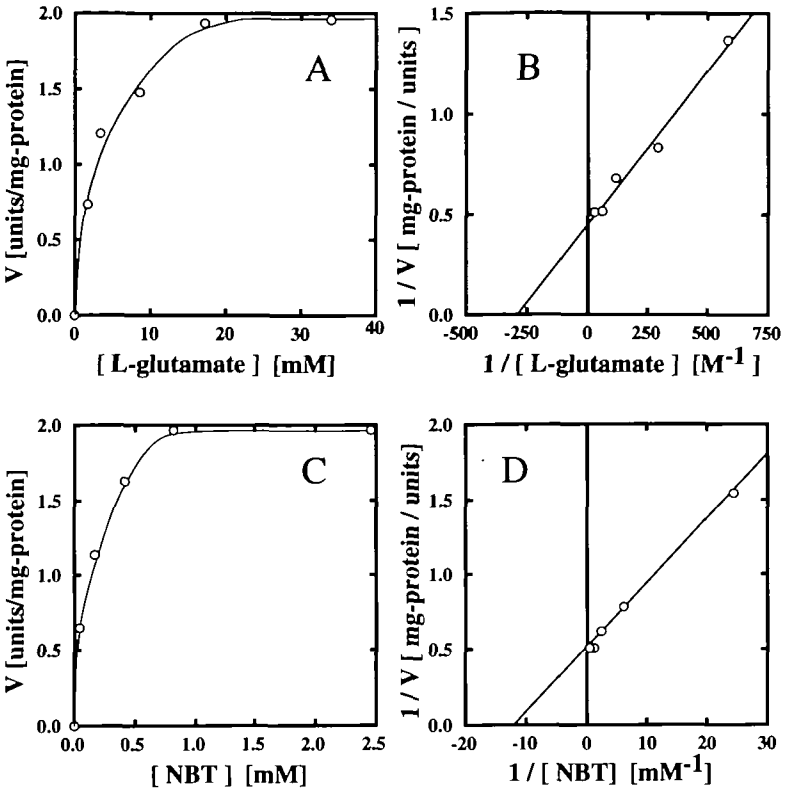

Fig. 6. **Effect of L-glutamate and NBT concentration on the activity of L-glutamate dehydrogenase from strain L101.** (A) Reaction velocity of our GLDH *versus* L-glutamate concentration at a fixed NBT concentration of 1 mM. Reaction velocity was measured by following the rate of reduction of NBT at 530 nm in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM NBT and L-glutamate at 20°C. (B) Lineweaver-Burk plots for L-glutamate at a fixed NBT concentration of 1 mM. (C) Reaction velocity of our GLDH *versus* L-glutamate concentration at a fixed L-glutamate concentration of 50 mM. Reaction velocity was measured by following the rate of reduction of NBT at 530 nm in 20 mM sodium phosphate buffer (pH 7.0) containing 50 mM L-glutamate and NBT at 20°C. (D) Lineweaver-Burk plots for NBT at a fixed L-glutamate concentration of 50 mM.

TABLE IV. **Substrate specificity of the L-glutamate dehydrogenase from strain L101.**[a]

Substrate	Oxidation rate (%)	Substrate	Oxidation rate (%)
L-Glutamate	100.0	L-Glycine	2.7
D-Glutamate	0.0	L-Asparagine	1.3
L-Aspartate	15.4	L-Arginine	0.0
L-Glutamine	10.3	L-Lysine	0.0
L-Alanine	8.9	L-Threonine	0.0
L-Leucine	8.4	L-Tyrosine	0.0
L-Methionine	8.4	L-Cysteine	0.0
L-Isoleucine	7.7	L-Histidine	0.0
L-Phenylalanine	6.8	L-Serine	0.0
L-Proline	6.6	L-Tryptophane	0.0
L-Valine	5.6		

[a]L-Glutamate dehydrogenase was estimated spectrophotometrically using 50 mM substrate and 1 mM NBT in 20 mM Tris-HCl buffer (pH 9.0) at 20°C. The linear absorbance increase was followed for ~2 min with a recording spectrophotometer, and the enzyme activity was calculated from the ΔA/min and the enzyme activity is calculated. The 100% activity is 1 μmol of NBT reduced per min per mg of protein.

completely lost within 5 h.

Catalytic Parameters and Substrate Specificity—Figure 6, A and C, shows the reaction velocity of our GLDH *versus* L-glutamate and NBT concentration, respectively. The maximum reaction velocity (V_{max}) was obtained at over 20 mM for L-glutamate and over 1 mM for NBT. Figure 6, B and D, shows Lineweaver-Burk plots of our GLDH for L-glutamate and NBT, respectively. The V_{max} values calculated from Fig. 6, B and C, were 2.23 and 1.92 units/mg-protein. The K_m of our GLDH for L-glutamate was 3.4 mM, similar to values for mesophilic enzymes, such as GLDH from beef liver (1.8 mM), from chicken liver (2.0 mM), from dogfish liver (2.5 mM), and from *Achya* (3.1 mM) (*1*). The K_m value of our GLDH for NBT was 82 μM.

Substrate specificity against L-amino acids and D-glutamate was examined (Table IV). The L-glutamate oxidation rate was the highest among L-amino acids. The enzyme also reacted with some other L-amino acids. The oxidation rate of L-aspartate and L-glutamine were 15.4 and 10.3% of the L-glutamate oxidation rate, but the enzyme showed no oxidation activity toward D-glutamate, L-threonine, L-tyrosine, L-cysteine, L-histidine, L-tryptophane, or L-serine. The substrate specificity of GLDH was as high as that of both GLDH from bovine liver (*12*) and GLOX from *Streptomyces endus* (*4*).

Oxidases and acceptor type dehydrogenases were electrochemically coupled with a ferrocene-modified electrode (*55*). The catalytic currents with the ferrocene-modified electrode were compared between mesophilic glutamate oxidase and our psychrophilic GLDH. Mesophilic glutamate oxidase gave no catalytic current below 15°C, while psychrophilic GLDH clearly expressed activity down to 5°C (data not shown). Glutamate sensors reported previously could not be previously reported glutamate sensors do not operate in a cold environment (*56-60*). Our GLDH can be used by biosensors and bioreactors operated at low temperatures.

REFERENCES

1. Smith, E.L., Austein, B.M., Blumenthal, K.M., and Nyc, J.F. (1975) Glutamate dehydrogenases in *The Enzymes* (3rd ed.) (Boyer, P.D., ed.) Vol. 11A, pp. 239-367, Academic Press, New York

2. Jurtshuk, P. and McManus, L. (1974) Non-pyridine nucleotide dependent L-(+)-glutamate oxidoreductase in *Azotobacter vinelandii*. *Biochim. Biophys. Acta* **368**, 158-172
3. Rocca, E. and Ghiretti, F. (1958) Purification of properties of D-glutamic acid oxidase from *Otopus vulgaris* Lam. *Arch. Biochem. Biophys.* **77**, 336-349
4. Böhmer, A., Müller, A., Passarge, M., Liebs, P., Honeck, H., and Müller, H.G. (1987) A novel L-glutamate oxidase from *Streptomyces endus* purification and properties. *Eur. J. Biochem.* **182**, 327-332
5. Venard, R. and Fourcade, A. (1972) Glutamate déshydrogénase de levure spécifique du NADP. *Biochimie* (*Paris*) **54**, 1381-1389
6. Neumann, P., Markau, K., and Sund, H. (1976) Studies of glutamate dehydrogenase. Regulation of glutamate dehydrogenase from *Candida utilis* by a pH and temperature-dependent conformational transition. *Eur. J. Biochem.* **65**, 465-472
7. Kimura, K., Miyakawa, A., Imai, T., and Sasakawa, T. (1977) Glutamate dehydrogenase from *Bacillus subtilis* PCI 219. I. Purification and properties. *J. Biochem.* **81**, 467-476
8. Sakamoto, N., Korte, A.M., and Savageau, M.A. (1975) Glutamate dehydrogenase from *Escherichia coli*: purification and properties. *J. Bacteriol.* **124**, 775-783
9. Glass, T.L. and Hylemon, P.B. (1980) Characterization of a pyridine nucleotide-nonspecific glutamate dehydrogenase from *Bacteroides thetaiotaomocron*. *J. Bacteriol.* **141**, 1320-1330
10. Phibbs, Jr. P.V. and Bernlohr, R.W. (1971) Purification, properties and regulation of glutamic dehydrogenase of *Bacillus licheniformis*. *J. Bacteriol.* **106**, 375-385
11. Hemmilä, I.A. and Mäntsälä, P.I. (1978) Purification and properties of glutamate synthase and glutamate dehydrogenase from *Bacillus megaterium*. *Biochem. J.* **173**, 45-52
12. Joannou, C.L. and Brown, P.R. (1992) NAD-dependent glutamate dehydrogenase from *Pseudomonas aeruginosa* is a membrane-bound enzyme. *FEMS Microbiol. Lett.* **90**, 205-210
13. Coulton, J.W. and Kapoor, M. (1973) Purification and some properties of the glutamate dehydrogenase of *Salmonella typhimurium*. *Can. J. Microbiol.* **19**, 427-438
14. Winnacker, E.L. and Barker, H.A. (1970) Purification and properties of a NAD-dependent glutamate dehydrogenase from *Clostridium* SB$_4$. *Biochim. Biophys. Acta* **212**, 225-242
15. Bartholmes, P. and Jaenicke, R. (1968) Glutamatdehydrogenase im photosynthetischen bakterium *Rhodospirillum rubrum*. *Arch. Mickrobiol.* **60**, 235-245
16. Chávez, S. and Candau, P. (1991) An NAD-specific glutamate dehydrogenase from cyanobacteria. Identification and properties. *FEBS Lett.* **285**, 35-38
17. Yarrison, G., Young, D.W., and Choules, G.L. (1972) Glutamate dehydrogenase from *Mycoplasma laidawii*. *J. Bacteriol.* **110**, 494-503
18. Maulik, P. and Ghosh, S. (1986) NADPH/NADH-dependent cold-labile glutamate dehydrogenase in *Azospirillum brasilense*. *Eur. J. Biochem.* **155**, 595-602
19. Jahns, T. (1992) Occurrence of cold-labile NAD-specific glutamate dehydrogenase in *Bacillus* species. *FEMS Microbiol. Lett.* **96**, 187-192
20. Jahns, T. and Kaltwasser, H. (1993) Properties of the cold-labile NAD-specific glutamate dehydrogenase from *Bacillus cereus* DSM 31. *J. Gen. Microbiol.* **139**, 775-780
21. Gounot, A.M. (1986) Psychrophilic and psychrotrophic microorganisms. *Experientia* **42**, 1192-1197
22. Margesin, R. and Schinner, F. (1994) Properties of cold-adapted microorganisms and their potential role in biotechnology. *J. Biotech.* **33**, 1-14
23. Morita, R.Y. (1975) Psychrophilic bacteria. *Bacteriol. Rev.* **39**, 144-167
24. Davail, S., Feller, G., Narinx, E., and Gerday, C. (1994) Cold adaptation of proteins, purification, characterization, and sequence of the heat-labile subtilisin from the antarctic psychrophile *Bacillus* TA41. *J. Biol. Chem.* **269**, 17448-17453
25. Hamamoto, T., Kanada, M., Horikoshi, K., and Kudo, T. (1994) Characterization of a protease from a psychrotroph, *Pseudomonas fluorescens* 114. *Appl. Environ. Microbiol.* **60**, 3878-3880
26. Morita, Y., Nakamura, T., Hasan, Q., Murakami, Y., Yokoyama, K., and Tamiya, E. (1997) Cold-active enzymes from cold-adapted bacteria. *JAOCS* **74**, 441-444
27. Ray, M.K., Devi, K.U., Kumar, G.S., and Shivaji, S. (1992) Extracellular protease from the antarctic yeast *Candida humicola*. *Appl. Environ. Microbiol.* **58**, 1918-1923
28. Feller, G., Lonhienne, T., Deronne, C., Libioulle, C., Beeumen, J.V., and Gerday, C. (1992) Purification, characterization, and nucleotide sequence of the thermolabile α-amylase from the antarctic psychrotroph *Alteromonas haloplanctis* A23. *J. Biol. Chem.* **267**, 5217-5221
29. Trimbur, D.E., Gutshall, K.R., Prema, P., and Brenchley, J.E. (1994) Characterization of a psychrotrophic *Arthrobacter* gene and its cold-active β-galactosidase. *Appl. Environ. Microbiol.* **60**, 4544-4552
30. Rentier, F., Mande, S.C., Moyens, S., Terpstra, P., Mainfroid, V., Goraj, K., Lion, M., Hol, W.G.J., and Martial, J.A. (1993) Cloning and overexpression of the triosephosphate isomerase gene from psychrophilic and thermophilic bacteria structural comparison of the predicted protein sequences. *J. Mol. Biol.* **229**, 85-93
31. Ishii, A., Imagawa, S., Fukunaga, N., Sasaki, S., Minowa, O., Mizuno, Y., and Shiokawa, H. (1987) Isozyme of isocitrate dehydrogenase from an obligately psychrophilic bacterium, *Vibrio* sp. strain ABE-1: purification, and modulation of activities by growth conditions. *J. Biochem.* **102**, 1489-1498
32. Ishii, A., Suzuki, M., Sahara, T., Takada, Y., Sasaki, S., and Fukunaga, N. (1993) Genes encoding two isocitrate dehydrogenase isozymes of a psychrophilic bacterium, *Vibrio* sp. strain ABE-1. *J. Bacteriol.* **175**, 6873-6880
33. Ochiai, T., Fukunaga, N., and Sasaki, S. (1984) Two structurally different NADP-specific isocitrate dehydrogenases in an obligately psychrophilic bacterium, *Vibrio* sp. strain ABE-1. *J. Gen. Appl. Microbiol.* **30**, 479-481
34. Lindsay, J.A. and Creaser, E.H. (1977) Purification and properties of histidinol dehydrogenases from psychrophilic, mesophilic and thermophilic bacilli. *Biochem. J.* **165**, 247-253
35. Vckovski, V., Schlatter, D., and Zuber, H. (1990) Structure and function of L-lactate dehydrogenases from thermophilic, mesophilic and psychrophilic bacteria, IX. *Biol. Chem. Hoppe-Seyler* **371**, 103-110
36. Zuber, H. (1988) Temperature adaptation of lactate dehydrogenase structural, functional and genetic aspects. *Biophys. Chem.* **29**, 171-179
37. Feinstein, R.N. and Lindahl, R. (1973) Detection of oxidases on polyacrylamide gels. *Anal. Biochem.* **56**, 353-360
38. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
39. Horn, D.B. and Squire, C.R. (1966) The estimation of ammonia using the indophenol blue reaction. *Clin. Chim. Acta* **14**, 185-194
40. Popoff, M. (1936) Genus III. *Aeromonas*. *Bergey's Manual of Systematic Bacteriology* **1**, 545-550
41. Feller, G., Narinx, E., Arpigny, J.L., Aittaleb, M., Baise, E., Genicot, S., and Gerday, C. (1996) Enzymes from psychrophilic organisms. *FEMS Microbiol. Rev.* **18**, 189-202
42. Pantaloni, D. and Dessen, P. (1969) Glutamate dehydrogenase. Attachment of NAD and NADP coenzymes and other nucleotides derived from adenosine-5′-phosphate. *Eur. J. Biochem.* **11**, 510-519
43. Veronese, F.M., Nyc, J.F., Degani, Y., Brown, D.M., and Smith, E.L. (1974) Nicotinamide adenine dinucleotide-specific glutamate dehydrogenase of *Neurospora*. I. Purification and molecular properties. *J. Biol. Chem.* **249**, 7922-7928
44. Yamanaka, T. and Okunuki, K. (1974) Cytochromes in *Microbial Iron Metabolism* (Yamanaka, T. and Okunuki, K., eds.) pp. 1-28, Academic Press, London/New York
45. Falk, J.E. (1964) *Porphyrins and Metalloporphyrins*, pp. 20-56, Elsevier, Amsterdam
46. Lemberg, R. and Barrett, J. (1973) *The Cytochromes*, Academic Press, London/New York
47. Olsiewski, P.J., Kaczorowski, G.J., and Walsh, C. (1980)

Purification and properties of D-amino acid dehydrogenase, an inducible membrane-bound iron-sulfur flavoenzyme from *Escherichia coli*. *J. Biol. Chem.* **255**, 4487-4494
48. Sharp, R.E., Chapman, S.K., and Reid, G.A. (1996) Deletions in the interdomain hinge region of flavocytochrome b2: effect on intraprotein electron transfer. *Biochemistry* **35**, 891-899
49. Durham, D.R. and Perry, J.J. (1978) Purification and characterization of a heme-containing amine dehydrogenase from *Pseudomonas putida*. *J. Bacteriol.* **134**, 837-843
50. Jong, G.A.H., Caldeira, J., Sun, J., Jongejan, J.A., Vries, S., Loehr, T.M., Moura, I., Moura, J.J.G., and Duine, J.A. (1995) Characterization of the interaction between PQQ and heme *c* in the quinohemoprotein ethanol dehydrogenase from *Comamonas testosteroni*. *Biochemistry* **34**, 9451-9458
51. Op den Camp, H.J., Liem, K.D., Meesters, P., Hermans, J.M., and Van der Drift, C. (1989) Purification and characterization of the NADP-dependent glutamate dehydrogenase from *Bacillus fastidiosus*. *Antonie Van Leeuwenhoek* **55**, 303-311
52. Moyano, E., Cardenas, J., and Munoz-Blanco, J. (1992) Purification and properties of three NAD(P)$^+$ isozymes of L-glutamate dehydrogenase of *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta* **1119**, 63-68
53. Ray, M.K., Devi, K.U., Kumar, G.S., and Shivaji, S. (1992) Extracellular protease from the antarctic yeast *Candida humicola*. *Appl. Environ. Microbiol.* **58**, 1918-1923
54. Martinez-Bilbao, M., Martinez, A., Urkijo, I., Llama, M.J., and Serra, J.L. (1988) Induction, isolation, and some properties of the NADPH-dependent glutamate dehydrogenase from the nonheterocystous cyanobacterium *Phormidium laminosum*. *J. Bacteriol.* **170**, 4897-4902
55. Turner, A.P.F. (1988) Amperometric biosensors based on mediator-modified electrodes. *Methods Enzymol.* **137**, 90-103
56. Tamiya, E. and Karube, I. (1992) Ultramicrobiosensors for monitoring of neurotransmitters. *Ann. N.Y. Acad. Sci.* **672**, 272-277
57. Tamiya, E., Sugiura, Y., Takeuchi, T., Suzuki, M., and Karube, I. (1993) Ultra micro glutamate sensor using platinized carbon fiber electrode and integrated counter electrode. *Sensor Actuators* **8**, 219-228
58. Tamiya, E., Sugiura, Y., Amou, Y., Karube, I., Kado, R.T., and Ito, M. (1995) Direct monitoring of glutamate released from cerebellar neurons using platinized carbon disk microelectrode modified with glutamate-oxidase-bound membrane. *Sensor Materials* **7**, 249-260
59. Wang, A.J. and Arnold, M.A. (1992) Dual-enzyme fiber-optic biosensor for glutamate based on reduced nicotinamide adenine dinucleotide luminescence. *Anal. Chem.* **64**, 1051-1055
60. Minami, H., Sugawara, M., Odashima, K., and Umezawa, Y. (1991) Ion channel sensors for glutamic acid. *Anal. Chem.* **63**, 2787-2795