Screening and Characterization of Cold-Active L-Glutamate Dehydrogenase

Akira Yamamura

School of Materials Science,
Japan Advanced Institute of Science and Technology

(Supervised by Kenji Yokoyama)

Keywords: psychrotrophic bacteria, L-glutamate dehydrogenase, heme-containing protein, heme *b*, biosensor

Chapter 1 Introduction

Enzyme is a biocatalyst with excellent reaction selectivity. It has been reported over 2,700 different types of enzyme, and enzymes have been classified by types of catalysis reaction into six groups, such as oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Enzymes generally catalyzes reaction under a mild condition (neutral pH, normal temperature, and atmospheric pressure). However, some enzymes adapt to extreme environments, such as halophilic, osmophilic, barophilic, acidic, alkalophilic, thermophilic, and psychrophilic conditions. One important factor for enzyme activity and stability is the temperature, and enzymes have been divided into three broad categories: thermophilic, mesophilic, and psychrophilic (cold-active) enzymes. 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 *[1]*. 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 *[2-5]*, α-amylase *[6]*, β-galactosidase *[7]*, triosephosphate isomerase *[8]* and a few dehydrogenases *[9-14]*, have been found from psychrophiles or psychrotrophes. We have accomplished the purification and characterization of novel cold-active serine protease from *Flavobacterium balustinum [4]* 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 *[9-11]*, histidinol dehydrogenase *[12]*, and lactate dehydrogenase *[13,14]*.

So far, enzymatic oxidation of L-glutamate have 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:

L-Glutamate + $NAD(P)^+$ + H_2O $\rightleftharpoons$ 2-Oxoglutarate + NH_4^+ + NAD(P)H.

This oxidation is generally considered to be a dehydrogenase type reaction (EC 1.4.1.2-4) *[15]*.The reaction can be distinguished from oxidase type reaction by L-glutamate oxidase (GLOX) in the utilization of molecular oxygen as given below (EC 1.4.3.11) *[16]*:

2 L-Glutamate + O_2 + H_2O → 2 2-Oxoglutarate +2 NH_4^+ + H_2O.

Stoichiometry, one molecule oxygen can oxidize two molecules of L-glutamate. Oxygen atom 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.) *[17]*:

D-Glutamate + O_2 + H_2O → 2-Oxoglutarate + NH_4^+ + H_2O_2.

In the oxidation of L-glutamate, a flavoenzyme, was purified from a gram positive bacteria, *Streptomyces endus*, forms hydrogen peroxide as a product *[18]*. However, there is no report on the presence of the glutamate dehydrogenase independent of $NAD(P)^+$ or molecular oxygen (acceptor type GLDH).

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

Chapter 2 Screening and identification of psychrotrophic bacteria containing L-glutamate dehydrogenase

In total, 136 strains of psychrotrophic bacteria were isolated from salmon, crab, and habitats, and their GLDH activities were tested using 3,3'-[3,3'-dimethoxy-(1,1'-biphenyl)-4,4'-diyl]-bis[2-(4-nitrophenyl-2H tetrazolium chloride] (nitroblue tetrazolium; NBT) without $NAD(P)^+$. Four strains (L101, P105, L301, and A105)showed high activity. The best cold-active GLDH producer, strain L101,isolated from the organs of salmon (*Oncorhynchus keta*).This bacterium is gram-negative and facultatively anaerobic. It is rod-shaped, and has a size approximately 0.8 x 2.0 μm and a single polar flagellum for motility, based on observations with ascanning 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. containing the family of *Vibrionaceae*. However, detailed analysis of the 16S rRNA sequence indicated that the strain L101 was most closely related to *Hafnia alvei* containing the family of *Enterobacteriaceae*.

Chapter 3 Purification of L-glutamate dehydrogenase

Strain L101 grows fastest at 15 °C, and the highest amount of GLDH activity was produced at 15 °C. Based on these results, the condition for the mass cultivation of strain L101 were determined at 15 °C. The GLDH was purified by several chromatographic steps, such as DEAE-Sepharose, Superdex 200pg, Q-Sepharose, CM-Sepharose and Phenyl-Sepharose. The final enzyme preparation obtained was purified about 70-fold over the crude extract.The molecular mass of the native GLDH was determined to be around 110 kDa by gel filtration. On the other hand, two different protein bands were observed on SDS-PAGE with apparent molecular masses of about 76 and 34 kDa, re-

spectively. This result suggests that the enzyme consists of two hetero-subunits. The isoelectric point of the GLDH from strain L101 was estimated to be 5.75.

Chapter 4 Characterization of L-glutamate dehydrogenase

Maximum activity was obtained at 55 °C and pH 8.5. The activities of GLDH at 4 °C and 20 °C were 38 % and 50 %, respectively, of that at 55 °C. GLDH was coupled to cytochrome *c* and several redox dyes including 1-methoxy-5-methylphenazinium methylsulfate (1-MethoxyPMS), 2,6-dichlorophenylindophenol (DCIP), 9-dimetylaminobenzo [α] phenoxazin-7-ium chloride (meldola's blue), NBT and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H tetrazolium (INT). NAD(P)$^+$ and oxygen gave no oxidation activity to GLDH.

Neither hydrogen peroxide production nor changes in the dissolved oxygen concentration were observed during the enzyme reaction. The indophenol blue test and HPLC indicated 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 mole NBT as electron acceptor. GLDH catalyzes the oxidative deamination of L-glutamate as shown by the following enzymatic reaction:

2 L-Glutamate + NBT^{2+} + 2 H_2O $\rightarrow$ 2 2-Oxoglutarate + 2 NH_4^+ + formazan.

The visible and ultraviolet absorption spectra of the purified enzyme were recorded in 0.1 M phosphate buffer (pH 7.0) at 20 °C with Beckman DU-640 spectrophotometer at a scan speed of 120 nm per minute. 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. 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 corresponded to the position of the β-band of cytochrome. An 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 *mu* M. 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 *[19-21]*. The alkaline pyridine ferrohemochrome spectrum of the enzyme showed α and β-peaks at 556 and 523 nm, respectively. This indicated that the enzyme contains heme *b [19-21]*. The enzyme had one iron atom per enzyme molecule, and also shows peroxidase activity like a heme-containing protein. This indicats that the enzyme has heme *b* as a prosthetic group.

Chapter 5 Application of biosensor using L-glutamate dehydrogenase

Oxidases and acceptor type dehydrogenases were electrochemically coupled with with a ferrocene-modified electrode *[22]*. 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. Glutamate sensors reported previously could not be previously reported glutamate sensors do not operate in a cold environment *[23-27]*. GLDH from strain L101 can be used by biosensors and bioreactors operated at low temperatures.

Chapter 6 Conclution

The best cold-active GLDH producer, strain L101,isolated from the organs of salmon, and strain L101 was novel psychrotrophic bacteria containing the family of *Enterobacteriaceae*.The molecular mass of the native GLDH was determined to be around 110 kDa, and GLDH was consisted by two hetero-subunits (about 76 and 34 kDa). GLDH was cold-active enzyme and acceptor type of dehydrogenase.GLDH had heme *b* as a prosthetic group. GLDH from strain L101 can be used by biosensors and bioreactors operated at low temperatures.

References

[1] Margesin, R. and Schinner, F. (1994) J. Biotech. **33**, 1-14

[2] Davail, S., Feller, G., Narinx, E., and Gerday, C. (1994) J. Biol. Chem. **269**, 17448-17453

[3] Hamamoto, T., Kanada, M., Horikoshi, K., and Kudo, T. (1994) Appl. Environ. Microbiol. **60**, 3878-3880

[4] 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

[5] 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

[6] Feller, G., Lonhienne, T., Deronne, C., Libioulle, C., Beeumen, J.V., and Gerday, C. (1992) J. Biol. Chem. **267**, 5217-5221

[7] Trimbur, D.E., Gutshall, K.R., Prema, P., and Brenchley, J.E. (1994) Appl. Environ. Microbiol. **60**, 4544-4552

[8] Rentier, F., Mande, S.C., Moyens, S., Terpstra, P., Mainfroid, V., Goraj, K., Lion, M., Hol, W.G.J., and Martial, J.A. (1993) J. Mol. Biol. **229**, 85-93

[9] Ishii, A., Imagawa, S., Fukunaga, N., Sasaki, S., Minowa, O., Mizuno, Y., and Shiokawa, H. (1987) J. Biochem. **102**, 1489-1498

[10] Ishii, A., Suzuki, M., Sahara, T., Takada, Y., Sasaki, S., and Fukunaga, N. (1993) J. Bacteriol. **175**, 6873-6880

[11] Ochiai, T., Fukunaga, N., and Sasaki, S. (1984) J. Gen. Appl. Microbiol. **30**, 479-481

[12] Lindsay, J.A., and Creaser, E.H. (1977) Biochem. J. **165**, 247-253

[13] Vckovski, V., Schlatter, D., and Zuber, H. (1990) Biol. Chem. Hoppe-Seyler. **371**, 103-110

[14] Zuber, H. (1988) Biophys. Chem. **29**, 171-179

[15] Smith, E.L., Austein, B.M., Blumenthal, K.M., and Nyc, J.F. (1975) In P. D. Boyer (ed.), The Enzymes (3rd edition) vol.11A. Academic Press, New York, 239-367

[16] Jurtshuk, P., and McManus, L. (1974) Biochim. Biophys. Acta **368**, 158-172

[17] Rocca, E., and Ghiretti, F. (1958) Arch. Biochem. Biophys. **77**, 336-349

[18] Böhmer, A., Müller, A., Passarge, M., Liebs, P., Honeck, H., and Müller, H.G. (1987) Eur. J. Biochem. **182**, 327-332

[19] Yamanaka, T., and Okunuki, K. (1974) Cytochromes. In: Microbial iron metabolism. Academic Press, London New York

[20] Falk, J.E. (1964) Porphyrins and metalloporphyrins. Elsevier, Amsterdam

[21] Lemberg, R., and Barrett, J. (1973) The cytochromes. Academic Press, London New York

[22] Turner, A.P.F. (1988) Methods Enzymol. **137**, 90-103

[23] Tamiya, E. and Karube, I. (1992) Ann. N.Y. Acad. Sci. **672**, 272-277

[24] Tamiya, E., Sugiura, Y., Takeuchi, T., Suzuki, M., and Karube, I. (1993) Sensor and Actuators **8**, 219-228

[25] Tamiya, E., Sugiura, Y., Amou, Y., Karube, I., Kado, R.T., and Ito, M. (1995) Sensor & Materials **7**, 249-260

[26] Wang, A.J. and Arnold, M.A. (1992) Anal. Chem. **64**, 1051-1055

[27] Minami, H., Sugawara, M., Odashima, K., and Umezawa, Y. (1991) Anal. Chem. **63**, 2787-2795

Publication list

[1] A. Yamamura, Y. Murakami, K. Yokoyama, and E. Tamiya (1995) DENKI KAGAKU **63**, 1195-1196

[2] A. Yamamura, T. Sakaguchi, Y. Murakami, K. Yokoyama, and E. Tamiya (1999) J. Biochem. **125**

Note: the thesis is written in Japanese.

February 12, 1999