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D-Lactate electrochemical biosensor prepared by immobilization of thermostable dye-linked D-lactate dehydrogenase from *Candidatus* Caldiarchaeum subterraneum

Takenori Satomura,[1,2,*] Junji Hayashi,[3] Hiroaki Sakamoto,[4] Takuro Nunoura,[5] Yoshihiro Takaki,[5] Ken Takai,[5] Hideto Takami,[5] Toshihisa Ohshima,[6] Haruhiko Sakuraba,[3] and Shin-ichiro Suye[1,2]

Division of Engineering, Faculty of Engineering, University of Fukui, 3-9-1 Bunkyo, Fukui 910-8507, Japan,[1] Organization for Life Science Advancement Programs, University of Fukui, 3-9-1 Bunkyo, Fukui 910-8507, Japan,[2] Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, 2393 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0795, Japan,[3] Tenure-Track Program for Innovative Research, University of Fukui, 3-9-1 Bunkyo, Fukui 910-8507, Japan,[4] Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan,[5] and Department of Biomedical Engineering, Faculty of Engineering, Osaka Institute of Technoligy, Ohmiya, 5-16-1 Asahi-ku, Ohsaka 535-8585 Japan[6]

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A stable D-lactate electrochemical sensing system was developed using a dye-linked D-lactate dehydrogenase (Dye-DLDH) from an uncultivated thermophilic archaeon, *Candidatus* Caldiarchaeum subterraneum. To develop the system, the putative gene encoding the Dye-DLDH from *Ca.* Caldiarchaeum subterraneum was overexpressed in *Escherichia coli*, and the expressed product was purified. The recombinant enzyme was a highly thermostable Dye-DLDH that retained full activity after incubation for 10 min at 70°C. The electrode for detection of D-lactate was prepared by immobilizing the thermostable Dye-DLDH and multi-walled carbon nanotube (MWCNT) within Nafion membrane. The electrocatalytic response of the electrode was clearly observed upon exposure to D-lactate. The electrode response to D-lactate was linear within the concentration range of 0.03–2.5 mM, and it showed little reduction in responsiveness after 50 days. This is the first report describing a D-lactate sensing system using a thermostable Dye-DLDH.

[**Key words:** D-Lactate; Electrochemical biosensor; Dye-linked dehydrogenase; Archaea; Thermophile]

D-Lactate is widely distributed within our bodies, and is also found in many of the foods we eat. Moreover, and the levels of D-lactate in human tissues are known to be closely associated with various disease conditions (1,2). For example, elevated D-lactate levels in plasma and urine may be related to diabetes (3–5). Accumulation of D-lactate is also triggered by appendicitis, short bowl syndrome, ischemia, and bacterial infection (6–9). Within the food industry, D-lactate is potentially useful as quality indicator. For example, elevated D-lactate concentrations in beer may indicate contamination with bacteria that cause undesirable changes in taste (10). D-Lactate could also be a good indicator of the quality of wine, milk, meat, and fruit juice (11,12). D-Lactate content is thus of great interest for both clinical diagnosis and food analysis.

Several analytical methods have been used to determine D-lactate levels in biological and food samples. One method is analysis using high performance liquid chromatography (HPLC). However, this method requires pretreatment of the samples to be analyzed and is time-consuming (5,13,14). As an alternative, an enzyme-based electrochemical system is useful, because it has high D-lactate selectivity and is a simpler procedure. Up to now, two types of D-lactate dehydrogenases (DLDHs) have been used as the elements in electrochemical D-lactate biosensors. The first is an NAD-dependent D-lactate dehydrogenase (NAD-DLDH), which catalyzes the oxidation of D-lactate to pyruvate in the presence of NAD as a cofactor (15–17). In this system, the D-lactate concentration is estimated from the increase in the amperometric current of NADH formed from NAD. This method has two major disadvantages, however: the cofactor is unstable in both its oxidized and reduced forms and the electrochemical oxidation of NADH produced by the NAD-DLDH-catalyzed reaction is necessary for electrochemical detection. As the direct electrochemical oxidation of NADH on conventional electrodes requires a high overvoltage and causes unwanted side reactions, additional redox mediators to decrease the overvoltage or an additional enzyme system coupled with NADH dehydrogenase is necessary.

The other type of DLDH is Dye-DLDH, which catalyzes the oxidation of D-lactate in the presence of an artificial dye such as 2,6-dichloroindophenole (DCIP). Dye-DLDH has an advantage over NAD-DLDH in that electrons from D-lactate can be introduced to an electrode using an artificial dye as the mediator. Dye-DLDHs are widely distributed in bacteria and eukarya (18–22). However, almost all Dye-DLDHs are associated with cell membranes from which they must be solubilized before their application to biosensors, as is the case with Dye-DLDH from baker's yeast (23). This solubilization entails several tedious procedures, including

* Corresponding author at: Division of Engineering, Faculty of Engineering, University of Fukui, 3-9-1 Bunkyo, Fukui 910-8507, Japan. Tel.: +81 776 27 8642; fax: +81 776 27 8747.
E-mail address: satomura@u-fukui.ac.jp (T. Satomura).

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detergent extraction and ultracentrifugation, and often results in a substantial loss of enzyme activity. Furthermore, difficulties are often encountered during purification of Dye-DLDH, even when produced as a recombinant enzyme. In the case of recombinant Dye-DLDH from *Hansenula polymorpha*, cell debris is directly used for immobilization without purification (24). Because of the associated disadvantages, the application of Dye-DLDH to D-lactate biosensors has so far been limited.

On the other hand, we recently detected two different kinds of Dye-DLDHs in the hyperthermophilic crenarchaeota, *Sulfolobus tokodaii* and *Aeropyrum pernix*, respectively (25,26). As these enzymes are located on the surface of the cytoplasmic membrane and are easily released, no special solubilization procedure is required. On the basis of its genomic information, in this study we identified Dye-DLDH from a thermophilic aigarchaeon, *Candidatus* Caldiarchaeum subterraneum (27). We then succeeded in producing the Dye-DLDH as a soluble protein in *Escherichia coli* and purified the enzyme with no solubilization process. After its characterization, we developed an electrochemical D-lactate sensing system using this Dye-DLDH as a sensor element.

MATERIALS AND METHODS

Materials Sodium D-lactate and Nafion perfluorinated resin solution were obtained from Sigma Aldrich (St. Louis, MO, USA). (*R*)-2-Hydroxybutyrate was from Santa Cruz Biotechnology (Dallas, TX, USA). Tryptone and yeast extract were from Difco Laboratories (Sparks, MD, USA). *p*-Iodonitrotetrazolium violet (INT) and DCIP were from MP Biomedicals, Inc. (Solom, OH, USA). Multi-walled carbon nanotubes (MWCNTs, 10–20 nm in diameter and 5–15 μm in length) were from Tokyo Chemical Industry (Tokyo, Japan). All other chemicals were of reagent grade.

Determination of enzyme activity and protein levels Enzyme activity was assayed spectrophotometrically using a Hitachi U-3210 spectrophotometer equipped with a thermostat. The standard reaction mixture contained 40 mM sodium D-lactate, 0.2 mM DCIP, 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–NaOH buffer (pH 7.0 at 25°C) and the enzyme in a total volume of 1 mL. The mixture was incubated at 60°C in a cuvette with a 0.4-cm light path. The reaction was started by addition of DCIP and followed by measuring the initial decrease in absorbance at 600 nm. One enzyme unit was defined as the amount catalyzing the reduction of 1 μmol of DCIP per min at 60°C. A millimolar absorption coefficient (ε mM) of 19.1 $mM^{-1}cm^{-1}$ at 600 nm was used for DCIP. Reduction of ferricyanide, INT and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were assayed at 405 nm (ε = 1.02 $mM^{-1}cm^{-1}$), 490 nm (ε = 15.0 $mM^{-1}cm^{-1}$) and 578 nm (ε = 13 $mM^{-1}cm^{-1}$), respectively (25). For reduction of INT, phenazine methosulfate (PMS) was used as an electron transfer intermediate. Protein concentrations were determined using a Coomassie (Bradford) Protein assay reagent kit supplied by Thermo Scientific (Rockford, IL USA) with bovine serum albumin serving as the standard.

Overexpression and purification of the recombinant protein To construct the expression plasmid, a 1.4-kbp gene fragment composed of the gene encoding Dye-DLDH and *Nde* I and *Bam* HI linkers was amplified by PCR using the following two primers. The first primer was designed to contain the N-terminal region of the Dye-DLDH gene and a *Nde* I cleavage sequence (5′-CATATGGATTTTGGGAAAGTCGC-3′), while the second was designed to contain the C-terminal region and a *Bam* HI cleavage sequence (5′-GGATCCCTACTGCGATTGCATCCATG-3′). The fosmid vector (JFF021-G03) containing the open reading frame of the gene (ORF ID: CSUB_C1080) served as the template (27). The amplified fragment was cleaved using *Nde* I and *Bam* HI and then ligated to the expression vector pCold I (Takara Bio Inc., Otsu, Japan) previously linearized with *Nde* I and *Bam* HI, which yielded pCsDLDH. Thereafter, *E. coli* strain BL21 (DE3) codon plus RIPL (Agilent Technologies, Santa Clara, CA, USA) was transformed with pCsDLDH, and the transformants were cultivated at 37°C in 1 L of Luria–Bertani medium containing 50 μg/mL ampicillin until the optical density at 600 nm reached 0.6. Expression was then induced by adding 1 mM isopropyl-β-D-thiogalacto-pyranoside to the medium, and cultivation was continued at 20°C for an additional 12 h. The cells were then harvested by centrifugation, washed twice with 0.85 % NaCl solution and stored at −20°C until used.

For the purification of the recombinant Dye-DLDH, the stored cells were suspended in 10 mM potassium phosphate buffer (pH 7.2) supplemented with 100 mM NaCl (buffer A) and then disrupted by ultrasonication. The crude extract was heated at 70°C for 10 min in the presence of 1 mM FAD, and the denatured protein was removed by centrifugation (10,000 × g for 10 min). The resultant supernatant was loaded onto a Ni^{2+}-charged chelating Sepharose column (100 mm x 26 mm i.d., GE Healthcare Bioscience, Buckinghamshire, UK) equilibrated with buffer A, after which the column was washed with three bed-volumes of the same buffer. The enzyme was then eluted with 100 mL of buffer A containing a linear gradient of 0–500 mM imidazole. The active fractions were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 7.2).

TABLE 1. Purification of recombinant Dye-DLDH.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	216	188	0.873	100
Heat treatment (70°C, 10 min)	37.4	122	3.26	64.9
Ni chelating column	30.3	96.4	3.18	51.3

One liter of cell culture was used for purification.

Polyacrylamide gel electrophoresis and molecular mass determination Sodium dodecyl sulfate (SDS)-PAGE was carried out according to the method of Laemmli (28) with 12.5% polyacrylamide gel. The molecular mass of the recombinant enzyme was determined using a Superdex 200 HR 10/30 column (GE healthcare) with 10 mM potassium phosphate buffer (pH7.0) containing 150 mM NaCl. A calibration curve was constructed using six standard proteins from a gel filtration calibration kit (GE Healthcare) (molecular mass range: 13.7–440 kDa).

Temperature and pH optima, stability and kinetic parameters The optimum temperature for the Dye-DLDH reaction was determined using the standard assay with 0.01 μg of enzyme at temperatures ranging from 50°C to 80°C. The effect of pH on the enzymatic activity was determined using the standard assay with 0.01 μg of enzyme at 60°C in 200 mM HEPES-NaOH buffer (pH 7.0–8.0 at 25°C), *N*,*N*-bis (2-hydroxyethyl) glycine-NaOH buffer (pH 8.0–9.0 at 25°C) and 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO)–NaOH buffer (pH 9.0–10.0 at 25°C). The thermostability of the enzyme was determined by measuring the remaining activity after incubating the enzyme (0.4 mg/mL) for 10 min at various temperatures. The pH stability of the enzyme was determined by measuring the remaining activity after incubating the enzyme (0.4 mg/mL) for 30 min at 50°C in buffers of various pHs. The buffers used were sodium acetate (pH 4.0–5.5), Bis-Tris-HCl (pH 5.5–7.0), HEPES–NaOH (pH 7.0–8.0), Tris–HCl (pH 8.0–9.0) and CAPSO–NaOH (pH 9.0–11.0). The Michaelis constant (K_m) was determined from Lineweaver–Burk plots of the data obtained from the initial rate analysis using D-lactate as the electron donor and DCIP as the electron acceptor at 60°C.

Extraction and determination of flavin compound The flavin compound from the enzyme was extracted with 1% (v/v) perchloric acid (PCA) (29). After removal of the precipitate formed by centrifugation, the supernatant was used to identify the flavin compound by HPLC with a COSMOSIL(R) 5C18-AR-II Packed Column (4.6 x 150 mm, Nacalai Tesque). FAD and FMN were monitored by the absorbance at 260 nm.

Preparation of a Dye-DLDH immobilized electrode A glassy carbon (GC) electrode (diameter: 3 mm) was continuously polished with 1.0 and 0.05 μm alumina powder until it was rinsed with ultrapure water. After sonication in ultrapure water, the electrode was scanned 50 times at 100 mVs^{-1} across potentials ranging from −1.0 V to 1.0 V in 50 mM sulfuric acid. Finally, the electrode was washed with ultrapure water. To prepare a Dye-DLDH/MWCNT/Nafion immobilized electrode, a suspension of Dye-DLDH-MWCNT-Nafion was prepared by suspending 500 μg of Dye-DLDH and 80 μg of MWCNT in 0.1 mL of 0.05% (w/v) Nafion solution using sonication, after which 10 μL of the suspension was cast onto the GC electrode surface and dried overnight at room temperature.

Electrochemical measurements Electrochemical measurements were made using an ALS electrochemical analyzer model 1205B (BAS Inc., Tokyo, Japan). A typical three-electrode system was applied using an Ag/AgCl (3 M KCl) as the reference electrode, a platinum wire as the counter electrode and a prepared GC electrode as the working electrode. The measurement temperature was set at 30°C or 50°C using a temperature-controlled water bath. Cyclic voltammograms (CVs) were recorded at a scan rate of 10 mV s^{-1} over a voltage range of 0.1 V–0.5 V. The standard reaction mixture contained 0.4 mM ferrocene carboxylate as a mediator, 0.5 M Tris–HCl buffer (pH 8.0) and 1 mM D-lactate. D-Lactate was detected using an amperometric method at a constant potential of 0.45 V vs. Ag/AgCl.

RESULTS AND DISCUSSION

Expression and purification of Dye-DLDHs Within the genome sequence of *Ca.* Caldiarchaeum subterraneum (http://www.genome.jp/kegg/), we identified a gene, CSUB_C1080 (1395 bp at position 1,048,195–1,049,589 of the entire genome), whose predicted amino acid sequence showed 43.5% identity with that of *S. tokodaii* Dye-DLDH. After transforming *E. coli* using pCsDLDH, an

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