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

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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

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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 5C18-AR-II Packed Column (4.6 × 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

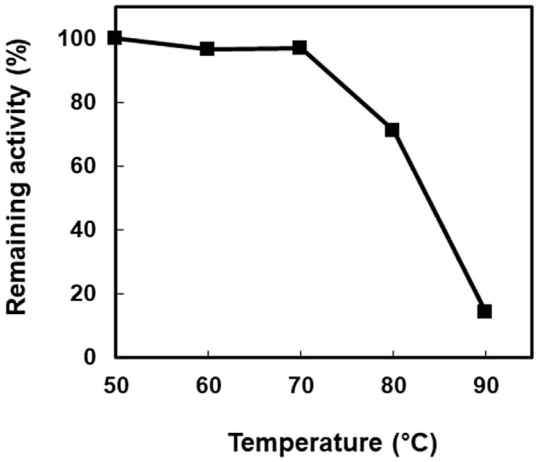

FIG. 1. Thermal stability of Dye-DLDH. The enzyme was incubated for 10 min at the indicated temperatures, and the remaining activity was determined using the standard assay method.

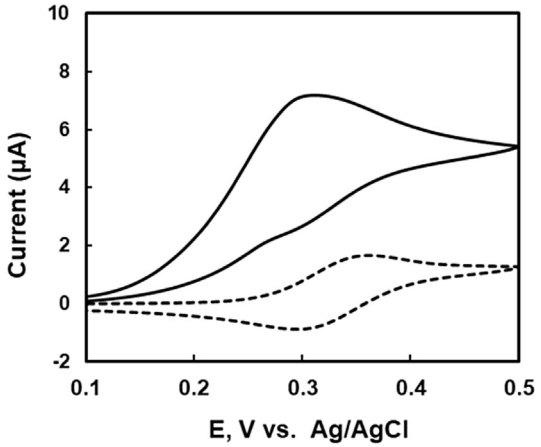

FIG. 3. CVs for electrocatalytic oxidation of D-lactate using a Dye-DLDH/MWCNT/Nafion immobilized electrode. The CVs were observed in Tris–HCl buffer (pH 8.0) containing 0.4 mM ferrocene carboxylate without (dashed line) and with (solid line) 1.0 mM D-lactate at 30°C. The scan rate was set at 10 mV s^{-1}.

expression vector harboring the gene, a high level of Dye-DLDH activity (0.873 units/mg) was detected in the extract of transformant cells, and the activity was retained, even after incubation for 10 min at 70°C (Table 1). In addition, the Dye-DLDH appeared in the soluble fraction after disrupting the cells with sonication, and no special solubilization process was required. This suggests the recombinant enzyme was easily released from the cell membrane, as is the case with Dye-DLDHs from *S. tokodaii* and *A. pernix*. The high thermostability and easy solubilization of the recombinant enzyme are important advantages for its preparation. In fact, 30.3 mg of pure recombinant Dye-DLDH was obtained from the crude extract of *E. coli* cells in one liter of medium through heat treatment and only a single column chromatography step (Table 1).

Properties of recombinant Dye-DLDHs We then examined molecular and catalytic properties of the Dye-DLDH from *Ca.* Caldiarchaeum subterraneum. Based on Superdex 200 gel filtration chromatography, the molecular mass of the native Dye-DLDH was estimated to be about 114 kDa (data not shown). Using SDS-PAGE, the subunit molecular mass of the Dye-DLDH was estimated to be about 51 kDa, indicating that the enzyme exists as a homodimer. When the enzyme activity was measured at various pHs in 200 mM buffer, the highest D-lactate dehydrogenation activity was observed at around pH 9.0. When we examined the effect of temperature on D-lactate dehydrogenation, we found that the enzyme activity increased linearly with increasing temperature below 70°C. Higher activity was observed above 70°C, but the assay was not correctly measuring enzyme activity, as there was non-enzymatic decolorization of DCIP. The enzyme retained its full activity after incubation for 10 min at temperatures ranging from 50°C to 70°C, but exhibited a marked loss of activity after incubation for 10 min at 90°C (Fig. 1). The enzyme also retained full activity after incubation for 30 min at pH 6.0 or 8.0 (50°C), but 40% of the activity was lost after incubation at pH 5.0 or 9.0. The spectrum of Dye-DLDH exhibited the properties of typical flavoprotein (Fig. 2). By the addition of 0.1 mM D-lactate to the purified enzyme, the specific spectrum of flavin compound transferred from the oxidized form to the reduced one. In addition, the flavin compound extracted from the purified enzyme was analyzed by HPLC. The flavin compound in the enzyme extract was identified to be FAD and but not FMN (data not shown). This indicates that the Dye-DLDH has FAD as its cofactor. Examination of the electron donor specificity of the enzyme revealed D-lactate to be the most preferred substrate, followed by (*R*)-2-hydroxybutyrate

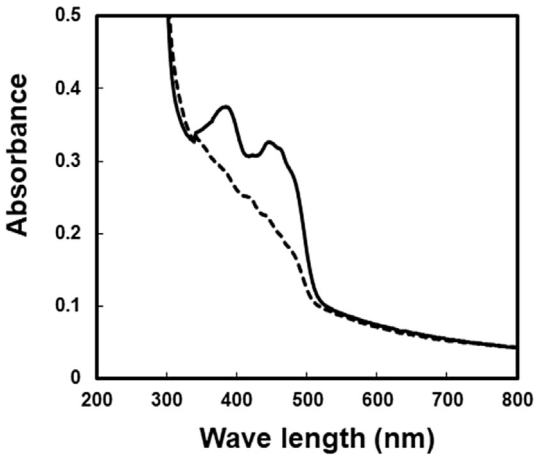

FIG. 2. Absorption spectra of recombinant Dye-DLDH in 10 mM potassium phosphate (pH 7.2). Solid line, Dye-DLDH; dashed line, after addition of 0.1 mM D-lactate.

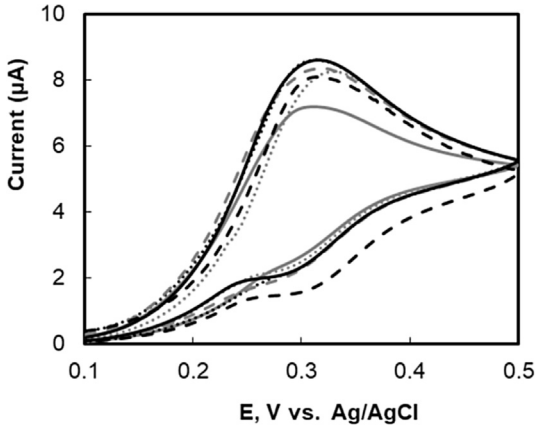

FIG. 4. CVs for the Dye-DLDH immobilized electrode without (solid gray line) and with 0.02% (w/v) (dotted black line), 0.04% (dotted gray line), 0.06% (w/v) (dashed gray line), 0.08% (w/v) (solid black line) or 0.1% (w/v) (dashed black line) MWCNT in standard reaction solution at 30°C. The scan rate was set at 10 mV s^{-1}.

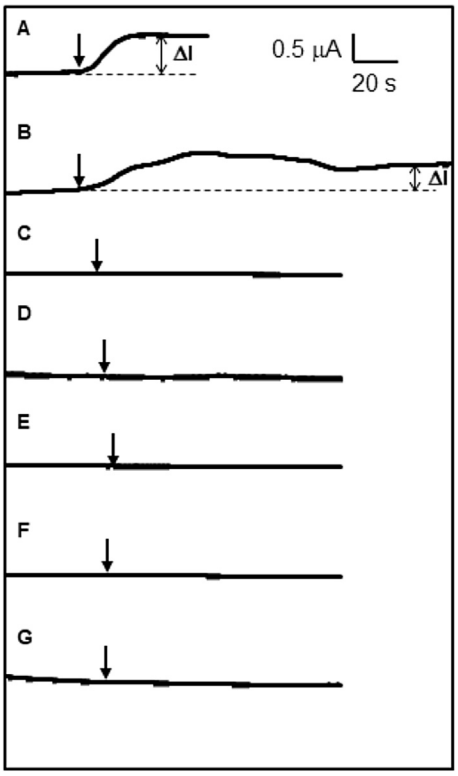

FIG. 5. Amperometric response of a Dye-DLDH/MWCNT/Nafion immobilized electrode upon addition of 0.1 mM D-lactate (A), (*R*)-2-hydroxybutyrate (B), L-lactate (C), L-ascorbate (D), ethanol (E), D-glucose (F) and pyruvate (G). The arrow indicates injection of substrate. ΔI indicates the increased current response after addition of substrate.

with a relative activity 46% compared to D-lactate. D-Alanine, pyruvate, D-glucose, L-alanine, ethanol and L-lactate were inert as electron donors. Examination of the electron acceptor specificity of the enzyme showed that PMS and an INT coupled system, ferricyanide and MTT, and DCIP all exhibited electron acceptor activity. The activities were 309%, 171% and 95%, respectively, relative to DCIP. Benzyl viologen and methylene blue were inert as electron acceptors. The apparent K_m for D-lactate with the recombinant enzyme was calculated to be 0.605 mM when DCIP (0.2 mM) was used as the electron acceptor.

Characterization of a Dye-DLDH/MWCNT/Nafion immobilized electrode The prepared Dye-DLDH immobilized electrode was characterized using CVs. The CVs were measured in Tris–HCl buffer (pH 8.0) containing 0.4 mM ferrocene carboxylate, which was used as the mediator for the Dye-DLDH because ferrocene and its derivatives are the most commonly used mediators for many kinds of dye-linked dehydrogenases (30–33). We observed a pair of redox peaks derived from the redox couple of Fe(III)/Fe(II) ions in ferrocene carboxylate at about +300 mV (vs. Ag/AgCl) when D-lactate was absent (Fig. 3, dashed line). However, in the presence of D-lactate, the observed peaks indicated the increase of oxidation current and the decrease of reduction current (Fig. 3, solid line). This indicates that the catalytic reaction through the D-lactate oxidation produces electrons, which reacts with the oxidized form of ferrocene carboxylate. As a result, the concentration of reduced ferrocene carboxylate increased and the oxidation current was amplified. Consequently, it was confirmed that ferrocene carboxylate could function as the electron acceptor for the Dye-DLDH and efficiently transfers electrons to the electrode as the mediator.

Because MWCNTs are highly conductivity and have a large surface area, the MWCNT concentration on the electrode strongly affects its current response (34). To assess the effect of the MWCNT concentration (0.02%, 0.04%, 0.06%, 0.08% and 0.1%, w/v) on the electrode's current response to D-lactate, CVs were measured in standard reaction solution. The oxidation currents upon addition of D-lactate using 0.02%, 0.04%, 0.06% and 0.08% (w/v) MWCNT-modified electrodes were similar. However, the oxidation current was less at 0.1% (w/v) MWCNT compared with those of other MWCNT concentrations (Fig. 4). This is likely because the thickness of the Dye-DLDH/MWCNT/Nafion membrane on the GC electrode increases in high MWCNT concentration, which may cause the current response to decrease due to less diffusion rates of both the substrate and mediator within the membrane. A similar phenomenon was reported in both the dye-linked D-proline dehydrogenase/carbon nanotube modified electrode (34) and the MWCNT/Nafion modified electrode (35). When MWCNT concentration is more than 0.1%, the reduction peak of the ferrocene carboxylate is markedly different from other cases due to the increase of membrane thickness. In addition, 0.08% (w/v) MWCNT -Nafion solution was much easier to prepare and handle due to the higher MWCNT content. Thus, 0.08% MWCNT was loaded onto the Dye-DLDH immobilized electrode in the subsequent experiments.

The selectivity of the Dye-DLDH/MWCNT/Nafion immobilized electrode was evaluated amperometrically in the presence of potential interferences such as ethanol, D-glucose, pyruvate, L-lactate, L-ascorbate and (*R*)-2-hydroxybutyrate. D-Lactate was added at the time indicated by arrow and the oxidation current was amplified immediately (Fig. 5A). The oxidation current after addition of D-lactate was detected after 50 s. The current response for the D-lactate oxidation by Dye-DLDH was estimated by the increased current response (ΔI). The current value after addition of (*R*)-2-hydroxybutyrate was static 580 s later. Therefore, the current response for the (*R*)-2-hydroxybutyrate oxidation by Dye-DLDH was calculated by the increased current response after 580 s (ΔI) (Fig. 5B). The developed electrode exhibited the highest

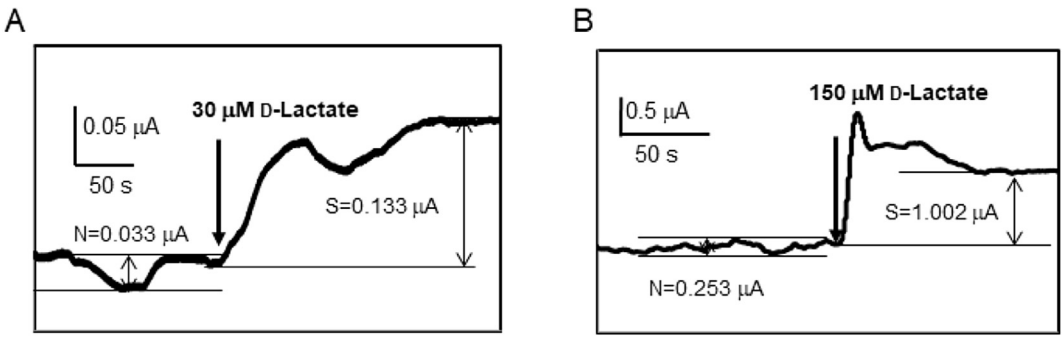

FIG. 6. Determination of detection limit for 30°C (A) and 50°C (B). N and S indicate the noise current and the signal current, respectively. The arrow indicates injection of D-lactate.

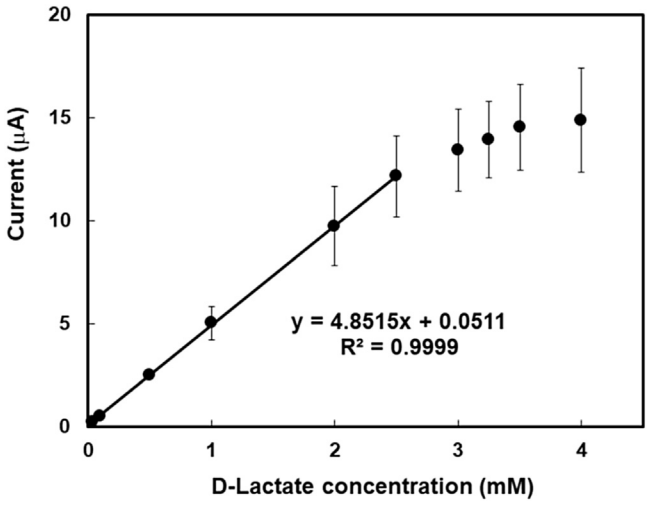

FIG. 7. Calibration curve for detection of D-lactate using a Dye-DLDH/MWCNT/Nafion immobilized electrode at 30°C. Error bars indicate standard deviations (N = 3).

amperometric current response to D-lactate, but also exhibited a response to (R)-2-hydroxybutyrate, which had an amplitude of 67% relative to D-lactate. No amperometric response was observed with L-lactate, L-ascorbate, ethanol, D-glucose and pyruvate (Fig. 5C–G). The selectivity of the Dye-DLDH/MWCNT/Nafion immobilized electrode was identical to that of the Dye-DLDH itself. This high selectivity is convenient for specific detection of D-lactate in biological tissues and foodstuffs.

The measurement temperature of a D-lactate detection system using a thermostable Dye-DLDH is a very important parameter that influences sensitivity. Therefore, the detection limits of the Dye-DLDH/MWCNT/Nafion immobilized electrode were estimated as triple the concentration corresponding to the background current observed in standard reaction solution at 30°C and 50°C. For D-lactate, the detection limits at 30°C and 50°C were estimated to be 30 μM and 150 μM, respectively (S/N = 3) (Fig. 6). While the current response measured at 50°C was higher than that at 30°C, greater sensitivity was observed at 30°C due to the high noise current at 50°C. Consequently, the optimum temperature for detection of D-lactate was determined to be 30°C, and the subsequent electrochemical D-lactate assay was carried out at 30°C. Under the optimum analytical conditions, amperometric determination of D-lactate using the Dye-DLDH/MWCNT/Nafion immobilized electrode yielded a linear relationship in the concentration range of 0.03–2.5 mM D-lactate (Fig. 7).

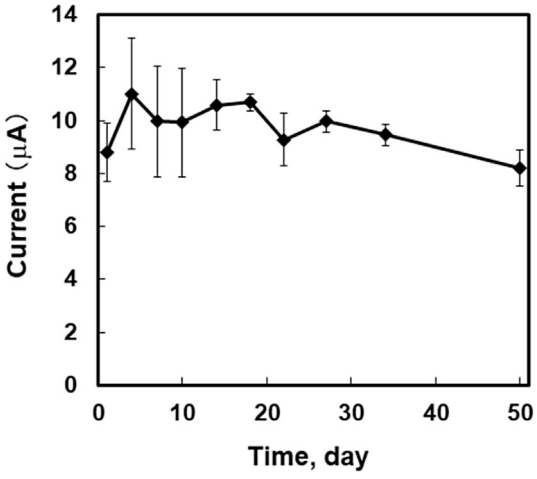

FIG. 8. Long-term stability of the Dye-DLDH/MWCNT/Nafion immobilized electrode. The current response was measured in standard reaction solution. The measurement temperature was 30°C. The electrode was stored in 50 mM potassium phosphate buffer (pH 7.0) at 4°C, except when in use for amperometric detection. Error bars indicate standard deviations (N = 3).

The long-term stability of the Dye-DLDH/MWCNT/Nafion immobilized electrode was evaluated by monitoring the current response to 2 mM D-lactate for 50 days. The electrode was stored in 50 mM PKB (pH 7.0) at 4°C when not in use. After 50 days, the current response of the electrode was more than 90% of the initial response measured on the first day of electrode preparation (Fig. 8). The measurement error at the initial stage of use was rather larger than that at later stage of use although the reason is not clear. This is in contrast to the baker's yeast Dye-DLDH immobilized carbon paste electrode, which retained 80% of its initial current response after storage at 4°C for 27 days (23). In addition, the cell debris Dye-DLDH from *H. polymorpha* immobilized on a graphite electrode retained only 71% of its initial current response after storage at 4°C for only 10 days (24). Thus, the Dye-DLDH/MWCNT/Nafion immobilized electrode exhibited the highest stability among Dye-DLDH immobilized electrodes reported so far. The high stability of the *Ca.* Caldiarchaeum subterraneum Dye-DLDH likely underlies the long-term stability of this electrode.

As the next step, we measured the D-lactate concentration in an untreated sample of white wine using the Dye-DLDH/MWCNT/Nafion immobilized electrode. The calibration curve was constructed with 2 mL of the standard reaction mixture containing the concentration range between 50 μM and 300 μM D-lactate. An aliquot (100 μL) white wine sample was added to 1.9 mL of the reaction mixture containing 500 mM Tirs-HCl (pH 8.0) buffer and 40 mM ferrocene carboxylate, and the increasing amperometric current was measured. Based on the constructed calibration curve, the D-lactate concentration in the white wine sample was estimated. As the result, the D-lactate concentration in the reaction mixture (2 mL) was 0.156 ± 0.00590 mM. Consequently, D-lactate concentration in the white swine sample was estimated as 3.12 ± 0.12 mM, which was comparable to the 3.05 ± 0.09 mM concentration spectrophotometrically determined by using the F-kit method (Roche Diagnostic GmbH, Mannheim, Germany). This indicates the thermophilic Dye-DLDH immobilized electrode is potentially applicable for measurement of D-lactate in real samples. We therefore suggest that a highly stable Dye-DLDH-immobilized electrode could be used in a portable device for long-term D-lactate detection.

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References

1. **Ewaschuk, J. B., Naylor, J. M., and Zello, G. A.:** D-Lactate in human and ruminant metabolism, J. Nutr., **135**, 1619–1625 (2005).
2. **Lord, R. S. and Bralley, J. A.:** Clinical applications of urinary organic acids. Part 2. Dysbiosis markers, Altern. Med. Rev., **13**, 292–306 (2008).
3. **Beisswenger, P. J., Howell, S. K., Touchette, A. D., Lal, S., and Szwergold, B. S.:** Metformin reduces systemic methylglyoxal levels in type 2 diabetes, Diabetes, **48**, 198–202 (1999).
4. **Talasniemi, J. P., Pennanen, S., Savolainen, H., Niskanen, L., and Liesivuori, J.:** Analytical investigation: assay of D-lactate in diabetic plasma and urine, Clin. Biochem., **41**, 1099–1103 (2008).
5. **Chou, C. K., Lee, Y. T., Chen, S. M., Hsieh, C. W., Huang, T. C., Li, Y. C., and Lee, J. A.:** Elevated urinary D-lactate levels in patients with diabetes and microalbuminuria, J. Pharm. Biomed. Anal., **116**, 65–70 (2015).

6. **Caglayan, F., Cakmak, M., Caglayan, O., and Cavusoglu, T.:** Plasma D-lactate levels in diagnosis of appendicitis, J. Invest. Surg., **16**, 233–237 (2003).
7. **Mayne, A. J., Handy, D. J., Preece, M. A., George, R. H., and Booth, I. W.:** Dietary management of D-lactic acidosis in short bowel syndrome, Arch. Dis. Child., **65**, 229–231 (1990).
8. **Bongaerts, G., Tolboom, J., Naber, T., Bakkeren, J., Severijnen, R., and Willems, H.:** D-Lactic acidemia and aciduria in pediatric and adult patients with short bowel syndrome, Clin. Chem., **41**, 107–110 (1995).
9. **Smith, S. M., Eng, R. H., Campos, J. M., and Chmel, H.:** D-Lactic acid measurements in the diagnosis of bacterial infections, J. Clin. Microbiol., **27**, 385–388 (1989).
10. **Girotti, S., Muratori, M., Fini, F., Ferri, E. N., Carrea, G., Koran, M., and Rauch, P.:** Luminescent enzymatic flow sensor for D- and L-lactate assay in beer, Eur. Food Res. Technol., **210**, 216–219 (2000).
11. **Shu, H. C. and Mattiasson, B.:** D-Lactic acid in pork as a freshness indicator monitored by immobilized D-lactate dehydrogenase using sequential injection analysis, Anal. Chim. Acta, **283**, 727–737 (1993).
12. **Soga, T. and Ross, G. A.:** Capillary electrophoretic determination of inorganic and organic anions using 2, 6-pyridinedicarboxylic acid: effect of electrolyte's complexing ability, J. Chromatogr. A, **767**, 223–230 (1997).
13. **Hasegawa, H., Fukushima, T., Lee, J. A., Tsukamoto, K., Moriya, K., Ono, Y., and Imai, K.:** Determination of serum D-lactic and L-lactic acids in normal subjects and diabetic patients by column-switching HPLC with pre-column fluorescence derivatization, Anal. Bioanal. Chem., **377**, 886–891 (2003).
14. **Scheijen, J. L., Hanssen, N. M., van de Waarenburg, M. P., Jonkers, D. M., Stehouwer, C. D., and Schalkwijk, C. G.:** L (+) and D (−) lactate are increased in plasma and urine samples of type 2 diabetes as measured by a simultaneous quantification of L (+) and D (−) lactate by reversed-phase liquid chromatography tandem mass spectrometry, Exp. Diabetes Res., **2012**, 234812 (2012).
15. **Montagne, M. and Marty, J. L.:** Bi-enzyme amperometric D-lactate sensor using macromolecular NAD^+, Anal. Chim. Acta, **315**, 297–302 (1995).
16. **Shu, H. C. and Wu, N. P.:** A chemically modified carbon paste electrode with D-lactate dehydrogenase and alanine aminotranferase enzyme sequences for D-lactic acid analysis, Talanta, **54**, 361–368 (2001).
17. **Gros, P. and Comtat, M.:** A bioelectrochemical polypyrrole-containing $Fe(CN)_6^{3-}$ interface for the design of a NAD-dependent reagentless biosensor, Biosens. Bioelectron., **20**, 204–210 (2004).
18. **Gregolin, C. and Singer, T. P.:** The lactic dehydrogenase of yeast: III. D (−) Lactic cytochrome *c* reductase, a zinc-flavoprotein from aerobic yeast, Biochim. Biophys. Acta, **67**, 201–218 (1963).
19. **Ho, C., Pratt, E. A., and Rule, G. S.:** Membrane-bound D-lactate dehydrogenase of *Escherichia coli*: a model for protein interactions in membranes, Biochim. Biophys. Acta, **988**, 173–184 (1989).
20. **Dym, O., Pratt, E. A., Ho, C., and Eisenberg, D.:** The crystal structure of D-lactate dehydrogenase, a peripheral membrane respiratory enzyme, Proc. Natl. Acad. Sci. USA, **97**, 9413–9418 (2000).
21. **Ma, C., Gao, C., Qiu, J., Hao, J., Liu, W., Wang, A., Zhang, Y., Wang, M., and Xu, P.:** Membrane-bound L-and D-lactate dehydrogenase activities of a newly isolated *Pseudomonas stutzeri* strain, Appl. Microbiol. Biotechnol., **77**, 91–98 (2007).
22. **Welchen, E., Schmitz, J., Fuchs, P., Garcia, L., Wagner, S., Wienstroer, J., Schertl, P., Braun, H. P., Schwarzlander, M., Gonzalez, D. H., and Maurino, V. G.:** D-Lactate dehydrogenase links methylglyoxal degradation and electron transport through cytochrome *c*, Plant Physiol., **172**, 901–912 (2016).
23. **Pohanka, M. and Zboril, P.:** Amperometric biosensor for D-lactate assay, Food Technol. Biotechnol., **46**, 107–110 (2008).
24. **Smutok, O. V., Dmytruk, K. V., Karkovska, M. I., Schuhmann, W., Gonchar, M. V., and Sibirny, A. A.:** D-Lactate-selective amperometric biosensor based on the cell debris of the recombinant yeast *Hansenula polymorpha*, Talanta, **125**, 227–232 (2014).
25. **Satomura, T., Kawakami, R., Sakuraba, H., and Ohshima, T.:** A novel flavin adenine dinucleotide (FAD) containing D-lactate dehydrogenase from the thermoacidophilic crenarchaeota *Sulfolobus tokodaii* strain 7: purification, characterization and expression in *Escherichia coli*, J. Biosci. Bioeng., **106**, 16–21 (2008).
26. **Shibahara, T., Satomura, T., Kawakami, R., Ohshima, T., and Sakuraba, H.:** Crystallization and preliminary X-ray analysis of a dye-linked D-lactate dehydrogenase from the aerobic hyperthermophilic archaeon *Aeropyrum pernix*, Acta Crystallogr F Struct. Biol. Commun, **67**, 1425–1427 (2011).
27. **Nunoura, T., Takaki, Y., Kakuta, J., Nishi, S., Sugahara, J., Kazama, H., Chee, G. J., Hattori, M., Kanai, A., Atomi, H., Takai, K., and Takami, H.:** Insights into the evolution of Archaea and eukaryotic protein modifier systems revealed by the genome of a novel archaeal group, Nucleic Acids Res., **39**, 3204–3223 (2011).
28. **Laemmli, U. K.:** Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, **227**, 680–685 (1970).
29. **Satomura, T., Zhang, X. D., Hara, Y., Doi, K., Sakuraba, H., and Ohshima, T.:** Characterization of a novel dye-linked L-proline dehydrogenase from an aerobic hyperthermophilic archaeon, *Pyrobaculum calidifontis*, Appl. Microbiol. Biotechnol., **89**, 1075–1082 (2011).
30. **Zheng, H., Hirose, Y., Kimura, T., Suye, S. I., Hori, T., Katayama, H., Arai, J., Kawakami, R., and Ohshima, T.:** L-Proline sensor based on layer-by-layer immobilization of thermostable dye-linked L-proline dehydrogenase and polymerized mediator, Sci. Technol. Adv. Mater., **7**, 243–248 (2006).
31. **Tani, Y., Tanaka, K., Yabutani, T., Mishima, Y., Sakuraba, H., Ohshima, T., and Motonaka, J.:** Development of a D-amino acids electrochemical sensor based on immobilization of thermostable D-proline dehydrogenase within agar gel membrane, Anal. Chim. Acta, **619**, 215–220 (2008).
32. **Yamada, Y., Hayashi, T., Sakuraba, H., Yabutani, T., and Takayanagi, T.:** Fabrication and characterization of a thermostable Quinoprotein aldose sugar dehydrogenase immobilized electrode, Anal. Sci., **29**, 79–83 (2013).
33. **Sakamoto, H., Watanabe, K., Koto, A., Koizumi, G., Satomura, T., Watanabe, S., and Suye, S. I.:** A bienzyme electrochemical biosensor for the detection of collagen L-hydroxyproline, Sens. Biosensing Res., **4**, 37–39 (2015).
34. **Tani, Y., Itoyama, Y., Nishi, K., Wada, C., Shoda, Y., Satomura, T., Sakuraba, H., Ohshima, T., Hayashi, Y., Yabutani, T., and Motonaka, J.:** An amperometric D-amino acid biosensor prepared with a thermostable D-proline dehydrogenase and a carbon nanotube-ionic liquid gel, Anal. Sci., **25**, 919–923 (2009).
35. **Cheng, Y., Liu, Y., Huang, J., Xian, Y., Zhang, W., Zhang, Z., and Jin, L.:** Rapid amperometric detection of coliforms based on MWNTs/Nafion composite film modified glass carbon electrode, Talanta, **75**, 167–171 (2008).