Journal of Bioscience and Bioengineering
VOL. xxx No. xxx, xxx, xxxx

www.elsevier.com/locate/jbiosc

Characterization of dye-linked D-amino acid dehydrogenase from *Sulfurisphaera tokodaii* expressed using an archaeal recombinant protein expression system

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Received 30 January 2020; accepted 22 April 2020
Available online xxx

A gene encoding a dye-linked D-amino acid dehydrogenase (Dye-DADH) homologue was found in a hyperthermophilic archaeon, *Sulfurisphaera tokodaii*. The predicted amino acid sequence suggested that the gene product is a membrane-bound type enzyme. The gene was overexpressed in *Escherichia coli*, but the recombinant protein was exclusively produced as an inclusion body. In order to avoid production of the inclusion body, an expression system using the thermoacidophilic archaeon *Sulfolobus acidocaldarius* instead of *E. coli* as the host cell was constructed. The gene was successfully expressed in *S. acidocaldarius*, and its product was purified to homogeneity and characterized. The purified enzyme catalyzed the dehydrogenation of various D-amino acids, with D-phenylalanine being the most preferred substrate. The enzyme retained its full activity after incubation at 90 °C for 30 min and after incubation at pH 4.0–11.0 for 30 min at 50 °C. This is the first report on membrane-bound Dye-DADH from thermophilic archaea that was successfully expressed in an archaeal host.

[**Keywords:** D-Amino acid; Dye-linked dehydrogenase; Archaea; Thermophile; Biosensor]

Dye-DADH is a kind of flavoenzyme containing FAD as a cofactor and catalyzes the oxidation of various D-amino acids to their corresponding 2-keto acids in the presence of electron acceptors such as ubiquinone and cytochrome *c* (1). Dye-DADH exists as a membrane-bound protein and functions as an enzyme involved in the electron transfer systems for energy generation in cells. Additionally, Dye-DADH has been known to utilize artificial electron acceptors such as 2,6-dichloroindophenol (DCIP) and potassium ferricyanide instead of their natural electron acceptor. These artificial electron acceptors efficiently mediate the electron transfer derived from the substrate to an electrode during the enzyme reaction. Therefore, Dye-DADH has a high utilization potential as a specific element for D-amino acid electrochemical detection.

D-Amino acids are widely distributed within biological tissues, and are also found in many of the foods (2). Moreover, the specific biological and physiological functions of D-amino acids within human tissues have been revealed. For example, D-serine is known to act as a co-agonist for the *N*-methyl-D-aspartate (NMDA) receptor which is widely distributed in the central nervous system. NMDA receptor plays an important role in memory and learning (3,4). D-Aspartic acid has been found in the nervous and endocrine tissues, where it functions to control the production of some hormones (5). D-Proline is distributed in the gastric juice, brain, and human saliva, and it specifically accumulates in stomach cancer and reportedly exhibits neural toxicity (6,7). In addition, D-proline has also been detected in foods such as vinegar and maple syrup (8–10), and its concentration in vinegars has been known to increase with their aging. The measurement of D-amino acid is thus of great interest for both clinical diagnosis and food analysis. Up to date, several analytical methods including gas chromatography, high performance liquid chromatography, and amino acid analyzer have been used for determination of D-amino acids (6). However, they need expensive and sophisticated systems. On the other hand, electrochemical biosensor using Dye-DADH can be easily operated with small equipment, and running cost is much less (11).

The presence of Dye-DADH has been identified in mesophilic bacteria including *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* (12–15). These Dye-DADHs exhibit broad substrate specificity and utilize D-alanine as the most preferred substrate. Despite their potential use as biosensor elements, the practical application of Dye-DADHs has been limited, because mesophilic enzymes are unstable and often lose their activities even at the room temperature. In order to overcome these limitations, thermophiles with highly stable Dye-DADH have been screened. Up till now, we have found two novel Dye-DADHs in a thermophilic bacterium *Rhodothermus marinus* and a hyperthermophilic archaeon *Pyrobaculum islandicum* (16,17). These enzymes

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2020.04.008

Please cite this article as: Satomura, T et al., Characterization of dye-linked D-amino acid dehydrogenase from *Sulfurisphaera tokodaii* expressed using an archaeal recombinant protein expression system, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2020.04.008

are highly stable, even at high temperatures and under both high and low pH conditions. However, the reaction mechanism of Dye-DADH including substrate binding site and catalytic amino acid residue has been unclarified because suitable crystal for determination of the three-dimensional structure has not yet been obtained.

As a crucial step toward the structural characterization of Dye-DADH, we have screened the gene encoding Dye-DADH based on available thermophiles genomic information. As a result, we found a Dye-DADH homologue (STK 16240 protein) from the thermoacidophilic archaeon, *Sf. tokodaii* that has 37 % similarity with the Dye-DADH in *Py. islandicum*. We expressed the gene encoding the Dye-DADH homologue in an archaeal host, *Sulfolobus acidocaldarius* SK1, and characterized the product. The expressed protein showed the highest stability among all reported thermophilic Dye-DADHs. In this study, we performed the detailed characterization of this enzyme for the potential utilization as a specific element for D-amino acid biosensor.

MATERIALS AND METHODS

Materials D-Phenylalanine, Tween-20 and DCIP were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Tryptone and yeast extract were obtained from Difco Laboratories (Sparks, MD, USA). All other chemicals were of reagent grade.

Strains and cultivation media *S. acidocaldarius* SK1 carrying *ΔpyrEF* and *ΔsauI* (Table 1) (18) was used for the construction of the recombinant protein expression system. *S. acidocaldarius* SK1 was cultivated in xylose and tryptone (XT) plates containing 0.65 % (w/v) Gelrite (Fujifilm Wako Pure Chemical Corporation), 1 mM $CaCl_2 \cdot 2H_2O$, and 2 mM $MgSO_4 \cdot 7H_2O$ (19). The recombinant protein in *S. acidocaldarius* SK1 was further expressed in a xylose free XT basal medium (T medium). In addition, medium containing maltose (MT) or dextrin (DT) (4 g/L) in the T medium were also used.

E. coli strains DH5α and BL21-CodonPlus(DE3)-RIPL (Agilent Technologies, Santa Clara, CA, USA) were used for the cloning of plasmids and expression of recombinant protein, respectively.

Plasmid construction for gene expression in *E. coli* and production of the recombinant proteins We constructed a plasmid for *E. coli* comprising a 1.1 kbp fragment containing the gene encoding Dye-DADH homologue (*stk16240*) and the *Nde*I and *Bam*HI linkers, which was then amplified by PCR using PrimeSTAR Max DNA Polymerase (Takara Bio, Shiga, Japan) with StDADHF and StDADHR as primers (Table 2). The amplified DNA fragment was further digested with *Nde* I and *Bam* HI, and then ligated with expression vector pET15b (Novagen, Madison, WI, USA), that has been linearized with *Nde*I and *Bam*HI, to generate pSTDADH. Transformed *E. coli* strain BL21(DE3) CodonPlus-RIPL containing pSTDADH was cultivated in 200 mL of Luria–Bertani (LB) medium containing ampicillin (50 μg/mL) at 37 °C. When OD_{600} of culture medium reached 0.6, expression of gene was induced by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside to the medium, and the cultivation was continued for 4 h at 37 °C. The cells were then harvested by centrifugation 10,000 ×*g*, at 4 °C, for 10 min and then stored at −20 °C.

Plasmid construction for gene expression in *S. acidocaldarius* SK1 Recombinant plasmid for *S. acidocaldarius* SK1 harboring the gene encoding Dye-DADH homologue with N-terminus histidine tag (His-STK16240) was amplified with primers ST16240F and ST16240R (Table 2) using PrimeSTAR Max DNA Polymerase and pSTDADH as template. Next, the *malE* (*saci_1165*) gene promotor region (−219 to −1) was amplified using the primer pair, PmalF and PmalR (Table 2). Genomic DNA was prepared using a MonoFas Bacterial Genomic DNA Isolation kit VII (GL Sciences, Tokyo, Japan) and then used as the template. The resultant PCR product was used as the template in subsequent PCR using primer pair, PmalF and Fusion-malR (Table 2). The reverse primer Fusion-malR was designed to contain an overhanging end of the His-STK16240 (Supplementary Fig. S1). The fusion between the *malE* promotor gene region and the His-STK16240 was performed by overlapping PCR using primer pair, pMalF and ST16240R to generate the fragment pMalE-STK16240 (Supplementary Fig. S1) (20).

The T-tailed pSAV2 plasmid vector with T-overhang was prepared according to the method of Hadjeb and Berkowitz (21). Plasmid vector pSAV2 (Table 1) was first digested with *Pst* I and then blunt-ended by Blunting high (TOYOBO, Osaka, Japan). Then, T-overhangs were attached to 3′ end of the blunted fragment with Takara Ex Taq. After the addition of 3′A-overhangs to the pMalE-STK16240 with Takara Ex Taq, the fragment was cloned into the T-tailed pSAV2 plasmid vector to generate the pSAVmal-STK16240 fragment. Correct construction was confirmed by DNA sequencing using pSAVexF and pSAVexR as primers (Table 2).

Gene expression in *S. acidocaldarius* SK1 Preparation of *S. acidocaldarius* SK1 competent cells and transformation procedure was applied as described previously (18). In brief, the cultured cells in XT medium containing 50 μg/mL uracil were washed once in 20 mM sucrose and then adjusted to 4 x 10^8 cells/mL of cell concentration in 20 mM sucrose. An amount of 200 μL competent cells (4 x 10^8 cells/mL) were transformed with 10 μg plasmid vector by electroporation using Gene Pulser Xcell (Bio-Rad, Richmond, CA, USA) with input parameters of 2500 V, 25 μF, and 1000 Ω in a 2 mm cuvette. The transformed cells were immediately spread onto XT-plate and incubated at 70 °C for 10 days. The transformants were then pre-cultivated at 70 °C for 4 days in XT medium, and further cultivated at 70 °C for 5 days in DT medium. The cells were then harvested by centrifugation 5000 ×*g*, at 4 °C, for 10 min and washed with 0.85 % NaCl solution. The washed cells were stored at −20 °C.

Determination of Dye-DADH activity and protein concentrations Dye-DADH activity was assayed spectrophotometrically as described previously (17). The reaction mixture contained 20 mM D-phenylalanine, 200 mM Tris–HCl buffer (pH 8.0), 0.2 mM DCIP, and the enzyme in a total volume of 1 mL. The mixture without DCIP was first incubated at 50 °C for 2 min in a cuvette with 4 mm-light path, and then the reaction was activated by the addition of DCIP and followed by measuring the initial decrease in absorbance at OD_{600}. One unit of enzyme activity was defined as the amount catalyzing the reduction of 1 μmol of DCIP per minute at 60 °C. A millimolar absorption coefficient of 19.1 mM^{-1} cm^{-1} at OD_{600} was used for DCIP. Protein concentration was determined using a Pierce BCA Protein assay reagent kit (Thermo Scientific, Rockford, IL, USA), with bovine serum albumin serving as the standard.

Purification of the recombinant Dye-DADH The recombinant *S. acidocaldarius* SK1 cells were suspended in 10 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl, 1% (w/v) Tween 20, and 20 mM imidazole (buffer A), and the cells were disrupted by ultrasonication. The cell debris was removed by centrifugation 10,000 × *g*, at 4 °C, for 10 min, and the supernatant was used as the crude extract. Protein purification was performed using AKTAprime Plus system (GE Healthcare, UK Ltd., Buckinghamshire, UK). The crude extract was loaded on a HisTrap FF Crude Column (5 mL, GE Healthcare) equilibrated with buffer A. The column was first washed with the same buffer, and then washed again with three bed volumes of the NaCl-free buffer A. The protein was eluted by a linear gradient of 20–500 mM imidazole in the same buffer with five bed volumes. The active fractions were collected, and the solution was then purified through a HiTrap Q HP column (1 mL, GE Healthcare) that was equilibrated with the NaCl/imidazole-free buffer A. After the column was washed with the same buffer, the bound protein was eluted with a linear gradient of 0–500 mM NaCl in the same buffer. The bound protein fractions were again pooled, and dialyzed with the NaCl/imidazole-free buffer A and used as purified enzyme in this study.

TABLE 1. *Sulfolobus* strains and vectors used in this study.

Strain or vector	Relevant characteristics	Source or reference
Sulfolobus strain		
S. acidocaldarius SK1	*S. acidocaldarius* MR31 with Δ*saiI*	18
Plasmid		
pSAV2	*Sulfolobus–E. coli* shuttle vector, based on pBluescript II KS (−) and pRN1, with the *ssopyrEF* marker	18
pSAVmal-STK16240	*Sulfolobus* expression vector derived from pSAV2, containing mal promotor (Pmal, −219 to −1) and *stk16240* with his-tag coding sequence.	This study

TABLE 2. Primers used in this study.

Primer	Sequence
StDADHF	CCGCGCGGCAGCCATATGAAAGTCGGAA TCGTAGG
StDADHR	TTAGCAGCCGGATCCTTAAAGGCTTAAA TTTCTTC
ST16240F	ATGGGCAGCAGCCATCATCATCATCATCAC
ST16240R	ATGCTAGTTATTGCTCAGCGG
PmalF	TTTTTTACCTACTCCTTGTGGTTTAAAAAT
PmalR	CGGGTTAACTTAATCACGTAATTATTTAT
Fusion-malR	[a]ATGGCTGCTGCCCATCGGGTTAACTTAA TCACGTA
pSAVexF	GAGGATAAGGTGATGAAATG TAAAGGAGCA
pSAVexR	CGTGTGAAAAGCTAATACCTGCAATTAACT

[a] Overlapping region with the PCR product amplified by primers, ST16240F and ST16240R is underlined.

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Determination of subunit molecular mass using SDS-PAGE and Western bolt analysis The subunit molecular mass of purified protein was carried out with SDS-PAGE of 12.5 % polyacrylamide gel and 0.1 % SDS according to Laemmli (22). WIDE-VIEW Prestained Protein Size Marker (Fujifilm Wako Pure Chemical Corporation) and XL-Ladder (APRO Science, Tokushima, Japan) were used as molecular mass standards. Protein bands were visualized by staining with Rapid CBB Kanto (Kanto Kagaku, Tokyo, Japan). Western blotting analysis was performed using the horseradish peroxidase-conjugated anti-His-tag antibody (Anti-His-tag mAb-HPR-DiercT, MBL, Nagoya, Japan). ImageQuant LAS 4000 (GE Healthcare) were used for signal detection.

Native molecular mass determination using gel filtration chromatography The molecular mass of the recombinant protein was determined using a Superdex 200 HR10/30 gel filtration column (GE Healthcare) with 10 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl and 0.1 % (w/v) Tween 20 as the elution buffer (3.46 mg of the enzyme was loaded). Gel filtration calibration kit (GE Healthcare) was used to make a calibration curve.

Kinetic parameters For the kinetic analysis, various concentrations of D-phenylalanine (1, 1.5, 2.5, 5.0, 10, 15, 20, and 30 mM) were used with the standard assay method. The Michaelis constant (*K*m) was determined from Michaelis–Menten plots using the Solver tool of Microsoft Excel.

RESULTS

Expression and purification of recombinant Dye-DADH An *E. coli* expression system for the *stk_16240* gene was successfully constructed using pET15b vector and expressed as a fusion protein with an N-terminal histidine tag. The overexpression of the recombinant protein in *E. coli* carrying pSTDADH was confirmed by both SDS-PAGE and detection of Dye-DADH enzyme activity. The deduced primary structure of ST16240 is composed of 372 amino acid residues with the predicted molecular mass of 42 kDa. As shown in Fig. 1, expression of the STK16240 was observed only in the insoluble fraction. In addition, Dye-DADH activity was detected in neither the crude extract nor the supernatant after heat treatment at 70 °C for 10 min. These results indicate that the recombinant STK16240 protein expressed exclusively as the inclusion body in *E. coli* carrying pSTDADH.

In this study, a *S. acidocaldarius* expression system for the *stk_16,240* gene using the *E. coli–Sulfolobus* shuttle vector, pSAV2 (Table 1) was constructed to avoid the inclusion body formation. Based on previous report (23), a maltose inducible promoter, *malE* (*saci_1165*) gene promoter was chosen for the expression of *stk_16,240* gene in *S. acidocaldarius* SK1 cells. The fusion gene cassette containing *malE* promoter and *stk_16,240* gene with histidine tag coding sequence was introduced into pSAV2, to produce the fusion pSAVmal-STK16240. *S. acidocaldarius* SK1 harboring pSAVmal-STK16240 (SK1/pSAVmal-STK16240) was cultivated in XT medium and the crude extract prepared from the cells exhibited strong Dye-DADH enzyme activity. The enzyme's specific activity from the crude extract of the SK1/pSAVmal-STK16240 was estimated to be about 0.029 units/mg (Fig. 2). The influence of different sugars on the Dye-DADH expression in SK1/pSAVmal-STK16240 was further investigated. SK1/pSAVmal-STK16240 was cultivated at 70 °C for 5 days in two different T media containing 0.4% maltose (MT) or dextrin (DT). The Dye-DADH activity of the crude extract prepared from the *S. acidocaldarius* transformants cultivated in T medium was 0.018 units/mg, while those from the transformants cultivated in MT and DT media were 0.051 and 0.065 units/mg (Fig. 2). The crude extract from the transformants cultured in DT medium exhibited the highest activity, and hence used for subsequent experiments.

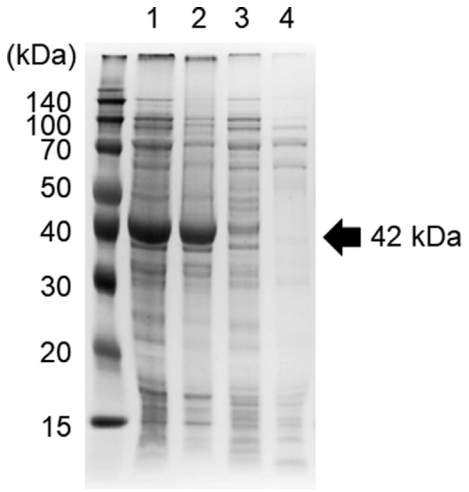

FIG. 1. SDS-PAGE analysis of recombinant STK16240 protein using *E. coli* expression system. Lanes represent the following: M, markers; 1, homogenate; 2, insoluble fraction after cell disruption; 3, soluble fraction after cell disruption; and 4, supernatant after incubation at 70 °C for 10 min. Two micrograms of the proteins were applied to each lane in SDS-PAGE.

Purification of recombinant Dye-DADH The purification process of the recombinant Dye-DADH from SK1/pSAVmal-STK16240 is summarized in Table 3. The final Dye-DADH was about 185-fold purified, with an overall yield of about 9.67%. When we superimposed the picture of the CBB stained SDS-PAGE onto that of the immunodetection, the CBB stained protein was coincided with immunodetection protein, indicating that the purified protein corresponded to the recombinant histidine tagged STK_16,240 protein (Fig. 3).

pH and temperature effects on the activity and stability Enzyme activity was measured at various pH, the highest Dye-DADH activity was observed at pH 8.0 (Fig. 4A). At temperatures between 40 and 80 °C, the enzyme activity was linearly increased with increasing temperature up to 80 °C. We observed with maximum activity at 80 °C (Fig. 4B). However, assay above 80 °C was not performed due to the non-enzymatic decolorization of DCIP. When the enzyme was incubated at pH range from 4.0 to 11.0 for 30 min at 50 °C, more than 75% of its full activity was retained (Fig. 4C). Enzyme retained its full

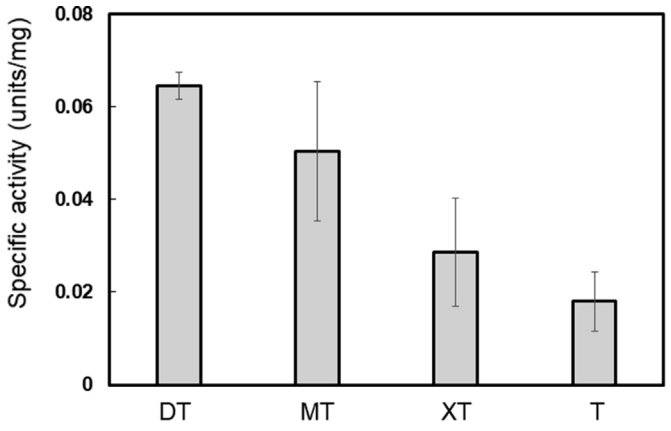

FIG. 2. Influence of different sugars on Dye-DADH activity under the control of *malE* promoter. T medium was used as the basal medium. All sugars were added at a final concentration of 0.4 %. Error bars indicate standard deviations (n= 3).

TABLE 3. Purification of recombinant *Sf. tokodaii* Dye-DADH.

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold
Crude extract	248	16.4	0.0660	100	1.00
HisTrap FF Crude	0.983	2.83	2.88	17.3	43.7
HiTrap Q HP	0.130	1.58	12.2	9.67	185

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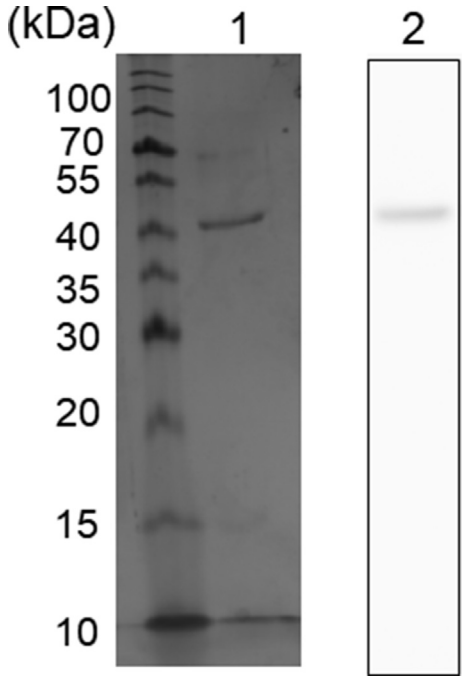

FIG. 3. SDS-PAGE and Western blotting analysis of the recombinant STK16240 protein using *S. acidocaldarius* as the expression system. Lanes represent the following: M, markers; 1, the Rapid CBB KANTO stained gel of the purified STK16240 protein; and 2, Western blotting of the purified STK16240 protein using Anti-His-tag mAb-HPR-DiercT. Protein (0.3 μg) was applied in SDS-PAGE.

activity after incubation for 30 min at temperature range from 50 to 90 °C (Fig. 4D).

Molecular mass and subunit structure Based on Superdex 200 gel filtration chromatography, the molecular mass of the native Dye-DADH was estimated to be about 205 kDa (data not shown). The molecular mass of subunit was estimated to be about 42 kDa from SDS-PAGE and this suggests that the native enzyme is a homopentamer.

Substrate and electron acceptor specificity When the ability of Dye-DADH to catalyze the dehydrogenation of various amino acids was examined, D-phenylalanine was found to be the most preferred substrate. The enzyme also exhibited activity for several amino acids including D-proline and D-histidine (Table 4). L-Phenylalanine, L-proline, and L-histidine were inert as substrates. The electron acceptor specificity of the enzyme was also examined. DCIP was the most preferred electron acceptor for the enzyme. Phenazine methosulfate (PMS) and an *p*-iodonitrotetrazolium violet (INT) coupled system also exhibited electron acceptor activity (the relative activity was 7.84 % of that observed with DCIP). Ferricyanide, O_2, benzyl viologen, and methylene blue were also inert as electron acceptors.

Kinetic parameter The apparent K_m values for D-phenylalanine was calculated to be 1.22 mM when 0.2 mM DCIP was used as the electron acceptor. The k_{cat} and k_{cat}/K_m values for D-phenylalanine was estimated to be 6.52 s^{-1} and 5.34 s^{-1} mM^{-1}, respectively.

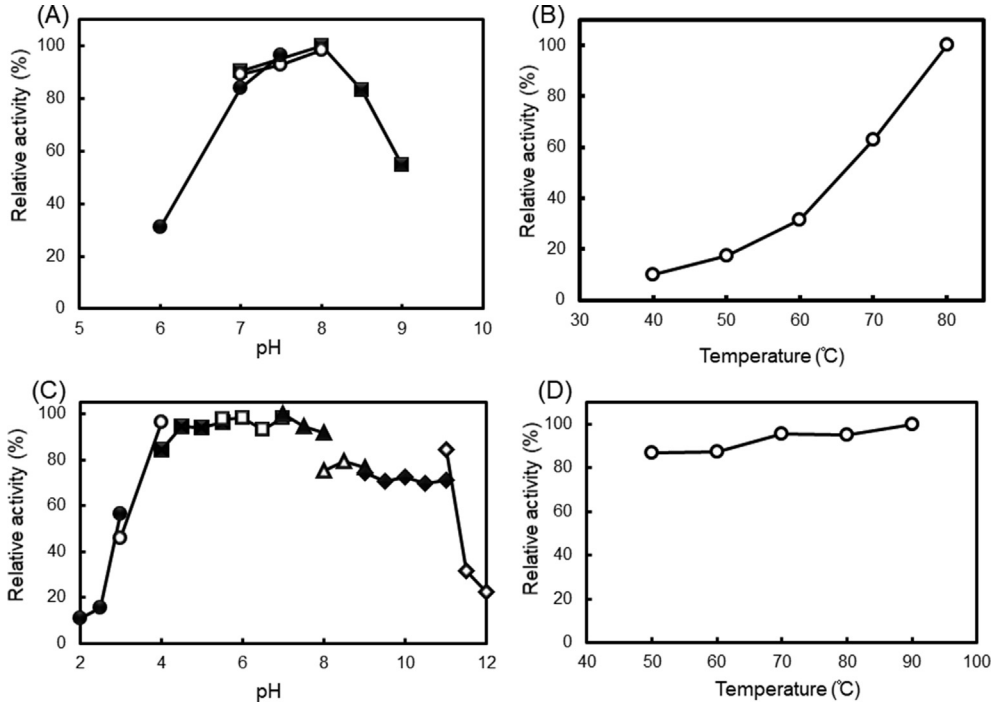

FIG. 4. Effect of pH and temperature on the stability and activity for Dye-DADH activity. (A) Optimal pH for enzyme activity. PIPES-NaOH (pH 6–7.5, closed circles), HEPES-NaOH (pH 7.0–8.0, open circles), and Tris–HCl (pH 8.0–9.0, closed squares) buffers (200 mM) at 50 °C were used to determine the optimal pH for enzyme activity. (B) Temperature profile of Dye-DADH activity. DCIP was used as an electron acceptor. Activity at 80 °C was defined as 100%. (C) Effect of pH on the stability of Dye-DADH. The enzyme was incubated for 30 min at 50 °C in buffers of various pH, after which residual activity was measured using the standard assay method. The buffers used were glycine-HCl (pH 2.0–3.0, closed circles), sodium citrate–citrate (pH 3.0–4.0, open circles), acetate-sodium acetate (pH 4.0–5.5, closed squares), MES-NaOH (pH 5.5–7.0, open squares), HEPES-NaOH (pH 7.0–8.0, closed triangles), Tris–HCl (pH 8.0–9.0, open triangles), glycine-NaOH (pH 9.0–11.0, closed rhombuses), and Na_2HPO_4–NaOH (pH 11.0–12.0, open rhombuses). (D) Effect of temperature on the stability of Dye-DADH. The enzyme was incubated for 30 min at the indicated temperatures, and the residual activity was determined using the standard assay method.

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TABLE 4. Substrate specificity of Dye-DADH from *Sf. tokodaii* and *R. marinus*.

Substrate	Relative activity (%)	
	Sf. tokodaii	*R. marinus*
D-Phenylalanine	100	100
D-Proline	85.5	93
D-Histidine	82.3	49
D-Alanine	76.5	8
D-Lysine	74.3	2
D-Arginine	69.9	22
D-Valine	68.2	26
D-Asparagine	67.7	N.D.[a]
D-Tryptophan	66.9	41
D-Serine	61.4	3
D-Leucine	60.6	36
D-Glutamine	59.4	N.D.
D-Isoleucine	56.3	37
D-Tyrosine	54.9	N.D.
D-Aspartate	25.7	0
D-Glutamate	18.1	3
D-Threonine	10.5	4
L-Phenylalanine	0	0
L-Proline	0	N.D.
L-Histidine	0	N.D.
Source of reference	This study	16

[a] Not determined.

DISCUSSION

In this study, we identified the gene encoding Dye-DADH from the thermoacidophilic archaeon, *Sf. tokodaii*. When we tried to express the gene encoding Dye-DADH in *E. coli*, Dye-DADH was produced as an inclusion body. Therefore, we constructed the recombinant protein expression system of Dye-DADH with an N-terminal histidine tag in *S. acidocaldarius* SK1. The recombinant protein was successfully expressed and purified, and exhibited thermostable Dye-DADH activity. In *Sulfolobales*, some recombinant protein expression systems have been developed (23–26). The ABCE1 protein from *Saccharolobus solfataricus* involved in recognition and modification of RNA assemblies has been successfully expressed under control of *malE* promoter and purified from 1 L of the *S. acidocardarius* culture with a yield of about 1 mg (23). The yield of the recombinant Dye-DADH from *Sf. tokodaii* was 0.13 mg per 4 L of SK1/pSAVmal-STK16240 culture. The expression level of the ABCE1 was much higher than that of Dye-DADH. This might be due to the ABCE1 protein is cytoplasmic protein, while Dye-DADH is membrane protein. However, to our best knowledge, this is the first example that membrane protein is successfully expressed in thermophilic archaea as a host.

The presence of Dye-DADH in hyperthermophilic archaeon, *Py. islandicum* and thermophilic bacterium, *R. marinus* was reported previously (16,17). When the two Dye-DADHs from thermophiles were expressed in *E. coli* as the fusion proteins with an N-terminal histidine tag, both the recombinant proteins could hardly bind to the nickel column (unpublished data). It has been reported that the N-terminal region of Dye-DADH contains a highly hydrophobic region which is postulated to be a membrane anchoring site (12,17). The predicted amino acids sequence of *Sf. tokodaii* Dye-DADH using SOSUI system (27) indicates that this enzyme also contains a membrane anchoring site at residues 1 to 23 (Supplementary Fig. S2). The N-terminal membrane binding region in Dye-DADH may disturb the correct folding of the enzyme in *E. coli* cells. On the other hand, the recombinant Dye-DADH with an N-terminal histidine tag expressed in *S. acidocaldarius* SK1 could bind to the nickel column and purified easily. This indicates that the recombinant Dye-DADH is correctly folded in *S. acidocaldarius* cells.

On the construction of highly sensitive electrochemical sensing system, the histidine tag for nickel binding is crucial to immobilize the enzyme onto electrode with a suitable orientation. We have already reported the highly orientated PQQ-dependent glucose dehydrogenase immobilized electrode utilizing the affinity between the histidine tag and Cu atom (28). The reported current density of the highly orientated electrode is 90-fold higher than that of the non-orientated enzyme immobilized electrode. Thus, the histidine tagged Dye-DADH from *Sf. tokodaii* has high potential use as a specific element for the application of the highly sensitive D-amino acid analysis.

Comparatively high sequence homology was detected in the N-terminal regions containing membrane binding site of all these Dye-DADHs (Supplementary Fig. S2). In their N-terminal regions,

TABLE 5. Comparison of the properties of Dye-DADHs.

Domain		*Sf. tokodaii*	*Py. islandicum*	*E. coli* B	*E. coli* K12	*Pseudomonas aeruginosa*	*R. marinus*
		Archaea	Archaea	Bacteria	Bacteria	Bacteria	Bacteria
Molecular mass	Native (kDa)	205	145	N.D.[a]	N.D.	200	115
	Subunit (kDa)	42	42	55, 42	N.D.	49	46
Electron acceptor		DCIP PMS-INT	DCIP PMS-INT	DCIP, CoQ1, CoQ2, CoQ3, CoQ4, CoQ5, CoQ6, CoQ7, CoQ8, Co Q1-DCIP	PMS-DCIP	PMS-DCIP	DCIP PMS-INT
Electron donor[b] (relative activity, %)		D-Phe (100) D-Pro (86) D-His (82) D-Ala (77) D-Lys (74) D-Arg (70)	D-Pro (100) *cis*-4-Hydroxy-D-proline (89)	D-Ala (100) D-Phe (100) D-Met (97) D-Amino butyrate (89)	D-Met (100) D-Ala (70)	D-Cycroserine (100) D-Ala (93) D-Asn (90) D-Lα-Amino-*n*-butyrate (76)	D-Phe (100) D-Pro (93)
K_m (mM)[c]		1.22	4.2	D-Ala: 3 D-Phe: 6	50	D-Cycroserin: N.D. D-Ala: 15	3.11
k_{cat} (s^{-1})[c]		6.52	N.D.	D-Ala: 15.5 D-Phe: 14.7	N.D.	D-Cycroserine: N.D. D-Ala: N.D.	N.D.
Optimum pH		8	7.5	N.D.	7.9	8.5	7.5
pH stability[d]		4-11	4-10	N.D.	N.D.	N.D.	4-11
Optimum temp. (°C)		80>	70>	N.D.	N.D.	N.D.	75
Thermal stability (°C)[e]		90	90	N.D.	25	37	75
Source or reference		This study	17	12	13	14	16

[a] Not determined.
[b] Substrates for which the relative activity of solubilized Dye-DADH is more than 70% relative to the best substrate.
[c] K_m and K_{cat} values for the best substrate.
[d] pH at which the enzyme retains its full activity after incubation for 10 min.
[e] Temperature at which the enzyme retains more than 80% of the initial enzyme activity after incubation for 10 min.

Please cite this article as: Satomura, T et al., Characterization of dye-linked D-amino acid dehydrogenase from *Sulfurisphaera tokodaii* expressed using an archaeal recombinant protein expression system, J. Biosci. Bioeng., https://doi.org/10.1016/j.jbiosc.2020.04.008

an FAD-binding motif (GXGXXG) was also contained, which was completely conserved among these Dye-DADHs (29). In addition, highly conserved amino acid residues were observed in C-terminal region (Supplementary Fig. S2). Although the functional amino acid residue involved in enzyme catalysis and substrate binding have not been identified, these amino acid residues in Dye-DADH might participate in catalytic reaction. To obtain further information, the three-dimensional structure analysis of the enzyme is required.

With regard to the substrate specificity, the preferred substrates for *Sf. tokodaii* Dye-DADH was similar to those of *R. marinus* Dye-DADH (Table 5). *Sf. tokodaii* Dye-DADH exhibited high against D-phenylalanine and D-proline as the electron donor (Table 5). As shown in Table 4, however, *Sf. tokodaii* Dye-DADH exhibited high activity toward other D-amino acids compared with *R. marinus* Dye-DADH: notably D-histidine, D-alanine, D-lysine, D-arginine, D-valine, D-asparagine, D-tryptophan, D-serine, D-leucine, D-glutamine, D-isoleucine, and D-tyrosine are the superior electron donors for *Sf. tokodaii* Dye-DADH. The high reactivity to broad D-amino acids is one of the unique properties of *Sf. tokodaii* Dye-DADH. D-amino acids including D-serine and D-proline are the important biomarkers on clinical diagnosis and food quality. Therefore, *Sf. tokodaii* Dye-DADH has a highly potential usefulness for application to the D-amino acid biosensor. This high reactivity against many D-amino acids indicates that *Sf. tokodaii* Dye-DADH has a high potential usefulness for enantioselective D-amino acid removal from the racemic mixtures of various amino acids as well as D-amino acid sensing.

In addition, the recombinant Dye-DADH expressed in *S. acidocaldarius* did not lose its activity after incubation at 90 °C for 30 min (Fig. 4). It has been reported that thermophilic Dye-DADHs from *Py. islandicum* and *R. marinus* lose about 80% and 20% of their activities by incubating at 90 °C for 10 min, respectively (16,17). To our best knowledge, Dye-DADH from *Sf. tokodaii* is the most stable Dye-DADH reported so far. The highly stable *Sf. tokodaii* Dye-DADH offers practical advantage for its use. In conclusion, the application of *Sf. tokodaii* Dye-DADH to a D-amino acid biosensor is now under way.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2020.04.008.

ACKNOWLEDGMENTS

We thank Ms. Rina Kitano and Mr. Nobutaka Yonehara for their technical assistance.

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