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Characterization of NAD-Dependent Formate Dehydrogenase from *Trametes versicolor* Using a Cell-Free Protein Expression System

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ABSTRACT

CO_2 emissions are the primary reason for global warming; hence, biological and chemical technologies for converting CO_2 into useful compounds are being actively studied. Biological methods using enzymes can convert CO_2 under mild conditions. Formate dehydrogenase (FDH) is a representative CO_2 conversion enzyme. Its function was revealed after isolation from bacteria, yeast, and plants. In this study, we evaluated the CO_2 conversion potential of FDH isolated from wood-rotting fungi. After isolating the FDH gene (TvFDH) from *Trametes versicolor*, we cloned the full-length FDH from *T. versicolor* and expressed it in a cell-free expression system. The gene encoding TvFDH was identified as 1,200 bp open reading frame (ORF) and the expected molecular weight of the protein was approximately 42 kDa. Overexpression of the recombinant crude protein including TvFDH was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Enzyme activities and metabolite analyses confirmed the efficiency of TvFDH for CO_2 reduction.

Keywords: CO_2 conversion, wood rot fungi, *Trametes versicolor*, formate dehydrogenase (FDH), formic acid

1. INTRODUCTION

Enzymatic CO_2 conversion has been researched as a potential method to greenhouse gas fixation and the production of renewable chemicals (Crable *et al*., 2011). Formate dehydrogenases (FDHs) are oxidoreductases that catalyzes the conversion of formate into CO_2; they have been extensively used for cofactor recycling in chemoenzymatic processes (Barin *et al*., 2019). Recently, several FDHs from bacteria, yeast, and plants have been shown to possess activity in the reverse reaction, that is, the mineralization of CO_2 into formate (Yu *et al*., 2019). FDHs can be classified into three categories, metal independent/NAD(nicotinamide adenine dinucleotide)(P)+-dependent FDHs, metal-containing/NAD(P)+ independent FDH, and metal-containing/NAD(P)+-dependent FDH (Maia *et al*., 2015). Although NAD-dependent FDHs have limited by drawbacks such as high cost and instability of reduced form of NAD (NADH), FDH (CbFDH) isolated from *Candida boidinii* is commercially available and has been adopted as a CO_2-reducing biocatalyst in enzymatic reaction systems (Calzadiaz-

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Ramirez and Meyer, 2022). However, the CO_2-reducing activity of CbFDH is still very low for practical application (Choe *et al*., 2014).

In this study, we research CO_2-reducing performance of wood rot fungus, *T. versicolor*. White rot fungi, a type of basidiomycetes, have been known as a major cause of wood biodegradation (Ham *et al*., 2021). They have also especially acid-producing abilities; oxalic acid and formic acid are the major acidic metabolites (Shimada *et al*., 1997). FDH is known as an enzyme that induces an oxidation of formate, a metabolite of wood rot fungi, can be decomposed into CO_2 (Watanabe *et al*., 2008). In the case of wood-rot fungi, studies have mainly focused on ligninolytic enzyme system for the lignin degradation (Hong *et al*., 2013; Kim and Bae, 2007; Lee *et al*., 2005). Laccase of ligninolytic enzymes has been shown effective in various biotechnological processes such as pulping and bleaching and bioremediation of environmental pollutants (Choi *et al*., 2006; Jung, 2019; Park *et al*., 2020). Also, enzyme related to secondary metabolism was reported in previous research (An *et al*., 2018). However, the reduction activity of FDH from wood-rotting fungi has not been characterized. Thus, we were motivated to characterize FDH from the white-rot fungus *T. versicolor*.

2. MATERIALS and METHODS

2.1. Chemical reagents

Commercial enzymes and high-pressure liquid chromatography (HPLC) standards were purchased from Sigma-Aldrich (Merk KGaA, Darmstadt, Germany). Expression vector (pBIVT) and AccuRapid™ protein expression kit were purchased from Bioneer (Daejeon, Korea).

2.2. Microorganism and culture conditions

Fungal culture was isolated from fruitbody collected by the National Institute of Forest Science (Seoul, Korea) in 2020. The fungal fruitbody was identified as *T. versicolor* based on morphological characteristics. The *T. versicolor* sample was inoculated on potato dextrose agar (PDA) with, 10 ppm of benomyl and 100 ppm of streptomycin sulfate, and cultured at room temperature.

The fungal isolate was identified based on internal transcribed spacer (ITS) sequences. Genomic DNA was extracted from the fungal cultures using AccuPrep genomic DNA extraction kit. The primers for polymerase chain reaction (PCR) were ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). PCR amplification conditions followed temperature cycling parameters; 95℃ for 5 min, followed by 30 cycles of 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 60 s. An elongation step of 72℃ for 5 min was performed at the end. The obtained ITS sequence was proofread and identified using Nucleotide BLAST (www.ncbi.nlm.nih.gov). Our ITS sequence was identified with the type specimen of *T. versicolor* (GenBank Acc. No.: NR_154494), showing high similarity (99.6%).

2.3. Resistance to formic acid and mRNA expression

The mycelia cultured on PDA medium were scraped and homogenized in sterile distilled water. The homogenized liquid containing the mycelium was oven-dried and inoculated into 100 mL of Potato Dextrose Broth medium so that the oven dried mycelia reached a concentration of 0.1%. Then, formic acid (5 and 10 mM) was added into an aqueous medium. The medium was shake-cultured at 100 rpm and 25℃ for 6, 9, or 12 days. The formic acid concentration was calculated by HPLC (1290 Infinity II, Agilent Technologies, Santa Clara, CA, USA). AccuPower reverse-transcription polymerase chain reaction (RT-PCR) Premix (Bioneer) was used to detect the expression level of mRNA encoding FDH. The reaction mixture was analyzed by agarose gel elec-

trophoresis. Gene-specific PCR primers were as follows: F: 5′-TGT ACT ATG CTC ACT TCC ACC A-3′ and R: 5′-CTC ATC CCA CGG TTA CAG G-3′.

2.4. Cloning

The TvFDH gene was amplified by PCR using *T. versicolor* cDNA with primers (F: 5′-TGT ACT ATG CTC ACT TCC ACC AGG ATC C-3′ and R: 5′-CTC ATC CCA CGG TTA CAG GCG CCG GCG-3′). The PCR-amplified DNA was gel-purified and sub-cloned into pCR2.1-Topo vector. And then, the open reading frame of TvFDH was digested with BamHI and NotI, and ligated using T4 DNA ligase, and was inserted into pBIVT vector.

2.5. Expression of recombinant TvFDH protein

The expression of recombinant TvFDH protein was conducted using the Accu Rapid™ cell-free protein expression kit (Bioneer). Cell-free protein synthesis is a valuable tool for understanding how mRNAs are translated into functional polypeptides (Katzen *et al*., 2005). The sample of recombinant TvFDH was concentrated using a centrifugal device according to the instructions suggested by the manufacturer. The expressed proteins were then visualized by electrophoresis on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue R-250 staining.

2.6. Enzyme activity

The FDH activity was determined by monitoring the absorbance change at 340 nm during the redox reaction catalyzed by cell-free recombinant proteins. The oxidation of formic acid was conducted using an assay solution (2 mL of a 100 mM sodium phosphate buffer at pH 7) containing 20 μL of cell-free proteins, 10 mM formic acid, and 2 mM NAD^+. The reduction of CO_2 was conducted using an assay solution (2 mL of a 100 mM sodium phosphate buffer at pH 7) containing 20 μL of recombinant protein, 50 mM $NaHCO_3$, and 0.15 mM NADH. In the case of the CO_2 reduction reaction, $NaHCO_3$ was used as a substrate to supply CO_2 because the concentration of gaseous CO_2 cannot be accurately determined.

2.7. Formic acid analysis

The amount of formic acid produced was determined by HPLC (1290 Infinity Ⅱ, Agilent Technologies) with a refractive index detector (RID). The column was an Aminex HPX-87H (300 × 7.8 mm, Bio-Lad Laboratories, Hercules, CA, USA). Sulfuric acid solution (0.01 N) was used as the mobile phase with a flow of 0.6 mL/min.

3. RESULTS and DISCUSSION

3.1. Oxidation of formic acid by *T. versicolor* mycelium

In our previous study, mycelium growth of 60 species under formic acid were identified to screen for biocatalysts from wood rot fungi. Of the 60 specimens collected, *T. versicolor* showed the highest growth rate (Jang *et al*., 2021).

To evaluate the oxidation activity of the *T. versicolor* whole cell, formic acid was added into mycelium cultures as a substrate. After 144 h of incubation, formic acid had decreased by more than 90% in the culture of *T. versicolor* treated with 5 mM formic acid. In cultures with 10 mM formic acid, substrate concentration was decreased by 7.7% after 72 h and 68.7% after 216 h. These results indicate that *T. versicolor* has the formic acid oxidation activity (Fig. 1b).

Gene expression of FDH gene was confirmed from the strain cultured at the same time by reverse trans-

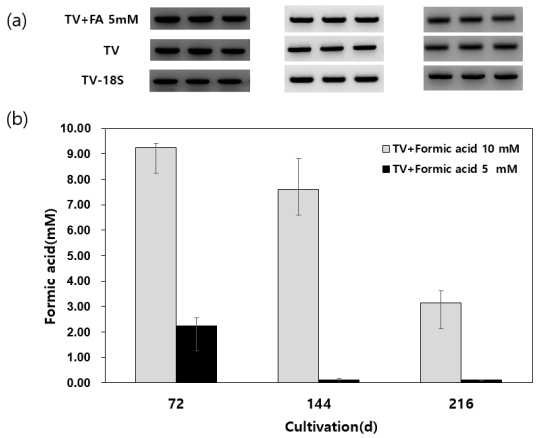

Fig. 1. Results of gene expression by RT-PCR (a) and formic acid consumption analyzed high-pressure liquid chromatography (b), respectively. The data of TV + FA 10 mM was not shown due to low growth rate. 18S: house keeping gene.

cription-polymerase chain reaction (RT-PCR). However, significant change in expression level was not observed by 5 mM formic acid (Fig. 1a). We assumed that FDH plays an essential biochemical role in *T. versicolor* during vegetative growth. According to the prior studies, FDH play an important role in decomposing oxalate to reduce NADH as an energy source during the vegetative growth of white-rot fungus *Ceriporiopsis subvermispora* (Watanabe *et al*., 2008). Wood rot fungi have been shown to produce NAD-dependent FDH as an intracellular enzyme, which decomposes formate, the reaction product of oxalate by oxalate decarboxylase, resulting in the formation of CO_2 (Mäkelä *et al*., 2014). However, the reduction activity of FDH from wood-rotting fungi has not been characterized.

3.2. Isolation of gene encoding TvFDH

The open reading frame (ORF) of TvFDH consisted of 1,200 bp nucleotides encoding 399 amino acid. The deduced protein sequence of TvFDH showed similarity with other wood rot fungi such as *Trametes* sp., *Lenzites betulinus*, *Pilatotrama ljubarskyi* etc. The BLAST search analysis for TvFDH revealed 56% identities with well-characterized FDH from the yeast *Candida boidinii* (GenBank accession no. AF004094). From the conserved domain architecture retrieval tool (CDART) analysis, NAD-binding domain were found in TvFDH at amino acid positions 174–234, 195, 256, 282, and 311–314 (Fig. 2). In CbFDH, conserved three-glycine pattern (G/A)XGXXG plays an important role in providing the specificity to NAD^+ versus $NADP^+$ (Schirwitz *et al*., 2007). This conserved motif was also identified in the TvFDH (Table 1). However, NAD binding site of TvFDH at position 233 was predicted differently than CbFDH. The roles depending on amino acid will be investigated using the purified proteins.

3.3. Expression of TvFDH recombinant protein by a cell-free expression system

TvFDH ORF of the pCR2.1-Topo clone was separated and was transferred to a Cell-free expression vector,

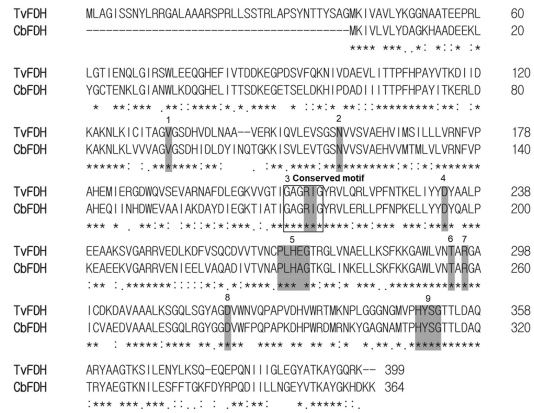

Fig. 2. Multiple alignment of amino acid sequences annotated as formate dehydrogenase from *Trametes versicolor* (TvFDH) from this study, *Candida boidnii* (CbFDH). Also, numbers (1–9) means the catalytic active site described in Table 1.

Table 1. Amino acid sequences of catalytic activity site in TvFDH and comparison of sequence alignment with CbFDH

No.	Position	Description	CbFDH	TvFDH	Similarity (%)
1	93	Substrate binding site	V	V	100
2	119	Substrate binding site	N	N	100
3	174–175	NAD binding site	RI	RI	100
4	195	NAD binding site	D	D	100
5	230–234	NAD binding site	PLHAG	PLHEG	80
6	256	NAD binding site	T	T	100
7	258	Important for catalytic activity site	R	R	100
8	282	NAD binding site	D	D	100
9	311–314	NAD binding site	HYSG	HYSG	100

FDH: formate dehydrogenase, NAD: nicotinamide adenine dinucleotide.

pBIVT vector tagged six-histidine (Fig. 3a). The recombinant gene in the plasmid was verified by PCR. Three recombinants (T-6, T-7, and T-8) of digested plasmids were obtained and showed gene expression at the expected size (Fig. 3b). These results show that the TvFDH gene had been successfully inserted into the pBIVT-N-His plasmid. The plasmids T-6, T-7, and T-8 were followed by the in vitro expression using a cell-free protein expression kit. The expression of the recombinant proteins of samples (T-6, T-7, and T-8) was visualized via SDS-page electrophoresis. The SDS-PAGE gel exhibited molecular mass (42 kDa) of the samples that were slightly over-expressed compared with the controls (Fig. 3c).

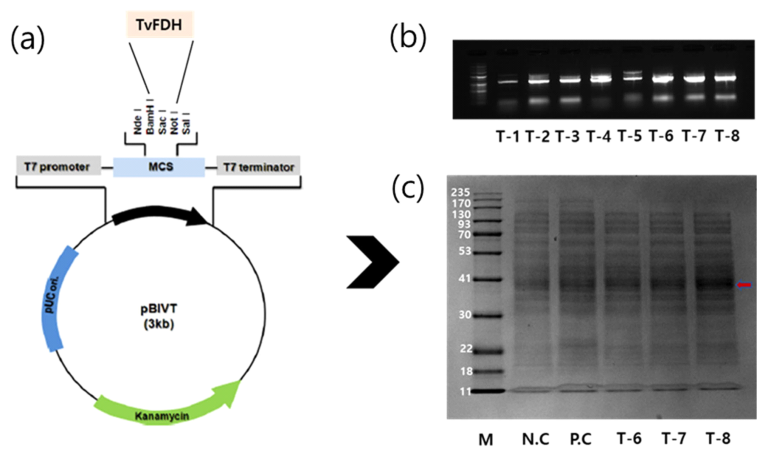

Fig. 3. Cell-free expression vector, pBIVT, and TvFDH expression. (a) schematic illustration of pBIVT vector, (b) RT-PCR of TvFDH in pBIVT-N-His-TvFDH, (c) the expressed cell-free recombinant TvFDH analyzed by SDS-PAGE. RT-PCR: reverse transcription-polymerase chain reaction.

3.4. Enzyme activity of cell-free recombinant proteins and identification of formic acid

As shown in Fig. 4, the sample of T-8 has the highest enzyme activity in oxidation and reduction reaction. The NADH measurement at 340 nm was increased from 0.10 to 0.33 (Fig. 4). In the reduction reaction, the NADH absorbance by T-8 was decreased from 0.39 to 0.29 (Fig. 5). After measuring enzyme activities, the products of samples (T-6 to T-8) were analyzed by HPLC. As the results, cell-free recombinant proteins produced 0.09–

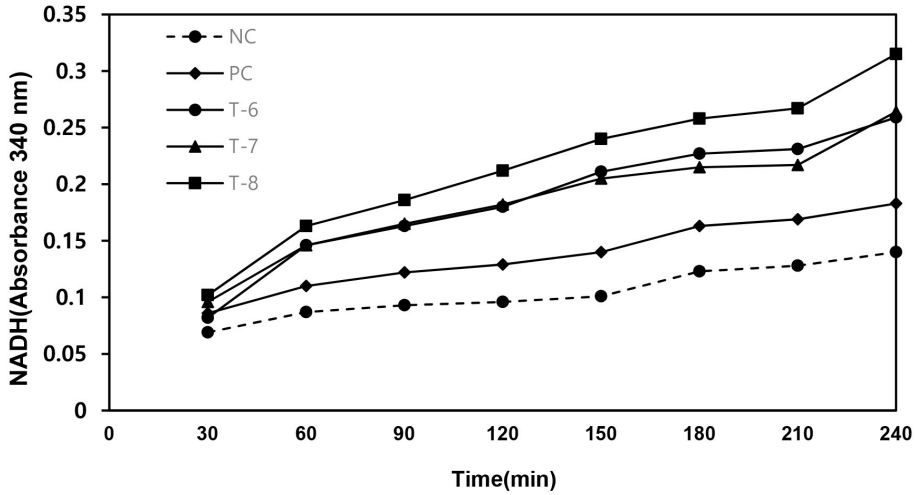

Fig. 4. Oxidation activity (absorbance at 340 nm) of cell-free recombinant proteins. NC: negative control (no DNA), PC: positive control (control DNA).

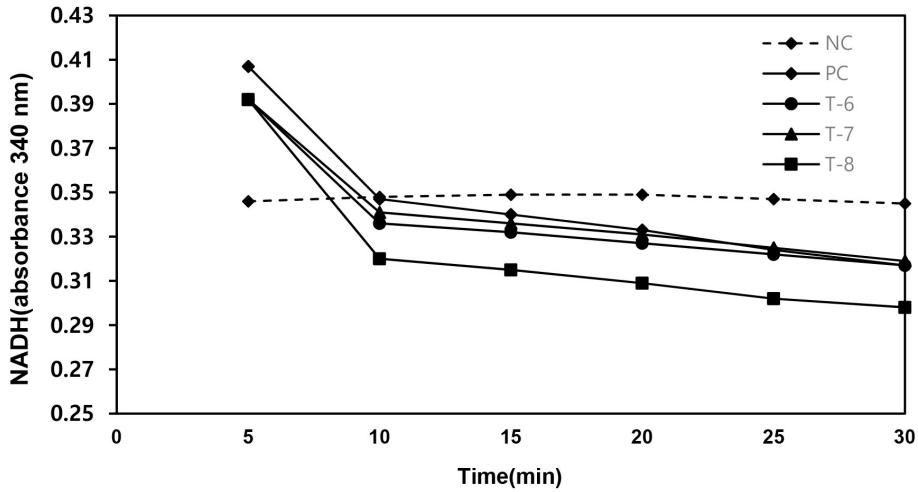

Fig. 5. Reduction activity (absorbance at 340 nm) of cell-free recombinant proteins. NC: negative control (no DNA), PC: positive control (control DNA).

Table 2. Reduction yield of formic acid by cell-free recombinant proteins by HPLC

	NC	PC	T-6	T-7	T-8
Reduction (mM)	ND	ND	0.09	0.12	0.17

HPLC: high-pressure liquid chromatography, NC: negative control (no DNA), PC: positive control (control DNA), ND: not detected.

0.17 mM of formic acid after 60 min (Table 2). These results of enzyme activities and metabolites analysis showed that the recombinant TvFDH proteins were expressed by a cell-free protein expression system.

FDHs are produced by bacteria, fungi, and plants, but few species of wood rot fungi have been characterized from *C. subvermispora* (Watanabe *et al*., 2005), and *Dichomitus squalenes* (Mäkelä *et al*., 2014). FDHs have been suggested to participate in the breakdown of oxalate metabolite by wood rot fungi (Shimada *et al*., 1994). More recently, FDH have been proposed to have a broader physiological role than detoxification. It has been suggested to participate in energy production using NADH inside the fungal cells (Mäkelä *et al*., 2014).

Reduction activity of FDH enzyme from white rot fungi has not been characterized in this study. We suggest CO_2 reduction by TvFDH isolated from *T. versicolor* based the previous suggested biological function of FDH.

4. CONCLUSIONS

Enzymatic conversion for CO_2 utilization offers a green and promising approach to reduce global warming and climate changes. In this study, we successfully cloned full-length TvFDH and expressed it in a cell-free protein expression system. We characterized the cell-free extract with NADH reduction and oxidation activity. TvFDH is a candidate enzyme for industrial applications of CO_2 conversion.

CONFLICT of INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENT

Not applicable.

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