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Research Article

DMSO tolerant NAD(P)H recycler enzyme from a pathogenic bacterium, *Burkholderia dolosa* PC543: effect of N-/C-terminal His Tag extension on protein solubility and activity

NAD(P)⁺ dependent formate dehydrogenase (FDH) is an oxidoreductase used as a biocatalyst to regenerate NAD(P)H in reductase-mediated chiral synthesis reactions. Solvent stability and the need to reduce NADP⁺ to NADPH, due to the high cost of NADPH, are required features in the industrial usage of FDHs. Therefore, we aimed to identify a novel, robust NADP⁺ dependent FDH and evaluate the effect of N- and C- terminus His tag extensions on protein solubility and activity. Herein, we report a novel, DMSO tolerant formate dehydrogenase (BdFDH), which has dual coenzyme specificity and tolerance to acidic pH, from *Burkholderia dolosa* PC543. N- and C-terminus His-tagged BdFDHs were expressed separately in *Escherichia coli* BL21 (DE3). The C-terminal His-tagged BdFDH was soluble and active whereas the N-terminal version was not. The enzyme displays dual coenzyme specificity and resistance to some organic solvents, particularly DMSO, and is able to tolerate acidic pH conditions. The apparent K_M values for NADP⁺, NAD⁺ and sodium formate (with NADP⁺), are 1.17, 14.7 and 5.66 mM, respectively. As a result, due to its DMSO tolerance and coenzyme preference, this enzyme can be utilized as an NAD(P)H recycler in several biotransformations particularly when carried out under acidic conditions. Moreover, it can be said that the position of the His tag extension may affect the enzyme solubility and functionality.

Keywords: *Burkholderia dolosa* PC543 / Cofactor regeneration / Formate dehydrogenase / N- and C- terminal His tag / DMSO tolerant

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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1 Introduction

Formate dehydrogenases (FDH) include several groups of enzymes that vary in quaternary structure, presence and type of prosthetic group and in substrate specificity. One of groups is represented by NAD(P)⁺ dependent FDHs (EC 1.2.1.2), which catalyse the oxidation of formate to carbon dioxide in the coupled reduction of NAD(P)⁺ to NAD(P)H. These FDHs contain two identical subunits, include no metal ions or prosthetic groups, and are highly specific for both formate and NAD⁺ [1].

FDH is an ideal model enzyme for the entire D-2-hydroxy-acid dehydrogenase superfamily because of its simple substrate structure and the absence of an acid-base catalysis stage in the reaction mechanism.

Because of the high cost of required coenzymes (NADH and particularly of NADPH), processes involving only dehydrogenases are economically unprofitable. The regeneration of the coenzyme *via* FDH allows low-cost and efficient industrial processes to be developed [1]. Hence, FDHs are extensively used in fine organic synthesis, being an ideal catalyst for cofactor regeneration due to its irreversible reaction and the wide optimum pH of the activity [1–4]. However, organic solvents are the most commonly used non-aqueous media for biocatalysis. Adequate

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Abbreviation: FDH, formate dehydrogenase

*Left from GTU in September 2016.

enzyme activity in such media is required for many synthetic applications. Thus, there is a need to identify solvent tolerant FDHs for industrial processes. Screening, characterization and application studies of solvent tolerant, NADP⁺ dependent FDHs has always been a hot topic in NADPH recycling research area.

Recombinant DNA technology is being widely used to express proteins in both prokaryotic and eukaryotic cells in order to obtain high levels of expression. Ultimately, these recombinant proteins are often expressed in the form of chimeric proteins. A frequent application consists in using a fusion partner to provide a tag, which can be used in the subsequent purification procedure [5]. As an example, His-tags are preferred in affinity purification, subcellular localization, ELISA, Western blotting and immobilization. However, His-tagging can sometimes affect the activity of the expressed enzyme [6]. Although, His-tag engineering has been extensively performed for protein purification, the comparative effect of His-tagging on different enzyme terminal regions has been studied only in a few papers [5–7]. Known NAD(P)⁺ dependent FDHs are usually generated as C-terminus His tagged forms in order to facilitate their purification. However, we do not have any information about the effect of polyhistidine groups on the N-terminus region of the protein.

In this study, we identified a new FDH with dual coenzyme specificity from a pathogenic bacterium, *Burkholderia dolosa* PC543, originally isolated from the lungs of cystic fibrosis patients [8]. The protein was successfully expressed with a His tag on either the N- and C-terminus of the polypeptide in *E. coli*, purified, and characterized. Activity experiments revealed the specific nature of the enzyme as a novel promising FDH, accepting both cofactors, with a remarkable dimethyl sulfoxide (DMSO) tolerance and adaptability to acidic pH. Being more active in a solvent used for substrate solubilisation, is an unusual and a significant feature for an industrial enzyme. Thus, this enzyme could be proposed as a new, promising natural candidate for NADPH regeneration systems and to guide further recombinant protein engineering studies. In addition, the effect of N-/C- terminus His tag extension on protein solubility and enzyme activity was investigated.

2 Materials and methods

2.1 Chemicals, reagents and plasmids

The plasmids pET14b and pET22b(+) used for both cloning and expression were provided by Novagen (Darmstadt, Germany). Restriction endonucleases and 2X Tag Master MixTM were obtained from New England Biolabs (NEB, GmbH, Germany) and T4 DNA ligase was from Fermentas. *E. coli* strains TOP 10 and BL21(DE3) used as host for cloning and expression, respectively were purchased from Invitrogen. IPTG (isopropyl- β -D thio-galactopyranoside) was supplied from Qiagen (ATQ Biotechnology, TR). Coomassie brilliant blue R-250, PMSF (phenylmethyl-sulfonyl fluoride), SDS (sodium dodecyl sulfate) were supplied by Sigma Aldrich. BSA was obtained from Merck Biosciences. Nicotinamide cofactors (NAD⁺ and NADP⁺; oxidized form) were purchased from Roche Applied Science. All other chemical reagents were purchased from various suppliers in analytical grade.

2.2 Mining protein sequences for NADP⁺ dependent FDH sources

The protein sequence of NADP⁺ dependent BstFDH from *Burkholderia stabilis* (ACF35003.1) was used as a probe to get novel NADP⁺ dependent FDH sources from Protein Blast (blastp) / Pattern-Hit Initiated BLAST (PHIBLAST) search [9] in GenBank hosted in the National Centre for Biotechnology Information (NCBI). An alignment of highly homolog protein sequences obtained from PHIBLAST search was performed by using Constraint Based Multiple Alignment Tool (COBALT) [10].

2.3 Cloning of BdfDH into N-terminus His tagged form

The whole genome of *B. dolosa* PC543 was sequenced by Workentine et al. and released in GenBank (NCBI accession no. NZ_LAWRY000000000). The putative FDH coding region, 1161 bp, from the *B. dolosa* PC543 genomic DNA was amplified by PCR using the following forward and reverse primers; FP1 (5'-CGGTGTCCATATGGCGAAAGTGCTTTGCG-3') and RP1 (5'-CGCTGGATCCCTACGTC AGGCTGTACG-3'). These primers include *NdeI* and *BamHI* restriction sites at their 5' end to facilitate N-terminus cloning. The PCR reaction mixture consisted of 2 $\times$ Taq Master MixTM (25 μ L, 1 $\times$), forward and reverse primers (4 μ L, 1.6 pmol/ μ L), genomic DNA (2 μ L, 3.2 ng/ μ L) and distilled water (15 μ L) in a reaction volume of 50 μ L. The PCR program involved an initial denaturation step of 5 min at 95°C and then 29 cycles of amplification (95°C, 1 min; 52°C, 1 min; 72°C, 1.5 min) followed by a final extension at 72°C for 10 min. After restriction reactions of PCR product and cloning vector, the amplified fragments were directionally cloned into the *NdeI* and *BamHI* site of pET14b to obtain the recombinant plasmids. After transformation into *E. coli* Top10 competent cells, a colony PCR reaction was performed to check the desired gene was inserted into the recombinant plasmid. In addition, the isolated recombinant plasmids were digested with restriction enzymes that were used in the cloning.

2.4 Expression and purification of N-terminus His tagged BdfDH

The recombinant plasmid was used to produce wild-type N-terminal His Tag BdfDH. This plasmid was transformed into the expression strain of *E. coli* BL21 (DE3) for FDH overexpression and plated on LB-Agar containing ampicillin for selection. A single colony was inoculated into starter cultures and grown in LB medium containing ampicillin at 37°C with agitation for 16 h. This culture was utilized for the induction of 750 mL of LB medium including ampicillin in a 2 L baffled flask. Protein expression was induced *via* IPTG (with 0.5 mM IPTG at 37°C and OD₆₀₀: 0.6–0.8). The induction was repeated at different temperatures (30, ~22 and 16°C) with shaking at 180 rpm for 6–8 h to obtain soluble protein. Aliquots from different temperatures were withdrawn at regular times and analyzed by SDS-PAGE. The auto induction procedure adapted from Studier

[11] was also carried out. The growth medium with recombinant strain was incubated for 16 h at 16°C and 180 rpm. Auto-induced cells were harvested by centrifugation, resuspended in lysis buffer comprising ice-cold buffer A (20 mM Tris/Cl, 0.5 M NaCl, 5 mM imidazole, pH 7.8), 0.1 M PMSF and lysozyme enzyme for 30 min on ice. The cell lysates were disrupted by sonication on ice. All further purification steps were performed at 4°C. The cell lysate was centrifuged and the supernatant was loaded onto a 0.5 mL Nickel HiTrap™ column (Amersham Biosciences).

The column was washed with buffer A several times to remove unbound material. The contaminating proteins were then removed by washing with 30 mM and 100 mM imidazole in buffer A before elution of the recombinant His-tagged BcFDH using 400 mM imidazole in buffer A. Subsequently, active fractions were collected and the buffer of the eluted proteins was exchanged to Buffer B (100 mM sodium phosphate, pH 7.0) using a PD-10 desalting column (Amersham Biosciences). The sample volume was reduced using a spin concentrator with a 30 kDa cut-off membrane (Millipore, Germany). Glycerol was added to enzyme at a final concentration of 50% (v/v) and the sample stored at –20°C.

2.5 Cloning of BcFDH into C-terminus His tagged form

The putative *fdh* gene was amplified from genomic DNA using the following forward and reverse primers for C-terminus cloning. FP2 (5'-CGGTGTC CATATGCGCAAAGTGCTTTGCG-3') and RP2 (5'-ATAAAT GCGGCCGCGACGTCAGGCTGTACGACGC-3'). *NdeI* and *NotI* restriction recognition sites were included in the 5' end of primers (underlined). The PCR reaction had the similar content and conditions with the aforementioned reaction with minor differences. The PCR product and pET22b(+) plasmid were digested with *NdeI/NotI* restriction enzymes. After restriction enzyme digestion, fragments was cloned into the *NdeI* and *NotI* site of pET22b(+) to construct the recombinant plasmids. After transformation into *E. coli* Top10 competent cells, plasmid DNA was purified from transformants and the insert in plasmid was completely sequenced on both strands by MEDSANTEK Company (Ankara, Turkey) to confirm sequence identity. DNA sequences were determined using T7 promoter and T7 terminator primers. Nucleotide sequence data were assembled and analysed using the BioEdit software packages (Ibis Therapeutics, CA) and checked for mutations. IPTG induction, auto-induction and purification procedures were carried out using a similar protocol to that described previously for obtaining soluble protein.

2.6 SDS/NATIVE-PAGE analysis and protein concentration determination

The purity of the recombinant native FDH was checked on 12% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) performed on Mini-PROTEAN II electrophoresis cell system (Bio-Rad). Resulting protein concen-

trations were determined using a Bradford protein quantification assay [12] with bovine serum albumin (BSA) as a standard. Native PAGE method was also used to determine the molecular mass of the native enzyme and to predict the quaternary structure of the enzyme.

2.7 Determination of steady state kinetics and enzyme assays

To determine the steady-state kinetic parameters of BcFDH for NAD⁺ or NADP⁺ separately in saturation concentrations, the final concentration of substrate (sodium formate) was maintained at 4 M while final cofactor concentration varied from 0.25 to 12.5 mM for NADP⁺ and 1.5 to 30 mM for NAD⁺ cofactor. To determine the kinetic constants for formate in the presence of NADP⁺ or NAD⁺ separately, the final concentrations of cofactors were maintained at 20 and 75 mM for NADP⁺ and NAD⁺, respectively, while the formate concentration varied from 4.25 to 403.75 mM. The reactions were monitored spectrophotometrically at 340 nm at 37°C for 3 min. All kinetics measurements were performed at least in triplicate using a microplate reader (SpectraMax Plus384™, Molecular Devices). Steady-state enzyme kinetic parameters, such as Michaelis–Menten constant (Half saturation constant; K_M), turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_M) values for substrates, were obtained by fitting the data to the Michaelis–Menten equation using non-linear regression analysis via Origin v.8 software (OriginLab, Northampton, MA). In addition, the standard activity assay was performed in a 96-well flat bottom plates in a final volume of 200 μ L based on the determination of NAD(P)H formation at 340 nm using a plate reader (SpectraMax Plus384™, Molecular Devices).

2.8 Properties of the recombinant BcFDH

The buffers used to determine the optimum pH of enzyme activity were acetic acid–sodium acetate buffer (pK₂, 50 mM, pH 3.8–5.8), citric acid–sodium citrate buffer (pK₂, 50 mM, pH 5.4–6.4), sodium phosphate buffer (pK₂, 50 mM, pH 6.2–8.0) Tris/HCl buffer (50 mM, pH 7.5–9.0) and ethanolamine buffer (50 mM, pH 8.5–10.5). The activity of the enzyme was performed at 30°C with NADP⁺ coenzyme. Also the optimum pH range was screened with the citric acid–sodium citrate buffer and sodium phosphate buffer in terms of both cofactors at optimum temperature (37°C). Activity values were determined in each of these buffers using 170 mM sodium formate, 20 μ g/mL BcFDH and 100 μ M NAD(P)⁺ at final concentrations. In a typical experiment, the buffer solution (680 μ L) containing sodium formate was mixed with enzyme sample (40 μ L) and the mixtures were incubated for 5 min. The reaction was initiated by the addition of NAD(P)⁺ (80 μ L) and monitored spectrophotometrically at 340 nm for 90 s using UV/vis spectrophotometer (Shimadzu UV-3600) equipped with a circulating water bath (VWR model 1162A). The initial rates of NAD(P)H formation were calculated using a molar extinction coefficient of 6.22 mM^{–1} cm^{–1} [13] and expressed as a percentage of the maximum activity obtained at 37°C.

The optimum temperature of the enzyme was measured by assaying the enzyme samples between the ranges of 15–60°C. Activity values were determined in 100 mM sodium phosphate buffer at pH 6.2. In a set of experiments, the buffer solution containing sodium formate (680 μ L) and enzyme sample (40 μ L) was incubated at the desired temperature for 5 min. The reaction was started by addition of NAD(P)⁺. The cofactor reduction was followed spectrophotometrically at 340 nm for 1.5–2 min. The initial rates of NADPH increment were calculated by using an extinction coefficient of 6,220 M⁻¹ cm⁻¹ and expressed as a percentage of the maximum activity obtained at 37°C. The temperature in UV/vis spectrometer was maintained using a circulating cooled water bath and temperature in the cuvette was constantly monitored using a thermometer. The thermal stability of enzyme was also determined. The protein samples (0.4 mg/mL, 400 μ L) in 75 mM sodium phosphate buffer (pH 6.2) were pre-incubated at 37, 45, 60°C for 0–60 min. After incubation for the determined time intervals (1, 5, 10, 20, 40, 60 min), samples were withdrawn and cooled. The residual enzyme activity was measured at 37°C using the standard assay with NADP⁺ by using a microplate reader and expressed as a percentage of the activity obtained without heat treatment.

Mass spectrometry of the soluble recombinant C-terminus His tagged BdfDH was performed by MALDI-TOF MS using MicroFlex instruments (Bruker Daltonics, Billerica, MA). The MS was calibrated with the Bruker's protein standards II kit including cytochrome c (12 360 Da), myoglobin (16 952 Da), trypsinogen (23 982 Da), protein A (44 613 Da) and bovine serum albumin (BSA, 66 527 Da).

2.9 Effects of metallic compounds and organic solvents on BdfDH activity and stability

To determine the effects of organic solvents on enzyme activity, BdfDH was assayed with different percentages of various, water miscible organic solvents (DMSO, acetonitrile, methanol, ethanol, isopropanol). The assay was performed in 96-well plate, in 200 μ L of the reaction mixture (170 mM sodium formate, 64 mM sodium phosphate buffer pH 6.2, 1 mM NADP⁺ at final concentrations) with organic solvents. The enzyme was added at a final concentration of 10 μ g/mL and the reaction was initiated after 5 min by addition of NADP⁺ at 37°C. Residual activity was compared in mixtures with and without co-solvent and expressed as a percentage. In addition, enzyme stability was measured by incubating the enzyme in the presence of 30% DMSO in 75 mM sodium phosphate buffer (pH 6.2) at room temperature (25°C) for 1–5 h. The remaining activity was measured as mentioned above.

To determine the effects of cations on BdfDH activity, the enzyme was incubated in the presence of various metal compounds (ZnCl₂, MgSO₄, MnCl₂, CaCl₂, NiSO₄, FeCl₂, FeCl₃ and CuSO₄) at a final concentration of 1 or 10 mM. The activity value of the assay without metal ions was arbitrarily set at 100% and resultant activities were compared with the activity of a reference reaction carried out under the same conditions.

3 Results

3.1 Screening of NADP⁺ dependent FDH sources

From pattern-based sequence screening and alignment results, some microorganisms which had a putative NADP⁺ dependent *fdh* gene sequence were identified. Among several putative FDH sources, the *Burkholderia dolosa* PC543 strain, a pathogenic bacterium isolated from the lungs of cystic fibrosis patients, was selected and the putative NADP⁺ dependent FDH (ETP61471.1) protein sequence was obtained (Supporting Information Fig. 1).

3.2 Cloning of BdfDH as N-terminus and C-terminus His tagged form

For the N- and C-terminus His tag cloning procedure, oligonucleotide primers were designed separately according to the selected vector and sequence data of the *fdh* gene. The whole *fdh* gene was amplified with the designed primers, FP1-RP1 and FP2-RP2, and then directly cloned into pET14b and pET22b(+), respectively. After transformation into chemically competent *E. coli* Top10 cells, colony PCR and restriction analysis of isolated plasmids was performed. Both analyses confirmed the desired gene insertion into the plasmid. The DNA sequencing results showed that the gene containing 1161 base pairs was successfully cloned with the polyhistidine tag. Recombinant plasmids (pET14b-*fdh* and pET22b-*fdh*) were transformed into *E. coli* BL21 (DE3) for expression of N- or C-terminus His tagged FDH protein at different temperatures. Cell extracts obtained from samples at specific time intervals were prepared and analyzed by SDS-PAGE.

3.3 Expression and purification of N- / C-terminus His tagged BdfDH

Both IPTG induction and auto-induction experiments showed that the N-terminal His tagged protein was over-expressed in inclusion bodies at different temperatures (16, ~22, 30°C). Only about 0.5 mg recombinant protein was recovered from purification at 16°C via IPTG induction for 10 hours (Fig. 1). Due to its low quantity and enzyme activity, C-terminal His tagged BdfDH was produced and further studies were performed. The C-terminal His tagged protein was soluble and the production at 30°C was stronger than at 16°C (Fig. 2A). The protein was purified by using a His-trap affinity chromatography column. SDS-PAGE analysis confirmed that C-terminus His tagged BdfDH was heterologously expressed in *E. coli* in a soluble form and the enzyme was ascertained to be pure, as seen by the homogeneous appearance of a single protein band. The protein of expected size, 43 kDa, was detected as seen by the appearance of a single protein band on a Coomassie-stained SDS-PAGE (Fig. 2A). The molecular mass of native BdfDH was estimated to be about 90 kDa by using a native PAGE procedure (Fig. 2B).

Figure 1. SDS-PAGE analysis of N-terminus His tagged BdfDH (Purification from IPTG induction at 16°C for 10 h). Lanes: M, marker protein (Bsp383FDH; molecular mass: 42 kDa); 1 and 2, washing solution; 3–5, eluted fractions containing 100, 200, and 400 mM imidazole, respectively.

3.4 Properties of the recombinant BdfDH

The theoretically calculated mass of the recombinant enzyme is 43 126 Da. The molecular mass of the monomer of enzyme was determined by MALDI-TOF and found to be 43 909 Da (Supporting Information Fig. 2). The calculated and measured masses of enzyme are in agreement with each other.

The effect of pH on BdfDH enzymatic activity was investigated at a pH range of 3.8–10.5 by ensuring the equality of ionic strength of buffers. The activity-pH profile indicated that optimum pH value is between 5.8 and 6.2 with sodium phosphate buffer for the reduction of both cofactor at 37°C (ionic strength, $\mu = 0.49$ M). Obtained data demonstrated that it keeps 50% of its activity with both cofactors in a particular pH range (pH 5.4–6.7 for NADP⁺; pH 5.7–7.2 for NAD⁺). At pH 3.4 and 8.0, the enzyme activity is almost close to zero (Fig. 3). We also observed that this enzyme is affected by the ionic strength value of the buffers. Thus, we examined the enzyme activity in terms of ionic strength. The experimental data showed that it has the highest activity between $\mu = 0.3$ –0.66 M and it lost its activity gradually outside this range.

The optimum temperature of BdfDH was determined by screening the change of enzymatic activity between the ranges of 15–60°C. As shown in Fig. 4A the enzyme exhibited an optimum activity at 37°C with both cofactors. The enzyme has activity in a broader range of temperatures with NADP⁺ cofactor (20–50°C with more than 75% activity) than with NAD⁺ (34–40°C) (4). The thermal stability of BdfDH was also detected by measuring its activity at different temperature points ranging from 37 to 60°C for incubation time of 0–60 min with NADP⁺ (Fig. 4B). The residual activity of enzyme after 1 h of exposure at 37, 45 and 60°C have been calculated as 94, 88 and 44%, respectively.

Figure 2. SDS-PAGE and Native PAGE analysis of C-terminus His tagged BdfDH. (A) The fractions from purification of recombinant native BdfDH after autoinduction at 30°C for 16 h analyzed by SDS-PAGE. Lanes: M, molecular mass marker (molecular masses in kDa are indicated on the left and right of images, Thermo Scientific); 1, cell lysate; 2, supernatant of cell lysate; 3 and 4, washing solution; 5 and 6, eluted fractions by 100 and 400 mM imidazole, respectively. (B) Native PAGE analysis of purified BdfDH. Lanes: M, molecular mass marker; lanes 1, purified BdfDH.

Figure 3. Activity-pH profile of BdfDH was determined using 50 mM citric acid/sodium citrate buffer (pH 3.0–6.2) and 100 mM sodium phosphate (pH 5.8–8.2) buffer. Each point is the mean of least triplicate assays.

3.5 Effect of metal ions and organic solvents on activity and stability

The relative activity of the enzyme was examined in the presence of various metal ions, which were added at a final concentration of 1 or 10 mM. All metal ions decreased the enzyme activity at both concentrations. However, the enzyme was completely inhibited in the presence of FeCl_2 , FeCl_3 regardless of its final concentration whereas CuSO_4 and MnCl_2 inhibited it only at 10 mM final concentration (Fig. 5).

We determined the organic solvent effect on enzyme activity and stability. To analyse the organic solvent effect on the enzyme, we used different percentages of solvents (5, 10, 20, 30, 40 and 50%) in the final reaction volume. Obtained data showed that some solvents at determined concentrations had a positive effect on the enzyme activity. For instance, ethanol, methanol and isopropanol up to 20% concentration increased the enzyme activity. In addition, enzyme in 30% DMSO had the best relative activity (about 170%) among other solvents

Figure 5. Effect of metal ions on BdfDH. Values are the means of at least three replications $\pm$ standard deviation (first columns indicate 1 mM and second ones are for 10 mM at final concentration for each metal ions).

and DMSO can enhance the enzyme activity up to a concentration of 40% (v/v) (Fig. 6). Moreover, co-solvent stability of the enzyme was checked in the 30% DMSO due to its activity enhancing property at various time intervals (0–5 h). After incubation of enzyme in 30% DMSO for 2 and 3 h, the enzyme activity was 86 and 41% that of the enzyme in aqueous solution, respectively.

3.6 Enzyme assay and steady state kinetics

The kinetic characterization of BdfDH indicated that this enzyme is able to use both cofactors with higher affinity to the NADP^+ . Kinetic constants of BdfDH for substrates and specific activities of enzyme with NADP^+ and NAD^+ cofactors were determined in 75 mM sodium phosphate buffer (pH 6.2) at 37°C. Ammonium formate and sodium formate were tested as substrates used by FDH in terms of both NADP^+ and NAD^+ . The enzyme acted on both substrates in a similar manner. The apparent

Figure 4. (A) Relative activity-temperature profile of BdfDH catalysed reduction of NAD(P)^+ . Each point is the mean of least triplicate assays. (B) Thermal stability of BdfDH. The enzyme (0.4 mg/mL) was incubated for 0–60 min at certain temperatures. The remaining residual activity was determined at 37°C using standard enzyme activity assay and expressed as a percentage of activity obtained without heat pre-treatment of the enzyme. Each point is the mean of least triplicate assays.

Figure 6. Residual activity of BdfDH in various concentrations of organic solvents.

K_M , k_{cat} and k_{cat}/K_M values of BdfDH for both cofactors and formate are expressed comparatively with reported native NADP⁺ dependent FDHs in Tables 1 and 2, respectively. As understood from kinetic parameters, the catalytic efficiency of BdfDH with NADP⁺ is about 7.5-fold more efficient with NAD⁺. K_M values for NADP⁺, NAD⁺ and formate (with NADP⁺) are 1.17, 14.7 and 5.66 mM, respectively (Table 1 and 2; Fig. 7A, B, C, D).

4 Discussion

For large-scale purification of recombinant proteins, affinity tags are usually preferred due to the relatively easy and efficient purification protocols. However, the decision regarding the relative positioning of the affinity tags remains difficult and depends on the primary structure and conformation of the protein [14]. In addition, the fusion of affinity tags has some perceived limitations, which are: misfolding and/or loss of activity or solubility of the protein, inability to use such proteins for X-ray crystallography or other physical characterization studies [15–18]. A polyhistidine-tag is an affinity tag comprising an amino acid motif made up of at least six consecutive histidine residues usually located at the N- or C-terminus of a protein [19, 20]. Although His-tag engineering has been extensively carried out for protein purification, the effect of His-tagging on different terminal regions of enzymes has not been well considered. In this study, we firstly aimed to identify a novel, robust and an efficient NADP⁺ dependent FDH and then, to evaluate the influence of the N- and C-terminal histidine tag extension on the solubility and activity of the recombinant enzyme. Therefore, we identified a new FDH with dual coenzyme specificity from a pathogenic bacterium, *Burkholderia dolosa* PC543. Both N- and C-terminus His tagged recombinant plasmids for the expression of this FDH were constructed and introduced into *E. coli*. Then, the expression levels of both were checked by SDS-PAGE and we concluded that the His tag position may not affect the expression level in a significant extent. Next, we attempted to purify the expressed N- and

Table 1. Kinetic parameters for native NADP⁺ dependent FDHs in terms of cofactors

	NADP ⁺				NAD ⁺		
	K_M (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_M	K_M (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_M	$(k_{cat}/K_M)^{NADP^+}/(k_{cat}/K_M)^{NAD^+}$
GraFDH ²⁶	0.85	3.96	4.66	6.5	5.77	0.88	5.30
Bsp184FDH ²⁷	1.1	3.07	2.79	15	0.57	0.04	73.66
Bsp383FDH ²⁷	0.7	2.77	3.96	7.6	0.82	0.11	36.76
BstFDH ²⁸	0.16	4.75	30	1.43	1.66	1.16	25.86
LbFDH ²⁹	0.12	3.51	29.25	1.68	1.76	1.05	27.9
BdfDH ^{b)}	1.17	4.04	3.45	14.7	6.68	0.46	7.5

a) Superscripts refer to references.

b) Refer to this study.

Table 2. Kinetic parameters for Native NADP⁺ dependent FDHs in terms of formate

	Formate (with NADP ⁺)			Formate (with NAD ⁺)		
	K_M (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_M Formate	K_M (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_M Formate
Bsp184FDH ²⁷	156.98	2.21	0.014	99.18	0.34	0.0034
Bsp383FDH ²⁷	126.79	6.12	0.048	50.13	0.77	0.015
LbFDH ²⁹	49.8	2.50	0.05	284.59	1.20	0.004
BstFDH ²⁸	55.5	—	—	—	—	—
GraFDH ²⁶	200	—	—	80	—	—
BdfDH ^{b)}	5.66	4.39	0.78	42.88	9.02	0.21

a) Superscripts refer to references.

b) Refer to this study.

Figure 7. A, B, C, D. Michaelis–Menten curves for NAD^+ and NADP^+ dependent reactions of BdfDH. Each point is the mean of least quadruple assays.

C-terminus His tagged recombinant BdfDH proteins by affinity chromatography. However, it became evident from these studies that the N-terminal his tagged protein could not be liberated into the supernatant after cell lysis, which was attributable to the protein being insoluble, in contrast to the C-terminus his tagged protein. Hence, in an attempt to produce this chimeric protein in a soluble form, we slowed the production by lowering the temperature and optimizing the expression time. Despite all these efforts, only a small quantity (~ 0.5 mg) of soluble protein was expressed at 16°C via IPTG induction for 10 hours was recovered from the purification process (Fig. 1). Due to poor activity and quantity of enzyme, we inferred that this tag may be inappropriate at this position. As known from literature, the N-terminal region of bacterial FDHs is an elongated loop, which covers a significant part of the enzyme subunit. There are multiple interactions with residues inside the subunit and also this loop is involved in formation of intersubunit contacts [1]. Moreover, Tishkov and Popov 2004 reported that FDH is a highly conservative enzyme. Analysis of spatial location of conservative residues in the structure of the FDH from *Pseudomonas* sp. 101 has shown that the functions of residues are different. Some of the residues provide stability of the subunit structure (e.g., ion pair Lys2-Asp89), others are involved in intersubunit interactions (Arg163, Asn164, Trp177, Ala180, Asp188), and only a few of them are involved in catalysis [1]. Therefore, we estimate that the

N-terminus his tag may have a negative effect on these inter-subunit interactions and stabilization effect, so that the polypeptide may be misfolded or unfolded and may have lost its three dimensional structure required for solubility and functionality. Then, the C-terminal His tagged plasmid (pET22b-*fdh*) was constructed and used for expression to increase the amount of purified protein in a soluble and active form. Analysis of the purified recombinant protein indicated that it was heterologously expressed in a soluble form and had retained FDH activity.

All NAD^+ dependent FDHs and other enzymes in this superfamily, D-isomer specific 2-hydroxy acid dehydrogenases, contain the coenzyme conserved nucleotide binding sequence Gly(Ala)XGlyXXGlyX₁₇Asp for NAD^+ [21]. Protein engineering studies on FDHs revealed that mutation of the conserved aspartate in the NAD^+ binding motif to alanine, serine or glutamine is particularly beneficial for NADP^+ binding [22–25]. We favoured the amino acid sequence that lacks aspartate at this position in order to obtain the NADP^+ dependent enzyme. The BdfDH has a serine residue in this conserved position. This residue has no net charge, which allows the enzyme to accept both the NADP^+ and NAD^+ . Our results are consistent with this inference and the coenzyme binding region of *B. dolosa* PC543 appears to possess this structure, which is suitable for dual coenzyme binding of NADP^+ and NAD^+ , although there is a preference for NADP^+ over NAD^+ . As understood from kinetic parameters indicated

in Table 1, the catalytic efficiency value of BdfDH with NADP^+ is about 7.5 times higher than the k_{cat}/K_M value of BdfDH in the presence of NAD^+ . On the other hand, to develop novel efficient NADP^+ dependent FDHs, Wu et al. 2009 performed simultaneous site-saturation mutagenesis of residues Asp195 and Tyr196 of CboFDH coupled with screening, which resulted in the identification of two mutant enzymes, D195Q/Y196R and D195S/Y196P that showed significant NADP^+ specificity. The ratio of catalytic efficiencies $(k_{\text{cat}}/K_M)^{\text{NADP}^+}/(k_{\text{cat}}/K_M)^{\text{NAD}^+}$ of D195Q/Y196R and D195S/Y196P are 2.1 and 0.2, respectively. Therefore, in our protein sequence mining process, we also utilized variations in these positions to get an efficient NADP^+ dependent FDH source. The protein sequence of the chosen source (BdfDH) has both alterations at these positions and our results were consistent with these inferences in terms of NADP^+ specificity. When the catalytic efficiency values are considered in the presence of both cofactors, BdfDH enzyme is more effective than the other natural NADP^+ dependent FDH enzymes except for BstFDH and LbFDH. The enzyme is active with both cofactors, however, it has a similar preference with GraFDH in favor of NADP^+ . The apparent kinetic parameters were given in Tables 1 and 2 (Fig. 7 A, B, C, D). In our opinion, it is significant to evaluate this enzyme in terms of its interaction with formate. As seen in Table 2, the K_M values for formate in the presence of both cofactors separately for BdfDH were the lowest among the reported native NADP^+ dependent FDHs [26–29]. That is, this enzyme has the best affinity to sodium formate and can act on the lowest formate concentration than the other native NADP^+ dependent FDHs. In addition, the k_{cat}/K_M value of the enzyme for formate was the highest among the native equivalents and it can be said that the interaction of BdfDH with sodium formate in the presence of NADP^+ is better than the interaction in the presence of NAD^+ .

The molecular mass of this protein was estimated to be approximately 43 kDa by SDS-PAGE and about 90 kDa using native PAGE, proving that it has a homodimer structure comprised of two identical subunits of approximately 43 kDa. The homodimer structure is also the natural form for many other reported FDHs, such as the enzyme from *Pseudomonas* sp. 101 [30], *M. vaccae* N10 [31], *C. boidinii* [32], *Bacillus* sp. F1 [33]. Bacterial FDHs that display a dependency on NAD(P)^+ have an optimum temperature of between 50 and 60°C. By comparison, the FDHs from *Burkholderia stabilis* [28], *Pseudomonas* sp. 101 [34], and *Bacillus* sp. F1 [33] showed optimum temperature at 60, 55, and 50°C, respectively. The optimum temperature for this enzyme was 37°C, the lowest value among the other reported NAD(P)^+ dependent FDHs. Therefore, it can be used in reactions that require moderate temperatures. The enzyme also has an optimum pH of between 5.8 and 6.2, making BdfDH an attractive enzyme for reactions that need to be performed under acidic conditions.

Moreover, BdfDH displays a remarkable DMSO tolerance. As we know, enzyme-catalysed reactions in organic solvents have found numerous potential applications due to their effects on enzymatic selectivity and substrate solubility. Therefore, technological utility of enzymes can be greatly improved by using them in organic solvents rather than aqueous reaction media [35]. The organic solvents including DMSO, methanol and ethanol are recognized as very effective denaturing reagents for most proteins,

because they can destroy the tertiary and secondary structures of proteins due to their high polarity and solubilisation capability [36]. However, the recombinant BdfDH was found to have high tolerance ability to the aforementioned organic solvents, particularly to DMSO (Fig. 6). High DMSO concentrations up to 40% (v/v) enhanced the BdfDH activity (at maximum 172% increasing in presence of 30% DMSO). Such enhancing ability of DMSO was not reported before for any NADP^+ dependent FDHs. It is an extraordinary feature to both increase substrate solubility and enhance the enzyme activity (Fig. 6). In terms of stability, BdfDH had nearly 86% of its initial activity after 2 h incubation in 30% DMSO. Therefore, this enzyme could be proposed for regeneration processes of both cofactors in certain enzymatic reactions (coupled with cytochrome P450, cyclohexanone monooxygenase etc.) including low water solubility substrates [27]. To benefit much more from this solvent stable protein, its high activity in DMSO can be further increased by either rational design or random mutagenesis.

5 Concluding remarks

In conclusion, we cloned, purified and characterized a novel, dual coenzyme specific and DMSO tolerant formate dehydrogenase from *Burkholderia dolosa* PC543. This enzyme can function at acidic pH values and has a relatively low optimum temperature. For these reasons, BdfDH is an attractive candidate for NAD(P)^+ recycling process in the synthesis of chiral chemicals and for pharmaceutical synthesis, particularly, for the catalysis performed in organic solvents or at lower pH values. We also conclude that addition of polyhistidine residues at the N-terminal portion of BdfDH can significantly interfere with its solubility and activity, therefore to decide the position of tag is crucial.

Practical application

This study emphasizes the utility of formate dehydrogenase from *Burkholderia dolosa* PC543 (BdfDH), which has dual coenzyme specificity and tolerance to acidic pH, for an NADPH regeneration process. This DMSO tolerant enzyme might be performed as an ideal recycler in different biotransformations containing low water solubility substrates. Since the DMSO, which is used to increase substrate solubility in the reaction systems that contains BdfDH as a recycler enzyme, will not only facilitate substrate solubility but will also increase enzyme activity. Moreover, BdfDH displays favourable characteristics for industrial utilization since it is active under acidic conditions and unlike other FDHs, possesses a lower optimum temperature range. Herein, we also examined the effect of His tag extension position on FDH functionality. By comparing the C- and N-terminus His tag effect results, it can be said that an interference on the N-terminus portion of the polypeptide may be significant for protein solubility and activity.

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