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ORIGINAL ARTICLE

Expression, purification and characterization of flavin reductase from *Citrobacter freundii* A1

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Abstract Flavin reductase plays an important biological role in catalyzing the reduction of flavin by NAD(P)H oxidation. The gene that codes for flavin reductase from *Citrobacter freundii* A1 was cloned and expressed in *Escherichia coli* BL21(DE3)pLysS. In this study, we aimed to characterize the purified recombinant flavin reductase of *C. freundii* A1. The recombinant enzyme was purified to homogeneity and the biochemical profiles, including the effect of pH, temperature, metal ions and anions on flavin reductase activity and stability, were determined. This enzyme exhibited optimum activity at 45 °C in a 10-min reaction at pH 7.5 and was stable at temperatures up to 30 °C. At 0.1 mM concentration of metal ions, flavin reductase activity was stimulated by divalent cations including Mn^{2+}, Sr^{2+}, Ni^{2+}, Sn^{2+}, Ba^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+} and Pb^{2+}. Ag^{+} was noticeably the strongest inhibitor of recombinant flavin reductase of *C. freundii* A1. This enzyme should not be defined as a standard flavoprotein. This is the first attempt to characterize flavin reductase of *C. freundii* origin.

Keywords *Citrobacter freundii* · Flavin reductase · NAD(P)H: flavin oxidoreductase · Ferric reductase · Enzyme characterization

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Introduction

Most living organisms contain multiple flavin reductases, which differ in enzymatic nature and molecular mass (Fieschi et al. 1995). Flavin reductase, known as NAD(P)H: flavin oxidoreductase, catalyzes the reduction of various flavins, including riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), at the expense of reduced pyridine nucleotides, namely NADH and NADPH (Spyrou et al. 1991). One class of bacterial flavin reductase, which is flavin-free protein, has been reported to play important biological roles as electron transfer mediator, for instance during ferric iron reduction, activation of ribonucleotide reductase, bioluminescence, and oxygen activation (Fieschi et al. 1995). This group of enzymes does not contain any prosthetic group and the enzyme thus should not be classified as a flavoprotein. There is no evidence of a chromophore or presence of flavins detected from the visible spectrum of the protein. Even when added, FMN and FAD would not bind tightly (Fieschi et al. 1995).

During assimilatory ferric iron reduction, bacterial flavin reductase reduces ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) either before or after iron transport into the bacterial cell (Schroder et al. 2003). As reviewed by Schroder et al. (2003), bacterial ferric iron reductases have been described from a variety of bacteria including *Agrobacterium tumefaciens*, *Azotobacter vinelandii*, *Bacillus megaterium*, *Bacillus subtilis*, *Escherichia coli*, *Legionella pneumophila*, *Listeria monocytogenes*, *Magnetospirillum magnetotacticum*, *Mycobacterium paratuberculosis*, *Mycobacterium smegmatis*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Rhodopseudomonas sphaeroides*, *Treponema denticola*, and *Vibrio vulnificus*. These flavin reductases are enzymes consisting of one polypeptide chain of a rather small size, generally between 10 and 40 kDa. The polypeptide chain displays an active site in which both the reduced pyridine nucleotide and the flavin can

transiently bind to allow rapid electron transfer (Fontecave et al. 1994). Until now, only a few of these reductases were purified to homogeneity and characterized in detail. The prototype for this group is Fre, the NAD(P)H: flavin oxidoreductase from *E. coli*, which consists of a single polypeptide chain of 233 amino acids, with a molecular mass of 26.2 kDa (Fieschi et al. 1995). Fre uses an electron donor, which could be either NADPH or NADH, and riboflavin is the best substrate (Nivière et al. 1999). In *E. coli*, Fre is naturally part of the ribonucleotide reductase complex, which is a key enzyme in DNA biosynthesis (Spyrou et al. 1991; Nivière et al. 1999). This enzyme was discovered as a component of this complex multiprotein system and the system catalyzes the *in vitro* transformation of an inactive form of ribonucleoside diphosphate reductase into an active enzyme. A specific tyrosine residue (Tyr-122) of the inactive ribonucleoside reductase was oxidized by the activity of flavin reductase into a stable tyrosyl-free radical. Thus, Fre functions in the first step, producing reduced flavins, which reduces the Fe^{3+} iron at the center of ribonucleotide reductase to Fe^{2+} iron. The reduced Fe^{2+} center subsequently oxidizes Tyr-122 into a radical. Meanwhile, oxygen regenerates the Fe^{3+} iron center (Fontecave et al. 1987; Spyrou et al. 1991).

In addition, similar flavin reductases were discovered in luminous marine bacteria that act by providing reduced flavins required for the luciferase reactions of the bioluminescence phenomenon. Luciferase then catalyzes the oxidation of reduced FMN by oxygen in the presence of a long-chain aldehyde with blue-green light emission (Nijvipakul et al. 2008). LuxG protein was reported as the flavin reductase from marine luminous *Photobacterium leiognathi* TH1 (Nijvipakul et al. 2008). In *V. harveyi*, at least three types of flavin reductases have been reported: one including enzymes specific for NADH, another specific for NADPH, and the third one for enzymes accepting both NADPH and NADH as electron donors (Wang et al. 2000; Campbell and Baldwin 2009).

Flavin reductase is also required for the activities of the two monooxygenases, DszC and DszA, involved in microbial dibenzothiophene (DBT) desulfurization with the sulfur-specific pathway (Ohshiro et al. 2002). DszC, which is DBT monooxygenase, catalyzes the first two steps of DBT desulfurization: DBT → DBT sulfoxide → DBT sulfone. Meanwhile, DszA is DBT sulfone monooxygenase that catalyzes the conversion of DBT sulfone to 2'-hydroxybiphenyl 2-sulfinate (HBPSi). Flavin reductase (DszD) catalyzes the reduction of flavin by NAD(P)H to form reduced flavin, which is required for DszC and DszA reactions (Ohshiro et al. 2002; Xi et al. 1997). An increase in the desulfurization rate has been caused by enhancement of reductase activity (Kertesz and Wietek 2001).

Russ et al. (2000) reported that the recombinant flavin reductase in the cell extract of *E. coli* strains, either under aerobic or anaerobic conditions, acted as an azo reductase. In the presence of NADH and FMN, several dyes and compounds were reduced by the reduced flavins generated by the flavin reductase. However, our previous study on the flavin reductase (*fre*) gene characterization revealed that the enzyme sequence is unique and does not exactly form monophyletic cluster with any of the other three grouping of azoreductases, though implies a similar functional role in azoreduction (Chan et al. 2012b). In this study, we attempted to express, purify, and characterize the flavin reductase from a dye-degrading bacterium, *C. freundii* A1. Until now, no flavin reductase enzyme from *C. freundii* has been characterized. *C. freundii* A1 was previously isolated from the oxidation pond in the vicinity of Universiti Teknologi Malaysia, and was found to be a good degrader of azo dyes (Rashid et al. 1999; Chan et al. 2012a). The *fre* gene was isolated from *C. freundii* A1, followed by directional cloning and overexpression in *E. coli* BL21(DE3) pLysS using pET-43.1c(+). The recombinant enzyme was purified to homogeneity. In this study, the biochemical characteristics of purified recombinant flavin reductase are presented. Understanding the biochemical profile of this enzyme is crucial prior to future exploration of the biological roles of flavin reductase from *C. freundii* A1.

Materials and methods

Microorganism

Citrobacter freundii A1 was streaked on nutrient agar and incubated at 37 °C. Glycerol stock of *C. freundii* A1 was maintained in nutrient broth with 15 % (v/v) glycerol and kept at −80 °C.

Molecular cloning of flavin reductase gene from *C. freundii* A1

Genomic DNA from *C. freundii* A1 was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA). The *fre* gene was amplified with designed primers, FREf*Bam*HI (5'-TAT GGA TCC GAC AGA GAG AGC GCA TG) and FREr*Pst*I (5'- TTT AAT AAC TGC AGA CGC ATC GCC AAA C). The PCR mixture (50 μL) contained 1.25 U of *Taq* DNA Polymerase (Invitrogen, USA) in PCR reaction buffer, $MgCl_2$ (2 mM), dNTPs (0.2 mM), forward and reverse primers (0.2 μM), and template DNA (1 ng). The gene was amplified in 25 cycles, with each cycle consisting of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 1 min, and chain extension at 72 °C for 2 min; with 3 min of initial denaturation and 8 min more of final chain extension. The purified 0.7-kb *fre* gene fragment was digested using restriction endonucleases *Bam*HI and *Pst*I and cloned into pET-43.1c(+) (Novagen, USA). The ligation mix was transformed into competent cells of *E. coli* strain NovaBlue (Novagen) and plated onto LB agar plates containing 100 μg mL^{-1} ampicillin (Sigma-Aldrich),

spread with 10 μL of 2 %w/v 5-bromo-4-chloro-3-indolyl-β-D-galactopyranisode (X-Gal; Promega) and 100 μL of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG; Promega). The recombinant plasmids were isolated and sequenced, subsequently transformed into *E. coli* strain BL21(DE3)pLysS (Novagen). Clones of *E. coli* BL21(DE3)pLysS containing pET43.1c(+)-*fre* were maintained on LB agar containing ampicillin (100 μg mL^{-1}), plus chloramphenicol (34 μg mL^{-1}).

Expression and purification of recombinant flavin reductase

The *E. coli* BL21(DE3)pLysS recombinant containing pET43.1c(+)-*fre* was induced with 0.5 mM IPTG for 4 h at 37 °C. The cells were harvested by centrifugation at 10,000 × *g* for 10 min. The cell pellet was thoroughly resuspended in 0.1 culture volume of 1X IB Wash Buffer (20 mM Tris-HCl, 10 mM EDTA, 1 % Triton X-100; pH 7.5) and lysed using lysozyme (100 μg mL^{-1}) after incubation at 30 °C for 30 min. Benzonase (5 U; Novagen) was added to digest bacterial nucleic acids. The cell lysate was incubated at 30 °C for 30 min. Subsequently, the inclusion bodies were collected by centrifugation at 10,000 × *g* for 10 min, followed by solubilization using Protein Refolding Kit (Novagen).

Following clarification, the solubilized fraction containing recombinant flavin reductase was dialyzed against Tris-HCl (20 mM; pH 7.5) containing 0.1 mM DTT as reducing agent to enhance correct disulfide bond formation. Dialysis was performed for at least 6 h at 4 °C with at least two buffer changes of greater than 50 times the sample volume. A second dialysis step using Tris-HCl (20 mM; pH 7.0) was carried out to remove excess DTT, and the recombinant fusion protein was transferred into this buffer of choice to promote greater stability of recombinant flavin reductase. Dialysis was continued through two additional changes of at least 3 h each. Any visible insoluble material seen following dialysis was removed prior to protein purification. The quantity of recombinant flavin reductase in the extract was initially estimated using S•Tag Rapid Assay (Novagen).

The recombinant flavin reductase contains internal His•Tag sequence in the polypeptide. Small polypropylene column (Bio-Rad Laboratories, USA) was used to hold 2.5 mL of settled His•Bind Resin (Novagen), which could purify up to 20 mg of target protein. The His•Bind resin slurry, containing immobilized Ni^{2+} cations, was added to the column and was packed at 2 mL min^{-1}. The column was washed at 1 mL min^{-1} with 3 column volumes of deionized water and equilibrated with 5 column volumes of Binding Buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole; pH 7.9). Flow rate was set at 0.5 mL min^{-1} for optimal protein loading. The column was washed with Binding Buffer (10 column volumes), followed by 6 column volumes of Wash Buffer (0.5 M NaCl, 20 mM Tris-HCl, 30 mM imidazole; pH 7.9). The bound protein was eluted with 6 column volumes of Elute Buffer (0.5 M NaCl, 20 mM Tris-HCl, 1 M imidazole; pH 7.9). The flow rate was set at 1.0 mL min^{-1} for these steps. The eluate was collected as 1-mL fractions and was dialyzed against Tris-HCl buffer (20 mM; pH 7.0) and concentrated using 30,000 MWCO Vivaspin 20 (Sartorius, Germany).

The cleavage of fusion tags from recombinant flavin reductase was performed using Thrombin Cleavage Capture Kit (Novagen). The concentration of purified recombinant flavin reductase was determined at 280 nm by Lowry assay with bovine serum albumin as standard (Bio-Rad Laboratories). The purified fraction was assayed and observed on 15 % SDS-PAGE after silver staining (Bio-Rad Laboratories).

UV-Vis spectrophotometric analysis of flavin reductase

The purified recombinant flavin reductase (0.2 mg mL^{-1}) from *C. freundii* A1 was observed using Cary 100 UV-Visible Spectrophotometer (Varian, USA) for particular chromophore visible at wavelengths above 300 nm (visible spectrum).

Flavin reductase assay

The purified recombinant enzyme was added to the reaction mixture (final volume, 1 mL) containing Tris-HCl buffer (15 mM; pH 7.5), NAD(P)H (0.2 mM) and riboflavin (15 μM). The activity of flavin reductase was determined spectrophotometrically at 30 °C for 10 min from the decrease in absorbance at 340 nm (ε_{340}=6.22 mM^{-1} cm^{-1}) due to the oxidation of NAD(P)H using Cary 100 UV-Visible Spectrophotometer (Varian). One unit (U) of enzyme activity is defined as the amount of enzyme that converts 1 nmol of substrate NAD(P)H per min.

Effect of pH on activity and stability

The optimum pH for flavin reductase activity was determined by flavin reductase assay at 30 °C in Tris-HCl buffer (20 mM) at varying pH values ranging from 3 to 11. Enzyme stability at different pH values was determined by measuring the residual activity after incubating the enzyme for 16 h at 4 °C. The effect of buffer molarity on the recombinant flavin reductase activity was also determined at various concentrations of Tris-HCl.

Effect of temperature on activity and stability

The optimum temperature for enzyme activity was determined by assaying the enzyme in Tris-HCl buffer (20 mM, pH 7.5) at varying temperature ranging from 10 to 80 °C. The activation energy (E_a) was determined from the Arrhenius plot (Segel 1976). Heat stability was measured after incubating the recombinant enzyme in Tris-HCl buffer (20 mM, pH 6.5) for 1 h

from 0 to 70 °C. The residual activity was measured at appropriate time interval (1 to 10 min) for each temperature tested. The samples were cooled on ice before flavin reductase assay. Effect of glycerol on heat stability was also measured after incubating the recombinant enzyme in Tris-HCl buffer (20 mM, pH 6.5) for 30 min at both 45 and 50 °C in the presence of 0 to 60 % (v/v) glycerol. All samples were cooled on ice for 15 min prior to absorbance determination.

Effect of metal ions, anions and other compounds on activity and stability

The effect of various metal ions (Na^+, K^+, Li^+, Rb^+, Ag^+, Mn^{2+}, Fe^{2+}, Sr^{2+}, Cd^{2+}, Ni^{2+}, Sn^{2+}, Ba^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, Pb^{2+}, Cu^{2+}, Hg^{2+}, Cr^{3+}, Fe^{3+}, and Al^{3+}; all as chloride salts except Ag^+, which was the sulfate salt) on flavin reductase activity were determined by assaying the enzyme in the presence of 0.1, 1.0 and 10.0 mM metal ions. The effect of various anions ($H_2PO_4^-$, HCO_3^-, NO_3^-, VO_3^-, IO_4^-, HPO_4^{2-}, $B_4O_7^{2-}$, $HAsO_4^{2-}$, CO_3^{2-}, SO_4^{2-}, MoO_4^{2-}, $S_2O_3^{2-}$, WO_4^{2-}, SO_3^{2-}, and PO_4^{3-}; all as sodium salts) and other compounds (EDTA, urea, SDS, DTT, guanidine-HCl, N-laurylsarcosine, Triton X-100 and Tween 20) on enzyme activity were also determined at 0.1, 1.0, or 10.0 mM (5, 10, or 20 % for N-laurylsarcosine, Triton X-100 and Tween 20). The activity obtained in the absence of ions or other compounds was taken as 100 %. The residual activity was also measured after incubating the recombinant enzyme in Tris-HCl buffer (20 mM, pH 6.5) for 2 h at 4 °C in presence of 2 mM (or 5 % for N-laurylsarcosine, Triton X-100 and Tween 20) metal ions, anions, or other compounds.

Results

Cloning, expression and purification of flavin reductase gene of *C. freundii* A1

The flavin reductase (*fre*) gene from *C. freundii* A1 of approximately 0.7 kb was successfully amplified using primers FREf*Bam*HI and FREr*Pst*I. These primers were designed based on the nucleotide sequence of *E. coli*'s flavin reductase (Accession No. M61182). The *fre* sequence of *C. freundii* A1was deposited into the GenBank with Accession No. AY163804. The *fre* nucleotide sequence revealed 81 % identity with the *fre* gene from *E. coli*, and has up to 93 % homology in amino acid sequence with *E. coli*'s flavin reductase. The *fre* gene was amplified to incorporate *Bam*HI and *Pst*I restriction sites for cloning into *E. coli* BL21(DE3)pLysS with pET-43.1c(+) as expression vector. Overexpression of the recombinant flavin reductase under T7 promoter led to intracellular accumulation in the form of inclusion bodies. An overexpression by up to 30 % of total cell extract was achieved after 4 h of induction with 0.5 mM IPTG. The hexahistidine-tagged flavin reductase enzyme was purified from the solubilized inclusion bodies followed by thrombin cleavage to remove fusion tags. The recombinant enzyme was purified to homogeneity at a 3.2-fold with yield of 17.4 % and specific activity of 170 Umg^{-1}. Figure 1 shows the SDS-PAGE analysis of recombinant flavin reductase purification from total cell extract to pure flavin reductase after immobilized metal affinity chromatography and thrombin cleavage. The result shown in lane 6 of Fig. 1 confirms that recombinant flavin reductase was preserved during thrombin proteolysis. The fusion tag and flavin reductase bands were observed at approximately 67 kDa and 26 kDa, respectively. Based on *in silico* analysis, the predicted molecular weight of flavin reductase from *C. freundii* A1 was estimated to be 26.2 kDa. The purified recombinant flavin reductase was used for characterization.

Grouping of recombinant flavin reductase

The purified recombinant flavin reductase of *C. freundii* A1 did not show the yellow color typical of flavoprotein or flavin containing enzyme. The enzyme displayed a typical protein spectrum at pH 6.5 with no particular chromophore visible at wavelengths above 300 nm (visible spectrum) at a protein concentration of 0.2 mg mL^{-1}. This excludes the presence of flavins bound to the enzyme. Therefore, the enzyme should not be classified as a flavoprotein.

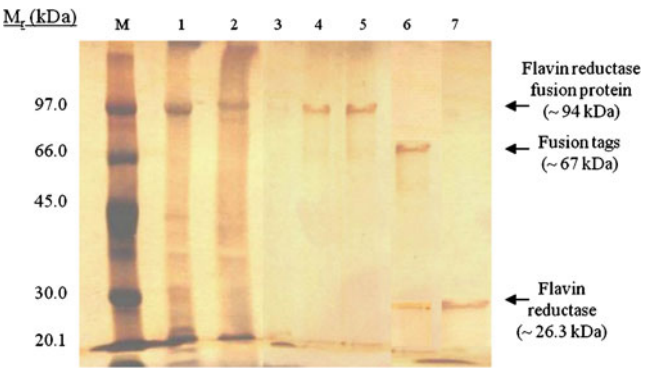

Fig. 1 Purification of recombinant flavin reductase from inclusion bodies of induced BL21(DE3)pLysS containing pET-43.1c(+)-*fre*. *M* Low Molecular Weight (LMW) standards phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.1 kDa); *1* total cell extract after 4 h of IPTG induction; *2* crude extract of solubilized inclusion bodies; *3* flow-through after wash with 30 mM imidazole; *4* eluate from elution with 1 M imidazole; *5* dialyzed and concentrated uncleaved recombinant flavin reductase fusion protein; *6* fragments produced by thrombin cleavage; *7* purified flavin reductase after thrombin cleavage. Samples were analyzed by SDS-PAGE (15 %) after silver staining

Effect of pH and temperature on flavin reductase activity and stability

From Fig. 2a, the optimal activity of recombinant flavin reductase was observed at pH 7.5. The pH stability profile is shown in Fig. 2b, suggesting that the enzyme is most stable at pH 6.5. The enzyme retained more than 90 % of its initial activity between the pH range of 6.0–7.0. The enzyme retained approximately 60–90 % of its initial activity when incubated at pH in the range of 3.0–6.0. Beyond pH 7.0, there was a drastic reduction in enzyme activity. At pH 7.5, the activity preserved was almost 58 %. Beyond pH 8.0, the activity was reduced drastically until it reached pH 11.0 where the activity was stalled.

The type of buffer used could influence the activity of the enzyme and Tris-HCl was found to be the most suitable buffer for the flavin reductase reaction. Figure 3 shows the effect of Tris-HCl buffer molarity on the activity of recombinant flavin reductase. Optimum activity was observed at 15 mM of Tris-HCl.

The activity of recombinant flavin reductase was also measured at varying temperature at pH 7.5. Figure 4a shows that the enzyme activity was optimal at 45 °C. The enzyme was completely inactivated below 10 °C and probably denatured above 80 °C. The enzyme activity increased with the increase in temperature ranges from 10 to 40 °C. Beyond 45 °C, the enzyme decreased in activity, possibly due to denaturation. Temperature may affect the higher order structure as well as the molecular motion of the enzyme in solution. The effect of temperature on the enzyme activity was further analyzed using the Arrhenius equation by plotting log V_{max} against the inverse of temperature 1/T ($K^{-1}\times10^3$). From Fig. 4b, the slope=-E_a/2.3R shows the temperature kinetic of recombinant flavin reductase. $-E_a/2.3R=-2.2813$ where the E_a is the activation energy and the R is the universal gas constants (R=8.3 JK^{-1} mol^{-1}). From the linear parts, the apparent activation energy for reduction of NAD(P)H was determined to be 43.55 kJ mol^{-1} for flavin reductase between the temperature range of 20–45 °C (Fig. 4). The high energy of activation value shows that high temperature favors the reduction of NAD(P)H by this enzyme. The Arrhenius plot clearly

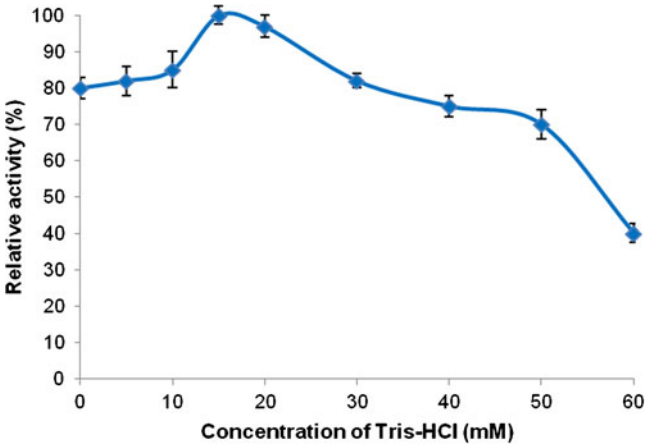

Fig. 3 Effect of Tris-HCl buffer molarity on the activity of flavin reductase of *C. freundii* A1. Values are means±standard deviation of triplicates

suggests that beyond transition point at 45 °C (1/T 3.145×10^3), V_{max} declined, indicating inactivation of the enzyme at higher temperature. The effect of temperature on the rate of reaction is expressed in terms of temperature coefficient, Q_{10}, and it was observed that the Q_{10} between 30 and 40 °C was 1.60. A rise in temperature from 30 to 40 °C resulted in a 1.6-fold increase in flavin reductase activity.

The effect of temperature on the stability of recombinant flavin reductase was also investigated and the result is shown in Fig. 5. The recombinant enzyme in 20 mM of Tris-HCl at pH 6.5 was incubated for 1 h at varying temperatures, and this was followed by measurement of the residual activity. The recombinant flavin reductase activity was practically stable for a period of 60 min at 30 °C. The enzyme showed a half-life of about 35 min at 45 °C under the conditions tested. At 65 °C, the flavin reductase activity was destroyed after 40 min of incubation. Flavin reductase of *C. freundii* A1 retained only 55 % activity at 45 °C after incubation for 30 min.

In addition, the effect of glycerol on thermal stability of purified recombinant flavin reductase was investigated. Our results indicate that the addition of glycerol at a concentration of 20 % (v/v) and below was able to increase the stability of

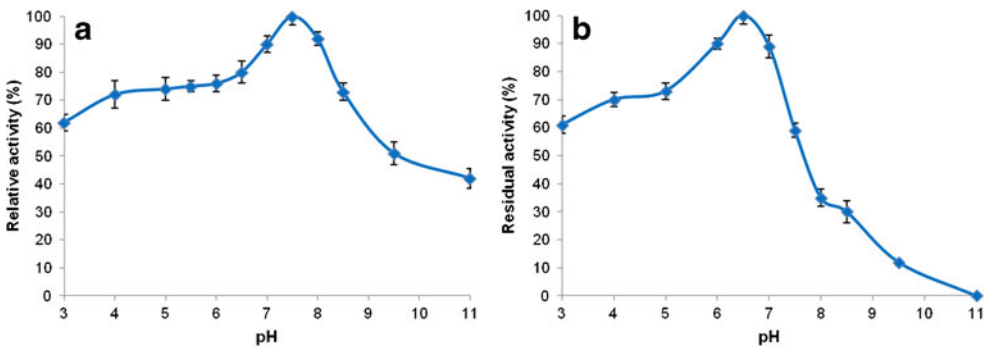

Fig. 2 Effect of pH on the **a** activity and **b** stability of flavin reductase of *C. freundii* A1. Values are means±standard deviation of triplicates

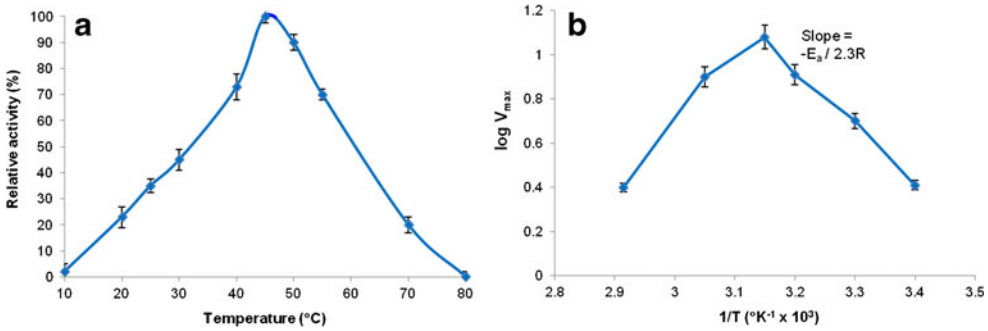

Fig. 4 **a** Effect of temperature on the activity of flavin reductase of *C. freundii* A1. **b** Arrhenius plot of log V^{max} versus 1/T ($K^{-1} \times 10^3$). The activation energy, E^a of the initial rate calculated from the Arrhenius plot was 43.55 kJ mol^{-1}. Values are means±standard deviation of triplicates

flavin reductase. In this study, higher residual activity was observed with addition of less than 40 % (v/v) glycerol. In fact, the activity of recombinant flavin reductase was enhanced with addition of glycerol between 5 and 40 % (v/v). Subsequently, the effect of 10 % (v/v) glycerol on thermal stability of purified recombinant flavin reductase was investigated at various temperatures (Fig. 6). The enzyme was found to be most stable when incubated for 10 min at 37 °C after 10 % (v/v) glycerol was added. Without the addition of glycerol, it is possible that the kinetic study of recombinant flavin reductase could be performed at 30 °C, which is within its thermostable range.

Effect of metal ions, anions and other compounds on recombinant flavin reductase activity and stability

External factors such as cations, anions, chelators, detergents, additives, and protein inhibitors could influence the flavin reductase activity. Metal ions were shown to activate or inhibit the enzymatic activity of recombinant flavin reductase (Table 1). Among the cations tested, Cu^{2+} was the best activator for recombinant flavin reductase. At 10 mM, the activity was increased to almost 1.3 times. Other cations that could stimulate the flavin reductase activity include Na^+, Li^+, Mn^{2+}, Sr^{2+}, Ni^{2+}, Ba^{2+}, Mg^{2+}, Ca^{2+}, and Cu^{2+}. Metal ions like Ag^+, Fe^{2+}, Sn^{2+}, and Fe^{3+} strongly inhibited flavin reductase activity. Ag^+ was noticeably the strongest inhibitor of recombinant flavin reductase of *C. freundii* A1 as indicated in this study. The rest of the metals in the list had no significant influence on flavin reductase activity. Although flavin reductase activity was stimulated by certain metal ions, none of these metals was absolutely required for its activity.

Among all the anions, only IO_4^- acted as activator at a 10 mM concentration. Most of the anions exhibited inhibitory effect on recombinant flavin reductase activity (Table 2). PO_4^{3-} showed the strongest inhibitory effect on the enzyme reaction. This explains the reason why phosphate buffer was probably not a suitable buffer for flavin reductase activity or in which to maintain the enzyme. SO_4^{2-} showed increasing inhibitory effect on enzymatic reaction with increasing concentration.

Most of the other compounds tested showed an inhibitory effect on recombinant flavin reductase activity (Table 3). Detergents like N-laurylsarcosine and Tween 20 showed a very strong inhibitory effect at 5 % (v/v) concentration.

The stability of recombinant flavin reductase was also analyzed after the enzyme was incubated for 2 h in 2 mM or

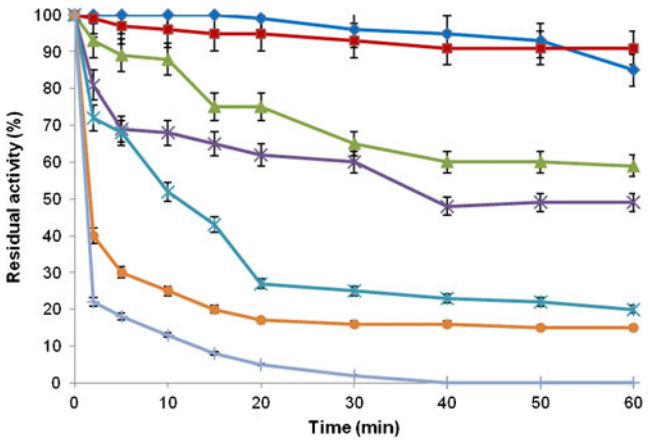

Fig. 5 Time course study on thermal stability of flavin reductase of *C. freundii* A1. The residual activity corresponds to the percentage flavin reductase activity of the non-incubated enzyme. The enzyme was initially incubated at 20 °C (♦), 30 °C (■), 37 °C (▲), 45 °C (x), 50 °C (*), 60 °C (●) and 65 °C (+).Values are means±standard deviation of triplicates

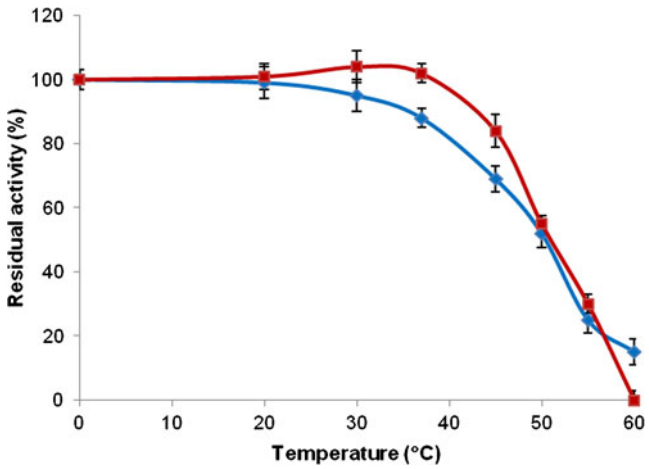

Fig. 6 Thermostability of purified recombinant flavin reductase. The enzyme was incubated in 20 mM Tris-HCl, pH 6.5, without glycerol (♦), and with 10 % (v/v) glycerol added (■), incubated at various temperatures for 10 min prior to flavin reductase assay. Values are means±standard deviation of triplicates

Table 1 Effect of metal ions on flavin reductase activity. Values are means of triplicates

	Relative activity[a] (%)		
Control	100.0		
Metal ions	0.1 mM	1.0 mM	10.0 mM
Na^{+}	113.0	95.6	118.4
K^{+}	110.3	98.0	65.2
Li^{+}	115.6	89.8	113.9
Rb^{+}	86.3	112.4	114.4
Ag^{+}	90.2	33.5	n.d.[c]
Mn^{2+}	115.6	103.6	n.d.[c]
Fe^{2+}	85.8	35.9	n.d. [c]
Cd^{2+}	93.2	87.4	75.8
Sr^{2+}	118.6	105.7	95.2
Ni^{2+}	109.5	109.3	103.2
Sn^{2+}	107.9	81.9	28.5
Ba^{2+}	110.2	88.6	116.0
Co^{2+}	107.3	106.0	97.7
Mg^{2+}	108.3	118.6	91.0
Zn^{2+}	57.6	61.9	68.8
Ca^{2+}	113.3	105.1	110.7
Pb^{2+}	113.8	81.0	64.6
Cu^{2+}	88.9	91.1	129.8
Hg^{2+}	76.8	79.9	64.8
Cr^{3+}	69.1	97.1	78.8
Fe^{3+}	52.3	37.9	n.d.[c]
Al^{3+}	88.2	68.2	59.1

[a] The enzyme activity was assayed in the presence of 0.1, 1.0, and 10.0 mM of metal ions in reaction mixture containing NADH (0.1 mM), riboflavin (0.5 μM) at pH 7.5, 30 °C for 10 min

[b] *n.d.* not determined

[c] Precipitation was observed

5 % (w/v) of the various metal ions or compounds in 20 mM of Tris-HCl (pH 6.5) at 4 °C. Subsequently, an aliquot of the mixture was used for the analysis of residual activity. The effect of metal ions, anions, and other compounds on the stability of recombinant flavin reductase enzyme is shown in Tables 4 and 5. Significant loss of activity was observed in the presence of the salts Fe(II) and Zn(II), followed by Co (II). The inhibitory effect was probably caused by the metal catalyzed oxidation on amino acid residues essential for the recombinant flavin reductase enzyme activity.

The stability of recombinant flavin reductase was maintained and enhanced in Tris-HCl buffer containing CO_3^{2-}, MoO_4^{2-}, $S_2O_3^{2-}$, WO_4^{2-}, PO_4^{3-}, SDS, and guanidine-HCl at a concentration of 2 mM (Table 5). SDS and guanidine-HCl has been shown to promote protein solubility in buffer, thus stabilizing the structural conformation of recombinant flavin reductase. Other

Table 2 Effect of anions on flavin reductase activity. Values are means of triplicates

	Relative activity[a] (%)		
Control	100.0		
Anions	0.1 mM	1.0 mM	10.0 mM
$H_2PO_4^{-}$	88.2	96.7	100.0
HCO_3^{-}	95.1	102.6	88.6
NO_3^{-}	95.4	71.6	97.2
VO_3^{-}	56.4	n.d.[c]	n.d.[c]
IO_4^{-}	94.8	98.2	134.9
HPO_4^{2-}	84.9	90.5	95.3
$B_4O_7^{2-}$	99.0	86.4	102.3
$HAsO_4^{2-}$	93.0	89.8	88.0
CO_3^{2-}	98.8	82.6	79.1
SO_4^{2-}	42.5	50.3	63.9
MoO_4^{2-}	93.3	94.2	96.8
$S_2O_3^{2-}$	82.0	96.5	88.8
WO_4^{2-}	92.5	76.8	88.0
SO_3^{2-}	99.5	96.2	94.8
PO_4^{3-}	68.4	52.1	41.6

[a] The enzyme activity was assayed in the presence of 0.1, 1.0, and 10.0 mM of anions in reaction mixture containing NADH (0.1 mM), riboflavin (0.5 μM) at pH 7.5, 30 °C for 10 min

[b] *n.d.* not determined

[c] Precipitation was observed

anions did not result in significant loss of activity, except for $B_4O_7^{2-}$.

Table 3 Effect of other compounds on flavin reductase activity. Values are means of triplicates

Other compounds	Relative activity[a] (%)		
	0.1 mM	1.0 mM	10.0 mM
EDTA	70.7	88.4	69.3
Urea	73.9	75.6	86.3
SDS	70.1	50.9	28.7
DTT	91.1	83.8	73.7
Guanidine-HCl	80.6	66.5	75.5
	5 %	**10 %**	**20 %**
N-laurylsarcosine	7.7	0	-
Triton X-100	69.8	-	-
Tween 20	14.9	0	0

[a] The enzyme activity was assayed in the presence of 0.1, 1.0, and 10.0 mM or 5, 10, and 20 % of other compounds in reaction mixture containing NADH (0.1 mM), riboflavin (0.5 μM) at pH 7.5, 30 °C for 10 min

Table 4 Effect of metal ions on flavin reductase stability. Values are means of triplicates

Metal Ions[a]	Residual activity (%)
Control	100.0
Na^{+}	81.1
K^{+}	97.6
Li^{+}	94.5
Ag^{+}	91.3
Mn^{2+}	59.6
Fe^{2+}	1.4
Cd^{2+}	21.0
Sr^{2+}	89.6
Ni^{2+}	44.6
Sn^{2+}	77.7
Ba^{2+}	94.2
Co^{2+}	14.6
Mg^{2+}	70.1
Zn^{2+}	9.2
Ca^{2+}	72.9
Rb^{2+}	98.5
Pb^{2+}	54.0
Cu^{2+}	57.4
Hg^{2+}	115.9
Cr^{3+}	54.8
Fe^{3+}	59.3
Al^{3+}	80.0

[a]2 mM

Table 5 Effect of anions and other compounds on flavin reductase stability. Values are means of triplicates

Anions and other compounds[a]	Residual activity (%)
Control	100.0
$H_2PO_4^{-}$	94.2
HCO_3^{-}	96.3
NO_3^{-}	95.4
VO_3^{-}	78.6
IO_4^{-}	82.5
HPO_4^{2-}	76.3
$B_4O_7^{2-}$	11.6
$HAsO_4^{2-}$	95.3
CO_3^{2-}	101.1
SO_4^{2-}	89.1
MoO_4^{2-}	100.8
$S_2O_3^{2-}$	100.9
WO_4^{2-}	103.7
SO_3^{2-}	87.4
PO_4^{3-}	102.4
EDTA	41.5
TRIS	112.6
Urea	24.0
SDS	118.8
DTT	59.8
N-laurylsarcosine[b]	73.7
Guanidine-HCl	120.6
Triton X-100[b]	86.5
Tween 20[b]	88.6

[a]2 mM
[b]5 %

Discussion

To the best of our knowledge, this is the first report on the flavin reductase enzyme from *Citrobacter* sp. Primers were designed based on the *fre* gene sequence reported earlier to contain restriction sites of *Bam*HI and *Pst*I that enabled directional cloning to be carried out (Chan et al. 2012b). This study has indicated that recombinant flavin reductase of *C. freundii* A1 can be efficiently expressed in *E. coli* BL21(DE3) pLysS and purified without affecting its activity. Overexpression of flavin reductase in *E. coli* BL21(DE3)pLysS is beneficial from the research and commercial standpoints, since it enables production of the desired enzyme in large quantities. A two-step purification protocol, using immobilized metal ion affinity chromatography (IMAC) followed by thrombin cleavage, has been carried out. This purification strategy takes advantage of the formation of insoluble inclusion bodies, which can sequester the recombinant protein from other soluble intracellular proteins and protect it from degradation in *E. coli* (Gupta et al. 2003; Vinogradov et al. 2003). Consequently, flavin reductase produced in high yield can be easily purified and characterized. Several research groups have reported on the use of recombinant flavin reductase for enzymatic characterization. For instance, in a biodesulfurization study, Matsubara et al. (2001) characterized the recombinant flavin reductase from *Rhodococcus erythropolis* D-1. Furuya et al. (2005) reported on the cloning of gene encoding flavin reductase from *Mycobacterium phlei* WU-F1 which was subsequently purified and characterized.

Flavin reductase from *C. freundii* A1 does not contain any flavin prostetic group, thus excluding the assumption that it is a flavoprotein. Optimum pH and temperature that enabled high enzyme activity and stability are presented here. The influence of cations, anions, chelators, detergents, additives, and protein inhibitors was observed. This study reveals that the enzyme exhibits a unique biochemical pattern. Flavin reductase from *C. freundii* A1 was found to be activated by 0.1 mM of divalent cations including Mn^{2+}, Sr^{2+}, Ni^{2+}, Sn^{2+}, Ba^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+}, and Pb^{2+}. This probably reveals the potential of flavin reductase in applications related to detection of metals and bioremediation of metals. Until now, pure flavin reductase is mainly applied in the development of enzyme-based amperometric biosensors. For instance, flavin reductase was co-immobilized with a lactate dehydrogenase in order to construct a dehydrogenase-based bioelectrode for the detection of lactate in the presence of NAD^{+} and riboflavin (Cosnier et al. 1997). Hence, there are more aspects and

applications of flavin reductase that could be explored. In addition, Ag^{+} was found to inhibit the activity of flavin reductase. Ag^{+} has been widely used as a bactericidal agent against both Gram-positive and Gram-negative bacteria (Feng et al. 2000). Silver inhibition of flavin reductase in this study inevitably reveals that this enzyme could possibly be one of the targets of the silver-based antibacterial agent.

In this study, the thermostability of flavin reductase from *C. freundii* A1 was studied to explore its industrial potential. Despite the stabilization with glycerol, the enzyme remains in the classification as a mesophilic enzyme. So far, only the flavin reductase from *Bacillus* sp. DSM411 has been reported to be the most thermophilic with an optimal activity at 70 °C, as well as the most thermostable that the enzyme retained 90 % of its activity after treatment at 70 °C for 30 min (Ohshiro et al. 2004).

This work has boosted information regarding the first ever reported flavin reductase from *C. freundii*. The comprehensive biochemical characterization of the enzyme, particularly on its temperature profile and the effect of metal ions and other compounds on flavin reductase, is important as it could pave the way to future applications of this enzyme. Substrate specificity and inhibition kinetics is underway to further characterize the enzyme activity.

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