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Production, Purification, and Characterization of Soluble NADH-Flavin Oxidoreductase (StyB) from *Pseudomonas putida* SN1

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In recombinant strains, many proteins and enzymes are expressed as inactive and insoluble inclusion bodies. For soluble expression of an active form of StyB, an NADH-flavin oxidoreductase, several recombinant *Escherichia coli* strains were developed and tested. Among them, strain BL21(DE3)pLysS effectively produced an active and soluble form of StyB as about 9% of the total protein content, when cultivated at 20°C with 0.5 mM IPTG. The solubly expressed StyB has the highest oxidoreductase activity at pH 6.5–7.5 and 37°C. Substrate dependence profiles of the StyB-catalyzed reaction showed that the maximum specific activity (V_m) and half saturation constant (K_m) were 1,867±148 U/mg protein and 51.6±11 μM for NADH, and 1,274±34 U/mg protein and 8.2±1.2 μM for FAD, respectively. This indicates that solubly produced StyB has 6- to 9-fold higher oxidoreductase activities than the *in vitro* refolded StyB from inclusion bodies.

Keywords: StyB, styrene monooxygenase, *Pseudomonas putida* SN1, BL21(DE3)pLysS, NADH, FAD

Styrene monooxygenase (SMO) catalyzes the enantioselective conversion of styrene to (*S*)-styrene oxide, an important chiral intermediate for the synthesis of many pharmaceuticals [5, 7, 12]. Several *Pseudomonas* strains that can grow on styrene as a sole carbon and energy source are known to have SMO activity, and have been studied for the genes and proteins responsible for the degradation of styrene [6, 8, 15, 27]. For example, *Pseudomonas fluorescens* ST has been reported to have four open reading frames coding for SMO (StyA and StyB), styrene oxide isomerase (StyC), and phenylacetaldehyde dehydrogenase (StyD) [3]. Recently, we have also found the presence of a similar set of genes in the *Pseudomonas putida* SN1 strain [21, 22]; these genes have high similarities in DNA and amino acid sequences with the corresponding genes and enzymes of the previously reported styrene-degrading *Pseudomonas* strains.

SMO consists of two separate enzymes, styrene epoxidase (StyA) and NADH-flavin oxidoreductase (StyB) [20]. Both enzymes are required for the bioconversion of styrene to styrene oxide, in the form of either SMO-expressing whole cells or purified enzymes [1, 17, 18, 20, 21]. For characterizing StyA and StyB and optimizing SMO reactions, the production and purification of both enzymes have been attempted from recombinant *E. coli* strains where *styA* and/or *styB* had been cloned under the control of *alk* or T7 promoter [11, 16, 19]. According to the relevant literature, however, only StyA protein (MW, ~46 kDa) could be expressed and obtained in a soluble and active form [11, 16]. StyB protein (MW, ~18 kDa) was always produced in inclusion bodies, and the inclusion bodies had to be renatured in order to create an active form of the StyB enzyme [11, 16].

It has been reported that the form of expression of recombinant proteins, either soluble or particulate, in *E. coli* is often affected by genetic characteristics of the host strain [23, 24]. In addition, it is well documented that the amount of enzymes produced in a soluble and active form varies greatly according to the induction temperature and inducer concentration during the cultivation of recombinant strains [14]. In this study, we report that the construction of a recombinant *E. coli* BL21(DE3)pLysS harboring *styB* of *P. putida* SN1 can produce StyB in a soluble and active form [21]. After the solubly produced StyB was purified, the kinetic properties were investigated and compared with those of the renatured StyB from inclusion bodies [9, 10]. This is the first report on the expression and purification of a soluble form of StyB protein originating from a *Pseudomonas* strain.

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MATERIALS AND METHODS

Construction of StyB Expression Vector

The DNA fragment containing the *styB* gene (0.53 kb) was obtained from the previously described pETAB2 vector [21], *via* PCR amplification with the forward primer 5'-AGGAATGCCATATGA-CGTTAAAAAAAAGATGTGGTGGTGGA-3', and the reverse primer 5'-TATGGCCTCGAGATTCAGTGGCAACGGGTTGCCGTGATA-ACG-3'. The PCR product was purified by electrophoresis, digested with NdeI/XhoI, and ligated with the similarly digested pET-31b(+) vector to yield the expression vector, pET31b-StyB, which has a His-tag at the C-terminus of the protein to facilitate protein purification.

Expression of StyB in Different Types of *E. coli* Competent Cells

The StyB expression vector, pET31b-StyB, was transformed into three different cells of *E. coli*, designated by BL21(DE3), Rosetta, and BL21(DE3)pLysS (Novagen), respectively. The cells were grown in LB medium supplemented with ampicillin (100 μg/ml) at 37°C. IPTG (final 0.5 mM) was added to induce protein expression at A_{600} = 0.6. The cultures were further cultivated at 37°C (4 h) or 20°C (14 h), and were then harvested by centrifugation. The cell pellet was resuspended in a buffer A [50 mM Tris/Cl (pH 7.5), 0.1 M NaCl, 1 mM PMSF, and 10% sucrose; 5 ml of buffer A/g of cell pellet] and disrupted by sonication. Lysed cells were centrifuged (13,000 rpm, 40 min), and the supernatant was then collected (crude extract).

Effect of Temperature on the Production of StyB in *E. coli* BL21(DE3)pLysS (pET31b-StyB)

The effect of temperature on the production of StyB in the recombinant BL21(DE3)pLysS (pET31b-StyB) was studied at induction temperatures ranging from 20–30°C. The cells were grown at 37°C until the bacterial culture reached A_{600}=0.6, and 0.5 mM IPTG was then added. The temperature was then changed to 30, 25, or 20°C, and the cultures were further cultivated for 5 h, 6 h, and 14 h, respectively. Crude extracts were prepared as described above and examined for the expression of soluble StyB.

Purification of Soluble StyB

The recombinant *E. coli* BL21(DE3)pLysS (pET31b-StyB) was cultured under the optimal overexpression conditions as described above (20°C and 14 h). The cells were then disrupted, and the crude extract was applied to a 10-ml Ni-charged chelating column equilibrated with a buffer B (pH 7.5; 50 mM Tris/Cl, 0.5 M NaCl, 5 mM imidazole, and 10% glycerol). The column was washed with 20 column volumes of buffer B, and the bound proteins were then eluted from the resin with a step gradient of 0.1, 0.2, and 0.3 M imidazole, respectively, in buffer B. The flow rate was maintained at 0.5 ml/min. Elutions containing StyB were pooled and concentrated using the Amicon membrane concentrator.

Measurement of StyB Activity

The StyB activity assay was performed spectrophotometrically by measuring the rate of NADH oxidation at 340 nm [11, 16]. The reaction was started by the addition of 200 μM NADH to a reaction mixture that contained 1 μg/ml StyB, 200 μM FAD, 1 mM DTT, and 5% glycerol in a proper buffer solution. The reaction volume was 0.5 ml. Assays under each condition were carried out in triplicate, and the data were averaged. Activities are represented in units (U), where 1 U is the activity of the enzyme that converts 1 μmol of substrates per minute.

The effect of pH on StyB activity was studied at 22°C in a pH range of 4.0 to 9.0. Several different buffer solutions (50 mM) were used as follows: sodium acetate (pH 4.0–5.0), MES (pH 5.5–6.0), sodium phosphate (pH 6.5–7.5), and Tris/Cl (pH 8.0–9.0). The effect of temperature was studied in phosphate buffer solution (pH 7.2, 50 mM) in the range of 20 to 40°C.

The dependence of StyB activity on the concentrations of NADH, FAD, and NADPH was studied at 37°C in a buffer C (50 mM sodium phosphate, pH 7.2, 1 mM DTT, and 5% glycerol) with 1 μg/ml StyB. The effect of NADH on StyB activity was investigated at 100 μM FAD and at NADH concentrations varying from 0 to 200 μM. The FAD dependence of StyB activity was examined at 200 μM NADH and at FAD concentrations varying from 0 to 200 μM. The NADPH dependence of StyB activity was studied at 200 μM FAD by measuring the rate of NADPH oxidation in the presence of NADPH concentrations ranging from 0 to 200 μM. In order to determine the maximum specific activity (V_m) and half saturation constant (K_m) values, the specific activity was plotted against the substrate concentration, and the curves were fitted to the Michaelis-Menten equation.

RESULTS AND DISCUSSION

Production of Soluble StyB in *E. coli* BL21(DE3)pLysS (pET31b-StyB)

For the production of soluble StyB protein, several *E. coli* strains were tested as hosts. Fig. 1 shows that StyB expression is dependent on both the *E. coli* host and the growth temperature following IPTG induction. Soluble expression of StyB protein was not appreciable at 37°C for any of the three strains (Fig. 1A), but at 20°C, the BL21(DE3)pLysS strain harboring pET31b-StyB abundantly expressed soluble StyB (Fig. 1B, lane 6). The two other strains could not produce soluble StyB even at 20°C. The BL21(DE3)pLysS strain is known to carry a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter, and also to contain a plasmid, pLysS, which carries the gene encoding the T7 lysozyme, a natural inhibitor of T7 RNA polymerase [13, 28]. This strain provides an extremely tight control for the expression of recombinant proteins cloned under the control of the T7 promoter, and has been used for the production of proteins with expression that is highly toxic to host cells. It is speculated that the production of StyB under a non-induced condition is more effectively suppressed in strain BL21(DE3)pLysS than in the other two *E. coli* strains. However, it is not clear how the tight control of the *styB* transcription helps the soluble production of StyB proteins in this study. The effect of induction temperature on the production of soluble StyB in *E. coli* BL21(DE3)pLysS (pET31b-StyB) was further studied by varying the culture temperature after IPTG induction in the

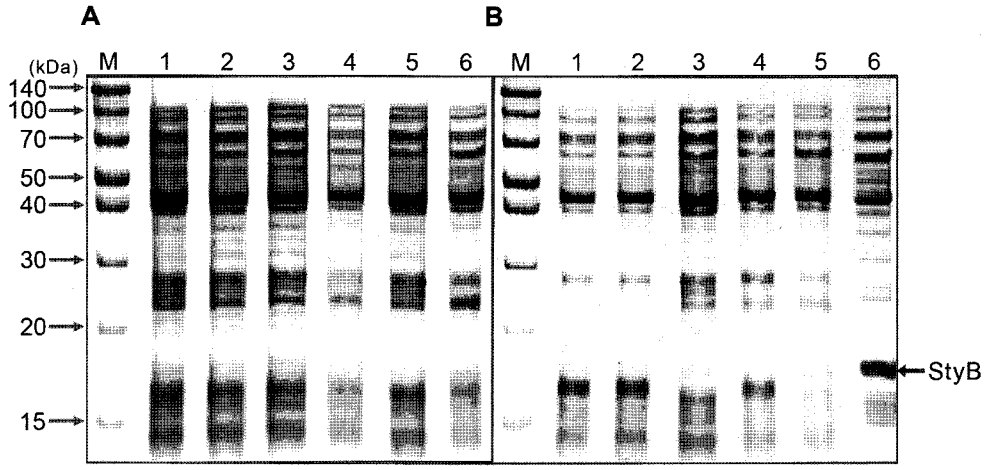

Fig. 1. SDS-PAGE (12%) analysis for the soluble crude extracts of three different kinds of transformed *E. coli* cells at growth temperatures of 37°C (**A**) and 20°C (**B**) following IPTG induction.
Lane M, molecular weight marker; lane 1, prior to induction with BL21(DE3); lane 2, following induction with BL21(DE3); lane 3, prior to induction with Rosetta; lane 4, following induction with Rosetta, lane 5, prior to induction with BL21(DE3)pLysS; lane 6, following induction with BL21(DE3)pLysS.

range of 20 to 37°C. Interestingly, StyB was not expressed as a soluble form at temperatures other than 20°C (data not shown). This finding suggests that, for soluble expression, StyB requires a slow synthesis rate, which is characteristic of a low growth temperature. This result agrees with numerous reports that the soluble production of many proteins increases as the culture temperature is decreased [25, 26].

The soluble production of StyA of the SMO enzyme in recombinant *E. coli* strains has been reported several times, including our study, which has employed the *E. coli* BL21(DE3) strain and the pETAB2 vector [21]. However, the production and purification of soluble StyB protein has not been possible thus far. The expression level of StyB was very low in the strains developed for whole-cell SMO biocatalysis, where both *styA* and *styB* were coexpressed [2, 21], or the StyB proteins easily formed aggregates in the strains that had been developed to produce StyB exclusively [11, 16]. The successful overexpression of soluble StyB in *E. coli* BL21(DE3)pLysS (pET31b-StyB) enabled us to purify the protein in a large quantity, and to study its characteristics further.

Purification and Characterization of Soluble StyB Protein

Fig. 2 shows the purification of His-tagged StyB as observed by SDS-PAGE. The purified StyB appeared almost 100% pure on a Coomassie-stained gel (Fig. 2, lane 5). The apparent size agreed with the expected molecular mass of StyB (~18 kDa), which was deduced from the DNA sequence. Western blot analysis also confirmed that the purified protein was StyB (data not shown). Approximately 10 mg of purified StyB was obtained from 1 l of culture broth.

The pH dependence of StyB activity in the presence of NADH and FAD shows that the optimal pH condition exists within the range of 6.5 to 7.5 (Fig. 3A). This is consistent with previous experiments in which the refolded StyB from inclusion bodies was tested. In the case of StyB from *P. putida* S12, the apparent maximum activity (V_{max}) was the highest at around pH 6, and the highest first-order reaction rate (V_{max}/K_m) was observed at pH 7 [11]. The temperature dependence of StyB activity was also investigated. Fig. 3B shows that StyB activity increases at temperatures up to 37°C, and decreases thereafter. The activation energy (E_a) was estimated as 2.72×10^4 J/mol from the Arrhenius plot (Fig. 3B inset).

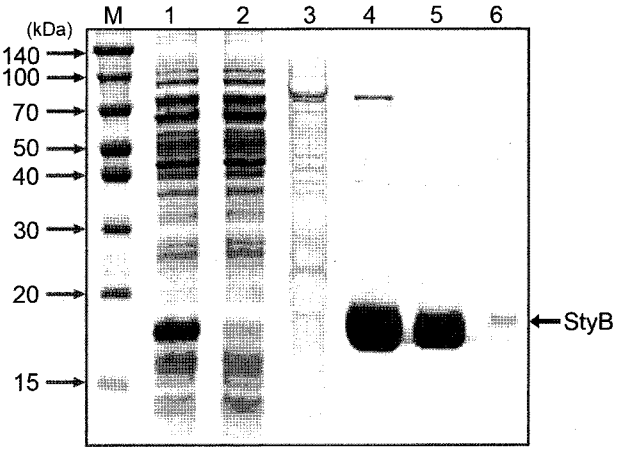

Fig. 2. SDS-PAGE (12%) analysis for the purification of StyB protein.
Lane M, molecular weight marker; lane 1, crude extract; lane 2, pool of fractions during loading of the crude extract sample to the Ni-charged chelating column; lane 3, pool of fractions during the washing step with buffer B; lane 4, elution with 0.1 M imidazole in buffer B; lane 5, elution with 0.2 M imidazole in buffer B; lane 6, elution with 0.3 M imidazole in buffer B.

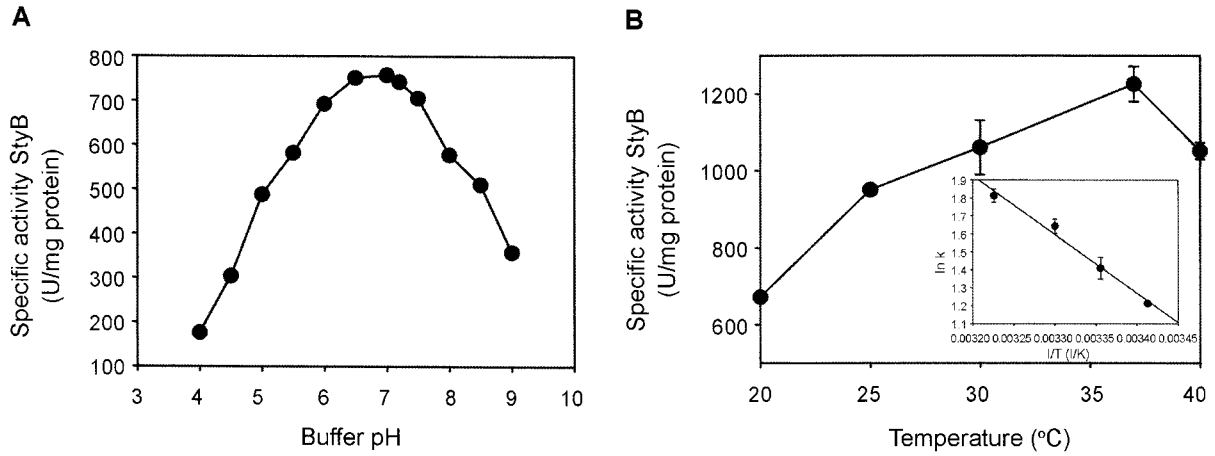

Fig. 3. PH and temperature dependence of StyB activity.
A. StyB activity was measured at a fixed concentration of StyB (1 μg/ml) in several buffer solutions at 22°C and at various pHs. The concentration of NADH and FAD was 200 μM. **B.** Enzyme activity was measured at a fixed concentration of StyB (1 μg/ml) by measuring the rate of NADH oxidation at various temperatures. The concentration of NADH and FAD was 200 μM. The Arrhenius plot provided an activation energy of 2.72×10^4 J/mol (inset).

The kinetic properties of soluble StyB were investigated at varying concentrations of NADH, FAD, and NADPH (Fig. 4). StyB shows the Michaelis-Menten kinetics of the tested substrates. When the StyB activity was measured at varying NADH concentrations in the presence of 100 μM FAD, the specific activity increased in a hyperbolic manner, with increasing NADH concentrations providing a saturating oxidoreductase activity (V_{max}) of 1,867±148 U/mg protein and a half saturation constant (K_m) of 51.6±11 μM (Fig. 4A). The effect of the FAD concentration on the activity of a StyB catalyzed reaction is shown in Fig. 4B. The specific activity also increased in a hyperbolic manner with increasing FAD concentrations, providing a V_{max} of 1,274± 34 U/mg protein and a K_m of 8.2±1.2 μM. From Michaelis-Menten kinetics of the tested substrates, the double-reciprocal plots were also obtained (Fig. 4 inset). These results are summarized in Table 1. Our results indicate that the specific activity of solubly produced StyB is significantly higher than that of the renatured StyB from inclusion bodies, whereas the K_m values do not differ to a significant degree. Otto *et al.* [16] reported that the specific activity of StyB refolded from inclusion bodies is about 200 U/mg protein at a StyB content of 1–2 μg of protein per ml, which is 6- to 9-fold lower than in our study. The significant difference in the V_m between solubly produced StyB and renatured StyB from inclusion bodies might be caused by structural differences between the two StyB types. However, it appeared more likely that it was related to the non productive folding of some StyB proteins during the processes of renaturation. Protein refolding is a complex process that often leads to

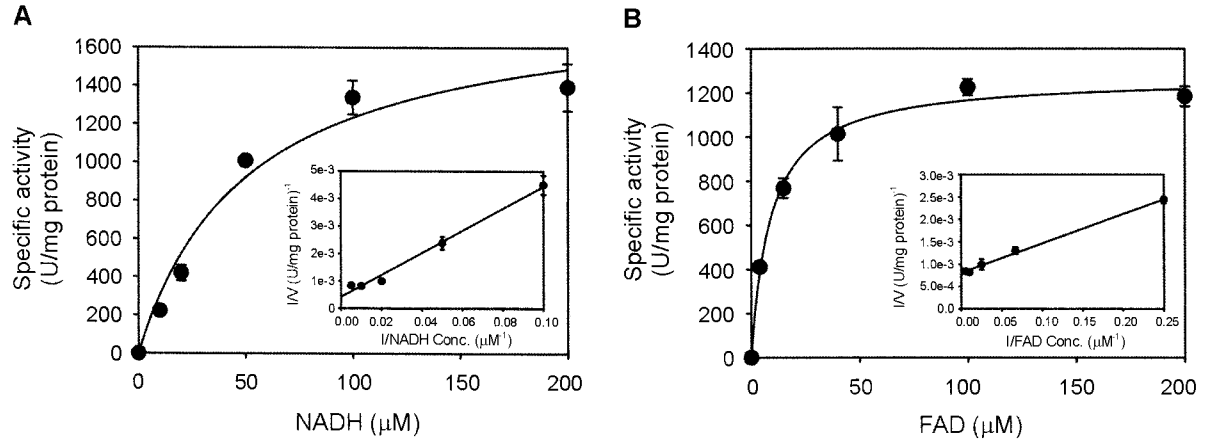

Fig. 4. Effect of substrate concentration on the rate of the StyB catalyzed reaction.
A. StyB activity was measured at a fixed concentration of StyB (1 μg/ml) and various concentrations of NADH at 37°C. The FAD concentration was 100 μM. The maximum specific activity and K_m values were 1,867±148 U/mg protein and 51.6±11 μM, respectively. **B.** StyB activity was measured at a fixed concentration of StyB (1 μ/ml) and various concentrations of FAD at 37°C. The NADH concentration was 200 μM. The maximum specific activity and K_m values were 1,274±34 U/mg protein and 8.2±1.2 μM, respectively. Double-reciprocal plots were depicted in (**A**) and (**B**) as inset.

Table 1. Saturating oxidoreductase activity (V_{max}) and half saturation constant (K_m) values for StyB in the presence of NADH and FAD.

Variable substrate	Fixed substrate (200 μM)	V_{max} (U/mg protein)	K_m (μM)
NADH	FAD	1,867±148	51.6±11
FAD	NADH	1,274±34	8.2±1.2

inactive conformation [4] and depends on many factors including metal ions, chaperone, and intermediates during the refolding process. Therefore, it can be suggested that the formation of a soluble enzyme by *in vitro* refolding does not guarantee the formation of an active enzyme in the case of StyB. If the specific StyB activity reflects the ratio of properly folded protein among the total soluble enzyme, we can suggest that only 11–16% of the refolded StyB has a proper conformation. NADPH was also tested as a reducing agent for StyB. No oxidoreductase activity was observed with NADPH (data not shown).

Here, we report the overexpression and purification of StyB as a soluble and active form in *E. coli* BL21(DE3)pLysS (pET31b-StyB). Biochemical studies show that solubly produced StyB has a much higher oxidoreductase activity than the previously reported renatured StyB. It is expected that the StyB produced in this study can be of great help for further investigation of the properties and biotechnological applications of SMO.

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