Purification and Characterization of 1-Naphthol-2-hydroxylase from Carbaryl Degrading *Pseudomonas* strain C4

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**Running Title:** 1-Naphthol-2-hydroxylase from *Pseudomonas* sp. C4
ABSTRACT

*Pseudomonas* sp. strain C4 metabolizes carbaryl (1-naphthyl-N-methyl carbamate) as the sole source of carbon and energy via 1-naphthol, 1,2-dihydroxynaphthalene, and gentisate. 1-Naphthol-2-hydroxylase (1-NH) was purified 9.1 fold to homogeneity from *Pseudomonas* sp. strain C4. Gel filtration and SDS-polyacrylamide gel electrophoresis showed that the enzyme is a homodimer with native molecular mass of 130 kDa and subunit molecular mass of 66 kDa. The enzyme was yellow in colour with absorption maxima at 274, 375 and 445 nm indicating a flavoprotein. HPLC analysis of the flavin moiety extracted from 1-NH suggests the presence of FAD. Based on the spectral properties and the molar extinction coefficient, it was determined that the enzyme contained 1.07 mol of FAD per mol of enzyme. Though, the enzyme accepts electrons from NADH, it showed maximum activity with NADPH and has pH optima of 8.0. Kinetic constants, $K_m$ and $V_{max}$ for 1-naphthol and NADPH were determined to be 9.6 and 34.2 µM; and 9.5 and 5.1 µmol. min$^{-1}$.mg$^{-1}$, respectively.

At higher concentration of 1-naphthol, the enzyme showed less activity, indicating substrate inhibition. $K_i$ for 1-naphthol was determined to be 79.8 µM. The enzyme showed maximum activity with 1-naphthol as compared to 4-chloro-1-naphthol (62 %) and 5-amino-1-naphthol (54 %). However, it failed to act on 2-naphthol, substituted naphthalenes and phenol derivatives. The enzyme utilized one mole of oxygen per mole of NADPH. Thin layer chromatographic analysis showed conversion of 1-naphthol to 1,2-dihydroxynaphthalene under aerobic condition but under anaerobic condition it failed to hydroxylate 1-naphthol. These results suggest that 1-NH belongs to the FAD containing external flavin mono-oxygenase group of oxido-reductase class.
**Key words:** 1-Naphthol-2-hydroxylase, Flavin hydroxylase, Purification and kinetic constants, Carbaryl metabolism, *Pseudomonas*

5 Abbreviations

The abbreviations used are 1-NH, 1-naphthol-2-hydroxylase; PAGE, poly acrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; TLC, thin layer chromatography; NADH, β-nicotinamide adenine dinucleotide reduced; NADPH, β-nicotinamide adenine dinucleotide phosphate reduced; FAD, flavin adenine dinucleotide; FMN, flavin adenine mononucleotide; EDTA, ethylene diamine tetra acetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N',N''-tetra acetic acid; TCA, trichloroacetic acid; BSA, bovine serum albumin.
INTRODUCTION

Polyaromatic compounds are highly reduced, toxic and recalcitrant in nature due to resonance stabilized benzene ring. However, microorganisms have evolved or adapted the ability to utilize polycyclic aromatic compounds as their sole source of carbon and energy. This is achieved by increasing the oxidation level of the compound followed by breaking the aromaticity by incorporating molecular oxygen, this reaction is catalyzed by the ‘oxygenase’ group of ‘oxido-reductase’ class of enzymes. Oxygenases are sub-grouped into ring-hydroxylating mono-oxygenases, and ring-cleaving or ring-hydroxylating dioxygenases (13, 16, 23-25). Hence, these enzymes are important in the metabolism of polyaromatic compounds and responsible for releasing the locked carbon from these pollutants. One such recalcitrant compound is 1-naphthol, a high volume industrial product, widely used in the production of synthetic dyes, perfumes and pesticides like Carbaryl or Sevin (8, 14). It is also released by microbes into the environment as a metabolic intermediate of various polycyclic aromatic compounds including carbaryl (6, 9, 32, 36).

Carbaryl (1-naphthyl-\textit{N}-methylcarbamate) is a pesticide used in the agriculture industry. The ester bond between \textit{N}-methylcarbamic acid and 1-naphthol is responsible for its toxicity. In aqueous solutions carbaryl hydrolyses to 1-naphthol, methylamine and CO$_2$ (41). A few microbes have been reported to transform 1-naphthol by hydroxylation either to 4-hydroxy-1-tetralone (5), 3,4-dihydro-dihydroxy-1(2H)-naphthalenone (42) or 1,4-naphthoquinone (33). \textit{Pseudomonas} sp. strain C4 isolated in our laboratory from soil by enrichment culture technique utilizes carbaryl as the sole source of carbon and energy via 1-naphthol and 1,2-dihydroxynaphthalene as shown in Fig. 1 (36). Involvement of
1,2-dihydroxynaphthalene as a metabolic intermediate was established by conducting a series of metabolic studies and demonstrating 1-naphthol-2-hydroxylase (1-NH) and 1,2-dihydroxynaphthalene dioxygenase activity in the cell-free extract of carbaryl grown cells (36). The enzyme 1-NH, responsible for the conversion of 1-naphthol to 1,2-dihydroxynaphthalene was found to be inducible and requires NAD(P)H plus FAD for its activity and has not been purified or characterized to date.

In the present study we report the purification of 1-naphthol-2-hydroxylase from *Pseudomonas* sp. strain C4 to homogeneity, biochemical characterization and kinetic properties of the enzyme.

**MATERIALS AND METHODS**

**Materials.** FAD, FMN, NADH, NADPH, 1-naphthol, 1,2-dihydroxynaphthalene, EDTA, EGTA, 1,10-phenanthroline, 2,2’-dipridyl, Q-sepharose (fast flow), Phenyl-Sepharose (fast flow), Sephacryl S-200 HR, acrylamide, bisacrylamide were purchased from Sigma-Aldrich (USA). Carbaryl™ was a gift from Bayer (India) Ltd. All other chemicals were of analytical grade and purchased locally.

**Bacterial strain and culture conditions.** *Pseudomonas* sp. strain C4 was grown in 150 ml minimal salt medium in 500 ml baffled Erlenmeyer flasks at 30°C on a rotary shaker at 200 rpm (36). The medium was supplemented aseptically with carbaryl (0.1 %) as the sole source of carbon.

**Enzyme assays.** 1-Naphthol-2-hydroxylase (1-NH) was monitored either spectrophotometrically or polarographically. The rate of disappearance of NAD(P)H at 340 nm was measured spectrophotometrically (Perkin-Elmer, Lambda 35). The reaction
mixture (1 ml) contained potassium phosphate buffer (50 mM, pH 7.5), FAD (6.25 µM), substrate (50 µM), NAD(P)H (300 µM) and an appropriate amount of enzyme. The enzyme activity was calculated using the molar extinction coefficient of NAD(P)H ($\varepsilon_{340\text{nm}}$ 6220 M$^{-1}$cm$^{-1}$) and expressed as µmoles of NAD(P)H oxidized per min. In the polarographic assay, rates of O$_2$ consumption were monitored at 30°C using an oxygraph (Hansatech, UK) fitted with a Clark’s O$_2$ electrode. The reaction mixture (2 ml) contained potassium phosphate buffer (50 mM, pH 7.5), substrate (50 µM), FAD (6.25 µM), NAD(P)H (300 µM) and an appropriate amount of enzyme. The enzyme activity was calculated as nmoles of O$_2$ consumed per min. The specific activity is reported as µmoles.min$^{-1}$.mg$^{-1}$ protein. Protein estimation was performed as described by Bradford (7) using BSA as the standard.

**Enzyme purification.** 1-Naphthol-2-hydroxylase was purified from carbaryl grown *Pseudomonas* sp. strain C4 cells to apparent homogeneity using following steps. All steps were performed at 4°C or on ice. During purification, the enzyme activity was monitored using NADH plus FAD as co-factors.

Step 1, Preparation of cell-free extract - Cells grown on carbaryl (~ 3.7 l) for 13 h were harvested by centrifugation (10,000 $\times$ g) and washed twice with Buffer A [potassium phosphate (50 mM, pH 7.5), glycerol (5 %), EDTA (0.5 mM)]. The cells (~ 4.5 g) were suspended in ice-cold Buffer A (1: 4, w/v) and sonicated with an ultrasonic processor (Ultrasonic processor, GE130, USA) on ice with 20 cycles of 15 pulses each and an output of 11 W with a 3-4 min interval. The cell homogenate was centrifuged at 40,000 $\times$ g for 30 min. The clear yellow coloured supernatant obtained was referred to as the cell-free extract.
Step 2, Q-sepharose anion exchange chromatography - The cell-free extract was loaded onto a Q-sepharose column (10 × 150 mm), pre-equilibrated with Buffer A and the matrix was washed with the same buffer to remove unbound protein. The enzyme was eluted with an increasing linear gradient (150 ml) of 0 - 1 M ammonium sulphate in Buffer A. Fractions (2 ml) were collected at a flow rate of 30 ml.h$^{-1}$ using a fraction collector (Redifrac 920, GE Health care). The enzyme was eluted in the range of 0.2 - 0.4 M ammonium sulphate. The fractions with activity greater than one unit were pooled and brought to 30 % ammonium sulphate saturation by adding solid ammonium sulphate. The suspension was centrifuged at 40,000 × g for 30 min. The supernatant containing the enzyme was processed further.

Step 3, Phenyl sepharose hydrophobic interaction column - The enzyme from step 2 was loaded onto a phenyl sepharose column (10 × 150 mm) pre-equilibrated with 30 % saturated ammonium sulphate in Buffer A. Unbound protein was washed with the equilibration buffer. The bound enzyme was eluted using an increasing linear gradient (100 ml) of 0 – 60 % ethylene glycol in Buffer A. Fractions (1.5 ml) were collected at a flow rate of 30 ml.h$^{-1}$. The active fractions were pooled and concentrated using ultrafiltration (membrane cut-off 30 kDa; Pall, USA or Millipore Amicon, USA).

Step 4, Gel filtration chromatography - The enzyme from Step 3 was loaded onto a Sephacryl S-200 HR gel filtration chromatography (10 × 950 mm, bed volume 76 ml, void volume 33 ml) equilibrated in Buffer A. The fractions (1 ml) were collected at a flow rate of 3 ml.h$^{-1}$. Active fractions were pooled and concentrated. The purified enzyme was stored at 4°C.
**Determination of molecular weight.** The native molecular weight of the enzyme was determined by gel filtration on a Sephacryl S-200 HR column equilibrated with Buffer A. The column was calibrated with β-Amylase (200 kDa), Alcohol dehydrogenase (150 kDa), BSA (66 kDa), Carbonic anhydrase (29 kDa) and Cytochrome c (12.4 kDa). The molecular mass of 1-NH were determined from a plot of log (molecular mass) versus $V_{\text{elution}}/V_{\text{void}}$ ($V_e/V_o$). The subunit composition and molecular weight was determined by discontinuous SDS-PAGE with a resolving (12 %) and stacking (5 %) gel as described by Laemmli (21). Electrophoresis was performed at a constant current of 8 mA along with standard molecular weight makers.

**Spectroscopy.** The UV-visible absorption spectrum of the pure 1-NH was recorded in the range of 200 - 700 nm in Buffer A (Perkin-Elmer, Lambda 35). The excitation and emission spectra of 1-NH was recorded using a fluorescence spectrometer (Jasco V-750).

**NH$_2$-terminal sequencing.** 1-NH from SDS-PAGE (12 %) was electro-blotted on to a PVDF membrane (0.45 µ, Pall, USA) in CAPS buffer (10 mM, pH 11) at 200 V for 8 h, stained with Coomassie Brilliant Blue R250 and subjected to automated Edman degradation (Applied Biosystems 470, USA).

**Identification of the prosthetic group.** The flavin co-factor was extracted from the protein by treating 1-NH (1 mg in 60 µl) in Buffer A with 10 µl of 70 % perchloric acid on ice for 5 min followed by centrifugation at 22,000 × g at 4°C (34). The supernatant was then subjected to HPLC analysis (Agilent, HP 1100 series) with a RP-C$_{18}$ column (250 × 4 mm) using an isocratic solvent system consisting of 40 % methanol and 60 %, 10 mM ortho-phosphoric acid (v/v) in water. The eluent was identified by comparison of
the retention time and UV-visible spectra to authentic FAD (retention time, 3.1 min) and FMN (retention time, 3.87 min).

**Identification of the reaction product.** The identification of the product was achieved by carrying out bulk-enzyme reactions. The reactions (10 ml) contained potassium phosphate buffer (50 mM, pH 7.5), 1-naphthol (50 µM), FAD (3.75 µM), NAD(P)H (50 µM) and an appropriate amount of enzyme. The reaction mixture was incubated for 1 h at 30°C. The substrate and co-factors were added intermittently at 15, 30 and 60 min. The reaction was terminated by adjusting the pH to 2 with HCL (2N), and the products were extracted in ethyl acetate, concentrated and dried over anhydrous sodium sulphate. The reaction products were resolved by TLC as described earlier (36) and identified by comparing Rf and UV-fluorescence properties with authentic 1-naphthol and 1,2-dihydroxynaphthalene.

**RESULTS**

**Purification of 1-naphthol-2-hydroxylase.** 1-Naphthol-2-hydroxylase was purified to homogeneity from carbaryl grown cells of strain C4 using anion exchange (Q-Sepharose), hydrophobic interaction (Phenyl Sepharose), and gel filtration (Sephacryl S-200 HR) column chromatography. The purification steps are summarized in Table 1. The enzyme was eluted as a single peak from the gel filtration chromatography (Fig 2A) and was bright yellow in colour. 1-NH was purified 9.1 fold with a 19 % yield and specific activity of 5.3 µmole min⁻¹.mg⁻¹ protein with NADH (specific activity with NADPH was 6.1 µmole min⁻¹.mg⁻¹). SDS-PAGE analysis of the enzyme showed a single band with a molecular mass of ~ 66 kDa (Fig 2B). The native molecular mass as determined by S-200
gel filtration chromatography was found to be ~ 130 kDa (Fig. 2A). The pure enzyme was found to be stable in Buffer A and retained ~ 90% activity when stored at 4°C for 90 days.

**NH$_2$-terminal sequencing.** The NH$_2$-terminal amino acid sequence of the purified 1-NH from strain C4 was determined by automated Edman degradation to be MLKNIFLXDEIRXVSV. A BLAST search performed with the N-terminal sequence failed to yield any significant similarity with existing proteins in the database (NCBI).

**Spectral properties of 1-NH.** The purified enzyme was yellow in colour. The UV-visible spectrum of 1-NH (0.6 mg.ml$^{-1}$) gave absorption maxima at 274, 375, and 445 nm (Fig. 3A). Above 320 nm, the absorption spectrum closely resembled that of authentic FAD (Fig 3A, Inset). The ratio of absorbance at 274 to 445 was found to be 13.1. Addition of NADPH or sodium dithionite led to the disappearance of absorption maxima at 445 nm (Fig 3A, Inset). Excitation of 1-NH (0.81 mg.ml$^{-1}$) at 450 nm showed an emission maximum at 529 nm, a similar emission spectrum was observed for authentic FAD (Fig. 3B). The absorbance for 1-NH (5.7 mg.ml$^{-1}$) at 445 nm was 0.54. Assuming a purity of 95%, relative molecular mass of 130 kDa, and an absorption coefficient of 11,300 M$^{-1}$ cm$^{-1}$ for the FAD moiety (43), it was estimated that 1 mol of enzyme contained 1.07 mol of FAD.

**Co-factor requirement and apoenzyme preparation.** The co-factor requirement for 1-NH is summarized in Table 2. The enzyme exhibited ~ 45% more activity with NADPH plus FAD as compared to NADH plus FAD (Table 2). In the presence of NADPH and 1-naphthol, 1-NH activity increased 17% upon the addition of external FAD but decreased slightly (1%) upon the addition of external FMN. HPLC analysis of the extracted flavin
moiety from 1-NH showed a retention time of 3.09 min, which closely corresponded to the retention time of authentic FAD (3.1 min). These results indicate the presence of FAD as the prosthetic group in the 1-NH. In the absence of substrate, the enzyme did not oxidize NAD(P)H or consume oxygen suggesting the absence of NAD(P)H oxidase activity (Table 2). Furthermore, in the presence of either NADPH or 1-naphthol, the enzyme did not produce hydrogen peroxide.

Preparation of apoenzyme (FAD-free protein) was attempted using various dialysis protocols. Purified 1-NH (2 mg.ml\(^{-1}\)) was dialyzed against Buffer A (250 ml × 4 changes, every 12 h) containing KBr, urea or ammonium sulphate at pH 6 or 7.5 for 36-48 h at 4°C. The enzyme dialyzed against KBr or ammonium sulphate at pH 6.0 lead to ~65 % and 30 % loss of the activity respectively, while dialysis against urea (2 and 4 M) lead to ~90 % loss in the activity. External addition of FAD or FMN in the assay mixture or dialysis against Buffer A containing FAD failed to reconstitute the activity. Dialysis against ammonium sulphate at 7.5 retained 92 % activity with absorption at 274, 375 and 445 nm comparable to non-dialyzed enzyme. Activity (100 %) could be recovered after external addition of FAD. Enzyme (2 mg) when treated with trichloroacetic acid (10 %) lost its activity with protein precipitation. The supernatant was pale yellow in colour with characteristic absorption maxima at 375 and 445 nm (Fig. 3A) while the precipitated protein in Buffer A was colourless and showed no characteristic FAD absorbance spectrum (Fig. 3A).

**pH optima and effect of metal ions and metal chelators.** The pH optima of the 1-NH reaction was investigated using Na-citrate buffer (50 mM, pH 4.0-6.0), KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\) buffer (50 mM, pH 6.0-8.0) and Tris-HCl (50 mM, pH 7.5-9.0). The enzyme exhibited
activity at pH 7-9 with maximal activity detected at pH 8.0. The activity in potassium phosphate buffer was two fold higher than that observed for Tris-Cl buffer.

Sulphate or chloride salts of metal ions Zn$^{2+}$, Mg$^{2+}$, Mn$^{2+}$ and Fe$^{2+}$ at 0.1 mM did not show any effect on the enzyme activity. Cu$^{2+}$ showed marginal decrease (35 %) in the activity at 0.1 mM concentration. NaCl at 50 and 100 mM showed 50 and 67 % inhibition, respectively. The metal chelators (2.5 mM) EDTA and EGTA did not inhibit the enzyme activity, however 1,10-phenanthroline and 2,2'-dipyridyl inhibited activity by ~ 60 %.

**Substrate specificity.** The activity of 1-NH was monitored spectrophotometrically as well as polarographically by using various mono- and di-aromatic compounds (Table 3). Compared to 1-naphthol (100 %), 1-NH exhibited activity with 4-chloro-1-naphthol (62 %) and 5-amino-1-naphthol (54 %). However, the enzyme failed to show any significant activity on carbaryl, naphthalene, substituted naphthalenes, phenol and its derivatives (Table 3).

**Kinetic constants.** The initial reaction velocities with varying concentrations of either 1-naphthol, NADH or NADPH were determined spectrophotometrically. Representative substrate saturation plot for 1-naphthol using NADPH as an electron donor is depicted in Fig. 4. Increasing the concentration of 1-naphthol showed linear increase in the activity till 20 µM. Further increase in 1-naphthol concentration up to 100 and 200 µM showed 25 and 40 % decrease in the activity, indicating the substrate inhibition. A similar pattern of inhibition was observed with 4-chloro-1-naphthol and 5-amino-1-naphthol. The kinetic constants for 1-NH are summarized in Table 4. 1-NH showed $K_m$ of 9.6 µM for 1-naphthol in the presence of NADPH compared to 10.05 µM in the presence of NADH.
The inhibition constant $K_i$ for 1-naphthol was determined to be 79.8 and 57.6 µM in the presence of NADPH and NADH, respectively. The enzyme showed $K_m$ of 34.2 and 118 µM for NADPH and NADH, respectively (Table 4) indicating that NADPH was the preferred co-enzyme. 1-NH showed highest $K_{cat}$ and $K_{cat}/K_m$ value for 1-naphthol plus NADPH compared to other combinations.

**Stoichiometry and reaction product identification.** The stoichiometry of NAD(P)H to oxygen consumption was monitored by varying the substrate (1-naphthol) concentration. The enzyme was found to consume one mole of oxygen per mole of NAD(P)H. To identify reaction product, the enzyme reaction was scaled up and performed under aerobic and anaerobic conditions using Thunberg tubes and appropriate controls as described earlier (36). The reaction product was isolated and identified by TLC. Under aerobic condition, two major spots with $R_f$ 0.10 (black quench when exposed to UV light) and 0.70 (brown black quench when exposed to UV light) were observed on TLC which were identical with the standard 1-naphthol ($R_f$ 0.71, brown-black quench) and 1,2-dihydroxynaphthalene ($R_f$ 0.11, black quench). Under anaerobic condition, spot corresponding to substrate, 1-naphthol ($R_f$ 0.70, brown-black quench) was detected, indicating that 1-NH failed to transform 1-naphthol. These results suggest that 1-NH requires molecular oxygen to catalyze hydroxylation of 1-naphthol to 1,2-dihydroxynaphthalene and can thus be classified as an oxygenase.
DISCUSSION

1-Naphthol-2-hydroxylase is the second enzyme of the carbaryl metabolic pathway in *Pseudomonas* strain C4. The enzyme hydroxylates 1-naphthol at *ortho* position to yield 1,2-dihydroxynaphthalene which is further metabolized to Kreb’s cycle intermediates through a series of enzymatic reactions. The enzyme, 1-NH, is inducible and showed maximum activity when cells were grown on carbaryl (36). The purification and characterization of the enzyme responsible for *ortho* hydroxylation of 1-naphthol to 1,2-dihydroxynaphthalene has not been reported. Here, we describe the purification and characterization 1-NH from *Pseudomonas* sp. strain C4.

1-Naphthol-2-hydroxylase was purified to homogeneity using conventional column chromatographic techniques. The enzyme is a homodimer with native molecular mass of 130 kDa and subunit molecular mass of 66 kDa. 1-NH showed optimum activity at pH 8.0. Besides 274 nm, pure enzyme showed absorption peaks at 375 and 445 nm, similar to other reported flavin hydroxylases. Aromatic flavin hydroxylases have been identified as either single or two component enzymes (3, 26-28, 38). These enzymes are known to initiate the biodegradation of several aromatic contaminants in the environment (17, 25, 27). Enzymes like orcinol hydroxylase, 2,4-dichlorophenol hydroxylase, hydroquinone hydroxylase, and 2-hydroxybiphenyl-3-monoxygenase are single component either monomeric, homodimeric or homotetrameric flavin monooxygenases and accept electrons from external electron donors including NADPH or NADH (4, 12, 30, 35). Phenol hydroxylase and *p*-hydroxyphenylacetate-3-hydroxylase are two component enzymes consisting of the catalytic ‘oxygenase’ component responsible for hydroxylation of substrate and the ‘reductase’ component responsible for electron
transfer from NAD(P)H to the oxygenase component (1, 2, 20, 31). The biochemical properties of 1-NH provide compelling evidence that the enzyme is a single component external flavin hydroxylase.

Spectral analysis indicates that 1-NH contain one mole of FAD per mole of protein. Preparation of apoenzyme by dialyzing enzyme against Urea, KBr or acid ammonium sulphate (pH 6) resulted in the complete or partial loss of the enzyme activity with the loss in the characteristic flavin absorption spectra. External addition of FAD or FMN failed to reactivate the enzyme. This could be due to partial denaturation or subtle changes in the protein structure leading to loss of the flavin moiety and the enzyme activity. On dialysis against ammonium sulphate in Buffer A at pH 7.5, 1-NH retained ~90% activity and external addition of FAD lead to 100% recovery. The enzyme exhibited significantly higher activity with FAD as compared to FMN. These observations were further supported by the identification of FAD by HPLC after treating 1-NH with perchloric acid. Gel filtration profile, dialysis experiments and identification of FAD as prosthetic group indicate that the flavin moiety is tightly but not covalently bound to the protein and its removal lead to loss of the enzyme activity. Some aromatic hydroxylases accept electrons exclusively from NADPH while others also utilize NADH for the reduction of flavin (37). Flavin hydroxylases like orcinol hydroxylase, 2,4-dichlorophenol hydroxylase, and 3-hydroxyphenylacetate 6-hydroxylase has been reported to accept electrons from NADPH as well as from NADH (4, 30, 39). 1-NH could accept electrons from both NADPH and NADH, however the enzyme exhibited significantly higher activity with NADPH (145%). The $K_m$ for NADPH was determined to be 34.2 µM compared to 118 µM for NADH suggesting that enzyme has high affinity
for NADPH. Addition of various chloride and sulphate salts of Fe, Zn, Mg and Mn or metal chelators like EDTA and EGTA did not show any effect on the 1-NH activity, suggesting that the external addition of metal ion is not required. Inhibition with 1,10-phenanthroline and 2,2’-dipyridyl (~ 60 % at 2.5 mM concentration) could be due to non-specific hydrophobic interaction between chelator and 1-NH.

Flavin monooxygenases have been reported to accept different substrates and substrate analogs thus exhibiting broad substrate specificity (4, 12, 22, 35, 44). 1-NH showed activity with 1-naphthol and partial activity with 4-chloro-1-naphthol and 5-amino-1-naphthol. It failed to show activity with phenol, 3-hydroxybenzoate and 4-hydroxybenzoate suggesting that 1-NH is a new enzyme and is different from earlier reported phenol or hydroxybenzoate hydroxylases. Inability of 1-NH to accept various other hydroxylated aromatic substrates suggest that the enzyme exhibited a limited substrate range. This property has previously been reported for other flavin hydroxylases (15, 18, 39). 1-NH showed $K_m$ of 9.6 µM for 1-naphthol. The affinity for 1-naphthol ($K_m$) remained unchanged irrespective of the electron donor used. However, the $K_{cat}$ value was higher in the presence of NADPH (20.58 s$^{-1}$). Increase in the 1-naphthol concentration above 20 µM lead to decrease in the activity indicating the substrate inhibition of the enzyme. Similar inhibition pattern was observed with 4-chloro-1-naphthol and 5-amino-1-naphthol. It has been reported that flavin hydroxylases are subjected to substrate or substrate analog inhibition, a commonly observed phenomenon (10, 11, 19, 29, 40). 1-NH showed a $K_i$ of 79.8 µM in the presence of NADPH compared to 57.6 µM in the presence of NADH. Comparison of kinetic constants for 1-naphthol plus NADPH and 1-naphthol plus NADH clearly indicate that the NADPH is a preferred co-enzyme over
NADH and enzyme is comparatively less sensitive to higher concentration of substrate. Detection of 1,2-dihydroxynaphthalene by TLC under aerobic condition and consumption of 1 mole of molecular oxygen per mole of NAD(P)H suggest that enzyme belong to oxygenase group of oxido-reductase class.

In conclusion, 1-naphthol-2-hydroxylase, responsible for conversion of 1-naphthol to 1,2-dihydroxynaphthalene was purified to homogeneity from carbaryl degrading *Pseudomonas* sp. strain C4. The enzyme was a homodimer, contains FAD as prosthetic group, prefers NADPH as co-factor, acts on 1-naphthol and consumes mole of O\(_2\) per mole of NADPH. These results suggest that the 1-NH belongs to the FAD containing external flavin mono-oxygenase group of oxido-reductase class. The results presented in this paper are good basis for further characterization of enzyme with respect to the events taking place during catalytic cycle at the active site and the structural features determining the substrate specificity of the enzyme.

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REFERENCES


FIGURE LEGENDS

FIG. 1. Pathway for degradation of carbaryl by *Pseudomonas* sp. strain C4. Enzymes involved are: 1, Carbaryl hydrolase; 2, 1-Naphthol-2-hydroxylase; 3, 1,2-Dihydroxy naphthalene dioxygenase; and 4, Gentisate dioxygenase.

FIG. 2. A, Elution profile of 1-naphthol-2-hydroxylase from Sephacryl S-200-HR gel filtration column chromatography. The hydroxylase activity is represented by crosses and protein elution by filled circles; Inset: Plot of log (molecular mass) versus $V_e/V_o$ for gel filtration standard molecular weight protein markers represented by open circles [markers: Alcohol dehydrogenase (150 kDa), BSA (66 kDa), Carbonic anhydrase (29 kDa) and Cytochrome c (12.4 kDa)] and the filled circle represents 1-NH. B, SDS-PAGE analysis of 1-NH during the different stages of purification: lane 1, Cell-free extract; lane 2, Q Sepharose fraction; lane 3, Phenyl Sepharose fraction; lane 4, Sephacryl S-200 HR fraction; lane 5 Molecular weight markers, Phosphorylase B (97.4 kDa), BSA (66 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), Soy-bean trypsin inhibitor (20.1 kDa) and Lysozyme (14.3 kDa).

FIG. 3. Spectral properties of 1-naphthol-2-hydroxylase from *Pseudomonas* sp. strain C4: A, the absorption spectrum of native 1-NH (0.6 mg.ml$^{-1}$) in Buffer A (solid line), TCA precipitated protein dissolved in Buffer A (dash line), and TCA supernatant (dotted line); Inset represents magnified visible region spectrum of oxidized 1-NH (0.6 mg.ml$^{-1}$, solid line) which disappeared when treated with NADPH (8 mM, dotted line) under anaerobic condition or with few milligrams of sodium dithionite (dash-dot-dash line) and authentic
FAD (100 µM, dash line). The lag between addition of NADPH or sodium dithionite and recording of spectra was ~15 sec and no absorbance detected beyond 500 nm. B, Fluorescence emission spectrum of native 1-NH (0.81 mg.ml\(^{-1}\), solid line) and authentic FAD (100 nM, dotted line) when excited at 445 nm in Buffer A.

FIG. 4. Reaction velocity (\(v\)) versus [S] plot for 1-naphthol-2-hydroxylase. The reactions were performed with enzyme (5 µg), NADPH (200 µM) and FAD (6.25 µM) with the varying concentration of 1-naphthol from 0.5 µM to 200 µM. The graph was fitted using the model for substrate inhibition (uncompetitive) with the equation 
\[
v = \frac{V_{\text{max}}}{K_m + \frac{S}{S^+} + \frac{1}{K_i}}
\]
FIG. 1.

Carbaryl

1-Naphthol

1,2-Dihydroxy naphthalene

2-Hydroxy benzal pyruvic acid

Gentisic acid

Kreb's Cycle
FIG. 2.
FIG. 3.
FIG. 4.
TABLE 1. Purification of 1-naphthol-2-hydroxylase from *Pseudomonas* sp. strain C4

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Sp. Act (U.mg⁻¹)</th>
<th>Fold</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>Cell- free extract</td>
<td>17</td>
<td>177</td>
<td>102</td>
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<td>100</td>
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<td>9.2</td>
<td>45</td>
<td>4.9</td>
<td>8.5</td>
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<td>3.7</td>
<td>19.6</td>
<td>5.3</td>
<td>9.1</td>
<td>19</td>
</tr>
</tbody>
</table>

*During purification, the enzyme activity was monitored spectrophotometrically using FAD and NADH; One unit (U) is the amount of enzyme that catalyses the oxidation of 1 µmol of NADH min⁻¹. The specific activity of the final purified 1-NH with NADPH plus FAD was 6.1 µmoles min⁻¹.mg⁻¹ of protein.*
TABLE 2. Co-factor requirement for 1-naphthol-2-hydroxylase

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Activity* (µmoles.min⁻¹)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NADH/NADPH) + FAD/FMN</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NADH + 1-Naphthol</td>
<td>1.60</td>
<td>88</td>
</tr>
<tr>
<td>NADH + FAD + 1-Naphthol</td>
<td>1.81</td>
<td>100</td>
</tr>
<tr>
<td>NADH + FMN + 1-Naphthol</td>
<td>1.72</td>
<td>95</td>
</tr>
<tr>
<td>NADPH + 1-Naphthol</td>
<td>2.30</td>
<td>127</td>
</tr>
<tr>
<td>NADPH + FAD + 1-Naphthol</td>
<td>2.68</td>
<td>148</td>
</tr>
<tr>
<td>NADPH + FMN + 1-Naphthol</td>
<td>2.27</td>
<td>125</td>
</tr>
</tbody>
</table>

*Activity was monitored spectrophotometrically, the reaction consisted of purified 1-NH (5 µg), 1-naphthol (10 µM), FAD or FMN (6.25 µM) and NADH or NADPH (200 µM) at pH 8.0.
TABLE 3. Activity of 1-naphthol-2-hydroxylase on various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Naphthol</td>
<td>100</td>
</tr>
<tr>
<td>4-Chloro-1-naphthol</td>
<td>62</td>
</tr>
<tr>
<td>5-Amino-1-naphthol</td>
<td>54</td>
</tr>
<tr>
<td>2-Naphthol</td>
<td>15</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>14</td>
</tr>
<tr>
<td>2-Hydroxybenzoate</td>
<td>12</td>
</tr>
</tbody>
</table>

*Activity was monitored spectrophotometrically, the reaction consisted of purified 1-NH (5 µg), substrate (50 µM), FAD (6.25 µM) and NADPH (200 µM) at pH 8.0. Compounds like carbaryl, methylnaphthalenes (1- and 2-), hydroxymethyl naphthalenes (1- and 2-), naphthoic acids (1- and 2-), 1-hydroxy-2-naphthoic acid, 3-hydroxy-2-naphthoic acid, 6-hydroxy-2-naphthoic acid, 2-naphthylamine, hydroxybenzoates (3- and 4-), and phenol showed activity in the range 0 - 4 %.
TABLE 4. Kinetic properties of the 1-naphthol-2-hydroxyalse

<table>
<thead>
<tr>
<th></th>
<th>( K_m ) (µM)</th>
<th>( V_{max} ) (µmoles. min(^{-1}).mg(^{-1}))</th>
<th>( K_i ) (µM)</th>
<th>( K_{cat} ) (s(^{-1}))</th>
<th>( K_{cat}/K_m ) (s(^{-1}).µM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Naphthol with NADPH</td>
<td>9.6</td>
<td>9.5</td>
<td>79.8</td>
<td>20.58</td>
<td>2.14</td>
</tr>
<tr>
<td>1-Naphthol with NADH</td>
<td>10.05</td>
<td>6.65</td>
<td>57.6</td>
<td>14.4</td>
<td>1.37</td>
</tr>
<tr>
<td>4-Chloro-1-naphthol with</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>4</td>
<td>5.16</td>
<td>95</td>
<td>11.2</td>
<td>2.72</td>
</tr>
<tr>
<td>5-Amino-1-Naphthol with</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>5.7</td>
<td>4.9</td>
<td>131</td>
<td>10.6</td>
<td>1.82</td>
</tr>
<tr>
<td>NADPH</td>
<td>34.2</td>
<td>5.1</td>
<td>-</td>
<td>11.04</td>
<td>0.32</td>
</tr>
<tr>
<td>NADH</td>
<td>118</td>
<td>5.3</td>
<td>-</td>
<td>11.47</td>
<td>0.097</td>
</tr>
</tbody>
</table>

Activity was monitored spectrophotometrically. The reactions were performed at pH 8.0 using enzyme (5 µg) and FAD (6.25 µM). \( K_m \), \( V_{max} \) and \( K_i \) values for 1-naphthol were derived by using fixed concentration of NADPH (200 µM) or NADH (300 µM). The concentration of 1-naphthol was varied from 0.5-200 µM. The kinetic constants for NADH and NADPH were determined by keeping 1-naphthol concentration at 10 µM and varying co-factor concentration in the range 20-300 µM.