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Research Paper

CHARACTERIZATION OF NITROPHENOL OXYGENASE PRODUCED BY *RHODOCOCCUS* SPECIES

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INTRODUCTION

Rapid industrialization in world today has made pollution a matter of prime concern. Nitroaromatic compounds are widely used in the manufacture of dyes, pesticides, plastics and pharmaceuticals (Nelson, 1982[1]; Konopka, 1993 [2]). These chemicals being toxic and mutagenic, their accumulation results in pollution of soil and ground water.

Nitroaromatic compounds are metabolized by two general catabolic pathways namely oxidative and reductive pathways. Many nitroaromatic compounds such as o- nitrophenol, m-nitrobenzoic acid, and 4-chloro-2-nitrophenol, have been reported to be mineralized by an oxygenase catalyzed oxidative pathways. In the oxidative pathway, the nitro group is eliminated as nitrite (Zeyer and Kearney, 1984 [3]; Nodeau and Spain, 1995 [4]; Bruhn *et al*., 1988 [5]). Reduction of aromatic compounds namely 4-nitrobenzoate, 4-nitrotoluene, and 2, 4-dinitrophenol results in hydroxyl amino aromatic derivatives which can occur under both aerobic and anaerobic conditions (Golab *et al*., 1979 [6]; Hallas and Alexander, 1983 [7]; Lenke and Knackmuss, 1996) [8].

The enzymatic systems reduce nitro groups independent of the position of the nitrogroup on the aromatic ring (Cartwright and Cain, 1959 [9]; Germanie and Wuhrmann, 1963 [10]). Initial hydrogenation of the aromatic ring of picric acid has been reported in Rhodococcus species (Zeyer, *et al*., 1986 [11]). Both monooxygenase and dioxygenase enzymes are reported to catalyze eli mination of nitrite from nitroaromatic compounds. These enzymes require electron donor and cofactors which could bring the oxygen and target carbon of aromatic nucleus together (Spain and Gibson, 1979 [12]; Zeyer and Kocher, 1988 [13]). For 2, 6 dinitrophenol a dioxygenase catalyzed elimination of nitrite has been reported as the initial reaction of the degradative pathway (Ecker *et al*., 1992 [14]). Nitrophenol oxygenase from a Morexella species which was able to grow on PNP, removed nitrite from PNP converting it to hydroquinone. This enzyme was found to be membrane bound (Spain and Gibson, 1979 [12]).

The phenols, Chloronitrophenols, even at a very low concentration are considered to be pollutants (Chakrabarti *et al*., 1988 [15]). To achieve the desired treatment for such pollutants, monitoring of genotype and its expression over a period of time is considered as the only option (Chakrabarti *et al*., 1988 [15]). Since this also involves the knowledge on the basic biological reactions and how these reactions are influenced, the information on the microorganisms and their enzymatic system needs to be studied. In the present investigation, purification and characterization of nitrophenol oxygenase responsible for utilization of 2-Chloro-4-nitrophenol (CNP) by indegenously isolated Rhodococcus species has been reported.

MATERIALS AND METHODS

CNP was obtained from E Merck (Germany). Protein standards, Coomassie brilliant blue, NADPH, DEAE Cellulose, were obtained from Sigma Chem. Co. USA. Sephadex G-75, Sephadex G-100 was obtained from Pharmacia fine chem. Uppasala, Sweden. All other chemicals were of analytical reagent grade.

MICROORGANISM:

Indigenously isolated strain of Rhodococcus species 1 (Haritha, *et. al*., 2003 [16]) was used. Culture was maintained on nutrient agar (N. A.) slant amended with 0.5mM CNP and sub cultured every month to keep it active and viable.

Growth condition: A loopful of culture of Rhodococcus species from N. A. slants was inoculated in nutrient broth (50 ml) and incubated at 37^{o}C for 12 hr. The cells in log phase ($3x10^{12}$ cells) were used as inoculums for 2 liter nutrient broth containing 0.5mM CNP. Inoculated flasks were incubated at 37^{o}C for 12 hr. The cells in the log phase ($3x10^{14}$ cells) were harvested by centrifugation at 15000 *g* for 15 min at 4^{o}C. The cell pellet was washed with 0.5 M sodium phosphate buffer (pH 7.4) and suspended in same buffer. The cells were disrupted by sonication (Sonifier, cell disrupter B-30, Danbury, Conn) at 150 rpm for 15 sec at 4^{o}C. Cell debris was removed by centrifugation at 20000 *g* for 20 min at 4^{o}C. Supernatant was used as crude nitrophenol oxygenase enzyme [16].

Enzyme assay:

Nitrophenol oxygenase (NPO) assay was carried out at room temperature using Gilford spectrophotometer (Germany) at 225 nm (Haritha, et al., 2003 [16]). Assay mixture contained 50 mM sodium phosphate buffer (pH 7.2), 40 mm NADPH, (in a final vol. of 2 ml), 0.1 mM CNP, and 0.1 mg enzyme source. The decrease in A_{225} (Corresponding λ_{max} of CNP) was monitored by spectrophotometer. One unit of enzyme activity was defined as the decrease in absorbance of 0.01/min at 225 nm under experimental conditions.

Estimation of protein:

Protein content was measured by the method of Bradford (1976 [17]) using BSA as standard protein.

PURIFICATION OF NITROPHENOL OXYGENASE:

Crude nitrophenol oxygenase (NPO) was centrifuged at 50000 *g* (Beckman Table top ultra centrifuge) for 1 hr. Clear supernatant was used for ammonium sulphate precipitation to 90% saturation at 4^{o}C (Patil and Shastri, 1982 [18]). Precipitated protein were collected by centrifugation at 15000 *g* for 15 min at 4^{o}C and suspended in 15 ml of buffer A (0.2 M sodium phosphate buffer, pH 7.2; buffer A) and the suspension was dialyzed against the same buffer for 18 hr. The dialyzed fraction was tested for NPO assay and proteins were measured by Bradford method.

Chromatography on DEAE-Cellulose:

Ammonium sulphate precipitated NPO was applied to a DEAE cellulose column (10x 1.5cm) preequilibrated with buffer by the method adopted by Patil and Shastri, (1982 [18]). DEAE Cellulose column was first eluted with the same buffer and then eluted with 0.1M sodium chloride mixed with buffer A. The adsorbed proteins were eluted with buffer a mixed with 0.2 M sodium chloride. Fractions of 3 ml each were collected in fraction collector (LKB Pharma) at a flow rate of 30ml/hr. NPO activity and absorbance at 280 nm were measured. Fractions containing enzyme activity were pooled together for further purification step.

Chromatography on sephadex G-75 column:

Fractions showing maximum NPO activity were mixed together and applied on to a column of sephadex G-75 column (15x1cm) preequilibrated with buffer A and proteins were eluted from the column with same buffer. Fractions containing 3 ml elute were collected by fraction collector at a flow rate of 30ml/hr. NPO activity and proteins were measured in all the fractions (Patil and Shastri, 1982 [18]).

Poly acryl amide gel electrophoresis

About 0.1 mg enzyme protein obtained from sephadex G-75 chromatography was subjected to simple PAGE using 7.5% gel. A constant current o 2.5 mA/gel was applied for 3 hr at 4^{o}C.

SDS PAGE and molecular weight

Molecular weight of purified NPO was determined by SDS PAGE using method of Weber & Osborne (1969 [19]). About 0.2 mg of enzyme protein was mixed with 1 ml tris glycine buffer (pH 8.3) containing 1% (w/v) each of SDS and 2-mercaptoethanol.The enzyme solution was placed in boiling water bath for two min. and then cooled to room temperature. About 0.1 mg of denatured enzyme protein was subjected SDS-PAGE. Electrophoresis was carried out using 7.5% polyacryl amide gel in tris-glycine buffer (pH 8.3) containing 1% SDS. A constant current of 1.5 mA per sq. cm of slab gel was applied for 8-12 hr at 4^{o}C. Bromophenol blue was used as a tracking dye during the electrophoretic process. After electrophoresis the gel was carefully lifted from the glass plate. Length of the gel was measured and the distance of migration of the dye was recorded.
Mobility of the known molecular weight protein samples and those of the enzyme protein were calculated as:

$$\text{Mobility} = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by the tracking dye}} \times \frac{\text{Length of the gel before staining}}{\text{Length of the gel after staining}}$$

Mobility of the standard proteins was plotted against logarithmic values of their molecular weights. Molecular weight of NPO was calculated from standard curve.

PHYSICO-CHEMICAL PROPERTIES OF NITROPHENOL OXYGENASE
Optimum temperature: NPO activity was assayed over the temperature range of 20 to 60^{o}C in 50 mM buffer A. The stock enzyme was suitably diluted and used in the assay mixture mentioned above.
Effect of metal ions: One mg enzyme protein in buffer A was mixed with 2 ml of 0.5 mM EDTA and incubated at 37^{o}C for 1 hr and the solution was dialyzed against buffer A for 18 hr for complete removal of EDTA. The demetalized NPO was used for studying the effect of various metal ions. Effect of different metal ions like Fe^{++}, Al^{+++}, Hg^{++}, Mn^{++}, Mg^{++}, Li^{++}, Co^{++}, Pb^{++} was studies by mixing the chloride salts of these metal ions at 0.5 mM concentration and NPO activity was measured as mentioned above.
Effect of various detergents: Detergents namely SDS, Tween 20, Tween 80 and Triton X-100 were added in reaction mixture at 0.05mM and effect on NPO was studied.
Effect of inhibitors: Enzyme inhibitors namely 2-mercaptoethanol, iodoacetate, PCMB, and KCN were used at 0.05 mM concentration.
Effect of cofactors: Various cofactors like NADH, NADPH, $NADP^{+}$, NAD^{+}, FAD and FMN were tested on NPO activity. Cofactors were mixed at 50 mM concentration and activity was measured as above.
Effect of substrate concentration: Purified NPO protein was added in reaction mixture at 0.1 mg and mixed with variable amount of substrate (CNP) concentration (0.025 mm to 0.1 mm). Enzyme activity was measured as above.
Nitrophenol oxygenase activity on various aromatic compounds: NPO activity was tested on various aromatic compounds namely 4-nitrophenol, 2-nitrophenol, 2,4-dinitrophenol, 4-chloro-2-nitrotoluene and 4-nitrotoluene. These aromatic compounds were added in reaction mixture in place of CNP at a concentration of 0.5mM and enzyme activity was measured.

RESULTS AND DISCUSSION:
Purification of NPO from Rhodococcus sp.1 started with ultracentrifugation (Table 1). The crude extract obtained by sonication of the cells was subjected to ultracentrifugation at 50000 *g* for 1 hr at 4^{o}C and the clear supernatant containing the enzyme protein was subjected to ammonium sulphate precipitation. The specific activity of the enzyme protein in the ultracentrifugation step was found to be 30.The enzyme proteins were precipitated by ammonium sulphate to 90%. Specific activity of the enzyme after ammonium sulphate precipitation reached to 116.
DEAE Cellulose chromatography: Ammonium sulphate precipitated proteins were loaded on to DEAE cellulose column (20×1.5 Cm). Bound proteins were eluted in NaCl gradient of 0.2 M at

these fractions. It was evident from the table that 24 fold purification of NPO was obtained. Sephadex G-75 gel filtration chromatography purified the enzyme to 42 fold as shown in Fig1.

SDSPAGE: The enzyme fraction was showing a single band coinciding with molecular weight of 65 KDa indicating the monomeric unit of enzyme NPO molecule.

Molecular weight by gel filtration chromatography was found around 65000 kD (Fig. 2).

CNP consumption by Rhodococcus sp.1 under aerobic conditions was accompanied by production of 4-clorocatechol which appeared in the culture medium. The cytosolic fraction of Rhodococcus sp.1 oxidized CNP to 4-chlorocatechol by using NADPH as an electron donor. The reaction appears to take place according to the following equation:

Nitrophenol oxygenase from Rhodococcus sp1 was found to have an optimum temperature at 37^{o}C and the enzyme was not stable beyond 40^{o}C. The optimum pH was at pH 7.0 and enzyme was stable up to ph 8.0 only. The optimum pH and temperature of NPO of Rhodococcus sp.1 were lower than the corresponding values described for NPO of *Pseudomonas putida* (Zeyer and Kocher, 1988 [13]).

The metal ions like $Fe^{++,}$ Mg^{++} and Mn^{++} showed inductive effect on enzyme activity (Table 2). Other metal ions like $Hg^{++,}$ Pb^{++}, Li^{++} were found to inhibit the activity. Like NPO from *P. putida*, the enzyme was also activated by Mg^{++} and Mn^{++} (Zeyer and Kocher, 1988 [13]).

Various detergents like SDS, Tween 20, Tween 80, and Triton X 100 inhibited enzyme activity at different levels (Table 3). NPO activity was inhibited by 2-mercaptoethanol, PCMB, Iodoacetate and KCN (Table 4). Like nitrophenol reductase of *Rhodobacter* capsulatus E1F1, NPO of Rhodococcus species was also inhibited by KCN. Various cofactors were studied on NPO activity. NPO used NADPH as electron donor but NPO activity was absent when other cofactors like NADH, NADPH, $NADP^{+}$, NAD^{+}, FAD and FMN were used in reaction mixture (Table 5). The Km and Vmax were calculated from the graphical analysis of the data represented by Lineweaver Burke plot (Fig.3). The enzyme reaction followed Michaelis Menten Kinetics with Km of 0.5mM and Vmax 12.5 units/mg protein towards CNP.

NPO activity was found in Rhodococcus cells grown in medium containing nitrophenols but not when nitroaromatic derivatives lacking phenolics were substituted for nitrophenols in the culture medium. The activity was observed when 4-nitrophenol, 2-nitrophenol etc. were used as substrates but absent when chloronitrotoluene were used as substrates (Table 6). In contrast other bacterial nitroreductases like nitroalkane reductase and nitropyrene reductase reduce nonphenolic nitroderivatives (Kido, *et al*., 1978[20]; Villanueva, 1964 [21]; Kinouchi and Ohinishi, 1983 [22]).

The NPO from Rhodococcus sp.1 was found to be similar like nitophenol oxygenase from *Pseudomonas putida* (Zeyer and Kocher, 1988 [13]). These two nitrophenol oxygenases were stimulated by Mg^{++} and Mn^{++} ions. The nitroalkane oxygenases from *Hansenula mrakii* and *Fusarium oxysporum* does not require NADPH but produce superoxides (Kido, *et al*., 1976 [23]; Kido, *et al*., 1978 [20]).

Table1: Purification chart of Nitrophenol Oxygenase from Rhodococcus sp-1

Fraction	Volume (ml)	Total protein (mg)	Total activity (Units)	Specific activity	Yield %	Fold purification
Crude extract	50	75	1250	16.6	100	1
Ultra centrifugation fraction	50	50	1500	30	120	1.8
Amm. Sulphate fraction	15	9	1050	116.6	84	7
DEAE Cellulose Fraction	9	2.7	1080	400	86.4	24
Sephadex G-75 fraction	6	1.2	840	700	67.5	42

Table 2: Effect of metal ions on Nitrophenol Oxygenase activity of Rhodococcus sp-1

Metal ions	Nitrophenol oxygenase activity (% activity)
Control	100
Al^{+++}	0
Ba^{++}	0
Co^{++}	0
Fe^{++}	180
Hg^{++}	0
Li^{++}	0
Mg^{++}	125
Mn^{++}	110
Mo^{++}	0
Pb^{++}	0

Table 3:Effect of detergents on Nitrophenol Oxygenase activity of Rhodococcus sp-1

Detergents	% activity
Control	100
SDS	10
Tween 20	40
Tween 80	35
Triton X-100	20

Table 4: Effect of inhibitors on Nitrophenol Oxygenase activity of Rhodococcus sp-1

Inhibitor	% activity
Control	100
B-mercaptoethanl	15
Iodoacetate	30
PCMB	35
KCN	10

Table 5: Nitrophenol Oxygenase activity of Rhodococcus sp-1with other cofactors

Cofactor	% activity
NADPH	100
NAD^{+}	0
NADH	0
FAD	0
FMN	0

Table 6: Nitrophenol Oxygenase activity of Rhodococcus sp-1with other aromatic compounds

Aromatic compound	% activity
Control (4-chloro-2-nitrophenol)	100
4-Nitrophenol	34
2-Nitrophenol	20
2,4-dinitrophenol	14
4-Chloro-2-nitrotoluene	0
4-nitrotoluene	0

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