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Nitroreductase II Involved in 2,4,6-Trinitrotoluene Degradation: Purification and Characterization from *Klebsiella* sp. C1

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Three 2,4,6-trinitrotoluene (TNT) nitroreductases from *Klebsiella* sp. C1 have different reduction capabilities that can degrade TNT by simultaneous utilization of two initial reduction pathways. Of these, nitroreductase II was purified to homogeneity by sequential chromatographies. Nitroreductase II is an oxygen-insensitive enzyme and reduces both TNT and nitroblue tetrazolium. The N-terminal amino acid sequence of the enzyme did not show any sequence similarity with those of other nitroreductases reported. However, it transformed TNT by the reduction of nitro groups like nitroreductase I. It had a higher substrate affinity and specific activity for TNT reduction than other nitroreductases, and it showed a higher oxidation rate of NADPH with the *ortho*-substituted isomers of TNT metabolites (2-hydroxylaminodinitrotoluene and 2-aminodinitrotoluene) than with *para*-substituted compounds (4-hydroxylaminodinitrotoluene and 4-aminodinitrotoluene).

Keywords: nitroreductase, *Klebsiella* sp., enzyme purification, trinitrotoluene

2,4,6-Trinitrotoluene (TNT) is the most widely used explosive. Because of the recalcitrance of nitroaromatics, TNT has accumulated in the areas of manufacturing, storage, and decommissioning over the past several decades (Stenuit *et al*., 2005). TNT and its metabolites are toxic, mutagenic, and carcinogenic to many organisms, including humans (Honeycutt *et al*., 1996), and should be removed from contaminated sites to prevent environmental and health problems. Recently, biodegradation of TNT by various microorganisms has been studied (Stenuit *et al*., 2005; Yin *et al*., 2005). Although the extent of TNT degradation and the major products formed depend on the type of microorganisms and the environmental conditions, initial reactions of TNT degradation by microorganisms are reductive rather than oxidative. This is due to the nitro groups which reduce the electron density of an aromatic ring and inhibit oxidative attack by the electrophilic oxygenases (Vorbeck *et al*., 1998). The initial reduction of TNT occurs via two different pathways: a direct ring reduction via hydride addition, and a successive reduction of nitro groups in an aromatic ring (Vorbeck *et al*., 1998).

To date, most nitroreductases from TNT degrading bacteria catalyze only one of these metabolic pathways (Caballero *et al*., 2005; Yin *et al*., 2005). Recently, Pak *et al*. (2000) reported that xenobiotic reductase B of *Pseudomonas fluorescens* catalyzed the reduction of TNT either by direct ring reduction or by nitro group reduction. However, TNT was not mineralized in these bacterial cultures. In our previous study *Klebsiella* sp. C1 isolated from activated sludge could metabolize TNT using both reduction routes, and had a higher mineralization rate than other TNT degrading bacteria (Kim *et al*., 2002). Among three nitroreductases detected in *Klebsiella* sp. C1, nitroreductase I had only the nitro group reduction ability (Kim and Song, 2005). In this study, another nitroreductase was purified and characterized, and its degradation of TNT was examined.

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Materials and Methods

Bacterial strain and chemicals

Klebsiella sp. C1 was isolated from activated sludge from a municipal sewage treatment plant in Chuncheon and showed TNT degrading capability (Kim *et al*., 2002). Analytical-grade TNT and degradation metabolites were purchased from Supelco Co. (USA) and AccuStandard Inc. (USA).

Purification and characterization of nitroreductase from *Klebsiella* sp. C1

Klebsiella sp. C1 grown in Nutrient Broth medium (Difco Lab., USA) for 24 h were harvested by centrifugation (7,080×g, 40 min). Cells were then disrupted by two passages through a French pressure cell at 105 kg/cm^2. Nitroreductase was purified by a series of chromatographic separations described elsewhere (Kim and Song, 2005), and the characteristics of purified enzyme were examined.

The relative molecular mass of the denatured nitroreductase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with molecular weight standards. The molecular mass of the native enzyme was measured by gel permeation chromatography (Sephadex G-100-120 resin) with molecular markers. The effects of pH and temperature on nitroreductase activity were determined by the methods described elsewhere (Kim and Song, 2005). To

Table 1. Purification steps of nitroreductase II from *Klebsiella* sp. C1

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Sp. activity (U/mg)	Purification (fold)	Yield (%)
Cell free extract supernatant	50	36.86	38745.5	1051	-	100.0
DEAE sepharose chromatography	30	3.04	14565.9	4791	4.5	37.0
Butyl HIC chromatography	15	0.35	7011.3	20032	19.0	18.0
Sephadex G-100 chromatography	5	0.01	1408.0	140800	133.9	3.6

examine the preference of electron donor for enzymatic reduction, 0.2 mM NADPH or NADH was added to 0.8 ml of 100 mM sodium phosphate buffer containing 0.44 mM TNT and 0.1 ml enzyme solution (20 μg/ml), and enzymatic reactions were carried out for 1 min. Preference of prosthetic groups of purified enzymes was examined with apoenzyme plus FMN, FAD, and riboflavin using a method of Watanabe *et al*. (1998). Effects of metals and inhibitors on the activity of the purified enzyme were examined with 1 mM metal ions ($FeSO_4$, $MgCl_2$, $MnSO_4$, $CuSO_4$, $HgCl_2$, $AgNO_3$) and 250 μM each of dicumarol, diphenyliodonium, phenol, acetate, lactate, and benzoate. The N-terminal sequence of purified nitroreductase was analyzed with an Automatic Protein Sequencer (Model 476A-01-120, Applied Biosystems, USA).

Enzymatic degradation of TNT and its metabolites

The kinetic parameters for NADPH as an electron donor was determined by changing its concentration under standard assay conditions with 0.15 mM TNT. The kinetic parameters for TNT was determined with the concentration of NADPH fixed at 0.3 mM. Degradation reactions of TNT and its metabolites were carried out in 1.5 ml vials containing 1 ml of 100 mM phosphate buffer (pH 6.0), 0.15 mM nitro-containing substrates and 0.3 mM of NADPH. Reactions were initiated by the addition of purified nitroreductase to give 1.4 μg of enzyme per reaction mixture. Reaction mixtures were incubated at 30°C, and reactions were stopped by heating the preparation at 100°C for 1 min. After cooling to room temperature, the samples were centrifuged at 28,500×g for 10 min at 4°C to precipitate suspended particulates. The supernatant was then analyzed by HPLC to measure nitroaromatic substrates and their degradation products.

Analytical methods

Nitroreductase activity was determined with an assay mixture containing 0.15 mM TNT and 0.30 mM NADPH in a final volume of 1.0 ml of 100 mM Na phosphate buffer (pH 6.0). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of NADPH per minute at 30°C (Kim and Song, 2005). Nitroreductase activity was also determined by spectrophotometry after enzymatic reduction of nitroblue tetrazolium (NBT). Nitroreductase activity of proteins on native gel was confirmed by protein staining for 30 min with NBT, menadione, NADPH, and 20 mM Tris-HCl buffer.

HPLC analysis for TNT and its intermediates was conducted using Waters HPLC (USA) under conditions described elsewhere (Kim *et al*., 2002). Protein was measured as described by Lowry *et al*. (1951).

Results and Discussion

Purification of nitroreductase II from *Klebsiella* sp. C1

Crude extract of *Klebsiella* sp. C1 showed 3 protein bands having nitroreductase activity in native-PAGE, which were designated as nitroreductase I, II, and III. Nitroreductase I had the highest TNT reduction activity, but low NBT reduction activity. Nitroreductase II showed somewhat lower reduction activity of TNT, but higher reduction activity of NBT than those of nitroreductase I. Nitroreductase III had the lowest reduction activity of both TNT and NBT among the three enzymes. Nitroreductase II was purified to homogeneity from crude extracts by sequential chromatographic separations. Nitroreductase II was purified about 134-fold and 3.6% of the total nitroreductase activity in the crude soluble extract remained (Table 1).

Characterization of nitroreductase II

The purified nitroreductase II from *Klebsiella* sp. C1 was composed of single polypeptide chain. The molecular mass of the denatured enzyme was 26 kDa (Fig. 1). The molecular masses of nitroreductases from enteric bacteria ranges from 24 to 28 kDa, including nitroreductase I (25 kDa) in this bacterium (Kim and Song, 2005).

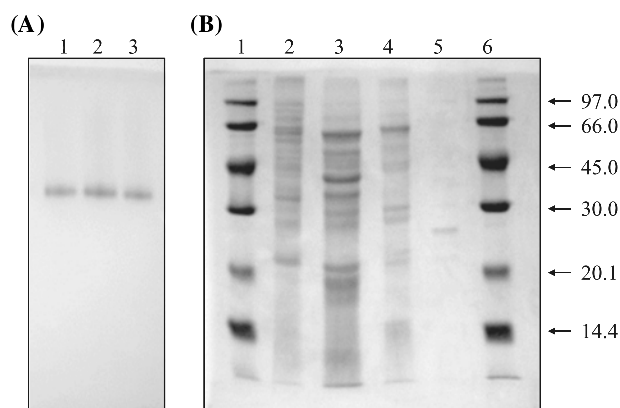

Fig. 1. Native-PAGE and SDS-PAGE of purified nitroreductase II. Left panel, native-PAGE and activity staining. (A) Lanes: 1, cell free extract; 2, DEAE-chromatography; 3, Butyl-chromatography; Right panel, SDS-PAGE and coomassie blue staining. (B) Lanes: 2, cell free extract; 3, DEAE-chromatography; 4, Butyl-chromatography; 5, Size-exclusion chromatography; lanes 1 and 6 contain molecular standards, rabbit muscle phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), chicken egg white ovalbumin (45.0 kDa), bovine erythrocyte carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Table 2. N-Terminal amino acid sequences of nitroreductase derived from various bacteria

Microorganism and enzyme proposed	N-terminal amino acid sequences															Reference
Klebsiella sp. C1, nitroreductase I	D	I	I	V	S	V	W									Kim and Song (2005)
Klebsiella sp. C1, nitroreductase II	A	D	T	N	V	G	G	G	Q	V	N	F	F	G	K	This work
E. coli, Nfs A	T	P	T	I	E	L	I	C	G	H	R	S	I	R	H	Whiteway *et al.* (1998)
E. coli, Nfs B	D	I	I	S	V	A	L	K	R	H	S	T	K	A	F	Whiteway *et al.* (1998)
E. cloacae, nitroreductase	D	I	I	S	V	A	L	K	R	H	S	T	K	A	F	Bryant and DeLuca (1991)
E. cloacae, PETN reductase	S	A	E	K	L	F	T	P	L	K	V	G	A	V	T	French *et al.* (1996)
S. typhimurium, nitroreductase	D	I	V	S	V	A	L	Q	R	Y	S	T	K	A	F	Watanabe *et al.* (1990)
P. putida, Xen A	S	A	L	F	E	P	Y	R	L	K	D	V	T	L	R	Blehert *et al.* (1999)
P. putida, Xen A	A	T	I	F	D	P	I	K	L	G	D	L	E	L	S	Blehert *et al.* (1999)

The N-terminal amino acid sequence of this enzyme does not show any sequence similarity to other bacteria, including nitroreductase I from same bacterium (Table 2). Although a high degree of amino acid sequence similarity has been reported among the nitroreductases of enteric bacteria (Lewis *et al.*, 1997), nitroreductase NfsA from *Escherichia coli* (Whiteway *et al.*, 1998), and pentaerythritol tetranitrate (PETN) reductase from *Enterobacter cloacae* PB2 (French *et al.*, 1996) have quite different amino acid sequences. Unexpectedly the amino acid sequence of the N-terminus of nitroreductase II was the same as the mrkA fimbrial protein precursor in *K. pneumoniae* (Gerlach *et al.*, 1988). Although the first 15 amino acids were same, their molecular masses were different. Since nitroreductases seem to be conserved among the enteric bacteria (Lewis *et al.*, 1997), the full amino acid sequence or nucleotide sequence of the structural gene of this nitroreductase II should be analyzed to define the difference and relationship between this enzyme and the mrkA fimbrial protein precursor.

When enzyme activity was measured after treatment of apoenzyme of purified nitroreductase II with FMN, FAD or riboflavin, addition of 0.1, 1, and 5 mM FMN recovered enzyme activity 30, 62, and over 100%, respectively, compared to the control (Fig. 2). The recovery of enzyme activity by FAD and riboflavin did not exceed to 55% which showed that the prosthetic group of nitroreductase II of *Klebsiella* sp. C1 is FMN. Nitroreductases of *E. cloacae* 96-3 (Bryant and DeLuca, 1991) and *Salmonella typhimurium* (Watanabe *et al.*, 1998) also showed much higher enzyme activities with FMN than with FAD and riboflavin. Severe activity loss occurred during the purification of nitroreductase II was overcome by the addition of FMN. However, incubation of the crude enzyme with FAD and riboflavin did not fully regain enzyme activity, indicating that FMN is most likely the natural flavin cofactor. This result also suggested that the flavin cofactor is loosely bound to the enzyme and that the flavin binding site is readily accessible to exoge-

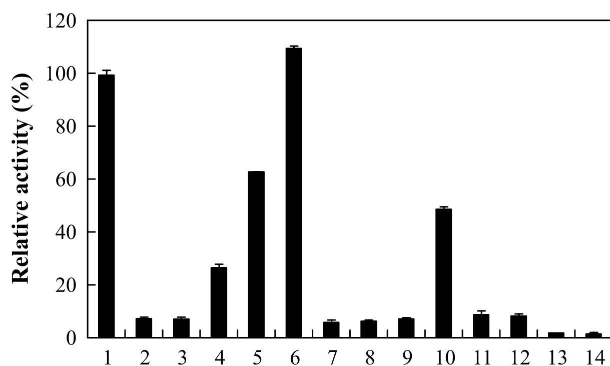

Fig. 2. Activity of nitroreductase II after reconstitution from the apoenzyme and flavins. Lanes: 1, nitroreductase II; 2, apoenzyme alone; 3, 4, 5, and 6, apoenzyme incubated with 0.01, 0.1, 1, and 5 mM FMN, respectively; 7, 8, 9, and 10, apoenzyme incubated with 0.01, 0.1, 1, and 5 mM FAD, respectively; 11, 12, 13, and 14, apoenzyme incubated with 0.01, 0.1, 1, and 5 mM riboflavin, respectively.

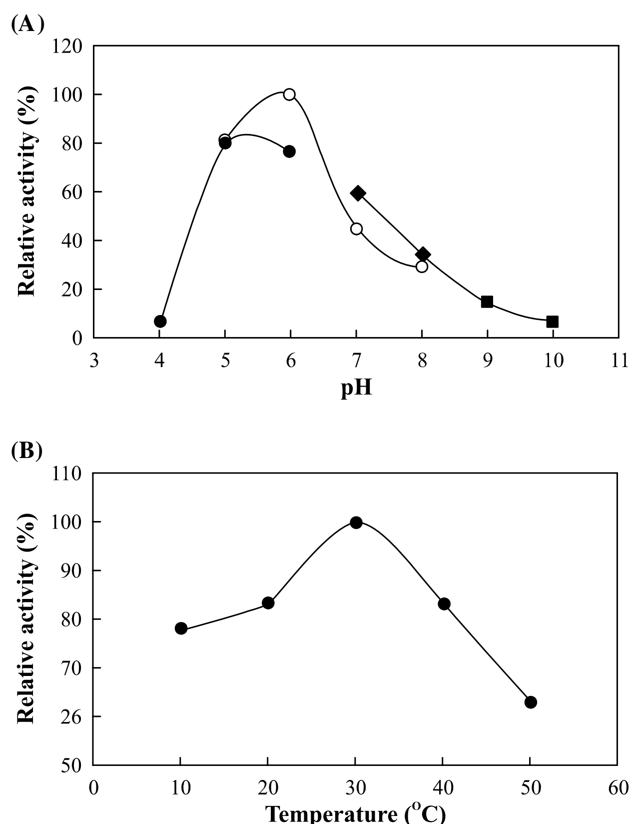

Fig. 3. Effect of pH (A) (●-●, citrate phosphate buffer; ○-○, sodium phosphate buffer; ◆-◆, Tris-HCl buffer) and temperature (B) on activity of purified nitroreductase II from *Klebsiella* sp. C1.

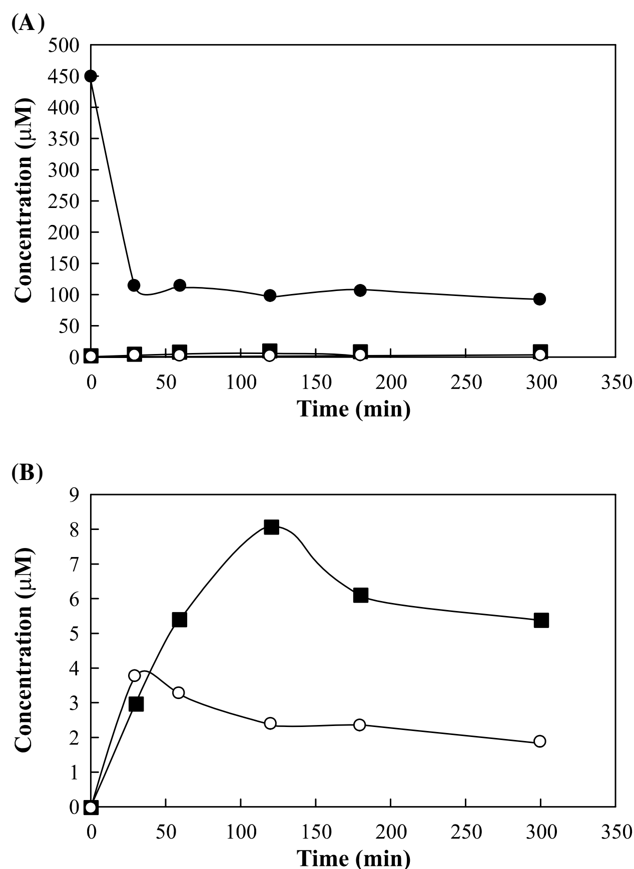

Fig. 4. Degradation of 2,4,6-trinitrotoluene by purified nitroreductase II from *Klebsiella* sp. C1 at different incubation time (●, TNT; ■, 2HA46DNT; ○, 2A46DNT).

nous flavins. When NADPH and NADH were added to the purified enzyme as an electron donor, enzyme activities were 3151 U/mg and 1837 U/mg, respectively. Nitroreductases of *E. coli* (Whiteway *et al*., 1998), *S. typhimurium* (Watanabe *et al*., 1998), and *Pseudomonas putida* JLR11 (Caballero *et al*., 2005), and pentaerythritol tetranitrate reductase of *E. cloacae* PB2 (French *et al*., 1996) were also preferred NADPH as an electron donor. However, other nitroreductase from *E. cloacae* exhibited much higher activity with NADH than NADPH (Bryant and DeLuca, 1991).

The purified nitroreductase II exhibited maximal activity at pH 5.0~6.0 (Fig. 3A), which is slightly lower than that of nitroreductase I (Kim and Song, 2005). In general, nitroreductases have optimal pH of around 7 (French *et al*., 1996). The optimal temperature of the nitroreductase II was 30°C (Fig. 3B). The enzyme was relatively stable from 10 to 40°C and had over 60% relative activity at 50°C. The effect of temperature on nitroreductase II activity was somewhat different from that of nitroreductase I from same organism. Nitroreductase I had maximal activity at 30 to 40°C and had a rapid decrease of activity at temperatures above 40°C (Kim and Song, 2005).

The addition of 1 mM Cu^{2+} and Hg^{2+} reduced enzyme activities to less than 60% compared to control, and the addition of 1 mM Fe^{3+}, Mg^{2+}, and Mn^{2+} decreased enzyme activities to lower than 30%. Among the metal tested, Ag^{+} exhibited the highest enzyme inhibition at 91%. Mercury inhibits the enzyme activity by binding to sulfhydryl group in protein (Haynes *et al*., 2002). Phenol, acetate, lactate, and benzoate (250 μM each) inhibitors of flavoproteins, exhibited over 40% inhibition of enzyme activities compared to the control, and diphenyliodinium and dicumarol showed 60 and 88% inhibition of enzyme activities, respectively. The higher inhibition by dicumarol indicated that the catalytic mechanism of this nitroreductase II may be similar to that of DT diaphorase [NAD(P)H oxidoreductase] (Brock *et al*., 1995).

Table 3. Specific activities of nitroreductase II from *Klebsiella* sp. C1 with different substrates determined by the change in absorbance at 340 nm due to the oxidation of NADPH (0.3 mM). All substrates were assayed at a final concentration of 150 μM

Substrate	Specific activity (U/mg)
2,4,6-Trinitrotoluen (TNT)	7.4
2-Hydroxyl-4,6-dinitrotoluene (2-HADNT)	1.3
4-Hydroxyl-2,6-dinitrotoluene (4-HADNT)	0.4
2-Amino-4,6-dinitrotoluene (2-ADNT)	1.1
4-Amino-2,6-dinitrotoluene (4-ADNT)	0.2
2,4-Dinitrotoluen (2,4-DNT)	0.8
2,6-Dinitrotoluen (2,6-DNT)	0.3
2-Nitrotoluen (2-NT)	0.0
4-Nitrotoluen (4-NT)	0.0

Enzymatic degradation of TNT and its metabolites

The optimum concentrations of NADPH and enzyme for the reduction of TNT by purified nitroreductase II were 0.3 mM and 20 μg/ml, respectively. Under these conditions, about 70% TNT was degraded. 2-Hydroxylamino-4,6-dinitrotoluene (2-HADNT) and 2-amino-4,6-dinitrotoluene (2-ADNT) were detected as the reduced metabolites, reaching 8.0 and 3.8 μM after 30 and 120 min, respectively (Fig. 4). The K_m and V_{max} of the purified enzyme were 56 μM and 1.2 mmol/mg/min, respectively, with TNT as a substrate and NADPH as an electron donor. The K_m of this enzyme for TNT was same as nitroreductase from *E. cloacae* (Bryant and DeLuca, 1991), but lower than of nitroreductase I from same organism (64 μM) (Kim and Song, 2005). Although nitroreductase II has a higher substrate affinity than nitroreductase I, it has a lower V_{max} than nitroreductase I (1.85 mmol/mg/min) (Kim and Song, 2005), but a higher V_{max} than nitroreductase from *E. cloacae* (1.13 mmol/min/mg) (Bryant and DeLuca, 1991).

The capability of purified nitroreductase II to reduce TNT and its metabolites was evaluated by measuring the NADPH consumption (Table 3). In the presence of 0.15 mM TNT, the NADPH oxidation rate was 7.4 μmol of NADPH mg^{-1} min^{-1}. Purified enzyme did not show higher specific activities with other TNT metabolites. TNT was the most efficient substrate among several nitrotoluene compounds tested as seen with PnrA nitroreductase in *P. putida* JLR11 (Caballero *et al*., 2005). The specific activity of this enzyme for TNT reduction was slightly lower than that of nitroreductase I from same organism (8.4 μmol/min/mg) but much higher

than those of *Enterobacter* PETN reductase (0.5 μmol/min/mg) (French *et al*., 1998) and *Pseudomonas fluorescens* xenobiotic reductase B (XenB), an NADPH-dependent flavoprotein oxidoreductase (2.6 μmol/min/mg) (Pak *et al*., 2000), under similar reaction conditions. This high specific activity of nitroreductase II is likely one of the reasons for the efficient transformation and mineralization of TNT by this bacterium (Kim *et al*., 2002). The purified nitroreductase also reacted with both isomers of HADNTs, ADNTs, and DNTs, but did not react with mononitrotoluenes (Table 3). Klausemeier *et al*. (2001) suggested that the substrate for nitroreductases must contain at least two nitro groups. XenB from *P. fluorescens* reacted with TNT and 2,4-DNT, but could not reduce any of the mononitrated aromatic compounds tested (Pak *et al*., 2000). French *et al*. (1998) also reported that NADPH oxidation by PETN reductase was not detected in the presence of nitrotoluenes or dinitrotoluenes, which differs from our results. In contrast, *E. cloacae* nitroreductase reacted on 4-nitrotoluene (Bryant and DeLuca, 1991). PnrA nitroreductase from *P. putida* JLR11 reacted on 3-nitrotoluene and 3- and 4-nitrobenzoate, but not on 2- and 4-nitrotoluene (Caballero *et al*., 2005). Other enzymes from *Klebsiella* sp. C1 showing nitroreductase activity should be examined further to determine if they can react with mononitrated aromatic substrates.

Nitroreductase II exhibited a higher specific activity with 2,4-DNT than with 2,6-DNT. Among other TNT metabolites such as HADNTs and ADNTs, the *ortho*-substituted isomers (2-HADNT and 2-ADNT) showed higher oxidation rate of NADPH than *para*-substituted compounds (4-HADNT and 4-ADNT). A nitro group at the *para* position is more easily reduced than one at the *ortho* position in TNT (Yin *et al*., 2005). Pak *et al*. (2000) also reported that purified XenB reacted with 2,4-DNT at a very low rate but did not react with 2,6-DNT. Nitroreductase II in this study showed a high specific activity with 2,4-DNT, but also could transform 2,6-DNT even at a low rate, which is similar to results with nitroreductase I from the same organism (Kim and Song, 2005). In contrast, PnrA nitroreductase from *P. putida* JLR11 showed similar V_{max} value with 2,4-DNT and 2,6-DNT (Cabarello *et al*., 2005). Among other TNT metabolites such as HADNTs and ADNTs, the *ortho*-substituted isomers (2-HADNT and 2-ADNT) showed much higher oxidation rate of NADPH than *para*-substituted compounds (4-HADNT and 4-ADNT) (Table 3). The same phenomenon of regioselectivity was also observed in the whole culture of white rot fungus *Phanerochaete chrysosporium*, with TNT (Hawari *et al*., 1999). Although nitroreductase of *P. chrysosporium* has not been purified and its degradation mechanism of TNT has not been elucidated, it may act in similar way as nitroreductase II in this study.

Klebsiella sp. C1 can metabolize TNT by simultaneous utilization of the nitro-group reduction pathway and the direct ring reduction pathways (Kim *et al*., 2002). In the enzymatic degradation of TNT, 2-HADNT, and 2-ADNT were detected (Fig. 4). However, hydride-Meisenheimer complex of TNT and 2,4-DNT were not detected as products of TNT degradation by this nitroreductase II. These results indicate that the nitroreductase II from *Klebsiella* sp. C1 was able to transform TNT using the nitro group reduction pathway, similar to nitroreductase I of same organism (Kim and Song, 2005). Other enzymes in *Klebsiella* sp. C1 may be responsible for the direct ring reduction and the denitration pathway, which are more favorable for the mineralization of TNT (Stenuit *et al*., 2005). Further study on those enzymes is necessary to elucidate the precise enzymatic TNT degradation in this bacterium. Other factors involved in the enzymatic reactions, such as pH and partial pressure of oxygen may affect the complex initial TNT degradation and inhibit the denitration metabolism of TNT.

Acknowledgements

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