The Journal of Microbiology, February 2005, p.21-27 Vol. 43, No. 1
Copyright © 2005, The Microbiological Society of Korea

Purification and Characterization of NADPH-Dependent Cr(VI) Reductase from *Escherichia coli* ATCC 33456

Woo-Chul Bae[1,†], Han-Ki Lee[1], Young-Chool Choe[1], Deok-Jin Jahng[2], Sang-Hee Lee[1], Sang-Jin Kim[3], Jung-Hyun Lee[3] and Byeong-Chul Jeong[1,*]

[1]*Division of Bioscience and Bioinformatics, Myongji University, Yongin, 449-728, Republic of Korea*
[2]*Department of Environmental Engineering and Biotechnology, Myongji University, Yongin, 449-728, Republic of Korea*
[3]*Microbiology Laboratory, Korea Ocean Research and Development Institute, Ansan, 425-600, Republic of Korea*

(Received September 13, 2004 / Accepted January 28, 2005)

A soluble Cr(VI) reductase was purified from the cytoplasm of *Escherichia coli* ATCC 33456. The molecular mass was estimated to be 84 and 42 kDa by gel filtration and SDS-polyacrylamide gel electrophoresis, respectively, indicating a dimeric structure. The pI was 4.66, and optimal enzyme activity was obtained at pH 6.5 and 37°C. The most stable condition existed at pH 7.0. The purified enzyme used both NADPH and NADH as electron donors for Cr(VI) reduction, while NADPH was the better, conferring 61% higher activity than NADH. The K_m values for NADPH and NADH were determined to be 47.5 and 17.2 μmol, and the V_{max} values 322.2 and 130.7 μmol Cr(VI) $min^{-1}mg^{-1}$ protein, respectively. The activity was strongly inhibited by N-ethylmalemide, Ag^{2+}, Cd^{2+}, Hg^{2+}, and Zn^{2+}. The antibody against the enzyme showed no immunological cross reaction with those of other Cr(VI) reducing strains.

***Key words*:** Cr(VI) reductase, *Escherichia coli* ATCC 33456, purification

Hexavalent chromium, Cr(VI), is highly water soluble and one of the most widely used metals in industry, resulting in large quantities being discharged into the environment. It is carcinogenic and mutagenic, thereby necessitating the treatment of wastewaters, soils and sediments (Pettrilli and Miller, 1977; Levis and Bianchi, 1982). However, trivalent chromium, Cr(III), is less toxic and readily forms insoluble chromium hydroxides at neutral pH, which can be easily removed from the environment (Wang and Shen, 1995).

The microbial reduction of toxic Cr(VI) to the less toxic Cr(III) provides a useful detoxification process. Several microorganisms that can reduce Cr(VI) have been isolated (Horitsu *et al.*, 1987; Bopp and Ehlich, 1988; Wang *et al.*, 1989; Lovely and Phillips, 1994; Oh and Choi, 1997) and the metabolism of Cr(VI) reduction has been extensively studied (Bopp and Ehlich, 1988; Wang *et al.*, 1990; Suzuki *et al.*, 1992; Shen and Wang, 1993; Myers *et al.*, 2000). The aerobic mechanism of Cr(VI) reduction is generally associated with a soluble fraction that utilizes NADH as an electron donor (Suzuki *et al.*, 1992; Shen and Wang, 1993; Oh and Choi, 1997). Conversely, anaerobic Cr(VI) reduction is mediated by membrane bound cytochrome b, c and d, (Bopp and Ehlich, 1988; Lovely and Phillips, 1994) or cytoplasmic membrane protein (Myers *et al.*, 2000). Although several bacterial Cr(VI) reductase systems have been developed, only a few enzymes have been fully purified and characterized (Suzuki *et al.*, 1992; Park *et al.*, 2000). *E. coli* ATCC 33456 has the ability to reduce Cr(VI) under both aerobic and anaerobic conditions, with the soluble reductase activity being a major Cr(VI) reducing mechanism (Shen and Wang, 1993; Wang and Shen, 1995). Previously, we reported the Cr(VI) reduction ability of this microorganism using batch and continuous cultures (Bae *et al.*, 2000). Accordingly, we tried to purify and characterize the Cr(VI) reductase, which is reported here.

Materials and Methods

Bacteria and culture conditions

The *Escherichia coli* ATCC 33456 was purchased from the American Type Culture Collection (ATCC). Cells were grown in 750 ml of nutrient broth (Difco, USA) for 8 h at 37°C, with agitation at 200 rpm, and transferred to 20 l carboys (Nalgen, USA) containing 15 l of nutrient broth. The cells were incubated for 12 h at 37°C, with air bubbled from the bottom of the carboy. The cells from 120 l culture were collected at 4°C by centrifugation at 6,000 × g for 15 min. *Pseudomonas aeruginosa* HP014 (Oh and Choi, 1997), *Pseudomonas fluorescens* LB300 (Bopp and Ehlich, 1988), *Bacillus* sp. (Wang and Xiao;

* To whom correspondence should be addressed.
(Tel) 82-31-330-6196; (Fax) 82-31-335-8249
(E-mail) bcjeong@mju.ac.kr
† Present address: Central Research Institute, Ilyang Pharm. Co. Yongin 449-904, Korea

1995) and *Desulfovibrio vulgaris* ATCC 29579 (Lovely and Phillips, 1994) were grown as previously reported.

Purification of Cr(VI) reductase

All purification procedures were performed at 4°C, with the exception of the Mono-Q ion exchange chromatography and Superdex 200 HR gel filtration chromatography, and all buffers were supplemented with 1 mM dithiothreitol to prevent enzyme oxidation. The cells (140 g, wet weight) were washed with 50 mM phosphate buffer (pH 7.0), resuspended in 750 ml of the same buffer, and disrupted in an ice bath by sonication with 20 times 1 min pulses at 100 W (Sonifier 450, Branson Ultrasonics Co., USA). The unbroken cells, cell debris and membrane fractions were removed by successive centrifugation at 12,000 × g for 60 min, and then at 100,000 × g for 90 min. The resulting supernatant was used as the crude extract for the enzyme purification. The protein precipitate obtained from 35 - 65% ammonium sulfate fractionation was collected by centrifugation (12,000 × g, 60 min) and resuspended in 100 ml 20 mM histidine buffer (pH 5.5). After desalting by dialysis against the same buffer (3 changes, 12 h, 5 l each), the protein sample (150 ml) was applied to a Q-Sepharose FF anion exchange (GE Healthcare Bioscience, USA) column (2.5 cm × 37 cm) that had been pre-equilibrated with histidine buffer. The protein was eluted with a 0 to 0.3 M linear gradient of NaCl in the same buffer at a flow rate of 3 ml/min. The active fractions (150 ml) were applied to a Cibacron blue 3GA dye affinity (Sigma, USA) column (15 ml) that had been equilibrated with histidine buffer. The column was washed with the same buffer, followed by elution with 1 mM NADH, 1 mM NADPH, and 5 M NaCl. The Cr(VI) reducing activity containing fractions were concentrated by 65% ammonium sulfate fractionation and applied to a Sephacryl S-100 HR (GE Healthcare Bioscience, USA) column (320 ml) that had been equilibrated with 20 mM phosphate buffer (pH 7.0), and the protein was eluted with the same buffer at a flow rate of 10 ml/h. The Cr(VI) reducing activity containing fractions were concentrated using an Amicon YM 10 membrane (Millipore, USA), and then applied to a Mono-Q HR 5/5 anion exchange column (GE Healthcare Bioscience, USA) using FPLC (GE Healthcare Bioscience, USA) that had been equilibrated with 20 mM phosphate buffer (pH 7.0). The column was washed with the same buffer, and the protein was eluted with a linear NaCl gradient (0-300 mM, flow rate 1 ml/min). The active fraction from the Mono-Q chromatography was concentrated to 0.5 ml, and the enzyme purified on a Superdex 200 HR 10/30 gel filtration (GE Healthcare Bioscience, USA) column using phosphate buffer at a flow rate of 0.25 ml/min.

Estimation of molecular weight and isoelectric point of purified enzyme

The molecular mass of the native form of the purified enzyme was estimated by gel filtration on a Superdex 200 HR 10/30 column that had been equilibrated with 20 mM KH_2PO_4 (pH 7.0) at a flow rate of 0.25 ml/min using a nondenatured protein molecular weight marker kit (Sigma, USA). The subunit size of the enzyme was determined by 12% SDS-PAGE (Laemmli, 1970). The protein bands were visualized with Coomassie brilliant blue R-250 (Sigma). Isoelectric focusing of the purified enzyme was performed on a precast isoelectric focusing gel (pH 3-9) (GE Healthcare Bioscience, USA). The sample preparation and isoelectric focusing were carried out following the instructions provided by Pharmacia. The gels were Coomassie Blue stained, as described in the PhastSystem manual (GE Healthcare Bioscience, USA).

Effects of pH and temperature on the activity and stability of purified enzyme

The optimum pH of the Cr(VI) reductase was determined over the range 4.0-9.0 using the following buffers (100 mM): citrate (4.0-6.0), phosphate (6.0-7.0) and Tris (7.0-9.0). For the pH stability test, the enzyme was stored at various pHs and 4°C for 40 h, and after adjusting the pH to 7.0, the residual activity assayed. The use of two buffers at overlapping pH compensated for the buffer associated effects. To determine the effect of temperature on the stability of enzyme and the optimum temperature for enzyme activity, the enzyme solution was incubated in 100 mM KH_2PO_4 for 14 h at various temperatures (4, 12, 20, 28, 37, 50 and 60°C), and the residual enzyme activity was then assayed.

Antiserum preparation and western blot analysis.

After the SDS-PAGE, the gel slice containing the Cr(VI) reductase was ground using a tissue grinder (5 ml, Iwaki, Japan). 20 μg of the enzyme contained in the ground gel was dissolved in 0.2 ml PBS (20 mM phosphate, 150 mM NaCl, pH 7.0) and injected into the peritoneal cavity of each mouse, every 14 day. On the 5th day after the third injection, blood was collected by the heart puncture method, the coagulated blood centrifuged at 10,000 × g and the supernatant collected as antiserum. To perform western blotting, 10 μg of the cell extracts of the *E. coli* and various Cr(VI) reducing strains were analyzed by 12% SDS-PAGE. After the proteins had been separated by SDS-PAGE, the western blots were carried out (Towbin *et al.*, 1979). The blots were blocked for 1 h in TBS buffer (10 mM Tris, 150 mM NaCl, pH 7.5), containing 3% skimmed milk, and then incubated with Cr(VI) reductase antiserum (1 : 1,000 dilution) for 14 h. After washing three times with TBS buffer for 5 min each, the blots were treated with horse radish peroxidase-conjugated anti-mouse IgG antibody (Sigma, USA) and developed with chloronaphthol substrate solution (15 mg chloronaphthol, 5 ml MeOH, 45 ml TBS and 10 μl H_2O_2).

Analytical methods

The Cr(VI) reducing activity was assayed by measuring the decrease in Cr(VI) using diphenylcarbazide and ion chromatography, as previously described (Urone, 1955; Bae *et al.*, 2000). The N-ethylmalemide reductase activity was measured as described earlier (Mizugaki *et al.*, 1981). The protein concentration was estimated by the method of Bradford, using bovine serum albumin as the standard (Bradford, 1976). The NAD(P)H (Sigma, USA) concentration was determined by measuring the decrease of NAD(P)H at 340 nm using a spectrophotometer (Ultrospec III, GE Healthcare Bioscience, USA). The K_m and V_{max} values were determined by the double-reciprocal plot method of Lineweaver-Burk using the Sigma Plot 4.01 software program. An amino acid analysis was performed by reverse phase Pico-Tag (Waters, USA) column chromatography following phenylisothiocyanate (PITC) derivatization. The N-terminal sequencing was performed by Edman degradation using an amino acid sequencer (Precise protein sequencing system, Applied Biosystem, USA).

Results

Purification of Cr(VI) reductase from E. coli ATCC 33456

The Cr(VI) reductase was purified 967.9 fold, with an overall yield of 3.5%, to homogeneity from the crude extract by ammonium sulfate fractionation, anion-exchange, dye affinity and gel filtration column chromatography (Table 1). Q-Sepharose FF anion exchange chromatography at pH 5.5 resulted in a significant decrease in the total activity, although the active fraction was not divided further. This suggested that Cr(VI) reductase was not stable at pH 5.5. The most effective purification step

Table 1. Purification of NADPH-dependent Cr(VI) reductase

Purification step	Volume (ml)	Total Protein (mg)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	750	4200	0.81	568	0.14	100	1
Ammonium sulfate fractionation	100	980	4.80	480	0.49	84.5	3.6
Q-Sepharose	150	84	0.57	86	1.02	15.1	7.6
Cibacron blue 3GA	90	22.65	0.50	45	1.99	7.9	14.7
Sephacryl S-100	13.2	1.39	1.89	25	18.05	4.4	133.7
Mono-Q	1	0.25	20.40	20.4	81.60	3.6	604.4
Superdex 200 HR	2	0.15	9.80	19.6	130.67	3.5	967.9

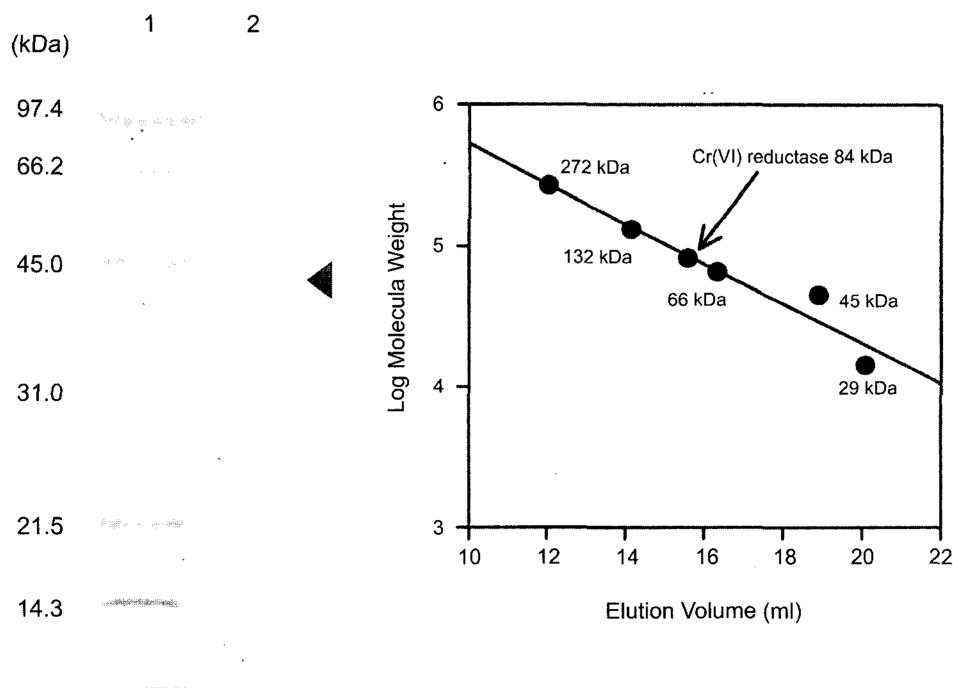

Fig. 1. Estimation of the molecular weight of the purified Cr(VI) reductase by SDS-PAGE (A) and gel filtration (B). Experimental conditions are described in Materials and Methods. Lane 1: standard size markers, lane 2: purified enzyme.

for the enzyme was the gel filtration chromatography using Sephacryl S-100, which alone yielded more than a 9-fold increase in purity. Gel filtration of Cr(VI) reductase yielded a single peak, corresponding to native molecular mass of 84 kDa, with Superdex 200HR 10/30. However, SDS-PAGE revealed that the purified enzyme consisted of 42 kDa subunits (Fig. 1), implying that the native enzyme was composed of two subunits of equal or very similar mol wt. The isoelectric point (pI) of the enzyme was 4.66.

Effects of pH and temperature on the activity and stability

As shown in Fig. 2, the purified enzyme was active over a narrow pH range, with an optimum pH of 6.5 at 37°C, whereas the activity was only 8% of the optimum value at pH 9.0 and 0% at pH 4.0. The enzyme was most stable at pH 6.5, but lost about 86 % of its initial activity after storage at pH 4.0 and 4°C for 40 h. When the pH of the reaction mixture was maintained at 7.0, the enzyme activity increased with temperature, peaking at 37°C. However, the activity decreased rapidly at 50°C, with only 23% of its activity remaining. Over 86% of the activity remained after storage below 20°C for 14 h.

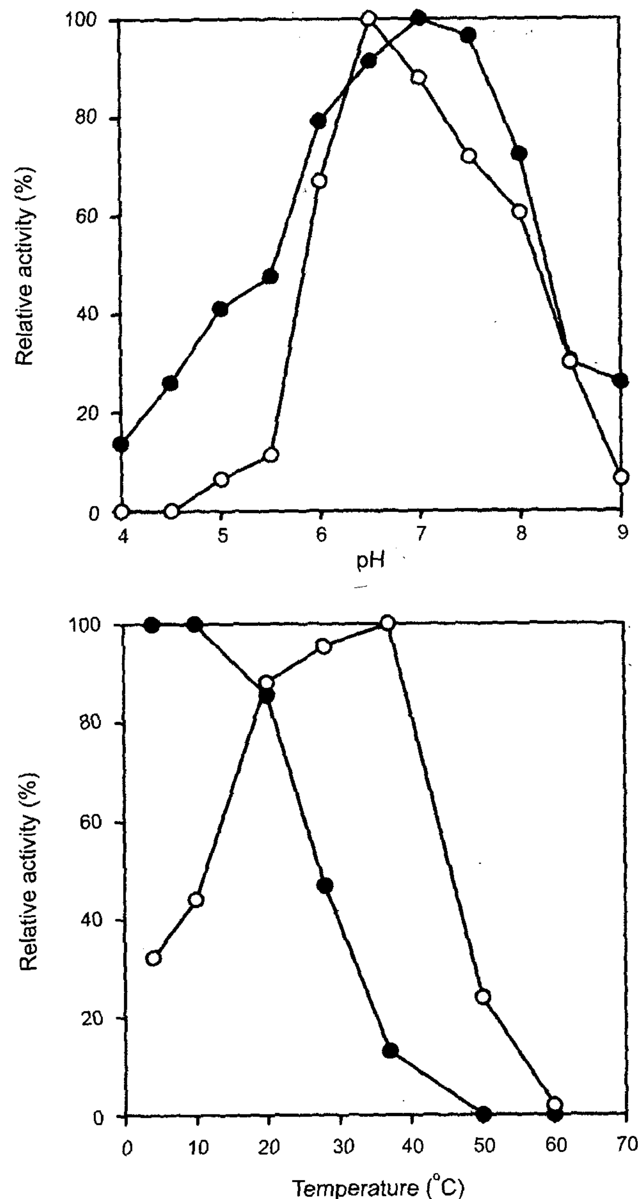

Fig. 2. Effects of pH and temperature on the stability and activity of the purified Cr(VI) reductase. ●: stability, ○: activity
Experimental conditions are described in Materials and Methods.

Electron donor and kinetic calculation

Both the NADH (0.4 mM) and NADPH (0.4 mM) were good electron donors, especially NADPH, which showed 161% of the activity compared to that of the NADH, while the enzyme was inactive in the absence of the cofactors. Interestingly, some other factors, such as ascorbic acid (0.02 mM), glutathione (0.4 mM), D-glucose (0.4 mM) and D-fructose (0.4 mM), which were effective in the reduction of Cr(VI) with whole cells (Bae *et al.*, 2000), did not support the Cr(VI) reducing activity of the purified enzyme. The Michaelis-Menten kinetic parameters determined in the range 5-400 μmol Cr(VI) showed an apparent V_{max} of 322.2 μmol Cr(VI) / min / mg protein and K_m of 47.5 μmol for NADPH, and a V_{max} of 130.7 μmol Cr(VI) / min / mg protein and K_m of 17.2 μmol for NADH. For the reduction of 1 mmol Cr(VI), 2.7 mmol of NADPH and 2.8 mmol NADH were consumed.

Effect of metal ions and metabolism inhibitors

When the influence of certain inhibitors on Cr(VI) reductase activity was studied (Table 2), all the metal cations tested inhibited the enzyme activity. The activity was

Table 2. Effect of metal ions and metabolism inhibitors on the Cr(VI) reductase activity

Effector (1 mM)	Relative activity (%)
None	100
Ag^{2+}	33.1
Ca^{2+}	91.2
Cd^{2+}	40.4
Hg^{2+}	0
Mg^{2+}	97.8
Mn^{2+}	61.8
Pb^{2+}	89.7
Zn^{2+}	28.4
Sodium azide	94.3
Imidazole	81.1
N-ethylmalemide	0
Potassium cyanide	86.2
Iodoacetic acid	96.2
Iodoacetamide	95.6
EDTA	76

completely abolished by 1 mM of Hg^{2+}, whereas Ag^{2+}, Cd^{2+} and Zn^{2+} showed more than 50% inhibitions. The addition of 1 mM of the thiol inhibitor, N-ethylmalemide (NEM), resulted in complete abolition of the enzyme activity.

N-terminal sequences, amino acid composition, and Western blot analysis

The sequence of the N-terminal amino acids of the enzyme was D-Y-E-K-L-Y-S-P-L-K. When the DDBJ/EMBL/Genbank was searched, the highest sequence identity (80%) was found with the N-ethylmalemide reductase from *E. coli* K12, with an N-terminal amino acid sequence of M-S-S-E-K-L-Y-S-P-L-K (Miura *et al.*, 1997). However, comparison with the N-terminal sequences of Cr(VI) reductases from *Pseudomonas ambigua* G-1(M-V-K-E-L-L-R-N-H-S-S, Suzuki, 1996) and *Pseudomonas putida* KT2440 (M-S-Q-V-Y-S-V-A-V-V, Ackerley *et al.*, 2004) showed no significant similarities. When the amino acid compositions were compared, it was evident that the purified enzyme con-

Table 3. Comparison of the amino acid compositions of the Cr(VI) reductase and the other reductases

Amino acid	*E. coli* ATCC 33456	*E. coli* K-12	*P. ambigua* G-1	*P. putida* KT2440
	Cr(VI) reductase	NEM reductase	Cr(VI) reductase	Cr(VI) reductase
Cys	10	0	2	2
Asp+Asn	38	27	26	16
Glu+Gln	44	43	30	16
Ser	31	22	14	18
Gly	56	37	15	13
His	10	11	4	1
Arg	17	25	15	13
Thr	19	17	8	4
Ala	39	48	20	20
Pro	20	21	10	12
Tyr	6	13	11	6
Val	26	16	21	19
Met	2	6	6	4
Ile	22	26	14	6
Leu	32	29	19	16
Phe	13	9	12	8
Trp	2	5	3	3
Lys	31	10	16	9
Total	418	365	243	186
Ref.	This study	Miura *et al.* 1997	Suzuki *et al.* 1996	Ackerley *et al.* 2004

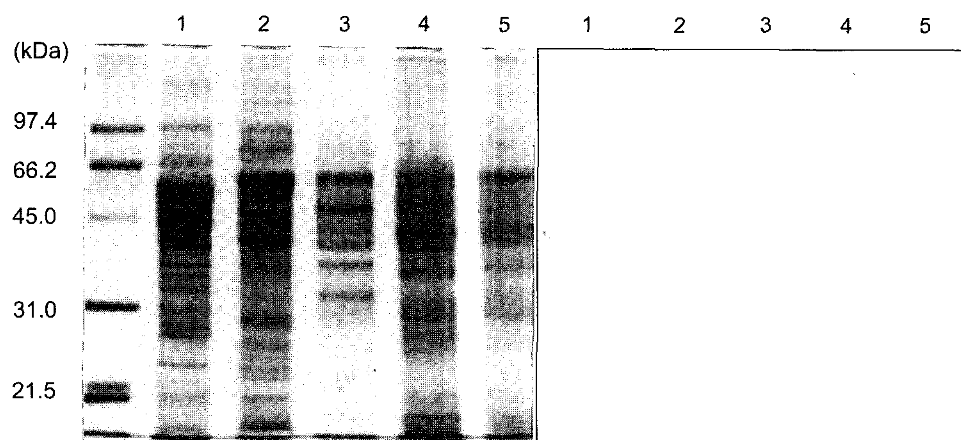

Fig. 3. Detection of the Cr(VI) reductase in the fractions from *E. coli* cells and the various Cr(VI) reducing strains. Crude extracts were separated by 12% SDS-PAGE (A) and subjected to Western blot analysis with antibodies raised against the purified Cr(VI) reductase (B). Lane 1: *E. coli* ATCC 33456, lane 2: *P. aeruginosa* HP014, lane 3: *D. vulgaris*, lane 4: *P. fluorescens* LB300, lane 5: *Bacillus* sp.

tained relatively higher portions of Cys, Gly and Lys, but smaller amounts of Tyr, Trp and Met than *P. ambigua* G-1 Cr(VI) reductase and *E. coli* K12 NEM reductase (Table 3).

As shown in Fig 3, there was no detectable antigenic cross responses between *E. coli* ATCC 33456 and other Cr(VI) reducing strains (*P. aeruginosa* HP014, *P. fluorescens* LB300, *Bacillus* sp., and *D. vulgaris*) when antiserum against the purified enzyme was used.

Discussion

Cr(VI) reductase is a central enzyme in the Cr(VI) reduction system ornate in many soils and enteric bacteria, which enables them to reduce Cr(VI) to Cr(III), which readily forms an insoluble less toxic chromium hydroxide at neutral pH, and thus, is relevant to an understanding of the detoxification and ultimate remediation of Cr(VI) pollution.

This paper describes the 967.9-fold purification of the Cr(VI) reductase from *E. coli* ATCC 33456. The purified enzyme required either NADH or NADPH as an electron donor for Cr(VI) reduction. The molecular weight was found to be 42 and 84 kDa on SDS-PAGE and gel filtration, respectively, indicating a dimeric structure. It was active within narrow temperature (10 to 37°C) and pH (6 to 8) ranges. These data suggested that this enzyme is active under physiological intracellular conditions, such as 37°C and pH 7.0.

Based on tested physico-chemical properties, including the mol wt, V_{max}, K_m, optimum temperature, electron donor usage, N-terminal sequence and amino acid composition, the purified enzyme in this study seems to be entirely different from other known Cr(VI) reductases from *P. ambigua* G-1 (Suzuki *et al.*, 1992) and *P. putida* (Park *et al.*, 2000, Ackerley *et al.*, 2004). The N-terminal amino acid sequence of the purified enzyme showed 80% sequence identity within the ten N-terminal amino acids with the sequence of the NEM reductase. However, the purified enzyme showed no NEM reducing activity (data not shown), and had a different amino acid composition and molecular weight. Thus, we presumed they are distinct enzymes.

Flavin compounds containing *Vibrio harveyi* nitroreductase (NfsA) (Kwak *et al*, 2003) and *E. coli* soluble flavoprotein (YieF) (Ackerley *et al.*, 2004) were recently reported to have chromate reducing activity. However, our enzyme showed no flavin compound from the absorption spectrum (data not shown) and no nitroreductase activity. Moreover, the molecular weight, N-terminal sequence (NfsA : M-S-P-I-L-G-Y-W-K-I, YieF : M-S-E-K-L-Q-V-V-T-L), amino acid compositions, V_{max} and K_m were different. This indicated that our enzyme was distinct from both NfsA and YieF.

When a comparative study was conducted using antiserum prepared from both Tn501- and R831- purified Hg(II) reductases, all the gram negative bacterial Hg(II) reductases tested were inactivated by the antiserum from the R831 enzyme (Silver and Kinscherf, 1982). With this result in mind, we investigated the antigenic relationship between the Cr(VI) reductase from *E. coli* ATCC 33456 and other Cr(VI) reducing strains by western blot. However, other strains *P. aeruginosa* HP014, *P. fluorescens* LB300, *Bacillus sp.* and *D. vulgaris* showed no reactivity to the antiserum raised against the purified enzyme. This result indicates that the Cr(VI) reductase from *E. coli* ATCC 33456 does not have an immunologically protein related to the other Cr(VI) reducing strains.

The enzyme was indeed greatly inhibited by Ag^{2+}, Cd^{2+}, Hg^{2+} and Zn^{2+}. Moreover, the activity was inhibited by NEM, a well-known thiol group inhibitor. This indicated that a sulfhydryl group might be involved in the active catalytic site. The chelating agent, EDTA, slightly inhibited the Cr(VI) reducing activity, indicating that divalent cations may be required for enzyme activation. However, the divalent cations, Ca^{2+}, Mg^{2+} and Mn^{2+}, did not significantly stimulate the enzyme activity. The mechanism of the inhibition; however, is still currently unknown, which should be clarified by further investigation. Further biophysical studies of the Cr(VI) reductase, as well as the cloning and DNA sequence analyses of the genes, are currently in progress, which should provide additional information regarding the mechanism and function of Cr(VI) reduction by *E. coli.*

Acknowledgments

This work was supported by a grant from the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science & Technology (Grant M102KK-010002-04K1101-00212), Republic of Korea, and from the RRC at Myongji University, KOSEF. We would like to thank Yi-Tin Wang and Henry L. Ehrlich for providing the Cr(VI) reducing strains (*Pseudomonas fluorescens* LB300, *Bacillus* sp.), and Sung Chan Choi for providing the *Pseudomonas aeruginosa* HP014.

References

Ackerley, D.F., C.F. Gonzalez, C.H. Park, R. Blake, M. Keyhan, and A. Matin. 2004. Chromate-reducing properties of soluble flavoproteins from *Pseudomonas putida* and *Escherichia coli. Appl. Environ. Microbiol.* 70, 873-882.

Bae, W.C., T.G. Kang, I.K. Kang, T.I. Won, and B.C. Jeong. 2000. Reduction of hexavalent chromium by *Escherichia coli* ATCC33456 in batch and continuous cultures. *J. Microbiol.* 38, 36-39.

Bopp, L.H. and H.L. Ehlich. 1988. Chromate resistance and reduction in *Pseudomonas fluorescens* strain LB300. *Arch. Microbiol.* 150, 426-431.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the princi-

ple of protein-dye binding. *Anal. Biochem.* 72, 248-254.

Horitsu, H., S. Futo, Y. Miyazawa, S. Ogai, and K. Kawai. 1987. Enzymatic reduction of hexavalent chromium by hexavalent chromium tolerant *P. ambigua* G-1. *Agric. Biol. Chem.* 51, 2417-2420.

Kwak, Y.H., D.K. Lee, and H. B. Kim. 2004. *Vibrio harveyi* nitroreductase is also a chromate reductase. *Appl. Environ. Microbiol.* 69, 4390-4395.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Levis, A.G. and V. Bianchi. 1982. Mutagenic and cytogenetic effects of chromium compounds, p. 171-208. *In* S. Langard (ed.), Biological and environmental aspect of chromium. Elsvier science, Amsterdam.

Llovera, S., R. Bonet, M.D. Simon-Pujol, and F. Congregado. 1993. Chromate reduction by resting cells of *Agrobacterium radiobacter* EPS-916. *Appl. Environ. Microbiol.* 59, 3516-3518.

Lovely, D.R. and E.J.P. Phillips. 1994. Reduction of chromate by *Desulfovibrio vulgaris* and its c3 cytochrome. *Appl. Environ. Microbiol.* 60, 726-728.

Miura, K., Y. Tomioka, H. Suzuki, M. Yonezawa, T. Hishinuma, and M. Mizugaki. 1997. Molecular cloning of the *nemA* gene encoding N-ethylmalemide reductase from *Escherichia coli*. *Biol. Pharm. Bull.* 20, 110-112.

Mizugaki, M., T. Unama, T. Shiraishi, T. Nishimaki, and H. Yamanaka. 1981. Studied on the metabolism of unsaturated fatty acid N-ethylmalemide reducing activity in *Escherichia coli* K-12. *Chem. Pharm. Bull.* 29, 570-573.

Myers, C.R., B.P. Carstens, W.E. Antholine, and J.M. Myers. 2000. Chromium(VI) reductase activity is associated with the cytoplasmic membrane of anaerobically grown *Shewanela putrefaciens* MR-1. *J. Appl. Microbiol.* 88, 98-106.

Oh, Y.S. and S.C. Choi. 1997. Reduction of hexavalent chromium by *P. aeruginosa* HP014. *J. Microbiol.* 35, 25-29.

Park, C.H., M. Keyhan, B. Wielinga, S. Fendorf, and A. Martin. 2000. Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Appl. Environ. Microbiol.* 66, 1788-1795.

Pettrilli, F.L. and W. Miller. 1977. Toxicity and mutagenicity of hexavalent chromium on *Salmonella typhimurium*. *Appl. Environ. Microbiol.* 33, 805-809.

Shen, H. and Y.T. Wang. 1993. Characterization of enzymatic reduction of hexavalent chromium by *Escherichia coli* ATCC 33456. *Appl. Environ. Microbiol.* 59, 3771-3777.

Silver, S. and T.G. Kinscherf. 1982. Genetic and biochemical basis for microbial transformations and detoxification of mercury and mercurial compounds, p. 85-103. *In* A.M. Chakrabarty, (ed.), Biodegradation and detoxification of environmental pollutants. CRC Press. Inc., Boca Raton. Florida.

Suzuki, T. 1996. *Pseudomonas* sp. *chrR* gene for Cr(VI) reductase. Unpublished, obtained from DDBJ/EMBL/Genbank., Acession No. D83142.1.

Suzuki, T., N. Miyata, H. Horitsu, K. Kawai, K. Takamizawa, Y. Tai, and M. Okazaki. 1992. NAD(P)H-dependent chromium(VI) reductase of *P. ambigua* G-1: a Cr(VI) intermediate is formed during the reduction of Cr(VI) to Cr(III). *J. Bacteriol.* 174, 5340-5345.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350-4354.

Urone, P.F. 1955. Stability of colorimetric reagent for chromium, S-diphenylcarbazide, in various solvents. *Anal. Chem.* 27, 1354-1355.

Wang, P.C., T. Mori, K. Komori, M. Sasatsu, K. Toda, and H. Ohtake. 1989. Isolation and characterization of an *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions. *Appl. Environ. Microbiol.* 55, 1665-1669.

Wang, P.C., T. Mori, K. Toda, and H. Ohtake. 1990. Membrane associated chromate reductase activity from *Enterobacter cloacae*. *J. Bacteriol.* 172, 1670-1672.

Wang, Y.T. and H. Shen. 1995. Bacterial reduction of hexavalent chromium. *J. Ind. Microbiol.* 14, 159-164.

Wang, Y.T. and C. Xiao. 1995. Factors affecting hexavalent chromium reduction in pure cultures of bacteria. *Water Res.* 29, 2467-2474.