Purification and Characterization of NADH-Dependent Cr(VI) Reductase from *Escherichia coli* ATCC33456

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Abstract A soluble Cr(VI) reductase was purified from the Cr(VI) reducing strain *Escherichia coli* ATCC33456 by ammonium sulfate fractionation, and chromatographies on Q-Sepharose FF, Cibacron blue 3GA dye affinity, Mono-Q 5/5, and Superdex 200 HR 10/30 columns. The estimated molecular mass of the purified enzyme was 27 kDa on SDS-polyacrylamide gel electrophoresis and 54 kDa on gel filtration, thus indicating a dimeric structure. The isoelectric point of the enzyme was pH 4.85. The optimum reaction pH and storage pH were both 7.0, the optimum reaction temperature was 37°C, and the storage temperature was 4°C. NADH and NADPH both served as electron donors for the reductase, with V_{max} of 68.3 μM Cr(VI)/min/mg protein and K_m of 7.6 μM using NADH, and V_{max} of 42.3 μM Cr(VI)/min/mg protein and K_m of 14.6 μM using NADPH. When 1 mM EDTA was added, the Cr(VI) reducing activity increased 4-fold.

Key words: Cr(VI), Cr(VI) reductase, purification, *Escherichia coli* ATCC33456

Hexavalent chromium, Cr(VI), is commonly released from the metal finishing industry, petroleum refining, leather tanning, iron and steel industries, inorganic chemical production, textile manufacturing, and pulp production [23]. Cr(VI) compounds are highly water soluble and toxic [16], thereby necessitating the treatment of wastewater, soil, and sediments. However, trivalent chromium, Cr(III), compounds are less toxic and readily form insoluble chromium hydroxides at neutral pH which can be easily removed from the environment [33].

There are several ways for microorganisms to survive in high concentration of heavy metals: resistance [26],

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accumulation [22], and reduction [11]. The microbial reduction of toxic Cr(VI) to the less toxic Cr(III) provides a useful detoxification process. Various microorganisms can reduce Cr(VI) using a wide range of substrates at near neutral pH [3, 7, 8, 9, 10, 12, 13, 15, 17, 18, 19, 23, 24, 25, 30, 32]. Two kinds of enzymatic mechanisms for Cr(VI) reduction have been proposed. The aerobic mechanism for Cr(VI) reduction is generally associated with a soluble fraction of cell extracts, which utilizes NADH as an electron donor [9, 10, 24, 32], whereas, the anaerobic activity for Cr(VI) reduction is mediated by the membrane bound cytochromes b, c, and d [18, 24, 31].

Cr(VI) reduction using a bioreactor has received much attention because of its possible practical application for *in situ* treatment [5, 6]. However, relatively few efforts have been directed toward the development of biological treatment of Cr(VI)-containing wastewater, due mainly to insufficient knowledge on the microbial Cr(VI) reductase. Previously, we reported Cr(VI) reductase activity in *E. coli* ATCC33456 using batch and continuous cultures [2]. Accordingly, the present study attempts to purify and characterize the Cr(VI) reductase in *E. coli* ATCC33456 and compare its biochemical properties with those of the *Pseudomonas* Cr(VI) reductases [21, 27]. This is the first report on the purification of Cr(VI) reductase from *E. coli*.

MATERIALS AND METHODS

Bacteria and Growth Conditions
E. coli ATCC33456 was purchased from the American Type Culture Collection (ATCC). The cells were grown in 14×750 ml of a nutrient broth (Difco, U.S.A.) for 8 h at 37°C with agitation at 200 rpm, and then transferred to 14 carboys (20 l capacity) containing 15 l each of the nutrient broth. The cells were incubated for 12 h at 37°C and air

was bubbled from the bottom of the carboy. The cells in 200 l of the culture were harvested at 4°C by centrifugation at 6,000 ×g for 15 min.

Purification of Cr(VI) Reductase

All purification steps were performed at 4°C except for Mono-Q chromatography, and all buffers were supplemented with 1 mM dithiothreitol to prevent enzyme oxidation. The cells (220 g, wet weight) were washed with 50 mM KH_2PO_4 buffer (pH 7.0), resuspended in 1 l of the same buffer, and disrupted in an ice bath by sonication for 1 min pulses at 100 W, 30 times. The unbroken cells, cell debris, and membrane fractions were removed by successive centrifugations 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 precipitates, obtained from a 35%–65% ammonium sulfate fractionation of the crude extract, were collected by centrifugation (12,000 ×g, 60 min) and resuspended in 120 ml of 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 (180 ml) was applied to a Q-Sepharose FF anion exchange column (2.5 cm×37 cm, Pharmacia, Sweden) pre-equilibrated with a histidine buffer. The proteins were 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 (355 ml) were pooled and then applied to a Cibacron blue 3GA dye affinity (Sigma, U.S.A.) column (15 ml). The column was washed with the same buffer followed by elution successively with 1 mM NADH, 1 mM NADPH, and 5 M NaCl. The Cr(VI) reducing activity containing fractions eluted by 1 mM NADH were concentrated using an Amicon YM 10 membrane (Millipore, U.S.A.), then a 10 ml sample was applied to a Mono-Q HR 5/5 anion exchange column (Pharmacia, Sweden) using FPLC. The column was washed with the same buffer and the proteins were eluted with a 0 to 0.3 M linear gradient of NaCl at a flow rate of 1 ml/min. Finally, the fractions containing the Cr(VI) reducing activity were concentrated, buffer was exchanged to 20 mM KH_2PO_4 (pH 7.0), and the fractions then purified on a Superdex 200 HR 10/30 column (Pharmacia, Sweden) in the same buffer at a flow rate of 0.25 ml/min.

Estimation of the Molecular Weight of Cr(VI) Reductase

The molecular mass of the native form of the purified Cr(VI) reductase was estimated by gel filtration on a Superdex 200 HR 10/30 (Pharmacia, Sweden) column 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, U.S.A.). The subunit size of Cr(VI) reductase was determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [14]. The enzyme preparation was boiled for 10 min in the presence of 1.6% (wt/vol) SDS and 4% (wt/vol) β-mercaptoethanol,

with 0.1% bromophenol blue as the sample buffer. The molecular weight standards (BioRad) used for the proteins were rabbit skeletal muscle myosin (200 kDa), E. coli β-galactosidase (116.25 kDa), rabbit muscle phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). The electrophoresis was performed at a constant current, using a vertical system (Mini Gel system, Bio-Rad Laboratories). The gels were stained at room temperature using Coomassie Brilliant Blue R250.

Determination of the Isoelectric Point

The isoelectric focusing of the purified enzyme was performed on a precast isoelectric focusing gel (pH 3–9) (Pharmacia, Sweden). The sample preparation and isoelectric focusing were carried out following the instructions provided by Pharmacia Biotech Inc., using a PhastSystem (Pharmacia, Sweden). The gels were Coomassie Blue stained as described in the PhastSystem manual.

Effects of pH and Temperature on the Activity and Stability of Enzyme

The optimal pH for the Cr(VI) reduction was determined at a pH range of 4.0 to 9.0 using the following buffers (100 mM); citrate (4.0–6.0), phosphate (6.0–7.0), Tris (7.0–9.0). For the pH stability test, the enzyme was incubated at different pHs at 4°C for 40 h, and then after adjusting the pH to 7.0, the residual activity was measured. The use of two buffers at pH overlap compensated for the buffer-associated effects.

To determine the effect of temperature on the stability of the enzyme, 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 then the residual enzyme activity was assayed. To examine the optimum temperature, the enzyme activity was measured at various temperatures (4, 12, 20, 28, 37, 50, and 60°C).

Effects of Various Compounds on Cr(VI) Reducing Activity

To examine the effect of various compounds on the reducing activity of the enzyme, purified enzyme was reacted in the presence of each compound (electron donors, metal cations, and metabolism inhibitors) and the activities were then assayed under the standard conditions.

Assay of Cr(VI) Reductase Activity

The Cr(VI) reducing activity was assayed by measuring the decrease in Cr(VI). The reaction mixture contained 20 μM Cr(VI), 0.4 mM NADH, and a suitable amount of the enzyme solution in 0.2 ml of a 50 mM phosphate buffer (pH 7.0). The Cr(VI) reduction was determined

colorimetrically using a spectrophotometer at 540 nm based on a reaction with diphenylcarbazide in an acid solution [28] and ion chromatography (Waters 626, U.S.A.) using the EPA method 218.6 [1]. The reaction was initiated by the addition of the enzyme solution. After incubating for 30–120 min at 37°C, the reaction was stopped by the addition of diphenylcarbazide in an acid solution. One unit of Cr(VI) reductase activity was defined as the amount of enzyme that catalyzed the reduction of 1 µM of Cr(VI) per min.

Analytical Method

The protein concentration was estimated by the method of Bradford using bovine serum albumin as the standard [4]. Protein concentration of the FPLC effluents was estimated by measuring absorbance at 280 mm. The NADPH (Sigma, U.S.A.) concentration was determined by measuring the absorbance of NADPH at 340 nm using a spectrophotometer (Ultrospec III, Pharmacia, Sweden). The K_m and V_{max} values were determined based on the double-reciprocal plot method of Lineweaver-Burk using the Sigma Plot 4.01 software program. The absorption spectrum of the enzyme was obtained at room temperature using a 1 cm quartz cuvette containing the enzyme dissolved in 20 mM KH_2PO_4 at pH 7.0.

RESULTS

Purification of Cr(VI) Reductase

Soluble Cr(VI) reductase was purified from the soluble fractions derived from 220 g of E. coli ATCC33456 cells by sonication, ammonium sulfate fractionation, and chromatographies on Q-Sepharose FF, Cibacron blue 3GA dye affinity, Mono-Q 5/5, and Superdex 200 HR 10/30 columns, as described in Materials and Methods. As shown in Table 1, the final purification resulted in a 515.9-fold purification with an overall yield of 1.2%. The Cibacron blue 3GA dye affinity chromatography (Fig. 1) was particularly effective for the purification of Cr(VI) reductase. However, it should be noted that most of the active Cr(VI) reductase did not bind to the resin. The most effective purification step for the Cr(VI) reductase was the anion exchange
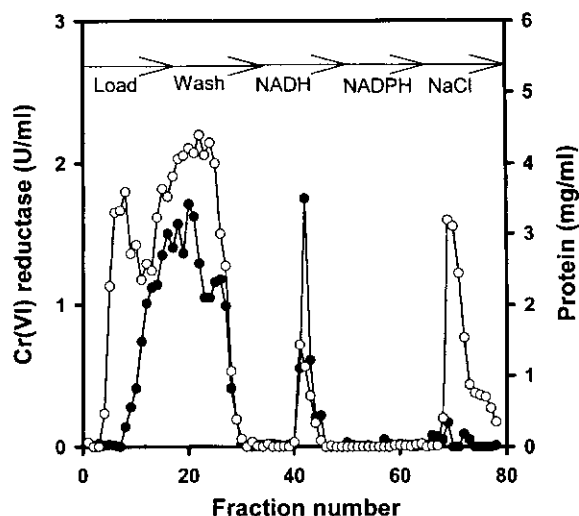

Fig. 1. Cibacron blue 3GA dye affinity chromatography of Cr(VI) reductase.

Active fractions from Q-Sepharose chromatography were pooled and then applied to a Cibacron blue 3GA dye affinity column. The fractions (39–46) eluted by 1 mM NADH were pooled. ● : Cr(VI) reductase activity; O: protein.

chromatography using Mono-Q, in which the enzyme was weakly absorbed and eluted with about 40 mM NaCl. Using Mono-Q, almost all remaining proteins were separated from the Cr(VI) reductase, thereby increasing the purification fold to about 666-fold (Table 1).

The purified enzyme appeared as a single protein band on SDS-PAGE by staining with Coomassie Brilliant Blue R250 (Fig. 2A). The apparent molecular mass of the Cr(VI) reductase was estimated to be 27 kDa by SDS-PAGE. The gel filtration of Cr(VI) reductase on Superdex 200 HR 10/30 indicated the native molecular mass of 54 kDa under nondenaturing conditions (Fig. 2B). The isoelectric point of the enzyme was pH 4.85.

pH and Temperature Dependency

As shown in Fig. 3A, the optimal pH for the Cr(VI) reductase activity was 7.0 at 37°C, whereas the activity showed 52% at pH 9.0 and only 2% at pH 4.0. The enzyme was most stable at neutral pH but lost about 70% of its initial activity after storage at pH 4.0, 4°C for 40 h.

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

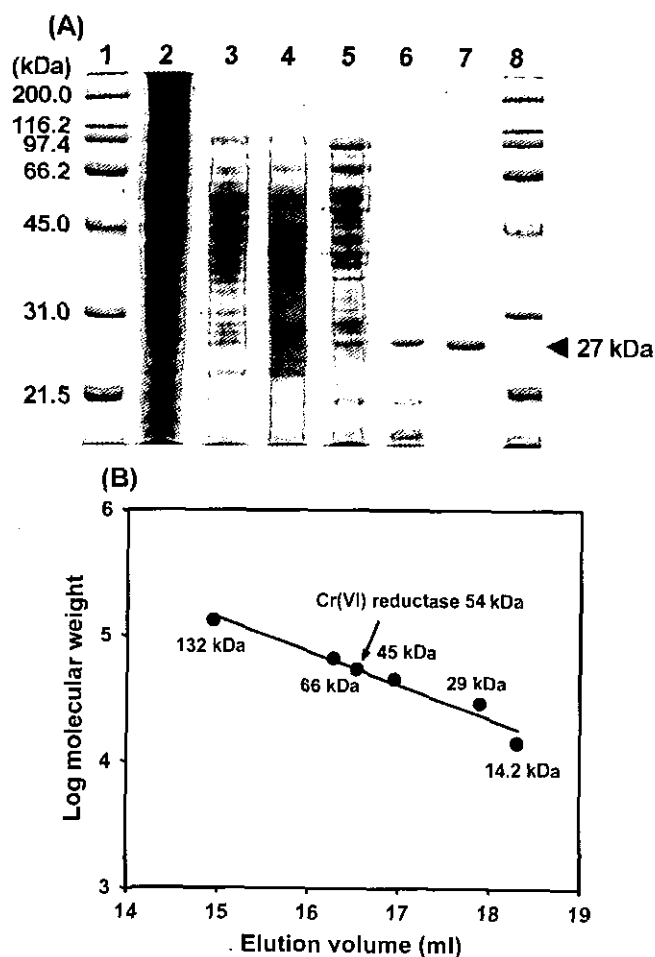| Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/ml)	Yield (%)	Purification (fold)
Crude extract	1,000	12,450	1,649.2	0.13	100.0	1.0
Ammonium sulfate fractionation	120	45,00	1,367.1	0.30	82.9	2.3
Q-Sepharose	365	670	823.5	1.23	49.9	9.3
Cibacron blue 3GA	85	50	71.9	1.44	4.4	10.9
Mono-Q	8	0.5	44.2	88.32	2.7	666.7
Superdex 200 HR	2	0.3	20.5	68.33	1.2	515.9

(A)

(B)

Fig. 2. Estimation of the molecular weight of purified Cr(VI) reductase by SDS-polyacrylamide gel electrophoresis (A) and gel filtration (B).

The conditions of SDS-PAGE and gel filtration are described in Materials and Methods. Lanes 1 and 8: size marker; lane 2: crude extract; lane 3: ammonium sulfate fractionation; lane 4: Q-Sepharose anion exchange; lane 5: Cibacron 3GA dye affinity; lane 6: Mono-Q HR 5/5; lane 7: Superdex 200 HR.

When the pH of the reaction mixture was maintained at pH 7.0, the Cr(VI) reducing activity of the purified enzyme increased with temperature and peaked at 37°C (Fig. 3B). However, it decreased rapidly at 50°C, and only 18% of its specific activity remained. Over 85% of its activity remained after storage at below 20°C for 14 h.

Electron Donor and Kinetic Calculation

The survey for a suitable electron donor for the Cr(VI) reduction activity of the purified enzyme showed that both NADH and NADPH were good electron donors, however, NADPH showed only 73.9% of activity compared with that of NADH (Table 2), while the enzyme was inactive in the absence of these cofactors. Interestingly, some other factors such as ascorbic acid, glutathione, D-glucose, and D-fructose, which were effective in the reduction of Cr(VI)
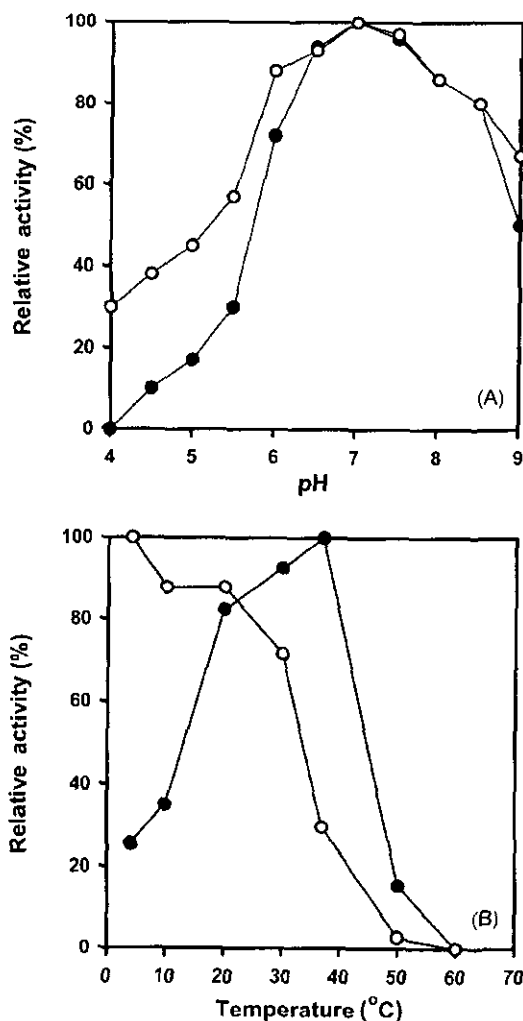

Fig. 3. Effects of pH and temperature on the stability and activity of purified Cr(VI) reductase.

● : activity; ○ : stability. (A) Enzyme activity was measured at various pHs at 37°C. For pH stability, residual activity was measured under standard conditions after incubation at various pHs at 4°C for 40 h. (B) Enzyme activity was measured at various temperatures at pH 7.0. For temperature stability, the enzyme solution was incubated at various temperatures at pH 7.0 for 14 h and the remaining activity was measured under standard conditions.

with whole cells [2], did not support the Cr(VI) reducing activity of the purified enzyme. The Michaelis-Menten

Table 2. Effect of electron donor on Cr(VI) reductae activity.

Electron donor (0.4 mM)	Relative activity (%)
None	0
NADH	100.0
NADPH	73.9
Ascorbic acid[a]	0
Glutathione	0
D-glucose	0
D-fructose	0

[a] 0.02 mM ascorbic acid was used.

Table 3. Comparison of Cr(VI) reductases.

Strain	Native (kDa)	Subunit (kDa)	pH optimum	V_{max} (μM/min/mg)	K_m (μM)	Electron donor	Electron donor to Cr(VI) ratio
E. coli ATCC33456	54	27	7.0	68.30	7.6	NADH	2.6:1
				42.30	14.6	NADPH	2.7:1
P. ambigua G-1	65	25	7.0	0.27	13.0	NADH	2.7:1
P. putida	50	20	5.0	1.72	374.0	NADH	nd[a]

[a]not determined.

kinetic parameters determined at the range of 5–80 μM Cr(VI) showed a V_{max} of 68.3 μM Cr(VI)/min/mg protein and K_m of 7.6 μM using NADH, and a V_{max} of 42.3 μM Cr(VI)/min/mg protein and K_m of 14.6 μM using NADPH (Table 3). When the Cr(VI) reduction and NADH/NADPH consumption were both analyzed, 2.6 mM of NADH was consumed per 1 mM of Cr(VI) and 2.7 mM of NADPH was consumed per 1 mM Cr(VI) (Table 3).

Effects of Metal Ions and Metabolism Inhibitors

The influence of certain inhibitors on the enzyme was studied. All the metal cations tested inhibited the enzyme activity (Table 4). The activity was completely abolished by 1 mM Hg^{2+}, whereas, Ca^{2+}, Cd^{2+}, and Mg^{2+} showed a more than 40% inhibition. As shown in Table 5, the addition of a thiol-modifying reagent (iodoacetic acid, N-ethylmalemide) and KCN resulted in a marked decrease in the enzyme activity, whereas the metal chelating agent, EDTA, stimulated the enzyme activity considerably.

Absorption Spectrum of Enzyme

The absorption spectrum of the purified Cr(VI) reductase is shown in Fig. 4. The enzyme exhibited an absorption spectrum at 369 nm and 457 nm indicating that this enzyme contains flavin compounds.

DISCUSSION

An intracellular soluble enzyme responsible for Cr(VI) reduction was purified approximately 515.9-fold and its

properties were studied. The absorption spectrum of the purified enzyme indicated that it contained a flavin compound. The Cr(VI) reducing activity required either NADH or NADPH as an electron donor. The estimated molecular weight of the intact enzyme was 54 kDa using gel filtration, whereas that of the denatured subunit was 27 kDa using SDS-PAGE. These data indicated that this enzyme was dimeric. The enzyme also required a narrow range of temperatures (20 to 37°C) and pHs (6 to 8) for its reducing activity, suggesting that it is only active under physiological conditions such as 37°C and pH 7.0.

Previously, two different types of Cr(VI) reductases have been purified from microbial origin (Table 3). Suzuki *et al.* [27] purified it from *P. ambigua* G-1 at about 38-fold, and Park *et al.* [21] purified the enzyme from *P. putida* at about 627-fold. Based on the known physico-chemical properties including the molecular weight, V_{max}, K_m, and optimum pH of these Cr(VI) reductases from *Pseudomonas* spp. (Table 3), the Cr(VI) reductase characterized from *E. coli* ATCC33456 in this study was identified as being distinct.

The inhibition of Cr(VI) reductase by a thiol-modifying reagent (iodoacetic acid, N-ethylmalemide) suggests that thiol groups might be involved in the active catalytic site. Since many metallic ions (Ca^{2+}, Cd^{2+}, Hg^{2+}, and Mg^{2+}) significantly inhibited this enzyme, a marked increase in the activity by the addition of EDTA was observed (Table 5). The exact mechanism of its inhibitory effect remains to be established.

Further biophysical studies of Cr(VI) reductase, as well as the cloning and DNA sequence analysis of the genes, are currently in progress and will provide us with additional

Table 4. Effect of metal ions on the Cr(VI) reductase activity.

Metal (1 mM)	Relative activity (%)
None	100.0
Ag^{2+}	72.4
Ca^{2+}	35.3
Cd^{2+}	57.9
Hg^{2+}	0
Mg^{2+}	56.0
Mn^{2+}	74.5
Pb^{2+}	87.1
Zn^{2+}	74.1

Table 5. Effect of metabolism inhibitors on Cr(VI) reductase activity.

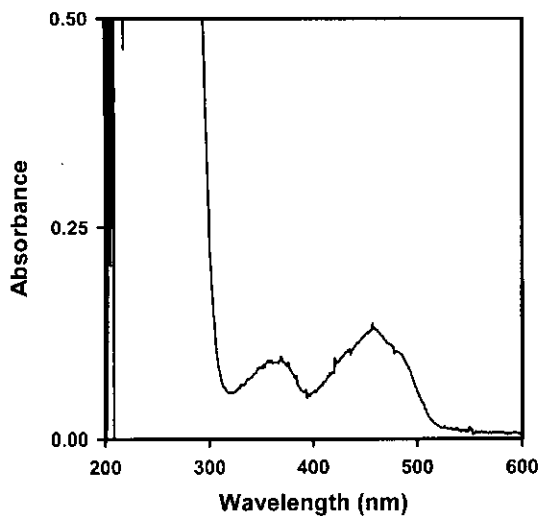| Inhibitor (1 mM)	Relative activity (%)
None	100.0
Sodium azide	88.9
Imidazole	69.4
N-ethylmalemide	50.0
Potassium cyanide	11.0
Iodoacetic acid	0
Iodoacetamide	80.5
EDTA	400.0

Fig. 4. Absorption spectrum of Cr(VI) reductase.
The absorbance of the enzyme dissolved in 20 mM KH_2PO_4, pH 7.0 was measured at 25°C.

information regarding the mechanism and function of Cr(VI) reduction in *E. coli*.

Acknowledgments

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