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Purification and Characterization of a Cyclohexanol Dehydrogenase from *Rhodococcus* sp. TK6

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Abstract Activity staining on the native polyacrylamide gel electrophoresis (PAGE) of a cell-free extract of *Rhodococcus* sp. TK6, grown in media containing alcohols as the carbon source, revealed at least seven isozyme bands, which were identified as alcohol dehydrogenases that oxidize cyclohexanol to cyclohexanone. Among the alcohol dehydrogenases, cyclohexanol dehydrogenase II (CDH II), which is the major enzyme involved in the oxidation of cyclohexanol, was purified to homogeneity. The molecular mass of the CDH II was determined to be 60 kDa by gel filtration, while the molecular mass of each subunit was estimated to be 28 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The CDH II was unstable in acidic and basic pHs, and rapidly inactivated at temperatures above 40°C. The CDH II activity was enhanced by the addition of divalent metal ions, like Ba^{2+} and Mg^{2+}. The purified enzyme catalyzed the oxidation of a broad range of alcohols, including cyclohexanol, *trans*-cyclohexane-1,2-diol, *trans*-cyclopentane-1,2-diol, cyclopentanol, and hexane-1,2-diol. The K_m values of the CDH II for cyclohexanol, *trans*-cyclohexane-1,2-diol, cyclopentanol, *trans*-cyclopentane-1,2-diol, and hexane-1,2-diol were 1.7, 2.8, 14.2, 13.7, and 13.5 mM, respectively. The CDH II would appear to be a major alcohol dehydrogenase for the oxidation of cyclohexanol. The N-terminal sequence of the CDH II was determined to be TVAHVTGAARGIGRA. Furthermore, based on a comparison of the determined sequence with other short chain alcohol dehydrogenases, the purified CDH II was suggested to be a new enzyme.

Key words: Cyclohexanol dehydrogenase, cyclohexanediol dehydrogenase, *Rhodococcus* sp., short chain alcohol dehydrogenase

Cyclohexanol is an intermediate in the breakdown of cyclohexane in fossil fuels and in the degradation of more complex alicyclic compounds, such as terpenes and sterols [11]. In contrast to the biodegradation of aliphatic and aromatic hydrocarbons, there have been relatively few reports of pure cultures capable of utilizing cycloaliphatics as sole carbon and energy sources [11, 19]. Studies have shown that the degradation of cyclohexanol proceeds via dehydrogenation to cyclohexanone, which is then oxygenated to 1-oxa-2-oxocycloheptane. The latter is hydrolyzed, yielding 6-hydroxycaproate, which undergoes further dehydrogenation reactions, yielding 6-oxohexanoate and finally adipate [6, 12, 16]. In this metabolic pathway, the dehydrogenation of cyclohexanol is the most recalcitrant reaction that is catalyzed by a specific alcohol dehydrogenase [16]. However, there is still a lack of detailed information on this enzyme, with only a few exceptions [4, 6, 16, 18].

Not many researchers have been successful in purifying cyclohexanol dehydrogenase (CDH), which oxidizes cyclohexanol to cyclohexanone. Stirling and Perry [16] reported on certain properties of CDH, which was partially purified from crude extracts from a *Nocardia* species using affinity chromatography. Their attempts to purify the enzyme by ion-exchange chromatography or gel filtration resulted in a significant loss of enzyme activity and the appearance of multiple protein bands on polyacrylamide gels, thereby suggesting that the enzyme was easily dissociated under the conditions employed.

Rhodococcus sp. TK6, isolated from the sludge in sewage from the Ulsan Industrial Complex for Petrochemicals, Korea [8], produced several isozymes that oxidized cyclohexanol to cyclohexanone. The current authors previously attempted to purify one of these isozymes, which was NAD^+-dependent CDH II [9]. The CDH II of *Rhodococcus* sp. TK6 on a cell-free extract was found to be extremely labile at 4°C. As such, it was very difficult to sustain its activity during the purification procedures. However, the current paper reports on the purification and characterization of the CDH II from *Rhodococcus* sp. TK6, which was able to utilize cyclohexanol as the sole source of carbon and energy.

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MATERIALS AND METHODS

Culture Medium and Growth of Bacteria

Rhodococcus sp. TK6, which can utilize cyclohexanol as the sole source of carbon and energy, has been described earlier [8]. The bacteria were grown at 30°C for 48 h in 1.5 l of a basal medium [8] with 0.4% cyclohexanol in a 3-l round flask on a reciprocal shaker.

Enzyme Assay and Protein Determination

CDH activity was assayed using the protocol described by Rodriguez and Trait [14] with certain modifications. The reaction was initiated by adding a 0.2-ml aliquot of an appropriately diluted enzyme to the reaction mixture, which was composed of a 0.4 ml dye mix containing five parts of 3.16 mM 2-*p*-iodophenyl-3-nitrophenyl-5-phenyltetrazolium chloride (INT) and one part of 0.65 mM phenazine methosulfate (PMS), 0.6 ml of 100 mM triethanolamine-HCl buffer (pH 7.6), 0.1 ml of 3 mM NAD^+, and 0.2 ml of 100 mM cyclohexanol. After incubation at 37°C for 10 min, the reaction was stopped by adding 1.5 ml of 0.2 N HCl. One unit of enzyme was defined as the increase of A_{520} per mg of protein under the above conditions. The protein concentrations were determined by the method of Bradford [1] using bovine serum albumin as the standard. During the purification of CDH II, the protein concentrations were determined by measuring the absorption at 280 nm.

Preparation of Cell Extract

The cells were grown in 1.5 l of a basal medium containing 0.4% (v/v) of cyclohexanol for 48 h at 30°C. The cells were harvested by centrifugation, washed three times, resuspended in buffer A [50 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM phenyl methyl sulfonyl fluoride (PMSF) and 1.0 mM dithiothreitol (DTT)], and sonicated at 90 μA five times for 2 min each using an ultrasonicator (Ultrasonics Ltd.; type 4523). The cell extract was centrifuged at 20,000 ×g for 10 min and any cell debris were removed. The supernatant of the cell extract was stored at −20°C for later use.

Enzyme Purification

All purification procedures were carried out at 0 to 4°C. The enzyme solution was more stable in buffer A than in 50 mM sodium phosphate buffer (pH 7.0), and more than 70% of its activity was sustained for a week at 4°C.

(I) DEAE Sephadex A-50 column chromatography. The supernatant was applied to a DEAE Sephadex A-50 column (Φ2.6×80 cm) previously equilibrated with buffer A. The column was washed with 250 ml of the same buffer and the CDH was eluted with 500 ml of a linear gradient formed by 300 and 750 mM NaCl at a flow rate of 24 ml/h, with 5 ml fractions.

(II) DEAE Sepharose CL-6B column chromatography. The fractions containing CDH activity collected from the DEAE Sephadex A-50 column were pooled, desalted, and concentrated using an ultrafiltration kit with a stirred Amicon cell (model; Amicon 8050) and a YM10 membrane (Amicon; lot no. K7MH7344). The resulting solution was applied to a DEAE Sepharose CL-6B column (Φ1.6×25 cm) previously equilibrated with buffer A. The CDH was eluted with 25 ml of 200 mM NaCl and 200 ml of a linear gradient formed by 200 and 300 mM NaCl in buffer A.

(III) FPLC with Mono Q, Mono P, and Superose 12. The fractions containing CDH activity from the DEAE Sepharose CL-6B column were pooled, desalted, and concentrated as described above. The concentrated enzyme solution was then applied to a Mono Q HR 5/5 column operated with an FPLC system (Pharmacia Biotech). The column was washed with buffer A and the CDH was eluted with a linear gradient formed between 0 to 300 mM NaCl in buffer A, at a flow rate of 0.5 ml/min. The fractions containing CDH activity were concentrated and desalted by the same ultrafiltration kit. The desalted enzyme was loaded onto a Mono P HR 5/5 column previously equilibrated with buffer A. The CDH was eluted with a linear gradient of 0 to 400 mM NaCl in buffer A, at a flow rate of 1.0 ml/min. The peak of the active protein fractions was pooled and concentrated with the same ultrafiltration kit. The concentrated enzyme solution was then applied to a Superose 12 HR 10/30 column equilibrated with buffer A and the CDH was eluted with buffer A, at a flow rate of 0.2 ml/min. Finally, the fractions containing CDH activity were concentrated as described above and stored at −20°C for later use.

SDS-PAGE and Activity Staining

SDS-PAGE was performed by the method of Laemmli [10]. For the activity staining of dehydrogenases, a native PAGE of the crude cell extract (12 μg of protein/well) was carried out with a running gel of 10% polyacrylamide according to the method of Davis [3], and the gels were stained with the reaction mixture used in the enzyme assay at 37°C for 60 min. A formazan band was formed at the site of enzyme activity. The reaction was stopped by dipping the gels in 7.0% acetic acid and the gel was cleared by washing with water five times.

Molecular Mass Determination

The molecular mass of the purified enzyme was determined by SDS-PAGE [10] using 10% polyacrylamide gels. A low-molecular-weight calibration kit (Pharmacia Biotech Co.; cat. no. 3040446011) was used for estimating the molecular mass. The relative molecular mass of the native protein was determinated by gel filtration using FPLC on a Superose 12 HR 10/30 column equilibrated with buffer A. The standard protein markers used to calibrate the column were bovine serum albumin (67 kDa), ovalbumin (45 kDa), and cytochrome *c* (12.5 kDa).

Effects of pH and Temperature on Activity

To determine the pH stability of the enzyme, the purified CDH II was incubated at different pHs at 4°C for 4 h. After adjusting the pH to 7.6, the residual activity was measured. To determine the heat stability, the purified enzyme was incubated in 50 mM sodium phosphate buffer (pH 7.0) for 20 min at various temperatures (10 to 50°C), and the residual activity was assayed, as described above.

Kinetic Studies

The apparent K_m and V_{max} values of the purified enzyme were determined graphically using Lineweaver-Burk plots. Linear regression analysis was performed to calculate the x and y intercepts. When the apparent K_m and V_{max} values of the purified enzymes were determined, the concentration of CDH II was 0.13 mg/ml in the presence of a fixed concentration of NAD^+ (3 mM).

Amino Acid Sequence Analysis

The amino-terminus of the purified CDH II was determined by the method of Edman degradation [7] using a protein sequencer (ABI Co.; model, Procise 476) at the Pusan branch of the Korea Basic Science Institute. The partial amino acid sequence obtained was compared with the sequences of proteins stored in BLAST at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). A sequence alignment was performed using SIM+LALNVIEW at expasy (http://www.expasy.ch/tools/sim-prot.html).

RESULTS

Extraction and Identification of CDH Isozymes

The crude cell extract of *Rhodococcus* sp. TK6 grown on a basal medium containing 0.3% alcohol was prepared as described in Materials and Methods. CDH activity was detected in the soluble fraction, but not in the pellets of the sonicated cells. A native PAGE of the cell-free extract followed by staining for *in situ* CDH activity demonstrated the presence of at least seven CDH activity bands in *Rhodococcus* sp. TK6, which was grown in a medium containing cyclohexanol, cyclohexanediol, and 1-pentanol as described previously [9].

The four major activity bands were previously designated as CDH I, CDH II, CDH III, and CDH IV [9]. CDH I was expressed in the medium containing the three alcohols, cyclohexanol, cyclohexane-1,2-diol, and 1-pentanol. CDH II and III were predominantly induced by cyclohexanol and cyclohexane-1,2-diol, respectively. CDH IV was also induced by cyclohexanol and cyclohexane-1,2-diol. CDH II, III, and IV were not induced by 1-pentanol.

Purification of CDH II

Among the alcohol dehydrogenases (CDH I, II, III, and IV), the CDH II specific to cyclohexanol was purified from the cell-free extract using DEAE Sephadex A-50 and DEAE Sepharose CL-6B column chromatography and FPLC with Mono Q, Mono P, and Superose 12.

Two active peaks that did not coincide with the protein peaks were eluted at 500 mM and 600 mM NaCl in a linear gradient in the DEAE Sephadex A-50 column. The protein peaks at 500 mM and 600 mM NaCl were specific to cyclohexane-1,2-diol and cyclohexanol, respectively. The enzyme fractions of the peak specific to cyclohexanol were then applied to the DEAE Sepharose CL-6B column. The major CDH peak that emerged at 250 mM NaCl in a linear gradient in the DEAE Sepharose CL-6B column was then applied to FPLC with Mono Q. An active peak with five protein peaks in FPLC with Mono Q, which remained unseparated after resolution, was loaded into a Mono P column for FPLC. The CDH in the Mono P column, which was packed with a weak anion exchanger designed for chromatofocusing (Pharmacia Biotech Co.), was eluted with a linear gradient of NaCl instead of a pH gradient. Because CDH II was very unstable with a change of pH, a salt gradient was chosen for the elution of the enzyme to avoid any loss of activity due to pH change in the column. One of four protein peaks with CDH II activity was separated from the other peaks in the FPLC with Mono P, pooled, and concentrated with an ultrafiltration kit. The concentrated enzyme was then loaded onto a Superose 12 column. A single protein peak with CDH II activity emerged from the Superose 12 column. The purification procedure for the enzyme is summarized in Table 1.

The enzyme was purified to homogeneity, evidenced by a single band on SDS-PAGE (Fig. 1A). The CDH II was purified about 9.8-fold from the supernatant. The specific activity of the purified enzyme was 127 U · mg of protein^{-1}, and the yield was 0.4%. The results of zymograms of

Table 1. Summary of CDH II purification.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
Cell-free extract	720.00	9,225	13	100.0
DEAE Sephadex A-50	42.00	1,605	38	17.0
DEAE Sepharose CL-6B	12.00	640	53	7.0
Mono Q	3.12	257	82	3.0
Mono P	1.66	170	102	2.0
Superose 12	0.26	33	127	0.4

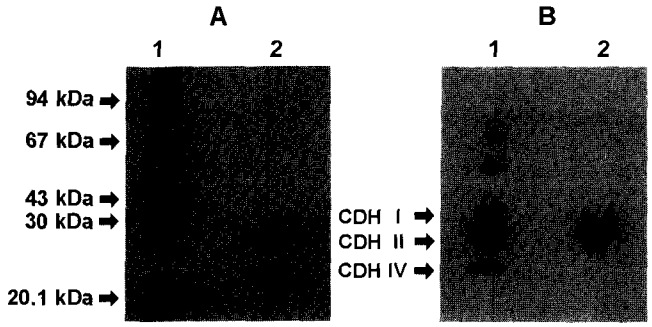

Fig. 1. Electrophoresis profile using SDS-PAGE (A) and activity staining with 15.4 mM cyclohexanol as substrate (B) of the purified CDH II.
Lane 1 in panels A and B is the molecular markers and CDH isozyme of the crude cell extract of *Rhodococcus* sp. TK6 induced with cyclohexanol, respectively. Lane 2 in panels A and B is the purified CDH II.

the purified enzyme and the cell-free extract showed that the purified enzyme was identical with CDH II in the CDH isozymes of the cell-free extract of *Rhodococcus* sp. TK6 grown in media containing cyclohexanol (Fig. 1B).

The CDH III band in the cell-free extract of *Rhodococcus* sp. TK6 induced with cyclohexanol did not appear densely in lane 1 of Fig. 1B, because CDH III may be dominantly induced by cyclohexane-1,2-diol.

Relative Molecular Mass and Subunit Structure

Using a calibrated column of Superose 12, it was estimated that the relative molecular mass of the CDH II was 60 kDa (data not shown), while the molecular mass of each subunit estimated by SDS-PAGE was approximately 28 kDa (Fig. 1A). These results suggest that the CDH II is a dimeric protein.

Properties of CDH II

The enzyme was stable at the pH between pH 7.5 and 8.5 (Fig. 2A). More than 90% of the purified CDH II activity

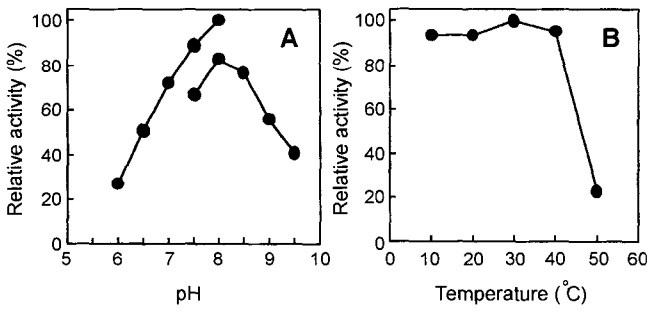

Fig. 2. pH stability (A) and thermal stability (B) of purified CDH II.
(A) Sodium phosphate buffer (pH 6.0 to 8.0) and Tris-HCl (pH 7.5 to 9.5) buffer were used. The purified enzyme was incubated at 4°C for 4 h in each buffer. After adjusting the enzyme solution to pH 7.6, the residual activity was measured. (B) The purified enzyme was incubated in a 50 mM sodium phosphate buffer (pH 7.0) for 20 min at various temperatures.

Table 2. Effect of metal ions on purified CDH II activity.

Metal salt (10 mM)	Relative activity (%)*
$CuSO_4$	4
$MgSO_4$	113
$ZnSO_4$	9
$AlCl_3$	4
$BaCl_2$	219
$CaCl_2$	49
$CoCl_2$	40
$MgCl_2$	119
$MnCl_2$	7
KCl	110
LiCl	104
NaCl	103
None	100

*The activity without metals (specific activity, 60 U/mg) was defined as 100%.

was retained with treatment at 40°C for 20 min, but dropped to less than 20% with treatment at 50°C for 20 min (Fig. 2B). The CDH II activity was enhanced by

Table 3. Substrate specificity of purified CDH II to alicyclic and aliphatic alcohols.

Substrate (14.8 mM)	Relative activity (%)*
Cyclohexane-1,2-diol (*cis*, *trans* mixture)	123
cis-Cyclohexane-1,2-diol	28
trans-Cyclohexane-1,2-diol	146
Cyclohexane-1,3-diol (*cis*, *trans* mixture)	28
Cyclohexane-1,4-diol (*cis*, *trans* mixture)	22
cis-Cyclopentane-1,2-diol	27
trans-Cyclopentane-1,2-diol	114
Cyclopentanol	77
Methanol	2
Ethanol	20
1-Propanol	20
1-Butanol	20
1-Pentanol	23
1-Hexanol	21
2-Propanol	17
2-Methyl-1-propanol	17
2-Methyl-1-butanol	26
2-Butanol	44
2-Metyl-2-propanol	16
Ethane-1,2-diol	5
Propane-1,2-diol	30
Propane-1,3-diol	7
Hexane-1,2-diol	84
Hexane-1,6-diol	21
Cyclohexanol	100

*The activity with cyclohexanol (specific activity, 60 U/mg) was defined as 100%.
The reactions were carried out under standard conditions based on the listed substrates.

Table 4. K_m and V_{max} values of purified CDH II.

	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m
Cyclohexanol	1.7	129.5	76.2
trans-Cyclohexane-1,2-diol	2.8	149.7	53.5
Cyclopentanol	14.2	110.8	7.8
trans-Cyclopentane-1,2-diol	13.7	163.5	11.9
Hexane-1,2-diol	13.5	123.1	9.1

the addition of Ba^{2+} and Mg^{2+} to the reaction mixture, but inhibited by the addition of Al^{3+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, and Zn^{2+} (Table 2).

As shown in Table 3, the purified enzyme catalyzed the oxidation of a broad range of alcohols including cyclohexanol, *trans*-cyclohexane-1,2-diol, *trans*-cyclopentane-1,2-diol, cyclopentanol, and hexane-1,2-diol. The relative activities of the purified CDH II toward *trans*-cyclohexane-1,2-diol and *trans*-cyclopentane-1,2-diol were 46% and 14% higher than that of cyclohexanol, respectively. However, the enzyme was much less active toward the *cis*-type of cyclohexane-1,2-diol and cyclopentane-1,2-diol than the *trans*-type of cycloalkane-diols and cyclohexanol. Short chain alcohols (C_2 to C_6), cyclohexane-1,3-diol, and cyclohexane-1,4-diol were hardly oxidized by CDH II.

The apparent K_m values of CDH II for cyclohexanol, *trans*-cyclohexane-1,2-diol, cyclopentanol, *trans*-cyclopentane-1,2-diol, and hexane-1,2-diol were 1.7, 2.8, 14.2, 13.7, and 13.5 mM, respectively (Table 4). The CDH II was more specific to cyclohexanol than *trans*-cyclohexane-1,2-diol, although the maximum velocity of CDH II for cyclohexanol was lower than that for *trans*-cyclohexane-1,2-diol.

The apparent K_m values of CDH II for cyclopentanol, *trans*-cyclopentane-1,2-diol, and hexane-1,2-diol were about eight-fold higher than that for cyclohexanol. The relative activities of CDH II for cyclopentanol, *trans*-cyclopentane-1,2-diol, and hexane-1,2-diol in Table 3 were found to be lower than those of their maximum velocities in Table 4, since the enzyme in Table 3 was not able to catalyze at the maximum velocity, due to low concentration of substrates (14.8 mM).

N-Terminal Amino Acid Sequence

The amino-terminus of the purified CDH II was determined to be TVAHVTGAARGIGRA by automated Edman degradation [7]. As shown in Fig. 3, the amino-terminus of the CDH II of *Rhodococcus* sp. TK6 is aligned with the putative cyclohexanol dehydrogenase of *Acinetobacter* sp. strain SE19 [2], *cis*-1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase of *Pseudomonas putida* [11], and carveol dehydrogenase of *Rhodococcus erythropolis* [20]. The CDH II sequence was similar to part of the N-terminal amino acid sequence of the putative cyclohexanol dehydrogenase, *cis*-1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase, and carveol dehydrogenase.

CDH II	T	V	A	H	V	T	G	A	A	R	G	I	G	R	A
ChnA	K	V	A	L	I	T	G	A	G	S	G	I	G	K	S
XYLL	K	V	A	V	I	T	G	A	A	Q	G	I	G	R	R
LIMC	Q	V	A	L	I	T	G	A	A	R	G	Q	G	R	S

Fig. 3. Comparison of N-terminal amino acid sequence of CDH II with those of other alcohol dehydrogenases.
The conserved residues are underlined. The residues strictly conserved for short chain alcohol dehydrogenase (SCAD) are in a solid box, whereas the residues commonly conserved for SCAD are in a broken box. CDH II, cyclohexanol dehydrogenase of *Rhodococcus* sp. TK6 in current study; ChnA, putative cyclohexanol dehydrogenase of *Acinetobacter* sp. strain SE19; XYLL, *cis*-1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase of *Pseudomonas putida*; LIMC, carveol dehydrogenase of *Rhodococcus erythropolis*.

Conserved residues (TGXXXGXG) for short chain alcohol dehydrogenases (SCADs) [15] were found in the N-terminal sequence of the CDH II. Five or more amino acid residues among fifteen residues in the N-terminal sequence of the CDH II were different from the other dehydrogenases. Therefore, based on the results of the N-terminal sequence analysis, the CDH II specific to cyclohexanol was identified as a new enzyme.

DISCUSSION

Rhodococcus sp. TK6, which is capable of mineralizing cyclohexanol, was isolated from sewage sludge from the Ulsan Industrial Complex for Petrochemicals, Korea [8] and was found to be very similar to *Rhodococcus* YH1 str. YH1 (GenBank database, accession no. AF103733), because of more than 98% similarity in DNA sequence analysis of the 16S rRNA gene. *Rhodococcus* sp. TK6 is also able to utilize primary and secondary alcohols with short chain (C_2 to C_6) as the sole source of carbon and energy, however, not tertiary alcohols such as 2-methyl-2-propanol and 2-methyl-2-butanol [8]. The current authors previously reported that *Rhodococcus* sp. TK6 can produce NAD^+-dependent cyclohexanol dehydrogenase and cyclohexane-1,2-diol dehydrogenase, including several alcohol dehydrogenases specific to short chain alcohols, ranging from 2 to 6 carbon atoms in chain length [9]. It was found that NAD^+-dependent cyclohexanol dehydrogenase (CDH II) and cyclohexane-1,2-diol dehydrogenase (CDH III) were induced by cyclohexanol and cyclohexane-1,2-diol, respectively [9]. Neither enzyme was induced by 1-pentanol.

The NAD^+-dependent CDH II of *Rhodococcus* sp. TK6 was found to be very unstable in a cell-free extract at 4°C, whereas cyclohexanediol dehydrogenase is thermostable (unpublished data). Since the CDH II retained only 20% of its activity after incubation for 20 min at 50°C, it was very difficult to keep its activity during the purification procedure.

The CDH II activity in *Rhodococcus* sp. TK6 was predominantly localized in the soluble fraction of cyclohexanol-

grown cells. The NAD^+-dependent alcohol dehydrogenase of *Acinetobacter* sp. strain M-1 was also found in the soluble fraction of the cells and was unstable during purification [17]. *Acinetobacter* TD63, which can utilize *trans*-cyclohexane-1,2-diol as the sole source of carbon, produces *trans*-cyclohexane-1,2-diol dehydrogenase that is responsible for the conversion of *trans*-cyclohexane-1,2-diol to 2-hydroxyl cyclohexane-1-one in the presence of NAD^+ [5]. Although *Acinetobacter* TD63 was unable to grow with cyclohexanol, *Rhodococcus* sp. TK6 was able to utilize cyclohexanol and *trans*-cyclohexane-1,2-diol, indicated by the growth spectrum of *Nocardia globerula* CL1 [12] and *Acinetobacter* NCIB9871 [6].

The relative molecular mass of the purified enzyme was estimated to be 60 kDa by gel filtration and 28 kDa by SDS-PAGE. The N-terminal amino acid sequence of TVAHVTGAARGIGRA was determined by Edman degradation. Judging from the results, the native CDH II would appear to be composed of two identical subunits. The NAD^+-dependent 4-hydroxycyclohexane carboxylate dehydrogenase of *Corynebacterium cyclohexanicum* grown on cyclohexane carboxylic acid as the sole carbon source also consists of two identical subunits of 27.6 kDa and has a relative molecular mass of 53.6 kDa [13]. The molecular mass of the subunit of the purified enzyme in *Rhodococcus* sp. TK6 was similar to that of the CDH deduced from the cloned gene in *Acinetobacter* sp. strain SE19, which is composed of 251 amino acid residues, but the properties of the enzyme protein was not characterized [2]. The molecular mass of the CDH in the *Nocardia* species is 145 kDa [16], which is different from the purified enzyme of *Rhodococcus* sp. TK6.

The relative activity of the purified enzyme toward *trans*-cyclohexane-1,2-diol and *trans*-cyclopentane-1,2-diol was higher than that of cyclohexanol. Also, the apparent V_{max} values for *trans*-cyclohexane-1,2-diol and *trans*-cyclopentane-1,2-diol were slightly higher than that for cyclohexanol. Still, the purified enzyme would appear to be cyclohexanol dehydrogenase rather than cyclohexane-1,2-diol dehydrogenase for the following reasons: (i) The affinity and catalytic efficiency of the purified enzyme against cyclohexanol, based on the K_m and V_{max} values, were the most favorable among substrates such as *trans*-cyclohexane-1,2-diol, *trans*-cyclopentane-1,2-diol, cyclopentanol, and hexane-1,2-diol. (ii) *trans*-Cyclohexane-1,2-diol was effectively oxidized by CDH II, since it contains two alcohol groups within one molecule. On the other hand, the *cis* type of cyclohexane-1,2-diol and cyclopentane-1,2-diol are unfavorable substrates for the purified CDH II. Hexane-1,2-diol seemed to be a good substrate for the purified CDH II, whereas 1-hexanol was very hard to oxidize, because it includes an alcohol group within one molecule. (iii) The purified enzyme was predominantly induced by cyclohexanol, rather than *trans*-cyclohexane-1,2-diol.

Rhodococcus sp. TK6 produced other CDHs which are specific to cyclohexane-1,2-diol and induced by cyclohexane-1,2-diol [9]. The CDH activity of *Rhodococcus* sp. TK6 grown in cyclohexane-1,2-diol as the growth substrate had less than 70% of the activity of the cells grown in cyclohexanol, which was the most favorable substrate among the cycloalkanols for the growth of *Rhodococcus* sp. TK6 [8]. Therefore, these results suggested that the preferred substrate for the purified enzyme from *Rhodococcus* sp. TK6 was cyclohexanol.

The CDH of *Xanthobacter* sp. grown on cyclohexane media can oxidize both cyclohexanol and cyclohexanediols [18]. The apparent K_m for cyclohexanol (1.3 μM) was two orders of magnitude lower than that for each cyclohexanediol (410 to 590 μM). The apparent K_m of the CDH II of *Rhodococcus* sp. TK6 for cyclohexanol was similar to that for *trans*-cyclohexane-1,2-diol. The K_m values of the 4-hydroxycyclohexane carboxylate dehydrogenase in *C. cyclohexanicum* for *trans*-4-hydroxycyclohexane carboxylic acid and NAD^+ in the oxidation reaction at pH 8.8 were previously established as 0.51 mM and 0.23 mM, respectively [13].

The N-terminal amino acid sequence of the purified CDH II was quite distinct from that of the putative CDH genes from *Acinetobacter* sp. strain SE19 [2], which is Gram negative. This is the first report on the purification of cyclohexanol dehydrogenase to homogeneity and identification of the CDH II of *Rhodococcus* sp. TK6 as a new enzyme, based on the N-terminal amino acid sequence. A detailed investigation of the CDH II properties was not possible due to the unstable nature of the purified enzyme. Accordingly, in the future, a gene for CDH II will be cloned and overexpressed in *Escherichia coli*, so as to investigate the detailed properties of CDH II.

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