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Characterization of Two Isozymes of Coniferyl Alcohol Dehydrogenase from *Streptomyces* sp. NL15-2K

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We purified two isozymes of coniferyl alcohol dehydrogenase (CADH I and II) to homogeneity from cell-free extracts of *Streptomyces* sp. NL15-2K. The apparent molecular masses of CADH I and II were determined to be 143 kDa and 151 kDa respectively by gel filtration, whereas their subunit molecular masses were determined to be 35,782.2 Da and 37,597.7 Da respectively by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Thus, it is probable that both isozymes are tetramers. The optimum pH and temperature for coniferyl alcohol dehydrogenase activity were pH 9.5 and 45 °C for CADH I and pH 8.5 and 40 °C for CADH II. CADH I oxidized various aromatic alcohols and allyl alcohol, and was most efficient on cinnamyl alcohol, whereas CADH II exhibited high substrate specificity for coniferyl alcohol, and showed no activity as to the other alcohols, except for cinnamyl alcohol and 3-(4-hydroxy-3-methoxyphenyl)-1-propanol. In the presence of NADH, CADH I and II reduced cinnamaldehyde and coniferyl aldehyde respectively to the corresponding alcohols.

Key words: coniferyl alcohol dehydrogenase; cinnamyl alcohol dehydrogenase; substrate specificity; *Streptomyces* sp.

Lignins are cross-linked phenylpropanoid polymers. They are the most abundant aromatic compounds in nature. Partial decay of lignins produces numerous aromatic monomers that have many applications in the cosmetics, food, pharmaceutical, and chemical industries. Hence lignins have attracted considerable attention as natural resources for the production of chemicals traditionally derived from petroleum.

In nature, lignins are assumed to be depolymerized initially by white rot fungi, followed by mineralization of the subsequent breakdown products by soil bacteria. Bacterial degradation pathways for lignin-related aromatic compounds have been characterized extensively for gram-negative bacteria, particularly *Pseudomonas* spp.[1–3] and *Sphingomonas paucimobilis* SYK-6.[4] Most of these compounds are degraded to protocatechuic acid or catechol, and further broken down *via* specific ring cleavage pathways. For example, in *Pseudomonas* sp. HR199, eugenol is degraded by side chain shortening to protocatechuic acid *via* coniferyl alcohol, coniferyl aldehyde, ferulic acid, vanillin, and vanillic acid,[1] and protocatechuic acid is further degraded by an *ortho*-cleavage pathway.[2] These sequential degradation pathways are found in a variety of bacterial species, including *Acinetobacter*,[5] *Corynebacterium*,[6] and *Streptomyces*.[7,8]

Streptomycetes are gram-positive bacteria abundant in soil. They are known to play important roles in biotransformation and biodegradation in nature, but the metabolic pathways involved, with the exception of those for the biosynthesis of bioactive secondary metabolites, have not been well studied. Studies done on streptomycetes include several reports on the degradation of lignin-related aromatic compounds.[7–10] Sutherland *et al.* described the processes by which *S. setonii* catabolizes cinnamic acid, *p*-coumaric acid, and ferulic acid.[7] Bioconversion of vanillin to vanillic acid and the catabolism of vanillic acid *via* guaiacol and catechol were identified in *S. viridosporus*[9] and *S. setonii*[10] respectively. In addition, we have isolated *Streptomyces* sp. NL15-2K from forest soil. We observed that it can demethylate veratric acid to vanillic acid.[8] Liquid chromatography-mass spectrometry (LC-MS) analysis indicated that NL15-2K has an extensive catabolic network for lignin-related aromatic compounds (Fig. 1).[8]

Although it is known that streptomycetes catabolize a variety of lignin-related aromatic compounds, only a few studies have attempted to characterize the catabolic pathways in question enzymatically or genetically.[11–13] Our project goal is to identify and elucidate the characteristics of the enzymes and genes responsible for the degradation of lignin-related aromatic compounds in *Streptomyces* sp. NL15-2K.

Coniferyl alcohol is the principal precursor of lignin biosynthesis in plants, and the sequential degradation of

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Abbreviations: ADH, alcohol dehydrogenase; CAD, cinnamyl alcohol dehydrogenase; CADH, coniferyl alcohol dehydrogenase; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; MALDI-TOF-MS, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride

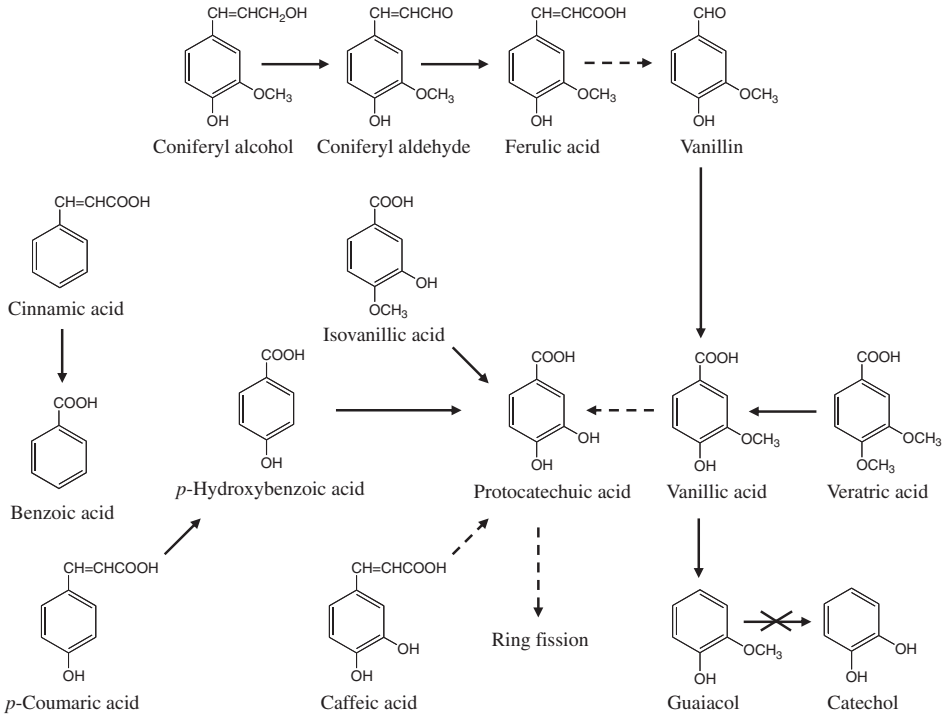

Fig. 1. Degradation Pathways for Lignin-Related Aromatic Compounds by *Streptomyces* sp. NL15-2K. Dotted arrows indicate unidentified steps.

lignin-related aromatic compounds in NL15-2K is initiated by the oxidation of coniferyl alcohol to coniferyl aldehyde, which is catalyzed by coniferyl alcohol dehydrogenase (EC 1.1.1.194). Three CADH enzymes have been found in bacteria[14,15] and plants,[16] but there is little information on their properties. In a preliminary study, we detected CADH activity in a cell-free extract of NL15-2K. Here we describe the purification and characterization of two isozymes of CADH isolated from *Streptomyces* sp. NL15-2K.

Materials and Methods

Materials and reagents. 3-(4-Hydroxy-3-methoxyphenyl)-1-propanol was purchased from Tokyo Chemical Industry (Tokyo). Coniferyl alcohol, cinnamyl alcohol, 3-phenyl-1-propanol, benzyl alcohol, allyl alcohol, and the other aromatic alcohols used were from Wako Pure Chemical Industries (Osaka, Japan). Coniferyl aldehyde and cinnamaldehyde were purchased from Sigma-Aldrich (St. Louis, MO) and Wako respectively. $NAD(P)^+$ and NAD(P)H were from Oriental Yeast (Osaka, Japan).

Microorganism culture conditions. Streptomyces sp. NL15-2K, isolated from forest soil,[8] was used throughout the study. The spores were grown on inorganic salts-starch agar (ISP4; Difco Laboratories, Detroit, MI), and then inoculated into and cultured in 100 mL of YEME medium (1% glucose, 0.5% polypeptone, 0.3% yeast extract, 0.3% malt extract, and 0.04% $MgCl_2 \cdot 6H_2O$, pH 7.0) at 30 °C. After 48 h in liquid culture, 10% of the cultured mycelia was transferred to 100 mL of fresh YEME medium and incubated for an appropriate time at 30 °C with reciprocal shaking.

Assay for CADH activity. CADH activity was determined by measuring the amount of coniferyl aldehyde formed from coniferyl alcohol as substrate. The reaction mixture (0.3 mL) containing 0.1 M Tris–HCl (pH 8.0), 1.5 mM coniferyl alcohol, 2.5 mM NAD^+, and variable amounts of enzyme, was incubated at 30 °C for 10 min. The formation of coniferyl aldehyde was monitored spectrophotometrically at 400 nm. One unit was defined as the amount of the enzyme that led to the formation of 1 µmol of coniferyl aldehyde per min under the assay conditions. The molar absorption coefficients at 400 nm, used in coniferyl aldehyde determination, were as follows: 13.9 $mM^{-1} \cdot cm^{-1}$ at pH 8.0, 20.7 $mM^{-1} \cdot cm^{-1}$ at pH 8.5, and 24.4 $mM^{-1} \cdot cm^{-1}$ at pH 9.5. Specific activity was expressed as units per mg of protein, where the protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) with bovine serum albumin as standard. The rates of oxidation of the other aromatic alcohols and allyl alcohol were determined by measuring the rate of production of NADH at 340 nm ($\varepsilon = 6.3\ mM^{-1} \cdot cm^{-1}$), and the rate of cinnamaldehyde formation was calculated as described previously,[17] using molar absorption coefficients at 340 nm of NADH and cinnamaldehyde (0.48 $mM^{-1} \cdot cm^{-1}$ at pH 8.5 and 0.34 $mM^{-1} \cdot cm^{-1}$ at pH 9.5). To determine the kinetic parameters of the purified isozymes, the initial velocities at various substrate concentrations were determined by the assay procedures described above, except that 0.1 M Tris–HCl buffer (pH 8.0) in the reaction mixture was replaced with 0.1 M glycine-NaOH buffer (pH 9.5) for CADH I and 0.1 M Tris–HCl buffer (pH 8.5) for CADH II. The K_m and k_{cat} values were calculated from a Hanes-Woolf plot.[18] The rates of reduction of coniferyl aldehyde and cinnamaldehyde to the corresponding alcohols were determined by measuring the rates of decrease in the absorbance at 400 nm of coniferyl aldehyde and at 340 nm of NADH and cinnamaldehyde, respectively.

Purification of CADH isozymes. All steps of purification were carried out at 4 °C. Sodium phosphate buffer (25 mM, pH 7.0) containing 1 mM dithiothreitol (DTT) was used as standard buffer in the enzyme purification procedures, unless otherwise stated.

(i) Preparation of cell-free extract: Cells (46 g) harvested from 2 L of culture were washed once with buffer I (standard buffer with 1 mM EDTA and 1 M KCl), and then twice with buffer II (standard buffer with 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride). The washed cells were resuspended in 120 mL of buffer II and disrupted using a sonicator (UD-201; TOMY, Tokyo). Cell debris was removed by centrifugation at 20,000 × *g* for 30 min, and the supernatant was collected as a cell-free extract.

(ii) DEAE-Sepharose column chromatography: The cell-free extract was applied to a column (2.1 × 20 cm) of DEAE-Sepharose FF (GE Healthcare Japan, Tokyo) equilibrated with standard buffer. The column was washed thoroughly with the same buffer, and the proteins were eluted with a linear gradient of 0 to 1 M NaCl. The active fractions were pooled and dialyzed against standard buffer containing 0.7 M

ammonium sulfate for CADH I and 1 M ammonium sulfate for CADH II. The subsequent purification steps for the CADH isozymes were performed separately with a fast protein liquid chromatography system (AKTApurifier 10; GE Healthcare).

(iii) Purification of CADH I: The dialysate was applied to a RESOURCE PHE column (6 mL; GE Healthcare) equilibrated with standard buffer containing 0.7 M ammonium sulfate. The column was washed with 3 column volumes of the same buffer, and CADH I was eluted with a 16-column-volume linear gradient of 0.7 to 0.1 M ammonium sulfate at a flow rate of 1.0 mL/min. The active fractions were pooled and concentrated by precipitation with 75% ammonium sulfate, followed by centrifugation at 20,000 × *g* for 20 min. The resulting pellet was resuspended and dialyzed against standard buffer containing 20% glycerol. The dialysate was applied to a Mono Q 4.6/100 PE column (1.7 mL; GE Healthcare) equilibrated and washed with 3 column volumes of the same buffer. CADH I was eluted with a 20-column-volume linear gradient of 0 to 0.6 M NaCl at a flow rate of 0.6 mL/min. The active fractions were pooled and stored at 4 °C until use.

(iv) Purification of CADH II: The dialysate was applied to a RESOURCE PHE column (6 mL) equilibrated with standard buffer containing 1 M ammonium sulfate, and washed with 3 column volumes of the same buffer. After washing, CADH II was eluted with a 20-column-volume linear gradient of 1 to 0.4 M ammonium sulfate at a flow rate of 1.0 mL/min. The active fractions were pooled and concentrated by precipitation with 75% ammonium sulfate. The precipitate was recovered by centrifugation and dissolved in a small volume of standard buffer, and the resulting solution was passed through a HiLoad 16/60 Superdex 200 pg column (120 mL; GE Healthcare) equilibrated with buffer containing 0.2 M NaCl at a flow rate of 0.5 mL/min. The active fractions were pooled and supplemented with glycerol to 20%. The enzyme solution was then applied to a Mono Q 4.6/100 PE column equilibrated with buffer containing 20% glycerol and 0.2 M NaCl. The column was washed with 3 column volumes of the same buffer, and the enzyme was eluted with a 20-column-volume linear gradient of 0.2 to 0.4 M NaCl at a flow rate of 0.6 mL/min. The active fractions were pooled and stored at −20 °C until use.

HPLC analysis. Oxidation of coniferyl alcohol to coniferyl aldehyde by CADH isozymes was checked by high-performance liquid chromatography (HPLC). After incubation for assay, the reaction mixture was diluted 20-fold with water, and a 5-μL portion of the dilute solution was subjected to HPLC using a Shim-pack XR-ODS column (3.0 × 100 mm; Shimadzu, Kyoto, Japan) at a flow rate of 0.40 mL/min at 45 °C. Elution was done with 30% methanol containing 0.1% phosphoric acid, and was monitored by the absorption at 220 nm and 400 nm. Compounds in the reaction mixture were identified by comparison of the retention times (3.70 min for coniferyl alcohol and 5.56 min for coniferyl aldehyde) with authentic standards and comparison of the chromatograms at 220 nm and 400 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was performed on pre-cast 10% polyacrylamide slab gels (e-PAGEL; Atto, Tokyo) under reducing conditions, and the proteins were stained with Coomassie Brilliant Blue G-250 (Bio-Safe Coomassie Stain; Bio-Rad). The molecular masses of the proteins observed were estimated using a molecular size marker (EzStandard; Atto).

Molecular mass determination. The molecular masses and quaternary structures of the CADH isozymes were determined by gel filtration, SDS–PAGE, and MALDI-TOF-MS. The molecular masses of the native isozymes were estimated by gel filtration on a HiLoad 16/60 Superdex 200 pg column equilibrated with standard buffer containing 0.2 M NaCl at a flow rate of 0.5 mL/min. A gel filtration calibration kit (GE Healthcare) for high-molecular-weight proteins, containing catalase (232 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (44 kDa), was used to provide approximate protein size markers. The MALDI-TOF mass spectra of the intact isozymes were acquired on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Each CADH solution was dialyzed against water and mixed with the matrix solution (1:4 v/v), a saturated solution of sinapinic acid (Bruker Daltonics) in 30% acetonitrile with 0.1% trifluoroacetic acid. The mixture (1 μL) was deposited onto a dried thin layer of the matrix on a MTP 384 target ground steel TF plate (Bruker Daltonics) and allowed to dry at room temperature. The measurements were performed in linear positive-ion mode with a nitrogen laser under the control of FLEXControl software (version 2.4; Bruker Daltonics). Spectra were obtained by accumulating up to 1,500 laser shots acquired at the minimum laser power necessary to ionize the samples. The instrument was calibrated using Protein Calibration Standard II (Bruker Daltonics).

N-Terminal amino acid sequencing. After SDS–PAGE, the proteins in the gel were electroblotted onto a pre-wetted polyvinylidene difluoride (PVDF) membrane (Sequi-Blot; Bio-Rad) using a Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad) and stained with Coomassie Brilliant Blue R-250. Protein bands of 37 kDa for CADH I and 39 kDa for CADH II were excised from the membrane and subjected to protein sequencing. The N-terminal amino acid sequences of the CADH isozymes were determined by the automated Edman method with a model 476A protein sequencer (Applied Biosystems, Foster City, CA).

Effects of pH and temperature on enzyme activity and stability. The effect of pH on CADH activity was examined by the standard assay method described above, except that the 0.1 M Tris–HCl buffer (pH 8.0) in the reaction mixture was replaced with 0.1 M sodium acetate buffer (pH 4.0–5.5), 0.1 M sodium phosphate buffer (pH 5.5–7.5), 0.1 M Tris–HCl buffer (pH 7.5–9.0), or 0.1 M glycine-NaOH buffer (pH 9.0–11.0). The pH stabilities of CADH isozymes were assessed by measuring residual activities after the enzymes were pre-incubated in the above buffers at 4 °C for 75 h. To investigate the effect of temperature on CADH activity, reactions were carried out at temperatures ranging from 25 °C to 55 °C for 10 min. The thermal stabilities of the CADH isozymes were assessed by measuring residual activities after the enzymes were pre-incubated in 25 mM sodium phosphate buffer (pH 7.0) at temperatures from 25 °C to 60 °C for 10 min.

Inhibition studies. The effects of metal ions and chemical reagents on CADH activity were determined by pre-incubating the enzyme at 37 °C for 15 min in 25 mM sodium phosphate buffer (pH 7.0) containing 1 mM of the various metal ions and reagents. Residual activities after pre-incubation were assayed by the standard assay method.

Results

Purification and properties of CADH I and II

Two isozymes of coniferyl alcohol dehydrogenase (CADH I and II) were separated by DEAE-Sepharose ion exchange chromatography. CADH I and II were eluted at NaCl concentrations of 0.25 M and 0.44 M respectively (Fig. 2). Both isozymes were confirmed to catalyze the oxidation of coniferyl alcohol to coniferyl aldehyde by HPLC analysis. The active fractions from DEAE-Sepharose column chromatography were purified independently. CADH I was purified by hydrophobic interaction chromatography on RESOURCE PHE and ion exchange chromatography on Mono Q. CADH II was purified by RESOURCE PHE, followed by gel filtration on Superdex 200 pg, and then by Mono Q. The results of the various courses of enzyme purification are summarized in Table 1. CADH I and II were purified up to 71-fold and 902-fold, with yields of 9% and 8% respectively. Approximately 1.6 mg of CADH I and 0.12 mg of CADH II were obtained per liter of culture. In a series of purification steps, hydrophobic chromatography on RESOURCE PHE resulted in high purification of both isozymes. The final preparations of CADH I and II gave single protein bands on SDS–

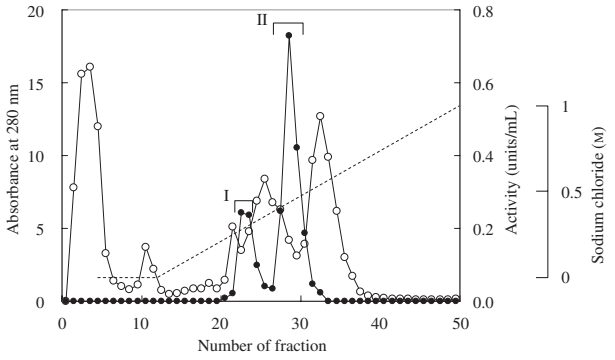

Fig. 2. Separation of CADH Isozymes by DEAE-Sepharose Column Chromatography.

Cell-free extract from *Streptomyces* sp. NL15-2K was applied to a DEAE-Sepharose column. Peaks I and II were collected as CADH I and CADH II respectively, and were further purified separately. ○, absorbance at 280 nm; ●, CADH activity; - - - -, NaCl concentration.

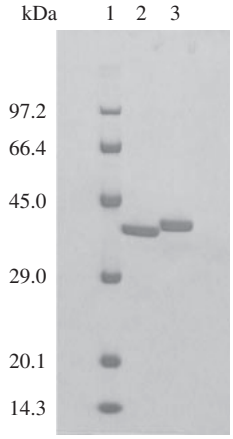

Fig. 3. SDS–PAGE Analysis of Purified CADH Isozymes.

The enzyme (6 μg) from the final purification step was denatured, reduced, and then subjected to SDS–PAGE. Lane 1, standard molecular mass markers (sizes indicated); lane 2, CADH I; lane 3, CADH II.

Table 1. Purification of CADH Isozymes from *Streptomyces* sp. NL15-2K

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Cell-free extract	56.2	2400	0.023	100	1
CADH I					
DEAE-Sepharose	20.8	378	0.055	37	2
RESOURCE PHE	12.9	8.5	1.5	23	66
Mono Q	5.2	3.2	1.6	9	71
CADH II					
DEAE-Sepharose	26.0	166	0.16	46	7
RESOURCE PHE	12.6	2.2	5.7	22	249
Superdex 200	8.9	0.49	18.2	16	792
Mono Q	4.8	0.23	20.8	8	902

PAGE, with molecular masses of 37 kDa and 39 kDa respectively (Fig. 3). The apparent molecular masses of the native isozymes as measured by gel filtration were 143 kDa for CADH I and 151 kDa for CADH II (data not shown), indicating that both isozymes are tetramers, with four identical subunits. For accurate determination of the molecular masses of the CADH isozymes, the purified CADH proteins were analyzed by MALDI-TOF-MS in linear positive-ion mode. The MALDI-TOF mass spectrum of CADH I showed a major peak at m/z 35,782.197, whereas that of CADH II showed a major peak at m/z 37,597.729 (Fig. 4). These molecular mass values are roughly equal to those estimated by SDS–PAGE, indicating that the two major peaks represented a single subunit of the various CADH isozymes. A minor peak at m/z 71,565.673 on the mass spectrum of CADH I was assumed to be a molecular ion of the dimer form, containing two subunits of 35,782.197 Da.

The 37- and 39-kDa proteins were blotted onto PVDF membranes and subjected to N-terminal protein sequencing. Seventeen and 18 amino acids were obtained from the N-terminal regions of CADH I and II respectively. The N-terminal amino acid sequence of CADH I was MKAAVVRAFGEPLVIEE. A BLAST search revealed that the N-terminal sequence of CADH I was identical to those of alcohol dehydrogenases (ADHs) from *S. scabiei* 87.22 (RefSeq accession no. YP_003486809), *S. sviceus* ATCC29083 (RefSeq accession no. ZP_05021059), and *Gemmatimonas aurantiaca* T-27 (RefSeq accession no. YP_002762200), which have molecular masses of 35,428, 35,311, and 35,631 Da respectively. The N-terminal amino acid sequence of CADH II was AQEVRGVIAPGKDEPVRM. A homology search revealed that the N-terminal sequence of CADH II showed 94% similarity to those of four oxidoreductases, from *S. ambofaciens* ATCC 23877 (Swiss-Prot accession no. A0ADE4), *S. coelicolor* A3(2) (RefSeq accession no. NP_625045), *S. ghanaensis* ATCC 14672 (RefSeq accession no. ZP_04684033), and *S. sviceus* ATCC 29083 (RefSeq accession no. ZP_05022334), a mycothiol-dependent formaldehyde dehydrogenase from *S. lividans* TK24 (RefSeq accession no. ZP_06532967), and an ADH from *S. coelicolor* A3(2) (Swiss-Prot accession no. Q9ZNB1), the molecular masses of which ranged from 37,616 to 37,868 Da. The calculated molecular masses of the CADH homologs were very close to those of the subunits of CADH I (35,782 Da) and CADH II (37,598 Da), suggesting that the amino acid sequences of the CADHs subunits are very similar to those of their homologs. The enzymatic properties of the homologs have not been described to date.

Effects of pH and temperature

The influence of pH on CADH activity and stability was examined over a pH range of 4.0–11.0. The

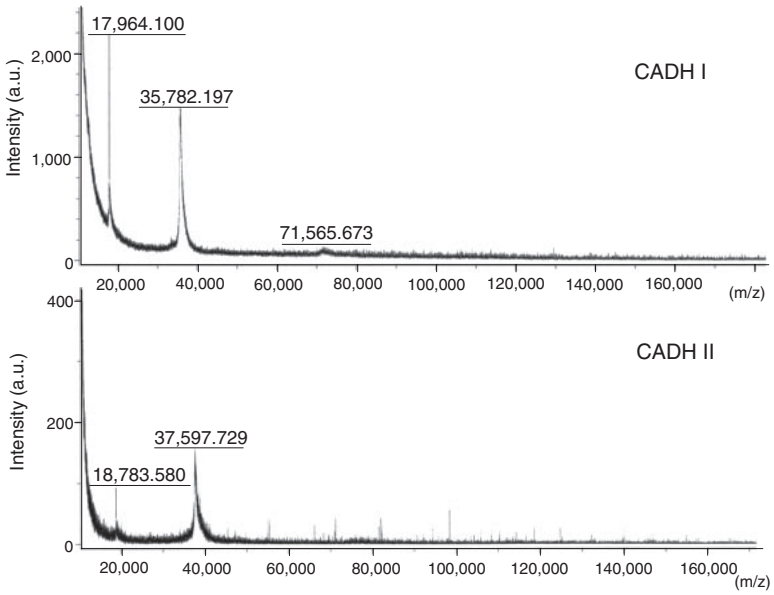

Fig. 4. MALDI-TOF-MS Analysis of CADH I and II.

The purified enzymes were subjected to MALDI-TOF-MS analysis. The peaks at 17,964.100 for CADH I and 18,783.580 for CADH II correspond to the double-charged molecular ions of their subunit proteins.

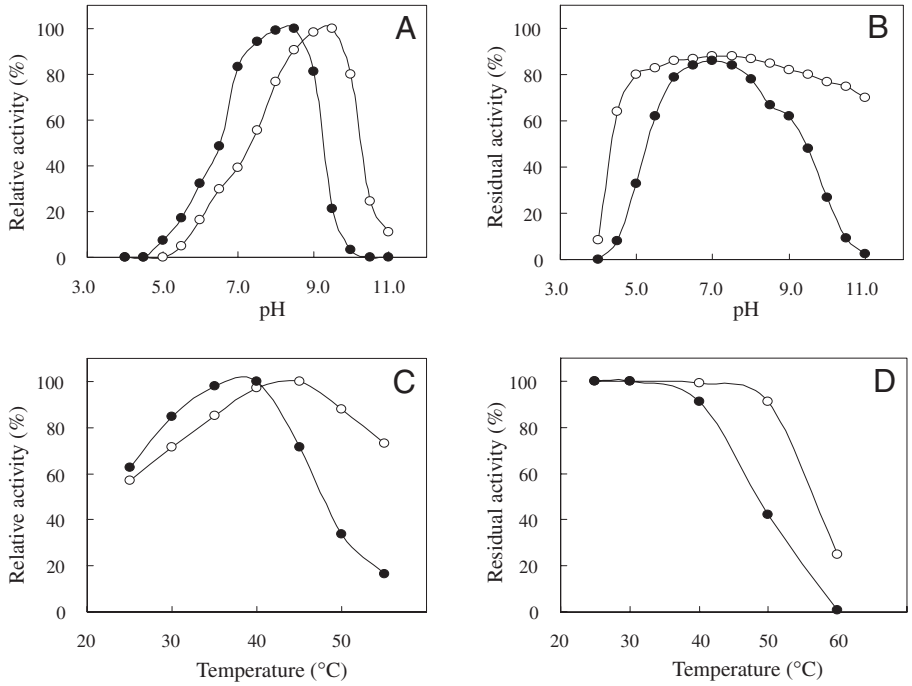

Fig. 5. Effects of pH and Temperature on CADH Activity and Stability.

A, Optimum pH. CADH activities were measured in 0.1 M buffer solutions at various pH values under otherwise standard assay conditions. The buffer systems used were sodium acetate buffer (pH 4.0–5.5), sodium phosphate buffer (pH 5.5–7.5), Tris–HCl buffer (pH 7.5–9.0), and glycine-NaOH buffer (pH 9.0–11.0). After incubation for assay, the pH was adjusted to 8.0 with 1 M Tris–HCl buffer (pH 8.0), and the absorbance at 400 nm was determined. The maximum activities, obtained at pH 9.5 for CADH I and at pH 8.5 for CADH II, were defined as 100%. B, pH stability. The enzyme solution was stored at 4 °C for 75 h in 25 mM buffer solutions at various pH values, and residual activities were measured by the standard assay method. The activity at the beginning was taken to be 100%. C, Optimum temperature. CADH activities were measured at various temperatures under otherwise standard assay conditions. The maximum activities, obtained at 45 °C for CADH I and at 40 °C for CADH II, were defined as 100%. D, Thermal stability. The enzyme solution was pre-incubated at various temperatures for 10 min in 25 mM sodium phosphate buffer (pH 7.0), and residual activities were measured by the standard assay method. The activity without pre-incubation was taken to be 100%. ○, CADH I; ●, CADH II.

optimum pHs for CADH I and II activities were 9.5 and 8.5 respectively (Fig. 5A). pH stability was studied by storing the enzymes at the indicated pH at 4 °C and monitoring changes in enzyme activity every 25 h for 75 h. Figure 5B shows the residual activities after 75 h. CADH I was stable over a broad pH range of 5.0–9.5, retaining over 80% of its activity, whereas CADH II was stable in only a narrow pH range of 6.5–7.5 (Fig. 5B). The optimum temperatures for CADH I and II activities were 45 °C and 40 °C respectively (Fig. 5C). Thermal stability was studied by measuring residual enzyme activity after pre-incubation at various temperatures for

Table 2. Effects of Metal Ions on CADH Activity

Metal ions (1.0 mM)	Residual activity (%)	
	CADH I	CADH II
None	100	100
$MgCl_2$	100	98
$CaCl_2$	93	90
$MnCl_2$	99	67
$FeCl_2$	94	23
$FeCl_3$	74	30
$ZnCl_2$	80	9
$CuCl_2$	0	0

Table 3. Effects of Chemical Reagents on CADH Activity

Reagents (1.0 mM)	Residual activity (%)	
	CADH I	CADH II
None	100	100
Diisopropyl fluorophosphate	100	100
Dithiothreitol	105	117
Phenylmethylsulfonyl fluoride	90	94
Ethylenediaminetetraacetic acid	98	81
Ethylenediaminetetraacetic acid (10 mM)	40	0
N-Ethylmaleimide	90	19
Monoiodoacetic acid	56	57
N-Bromosuccinimide	6	3

Table 4. Substrate Specificity of CADH Isozymes from *Streptomyces* sp. NL15-2K

Substrate	CADH I			CADH II		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Coniferyl alcohol	21.1	25.9	1.23	0.13	69.7	536
Cinnamyl alcohol	0.17	18.1	106	1.70	116	68.3
3-(4-Hydroxy-3-methoxyphenyl)-1-propanol	20.3	9.13	0.45	74.6	21.5	0.29
3-Phenyl-1-propanol	0.96	11.2	11.6	*n.d.*		
Benzyl alcohol	36.1	37.8	1.05	*n.d.*		
Allyl alcohol	0.50	32.9	66.5	*n.d.*		
NAD^+	0.052	19.9	387	0.40	53.7	134
$NADP^+$	17.2	1.53	0.089	*n.d.*		
Coniferyl aldehyde	—			5.10	354	69.3
Cinnamaldehyde	0.92	14.4	15.6	—		

A dash indicates that values were not determined. *n.d.*, not detectable.

10 min. At pH 7.0, CADH I and II were stable at temperatures of up to 50 °C and 40 °C, but their activities decreased rapidly after incubation above 50 °C and 40 °C respectively (Fig. 5D).

Effects of metal ions and various reagents

We examined the effects of metal ions and other reagents on CADH activity. CADH I was completely inhibited by Cu^{2+} and mildly inhibited by Fe^{3+} and Zn^{2+}, while CADH II was markedly inhibited by Cu^{2+}, Zn^{2+}, Fe^{2+}, and Fe^{3+}, and mildly inhibited by Mn^{2+} (Table 2). The other metal ions did not significantly affect the activity of either CADH. Both enzymes were strongly inhibited by *N*-bromosuccinimide and partially inhibited by monoiodoacetic acid (Table 3). In addition, CADH II was inhibited by *N*-ethylmaleimide. The residual activities of CADH I and II were about 40% and 0% respectively after pre-incubation with 10 mM EDTA.

Substrate specificity

Next we investigated the substrate specificities of CADH I and II for various aromatic alcohols and allyl alcohol. The kinetic parameters are summarized in Table 4. CADH isozymes showed clear differences in substrate preference. CADH I had higher affinity for cinnamyl alcohol ($K_m = 0.17$ mM) and 3-phenyl-1-propanol ($K_m = 0.96$ mM) than for coniferyl alcohol ($K_m = 21.1$ mM) or 3-(4-hydroxy-3-methoxyphenyl)-1-propanol ($K_m = 20.3$ mM). The k_{cat}/K_m (catalytic efficiency) ratios for cinnamyl alcohol and 3-phenyl-1-propanol were approximately 86- and 26-fold higher than those for coniferyl alcohol and 3-(4-hydroxy-3-methoxyphenyl)-1-propanol respectively. Similarly, benzyl alcohol was recognized as a substrate by CADH I, whereas vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol) was not oxidized (data not shown). This suggests that CADH I favors aromatic alcohols without 4-hydroxy-3-methoxy groups. Allyl alcohol was the next-best CADH I substrate after cinnamyl alcohol (3-phenyl allyl alcohol). On the other hand, CADH II exhibited a narrow substrate specificity, acting only on coniferyl alcohol, cinnamyl alcohol, and 3-(4-hydroxy-3-methoxyphenyl)-1-propanol. In contrast to CADH I, CADH II displayed a k_{cat}/K_m ratio for coniferyl alcohol that was 8-fold higher than that for cinnamyl alcohol. Neither phenethyl alcohol (2-phenyl-1-ethanol) nor homovanillyl alcohol (4-hydroxy-3-methoxyphenethyl alcohol) were oxidized by either enzyme (data not shown). Both CADH I and II catalyzed the reverse reaction in the presence of NADH, reducing cinnamaldehyde and coniferyl aldehyde respectively to the corresponding alcohols, but the k_{cat}/K_m ratios of these enzymes were 7–8-fold lower for the aldehydes than for the alcohols (Table 4), suggesting that CADH I- and II-mediated reduction of the aldehydes was less efficient than oxidation of the alcohols. The differences in catalytic efficiencies were due mostly to the lower affinities (increased K_m values) of the enzymes for the substrates.

The cofactor specificities of CADH I and II were investigated with 1.5 mM of cinnamyl alcohol and coniferyl alcohol respectively as substrate. Both NAD^+ and $NADP^+$ supported CADH I activity, but a comparison of the k_{cat}/K_m ratios showed that $NADP^+$ ($0.089\ s^{-1}\cdot mM^{-1}$) was a much less efficient cofactor for CADH I than NAD^+ ($387\ s^{-1}\cdot mM^{-1}$). CADH II was active only in the presence of NAD^+.

Discussion

In this study, we purified two isozymes (CADH I and II) of coniferyl alcohol dehydrogenase from cell-free extracts of *Streptomyces* sp. NL15-2K. Substrate specificity studies with aromatic alcohols and allyl alcohol revealed that CADH I oxidized many of the alcohols tested, whereas CADH II oxidized only coniferyl alcohol, cinnamyl alcohol, and 3-(4-hydroxy-3-methoxyphenyl)-1-propanol. A comparison of K_m values showed that the highest substrate affinities of CADH I and II were for cinnamyl alcohol and coniferyl alcohol respectively. Similarly, the k_{cat}/K_m ratios indicated that cinnamyl alcohol and coniferyl alcohol were the preferred substrates of CADH I and II respectively. These results suggest that CADH I should be considered a cinnamyl alcohol dehydrogenase rather than a coniferyl alcohol dehydrogenase.

Cinnamyl alcohol dehydrogenases (CADs; EC 1.1.1.195) also catalyze the reverse reaction, the reduction of cinnamaldehyde to cinnamyl alcohol. A number of CADs in plants catalyze the reduction of *p*-hydroxycinnamaldehydes to their corresponding alcohols; this is the final step in the biosynthesis of monolignols before their polymerization in the cell walls.[19,20] Outside of the plant kingdom, CAD enzymes have been characterized from three microorganisms, *Helicobacter pylori*,[21] *Mycobacterium bovis* BCG,[22,23] and *Saccharomyces cerevisiae*.[24] These enzymes as well as plant CADs show strong substrate preferences for aldehydes to their corresponding alcohols and high dependence on NADP(H). For example, the k_{cat}/K_m ratios of the microbial CADs for cinnamaldehyde and cinnamyl alcohol respectively are as follows: 5,480 and 126 $s^{-1}\cdot mM^{-1}$ for *H. pylori* CAD; 42 and 0.2 $s^{-1}\cdot mM^{-1}$ for *M. bovis* BCG CAD; and 3,035 and 706 $s^{-1}\cdot mM^{-1}$ for *S. cerevisiae* CAD. In contrast, the k_{cat}/K_m ratios of CADH I for cinnamaldehyde and cinnamyl alcohol were 15.6 and 106 $s^{-1}\cdot mM^{-1}$ respectively. CADH I also showed a marked preference for NAD^+, the k_{cat}/K_m ratio being 4,348-fold higher than for $NADP^+$. These results indicate that CADH I is different from previously described CAD enzymes. In addition, the N-terminal amino acid sequence (17 amino acids) of the CADH I subunit showed identity with those of three ADHs from *Streptomyces* and *Gemmatimonas*, but no significant homology with those of plant and microbial CADs. Hence CADH I was assumed to be an ADH with a high substrate preference for cinnamyl alcohol.

In contrast to CAD enzymes, little is known about the characteristics of coniferyl alcohol dehydrogenases. To date, only three CADH enzymes have been reported, from *Rhodococcus erythropolis*,[14] *Pseudomonas* sp. HR199,[15] and plants,[16] but a comparison of enzymatic properties among these CADHs and CADH II is difficult, because there is little information on the properties of previously reported CADHs. The molecular masses of the native CADHs from *Streptomyces* (CADH II), *Rhodococcus*, and *Pseudomonas* were 151 kDa, 200 kDa, and 54.9 kDa, respectively. The CADH from *Pseudomonas* is a dimer composed of two identical subunits with individual molecular masses of 27 kDa, whereas CADH II is tetrameric. This suggests that the molecular masses and quaternary structures vary among these bacterial enzymes, although the *Rhodococcus* CADH subunit has not been identified. The molecular masses of plant CADHs are unknown, because purified enzymes are not available. CADH II exhibited a very narrow substrate specificity, and oxidized only coniferyl alcohol, cinnamyl alcohol, and 3-(4-hydroxy-3-methoxyphenyl)-1-propanol. *Rhodococcus* CADH demonstrated a similarly limited substrate specificity and was active only on coniferyl alcohol, cinnamyl alcohol, vanillyl alcohol, 4-(4-methoxyphenyl)-1-butanol, and 3-(3,4-dimethoxyphenyl)-1-propanol. Coniferyl alcohol was the best substrate for the enzymes from both *Streptomyces* and *Rhodococcus*, and its K_m values were 0.13 mM for CADH II and 0.65 mM for *Rhodococcus* CADH. Both enzymes were also NAD^+-dependent, and the K_m values of CADH II and *Rhodococcus* CADH for NAD^+ were 0.40 mM and 0.22 mM respectively. In contrast, plant CADH activities were detected only with $NADP^+$ in 86 of the 89 plants species tested.[16] The N-terminal amino acid sequence of *Pseudomonas* CADH was found to be MQLTNKKIVVV. This sequence shows no significant homology with the N-terminal sequence of CADH II. A homology search revealed that the N-terminal amino acid sequence of the CADH II subunit showed 94% similarity to those of four different oxidoreductases, a mycothiol-dependent formaldehyde dehydrogenase, and an ADH. These are all from *Streptomyces* spp. and have not been enzymatically characterized. However, as described in "Results" above, the molecular masses of these homologs were almost equal to that of CADH II subunit. Judging from these results, CADH II might be closely related to these homologs. To evaluate the similarities in amino acid sequence between CADH II and its homologs, it will be necessary to analyze further the amino acid sequence of the CADH II subunit.

CADH I and II were unstable in buffers without DTT and were sensitive to thiol-blocking reagents such as *N*-ethylmaleimide and monoiodoacetic acid, as well as to divalent heavy metal ions Zn^{2+} and Cu^{2+}. This suggests that their active sites contain a sulfhydryl group. In streptomycetes, mycothiol is the major thiol and plays a key role in maintaining a reducing environment in the cells.[25] Therefore, the high similarity in N-terminal amino acid sequence between the CADH II subunit and a mycothiol-dependent formaldehyde dehydrogenase suggests the presence of a sulfhydryl group in the active site of CADH II. CADH I and II were also markedly inhibited by *N*-bromosuccinimide, suggesting that a tryptophan residue is also present in the active sites of these enzymes. In the presence of 10 mM EDTA, the activity of CADH I was reduced to 40% of the baseline, and CADH II was completely inhibited. Therefore, some metal ions are assumed to be involved in the enzymatic activities or structural stabilities of CADH I and II. There are three major classes of microbial alcohol dehydrogenases as categorized by cofactor specificities:[26] (i) $NAD(P)^+$-dependent ADHs, which are further sub-divided into zinc-dependent ADHs, short-chain zinc-independent ADHs, and iron-activated ADHs; (ii) $NAD(P)^+$-independent enzymes, which use pyrroloquinoline quinone, heme, or cofactor F_{420}; and (iii) FAD-dependent

ADHs. Since both CADH I and II showed marked preference for NAD^+, they should be classified as members of the NAD^+-dependent group of ADHs. However, further information on the amino acid sequences and/or sensitivities to Zn^{2+} or Fe^{2+} of CADH I and II are required to assign them to one of the $NAD(P)^+$-dependent ADH sub-groups.

In this study, we obtained two dehydrogenases that showed different substrate specificities for aromatic alcohols and allyl alcohol. These enzymes performed 2-way conversions between coniferyl alcohol or cinnamyl alcohol and the corresponding aldehydes. We are in the process of cloning the genes encoding the CADH I and II from the chromosomal DNA of *Streptomyces* sp. NL15-2K to characterize these enzymes further.

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