

A New NAD⁺-Dependent Opine Dehydrogenase from *Arthrobacter* sp. Strain 1C

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A new NAD⁺-dependent opine dehydrogenase was purified to homogeneity from *Arthrobacter* sp. strain 1C isolated from soil by an enrichment culture technique. The enzyme has a molecular weight of about 70,000 and consists of two identical subunits with molecular weights of about 36,000. The enzyme catalyzed a reversible oxidation-reduction reaction of opine-type secondary amine dicarboxylic acids. In the oxidative deamination reaction, the enzyme was active toward unusual opines, such as *N*-[1-*R*-(carboxyl)ethyl]-*S*-methionine and *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine. In the reductive secondary amine-forming reaction with NADH as a cofactor, the enzyme utilized *L*-amino acids such as *L*-methionine, *L*-isoleucine, *L*-valine, *L*-phenylalanine, *L*-leucine, *L*-alanine, and *L*-threonine as amino donors and α -keto acids such as pyruvate, oxaloacetate, glyoxylate, and α -ketobutyrate as amino acceptors. The product enzymatically synthesized from *L*-phenylalanine and pyruvate in the presence of NADH was identified as *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine.

Opines are unusual compounds found in crown gall tumor tissues induced by infection with *Agrobacterium tumefaciens* (26); they are structurally classified into several groups (6, 26). Two of these groups have a common secondary amine dicarboxylic acid structure, HOOCRHC-NHC HR'COOH. One group can be categorized as *N*-substituted *D*-alanine and includes lysopine, octopine, octopinic acid, and histopine (4, 14), and the other can be categorized as *N*-substituted *D*-glutamic acid and includes nopaline and nopalinic acid (16). Octopine is found in molluscan organisms, together with structurally related alanopine and strombine (10). Octopine dehydrogenase (EC 1.5.1.11) (9, 13, 15, 37), lysopine dehydrogenase (EC 1.5.1.16) (30), alanopine dehydrogenase (EC 1.5.1.17) (11), and nopaline dehydrogenase (synthase) (EC 1.5.1.19) (20), involved in the metabolism of these compounds, have been characterized from crown gall tumor tissues and molluscs. These NAD(P)⁺-dependent opine dehydrogenases appear to share a similar reaction mechanism with the NAD(P)⁺-dependent amino acid dehydrogenases (33, 34). It is peculiar that the NAD(P)⁺-dependent secondary amino acid dehydrogenases form a *D*-configuration from α -keto acid in the reductive amine-forming reaction, since no NAD(P)⁺-dependent amino acid dehydrogenase forms a *D*-amino acid from the keto analog in the reductive amination reaction, with NADP⁺-dependent *meso*- α,ϵ -diaminopimelate *D*-dehydrogenase as an exception (24).

We have recently purified and characterized phenylalanine dehydrogenases in crystalline form or to homogeneity from *Sporosarcina ureae* SCRC-R04, *Bacillus sphaericus* SCRC-R79a (1), and *B.adius* IAM 11059 (2). The *pdh* genes have been cloned into plasmids and expressed in *Escherichia coli*, and the *B. sphaericus pdh* gene has been sequenced (28). A comparison of the primary structure of an opine dehydrogenase with those of the known amino acid dehydrogenases would provide valuable information on the structure and function of this class of enzymes. Furthermore, the reversible nature of the enzyme-catalyzed reaction would enable us to synthesize new chiral opine derivatives, the chemical synthesis of which is rather laborious.

We screened for a novel NAD⁺-dependent dehydrogenase acting on opine-type secondary amine dicarboxylic acids from microbial origins with synthetic *N*-[1-*RS*-(carboxyl)ethyl]-*S*-phenylalanine (Fig. 1) as a substrate and isolated a new enzyme producer from soil samples. Here we report the purification and characterization of a novel opine dehydrogenase from *Arthrobacter* sp. strain 1C (Fig. 2).

MATERIALS AND METHODS

Materials. DEAE-Toyopearl, Butyl-Toyopearl, and high-performance liquid chromatography (HPLC) G-3000 SW and DEAE-5PW columns were purchased from Tosoh Corp., Tokyo, Japan, and an HPLC Crownpack CR (+) column was purchased from Daicel Chemical Industries, Tokyo, Japan. Sephadex G-200 was purchased from Pharmacia, Uppsala, Sweden; alanine dehydrogenase (EC 1.4.1.1; *B. sphaericus*), *D*-amino-acid oxidase (EC 1.4.3.3; hog kidney), *L*-amino-acid oxidase (EC 1.4.3.2; *Bothrops atrox*), saccharopine, *D*-octopine, and *L*-*allo*-octopine were purchased from Sigma Chemical Co., St. Louis, Mo.; and marker proteins for molecular weight determination were purchased from Oriental Yeast, Tokyo, Japan. Membrane filters (Diaflo PM 30) were obtained from Amicon Corp., Lexington, Mass. 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride was purchased from Dojin Chemicals, Kumamoto, Japan, and Coomassie brilliant blue R-250 was purchased from Fluka AG, Buchs, Switzerland. Peroxidase (EC 1.11.1.7; horseradish) was purchased from Toyobo, Osaka, Japan, and hydroxyapatite was purchased from Wako Chemicals, Osaka, Japan. *R*-Methylactate and *S*-methylactate were purchased from Tokyo Kasei, Tokyo, Japan, and Aldrich Chemical Co., Inc., Milwaukee, Wis., respectively. Formate dehydrogenase (EC 1.2.1.2) was purified from *Candida boidinii* (formerly *Kloeckera* sp., no. 2201) by the method of Kato et al. (18). Phenylalanine dehydrogenase (EC 1.4.1.-) was purified to homogeneity from *B. sphaericus* SCRC-R79a (1). 2-Keto-4-phenylbutyric acid was synthesized from bromoethylbenzene and diethyloxalate (38). *L*-Homophenylalanine (*L*-2-amino-4-phenylbutyric acid) was enzymatically synthesized from sodium 2-keto-4-phenylbutyrate, ammonium formate, and NAD⁺ with phenylalanine dehydrogenase and formate dehydrogenase as catalysts (1).

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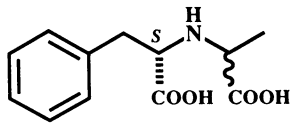


FIG. 1. Structure of *N*-[1-*RS*-(carboxyl)ethyl]-*S*-phenylalanine.

Diazomethane was evolved from *N*-nitrosomethylurea, which was purchased from ICN K & K Laboratories, Inc. All other chemicals were purchased from commercial sources.

Synthesis of opines. *N*-[1-*S*-(Carboxymethyl)-ethyl]*S*-phenylalanine methyl ester, *N*-[1-*R*-(carboxymethyl)ethyl]-*S*-phenylalanine methyl ester, *N*-[1-*S*-(carboxymethyl)ethyl]-*R*-phenylalanine methyl ester, *N*-[1-*R*-(carboxymethyl)ethyl]-*R*-phenylalanine methyl ester, *N*-[1-*S*-(carboxymethyl)ethyl]-*S*-methionine methyl ester, and *N*-[1-*R*-(carboxymethyl)ethyl]-*S*-methionine methyl ester were synthesized by the method of Effenberger and Burkard (8). The dimethyl esters were purified by silica gel column chromatography with an *n*-hexane-ethyl acetate (9:1 [vol/vol]) solvent system to remove the diastereomer by-product. Hydrolysis of the dimethyl esters was carried out in 1 M potassium hydroxide containing 50% tetrahydrofuran at room temperature for 6 h. The reaction mixture was neutralized with 2 M hydrochloric acid and then evaporated. To further remove the contaminating diastereomer, we dissolved the potassium salt of the hydrolysate in water, applied it to a Crownpack CR (+) column (0.40 by 15 cm) in an HPLC system (HLC 803D; Tosoh), eluted it at room temperature with 0.05 M perchloric acid at a flow rate of 0.4 ml/min, and collected the fractions. *N*-[1-*RS*-(Carboxyl)ethyl]-*S*-phenylalanine for culture medium was synthesized from *L*-phenylalanine and sodium pyruvate with sodium cyanoborohydride (21).

Isolation of opine dehydrogenase-producing strains. Microorganisms were isolated from soil samples from the Kanagawa Prefecture, Japan, with a medium containing 5 g of *N*-[1-*RS*-(carboxyl)ethyl]-*S*-phenylalanine as a sole carbon source per liter of the basal medium described previously (3). Several strains belonging to the genus *Arthrobacter* were obtained from the stock cultures of the Institute for Applied Microbiology (IAM), The University of Tokyo, Tokyo, Japan, the Japan Collection of Microorganisms (JCM), Wako, Japan, and the Institute for Fermentation (IFO), Osaka, Japan. The buffer used in this study was potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol unless otherwise stated. The strains were cultured aerobically at 30°C for 48 h in the basal medium described above. The cells were washed once with physiological saline and suspended in 0.1 M buffer. The cells were disrupted for 8 to 15 min with a 20-kHz ultrasonic oscillator (UCD-130; Tosho Denki, Tokyo, Japan). The disrupted cells were centrifuged at 1,500 × *g* for 20 min. The enzyme activity in the supernatant was measured after dialysis against 0.01 M buffer.

Identification of strain 1C. The taxonomic characteristics of strain 1C were as follows. The cells were rods (0.6 by 1.5

μm) occurring singly, nonmotile, non-sporeforming, and gram positive. A typical rod-coccus growth cycle was observed: fresh cultures were composed largely of irregular rods, and older cultures were composed of coccoid cells. When grown on nutrient agar, the colonies were smooth, entire, low convex, round, opaque, and off-white to yellow. Starch hydrolysis was negative. Nitrate reduction and denitrification were negative. Indole formation and hydrogen sulfide formation were negative. Catalase was positive, and oxidase was negative. Citrate utilization (Koser and Christensen) was positive. The Hugh-Leifson reaction was aerobic. Pigment production on King A and B media was negative. Acid without gas was produced from arabinose, sorbitol, mannitol, glycerol, and inulin. No acid or gas was produced from glucose, fructose, xylose, mannose, galactose, maltose, sucrose, lactose, trehalose, starch, raffinose, ribose, sorbose, glycogen, or carboxymethyl cellulose. The cells did not survive heating at 63°C for 30 min in skim milk. The growth temperature ranged between 7 and 41°C. The growth pH ranged between 5.0 and 10.0. Growth in the presence of 7% NaCl was positive. The cell wall peptidoglycan contained lysine as a diamino acid. Mycolic acid was not found. Major fatty acids present were anteiso C_{15:0}, iso C_{15:0}, iso C_{16:0}, and anteiso C_{17:0}.

Enzyme assay. Enzyme activity was routinely assayed by the reduction of NAD⁺ monitored at 340 nm with a Hitachi 228A spectrophotometer, with *N*-[1-*RS*-(carboxyl)ethyl]-*S*-phenylalanine as a substrate. Enzyme activity in the oxidative deamination reaction was measured at 25°C in a reaction mixture (1.0 ml) containing 10 mM *N*-[1-*RS*-(carboxyl)ethyl]-*S*-phenylalanine, 100 mM Na₂CO₃-NaHCO₃ (pH 9.5), 2.5 mM NAD⁺, and the enzyme. A reaction mixture that resulted in a linear change in absorbance for at least 1 min was used in the kinetic study, and the absorbance change for the initial 5 s was used for the calculation. The reaction mixture used for the kinetic study of the oxidative deamination reaction contained 100 mM Na₂CO₃-NaHCO₃ (pH 9.5), various concentrations of opines and NAD⁺, and the enzyme. Enzyme activity in the reductive secondary amine-forming reaction was assayed by the oxidation of NADH in a reaction mixture (1.0 ml) containing 100 mM Tris hydrochloride (pH 8.0), 10 mM α-keto acid, 10 mM amino acid, 0.1 mM NADH, and the enzyme. The reaction mixture (1.0 ml) used for the kinetic study of the reductive secondary amine-forming reaction contained 100 mM Tris hydrochloride (pH 8.0), various concentrations of pyruvate, amino acid or amine, and NADH, and the enzyme. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of NADH per min in the oxidative deamination reaction. Protein was determined by the method of Lowry et al. (23) or by measurement of A₂₈₀.

Purification of opine dehydrogenase from *Arthrobacter* sp. strain 1C. *Arthrobacter* sp. strain 1C was cultivated aerobically at 30°C for 48 h in basal medium (3) containing 0.5% soybean hydrolysate, 0.5% sodium succinate, and 0.2% *N*-[1-*RS*-(carboxyl)ethyl]-*S*-phenylalanine. Cells harvested from 3.3 liters of culture (33.2 g [wet weight]) were washed and suspended in 200 ml of 0.1 M buffer. The cells were disrupted for 20 min with a 9-kHz ultrasonic oscillator (Kubota Syoji, Tokyo, Japan). The disrupted cells were centrifuged at 14,000 × *g* for 20 min. The enzyme solution was fractionated with ammonium sulfate (30 to 60% saturation). The active precipitate was dialyzed against 0.01 M buffer, applied to a DEAE-Toyopearl column (2.6 by 17 cm) equilibrated with 0.01 M buffer, and eluted with 0.1 M buffer containing 0.1 M NaCl. Ammonium sulfate was added (30%

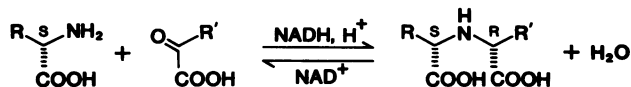


FIG. 2. Reaction catalyzed by a novel opine dehydrogenase from *Arthrobacter* sp. strain 1C.

TABLE 1. Purification of the opine dehydrogenase from *Arthrobacter* sp. strain 1C

Step	Total activity (U)	Total ^a protein (mg)	Sp act (U/mg)
Cell extract	544	1,850	0.294
Ammonium sulfate	579	1,510	0.383
DEAE-Toyopearl	433	135	3.21
Butyl-Toyopearl	349	26.1	13.4
Sephadex G-200	194	11.6	16.7

^a The concentration of the purified enzyme after the DEAE-Toyopearl step was determined from the A_{280} by assuming an extinction coefficient of $A_{1\text{cm}}^{1\%} = 10.0$.

saturation) to the active fractions, which were applied to a Butyl-Toyopearl column (2.6 by 19 cm) equilibrated with 0.01 M buffer containing ammonium sulfate (30% saturation). The active fractions were eluted with a linear gradient of ammonium sulfate (30 to 0% saturation) in 0.01 M buffer. The active fractions were combined, dialyzed, concentrated by ultrafiltration, and applied to a Sephadex G-200 column (2.4 by 120 cm) equilibrated with 0.05 M buffer containing 0.1 M NaCl. The eluted active fractions were concentrated by ultrafiltration.

Other methods. The molecular weight of the enzyme was determined by gel filtration with G-3000 SW eluted under the same conditions as those described previously (2). Electrophoresis and determination of the molecular weight of the subunit of the enzyme were carried out on polyacrylamide gels with or without sodium dodecyl sulfate (SDS) as described previously (2). To check the purity of the enzyme, we applied the enzyme to a DEAE-5PW column (0.75 by 7.5 cm) in an HPLC system (SP-8700; Tosoh). The conditions were as described previously (2). The opine dehydrogenase was stained for activity with a solution containing 10 mM *N*-[1-*RS*-(carboxyl)ethyl]-*S*-phenylalanine, 0.42 mM NAD^+ , 0.60 mM 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, and 0.33 mM phenazine methosulfate in 0.1 M Tris hydrochloride (pH 8.5).

RESULTS

Isolation and identification of microorganisms. Strain 1C was chosen as a likely source for the NAD^+ -dependent opine dehydrogenase from about 140 isolates from soil. Taxonomic studies of strain 1C indicated that it belongs to the genus *Arthrobacter* because it is nonmotile, never fermentative, and occurs in rod-coccus shape, the peptidoglycan contains lysine as the diamino acid, and no mycelium formation is observed (19). The following species did not grow on a medium containing *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine as a sole source of carbon: *Arthrobacter pascens* IAM 12343, *A. aurescens* JCM 1330, *A. prothormiae* JCM 1973, *A. variabilis* JCM 2154, *A. histidinolorans* JCM 2520, *A. polychromogenes* JCM 2523, *A. atrocyaneus* IFO 12956, *A. crystallopoietes* IFO 14235, *A. nicotianae* IFO 14234, *A. oxydans* IFO 12138, *A. ureafaciens* IFO 12140, *A. viscosus* IFO 13497, *A. duodecadis* IFO 12959, *A. radiotolerans* JCM 2153, and *Pimelobacter tumescens* (formerly *A. tumescens*) IFO 12960. Only *A. ramosus* JCM 1334 and *A. sulfureus* JCM 1338 grew on the medium, although they had no NAD(P)^+ -dependent dehydrogenase acting on the substrate. Thus, strain 1C appears to differ from other *Arthrobacter* species with respect to production of the NAD^+ -dependent opine dehydrogenase and

utilization of *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine as a carbon source.

Purification of opine dehydrogenase from *Arthrobacter* sp. strain 1C. The enzyme activity found in *Arthrobacter* sp. strain 1C was inducible in the presence of *N*-[1-*RS*-(carboxyl)ethyl]-*S*-phenylalanine: only 1/10 of the total enzyme activity per culture was obtained in the absence of the substrate. Table 1 summarizes the purification of the enzyme from *Arthrobacter* sp. 1C. The enzyme was purified about 57-fold, with a 36% yield from the cell extract.

Purity and absorption spectrum. The enzyme was found to be homogeneous by polyacrylamide and SDS-polyacrylamide disc gel electrophoreses (Fig. 3A and B). The protein band visualized with Coomassie brilliant blue R-250 coincided well with that visualized by activity staining. It was eluted as a single peak by the HPLC system with a DEAE-5PW column (Fig. 3C). The purified enzyme showed an absorption maximum at 278 nm and no absorption in the visible region.

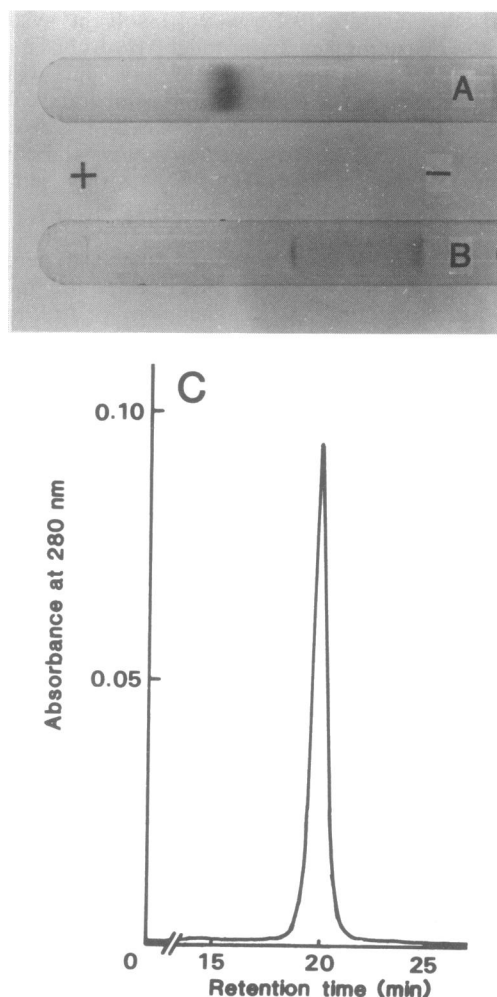


FIG. 3. Polyacrylamide disc gel electrophoresis and HPLC of the purified enzyme from *Arthrobacter* sp. strain 1C. (A) Purified enzyme (10 μg) was electrophoresed in the absence of SDS at a current of 2 mA. (B) Purified enzyme was incubated in the presence of 1% SDS and 3% 2-mercaptoethanol at 95°C for 3 min, and 2 μg was electrophoresed in the presence of 0.1% SDS at a current of 8 mA. The gels were stained with Coomassie brilliant blue R-250. (C) Elution profile of the enzyme.

Molecular weight and subunit structure. The molecular weight of the enzyme was calculated to be about 70,000 by gel filtration with an HPLC G-3000 SW column. The molecular weight of the subunit of the enzyme was estimated to be about 36,000 by SDS-polyacrylamide disc gel electrophoresis. The native enzyme probably has a dimeric structure consisting of two identical subunits.

Identification of the reaction product. The enzyme-catalyzed reductive secondary amine-forming reaction was carried out with L-phenylalanine, sodium pyruvate, and NADH simultaneously regenerated from NAD⁺ with formate dehydrogenase. The reaction mixture contained 2.7 mmol of sodium pyruvate, 2.7 mmol of L-phenylalanine, 2.8 mmol of sodium formate, 1.0 mmol of NAD⁺, 1.0 mmol of Tris hydrochloride (pH 8.5), 40 U of the purified enzyme after the DEAE-Toyopearl step, and 40 U of purified formate dehydrogenase in a total volume of 10 ml. When the mixture was incubated at 30°C for 21 h and the disappearance of L-phenylalanine was confirmed, the product formed in the mixture was adsorbed to Dowex 50 × 8W (H⁺) and eluted with 1 M ammonia-water. The released compound was dissolved in a small amount of water and methylated with ethereal diazomethane on ice, followed by silica gel column chromatography. Nuclear magnetic resonance, infrared, and mass spectrum analyses of the dimethyl ester of the product revealed that the compound had the structure of *N*-[1-(carboxymethyl)ethyl]phenylalanine methyl ester. Nuclear magnetic resonance (90 MHz, CDCl₃) δ ppm: 1.2 (3H, d, CH₃), 1.9 (1H, broad s, NH), 3.0 (2H, d, CH₂Ph), 3.3–3.6 (2H, m, CHNH × 2), 3.7 (6H, two s, OCH₃ × 2), 7.1–7.4 (5H, m, CH₂Ph). Infrared (neat) ν cm⁻¹: 2970, 1735, 1435, 1200, 1170. Mass spectrum: *m/z* 266 (M + 1, 6.3%), 206 (49), 174 (100), 146 (40), 114 (48), 91 (26). Elementary analysis, C₁₄H₁₉NO₄, calculated: C, 63.38; H, 7.22; N, 5.28; found: C, 62.98; H, 7.38; N, 5.19. The product synthesized by the action of the enzyme (30 mg) was subjected to an oxidation reaction with 1% KMnO₄ by the method of Hatanaka et al. (16). Paper chromatography of the oxidized products revealed two kinds of amino acids at the positions of authentic phenylalanine and alanine when the paper chromatogram was developed with a solvent system of *n*-butanol–acetic acid–H₂O (4:1:1 [vol/vol]). Each of the amino acids was extracted from the paper chromatogram, and the configuration was analyzed with L-amino-acid oxidase, D-amino-acid oxidase, and alanine dehydrogenase. The amino acids were identified as L-phenylalanine and D-alanine. Thus, the reaction product enzymatically synthesized from L-phenylalanine and sodium pyruvate was identified as *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine.

Substrate specificity and kinetic properties. The enzyme was specific for *N*-[1-*R*-(carboxyl)ethyl]-*S*-methionine and *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine in the oxidative deamination reaction. The enzyme did not act on saccharopine, D-octopine, L-*allo*-octopine, *N*-[1-*S*-(carboxyl)ethyl]-*S*-methionine, *N*-[1-*S*-(carboxyl)ethyl]-*S*-phenylalanine, *N*-[1-*S*-(carboxyl)ethyl]-*R*-phenylalanine, and *N*-[1-*R*-(carboxyl)ethyl]-*R*-phenylalanine. Kinetic studies were carried out to determine the Michaelis constant (K_m) and maximum reaction velocity (V_{max}). The apparent K_m values for *N*-[1-*R*-(carboxyl)ethyl]-*S*-methionine and *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine were calculated from Woolf plots to be 9.0 and 14 mM, respectively, with a fixed NAD⁺ concentration of 2.5 mM. The apparent K_m value for NAD⁺ was similarly calculated to be 0.76 mM with a fixed *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine concentration of 10 mM. The V_{max} values of the enzyme activity in the oxidative

TABLE 2. Substrate specificity of the opine dehydrogenase in the reductive secondary amine-forming reaction with pyruvate as a fixed substrate

Amino acid	Relative activity ^{a,b} (%)	K_m ^b (mM)
L-Methionine	100	4.1
L-Isoleucine	94	6.2
L-Valine	92	3.0
L-Phenylalanine	91	7.5
L-Leucine	89	2.9
L-Alanine	67	5.1
L-Threonine	50	7.4
L-Cysteine	39	2.5
L-Serine	39	3.6
Glycine	5.3	
L-Asparagine	1.2	

^a No activity was seen with β-alanine, L-histidine, L-glutamine, L-glutamic acid, L-lysine, L-ornithine, L-tryptophan, L-proline, L-tyrosine, L-arginine, L-aspartic acid, L-citrulline, ammonium chloride, methylamine, L-phenylalanine ethyl ester, and D-amino acids such as D-methionine, D-isoleucine, D-valine, D-phenylalanine, D-alanine, D-threonine, D-cysteine, and D-serine. L-Homophenylalanine was active as a substrate.

^b Assayed with pyruvate and NADH concentrations fixed at 10 and 0.1 mM, respectively.

deimination reaction with *N*-[1-*R*-(carboxyl)ethyl]-*S*-methionine and *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine as substrates were calculated from Woolf plots to be 86 and 110 U/mg, respectively. The latter value represents the specific activity at the infinite concentration of the substrate and is much higher than the final specific activity of the purified enzyme (16.7 U/mg) (Table 1), because the value was measured under standard assay condition in which the *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine (substrate) concentration (5.0 mM) was considerably lower than the apparent K_m value (14 mM). The enzyme was also susceptible to inhibition by the diastereomer *N*-[1-*S*-(carboxyl)ethyl]-*S*-phenylalanine, as discussed below. NADP⁺ did not serve as a cofactor.

The substrate specificity of the enzyme in the reductive secondary amine-forming reaction was examined with pyruvate as a fixed substrate (Table 2). The enzyme showed relatively wide substrate specificity with hydrophobic L-amino acids as major substrates. It is noteworthy that of the D-amino acids tested only D-leucine acted as a substrate and only for 3.4% of the activity found with L-methionine. The K_m values for the amino acid substrates were in the range of 2 to mM. The substrate specificity of the enzyme with

TABLE 3. Substrate specificity of the opine dehydrogenase in the reductive secondary amine-forming reaction with L-methionine as a fixed substrate

Keto acid	Relative activity ^{a,b} (%)	K_m ^b (mM)
Pyruvate ^c	100	3.0
Oxaloacetate	30	3.5
Glyoxylate	11	8.8
α-Ketobutyrate	11	28
Hydroxypyruvate	6.1	
α-Ketocaproate	2.1	

^a 2-Keto-4-phenylbutyrate, glyoxal, α-ketoglutarate, phenylpyruvate, and acetone were inert as substrates.

^b Assayed with L-methionine and NADH concentrations fixed at 10 and 0.1 mM, respectively.

^c The apparent K_m value for NADH was calculated to be 0.029 mM at L-methionine and pyruvate concentrations fixed at 10 mM each.

L-methionine as a fixed substrate in the secondary amine-forming reaction was also studied (Table 3). Lower-molecular-weight α -keto acids such as pyruvate, oxaloacetate, glyoxylate, and α -ketobutyrate were substrates for the enzyme.

Other properties. The enzyme had maximal activity at pH 8.0 for the reductive secondary amine-forming reaction, whereas for the oxidative deamination reaction, maximal activity was seen at pH 10.0. The velocity for the reductive secondary amine-forming reaction was about six times greater than that for the oxidative deamination reaction when the same amount of enzyme was used. When the enzyme was incubated at 30°C for 1 h with buffers of various pHs, more than 85% of the initial activity was retained at pH 5.0 to pH 9.5. No loss of activity was observed when the enzyme was incubated at 37°C and pH 8.0 for 10 min; 80% of the activity remained after incubation at 50°C, and about half was lost after incubation at 52°C. Maximal initial activity was observed at 55°C.

The effect of metal ions and inhibitors (at 1.0 mM unless otherwise noted) on enzyme activity was investigated. Enzyme activity was measured after the enzyme was preincubated at 25°C for 10 min. The enzyme was not affected by Li^+ , Na^+ , Ba^{2+} , Ca^{2+} , Mg^{2+} , Sn^{2+} (at 0.4 mM), Al^{3+} , Fe^{3+} , EDTA, 8-oxyquinoline, α, α' -dipyridyl, *o*-phenanthroline, sodium azide, KCN (at 0.4 mM), hydroxylamine, semicarbazide, and monoiodoacetate. Enzyme activity was inhibited to 10 to 60% by Mn^{2+} , Fe^{2+} , Pb^{2+} , Zn^{2+} , Co^{2+} , and *p*-chloromercuribenzoate (at 0.24 mM) and to 100% by Ag^+ , Hg^{2+} , Cu^{2+} , Ni^{2+} , Cd^{2+} , *N*-ethylmaleimide, and 5,5'-dithio-bis(2-nitrobenzoic acid) (at 0.13 mM).

When the *N*-[1-*R*-(carboxyl)ethyl]-*S*-phenylalanine (substrate) concentrations were 1, 2, 5, 10, and 20 mM, the enzyme was inhibited by the diastereomer *N*-[1-*S*-(carboxyl)ethyl]-*S*-phenylalanine (at a fixed concentration of 5 mM) by 26, 30, 33, 41, and 46%, respectively. Other diastereomers, such as *N*-[1-*S*-(carboxyl)ethyl]-*R*-phenylalanine and *N*-[1-*R*-(carboxyl)ethyl]-*R*-phenylalanine, showed no inhibitory effect.

DISCUSSION

In this paper, we described the screening, purification, and characterization of a new NAD^+ -dependent opine dehydrogenase from *Arthrobacter* sp. strain 1C isolated from soil by the use of a synthetic opine as a probe.

The enzyme isolated from *Arthrobacter* sp. strain 1C had a substrate specificity completely different from those of known opine dehydrogenases from both molluscan and plant origins (9, 11, 13, 15, 20, 27, 30, 31, 37, 39), although the configuration of the secondary amine dicarboxylic acid product was the same as that of nonsugar opines [*N*-[1-*R*-(carboxyl)alkyl]-*S*-amino acid].

The molecular weight of the enzyme was calculated to be 70,000, and the molecular weight of the subunit of the enzyme was about 36,000. The previously reported octopine dehydrogenases, alanopine dehydrogenase, and strombine dehydrogenase from marine molluscs are exclusively monomeric proteins with molecular weights of 35,000 to 45,000 and are NAD^+ dependent (9, 11, 13, 31, 35, 37, 39). The molecular weight of the polypeptide chain of octopine dehydrogenase (octopine synthase) and nopaline dehydrogenase (nopaline synthase) from crown gall tumor tissue induced by *A. tumefaciens* is also about 40,000 (15, 20, 37), although the latter is a tetrameric enzyme with a molecular weight of 158,000 (30). These enzymes are different from the molluscan enzymes in that they utilize both NADP^+ and NAD^+ as

cofactors. Firmin et al. have proposed that *N*-[1-(carboxyl)ethyl]methionine (methiopine) be categorized as a "pseudo-opine" (12), although the configuration of methiopine is not known (29). We propose that the enzyme in the present study be tentatively called pseudo-opine dehydrogenase or phenylalanine-pyruvate reductase, with the systematic name *N*-[1-*D*-(carboxyl)ethyl]-*L*-phenylalanine: NAD^+ oxidoreductase (*L*-phenylalanine forming) (EC 1.5.1.—) (27). The physiological role of this enzyme might be to oxidize opines of plant origin, such as methiopine, to yield NADH , α -keto acid, and *L*-amino acid, which can support the growth of the organism.

It has been reported that *Agrobacterium* strains that cause crown gall tumors (26) and some *Pseudomonas* species have the ability to utilize opines as sole carbon or nitrogen sources (5, 22). Recently, Tremblay et al. showed that not only gram-negative *Agrobacterium* and *Pseudomonas* species but also gram-positive coryneform species utilize opines (36). The coryneform species have been characterized to utilize mannopine, agropinic acid, succinamopine, or nopaline. However, from an enzymological point of view, it has only been found in a preliminary communication that a membrane-bound oxidase is responsible for the degradation of lysopine in *A. tumefaciens* (17). Montoya et al. have clearly shown that *Agrobacterium* mutants unable to degrade octopine are still virulent for plants, inducing not only crown gall tumors but also the synthesis of opines (25). These results indicate that *Agrobacterium* species have two genes concerned with the synthesis and degradation of opines; one is the gene for the opine dehydrogenase (synthase) (7), and the other is the gene for the opine oxidase (17, 32). This study is the first to show that opines can be degraded by an NAD^+ -dependent opine dehydrogenase from an *Arthrobacter* sp. The enzyme could be utilized in the stereospecific in vitro enzymatic synthesis of a wide variety of unusual opines containing two chiral centers from free *L*-amino acids and α -keto acids as substrates, without any laborious protection and deprotection or kinetic resolution procedures.

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