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Stereoselective synthesis of opine-type secondary amine carboxylic acids by a new enzyme opine dehydrogenase Use of recombinant enzymes

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Abstract

The substrate specificity of the recently discovered enzyme, opine dehydrogenase (ODH) from *Arthrobacter* sp. strain 1C for amino donors in the reaction that forms secondary amines using pyruvate as a fixed amino acceptor is examined. The enzyme was active toward short-chain aliphatic (*S*)-amino acids and those substituted with acyloxy, phosphonooxy, and halogen groups. The enzyme was named N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-norvaline: NAD^+ oxidoreductase (L-norvaline forming). Other substrates for the enzyme were 3-aminobutyric acid and (*S*)-phenylalaninol. Optically pure opine-type secondary amine carboxylic acids were synthesized from amino acids and their analogs such as (*S*)-methionine, (*S*)-isoleucine, (*S*)-leucine, (*S*)-valine, (*S*)-phenylalanine, (*S*)-alanine, (*S*)-threonine, (*S*)-serine, and (*S*)-phenylalaninol, and α-keto acids such as glyoxylate, pyruvate, and 2-oxobutyrate using the enzyme, with regeneration of NADH by formate dehydrogenase (FDH) from *Moraxella* sp. C-1. The absolute configuration of the nascent asymmetric center of the opines was of the (*R*) stereochemistry with > 99.9% e.e. One-pot synthesis of N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalanine from phenylpyruvate and pyruvate by using ODH, FDH, and phenylalanine dehydrogenase (PheDH) from *Bacillus sphaericus*, is also described.

Keywords: Enzymatic synthesis; Opine dehydrogenase; Dehydrogenase; Phenylalanine dehydrogenase; Formate dehydrogenase; *Arthrobacter* sp.

1. Introduction

NAD^+-dependent amino acid dehydrogenase (EC 1.4.1) catalyzes reversible amination–deamination reactions between (*S*)-amino acids and α-keto acids [1]. We have used phenylalanine dehydrogenase and other amino acid dehydrogenases to enzymatically synthesize optically pure natural and unnatural amino acids from their corresponding α-keto acids [2–5].

Opine-type secondary amine dicarboxylic acids are useful chiral intermediates of angiotensin converting enzyme (ACE)-inhibitors such as enalapril and lysinopril [6–8]. In order to extend the use of enzymes in stereoselective synthesis, we screened for an enzyme catalyzing the reversible oxidation–reduction of opine-type secondary amine dicarboxylic acids and isolated the bacterial producer, *Arthrobacter* sp. strain

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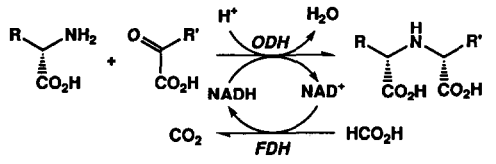


ODH, **Opine dehydrogenase from *Arthrobacter* sp. 1-C**
FDH, **Formate dehydrogenase from *Moraxella* sp. C1**

Fig. 1. Synthesis of opine-type secondary amine dicarboxylic acids from (*S*)-amino acids and α-keto acids by opine dehydrogenase with regeneration of NADH by formate dehydrogenase.

1C. We purified and characterized an NAD^+-dependent secondary amine dicarboxylic acid dehydrogenase, and named it opine dehydrogenase (ODH) [9]. We then cloned and sequenced the *odh* gene, and overproduced the enzyme for use in synthesis, since the enzyme is only induced by opine-type secondary amine dicarboxylic acids, the preparation of which, is rather laborious [10]. *Escherichia coli* JM109/pODH1 expresses about 6.6-fold higher activity of the enzyme per liter of culture than the wild type *Arthrobacter* sp. strain 1C without the addition of the chemically synthesized growth substrate, N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalanine.

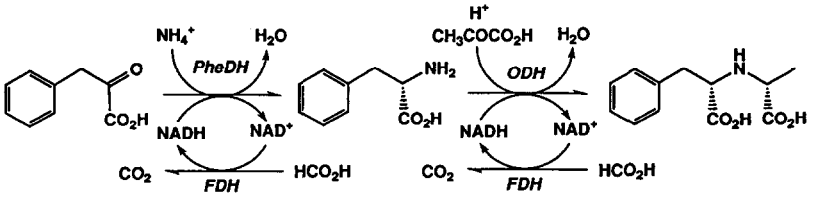
Optically active secondary amine dicarboxylic acids have been chemically synthesized as follows: (i) reductive condensation reaction of α-keto acids or their esters and amino acid derivatives using sodium cyanoborohydride [11], Raney–Ni [12], or catecholborane [13], (ii) SN_2 reaction of optically active 2-halo [14] and 2-trifluoromethanesulfonyloxy esters [15] with amino acid derivatives. However, these methods generally require protection of the functional groups and the stereoselectivity is not always high. We chose ODH to apply to the synthesis of secondary amine dicarboxylic acids without protection of the substrates.

In this report, we clarified the substrate specificity for amino donors of *Arthrobacter* ODH with several unnatural amino acids and amino compounds. We applied the enzyme to the synthesis of secondary amine dicarboxylic acids from (*S*)-amino acids and α-keto acids with a regeneration of NADH by formate dehydrogenase (FDH) [2–5] (Fig. 1). A secondary amine carboxylic acid from (*S*)-phenylalaninol and pyruvate was also synthesized. Optically pure N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalanine was synthesized in one-pot reaction from phenylpyruvate and pyruvate using ODH, FDH, and phenylalanine dehydrogenase (PheDH) from *Bacillus sphaericus* [3] (Fig. 2).

2. Experimental

2.1. Materials

^1H- and ^{13}C-NMR spectra were recorded in D_2O using a JEOL JNM-EX400 spectrometer (Tokyo, Japan), with tetramethylsilane as the internal standard. Mass spectra were recorded on a JEOL JMS-AX500 mass spectrometer (Tokyo, Japan) under fast atom bombardment (FAB) conditions. Optical rotations were recorded on a Horiba SEPA-200 polarimeter (Kyoto, Japan). DEAE-Toyopearl 650 M,



PheDH, **Phenylalanine dehydrogenase from *Bacillus sphaericus* R79a**
ODH, **Opine dehydrogenase from *Arthrobacter* sp. 1-C**
FDH, **Formate dehydrogenase from *Moraxella* sp. C1**

Fig. 2. One-pot synthesis of N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalanine from phenylpyruvate and pyruvate by phenylalanine and opine dehydrogenases with NADH regeneration by formate dehydrogenase.

Butyl-Toyopearl 650 M, and HPLC column ODS-80Ts were purchased from Tosoh Corp. (Tokyo, Japan), ion-exchange resins DIAION SK1B and SA20A were from Mitsubishi Chemicals (Tokyo, Japan), and the HPLC column Crownpak CR(+) was from Daicel Chem. Ind. Ltd. (Tokyo, Japan). All other chemicals were from commercial sources and used without further purification.

2.2. Enzyme preparations

Amounts of ODH were purified from *E. coli* JM109/pODH1 up to 45.8 units/mg as described previously [9,10], by means of ammonium sulfate fractionation, DEAE-Toyopearl and Butyl-Toyopearl column chromatography. Amounts of FDH were purified from *E. coli* JM109/pMxFDH2 [16,17] up to 4.1 units/mg in a similar manner. Amounts of PheDH were purified to homogeneity from *E. coli* JM109/pBPDH1-DBL [3] as described [4].

2.3. Enzyme assay

ODH activity in the reductive secondary-amine forming reaction was assayed at 25°C by measuring the oxidation of NADH at 340 nm in a reaction mixture (1 ml) containing 100 μmol of Tris–HCl (pH 8.0), 10 μmol of sodium pyruvate, 0.1 μmol of NADH, 10 μmol of amino compound, and the enzyme (0.5–3 units) with a Hitachi U-3210 spectrophotometer (Tokyo, Japan). A linear change in absorbance for the initial 10 s was used for the calculation. One unit of the enzyme was defined as the amount of the enzyme that catalyzed the consumption of 1 μmol of NADH per min. FDH [16] and PheDH [3] activities were measured as described. One unit of these enzymes was defined as the amount that catalyzed the formation of 1 μmol of NADH in the oxidation reaction at pH 7.5 with sodium formate and at pH 10.5 with (*S*)-phenylalanine as substrates for FDH and PDH, respectively. Protein was assayed by measuring the absorbance at 280 nm.

2.4. General procedure for the secondary amine-forming reaction from (S)-amino acids and pyruvate or glyoxylate

The reaction mixture contained 0.5 mmol of (*S*)-amino acid, 0.75 mmol of sodium salt of α-keto acid, 0.75 mmol of sodium formate, 25 μmol of NAD^+, 0.5 mmol of Tris–HCl (pH 8.5), ODH (48 units), and FDH (40 units) in a total volume of 5 ml. The reaction mixture was incubated at 30°C for 16 h and the disappearance of (*S*)-amino acid was confirmed using a Hitachi L-8500 amino acid analyzer (Tokyo, Japan). The reaction mixture was boiled for 10 min and centrifuged to remove denatured protein. The supernatant was applied to a column of DIAION SK-1B (H^+) and eluted with 1 N NH_4OH. The effluent was evaporated to dryness, applied to a column of DIAION SA-20A (Cl^-) and eluted with 1 N formic acid. The eluate was concentrated and lyophilized.

2.5. Determination of enantiomeric excess (e.e.) and absolute configuration of synthesized secondary amine carboxylic acids

The compounds synthesized (10 mg) by the secondary amine-forming reaction were oxidized with 1% $KMnO_4$ as described by Hatanaka et al. [18]. Amino acids formed were purified from the reaction mixture by paper chromatography (Advantec 51B, 20 × 20 cm, Tokyo, Japan) using 70% n-propanol as the developing solvent. The amino acids were analyzed using a Waters HPLC system (Millipore Corp., Bedford, MA, USA) with a Crownpak CR(+) (4.6 × 150 mm) at a flow rate of 0.5 ml/min using aqueous $HClO_4$ (pH 1.0) as the mobile phase at 4°C to determine the optical purity and absolute configuration.

2.6. N-[1-(R)-(Carboxyl)ethyl]-(S)-methionine

This was synthesized from (*S*)-methionine and pyruvate. Yield, 15.7% (17.3 mg) based on (*S*)-methionine (75 mg), ^{1}H-NMR (D_2O) δ_{ppm}

3.969–4.037 (m, 1 + 1 H), 2.652–2.741 (m, 2H), 2.199–2.252, (m, 2H), 2.126 (s, 3H), 1.557 (d, 3H, $J = 7.3$ Hz); ^{13}C-NMR (D_2O) δ_{ppm} 171.48, 170.73, 58.14, 55.86, 30.02, 29.63, 14.69, 14.62; FAB-MS: m/z 221 (rel. int. 33%, M + H), 93 (100); $[\alpha]_D^{22}$ +8.3° $c = 1.0$, H_2O).

2.7. *N-[1-(R)-(Carboxyl)ethyl]-(S)-isoleucine*

This was synthesized from (*S*)-isoleucine and pyruvate. Yield, 56.7% (57.6 mg) based on (*S*)-isoleucine (65 mg), ^{1}H-NMR (D_2O) δ_{ppm} 4.18 (q, 1H, $J = 7.2$ Hz), 4.02 (d, 1H, $J = 3.9$ Hz), 2.062–2.124 (m, 1H), 1.556–1.624 (m, 1H), 1.584 (d, 3H, $J = 7.4$ Hz), 1.370–1.443 (m, 1H), 1.010 (d, 3H, $J = 6.9$ Hz), 0.959 (t, 3H, $J = 7.3$ Hz); ^{13}C-NMR (D_2O) δ_{ppm} 174.93, 173.88, 65.76, 58.69, 38.92, 28.79, 16.75, 16.35, 13.95; FAB-MS: m/z 204 (rel. int. 100%, M + H), 158 (88), 112 (25), 86 (43), 69 (23), 44 (82); $[\alpha]_D^{21}$ +19.1° ($c = 1.0$, H_2O).

2.8. *N-[1-(R)-(Carboxyl)ethyl]-(S)-leucine*

This was synthesized from (*S*)-leucine and pyruvate. Yield, 68.2% (69.2 mg) based on (*S*)-leucine (65 mg), ^{1}H-NMR (D_2O) δ_{ppm} 4.135 (q, 1H, $J = 7.3$ Hz), 3.996–4.029 (m, 1H), 1.860–1.910 (m, 1H), 1.682–1.816 (m, 1 + 1 H), 1.592 (d, 3H, $J = 7.3$ Hz), 0.963 (d, 6H, $J = 6.3$ Hz); ^{13}C-NMR (D_2O) δ_{ppm} 175.07, 175.04, 60.59, 58.03, 41.42, 27.41, 24.84, 23.9, 16.84; FAB-MS: m/z 204 (rel. int. 83%, M + H), 158 (77), 132 (34), 112 (29), 86 (77), 70 (42), 44 (100); $[\alpha]_D^{21}$ +9.3° ($c = 1.0$, H_2O).

2.9. *N-[1-(R)-(Carboxyl)ethyl]-(S)-valine*

This was synthesized from (*S*)-valine and pyruvate. Yield, 58.9% (55.7 mg) based on (*S*)-valine (55 mg), ^{1}H-NMR (D_2O) δ_{ppm} 4.148 (q, 1H, $J = 7.3$ Hz), 3.913 (d, 1H, $J = 4.4$ Hz), 2.326–2.405 (m, 1H), 1.576 (d, 3H, $J = 7.3$ Hz), 1.116 (d, 3H, $J = 6.9$ Hz), 1.046 (d, 3H, $J = 7.3$ Hz); ^{13}C-NMR (D_2O) δ_{ppm} 175.04, 174.14, 67.93, 58.91, 32.32, 21.18, 19.73, 16.26; FAB-MS: m/z 190 (rel. int. 100%, M + H), 144 (77), 98 (37), 72 (34), 44 (56); $[\alpha]_D^{22}$ +9.3° ($c = 1.0$, H_2O).

2.10. *N-[1-(R)-(Carboxyl)ethyl]-(S)-phenylalanine*

This was synthesized from (*S*)-phenylalanine and pyruvate. Yield, 29.4% (34.8 mg) based on (*S*)-phenylalanine (80 mg), ^{1}H-NMR (D_2O) δ_{ppm} 7.329–7.439 (m, 5H), 4.215 (dd, 1H, $J =$ 4.4, 6.4 Hz), 3.927 (q, 1H, $J = 7.3$ Hz), 3.313 (d, 2H, $J = 6.4$ Hz), 1.487 (d, 3H, $J = 7.3$ Hz); ^{13}C-NMR (D_2O) δ_{ppm} 175.58, 174.61, 137.36, 132.22, 132.15, 130.87, 63.66, 58.60, 38.53, 16.92; FAB-MS: m/z 260 (rel. int. 24%, M + H), 238 (40), 192 (46), 146 (30), 120 (38), 91 (69), 44 (100); $[\alpha]_D^{22}$ +7.7° ($c = 1.0$, H_2O)

2.11. *meso-N-[1-(Carboxyl)ethyl]-alanine*

This was synthesized from (*S*)-alanine and pyruvate. Yield, 61.1% (49.2 mg) based on (*S*)-alanine (45 mg), ^{1}H-NMR (D_2O) δ_{ppm} 4.181 (q, 2H, $J = 6.9$ Hz), 1.596 (d, 6H, $J = 6.9$ Hz); ^{13}C-NMR (D_2O) δ_{ppm} 175.29, 57.42, 17.32; FAB-MS: m/z 162 (rel. int. 56%, M + H), 116 (48), 90 (30), 70 (35), 44 (100); $[\alpha]_D^{15}$ +0° ($c = 1.0$, H_2O).

2.12. *N-[1-(R)-(Carboxyl)ethyl]-(S)-threonine*

This was synthesized from (*S*)-threonine and pyruvate. Yield, 71.1% (67.9 mg) based on (*S*)-threonine (60 mg), ^{1}H-NMR (D_2O) δ_{ppm} 4.209–4.285 (m, 1 + 1 H), 3.930 (d, 2H, $J = 6.8$ Hz), 1.61 (d, 3H, $J = 6.8$ Hz), 1.388 (d, 3H, $J = 6.4$ Hz); ^{13}C-NMR (D_2O) δ_{ppm} 174.74, 172.95, 68.89, 68.03, 59.07, 22.39, 16.35; FAB-MS: m/z 192 (rel. int. 100%, M + H), 146 (38), 120 (21), 100 (20), 44 (100); $[\alpha]_D^{15}$ −14.4° ($c = 1.0$, H_2O).

2.13. *N-[1-(R)-(Carboxyl)ethyl]-(S)-serine*

This was synthesized from (*S*)-serine and pyruvate. Yield, 65.3% (57.8 mg) based on

(*S*)-serine (50 mg), ^{1}H-NMR (D_2O) δ_{ppm} 4.169–4.452 (m, 1 + 1 H), 4.119–4.136 (m, 2H), 1.629 (d, 3H, J = 6.4 Hz); ^{13}C-NMR (D_2O) δ_{ppm} 175.09, 172.77, 63.2, 61.56, 57.77, 17.26; FAB-MS: m/z 178 (rel. int. 100%, M + H), 132 (41), 93 (39), 44 (46); $[\alpha]_D^{15}$ +5.9° (c = 1.0, H_2O).

2.14. N-Carboxylmethyl-(S)-isoleucine

This was synthesized from (*S*)-isoleucine and glyoxylate. Yield, 66.3% (62.7 mg) based on (*S*)-isoleucine (65 mg), ^{1}H-NMR (D_2O) δ_{ppm} 4.058 (m, 1H), 3.989 (dd, 2H, J = 13.4, 17.1 Hz), 2.122–2.129 (m, 1H), 1.532–1.602 (m, 1H), 1.339–1.442 (m, 1H), 1.039 (d, 3H, J = 3.9 Hz), 0.962 (t, 3H, J = 7.3 Hz); ^{13}C-NMR (D_2O) δ_{ppm} 173.57, 171.94, 67.83, 49.93, 38.81, 28.49, 16.9, 13.99; FAB-MS: m/z 190 (rel. int. 100%, M + H), 144 (58), 98 (10), 86 (15), 30 (24); $[\alpha]_D^{17}$ +7.9° (c = 1.0, H_2O).

2.15. N-Carboxylmethyl-(S)-leucine

This was synthesized from (*S*)-leucine and glyoxylate. Yield, 69.6% (65.8 mg) based on (*S*)-leucine (65 mg), ^{1}H-NMR (D_2O) δ_{ppm} 4.075–4.165 (m, 1H), 4.012 (dd, 2H, J = 4.4, 17.1 Hz), 1.857–1.943 (m, 1H), 1.731–1.839 (m, 1 + 1 H), 0.974 (d, 6H, J = 5.8 Hz); ^{13}C-NMR (D_2O) δ_{ppm} 174.71, 171.85, 61.76, 49.2, 41.15, 27.25, 24.69, 24.01; FAB-MS: m/z 190 (rel. int. 100%, M + H), 144 (78), 88 (31), 86 (43), 44 (44), 30 (64); $[\alpha]_D^{17}$ +8.1° (c = 1.0, H_2O).

2.16. N-Carboxylmethyl-(S)-valine

This was synthesized from (*S*)-valine and glyoxylate. Yield, 65.7% (57.5 mg) based on (*S*)-valine (55 mg), ^{1}H-NMR (D_2O) δ_{ppm} 3.962 (dd, 2H, J = 7.1, 27.8 Hz), 3.943 (d, 1H, J = 3.9 Hz), 2.376–2.435 (m, 1H), 1.090 (dd, 6H, J = 6.3, 6.9 Hz), ^{13}C-NMR (D_2O) δ_{ppm} 173.87, 172.07, 69.33, 50.05, 32.11, 20.68, 19.9; FAB-MS: m/z 176 (rel. int. 100%, M + H), 130 (80), 84 (31), 72 (22), 30 (31); $[\alpha]_D^{17}$ +2.2° (c = 1.0, H_2O).

2.17. General procedure for the secondary amine-forming reaction from (S)-amino acids and 2-ketobutyrate

A reaction mixture (5 ml) containing 0.5 mmol of (*S*)-amino acid, 0.75 mmol of sodium 2-ketobutyrate, 0.75 mmol of sodium formate, 25 μmol of NAD^+, 0.5 mmol of Tris–HCl (pH 8.5), ODH (110 units), and FDH (40 units) was incubated at 30°C for 21 h and the disappearance of the (*S*)-amino acid was confirmed as described above. Six hours after starting the reaction, ODH (220 units), FDH (17 units) and sodium formate (1.5 mmol) was added. The reaction product was purified as described above.

2.18. N-[1-(R)-(Carboxyl)propyl]-(S)-isoleucine

This was synthesized from (*S*)-isoleucine and 2-ketobutyrate. Yield, 63.9% (69.3 mg) based on (*S*)-isoleucine (65 mg), ^{1}H-NMR (D_2O) δ_{ppm} 4.117 (d, 1H and q, 1H, J = 2.9 Hz) 2.054–2.161 (m, 2H), 1.999–2.062 (m, 1H), 1.566–1.634 (m, 1H), 1.401–1.473 (m, 1H), 1.036 (t, 3H, J = 7.3 Hz), 1.015 (d, 3H, J = 6.8 Hz), 0.962 (t, 3H, J = 7.3 Hz); ^{13}C-NMR (D_2O) δ_{ppm} 173.81, 173.04, 67.28, 63.88, 31.73, 24.65, 21.54, 19.28, 11.70; FAB-MS: m/z 218 (rel. int. 100%, M + H), 172 (69), 86 (25), 58 (42), 41 (28); $[\alpha]_D^{19}$ +16.2° (c = 1.0, H_2O).

2.19. N-[1-(R)-(Carboxyl)propyl]-(S)-valine

This was synthesized from (*S*)-valine and 2-ketobutyrate. Yield, 62.7% (63.6 mg) based on (*S*)-valine (55 mg), ^{1}H-NMR (D_2O) δ_{ppm} 4.084 (dd, 1H, J = 6.9, 7.3 Hz), 4.025 (d, 1H, J = 4.4 Hz), 2.394–2.439 (m, 1H), 1.974–2.093 (m, 1 + 1H), 1.143 (d, 3H, J = 6.8 Hz), 1.046 (d, 3H, J = 7.3 Hz), 1.037 (t, 3H, J = 7.8 Hz); ^{13}C-NMR (D_2O) δ_{ppm} 173.96, 173.24, 67.28, 63.88, 31.73, 24.65, 21.54, 19.28, 11.7; FAB-

MS: m/z 204 (rel. int. 100%, M + H), 158 (81), 112 (39), 72 (39), 58 (43), 55 (27), 41 (22); $[\alpha]_D^{19}$ +5.0° (c = 1.0, H_2O).

2.20. Asymmetric synthesis of N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalaninol from (S)-phenylalaninol and pyruvate

A reaction mixture (5 ml) containing 0.5 mmol of (*S*)-phenylalaninol, 0.75 mmol of sodium pyruvate, 0.75 mmol of sodium formate, 25 μmol of NAD^+, 0.5 mmol of Tris–HCl (pH 8.5), ODH (110 units), and FDH (40 units) was incubated at 30°C for 26 h. The reaction product was purified by ion exchange column chromatography and acidified with hydrochloric acid followed by lyophilization. N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalaninol was obtained as a hydrochloride salt in a yield of 87.5% (113.5 mg).^{1}H-NMR (D_2O) δ_{ppm} 7.326–7.442 (m, 5H), 4.123 (q, 1H, J = 7.4 Hz), 3.834 (m, 1H), 3.639–3.707 (m, 1 + 1H), 3.139 (dd, 1H, J = 4.8, 5.8 Hz), 3.027 (dd, 1H, J = 4.8, 8.5 Hz), 1.585 (d, 3H, J = 7.4 Hz); ^{13}C-NMR (D_2O) δ_{ppm} 175.15, 138.39, 132.17, 132.08, 130.47, 62.73, 61.91, 57.04, 36.39, 17.78; FAB-MS: m/z 224 (rel. int. 90.6%, M + H), 91 (100), 44 (86.5); $[\alpha]_D^{19}$ −13.7° (c = 1.0, H_2O).

2.21. One-pot synthesis of N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalanine by ODH and PheDH from phenylpyruvate and pyruvate

A reaction mixture (5 ml) containing 0.25 mmol of sodium phenylpyruvate, 0.75 mmol of sodium pyruvate, 2.5 μmol of ammonium formate, 25 μmol of NAD^+, 0.5 mmol of Tris–HCl (pH 8.5), ODH (110 units), PheDH (35 units),

Table 1
Substrate specificity of opine dehydrogenase from *Arthrobacter* sp. strain 1C [a]

Amino donor [b]	K_m (mM)	V_{max} (units/mg)	V_{max}/K_m (units/mg/mM)	Relative activity (%) [c]
(*S*)-Norvaline	2.17	607	280	100
(*S*)-2-Aminobutyric acid	20.0	519	26.0	83.7
(*S*)-Norleucine	3.72	523	141	72.5
β-Chloro-(*S*)-alanine	3.19	362	113	53.1
o-Acetyl-(*S*)-serine	5.94	294	49.5	35.8
(*S*)-Methionine	4.10	–	–	23.9 [d]
(*S*)-Isoleucine	6.20	–	–	22.3 [d]
(*S*)-Valine	3.00	–	–	21.9 [d]
(*S*)-Phenylalanine	8.70	222	25.5	21.7
(*S*)-Leucine	2.90	–	–	21.2 [d]
(*S*)-Alanine	5.10	–	–	16.0 [d]
o-Phospho-(*S*)-serine	8.69	153	17.6	15.8
(*R,S*)-2,3-Diaminopropionic acid	11.9	105	8.82	9.67
(*S*)-Phenylglycine	28.3	82.2	2.90	4.40
(*R,S*)-3-Aminobutyric acid	7.73	6.54	0.846	0.683
o-Phospho-(*S*)-threonine	9.01	6.54	0.726	0.640
(*S*)-Phenylalaninol	46.0	18.2	0.396	0.631

[a] The reaction with various amine-containing compounds was tested in 1 ml of the same reaction mixture as described in Section 2 except that the substrate concentration was varied from 10 to 100 mM.
[b] The following compounds were inert as substrates: 4-amino-3-hydroxybutyric acid, 4-aminobutyric acid, 6-aminohexanoic acid, (*S*)-phenylalanine methyl ester, (*S*)-phenylglycine methyl ester, (*S*)-norvaline methyl ester, (*S*)-alanine methyl ester, (*S*)-phenylalanine amide, (*S*)-phenylglycine amide, (*S*)-alanine amide, (*S*)-isoleucinol, (*S*)-valinol, (*S*)-alaninol, (*S*)-serinol, 1-amino-2,3-propanediol, 1-amino-2-propanol, (2*S*,3*S*)-2-amino-1-phenyl-1,3-propanediol, 3-amino-4-phenyl-4-butanol, ethanolamine, 2-amino-2-methyl-1,3-propanediol, methylamine, ethylamine, isopropylamine, butylamine, benzylamine, 3-aminopentane, 2-phenylethylamine, methoxyamine, dimethylamine, hydrazine, phenylhydrazine, and hydroxylamine.
[c] Assayed with pyruvate concentrations fixed at 10 mM.
[d] Reported values [9].

and FDH (40 units) was incubated at 30°C for 22 h. Seven hours after starting the reaction, sodium phenylpyruvate (0.25 mmol), ODH (110 units), FDH (35 units), PheDH (35 units), and ammonium formate (2.5 mmol) was added. The reaction product was purified as described above. ^{1}H-NMR (D_2O) δ_{ppm} 7.332–7.435 (m, 5H), 4.215 (dd, 1H, $J = 4.4$, 6.4 Hz), 3.929 (q, 1H, $J = 7.3$ Hz), 3.311 (d, 2H, $J = 6.4$ Hz), 1.489 (d, 3H, $J = 7.3$ Hz); ^{13}C-NMR (D_2O) δ_{ppm} 175.57, 174.65, 137.39, 132.25, 132.21, 130.89, 63.66, 58.63, 38.50, 16.91; FAB-MS: m/z 260 (rel. int. 18%, M + H), 238 (38), 192 (40), 146 (19), 120(22), 91 (70), 44 (100); $[\alpha]_D^{22}$ +7.7° ($c = 1.0$, H_2O).

3. Results and discussion

3.1. Substrate specificity of Arthrobacter opine dehydrogenase

ODH utilizes hydrophobic (*S*)-amino acids such as (*S*)-methionine, (*S*)-isoleucine, (*S*)-valine, (*S*)-phenylalanine, and (*S*)-leucine etc. as amino donors [9], although the substrate specificity of unnatural-type amino acids have not been examined. We screened for a substrate among several amino compounds, which can act as amino donors in the secondary amine-forming reaction, using pyruvate as the fixed amino acceptor. Among the unnatural types α-amino acids tested, short-chain neutral amino acids such as (*S*)-2-aminobutyric acid, (*S*)-norvaline, and (*S*)-norleucine, and aromatic ring containing (*S*)-phenylglycine were good substrates for the enzyme. 3-Chloro-(*S*)-alanine, 2,3-diaminopropionic acid, as well as serine and threonine derivatives such as *o*-phospho-(*S*)-serine, *o*-phospho-(*S*)-threonine, and *o*-acetyl-(*S*)-serine also acted as substrates. Other amino compounds such as ω-amino acids, amino acid esters and amides, amino alcohols, organic amines, hydroxylamines, and hydrazines were inactive as substrates.

Kinetic studies were performed to determine the Michaelis constant (K_m) and the maximum reaction velocity (V_{max}). As shown in Table 1, the presence of a hydrophilic group, such as an amino or phosphonooxy group in the substrate, caused a decrease of the V_{max} value, but not of the K_m value. The V_{max}/K_m value for (*S*)-phenylglycine was one-tenth of that for (*S*)-phenylalanine. We showed that (*S*)-methionine was the most suitable amino donor in the secondary amine-forming reaction and the relative activity compared with that of (*S*)-phenylalanine was 110% [9]. This study showed that the relative activity for (*S*)-norvaline was four times higher than that for (*S*)-methionine. Therefore, the enzyme should be called opine dehydrogenase (N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-norvaline: NAD^+ oxidoreductase (L-norvaline forming)). The β-amino alcohol, (*S*)-phenylalaninol, and the β-amino acid, 3-aminobutyric acid, were active as substrates but their relative activities were very low.

The enzyme showed a relatively wide substrate specificity for several (*S*)-α-amino acids as amino donors, whereas it did not utilize other amino compounds, including amino acid esters and amides, indicating that a free carboxylic acid moiety is needed for recognition as an amino donor. However, (*S*)-phenylalaninol, which has no carboxylic acid moiety, exceptionally acted as an amino donor for the enzyme. This finding will help elucidate the reaction mechanism of the enzyme. On the other hand, amino alcohols corresponding to aliphatic (*S*)-amino acids such as (*S*)-isoleucine, (*S*)-valine, (*S*)-alanine, and (*S*)-serine were inert as substrates, whereas the amino acids were good substrates. The reason for the requirement of an aromatic ring in the substrate remains unknown. Probably the affinity of the aromatic moiety for the enzyme compensated for the loss of a carboxylic acid in (*S*)-phenylalaninol.

3.2. Synthesis of secondary amine dicarboxylic acids from (S)-amino acids and α-keto acids

Several opine-type secondary amine dicarboxylic acids were synthesized from natural-type

(*S*)-amino acids and α-keto acids with NADH regeneration by FDH. (*S*)-Methionine, (*S*)-isoleucine, (*S*)-leucine, (*S*)-valine, (*S*)-phenylalanine, (*S*)-alanine, (*S*)-threonine, and (*S*)-serine were used as amino donors and glyoxylic, pyruvic, and 2-ketobutyric acids were used as amino acceptors. As shown in Table 2, several secondary amine dicarboxylic acids were synthesized almost quantitatively. However, isolated yields were low because of losses during purification. The diastereomeric excess of the product from pyruvate and 2-ketobutyrate was over 99.9% according to high-resolution ^{1}H- and ^{13}C-NMR analysis. To confirm the absolute configuration, $KMnO_4$ oxidation, followed by HPLC analysis, proceeded as described in Section 2. When the secondary amine dicarboxylic acid was synthesized from (*S*)-isoleucine and pyruvate, the amino acids obtained from the oxidation reaction were optically pure (*S*)-isoleucine and (*R*)-alanine. In a similar manner, the absolute configurations of the new asymmetric centers of the synthesized secondary amine dicarboxylic acids were all of the (*R*) stereochemistry with > 99.9% enantiomeric excess (% e.e.). This one step enzymatic synthesis seems to be a most simple method for the stereoselective synthesis of opine-type secondary amine dicarboxylic acids without protection of the substrates, because chemical procedures generally require five to eight steps [10–15].

3.3. Synthesis of N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalaninol from pyruvate and (S)-phenylalaninol

Based on the substrate specificity of the enzyme, the reductive condensation between pyruvate and the α-amino group of (*S*)-phenylalaninol was investigated. The reaction proceeded smoothly using three-fold more ODH than that used to synthesize amine dicarboxylic acids, and it quantitatively yielded a secondary amino acid according to HPLC. The compound was proven to be diastereomerically pure N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalaninol.

3.4. One-pot synthesis of N-[1-(R)-(carboxyl)ethyl]-(S)-phenylalanine using ODH and PheDH

We showed that ODH acts on pyruvate but not on phenylpyruvate [9], whereas PheDH acts

Table 2
Synthesis of opine-type secondary amine dicarboxylic acids from (*S*)-amino acids and α-keto acids using opine dehydrogenase and formate dehydrogenase [a]

Amino acid	α-Keto acid						
	Glyoxylate	Pyruvate			2-Ketobutyrate		
	Yield	Yield	Purity		Yield	Purity	
	(%)	(%)	(% d.e.) [b]	(% e.e.) [c]	(%)	(% d.e.) [b]	(% e.e.) [c]
(*S*)-Methionine	–	96	> 99.9	> 99.9	–	–	–
(*S*)-Isoleucine	> 99	> 99	> 99.9	> 99.9	97	> 99.9	> 99.9
(*S*)-Leucine	> 99	> 99	> 99.9	> 99.9	–	–	–
(*S*)-Valine	> 99	> 99	> 99.9	> 99.9	> 99	> 99.9	> 99.9
(*S*)-Phenylalanine	–	95	> 99.9	> 99.9	–	–	–
(*S*)-Alanine	–	98 [d]	> 99.9	> 99.9	–	–	–
(*S*)-Threonine	–	> 99	> 99.9	> 99.9	–	–	–
(*S*)-Serine	–	97	> 99.9	> 99.9	–	–	–

[a] The yields were determined by HPLC on a ODS-80Ts column at 200 nm with a mobile phase of 40% acetonitrile containing 20 mM of $HClO_4$ at a flow rate of 1.0 ml/min using their standard curves.
[b] Determined by 400 MHz ^{1}H- and ^{13}C-NMR.
[c] Determined by HPLC as described in Section 2.
[d] The obtained compound was in the *meso*-form.

on phenylpyruvate but not on pyruvate [3]. Based on these findings, we attempted the one-pot synthesis of N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalanine from phenylpyruvate and pyruvate by using ODH and PheDH (Fig. 2). Fig. 3 shows the effect of the phenylpyruvate concentration in the reaction mixture. At any phenylpyruvate concentration, the secondary amine dicarboxylic acid was formed quantitatively within 2 h, but the yield was decreased by further incubation when the substrate concentration was over 100 mM. We therefore set the concentration of phenylpyruvate to 50 to 75 mM. The concentration of ammonium formate was then varied while the concentration of phenylpyruvate was kept constant at 50 mM. As shown in Fig. 4, the duration of the reaction time led to a decrease in yield when the ammonium formate concentration was low (up to 125 mM). The concentration of ammonium formate was set to 375 to 500 mM.

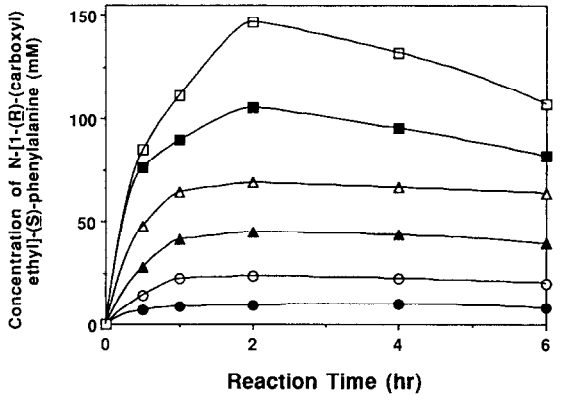


Fig. 3. Effect of phenylpyruvate concentration on the synthesis of N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalanine. A reaction mixture (1 ml) containing 500 μmol of Tris-HCl buffer (pH 8.5), 150 μmol of sodium pyruvate, 5 μmol of NAD^+, 500 μmol of ammonium formate with 21.9, 12.1, and 5.5 units of ODH, PheDH, and FDH, respectively, was incubated at 30°C in the presence of 10 (●), 25 (○), 50 (▲), 75 (△), 100 (■), and 150 μmol (□) of phenylpyruvate. After 0.5, 1, 2, 4 and 6 h incubations, 100 μl of the reaction mixture was removed and 900 μl of 0.2 M $HClO_4$ was added to each aliquot. After centrifugation (15,000× *g*, 10 min), the amount of N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalanine in the supernatant was determined by HPLC (equipped with a column ODS-80Ts) with a solvent system of 40% acetonitrile containing 20 mM of $HClO_4$ using a standard curve.

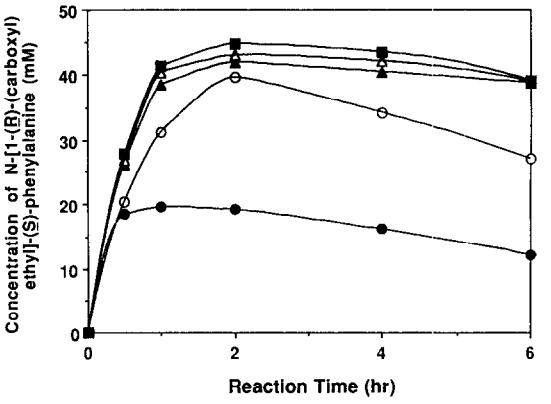


Fig. 4. Effect of ammonium formate concentration on the synthesis of N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalanine. The mixture was incubated at 30°C as described in Fig. 3, except 50 μmol of phenylpyruvate and 50 (●), 125 (○), 250 (▲), 375 (△) and 500 μmol (■) of ammonium formate were added. The concentrations of N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalanine was determined as described in the legend to Fig. 3.

Thus, the reaction conditions were optimized as summarized in Section 2 and the secondary amine dicarboxylic acid was synthesized at a yield of 98%. We identified the compound as optically pure N-[1-(*R*)-(carboxyl)ethyl]-(*S*)-phenylalanine.

There are no reports describing the enzyme-catalyzed in vitro asymmetric synthesis of opine-type secondary amine carboxylic acids. When used in combination with FDH, ODH effectively synthesized them. The reductive amination reaction of the enzyme proceeded D-(*R*)-stereospecifically. Generally, the known $NAD(P)^+$ dependent amino acid dehydrogenases, which appear to share a reaction mechanism similar to that of ODH, are all L-stereospecific [1–5], except for $NADP^+$-dependent *meso*-α,ε-diaminopimelate D-dehydrogenase [19]. The sequence and three-dimensional structural homology among amino acid dehydrogenases indicates the existence of an enzyme superfamily related by divergent evolution [20,21]. A comparison of the structure of ODH with other amino acid dehydrogenases would provide insight to the catalytic, structural, and evolutionary relationships among members of the dehydrogenase families.

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