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Characterization of novel alcohol dehydrogenase of *Devosia riboflavina* involved in stereoselective reduction of 3-pyrrolidinone derivatives

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Abstract

Optically active *N*-benzyl-3-pyrrolidinols are versatile chiral building blocks. Stereoselective reduction of *N*-benzyl-3-pyrrolidinone is an economical and environmentally friend means of synthesizing these compounds. *Devosia riboflavina* KNK10702 was discovered on screening as a source of a reducing enzyme giving the (*R*)-form *N*-benzyl-3-pyrrolidinol. An NADH-dependent alcohol dehydrogenase was purified to homogeneity through five steps from this microorganism. The relative molecular mass of the enzyme was estimated to be 58,000 on gel filtration and 28,000 on SDS-polyacrylamide gel electrophoresis. This enzyme reduced a broad range of carbonyl compounds in addition to *N*-substituted-3-pyrrolidinones. Some properties of the enzyme are reported herein.

Keywords: Alcohol dehydrogenase; NADH; Pyrrolidinol; Reduction; Chiral alcohols

1. Introduction

Optically active compounds such as chiral alcohols have been widely recognized as important synthetic intermediates for pharmaceuticals, agrochemicals, and flavors. Optically active 3-pyrrolidinol derivatives are also versatile building blocks for the synthesis of pharmaceuticals such as quinolones and β-lactam antimicrobial agents and calcium antagonists [1–3]. There are two industrial methods for the preparation of (*R*)-3-pyrrolidinol derivatives: one is a chemical decarboxylation of (2*S*,4*R*)-4-hydroxy-2-proline [4]; the other is an enzymatic optical resolution of *N*-benzyl-3-acyloxypyrrolidinol [5]. In the latter method, both isomers are easily synthesized, but the maximum yield of each isomer is at most 50% (Scheme 1). As a more advisable methodology, the asymmetric microbial hydroxylation of *N*-benzylpyrrolidine, a prochiral compound, has been attempted, but the stereoselectivity of the product has not been achieved [6].

Enzymatic asymmetric reduction is also an attractive methodology for the synthesis of optically active alcohols. This reaction will proceed almost stoichiometrically and stereoselectively under ambient temperature and atmospheric pressure. An alcohol dehydrogenase reducing *N*-benzyl-3-pyrrolidinone to (*S*)-*N*-benzyl-3-pyrrolidinol has been purified and characterized from *Geotrichum capitatum* JCM3908 [7]. This was NADH-dependent and composed of two subunits, and it reduced various kinds of carbonyl compounds and oxidized some secondary alcohols. However, the reducing enzyme of *N*-benzyl-3-pyrrolidinone to (*R*)-form alcohol has not been characterized, and then this paper provides the first description of the (*R*)-specific *N*-benzyl-3-pyrrolidinone reducing enzyme.

2. Materials and methods

2.1. Chemicals

N-benzyl-3-pyrrolidinone and *N*-benzyl-3-pyrrolidinol were purchased from Sigma–Aldrich Japan, Tokyo. *N*-substituted-3-pyrrolidinones were synthesized from acrylic acid esters [8]. Glucose dehydrogenase was purchased from Amano Enzyme, Nagoya, Japan. All other chemicals used in this study were of analytical grade and commercially available.

2.2. Microorganisms and cultivation

Microorganisms were obtained from our laboratory collection: the collection of the National Institute of Technology

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and Evaluation of Japan (NBRC), and the American Type Culture Collection (ATCC), and our laboratory culture collection (KNK). The medium for yeast was composed of 0.7% KH_2PO_4, 1.3% $(NH_4)_2HPO_4$, 0.08% $MgSO_4{\cdot}7H_2O$, 0.007% $ZnSO_4{\cdot}7H_2O$, 0.009% $FeSO_4{\cdot}7H_2O$, 0.0005% $CuSO_4{\cdot}5H_2O$, 0.001% $MnSO_4{\cdot}4H_2O$, 0.01% NaCl, 0.3% yeast extract, and 4% glucose, pH 7.0. The medium for bacterium was composed of 1.0% meat extract, 1.0% polypeptone, 0.5% yeast extract, and 0.3% NaCl, pH 7.0. In the screening experiments, each strain was inoculated into 5 ml of medium in a test tube (24 mm Ø × 200 mm), followed by incubation at 30 °C with reciprocal shaking, usually for 24 h.

2.3. Screening method

Each reaction mixture, composed of cells from 5 ml of culture broth, 5 mg of *N*-benzyl-3-pyrrolidinone, 80 mg of glucose, and 1 ml of *n*-butyl acetate in 1 ml of 100 mM potassium phosphate buffer (pH 7.0), was shaken for 24 h at 30 °C. Ethyl acetate (5 ml) was added to the reaction mixture, followed by centrifugation. The organic layer was then analyzed to determine the yield and optical purity of the product.

2.4. Enzymatic reduction of N-benzyl-3-pyrrolidinone

Devosia riboflavina KNK10702 was cultured in 18 l of the above medium at 30 °C using a 30-l fermenter. Cells obtained from 5.2 l of the cultured broth by centrifugation were suspended in 110 ml of 100 mM potassium phosphate buffer (pH 6.5) and disrupted by ultrasonic disintegration (Branson cell disruptor model 450, Danbury, CT, U.S.A.) at 4 °C. After centrifugation, the resulting supernatant was used as the cell-free extract. The cell-free extract was stirred at 60 °C for 20 min, and the precipitate was discarded after centrifugation. The resulting supernatant solution was then brought to 60% saturation with ammonium sulfate and centrifuged again. The supernatant was then discarded, while the precipitate was dissolved in a 10 mM potassium phosphate buffer (pH 7.5) and dialyzed against the same buffer. A reaction mixture of 22.5 ml of the dialyzed solution, 750 mg of *N*-benzyl-3-pyrrolidinone, 1.5 g of glucose, 18 mg of NAD^+, 10 ml of *n*-butyl acetate, and 675 units of glucose dehydrogenase was stirred at 30 °C under nitrogen gas atmosphere.

2.5. Synthesis of (R)-N-benzyl-3-pyrrolidinol

The enzymatic reaction mixture (225 ml) was extracted twice with toluene. The organic layer was evaporated under a vacuum. The oily residue was distilled to obtain (*R*)-*N*-benzyl-3-pyrrolidinol (132–137 °C/3 mmHg, 6.75 g, 90% yield, more than 99% e.e.; NMR δ_H ($CDCl_3$, 400 MHz): 1.63–1.76 (1H, m), 2.09–2.21 (1H, m), 2.51–2.64 (2H, m), 2.75–2.85 (1H, m), 3.38 (1H, brs), 3.61 (2H, s), 4.24–4.33 (1H, m), 7.19–7.37 (5H, m); $[\alpha]_D^{20} + 3.73$ (c 5, CH_3OH). The reported value for the (*R*)-isomer is $[\alpha]_D^{20} + 3.73$ [5].

2.6. Enzyme assays and protein determination

Reductive reaction of the enzyme was assayed spectrophotometrically at 30 °C by the decrease in the absorbance of NADH at 340 nm. The reaction mixture containing 100 mM potassium phosphate buffer (pH 6.5), 5 mM of *N*-benzyl-3-pyrrolidinone as the substrate, 0.167 mM of NADH, and the enzyme solution in a total volume of 3 ml. One unit of the enzyme was defined as the amount catalyzing the oxidation of 1 μmol of coenzyme/min. Oxidative reaction of the enzyme was also measured at 340 nm in 3 ml of reaction mixture containing 100 mM potassium Tris–HCl buffer (pH 8.0), 5 mM of *N*-benzyl-3-pyrrolidinol as the substrate, 0.167 mM of NAD, and the enzyme solution. Specific activity was expressed as units per milligram of protein. Protein was measured using the protein-dye binding method using bovine serum albumin as a standard [9].

2.7. Purification of the reducing enzyme

The purification procedure was performed at 0–4 °C. *D. riboflavina* KNK10702 was cultured in 18 l of the above medium at 30 °C using a 30-l fermenter. Cells obtained by centrifugation from 7.2 l of the cultured broth were suspended with 500 ml of a 100 mM potassium phosphate buffer (pH 6.5) and disrupted by ultrasonic disintegration at 4 °C. After centrifugation, the resulting supernatant was used as the cell-free extract. The cell-free extract was stirred at 60 °C for 25 min, and the precipitate was discarded after centrifugation. The pH of the crude enzyme solution was adjusted to 7.0 with aqueous ammonia, solid of ammonium sulfate was added to attain 35% saturation, and the resultant precipitate was removed by centrifugation. With maintaining the pH at 7.0, solid of ammonium sulfate was further added and dissolved in the supernatant resulting from centrifugation to attain 55% saturation, and the resultant precipitate was collected by centrifugation. The precipitate was dissolved in 50 ml of 10 mM potassium phosphate buffer (pH 6.8), and the solution was then dialyzed overnight with the same buffer. The dialyzed solution was applied to the DEAE-Toyopearl 650 M column (Tosoh, Tokyo, Japan) and equilibrated with 10 mM potassium phosphate buffer (pH 8.0) to elute an active fraction with the same buffer. The active fraction was collected and added with phosphoric acid to adjust the pH to 7.0. Solid ammonium sulfate was dissolved in the crude enzyme solution obtained in the above chromatography to a final concentration of 1 M, and the solution was applied to a Phenyl-Toyopearl 650M column (Tosoh) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 1 M ammonium sulfate. The enzyme was eluted with an ammonium sulfate linear gradient solution (1–0 M). The active fractions were collected and dialyzed against 10 mM potassium phosphate buffer (pH 7.0) to obtain a purified enzyme preparation showing a single spot in SDS-polyacrylamide gel electrophoresis (Fig. 1).

2.8. Determination of the enzyme molecular mass

The molecular mass of the native enzyme was estimated by column chromatography using a TSK-GEL G3000

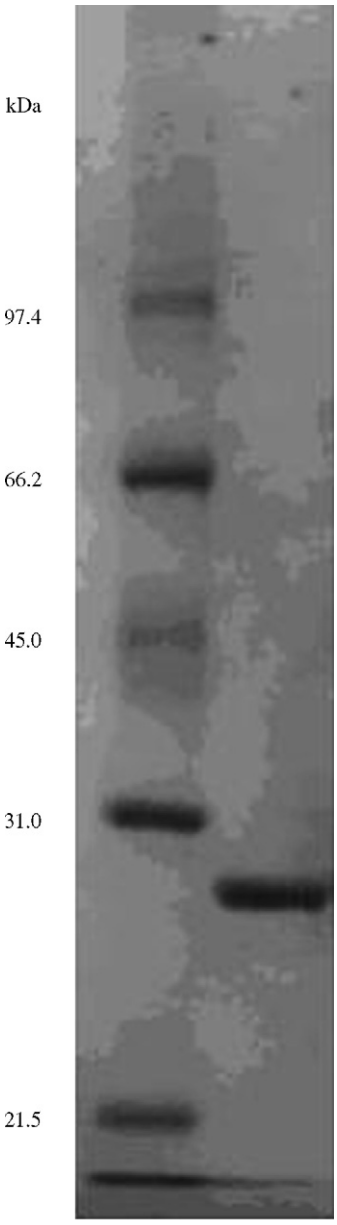


Fig. 1. SDS-PAGE of the purified reducing enzyme of *N*-benzyl-3-pyrrolidinone from *D. riboflavina*. Lane 1: molecular mass marker proteins; lane 2: purified enzyme (2 μg). The gel was stained with Coomassie Brilliant Blue.

SWXL column and a standard molecular marker with 50 mM potassium phosphate buffer (pH 7.0) containing 150 mM sodium chloride. The molecular mass of the subunit was estimated by SDS-polyacrylamide gel electrophoresis (10%) with an SDS-PAGE marker as the standard.

2.9. N-terminal amino acid sequence analysis

The *N*-terminal amino acid sequence was determined with a model ABI492 pulsed liquid protein sequencer equipped with an online phenylthiohydantoin analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

2.10. Determination of substrate specificity

The substrate specificity of the enzyme was determined spectrophotometrically by measuring the decrease in absorbance of NADH at 340 nm. The reaction conditions were the same as those for the enzyme assay system, except that different substrate and enzyme concentrations were used.

2.11. Reduction of various carbonyl compounds

Each reaction mixture comprising 2.5 mg of substrate, 14 mg of NADH, and the enzyme in 0.5 ml of 100 mM potassium phosphate buffer (pH 6.5) was stirred at 30 °C for 20 h. Ethyl acetate (5 ml) was added, and the reaction mixture was centrifuged. The organic layer was then analyzed for the optical purity of the product.

2.12. Analysis

The amounts of *N*-benzyl-3-pyrrolidinone and *N*-benzyl-3-pyrrolidinol were determined with a gas chromatograph equipped with a Uniport B 10% PEG-20 M (3.0 mm Ø × 1.0 m) column (GL Science, Tokyo). The optical purities of *N*-benzyl-3-pyrrolidinol and ethyl 4-chloro-3-hydroxybutyrate were determined using an HPLC equipped with a Chiralcel OB column (Daicel, Osaka, Japan). The optical purities of 7-metoxy-2-tetralol, 2-chloro-(*p*-fluorophenyl)ethanol, and 2-chloro-(*m*-chlorophenyl)ethanol were determined using an HPLC equipped with a Chiralcel OJ column (Daicel). The optical purities of *N*-benzyloxy-3-pyrrolidinol and *N*-*p*-nitrobenzyloxy-3-pyrrolidinol were determined using an HPLC equipped with a Chiralcel OF column and a Chiralpak AD-H column (Daicel), respectively. ^{1}H NHR spectra were recorded in $CDCl_3$ with an FT NMR JM-400 spectrometer (JEOL, Tokyo, Japan). Chemical shifts are expressed in parts/million (ppm), with tetramethylsilane as the internal standard. Optical rotation was measured with a SEPA-200 digital polarimeter (Horiba, Kyoto, Japan).

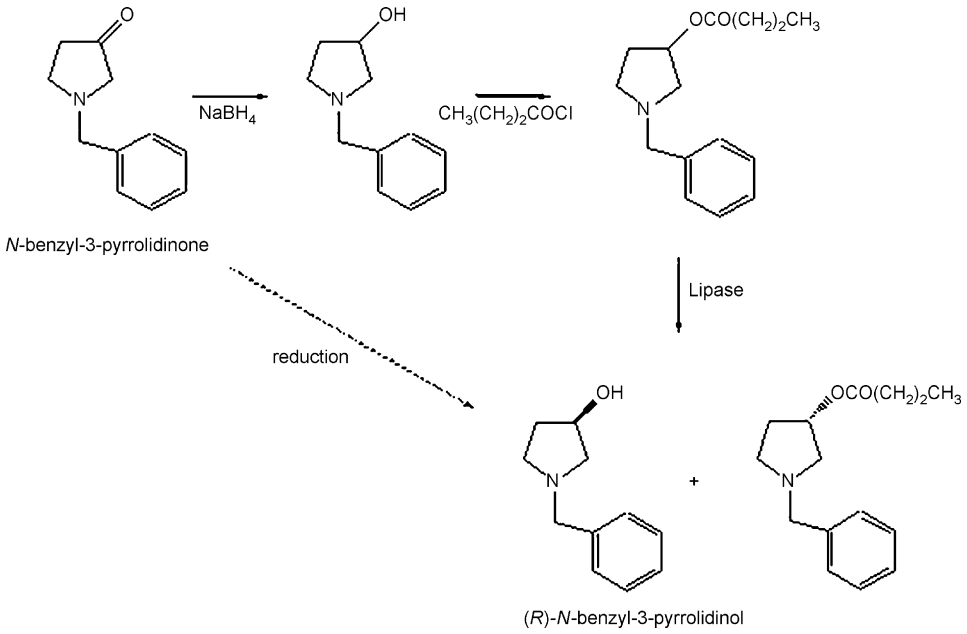
3. Results

3.1. Stability of the substrate

N-Benzyl-3-pyrrolidinone is an unstable compound in aqueous solution. Decomposition of the substrate in the reduction may result in a low yield and the formation of byproducts. The instability of *N*-benzyl-3-pyrrolidinone was found to be improved by addition of organic solvent to the aqueous solution to form a two-phase system (Fig. 2).

3.2. Screening of N-benzyl 3-pyrrolidinone reducing microorganism

The intact cell reaction was carried out in the presence of butyl acetate to avoid decomposition of the substrate. *N*-benzyl-3-pyrrolidinone reducing activity was found to be widely distributed in various microorganisms, although the activity was



Scheme 1. Synthesis of optically active *N*-benzyl-3-pyrrolidinol.

weak. Among over 700 strains tested, 22 strains accumulated *N*-benzyl-3-pyrrolidinol. All the stains accumulated (*S*)-isomer except *Pseudomonas diminuta* KNK10201. The stereoselectivity of the reduction carried out with the heat-treated cells of the six bacteria created (*R*)-rich selectiveity, as shown in Table 1. Of these, *D. riboflavina* KNK10702 and *P. diminuta* KNK10201 showed the highest stereoselectivity. The former strain, however, has higher stereoselectivity than the latter one. *D. riboflavina* was therefore selected as the reducing enzyme source.

Table 1
Screening of *N*-benzyl-3-pyrrolidinone reducing enzyme

Microorganism	Heating condition	Yield (%)	Stereoselectivity (%e.e.)	
Arthrobacter protophormiae NBRC12128	None	31	87.8	For (*S*)
	55 °C	2	76.0	For (*S*)
	60 °C	1	25.2	For (*R*)
Corynebacterium acetoacidophilum ATCC21476	None	55	93.5	For (*S*)
	55 °C	1	23.1	For (*R*)
	60 °C	0.4	44.3	For (*R*)
Devosia riboflavina KNK10702	None	4	29.1	For (*S*)
	55 °C	4	91.2	For (*R*)
	60 °C	6	99.3	For (*R*)
Erwinia carotovora KNK10103	None	26	85.4	For (*S*)
	55 °C	4	32.9	For (*S*)
	60 °C	2	15.8	For (*S*)
Micrococcus glutamicus ATCC13287	None	25	88.6	For (*S*)
	55 °C	17	82.2	For (*S*)
	60 °C	18	77.4	For (*S*)
Pseudomonas caryphilli NBRC12950	None	70	44.4	For (*S*)
	55 °C	2	16.4	For (*S*)
	60 °C	0.4	22.2	For (*R*)
Pseudomonas diminuta KNK10201	None	73	86.0	For (*R*)
	55 °C	73	96.8	For (*R*)
	60 °C	88	97.5	For (*R*)
Pseudomonas stutzeri KNK10301	None	17	60.1	For (*S*)
	55 °C	4	13.9	For (*S*)
	60 °C	3	4.0	For (*R*)

The assay conditions are given in Section 2.

Table 2
Purification of alcohol dehydrogenase from *D. riboflavina* KNK10702

Step	Total protein[a] (mg)	Total activity[a] (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-free extract	39,050	1549	0.040	100	1
Heating	11,350	1525	0.134	98	3
Acidification	8670	1471	0.170	95	4
Ammonium sulfate	4440	1454	0.327	94	8
DEAE-Toyopearl	290	860	2.97	56	74
Phenyl-Toyopearl	26	81	3.12	5	78

[a] The assay conditions are given in Section 2.

3.3. Enzymatic reduction of N-benzyl-3-pyrrolidinone

(*R*)-*N*-benzyl-3-pyrrolidinol was synthesized by using a crude enzyme solution of *D. riboflavina* prepared as described in Section 2. The reaction required NADH as a cofactor. NADH was regenerated by the addition of commercial glucose dehydrogenase and glucose. The enzyme could accumulate around 30 g/l (168 mM) of the alcohol. The turnover number of NADH for the alcohol production was around 170. The formed alcohol was extracted with organic solvent from the reaction mixture and purified by distillation. The optical purity of the formed alcohol showed 99.7%e.e.

3.4. Purification of the reducing enzyme

The *N*-benzyl-3-pyrrolidinone reducing enzyme was purified from *D. riboflavina* KNK10702 (Table 2). The enzyme was purified 78-fold to homogeneity with an overall recovery of 5%. As the enzyme was not absorbed on the resin, DEAE-Toyopearl, this column chromatography was an effective step for its purification. The molecular weight of the enzyme was found to be 58,000 by gel filtration. The relative molecular mass weight of the subunit was estimated to be approximately 28,000 on SDS-polyacrylamide gel electrophoresis.

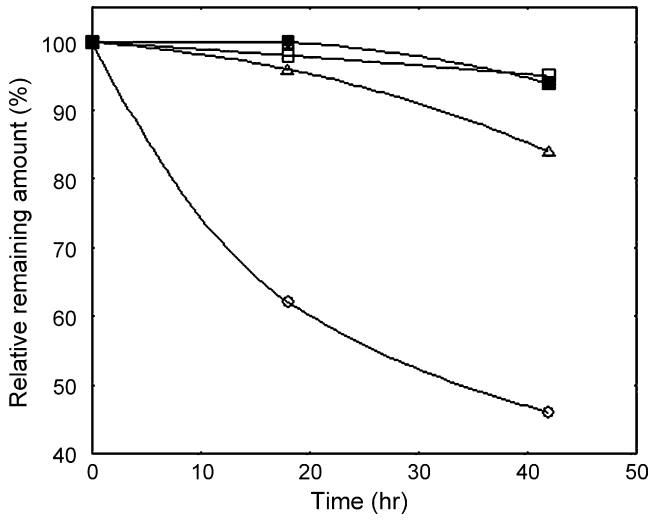


Fig. 2. Stabilities of *N*-benzyl-3-pyrrolidinone in an aqueous organic two-phase system. The remaining amount was measured after incubation in aqueous-organic solvent. Open circles: aqueous solution only; open squares: aqueous-*n*-butyl acetate; closed squares: aqueous-toluene; open triangles: aqueous-1-octanol.

3.5. N-terminal amino acid sequence analysis

Automated Edman degradation of the enzyme protein with a pulsed liquid phase sequencer showed that the *N*-terminal amino acid sequence was MQDFSGKVAFVTGGASGIGE. A BLAST program search found this sequence to be significantly similar to the partial amino acid sequences of some dehydrogenases.

Table 3
Effect of chemicals on the enzyme activity

Compound	Relative activity[a] (%)
None	100
EDTA 2Na	69
2,2'-Bipyridyl	89
Iodoacetate	110
1,10-Phenanthroline	94
5,5'-Dinitrobis(2-nitrobenzoate)	0
N-ethylmaleimide	89
2,4-Dinitrophenol	0
2-Mercaptoethanol	81
Dithiothreitol	94
$CaCl_2$	106
$CuSO_4$	167
$FeSO_4$	0
$MgSO_4$	94
$MnCl_2$	97
$ZnSO_4$	128
LiCl	109
$CoCl_2$	101
$NiCl_2$	119
$PbCl_2$	0
$BaCl_2$	0
$HgCl_2$	0
$AgNO_3$	63

[a] Enzyme activity was spectrophotometrically measured in the presence of additional test substance (1 mM) under the standard conditions.

Table 4
Substrate specificity of the alcohol dehydrogenase from *D. rivoflavina* for ketones

Substrate[a]	Relative activity (%)[b]	Substrate[a]	Relative activity (%)[b]
N-benzyl-3-pyrrolidinone	100	Diacetyl	1614
N-benzyl-4-piperidone	1070	Cyclopentanone	103
N-benzyl-3-piperidone	228	Dihydro-4,4-dimethyl-2,3-furandione	1314
N-benzyl-2-piperidone	0	Methyl pyruvate	1889
N-benzyl-2-pyrrolidinone	0	Ethyl acetoacetate	4236
Tetrahydrothiophene-3-one	8	Ethyl 4-chloroacetoacetate	683
3-Tetrahydrofuranone	2	Benzaldehyde	21
2-Acetylpyridine	13	Pyridine-3-aldehyde	82
3-Acetylpyridine	788	Pyridine-4-aldehyde	441
4-Acetylpyridine	6448	*p*-Chlorobenzaldehyde	117
2-Acetylpyrrole	70	o-Nitrobenzaldehyde	33
Acetophenone	181	*n*-Butyraldehyde	728
m-Chlorophenacyl chloride	463	Hexanal	2225
1-Phenyl-2-butanone	147	3-Phenylpropionaldehyde	685
Propiophenone	27	Methyl glyoxal	345
Benzoin	13	Glutaraldehyde	1339
Acetone	194	7-Metoxy-2-tetralone	7084
2-Butanone	280	2-Keto-butyric acid	15
2-Hexanone	5043	Oxalacetic acid	14
Chloroacetone	4170	Levulinic acid	11

Enzyme activity was measured as described in Section 2.
[a] The substrate concentration was 1 mM.
[b] To calculate the relative activity, the activity for *N*-benzyl-3-pyrrolidinone was taken as 100%.

Table 5
Substrate specificity of the alcohol dehydrogenase from *D. rivoflavina* for alcohols

Substrate[a]	Relative activity (%)[b]	Substrate[a]	Relative activity (%)[b]
N-benzyl-3-pyrrolidinol	100	Phenylethanol	15
Methanol	0	(*S*)-phenylethanol	0
Ethanol	0	1-Octanol	6
Propanol	0	1,2-Butanediol	0
Isopropanol	0	Cyclohexanol	0
1-Butanol	1	1,2-Pentanediol	1
2-Butanol	34	3-Chloro-1,2-propanediol	0
(*R*)-2-butanol	55	Galactose	0
(*S*)-2-butanol	6	Arabinose	0
2-Pentanol	45	Mannose	0
Glycerol	5	Glucose	0

Enzyme activity was measured as described in Section 2.
[a] The substrate concentration was 1 mM.
[b] To calculate the relative activity, the activity for *N*-benzyl-3-pyrrolidinone was taken as 100%.

3.6. Effects of temperature and pH

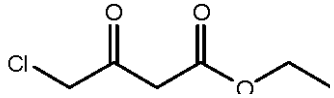
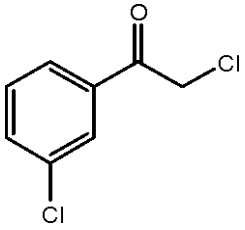
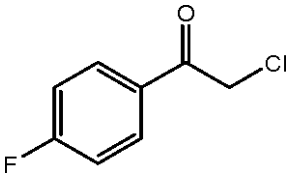
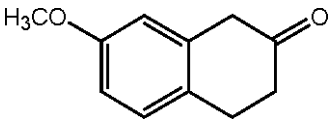
The enzyme activity was measured at various temperatures, with the optimum temperature being observed at 55 °C at pH 6.5: 36% activity at 30 °C, and 24% activity at 65 °C. The thermal stability of the enzyme was also examined by incubation for 1 h in the 90 mM potassium phosphate buffer (pH 6.5). The enzyme was stable below 30 °C and retained 30% activity at 45 °C and 0% activity at 50 °C. The effects of pH on the activity were measured in sodium acetate buffer (pH 4.0–5.7) and potassium

Table 6
Stereo selectivity for 3-pyrrolidinone derivatives of the alcohol dehydrogenase from *D. rivoflavina*

Protection group	Relative activity (%)	Stereoselectivity (%e.e.)		Yield (%)
Benzyl	100	99.7	For (*S*)	100
tert-Butoxycarbonyl	36	99.6	For (*S*)	100
Benzyloxycarbonyl	20	43.9	For (*S*)	80
p-Nitrobenzyloxycarbonyl	16	90.7	For (*S*)	50

Enzyme activity was measured as described in Section 2. The substrate concentration was 1 mM. Reaction was carried out for 5 h. To calculate the relative activity, the activity for *N*-benzyl-3-pyrrolidinone was taken as 100%.

Table 7
Stereoselectivity of *Devosia* dehydrogenase for various carbonyl compounds

Substrate	Optical purity (%e.e.)
	99
	99
	99
	99

The reaction conditions are given in Section 2.

phosphate buffer (pH 5.5–8.5). The enzyme showed maximum activity at pH 5.7.

3.7. Effects of chemicals

The effects of various compounds on the enzyme activity were examined by adding each compound to the reaction mixture. Metal ions such as Fe^{2+}, Pb^{2+}, Ba^{2+}, and Hg^{2+} inhibited the reaction (Table 3).

3.8. Substrate specificity

NADH was absolutely required as a cofactor; when NADH was replaced with NADPH, the reaction rate was reduced by 0.6%. Kinetic parameters were measured by Lineweaver-Burk plots. For *N*-benzyl-3-pyrrolidinone, K_m and V_{max} were 0.58 mM and 3.4 μmol/min/mg, respectively. As shown in Table 4, a broad range of carbonyl compounds was used to investigate substrate specificities. The enzyme reduced various kinds of aromatic and aliphatic aldehydes and ketones. In particular, β-ketoesters such as methyl acetoacetate and ethyl 4-bromoacetoacetate, and methylketone such as 4-acetylpyridine and hexanone were good substrates for the enzyme. Despite these results, *N*-benzyl-3-pyrrolidinine was not a good substrate compared to the substrates described above. *N*-benzyl-3-piperidone and *N*-benzyl-4-piperidone were also reduced by this enzyme. Cyclic amides such as *N*-benzyl-2-piperidone and *N*-benzyl-2-pyrrolidinone were not reduced. Thiophenone and furanone were reduced slightly. Other derivatives of 3-pyrrolidinone such as *N*-*tert*-butoxycarbonyl-3-pyrrolidinone were reduced by this enzyme (Table 6). This enzyme also oxidized secondary alcohols such as 2-butanol and 2-pentanol in the presence of NADH (Table 5). Primary alcohols and sugars, however, were not oxidized.

3.9. Stereoselectivity

The stereospecificities for some carbamates of 3-pyrrolidinone were studied. As shown in Table 6, *N*-*tert*-butoxycarbonyl-3-pyrrolidinone was reduced with a high enantioselectivity as well as *N*-benzyl-3-pyrrolidinone. When the nitrogen atom of the substrate was protected with a benzyloxycarbonyl group, the stereoselectivity of the enzyme was not high enough. Also the stereoselectivities for some useful carbonyl compounds for chiral industry were examined, and Table 7 shows the enantioselectivities of the enzymes.

4. Discussion

Optically active pyrrolidinone derivatives are useful versatile alcohols for synthesis of various kinds of chiral compounds. Enzymatic optical resolution of racemic compounds is convenient, as both isomers are prepared, however, the undesired isomer has been wasted if the demand of the isomers is not balanced. Asymmetric enzymatic reduction of prochiral ketone using a suitable redutase is a desirable method from an environmental perspective. An alcohol dehydrogenase was purified and characterized from *Geotrichum caspitatum* JCM3908 as the reducing enzyme *N*-benzyl-3-pyrrolidinone to (*S*)-*N*-benzyl-3-pyrrolidinone [7]. However, the reducing enzyme of *N*-benzyl-3-pyrrolidinone to (*R*)-form alcohol has not been characterized.

In this study, we searched for a reducing enzyme to convert *N*-benzyl-3-pyrrolidine to the optically active (*R*)-*N*-benzyl-3-pyrrolidinol. The microbial screening for the reductase was carried out by using a water–organic solvent two-phase system, as the substrate is not stable in an aqueous solution. This method is useful because the decomposition of the substrate can be avoided and even weak enzyme activity can be detected. Only one bacterium *P. diminuta* KNK10201 showed the (*R*)-specific reducing activity. Six bacteria were, however, identified as (*R*)-specific enzyme producers by a test of heat-treatment cells. In these microbes, the (*S*)-specific enzymes were inactivated by heating, and the tough enzyme in cells indicated (*R*)-specific activity. The use of heat-treated microbial cells in the screening experiment is an effective method for identifying stereoselective reductases because microbial cells often have several reductases with different stereoselectivities. The stereoselectivity of the reduction of *N*-benzyl-3-pyrrolidinone is an important factor because it is difficult to raise the optical purity of *N*-benzyl-3-pyrrolidinol in the following purification process. Then *D. riboflavina* KNK10702 which showed the highest stereselectivity was selected as the (*R*)-specific enzyme producer.

(*R*)-specific *N*-benzyl-3-pyrrolidinone reducing enzyme was purified to homogeneity on SDS-PAGE for the first time from the cell-free extract of *D. riboflavina* KNK10702 by sequential column chromatography. The purified enzyme consisted of

two subunits (28,000 Da). The *N*-terminal amino acid sequence of the enzyme was similar to putative dehydrogenases. The sequence contained a conservative glycine-rich domain (GXXXGXG) that is found in most of the short-chain dehydrogenases [10]. This enzyme also reduced *p*-nitrobenzaldehyde, *p*-chlorobenzaldehyde, and pyridine aldehyde, which are typical substrates for aldo-keto-reductase family enzymes [11]. The enzyme may indeed belong to such a short-chain dehydrogenase/reductase superfamily according to its molecular weight, its independence of metals, its response to some inhibitors, and the conservative domain in its *N*-terminus.

The *Devosia* enzyme was found to widely reduce various kinds of carbonyl compounds such as aliphatic ketones and aldehydes. In particular, 4-acetylpyridine, 2-hexanone, ethyl acetoacetate, and 7-methoxy-2-tetralone are good substrates for the enzyme compared with *N*-benzyl-3-pyrrolidinone. In addition, this enzyme has unique substrate selectivity for nitrogen-containing cyclic carbonyl compounds such as pyrrolidinone and piperidone. The carbonyl groups of the 3- and 4-positions for nitrogen atoms could be reduced, but that of the 2-position was not reduced. It also catalyzed oxidation of secondary alcohol to the corresponding ketone. But the stereoselectivity was not high, as both isomers of 2-butanol were oxidized.

Nitrogen-protected derivatives of 3-pyrrolidinone reductions by the *Devosia* dehydrogenase were examined. *N*-Boc (*tert*-butoxycarbonyl) compounds were reduced to (*R*)-form alcohol with over 99%e.e. This reaction is catalyzed by a bacterial NADH-dependent alcohol dehydrogenase from *Leifsonia* sp. [12], but low optically active *N*-Z (benzyloxycarbonyl)-3-pyrrolidinol is formed. It is worthwhile to synthesize various kinds of chiral nitrogen-protected derivatives of the pyrrolidinol, as each protecting group has special properties such as cleavage conditions. For example, the Boc group can be cleaved under acidic conditions, although the benzyl group can be cleaved by hydrogenation catalyzed by a metal. The substrate specificity and stereoselectivity for heterocyclic ketone may also provide important information.

The *Devosia* alcohol dehydrogenase could give some useful chiral alcohols with high optical purity from the corresponding carbonyl compounds. In particular, (*S*)-ethyl 4-chloro-3-hydroxybutyrate is a useful, representative chiral building block for synthesis of optically active pharmaceuticals [13]. Optically active 2-chloro-1-(3′-chlorophenyl)ethanol is also an important intermediate for pharmaceuticals [14]. The (*R*)-form of the compound is synthesized from the corresponding phenacyl chloride by enzymatic reduction. The *Devosia* enzyme gave (*S*)-form compound with over 99%e.e. Synthesis of the (*S*)-form compound by NADH-dependent *Leifsonia* dehydrogenase [12] and NADPH-dependent *Hansenula* keto-reductase [15] has been reported. The former enzyme has similar substrate specificity and stereoselectivity to *Devosia* enzyme, but some biochemical properties are different such as the number of subunits.

Some examples of an effective enzymatic reduction coupled with enzymatic coenzyme regeneration such as the use of glucose dehydrogenase have been reported. Enzymatic NADH regeneration can take place by not only glucose dehydrogenase, but also formate dehydrogenase or phosphite dehydrogenase [16], and recombinant cells over-producing two enzymes, reducing enzyme and NADH regenerating enzyme, will be powerful tools for the industrial asymmetric synthesis of chiral alcohols [17]. Some alcohol dehydrogenases can catalyze both asymmetric reduction and reduction of NAD^+ via cheap alcohols; in these cases, the second enzyme for NADH regeneration is not required [12], but *Devosia* alcohol dehydrogenase was found to not oxidize isopropanol to acetone.

Various kinds of versatile optically active compounds, not to mention *N*-benzyl-3-pyrrolidinol, may be produced by the suitable combination of this *Devosia* dehydrogenase and an enzymatic coenzyme regeneration system.

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