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Purification and Characterization of Alcohol Dehydrogenase Reducing *N*-Benzyl-3-Pyrrolidinone from *Geotrichum capitatum*

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(*S*)-*N*-Benzyl-3-pyrrolidinol is widely used in the synthesis of pharmaceuticals as a chiral building block. We produced 30 mM (*S*)-*N*-benzyl-3-pyrrolidinol (enantiometric excess > 99.9%) from the corresponding ketone *N*-benzyl-3-pyrrolidinone with more than 99.9% yield in 28 h of the resting-cell reaction of *Geotrichum capitatum* JCM 3908. NAD^+-dependent alcohol dehydrogenase reducing *N*-benzyl-3-pyrrolidinone from *G. capitatum* JCM 3908 was purified to homogeneity by ammonium sulfate fractionation and a series of DEAE-Toyopearl, Butyl-Toyopearl, Superdex 200, and Hydroxyapatite column chromatographies. The results of SDS–PAGE and HPLC showed the enzyme to be a dimer with a molecular mass of 78 kDa. The purified enzyme produced (*S*)-*N*-benzyl-3-pyrrolidinol (*e.e.* > 99.9%) from *N*-benzyl-3-pyrrolidinone. The enzyme reduced 2,3-butanedione, 2-hexanone, cyclohexanone, propionaldehyde, *n*-butylaldehyde, *n*-hexylaldehyde, *n*-octylaldehyde, *n*-valeraldehyde, and benzylacetone more effectively than it did *N*-benzyl-3-pyrrolidinone. No activity was detected towards *N*-benzyl-2-pyrrolidinone or 2-pyrrolidinone. The activity towards (*R*)-*N*-benzyl-3-pyrrolidinol was not detected under the assay conditions employed. The oxidizing activity of the enzyme was higher towards 2-propanol, 2-butanol, 2-pentanol, 2-hexanol, 3-hexanol, and 1-phenyl-2-propanol than towards (*S*)-*N*-benzyl-3-pyrrolidinol. The K_m values for *N*-benzyl-3-pyrrolidinone reduction and (*S*)-*N*-benzyl-3-pyrrolidinol oxidation were 0.13 and 8.47 mM, respectively. To our knowledge, this is the first time that an *N*-benzyl-3-pyrrolidinol/*N*-benzyl-3-pyrrolidinone oxidoreductase was purified from a eukaryote; moreover, this is the first report of (*S*)-*N*-benzyl-3-pyrrolidinol dehydrogenase activity in microorganisms. This enzyme showed features different from those of known prokaryotic *N*-benzyl-3-pyrrolidinone reductases. This enzyme will be very useful for the production of chiral compounds.

[**Key words:** *N*-benzyl-3-pyrrolidinol dehydrogenase, (*S*)-*N*-benzyl-3-pyrrolidinol production, *N*-benzyl-3-pyrrolidinone reductase, optically pure (*S*)-*N*-benzyl-3-pyrrolidinol, *Geotrichum capitatum*]

(*S*)-*N*-Benzyl-3-pyrrolidinol is an important enantiometric intermediate for optically active drugs including antitumor, anesthetic, antispasmodic, hepatotoxic, anti-inflammatory, and anti-HIV products. Two methods of synthesizing this compound chemically have been developed: synthesizing from optically active compounds such as hydroxyproline (Okada, Y. *et al*., Japanese patent application S60-23328, 1985), malate (1), and glutamate (2); and that from prochiral compounds such as 4-hydroxy-2-pyrrolidone (Iriuchijima, S. and Masuda, T., Japanese patent H01-207266, 1988) and aminobutanol relatives (Takahashi, S. *et al*., Japanese patent H03-176462, 1991).

Enzyme-catalyzed reactions are often highly enantioselective and regioselective. They can be carried out at ambient temperature and atmospheric pressure, avoiding the use of harsh conditions associated with chemical synthesis that can lead to isomerization, racemization, epimerization, and rearrangement of products.

Many microorganisms have been identified to produce (*S*)-*N*-benzyl-3-pyrrolidinol from *N*-benzyl-3-pyrrolidinone (Mochida, K. and Fujimoto, T., Japanese patent H06-141876, 1994; Yasohara, Y. and Hasegawa, J., Japanese patent H10-150997, 1998; Nikaido, T., Japanese patent 2005-117905, 2005). Enzymes that have the reducing activity of *N*-benzyl-3-pyrrolidinone to (*S*)-*N*-benzyl-3-pyrrolidinol were isolated from bacteria (9, 10), but never from eukaryotes. In the present study, we produced (*S*)-*N*-benzyl-3-pyrrolidinol from *N*-benzyl-3-pyrrolidinone using *Geotrichum capitatum* JCM 3908, and purified and characterized the *N*-benzyl-3-pyrrolidinol dehydrogenase from this strain.

MATERIALS AND METHODS

Chemicals Chemicals were obtained from usual commercial sources and used without further purification unless otherwise

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noted.

Microorganism and culture conditions *G. capitatum* JCM 3908 was supplied by Daicel Chemical Industries (Tokyo), which is a strain that has reducing activity of *N*-benzyl-3-pyrrolidinone (Nikaido, T., Japanese patent 2005-117905, 2005). *G. capitatum* JCM 3908 was cultivated in a 16.5×165-mm test tube containing 5 ml of a culture medium (pH 6.0) with 2% glucose, 0.5% Polypepton, 0.3% Bacto-malt extract, and 0.3% Bacto-yeast extract at 30°C with reciprocal shaking at 300 rpm for 1 d. It was transferred into 100 ml of the same medium in a 500-ml shaking flask. Cultivation was performed at 30°C with rotary shaking at 150 rpm for 1 d. Cells were harvested by centrifugation at 5200×*g* for 20 min and stored at −20°C until use for resting-cell reaction and purification of the enzyme.

(*S*)-*N*-Benzyl-3-pyrrolidinol production by resting-cell and enzyme reaction For the enantioselective reduction of *N*-benzyl-3-pyrrolidinone, harvested cells (10 mg as dry cell weight) were added to 1 ml of a reaction mixture containing 30 mM *N*-benzyl-3-pyrrolidinone in 50 mM potassium phosphate buffer (KPB; pH 6.5). For enzyme reaction, a reaction mixture contained 20 mM NAD^+, 60 mM glucose, 2 U of glucose dehydrogenase (Toyobo, Tokyo), 30 mM *N*-benzyl-3-pyrrolidinone, and 4 U of the purified enzyme in 600 mM KPB (pH 6.5). The reaction medium was purged with nitrogen gas before incubation to prevent the decomposition of *N*-benzyl-3-pyrrolidinone by oxygen. The reaction was performed at 30°C with reciprocal shaking at 300 rpm. The reaction medium was centrifuged at 8000×*g* and the supernatant was employed for the determination of reaction products by HPLC.

Detection of *N*-benzyl-3-pyrrolidinol and *N*-benzyl-3-pyrrolidinone *N*-Benzyl-3-pyrrolidinol and *N*-benzyl-3-pyrrolidinone concentrations were measured by HPLC. The sample was applied to a Hitachi L-7100 system (Tokyo) equipped with a column (Chiralcel OB-H, 0.46×15 cm; Daicel). Chromatography was carried out using *n*-hexane/isopropanol (19/1, v/v) as the solvent at a flow rate of 1 ml/min at 40°C, with detection at 220 nm using a photodiode array detector (Waters 996; Tokyo). The retention times of the substrates were as follows: (*R*)-*N*-benzyl-3-pyrrolidinol, 6.0 min; (*S*)-*N*-benzyl-3-pyrrolidinol, 8.0 min; and *N*-benzyl-3-pyrrolidinone, 11 min. Enantiometric excess (*e.e.*) and conversion rate were calculated from peak areas.

Enzyme assay *N*-Benzyl-3-pyrrolidinone reducing activity was measured in a standard assay mixture that contained 50 mM KPB (pH 6.0), 0.2 mM NAD(P)H, 3 mM *N*-benzyl-3-pyrrolidinone, and an appropriate amount of the enzyme solution in a total volume of 1 ml. The reaction mixture without *N*-benzyl-3-pyrrolidinone was used as the reference. Enzyme activity was assayed at 25°C by measuring the change in NAD(P)H absorbance at 340 nm. One unit of the enzyme is defined as the amount of the enzyme that catalyzes a change of 1 μmol of NAD(P)H per min, using a molar absorption coefficient of 6220 $M^{-1} \cdot cm^{-1}$ at 340 nm. During purification, *N*-benzyl-3-pyrrolidinone reducing activity was monitored using NADH as a cofactor. (*S*)-*N*-benzyl-3-pyrrolidinol oxidizing activity was determined in the reaction mixture that contained 10 mM (*S*)-*N*-benzyl-3-pyrrolidinol, 2 mM NAD^+, and an appropriate amount of the enzyme in 50 mM KPB (pH 8.0). The K_m values for *N*-benzyl-3-pyrrolidinone, (*S*)-*N*-benzyl-3-pyrrolidinol, NADH, and NAD^+ were calculated from Lineweaver–Burk plots. Enzyme activity was determined as described above except that various concentrations of *N*-benzyl-3-pyrrolidinone, (*S*)-*N*-benzyl-3-pyrrolidinol, NADH, and NAD^+ were used.

Analyses Protein concentration was determined using a commercial protein assay kit (Nippon Bio-Rad Laboratories, Tokyo) with bovine serum albumin as a standard. The molecular mass of the purified enzyme was determined using an HPLC system (LC-10AD; Shimadzu, Kyoto) equipped with a TSKgel G3000SWXL (0.78×30 cm; Tosoh, Tokyo) and a TSK guard column SWXL. The solvent was 100 mM KPB (pH 7.0) and its flow rate was 1.0 ml/min. The molecular mass of the purified enzyme was estimated by comparing its retention time with those of marker proteins (Boehringer, Mannheim, Germany). To determine the molecular masses of the subunits of the purified enzyme, SDS–PAGE was carried out in a 12.5% gel (3), and the protein was stained with Coomassie Brilliant Blue R-250. The amino acid residues of the N-terminal region of the purified enzyme were analyzed using a protein sequencer as described previously (4).

Purification of enzyme The enzyme was purified at 4°C. Frozen cells (180 g as wet weight) were thawed and mixed with 400 ml of glass beads (diameter, 0.25–0.5 mm), and the mixture was suspended in 600 ml of buffer A (50 mM Tris–HCl buffer [pH 8.0] containing 1 mM 2-mercaptoethanol). The cells were disrupted using a homogenizer (Bead-beater model 11079S; Biospec Products, Bartlesville, OK, USA). The homogenate was centrifuged at 8000×*g* for 30 min to remove unbroken cells and debris. The supernatant was used as the cell extract. Solid ammonium sulfate was added to the supernatant, and the 50%-saturated precipitate was discarded. After solid ammonium sulfate was added onto the supernatant up to 70% saturation, the precipitate was dissolved in 3 ml of buffer A. The supernatant was dialyzed against buffer A and centrifuged to remove the precipitate. The supernatant was applied onto a DEAE-Toyopearl column (2.2×30 cm; Tosoh) equilibrated with buffer A. After washing with buffer A, the active fraction was eluted with NaCl in a linear gradient from 0 to 0.5 M in Buffer A. Solid ammonium sulfate was added onto the active fraction up to 40% saturation, and the supernatant was applied to a Butyl-Toyopearl column (1.6×10 cm; Tosoh) equilibrated with buffer A containing 40% ammonium sulfate. After washing with the same buffer, the enzyme was eluted with ammonium sulfate in a linear gradient from 40 to 0% in buffer A. The active solution was dialyzed in Buffer A containing 0.1 M NaCl and concentrated using an ultrafiltration membrane (Centricon YM30; Nippon Millipore, Tokyo). The dialyzed solution was applied to a Hiload 16/60 Superdex 200 pg column (1.6×30 cm; GE Health Care Biosciences, Tokyo) equilibrated with the same buffer. The active fraction eluted with the same buffer was dialyzed against 10 mM of Buffer B (KPB, pH 7.0 containing 1 mM 2-mercaptoethanol), and applied onto a Hydroxyapatite column (0.64×3 cm; Nippon Bio-Rad Laboratories). After washing with buffer B, the active fraction was eluted with buffer B in a linear gradient from 0.01 to 0.5 M and precipitated by adding ammonium sulfate. The 80%-saturated precipitate was dissolved in 1 ml of 100 mM buffer B.

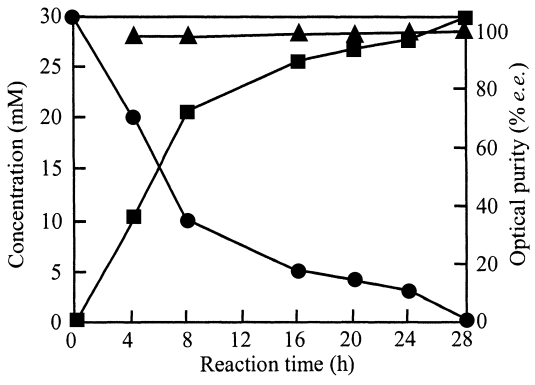

FIG. 1. (*S*)-*N*-Benzyl-3-pyrrolidinol production from *N*-benzyl-3-pyrrolidinone by *G. capitatum* JCM 3908. Circles, *N*-Benzyl-3-pyrrolidinone; squares, (*S*)-*N*-benzyl-3-pyrrolidinol; triangles, optical purity.

TABLE 1. Purification of *N*-benzyl-3-pyrrolidinol dehydrogenase from *G. capitatum* JCM 3908

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Cell-free extract	360	11000	0.033	1	100
Ammonium sulfate	330	5300	0.062	2	92
DEAE-Toyopearl	47	44	1.1	33	13
Butyl-Toyopearl	6.4	1.8	3.6	110	1.8
Superdex 200	2.5	0.45	5.6	170	0.7
Hydroxyapatite	0.87	0.028	31	940	0.2

RESULTS AND DISCUSSION

G. capitatum JCM 3908 produced (*S*)-*N*-benzyl-3-pyrrolidinol (*e.e.*>99.9%) from 30 mM *N*-benzyl-3-pyrrolidinone with more than 99.9% yield (Fig. 1) under anaerobic conditions in the resting-cell reaction. *N*-Benzyl-3-pyrrolidinone reducing activity was detected when NADH was used as a cofactor in the cell extract of *G. capitatum* JCM 3908 grown on the culture medium, whereas NADPH did not work. The *N*-benzyl-3-pyrrolidinone-reducing enzyme was purified 940-fold from the cell extract (Table 1). The active fraction showed a single peak on a gel permeation chromatogram (Fig. 2a), and the molecular mass of the enzyme was estimated to be 78 kDa (Fig. 2b). The molecular masses of the enzyme subunits were 39 kDa and 41 kDa (Fig. 2c). The N-terminal amino acid residues of these subunits could not be sequenced, suggesting that they might be blocked.

The purified enzyme catalyzed the conversion of *N*-benzyl-3-pyrrolidinone to (*S*)-*N*-benzyl-3-pyrrolidinol (*e.e.*> 99.9%) (Fig. 3). The enzyme activity was optimized at 30°C and pH 7 in McIlvaine buffer (Fig. 4). $ZnSO_4$, $HgCl_2$, $CuCl_2$, and $NiCl_2$ were strongly inhibitory (Table 2). $MgSO_4$, $BaCl_2$, $CaCl_2$, $MnCl_2$, and LiCl at 1 mM had almost no effect on the activity. 2,2′-Bipyridyl and *o*-phenanthroline, which are inhibitors of Fe^{2+}- or Cu^{2+}-enzymes, decreased the enzyme activity up to 22% and 0%, respectively, whereas EDTA and sodium azide were not strongly inhibitory. An SH reagent, *p*-chloromercuribenzoate, was strongly inhibitory at 0.1 mM, whereas 5,5′-dithiobis(2-nitrobenzoic acid) at 0.05 mM was not.

The purified enzyme showed greater activity towards aliphatic ketone and aldehyde than towards *N*-benzyl-3-pyrrolidinone (Table 3). No activity was detected towards *N*-benzyl-2-pyrrolidinone or 2-pyrrolidinone. The purified enzyme preferably reduced cyclohexanone compared with *N*-benzyl-3-pyrrolidinone. 2-Pentanol and 2-hexanol oxidizing activities of the enzyme were 9- and 11-fold the (*S*)-*N*-benzyl-3-pyrrolidinol oxidizing activity of the enzyme, respectively. Compounds such as 2-propanol, 2-butanol, 3-hexanol, 2,5-hexanediol, and 1-phenyl-2-propanol were also good substrates.

The K_m values for *N*-benzyl-3-pyrrolidinone and NADH

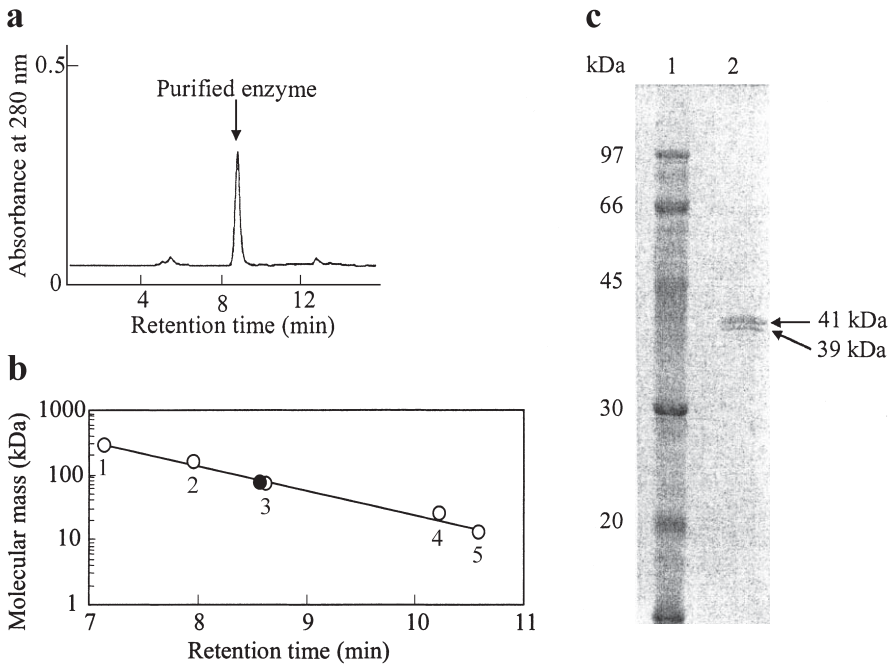

FIG. 2. (a) Gel permeation chromatogram of purified enzyme. (b) Molecular mass calibration pattern. Closed circle, Purified enzyme. Open circles, Marker proteins: 1, glutamate dehydrogenase (290 kDa); 2, glucose dehydrogenase (160 kDa); 3, bovine serum albumin (68 kDa); 4, chymotrypsinogen A (25 kDa); 5, cytochrom *c* (12.5 kDa). (c) SDS–PAGE for determining molecular mass of purified enzyme subunits. Lane 1, Marker proteins: phosphorylase *b* (subunit molecular mass, 97 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (20.1 kDa). Lane 2, Purified enzyme.

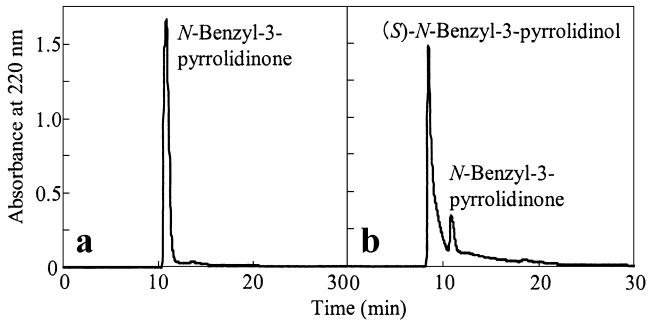

FIG. 3. HPLC pattern of products of enantioselective reduction of *N*-benzyl-3-pyrrolidinone by purified enzyme. (a) Before enzyme reaction; (b) after 15-h enzyme reaction.

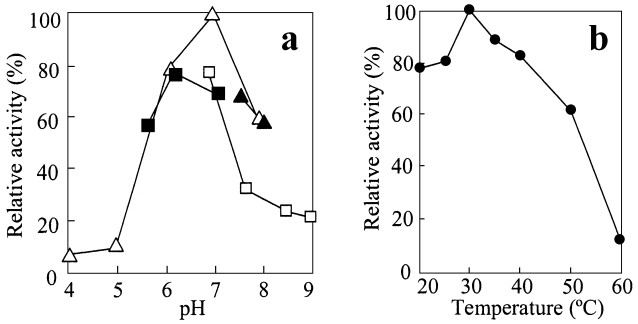

FIG. 4. Effect of pH (a) and temperature (b) on *N*-benzyl-3-pyrrolidinone reducing activity of purified enzyme. *N*-Benzyl-3-pyrrolidinone reducing activity was measured as described in Materials and Methods except that different buffers and temperatures were used. For determining of the effect of pH on activity, 50 mM McIlvaine buffer (open triangles), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–NaOH buffer (closed triangles), Tris–HCl buffer (open squares), and MES (2-[*N*-morpholino]ethanesulfonic acid)–NaOH buffer (closed squares), were used instead of 50 mM KPB (pH 6.0). For determining the effect of temperature on activity, enzyme activity was measured at 20–60°C in the standard assay mixture. The activity is expressed as the value relative to the highest activity at various pHs and temperatures.

were 0.13 and 0.87 mM at pH 6.0, respectively. For oxidation, the K_m values for (*S*)-*N*-benzyl-3-pyrrolidinol and NAD^+ were 8.47 and 0.79 mM at pH 8.0, respectively.

Our enzyme produced (*S*)-*N*-benzyl-3-pyrrolidinol by stereoselective reduction of *N*-benzyl-3-pyrrolidinone. Enzymes from *Micrococcus luteus* (Kizaki, N. *et al*., WO 02/10399, 2002) and *Corynebacterium pseudodiphteriticum* (Asako, H. *et al*., Japanese patent 2004-350625, 2004) were also reported to have this activity. *Corynebacterium*'s enzyme was originally reported as a phenylacetaldehyde reductase (5). Our enzyme was inhibited by Cu^{2+}, as were *M. luteus*'s and *Corynebacterium*'s enzymes. Metal ions such as Ni^{2+} and Hg^{2+} also strongly inhibited our enzyme as well as *Corynebacterium*'s enzyme. The enzyme from *M. luteus* requires NADPH as a cofactor, but not NADH. The cofactor required for our enzyme and *Corynebacterium*'s enzyme is the opposite. The ranges of optimum pH and temperature for the reduction of *N*-benzyl-3-pyrrolidinone by *M. luteus*' enzyme are 4.5 to 5.5 and 40°C to 45°C, respectively, whereas those by our enzyme were 6.0 to 7.0 and 30°C, respectively. *Corynebacterium*'s enzyme showed maximum activity at pH

TABLE 2. Effects of metal salts and inhibitors on enzyme activity

Compounds	Relative activity (%)
None	100
$ZnSO_4$	ND
$MgSO_4$	99
$HgCl_2$	ND
$FeCl_3$	87
$CuCl_2$	ND
$BaCl_2$	109
$CoCl_2$	63
$CaCl_2$	104
$MnCl_2$	101
$FeCl_2$	93
$MgCl_2$	90
LiCl	104
$NiCl_2$	ND
2,2′-Bipyridyl	22
o-Phenanthroline	ND
EDTA	96
Sodium azide	99
Potassium cyanide	106
Iodoacetic acid	90
p-Chloromercuribenzoate	ND
5,5′-Dithiobis(2-nitrobenzoic acid)	100

Enzyme reducing activity was determined as described in the Materials and Methods except that metal salts or inhibitors were added. Each metal salt or inhibitor, except for $FeCl_3$, $FeCl_2$, *p*-chloromercuribenzoate, and 5,5′-dithiobis(2-nitrobenzoic acid), was added at 1 mM. $FeCl_3$, $FeCl_2$, and *p*-chloromercuribenzoate were added at 0.1 mM; 5,5′-dithiobis(2-nitrobenzoic acid) at 0.05 mM. The activity is expressed as the value relative to that observed without metal salts or inhibitors. ND, Not detected.

values between 6 and 6.5. No optimum temperature was reported. *M. luteus*'s enzyme has a molecular weight of 29 kDa and is composed of one subunit; *Corynebacterium*'s enzyme has a molecular weight of 155 kDa and is composed of four subunits; our enzyme had a molecular weight of 78 kDa and was composed of two subunits. Our enzyme oxidized (*S*)-*N*-benzyl-3-pyrrolidinol. (*S*)-*N*-benzyl-3-pyrrolidinol oxidizing activity has never been reported before. Our enzyme may catalyze the production of (*R*)-*N*-benzyl-3-pyrrolidinol from the racemate of *N*-benzyl-3-pyrrolidinol after the removal of the (*S*)-isomer by stereoselective oxidation. Our enzyme reduced cyclohexanone, which has not been reported for *M. luteus*'s and *C. pseudodiphteriticum*'s enzymes. *Corynebacterium*'s enzyme has reducing activity towards 2-hexanone that was comparable to that of our enzyme, but not towards propionaldehyde and *n*-butylaldehyde, which differs from our enzyme. Our enzyme showed a broad substrate range and had different features from those of bacterial origin. *N*-Benzyl-3-pyrrolidinol dehydrogenase from *G. capitatum* JCM 3908 might be a promising tool for the production of chiral compounds. The stereospecificity of our enzyme for chiral compounds other than *N*-benzyl-3-pyrrolidinol will be investigated in our future studies.

ACKNOWLEDGMENTS

We are grateful to Dr. Yamamoto for valuable advice and kindly providing *G. capitatum* JCM 3908.

TABLE 3. Substrate specificity of purified enzyme

Substrate	Relative activity (%)
For reduction	
N-Benzyl-3-pyrrolidinone	100
N-Benzyl-2-pyrrolidinone	ND
2-Pyrrolidinone	ND
2-Butanone	40
2,3-Butanedione	161
2-Hexanone	159
3-Hexanone	ND
3,4-Hexanedione	77
Cyclohexanone	212
Propionaldehyde	112
n-Butylaldehyde	182
n-Hexylaldehyde	163
n-Octylaldehyde	167
n-Valeraldehyde	277
Benzaldehyde	74
Hydoxyacetone	27
1-Hydroxy-2-butanone	59
4-Hydroxy-2-butanone	ND
Acetylacetone	63
Benzylacetone	128
Acetophenone	40
For oxidation	
(*S*)-*N*-Benzyl-3-pyrrolidinol	100
(*R*)-*N*-Benzyl-3-pyrrolidinol	ND
racemic-*N*-Benzyl-3-pyrrolidinol	33
Ethanol	ND
2-Propanol	312
1,3-Propanediol	ND
1-Butanol	ND
2-Butanol	374
1,2-Butanediol	35
1,3-Butanediol	39
1,4-Butanediol	ND
2,3-Butanediol	83
1,2,3-Butanetriol	ND
1,2,4-Butanetriol	ND
Acetol	ND
1-Pentanol	8
2-Pentanol	895
1,2-Pentanediol	12
2,4-Pentanediol	21
1-Hexanol	15
2-Hexanol	1121
3-Hexanol	184
1,2-Hexanediol	20
2,5-Hexanediol	102
1-Phenyl-1-propanol	31
1-Phenyl-2-propanol	161
2-Phenyl-1-propanol	ND
3-Phenyl-1-propanol	ND
Benzyl alcohol	ND
Glycerol	ND

Reducing and oxidizing activities were determined as described in Materials and Methods except that each substrate was used instead of *N*-benzyl-3-pyrrolidinone and (*S*)-*N*-benzyl-3-pyrrolidinol at 10 mM except for benzaldehyde and benzylacetone. Benzaldehyde and benzylacetone were added at 5 mM. Except for (*S*)- and (*R*)-*N*-benzyl-3-pyrrolidinol, the racemate of chiral compounds was used as a substrate. Activity is expressed as the value relative to that observed using *N*-benzyl-3-pyrrolidinone and (*S*)-*N*-benzyl-3-pyrrolidinol for reduction and oxidation, respectively. ND, Not detected.

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