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Purification and Characterization of a Novel (*R*)-Imine Reductase from *Streptomyces* sp. GF3587

Koichi MITSUKURA,† Mai SUZUKI, Sho SHINODA, Tatsuya KURAMOTO, Toyokazu YOSHIDA, and Toru NAGASAWA

Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

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The (*R*)-imine reductase (RIR) of *Streptomyces* sp. GF3587 was purified and characterized. It was found to be a NADPH-dependent enzyme, and was found to be a homodimer consisting of 32 kDa subunits. Enzymatic reduction of 10 mM 2-methyl-1-pyrroline (2-MPN) resulted in the formation of 9.8 mM (*R*)-2-methylpyrrolidine ((*R*)-2-MP) with 99% e.e. The enzyme showed not only reduction activity for 2-MPN at neutral pH (6.5–8.0), but also oxidation activity for (*R*)-2-MP under alkaline pH (10–11.5) conditions. It appeared to be a sulfhydryl enzyme based on the sensitivity to sulfhydryl specific inhibitors. It was very specific to 2-MPN as substrate.

Key words: (*R*)-imine reductase; *Streptomyces* sp. GF3587; 2-methyl-1-pyrroline; 2-methylpyrrolidine

Recently the catalytic synthesis of chiral amines has received much attention due to wide application as pharmaceutical and agrochemical intermediates. Various catalysts have been developed for the asymmetric reduction of imines, enamines, and oximes, and applications have been reported.[1,2] As for biocatalysts, hydrolases and ω-transaminases have been examined for the synthesis of optically active primary amines.[3,4] Recently, efficient production systems have been designed using ω-transaminase coupled with amine oxidase, amino acid dehydrogenase, lactate dehydrogenase, pyruvate decarboxylase, or α-transaminase.[5,6] As for optically active cyclic secondary amines, optically active pyrrolidine and piperidine derivatives were synthesized from their corresponding racemic oxalamic esters through steps of protease-catalyzed hydrolysis and chemical deprotection.[7] In the enantioselective synthesis of cyclic tertiary amines, monoamine oxidase variant N-5 (MAO-N-5), which oxidizes one enantiomer of racemic cyclic tertiary amine to the corresponding imine, was developed by directed evolution of MAO-N from *Aspergillus niger*.[8,9] The cyclic imine thus formed was reduced to racemic amine with ammonia borane. Through repetition of the consecutive reaction process, the racemic cyclic tertiary amines was converted to enantiomerically-pure tertiary amines.[10]

Enzymatic asymmetric reduction of imine is a promising alternative for chiral amine synthesis, but there have been no reports of stereospecific imine reductase. It is not easy to carry out screening of imine reductase using imines, because most imines are very labile in aqueous media. Recently, however, asymmetric reduction of aryl imines with whole cells of *Candida parapsilosis* ATCC 7330, which show carbonyl reductase activity, has been reported.[11] Although the *Candida* enzyme showed high (*R*)-selectivity, the productivity of chiral amine using whole cells was very low.

In a previous study,[12] we attempted to survey a novel imine reductase with high activity and excellent enantioselectivity using a cyclic imine, 2-methyl-1-pyrroline (2-MPN), which is extremely stable in neutral aqueous media. We found (*R*)- and (*S*)-selective imine-reducing activity in cells of *Streptomyces* sp. GF3587 and GF3546 respectively. (*R*)-2-Methylpyrrolidine ((*R*)-2-MP) and (*S*)-2-MP were synthesized from 2-MPN with 99.2% e.e and 92.3% e.e using the whole-cell catalysts of strains GF3587 and GF3546 respectively.[12] In the present study, we focused on the purification and characterization of the (*R*)-imine reductase (RIR) from *Stretptomyces* sp. GF3587 (Fig. 1).

Materials and Methods

Chemicals. 2-Methyl-1-pyrroline (2-MPN), racemic, (*R*)- and (*S*)-2-methylpyrrolidine (2-MP), and an assay kit for dihydrofolate reductase (DHFR) activity were purchased from Sigma-Aldrich (Tokyo). The other chemicals used were of guaranteed reagent grade.

Cultivation. Streptomyces sp. GF3587 was cultivated for 24 h at 28 °C with reciprocal shaking (115 strokes/min) in a nutrient medium (400 mL) containing 0.8% w/v glucose, 2% w/v malt extract (Difco, New Jersey, USA), 1% w/v NZ amine (Wako Pure Chemical Industries, Osaka, Japan), and 0.0005% w/v $FeSO_4 \cdot 7H_2O$ at pH 7.3. Cells, harvested by centrifugation at 16,000 *g* for 10 min at 4 °C, were stored at −30 °C until just before use.

Enzyme assay. Imine reductase activity was assayed in a quartz cuvette (1-cm path) by measuring the oxidation of NADPH (molecular extinction coefficient, 6,200/M/cm) at 340 nm (A_{340}) at 30 °C. The reaction mixture (1 mL) consisted of 10 mM 2-MPN or an other tested compound, 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM NADPH, and an appropriate amount of enzyme. The enzyme reaction was started by the addition of the enzyme solution, and the value of A_{340} was measured at 30 °C for 2 min with UV1600PC (Shimadzu, Tokyo, Japan). One unit of enzyme was defined as the amount that catalyzes the oxidation of 1 μmol of NADPH per min. Reduction activity for 2-MPN was also examined using human DHFR in a

† To whom correspondence should be addressed. Tel: +81-58-293-2649; Fax: +81-58-293-2794; E-mail: mitukura@gifu-u.ac.jp
Abbreviations: 2-MPN, 2-methyl-1-pyrroline; 2-MP, 2-methylpyrrolidine; RIR, (*R*)-imine reductase

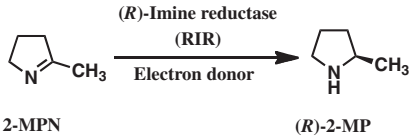

Fig. 1. Asymmetric Reduction of 2-MPN Using RIR in the Presence of an Electron Donor.

manner similar to that described above. The kinetic constants K_m of the enzyme for 2-MPN and NADPH were determined from the double reciprocal plots of the substrate concentration against the reduction or oxidation reaction rate under standard assay conditions.

Enzyme purification. Throughout purification, potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol (DTT) was used, unless otherwise specified, and all purification steps were performed at 4 °C. The enzyme was dialyzed against the various equilibration buffers at the next chromatographic purification step. Cells harvested from 4 L of culture broth were washed with 100 mM of the buffer and suspended in the same buffer. Cell suspension was disrupted by ultra-sonication (Kubota Insonator 201M, Tokyo, Japan) for 20 min. After centrifugation for 30 min at 16,000 *g*, the supernatant was used as a cell-free extract. The protein concentration was determined by the Bradford method[13] using bovine serum albumin as standard.

Step 1: Ammonium sulfate fractionation

Fractionation of the cell-free extract was carried out with ammonium sulfate (40–70% saturation) by adjustment to pH 7.0 with ammonium solution.

Step 2: Affinity column chromatography

The precipitate was suspended in phosphate buffer, and then the suspension was dialyzed against the same buffer. The enzyme solution was loaded on a 2′5′-ADP Sepharose 4B column (20 × 90 mm; GE Healthcare, Tokyo, Japan) equilibrated with 100 mM of the buffer. After the column was washed with the buffer, the enzyme was eluted with the same buffer containing 0.5 mM $NADP^+$.

Step 3: Hydrophobic column chromatography

The eluted proteins were loaded on a butyl-Toyopearl column (10 × 120 mm; Tosoh, Tokyo, Japan) equilibrated with 100 mM of the buffer containing 30% saturated ammonium sulfate. The column was washed with the same buffer, and the enzyme was eluted by decreasing the concentration of ammonium sulfate from 30% to 15% saturation. The enzyme was eluted with 100 mM of the buffer containing 10% saturated ammonium sulfate.

SDS–PAGE and gel-permeation HPLC. To determine the purity and subunit molecular mass of the enzyme, SDS–PAGE was carried out using 10% w/v polyacrylamide gel by Laemmli's method.[14] The subunit molecular mass of the purified enzyme was calculated using a linear regression curve obtained from its mobility and the molecular masses of phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The native molecular mass of the enzyme was estimated by gel-permeation HPLC using a TSK G-3000SW column (7.5 × 600 mm; Tosoh, Japan) at 0.7 mL/min with 100 mM sodium phosphate buffer (pH 7.0) containing 200 mM NaCl as eluent. The molecular mass of the purified enzyme was calculated using a linear regression curve obtained from its mobility, and the molecular masses of glutamate dehydrogenase (292 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome *c* (12.4 kDa).

N-Terminal amino acid sequence analysis. The purified RIR was transferred to a PVDF membrane by Western blotting. The N-terminal amino acid sequence of the purified RIR was determined by the Procise 494 HT Protein Sequencing system by Apro Science (Tokushima, Japan).

Enzymatic reduction of 2-MPN. The reaction mixture (0.5 mL) comprised 10 mM 2-MPN, 100 mM potassium phosphate buffer (pH 7.0), 15 mM NADPH, and 0.032 units of the purified enzyme. After incubation at 30 °C, the reaction mixture was heat-treated for

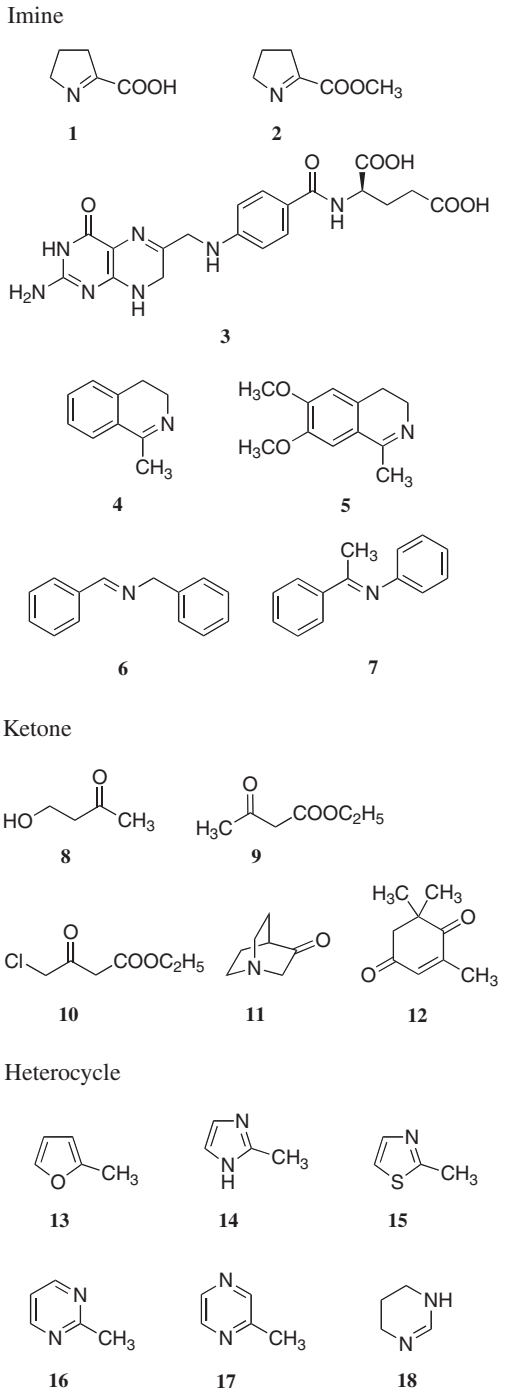

Fig. 2. Tested Compounds.

Imines; **1**, Δ^1-Pyrroline-2-carboxylic acid; **2**, Methyl Δ^1-pyrroline-2-carboxylate; **3**, Dihydrofolic acid; **4**, 1-Methyl-3,4-dihydroisoquinoline; **5**, 1-Methyl-6,7-dimethoxy-3,4-dihydroisoquinoline; **6**, *N*-Benzylidenebenzylamine; **7**, (*E*)-*N*-(1-Phenylethylidene)benzeneamine Ketone; **8**, 4-Hydroxy-2-butanone; **9**, Ethyl 3-oxobutanoate; **10**, Ethyl 4-chloro-3-oxobutanoate; **11**, 3-Quinoqulidinone; **12**, 2,2,6-Trimethyl-2-cyclohexen-1,4-dione (Ketoisophorone) Heterocycle; **13**, 2-Methylfuran; **14**, 2-Methylimidazole; **15**, 2-Methylthiazole; **16**, 2-Methylpyrimidine; **17**, 2-Methylpyrazine; **18**, 1,4,5,6-Tetrahydropyrimidine.

1 min at 100 °C and then centrifuged at 10,000 *g*. Derivatization of the supernatant using 2,3,4,6-tetra-*O*-acetyl-b-D-glucopyranosyl isothiocyanate (GITC) was performed for 1 h at 40 °C, and then the concentration and the optical purity of 2-MP were determined by HPLC, as described previously.[12]

Effects of temperatures, pH values, metal ions, and inhibitors. Imine reductase activity was examined using an appropriate amount of the

Table 1. Purification of RIR from *Streptomyces* sp. GF3587

Step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	644	84.3	0.131	100	1
$(NH_4)_2SO_4$ 40–70%	334	96.7	0.290	115	2.2
2′,5′-ADP Sepharose 4B	7.4	54.4	7.35	64	56
Butyl-Toyopearl	1.1	11.2	10.2	13	78

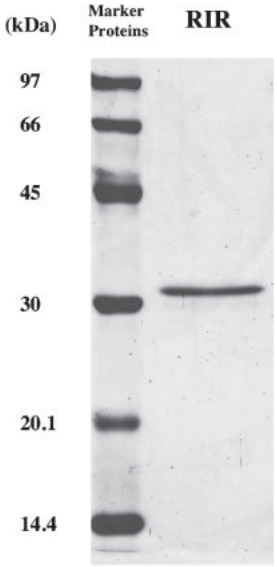

Fig. 3. SDS–PAGE of RIR.

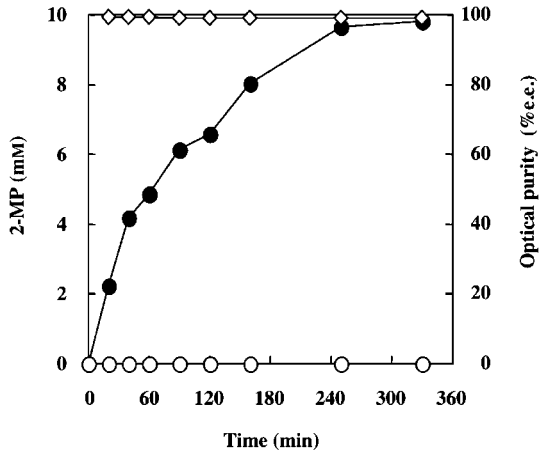

Fig. 4. Enzymatic Asymmetric Reduction of 2-MPN.
Filled circles and hollow diamonds represent 2-MP concentration and optical purity respectively. Hollow circles represent 2-MP concentration in the absence of the purified enzyme.

purified enzyme. The reactions were carried out under the following conditions: temperature range from 10 to 80 °C and pH range from 3 to 11 with 100 mM of various buffers. The effects of metal compounds and inhibitors on reductase activity were examined at 1 mM, except that *p*-chloromercuribenzoate (PCMB), phenylmethylsulfonyl fluoride (PMSF), and cuprizone were used at 0.1 mM. After incubation of appropriate amounts of the enzyme with the various reagents for 30 min for 30 °C, reduction activity was calculated from the decrease in NADPH using a spectrometer.

Substrate specificity. Reduction activity for compounds 1–18 was determined using appropriate amounts of the purified enzyme. The tested compounds, except for 2-MPN, are shown in Fig. 2. Compound 3 was used at 1 mM, and compounds 6 and 7 were used at 25 mM. All the other compounds were used at 10 mM. Reduction activity was determined by the same method as in the enzyme assay described above.

Results and Discussion

Purification and properties of RIR

RIR of *Streptomyces* sp. GF3587 was purified homogeneity from the cell-free extract at an overall yield of 13% (Table 1). The purified enzyme catalyzed the reduction of 2-MPN with a V_{max} of 10.2 μmol/min/mg in the presence of NADPH. NADPH acted as electron donor, but NADH was inert. The subunit molecular mass of the purified enzyme was determined to be 32 kDa by SDS–PAGE (Fig. 3). The molecular mass of the native enzyme was determined to be 65 kDa by gel-permeation HPLC. The kinetic constants K_m of the enzyme for 2-MPN and NADPH, calculated based on Lineweaver-Burk plots at 30 °C in 100 mM potassium phosphate buffer (pH 7.0), were 3.52 mM and 3.7 μM respectively. Enzymatic asymmetric reduction of 10 mM 2-MPN was performed in the presence of an excess amount of NADPH for 360 min at 30 °C. The purified enzyme catalyzed the formation of 9.8 mM (*R*)-2-MP with 99% e.e. (Fig. 4).

Effects of temperature and pH

RIR also catalyzed the oxidation of (*R*)-2-MP to 2-MPN. We examined both reduction and oxidation activities at various pH values. The highest reduction and oxidation activities were at about pH 6.5 and pH 11.0 respectively (Fig. 5). Reduction activity (10.2 μmol/min/mg) was 30 times higher than oxidation activity (0.32 μmol/min/mg) under each of the various optimized pH conditions. The optimum temperature was 50 °C. Reduction activity remained stable below 35 °C after 30-min of incubation.

Effects of metal ions and inhibitors

The effects of various metal ions and inhibitors on reduction activity were examined. $AgNO_3$, $CuCl_2$, and $HgCl_2$ inhibited 97%, 41%, and 75% of the activity (100%) respectively. The following metal compounds did not exhibit any inhibitory effect: $CaCl_2$, $CdCl_2$, $CoCl_2$, $FeCl_3$, LiOH, $MgCl_2$, $NiCl_2$, and $ZnSO_4$. PCMB inhibited 97% of the activity. These results strongly suggest that the enzyme is to be classified as thiol-sensitive. A specific inhibitor of serine hydrolases, PMSF, also inhibited 93% of the activity. The enzyme was not sensitive to chelating agents, such as EDTA and Tiron. Typical chelating agents *o*-phenanthroline, 2,2′-bipyridyl, 8-hydroquinoline, and cuprizone inhibited 78%, 75%, 94%, and 87% of the activity respectively, but it is unlikely that the enzyme contained metal ions

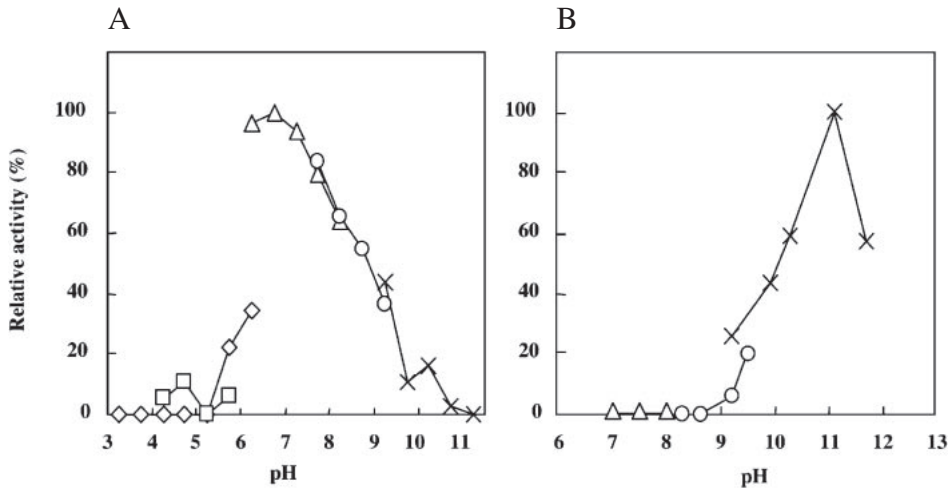

Fig. 5. Effects of pH on 2-MPN Reduction (A) and (*R*)-2-MP Oxidation (B).
The following buffers were used at a concentration of 100 mM: sodium citrate buffer (diamond), sodium acetate buffer (square), potassium phosphate buffer (triangle), Tris/HCl buffer (circle), and glycine/NaOH buffer (x-mark).

Table 2. Properties of RIR and Other NAD(P)H Reductases

Characteristics	*Streptomyces* sp. RIR (this work)	*P. putida* Pip2CR[16]	*C. macedoniensis* OYE[18]
Molecular mass of subunits	32 kDa	—	45 kDa
Molecular mass	65 kDa	200 kDa	61.5 kDa or 65 kDa
Coenzyme	NADPH	NADPH	NAD(P)H
Optimum temperature (reduction)	50 °C	30 °C	60 °C
Optimum pH (reduction)	6.5	8.0	4.5–8.5
(oxidation)	11.0	10.0	—
Substrate (preferable)	2-MPN	Pip2C, Pyr2C	KIP, 3-buten-2-one
Inhibitor	PCMB	PCMB	Ag^{+}, Cu^{2+}, Hg^{2+}, Pb^{2+}
	PMSF	Co^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+}	
	o-Phenanthroline		
	2,2′-Bipyridyl		
	Cuprizone		
	Ag^{+}, Cu^{2+}, Hg^{2+}		

as a cofactor. The strong inhibition of the enzyme by *o*-phenanthroline, 2,2′-bipyridyl, and cuprizone might be ascribed to its high affinity for their bulky heterocyclic structures rather than their chelating function. The inhibition of the enzyme caused by PMSF might be explained by its high affinity to the bulky hydrophobic structure rather than its serine-specific inhibition. Semicarbazide, diethylamine cysteamine, 3-(aminomethyl)pyridine, iodoacetic acid, (*R*)-(+)-cycloserine, diethyldithiocarbamic acid, and *N*-ethylmaleimide were inert.

Substrate specificity

RIR can synthesize (*R*)-2-MP from the prochiral cyclic imine, 2-MPN. To assess response to other imines, reduction of Δ^1-Pyrroline-2-carboxylic acid (Pyr2C) and dihydrofolic acid (DHF) by the purified RIR was also examined. Pyr2C is a substrate for Δ^1-pyrroline-2-carboxylate reductase, Pyr2CR (EC 1.5.1.1), and Δ^1-piperidine-2-carboxylate reductase, Pip2CR (EC 1.5.1.21). DHF is a substrate for dihydrofolate reductase, DHFR (EC 1.5.1.3). We synthesized Pyr2C from L-proline methyl ester, since Pyr2C is not commercially available.[15] We examined the reduction activity of RIR for those imines, and found that it did not act on the tested imines at all. RIR was different from Pyr2CR, Pip2CR, and DHFR. The reduction of (*E*)-*N*-(1-phenylethylidene)benzeneamine, for which whole cells of *C. parapsilosis* ATCC 7330 can act as substrate, was examined.[11] RIR did not catalyze the reduction of its arylimine.

Next we expanded the scope of the inquiry to other imines, carbonyl compounds including ketoisophorone (KIP) and heterocyclic compounds to check reduction activity. The compounds shown in Fig. 2 were inert as substrates. The substrate specificity of purified RIR appeared to be very narrow. As for the oxidation reaction, (*R*)-2-MP was accepted as substrate, but (*S*)-2-MP was inert. The reduction of 2-MPN by human DHFR was also examined, but DHFR did not act on 2-MPN.

Comparison of RIR with other NAD(P)H-dependent oxidoreductases

The properties of RIR were compared with the following NADPH-dependent reductases: Pip2CR from *Pseudomonas putida*,[16,17] which catalyzes the enantioselective reduction of the C=N bond of Pyr2C and Pip2C to L-proline and L-pipecolic acid, and old yellow enzyme (OYE) from *Candida macedoniensis*,[18] which catalyzes the stereospecific reduction of the C=C bond of KIP to form (6*R*)-levodione. RIR is similar to these reductases as for coenzyme, inhibitor sensitivity and

Table 3. Comparison of Partial Amino Acid Sequences of RIR and Other Oxidoreductases

Microorganism	Function	Amino acid sequence
Streptomyces sp. GF3587	RIR	GDNRTPVTVIGLGLMGQALAAAFLEAGHTT
Frankia sp.	6-PGDH	DGTDTPVTVIGLGLMGQALAGAFLKAGHPT
Pseudomonas putida[17]	Pip2CR	MSAPSTSTVVRVPFTELQSLLQAIFQRHGC
Candida macedoniensis[19]	OYE	MSYMNFDPKPLGDTNIFKPIKIGNNELKHR

optimum pH. However, the substrate specificity of RIR was different from the reductases (Table 2). An N-terminal amino acid sequence (30 residues) was carried out. The $NADP^+$-binding motif (GXGXXG) was found in this sequence. The partial amino acid sequence of the RIR showed 77% identity to those of 6-phosphogluconate dehydrogenase (6-PGDH) from *Frankia* sp. on a homology search using a BLAST program (Table 3). Hence, we examined the 6-PGDH activity of RIR. It did not exhibit any 6-PGDH activity at all. No homologies with NAD(P)H-dependent reductases such as Pip2CR,[17] and OYE[19] were found (Table 3). RIR appears to be different from any reductases reported to date. Further studies on the cloning and overexpression of the RIR gene are in progress. A comparison of the primary structure of the RIR with other NADPH-dependent reductases will be reported in the near future.

Acknowledgments

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