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Purification and Characterization of a Yeast Carbonyl Reductase for Synthesis of Optically Active (*R*)-Styrene Oxide Derivatives

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Optically active styrene oxide derivatives are versatile chiral building blocks. Stereoselective reduction of phenacyl halide to chiral 2-halo-1-phenylethanol is the key reaction of the most economical synthetic route. *Rhodotorula glutinis* var. *dairenensis* IFO415 was discovered on screening as a potent microorganism reducing a phenacyl halide to the (*R*)-form of the corresponding alcohol. An NADPH-dependent carbonyl reductase was purified to homogeneity through four steps from this strain. The relative molecular mass of the enzyme was estimated to be 40,000 on gel filtration and 30,000 on SDS-polyacrylamide gel electrophoresis. This enzyme reduced a broad range of carbonyl compounds in addition to phenacyl halides. Some properties of the enzyme and preparation of a chiral styrene oxide using the crude enzyme are reported herein.

Key words: enantioselective reduction; carbonyl reductase; styrene oxide; *Rhodotorula glutinis*

The importance of optically active compounds has increasingly been recognized in the field of pharmaceuticals. Optically active styrene oxide derivatives are useful as versatile chiral building blocks. For example, (*R*)-3-chlorostyrene oxide is an important intermediate for antidiabetic and antiobesity drugs.[1)]

There are several methodologies for synthesis of optically active styrene oxide. Microbial enantioselective hydrolysis of racemic oxide to chiral oxide and the corresponding diol has been reported, but the stereoselectivity was not sufficiently high.[2)] One of the most practical methods is the use of optically active 2-halo-1-phenylethanol as an intermediate; it is smoothly converted to an oxide without loss of optical purity (Fig. 1). Enzymatic stereoselective hydrolysis[3)] and transesterification including the practical preparation of racemic substrate[4)] for synthesis of optically active 2-halo-1-phenylethanol (halohydrin) have been reported, but the theoretical yield of the product by optical resolution is 50%, making it necessary to reuse the undesired isomer in order to be economical.

Asymmetric reductions of phenacyl halide by *Geotrichum*[5,6)] baker's yeast[7,8)] and phenylacetaldehyde reductase[9)] from *Corynebacterium* sp. have been reported. Enzymatic reduction has been one of the most powerful and practical methodologies for the synthesis of optically active alcohols.[10)] In this paper, we describe the screening and isolation of a novel carbonyl reductase catalyzing the conversion of phenacyl halide to optically active halohydrin from a *Rhodotorula* yeast.

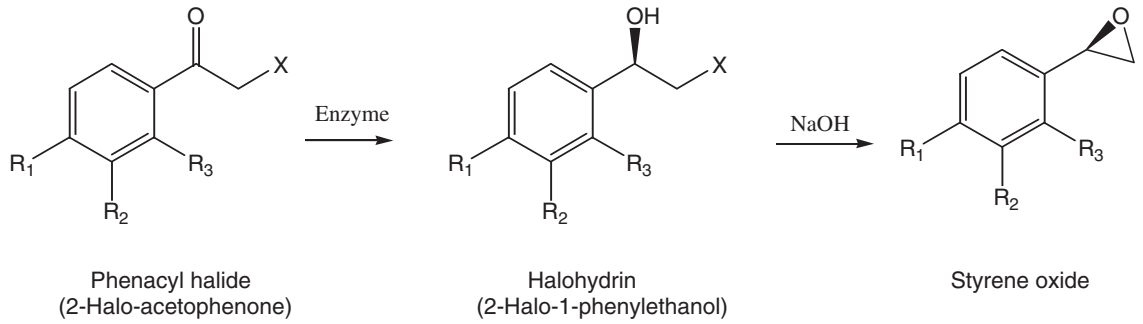

Fig. 1. Synthesis of Optically Active Styrene Oxide Derivatives.

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Abbreviations: COBE, ethyl 4-chloro-3-oxobutanoate; CPH, 2-chloro-1-(3-chlorophenyl)ethanol; CPN, 3-chlorophenacyl chloride; e.e., enantiomeric excess; KPL, ketopantoyl lactone

Materials and Methods

Chemicals. Substituted phenacyl bromides were prepared from substituted acetophenones using bromine. Substituted phenacyl chlorides were prepared from substituted acetophenones using sulfuryl chloride. Phenacyl chloride and phenacyl bromide were purchased from Tokyo Kasei, Japan. Racemic halohydrins were prepared by $NaBH_4$ reduction. Glucose dehydrogenase was purchased from Amano Enzyme, Nagoya, Japan. All other chemicals used in this study were of analytical grade and commercially available.

Microorganisms and cultivation. Microorganisms were obtained from our laboratory collection, the collection of the National Institute of Technology and Evaluation of Japan (IFO), and the Centraalbureau voor Schimmelcultures of the Netherlands (CBS). The medium 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. In the screening experiments, each strain was inoculated into 5 ml of medium in a test tube (24 mmϕ × 200 mm), followed by incubation at 30 °C with reciprocal shaking, usually for 24 h.

Screening method. Each reaction mixture, composed of cells from 5 ml of culture broth, 5 mg of one of three substituted monochlorophenacyl bromides or phenacyl bromide, and 80 mg glucose in 1 ml of 100 mM potassium phosphate buffer (pH 7.0), was shaken for 24 to 144 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.

Enzymatic reduction of 3-chlorophenacyl chloride. Rhodotorula glutinis var. *dairenensis* IFO415 was cultured in 18-liter of the above medium at 30 °C using a 30-liter fermenter. Cells obtained from 500 ml of the cultured broth by centrifugation were suspended in 150 ml of 100 mM potassium phosphate buffer (pH 7.0) and disrupted with 0.25 mm-diameter glass beads (Dyno Mill KDL-A, W.A. Bachofen, Basel, Switzerland) at 4 °C. After centrifugation, the resulting supernatant was used as the cell-free extract. In the case of the aqueous reaction, a reaction mixture of 18 ml of the cell-free extract, 2.7 g of 3-chlorophenacyl chloride (CPN), 2.7 g of glucose, 6 mg of $NADP^+$, and 1,200 units of glucose dehydrogenase was stirred at 30 °C. The pH of the mixture was kept at 6.5 with 5 N sodium hydroxide. In the case of the water/organic solvent two-phase reaction, 18 ml of *n*-butyl acetate was added to the above reaction mixture.

Synthesis of (R)-3-chlorostyrene oxide. The enzymatic reaction mixture (2,000 ml) was extracted with 2,000 ml of toluene twice. The organic layer was evaporated under a vacuum. The oily residue was distilled to obtain (*R*)-2-chloro-1-(3-chlorophenyl)ethanol (CPH) (110–120 °C/1 mmHg, 24.5 g, 90% yield, over 99% e.e; NMR δ_H ($CDCl_3$): 2.71 (1H, s, OH), 3.62 (1H, dd, J = 11.3 and 8.5 Hz, CH_2Cl), 3.75 (1H, dd, J = 11.2 and 3.6 Hz, CH_2Cl), 4.90 (1H, dd, J = 8.3 and 3.4 Hz, CH), 7.25–7.30 (3H, m, Ph), 7.42 (1H, s, Ph); $[\alpha]_D^{20}$ −33.62 (c 1.0, CH_3OH). The mixture of 20 g of (*R*)-CPH dissolved in 100 ml of toluene, and 6.5 g of NaOH dissolved in water was stirred at 40 °C for 3 h. Then the reaction mixture was separated into two phases: the organic phase was evaporated under a vacuum, and the oily residue was distilled to obtain (*R*)-3-chlorostyrene oxide (54–56 °C/1 mmHg, 15.3 g, 95% yield, over 99% e.e.); NMR δ_H ($CDCl_3$): 2.75 (1H, dd, J = 2.4 and 5.4 Hz, CH_2O), 3.15 (1H, dd, J = 4.2 and 5.4 Hz, CH_2O), 3.82 (1H, dd, J = 2.4 and 4.2 Hz, CH), 7.10 (1H, m, Ph), 7.20–7.26 (3H, m, Ph); $[\alpha]_D^{20}$ +15.98 (c 1.0, CH_3OH). The reported value for the (*R*)-isomer is $[\alpha]_D^{25}$ +10.8.[1)]

Enzyme assays and protein determination. Carbonyl reductase activity for CPN was assayed spectrophotometrically. The standard assay mixture, which was composed of 100 mM potassium phosphate buffer (pH 6.5), 1 mM of CPN, 0.25 mM of NADPH, 0.3% (v/v) of dimethylsulfoxide, and the enzyme at 30 °C in a total volume of 3 ml, was monitored for decrease in absorbance at 340 nm. One unit of the enzyme was defined as the amount catalyzing the oxidation of 1 μmol of coenzyme per min. 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.[11)]

Purification of carbonyl reductase. The purification procedure was performed at 0–4 °C. *Rhodotorula glutinis* var. *dairenensis* IFO415 was cultured in 18-liter of the above medium at 30 °C using a 30-liter fermenter. Cells obtained by centrifugation of 5.6-liter of the cultured broth were suspended with 1,000 ml of a 100 mM potassium phosphate buffer (pH 8.2) and disrupted with 0.25 mm-diameter glass beads at 4 °C. After centrifugation, the resulting supernatant was used as the cell-free extract. The cell-free extract was fractionated by the addition of solid ammonium sulfate to give a 45% saturated solution while maintaining the pH at 7.5, and the precipitate was discarded after centrifugation. The resultant supernatant solution was then brought to 60% saturation with ammonium sulfate and centrifuged again. The supernatant was discarded while the precipitate was dissolved in a 10 mM potassium phosphate buffer (pH 7.5) and dialyzed against the same buffer. The dialyzed solution was applied to the DEAE-Toyopearl 650M column (Tosoh, Tokyo, Japan) and equilibrated with 10 mM potassium phosphate buffer (pH 7.5). The enzyme was eluted with a linear NaCl

gradient solution (0–0.3 M). Active fractions were collected and dialyzed against 10 mM potassium phosphate buffer (pH 7.5). Solid ammonium sulfate was dissolved in the dialyzed solution (the final concentration was 1 M) and applied to the Phenyl-Toyopearl 650M column (Tosoh, Tokyo, Japan) equilibrated with 10 mM potassium phosphate buffer (pH 7.5) containing 1 M of 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 7.5). The dialyzed solution was applied to a Blue-Sepharose CL-6B column (Amersham Biosciences, Piscataway, NJ, U.S.A.). The enzyme was eluted with a linear NaCl gradient (0–1 M). The active fractions were collected and dialyzed against 10 mM potassium phosphate buffer (pH 7.5). The active fraction was pooled and used as the purified enzyme for characterization.

Determination of molecular mass of enzyme. The molecular mass of the native carbonyl reductase was estimated by column chromatography using a Superdex 200 HR 10/30 column and 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.

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

Reduction of various carbonyl compounds. Each reaction mixture comprising 2.5 mg of substrate, 14 mg of NADPH, 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 optical purity of the product.

Analysis. The amounts and the optical purity of the halohydrins were determined with an HPLC equipped with a Chiralcel OJ (4.6 mmϕ × 250 mm) column (Daicel Chemicals, Osaka, Japan). The HPLC conditions included *n*-hexane/isopropanol (39/1 (v/v)) as a mobile phase, a flow rate of 1 ml/min, ambient column temperature, and detection at 210 nm. The optical purity of various halohydrins produced by the enzyme was determined using an HPLC equipped with the column (Daicel) described in Table 5. The optical purity of pantolactone, produced from ketopantoyl lactone by the enzyme, was determined using an HPLC equipped with a Chiralpak AS (4.6 mmϕ × 250 mm) column (Daicel). And the optical purity of ethyl 4-chloro-3-hydroxybutanoate produced from 4-chloro-3-hydroxybutanoate was determined using an HPLC equipped with a Chiralcel OB (4.6 mmϕ × 250 mm) column (Daicel). ^{1}H-NHR spectra were recorded in $CDCl_3$, with a FT-NMR JM-400 spectrometer (JEOL, Tokyo, Japan). Chemical shifts were expressed in parts/million (ppm), with trimethylsilane as the internal standard. Optical rotation was measured with a SEPA-200 digital polarimeter (Horiba, Kyoto, Japan).

Results

Screening of chlorophenacyl halide-reducing microorganisms

Phenacyl halide-reducing activity was widely distributed in various microorganisms, although the activity was very weak. Approximately 430 yeast strains were examined for phenacyl halide-reducing activity. For 3-chlorophenyl bromide, 63 strains had reducing activity and 47 strains reduced to the corresponding (*R*)-form alcohol. For phenacyl bromide, 21 strains produced the (*R*)-form alcohol. The strains producing the alcohol with a high optical purity are listed in Table 1. The *Candida* and *Rhodotorula* genera had especially high stereoselectivities for the desired (*R*)-isomer. The stereoselectivity of the reduction depended on the structure of chlorophenacyl bromide. *Candida blankii* CBS2774 reduced 3-chlorophenacyl bromide to the (*R*)-form alcohol and phenacyl bromide to the (*S*)-form alcohol. *Rhodotorula glutinis* var. *dairenensis* IFO415 was selected as the best enzyme source because it showed high stereoselectivity for all tested substrates.

Enzymatic reduction of CPN and synthesis of (R)-3-styrene oxide

For the synthesis of (*R*)-3-chlorostyrene oxide, optically active 2-chloro-1-(3-chlorophenyl)ethanol (CPH) was synthesized by cell-free extract from *R. glutinis* IFO415. 3-Chlorophenacyl chloride (CPN) was a better substrate because it was more stable than 3-chlorophenacyl bromide. For reduction of CPN by this cell-free extract, NADPH was required as a cofactor. Then NADPH was regenerated by the addition of glucose and commercial glucose dehydrogenase. The cell-free extract accumulated approximately 130 g/l (680 mM) of (*R*)-CPH. The turnover number of NADPH for CPH was about 1,800. (*R*)-CPH was extracted from the reaction mixture and purified by distillation in a high yield. This alcohol was easily converted to (*R*)-3-chlorostyrene oxide with sodium hydroxide. In the case of the butyl acetate-water two-phase reaction, 90 g/l (471 mM) of (*R*)-CPH was accumulated.

Purification of the carbonyl reductase

The CPN-reducing enzyme was purified from *R. glutinis* var. *dairenensis* IFO415 (Table 2). The enzyme was purified 487-fold to homogeneity with an overall recovery of 4%. The molecular weight of the enzyme was found to be 40,000 by gel filtration. The relative

Table 1. Microbial Reduction of Chlorophenacyl Bromides and Phenacyl Bromide[a)]

Micoorganism	Product[b)]							
	o-Cl (R_1, R_2 = H, R_3 = Cl, X = Br)		*m*-Cl (R_1, R_3 = H, R_2 = Cl, X = Br)		*p*-Cl (R_2, R_3 = H, R_1 = Cl, X = Br)		(R_1, R_2, R_3 = H, X = Br)	
	Yield	% e.e.	Yield	% e.e.	Yield	% e.e.	Yield	% e.e.
Ashbya gossypii IFO560	n.t.[c)]	—	55	72	47	95	59	45
Brettanomyces custersianus IFO1585	n.t.	—	10	99	11	99	n.t.	—
Candida blankii CBS2774	23	92	40	90	51	73	49	(*S*) 98
Candida intermedia IFO761	23	92	54	99	26	72	n.t.	—
Candida krusei IFO11	5	99	31	99	8	87	n.t.	—
Candida magnoliae IFO705	98	96	80	81	89	68	99	11
Candida pini IFO1327	32	96	44	92	25	96	99	99
Candida saitoana IFO768	13	91	n.t.	—	n.t.	—	n.t.	—
Candida tropicalis IFO1403	32	86	n.t.	—	n.t.	—	n.t.	—
Cryptococcus albidus IFO378	15	98	59	92	24	97	n.t.	—
Cryptococcus terreus IFO727	39	99	63	98	9	99	n.t.	—
Geotrichum hirtum ATCC56047	75	92	n.t.	—	n.t.	—	n.t.	—
Pichia membranefaciens IFO461	n.t.	—	21	99	n.t.	—	n.t.	—
Rhodosporidium toruloides IFO871	62	98	79	99	17	88	60	3
Rhodotorula glutinis IFO1099	87	99	56	67	55	93	66	99
Rhodotorula glutinis var. *dairenensis* IFO415	15	99	75	99	67	99	60	99
Rhodotorula glutinis IFO190	31	98	51	99	31	89	n.t.	—
Rhodotorula minuta IFO387	n.t.	—	8	99	n.t.	—	n.t.	—
Trigonopsis variabilis IFO671	7	99	76	88	42	76	n.t.	—
Trichosporon loubieri var.*loubieri* CBS7065	27	92	n.t.	—	n.t.	—	n.t.	—
Yamadazyma farinosa IFO574	34	88	44	81	51	84	n.t.	—

[a)]The assay conditions are given in "Materials and Methods".
[b)]The absolute configilation of the product is (*R*) form.
[c)]Not tested.

Table 2. Purification of Carbonyl Reductase from *R. glutinis* var. *dairenensis* IFO415

Step	Total protein[a)] (mg)	Total activity[a)] (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-free extract	23,500	1,840	0.078	100	1
Ammonium sulfate	4,200	775	0.18	42	2.30
DEAE-Toyopearl	840	732	0.87	40	11.1
Phenyl-Toyopearl	21.2	508	24	28	308
Blue-Sepharose	1.77	66	38	4	487

[a)]The assay conditions are given in "Materials and Methods".

molecular mass weight of the subunit was estimated to be about 30,000 on SDS–polyacrylamide gel electrophoresis. The optimum temperature of the enzyme was 40–50 °C. The optimum pH was 5.0–6.0 (potassium phosphate).

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 Pro–Ala–Ala–Lys–The–Tyr–Phe–Ile–Ser–Gly–Ala–Ser–Arg–Gly–Leu–Gly–Leu–Gly–Tyr–Thr–Arg–Glu–Leu–Leu. A BLAST program search found this sequence to be significantly similar to the partial amino acid sequences of five putative dehydrogenases.

Effects of chemicals

The various compounds listed in Table 3 were added to the reaction mixture, and then their relative activities were measured with CPN as substrate. The enzyme was inhibited by several sulfhydryl reagents, *viz.*, 5,5′-dithiobis(2-nitrobenzoate), $HgCl_2$, and *p*-chloromercuribenzoate. Quercetin, a nonspecific inhibitor of human brain carbonyl reductase,[12)] diphenylhydantoin, a potent inhibitor of aldehyde dehydrogenase,[13)] and 2,4-dinitrophenol, an inhibitor of NADPH dehydrogenase (quinone),[14)] were also inhibitory. Among the metallic compounds tested, Cu^{2+}, Fe^{2+}, and Mg^{2+} had no effect on enzyme activity, while Zn^{2+} and Ag^{+} did. Chelating reagents such as EDTA and 1,10-phenanthroline also had no effect.

Table 3. Effects of Various Chemicals on Enzyme Activity[a)]

Compound	Concentration (mM)	Activity (%)
Quercetin	1	0
	0.1	0
Diphenylhydantoin	0.5	32
5,5′-dithiobis(2-nitrobenoate)	1	0
	0.1	89
Iodoacetate	1	81
p-Chloromercuribenzoate	1	24
N-Ethylmaleimide	1	70
EDTA	1	96
1,10-Phenanthroline	1	98
2,4-Dinitrophenol	1	0
	0.1	7
$CaCl_2$	1	96
$CuSO_4$	1	101
$FeSO_4$	1	114
$HgCl_2$	1	0
	0.1	52
$MgSO_4$	1	96
$MnCl_2$	1	120
$ZnSO_4$	1	57
$AgNO_3$	1	0
	0.1	39

[a)]Enzyme activity was measured as described in "Materials and Methods", except for the addition of the indicated compounds.

Substrate specificity

As shown in Table 4, a broad range of carbonyl compounds was used to investigate substrate specificity. The enzyme catalyzed some aromatic and aliphatic aldehydes, aliphatic ketones and keto esters, and keto acids. *p*-Nitrobenzaldehyde, ketopantoyl lactone (KPL) and methyl pyruvate, and ethyl 2-chloro-3-oxobutanoate were good substrates. 4-Acetylpyridine and *m*-nitroacetophenone were reduced while acetophenone and *m*-chloroacetophenone were not reduced by the enzyme. 3-Oxoesters were reduced but 2-oxoesters did not serve as substrates. Moreover, the enzyme reduced KPL and ethyl 4-chloro-3-oxobutanoate (COBE). Asymmetric reductions of these compounds are interesting reactions from the standpoint of industrial applications. NADPH was almost absolutely required as a cofactor; when NADPH was replaced with NADH, the reaction rate was reduced by 93%. Kinetic parameters were measured by Lineweaver–Burk plots. For NADPH and NADH, K_m and V_{max} were 0.056 mM and 70.4 μmol/min/mg, and 0.54 mM and 15.0 μmol/min/mg, respectively. For CPN, K_m and V_{max} were 0.060 mM and 54.1 μmol/min/mg.

Stereospecificity

The stereospecificities for some useful carbonyl compounds were examined. The enzyme reduced various phenacyl halides with high enantioselectivity for the (*R*)-isomer (Table 5). Ethyl 4-chloro-3-oxobutanoate (COBE) was reduced to the useful (*R*)-form optically pure active alcohol, which is versatile for the synthesis of pharmaceutical compounds.[15)] On the other hand, ketopantoyl lactone (KPL) was reduced to (*R*)-pantolactone, the synthetic starting material of D-pantothenic acid,[16)] with only 52% e.e.

Table 4. Substrate Specificity of the Carbonyl Reductase from *R. glutinis* var. *dairenensis*[a)]

Substrate[b)]	Relative activity (%)[c)]	Substrate	Relative activity (%)
3-Chlorophenacyl chloride (CPN)	100	Acetone	11
		2-Butanone	10
Propionaldehyde	4	2-Heptanone	11
n-Butylaldehyde	20	Diacetyl	4
n-Hexylaldehyde	10	Camphorquinone	33
Methy glyoxal	13	Cyclopentanone	9
Glutaraldehyde	2	*p*-Chloroacetophenone	5
o-Nitrobenzaldehyde	31	4-Acetylpyridine	44
m-Nitrobenzaldehyde	64	*m*-Nitroacetophenone	35
p-Nitrobenzaldehyde	162	Propiophenone	14
Benzaldehyde	25		
o-chlorobenzaldehyde	24	Ketopantoyl lactone (KPL)	238
m-chlorobenzaldehyde	45	Methy pyruvate	208
p-chlorobenzaldehyde	11	Ethyl 4-chloro-3-oxobutanoate (COBE)	12
Pyridine-4-aldehyde	55	Octyl 4-chloro-3-oxobutanoate	72
		Ethyl 2-chloro-3-oxobutanoate	111
2-Ketobutyric acid	7		
Oxalactic acid	24		

[a)]Enzyme activity was measured as described in "Materials and Methods".
[b)]The substrate concentration was 1 mM.
[c)]To calculate the relative activity, the activity for CPN was taken as 100%.
The following compounds did not serve as substrates: acetophenone, *m*-chloroacetophenone, benzylacetone, 2-octanone, ethyl 2-oxopentanoate, methyl 2-oxodecanoate, methyl propionylacetate, nicotinaldehyde, glyoxal, and 2-oxo-*n*-valeric acid.

Table 5. Stereoselectivity of the Carbonyl Reductase from *R. glutinis* var. *dairenensis*[a)]

Substrate[b)]		Product		Analysis[c)]
		Optical purity (% e.e.)	Configulation	
Phenacyl chloride	(R_1, R_2, R_3 = H, X = Cl)	>99	(*R*)	OB
Phenacyl bromide	(R_1, R_2, R_3 = H, X = Br)	>99	(*R*)	OJ
3-Chlorophenacyl chloride (CPN)	(R_1, R_3 = H, R_2 = Cl, X = Cl)	>99	(*R*)	OJ
4-Chlorophenacyl chloride	(R_2, R_3 = H, R_1 = Cl, X = Cl)	>99	(*R*)	OJ-H
4-Chlorophenacyl bromide	(R_2, R_3 = H, R_1 = Cl, X = Br)	>99	(*R*)	AD

[a)]The reaction conditions are given in "Materials and Methods".
[b)]The structures are described in Fig. 1.
[c)]The HPLC columns used for the determination of optical purity are shown. The assay conditions are given in "Materials and Methods".

Discussion

Optically active styrene oxide derivatives are useful chiral building blocks for the synthesis of pharmaceuticals. In turn, a combination of biological asymmetric reduction of phenacyl halide and chemical epoxidation of 2-halo-1-phenylethanol is one of the most practical and economical strategies for their synthesis.

Several microorganisms having the ability to reduce phenacyl halide derivatives to optically active 2-halo-1-phenylethanol derivatives have been reported. But no phenacyl halide-reducing enzyme have been characterized except for phenylacetaldehyde reductase from *Corynebacterium* sp.[17)] This is a tetrameric NADH-dependent alcohol dehydrogenase (the subunit M_r is 40,299) that catalyzes various asymmetric reductions of carbonyl compounds. We purified an NADPH-dependent carbonyl reductase that catalyzed asymmetric reduction of phenacyl halide to homogeneity from *R. glutinis* var. *dairenensis* IFO415. The purified enzyme readily reduced several aldehydes, such as *p*-nitrobenzaldehyde, *p*-chlorobezaldehyde, and pyridine aldehyde. These aldehydes are typical substrates for aldo-keto reductase family enzymes.[12)] Also, this enzyme reduced ethyl 4-chloro-3-oxobutanoate (COBE) to (*R*)-formed alcohol with a 99% e.e., and ketopantoyl lactone (KPL) to the (*S*)-form alcohol with 52% e.e., respectively. These optically active alcohols are interesting compounds from an industrial standpoint.[15,16)]

The *N*-terminal amino acid sequence of the enzyme was similar to putative dehydrogenases. Also, the sequence contained a conservative glycine-rich domain (Gly–X–X–X–Gly–X–Gly) that is found in most of the short chain dehydrogenases.[18)] This enzyme might belong to the short-chain dehydrogenase/reductase family judging by its molecular weight, its independence of metals, its response to certain inhibitors, and the conservative domain in its *N*-terminus.

Table 6. Comparison of Stereoselectivities of Various Yeast Carbonyl Reductases[a)]

Substrate		3-Chlorophenacyl chloride[b)] (CPN)	Ethyl 4-chloro-3-oxobutanoate (COBE)	Ketopantoyl lactone (KPL)
Enzyme	Origin			
Carbonyl reductase	*R. glutinis*	(*R*)	(*R*)[c)]	(*S*) 52% e.e[c)]
Menadione reductase	*C. macedoniensis*	n.t.[d)]	(*S*)	(*R*) 67% e.e.
Aldehyde reductase	*S. salmonicolor*	n.r.[e)]	(*R*)	n.r.
Conjugated polyketone reductase C1	*C. parapsilosis*	n.r.	n.r.	(*R*)
Conjugated polyketone reductase C2	*C. parapsilosis*	n.r.	n.r.	(*R*)

[a)]The configulation and optical purity of the product are listed.
[b)]The assay conditions are given in "Materials and Methods".
[c)]The analytical methods are given in "Materials and Methods".
[d)]Not tested.
[e)]Not reacted.

Several monomeric NADPH-dependent carbonyl reductases have been purified from yeast and characterized. The stereoselectivities of several enzymes for CPN, COBE, and KPL are compared in Table 6. Aldehyde reductase from *Sporobolomyces salmonicolor* reduces COBE to (*R*)-form alcohol but does not reduce CPN or KPL.[19)] Two conjugated polyketone reductases from *Candida parapsilosis* reduce KPL to (*R*)-form alcohol but do not reduce CPN or COBE.[20)] Moreover, carbonyl reductase from *R. glutinis* and menadione reductase from *Candida macedoniensis* reduce COBE and KPL to the corresponding alcohols with opposite stereoselectivities.[21,22)] The wide variation among these useful compounds in terms of their reactivities and stereoselectivities for reductases suggests that the each suitable reductase is necessary for the preparation of the desired optically active compound.

Preparations of optically active 2-halo-1-phenylethanol derivatives using microbial cells have been reported. For enzymatic reduction, the reduced form of the coenzyme is needed; therefore, an effective coenzyme regeneration system is essential. But the enzymes required for regeneration of coenzymes in microbial cells are often weak and complicated. Further, these enzymes might be damaged by phenacyl halide, which is a potent acylating reagent. Previous studies have therefore examined the effects of the addition of an organic solvent,[23)] surfactant,[24)] or absorbing resin[6)] to reduce the damage, but none of these trials has resulted in a fundamental solution to the problem. Construction of an effective enzymatic reduction system using a transformant overexpressing both reductase and glucose dehydrogenase genes has been reported.[25)] The use of this system for the preparation of optically active styrene oxide derivatives as explained in the present study has great promise for industrial applications.

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